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Human T-cell responses to *Aspergillus fumigatus*: In healthy individuals and patients with *Aspergillus*-related disease

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Human T-cell responses to *Aspergillus fumigatus*

In healthy individuals and patients
with *Aspergillus*-related disease

Hetty Jolink

Colophon

Human T-cell responses to *Aspergillus fumigatus* – in healthy individuals and patients with *Aspergillus*-related disease

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In healthy individuals and patients
with *Aspergillus*-related disease

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de graad van Doctor aan de Universiteit van Leiden,
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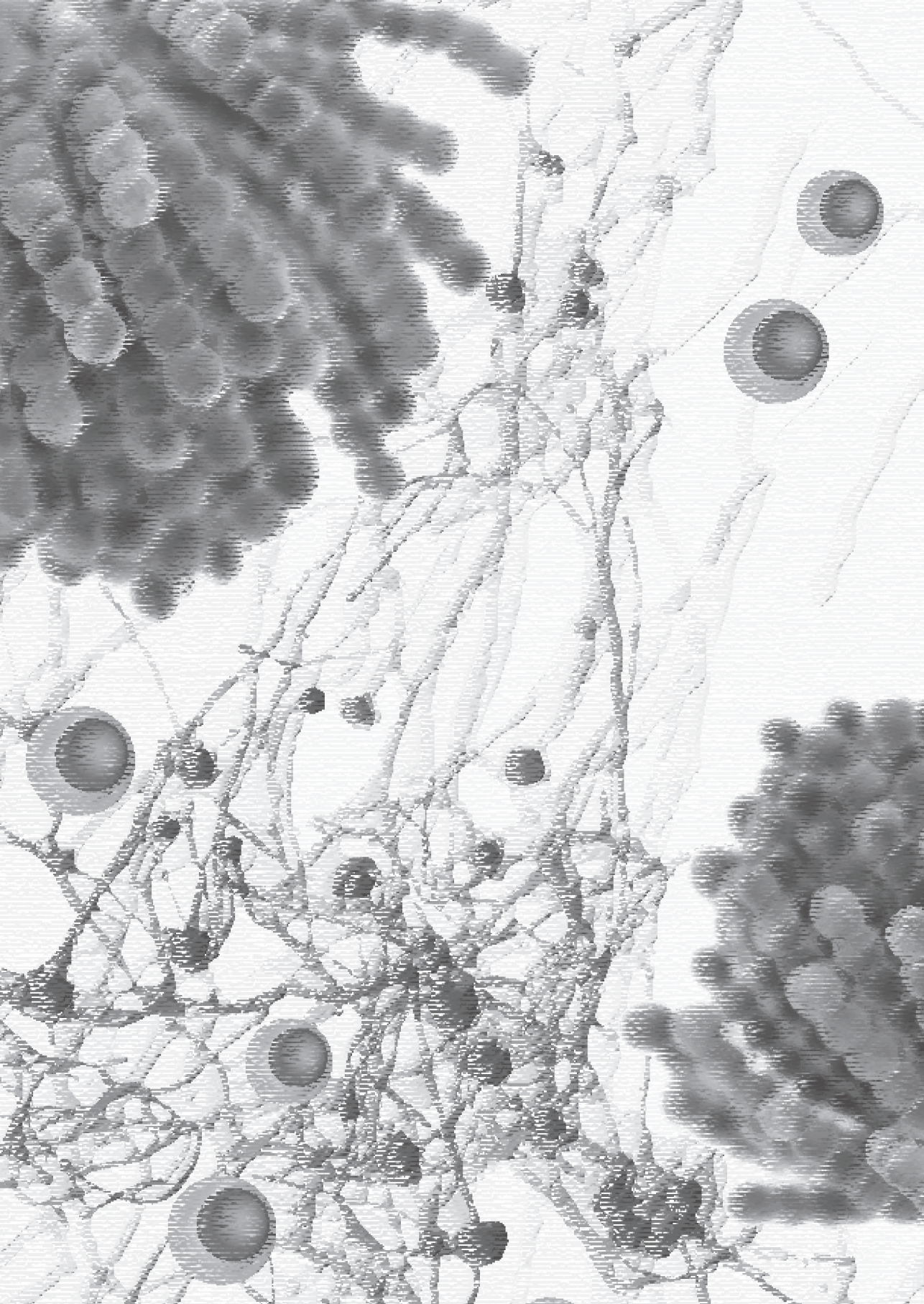
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Contents

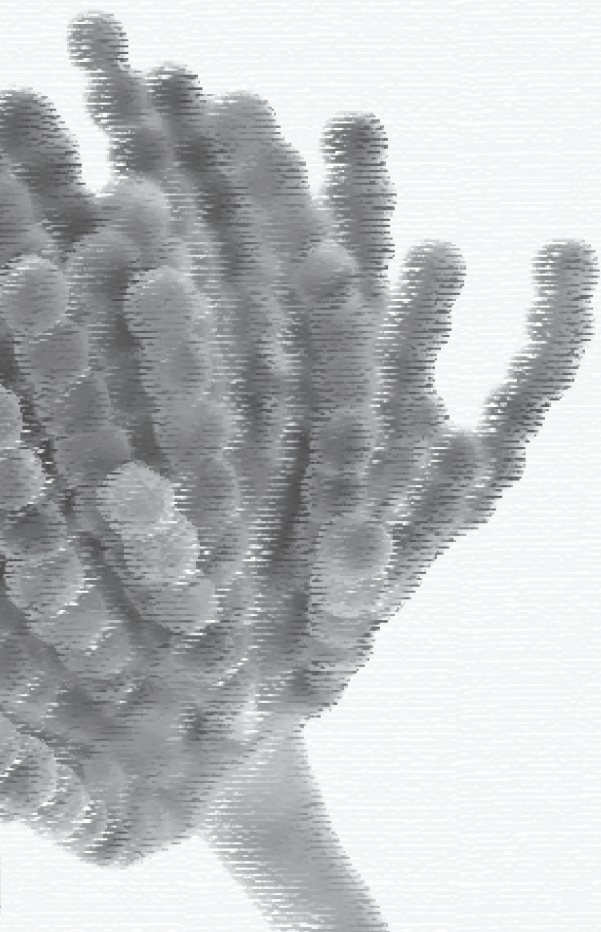
Chapter 1	Introduction and aim of the thesis	7
Chapter 2	Characterization of the T-cell mediated immune response against the <i>Aspergillus fumigatus</i> proteins Crf1 and Catalase1 in healthy individuals	23
Chapter 3	Induction of <i>A. fumigatus</i> -specific CD4+ T-cells in patients recovering from invasive aspergillosis	43
Chapter 4	Pulmonary immune responses against <i>Aspergillus fumigatus</i> are characterized by high frequencies of IL-17 producing T-cells	73
Chapter 5	Thelper2 response in allergic bronchopulmonary aspergillosis is not driven by specific <i>Aspergillus</i> antigens	91
Chapter 6	Phagocytosis of <i>A. fumigatus</i> spores by neutrophil granulocytes is improved by activated CD4+ T-cells	101
Chapter 7	Summary	117
Addendum	Nederlandse samenvatting	132
	Dankwoord	138
	Curriculum Vitae	140
	List of abbreviations	141
	List of publications	143





Chapter 1

Introduction and aim of the thesis



Introduction

Aspergillus fumigatus: a variety of clinical syndromes

Aspergillus fumigatus is a mould that is ubiquitously present and can be isolated in large amounts from compost heaps and soil¹, but also by sampling air from the outdoor or indoor environment, including apartments² and hospitals³. As a consequence, humans inhale hundreds of *A. fumigatus* spores per day, mostly without clinical consequences. However, dependent on specific host factors exposure to *A. fumigatus* can give rise to a variety of clinical syndromes⁴ as depicted in figure 1.1.

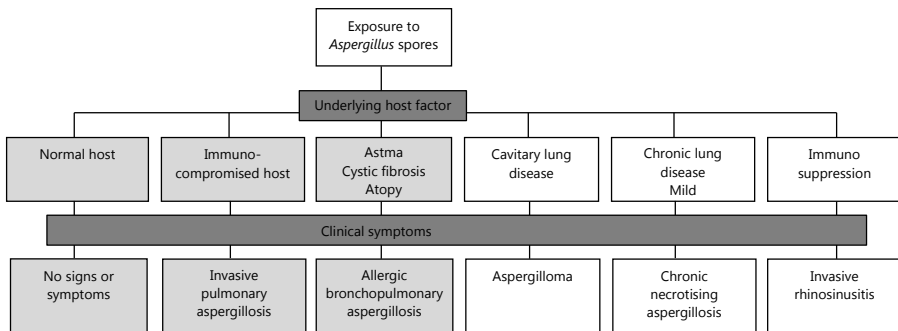


Figure 1.1 Adapted from Kousha et al 2011. This thesis will focus on T-cell mediated immune responses in the clinical situations indicated in the light grey boxes.

Invasive pulmonary aspergillosis is a severe infection affecting immunocompromised patients, such as patients after hematopoietic stem cell transplantation⁵, patients with haematological malignancies^{6,7}, solid organ transplant recipients⁸, patients with chronic granulomatous disease⁹ or patients with long-term use of corticosteroids⁹. Symptoms of invasive aspergillosis can vary; some patients have fever only, other patients present with pulmonary symptoms like cough, chest pain or dyspnea or with neurological symptoms when the aspergillus has disseminated to the brain. Invasive pulmonary aspergillosis is diagnosed according to the revised definitions of invasive fungal disease from the EORTC/MSG consensus group¹⁰. Proven invasive aspergillosis is defined by a histopathological examination with hyphae and associated tissue damage or a positive culture for *A. fumigatus* from a normally sterile site that shows clinical or radiological evidence of mould infection. However, since the introduction of less invasive tests like Galactomannan (*Aspergillus* antigen) in serum and bronchoalveolar lavage fluid and β -glucan, proven invasive aspergillosis is seldom diagnosed. A positive Galactomannan or β -glucan test combined with a clinical and radiological criterium leads to a probable invasive aspergillosis diagnosis and initiation of empirical treatment. Invasive pulmonary aspergillosis is a serious

disease; the mortality rate in patients with invasive aspergillosis can be as high as 50–80%^{5,6,11}.

Allergic bronchopulmonary aspergillosis (ABPA) is a hypersensitivity disorder that is characterized by an allergic reaction upon exposure to *A. fumigatus*¹². ABPA is only present in patients with a history of asthma or cystic fibrosis. Clinical symptoms include worsening of asthma symptoms, i.e. increase of cough, sputum production or wheezing. The sputum can be very thick and difficult to evacuate from the lungs. Constitutional symptoms like weight loss, low-grade fever or malaise may be part of the clinical picture¹³. The diagnosis ABPA cannot be made by a single diagnostic test, but relies on different criteria, as described by Rosenberg and later modified by Greenberger: asthma or cystic fibrosis, elevated total serum IgE (> 417 IU/ml), elevated IgE and IgG anti-*A. fumigatus* antibodies, positive precipitating antibodies to *A. fumigatus*, positive *A. fumigatus* skin reactivity, pulmonary infiltrates, peripheral blood eosinophilia (>500/mm³), central bronchiectasis¹³.

Other clinical syndromes caused by *A. fumigatus* are aspergilloma, chronic necrotising aspergillosis and invasive rhinosinusitis. Aspergilloma is a fungus ball that can arise in a cavitary lung lesion. It is most often associated with pulmonary tuberculosis, but can also be present in other cavitary pulmonary diseases like sarcoidosis, bronchiectasis or neoplasms⁴. *Aspergillus* hyphae in aspergilloma seldom become invasive but confine to the cavity in the lung. Chronic necrotising aspergillosis is a rare disease only described in case reports and case series. It presents as a slowly progressive invasive aspergillosis over months, and rarely disseminates throughout the body. Chronic necrotising aspergillosis is diagnosed in patients with chronic lung disease like COPD or in mildly immunosuppressed patients, like patients with diabetes mellitus or alcohol abuse⁴. Invasive rhinosinusitis can occur in severely immunocompromised patients with haematological malignancies or patients after allogeneic stem cell transplantation, but also in the mildly immunosuppressed patient with diabetes mellitus¹⁴.

The focus of this thesis is the *Aspergillus*-specific T-cell immune response in the normal host and in two patient groups with *Aspergillus*-related disease. With these groups we were able to study both extremes of the spectrum of T-cell immunity in *Aspergillus fumigatus*: on the one hand we studied patients after an allogeneic stem cell transplantation, who are immunocompromised and exhibit an impaired immune reaction to *Aspergillus fumigatus*, making them at risk for invasive pulmonary aspergillosis. On the other hand we studied patients with an overreaction after exposure to *A. fumigatus*; the atopic host with ABPA. The T-cell immune response in these patients were compared to the situation in the healthy human host with a normal T-cell immune response to *A. fumigatus*.

Microbiology of *Aspergillus fumigatus*

Aspergillus fumigatus is a fast growing saprophytic fungus. Microscopically it can be recognized by septate hyphae, branching at a 45° angle¹⁵. *Aspergillus fumigatus* sporulates and the spores, also called conidia are easily spread in

the air. *Aspergillus fumigatus* grows at a broad temperature range from 30°C to 50°C¹⁵, and can even survive at temperatures up to 70°C. The complete genome of *Aspergillus fumigatus* consists of 8 chromosomes containing almost 10.000 genes. Genes are differentially expressed dependent on temperature, leading to a different expression of proteins at different temperatures.. Several genes have a higher expression level at 48°C than at 37°C, for other genes a higher expression level is seen at 37°C compared to 48°C¹⁶. Furthermore, certain genes and proteins are only expressed in hyphae and others only in conidia¹⁷⁻¹⁹.

Host immune defence against *Aspergillus fumigatus*

The immune system helps to protect the host against invading micro-organisms, when the barrier function, the first line of defence, has failed. In humans the immune system is divided in the innate and adaptive immune system. For a long time it has been known that the innate immune system plays an important role in the immune response against *A. fumigatus*. Patients with prolonged neutropenia have a significant increased risk for the development of invasive aspergillosis²⁰⁻²². *In vitro* data support the notion that neutrophil granulocytes are important for the clearance of aspergillus infection. Lehrer et al. showed already in the 1970s that neutrophil granulocytes are capable of phagocytosing *A. fumigatus* spores, although the viability of conidia appeared not to be affected²³. When neutrophil granulocytes were coincubated with *A. fumigatus* hyphae, neutrophils attached to the hyphal surface and degranulated, which ultimately led to severe damage of *Aspergillus* hyphae²⁴. More recently additional mechanisms that can be used by neutrophil granulocytes have been described. These include ion fluxes that may be involved in the regulation of the granule enzyme function of neutrophil granulocytes²⁵, and the formation of neutrophil extracellular traps (NETs)²⁶, consisting of chromatin and cytoplasmic proteins released by neutrophil granulocytes to kill extracellular micro-organisms without the process of phagocytosis. NET formation is induced by *A. fumigatus* conidia and hyphae and leads to reduced fungal growth, but not fungal killing²⁷.

Besides neutrophil granulocytes, also monocytes, macrophages and dendritic cells (DC) belong to the innate immune system and play an important role in the immune response against *A. fumigatus*. In the lungs, the first location of exposure to *A. fumigatus*, alveolar macrophages are present to control the spread of *Aspergillus*. *A. fumigatus* conidia and hyphae are recognized by pathogen recognition receptors (PRRs) on the membrane surface of mononuclear cells, including the toll-like receptors (TLRs) TLR2^{28,29} and TLR4^{29,30} and the C-type lectin receptors Dectin-1³¹ and DC-SIGN³². When pathogen-associated molecular patterns (PAMPs) of *Aspergillus fumigatus* are recognized by one of these PRRs, *A. fumigatus* conidia will be ingested by the phagocytes and the phagocytes become activated. This will lead to the release of cytokines and chemokines and activation of the Reactive Oxygen Species, important for the killing of *A. fumigatus* conidia³³. Several studies have shown that patients with polymorphisms in the

genes encoding TLRs, Dectin-1 and cytokines related to innate immunity have an increased risk of invasive aspergillosis³⁴⁻³⁶, underscoring the role of monocytes, macrophages and DC in the immune defence against *Aspergillus*.

Only recently, attention has shifted to the role of the adaptive immune system in the immune response against *A. fumigatus*. Because in several patients, especially after hematopoietic stem cell transplantation, neutropenia was not a prominent feature, it was postulated that T-cells might be of importance. After hematopoietic stem cell transplantation the recovery of neutrophil granulocytes is fast, around 4 weeks, whereas T-cell mediated immunity stays impaired for several months.

T-cell mediated immunity

T-cells are part of the adaptive immune system and can be distinguished by expressed coreceptors. Cytotoxic T-cells express the CD8 coreceptor and mainly recognize short amino acid fragments (peptides) derived from intracellular proteins in the context of human leukocyte antigen (HLA) class I. Helper T-cells (Th cells) express the CD4 coreceptor and mainly recognize extracellular foreign peptides in the context of HLA class II. Peptide length can vary; in the context of an HLA class II molecule peptide length is typically 10-20 amino acids. This recognized amino acid sequence is termed T-cell epitope and is HLA-restricted, meaning that a particular T-cell epitope can only be recognized in the context of a particular HLA-molecule. HLA class I is expressed by all nucleated cells in humans, whereas HLA class II is only expressed by cells from the hematopoietic system, by professional antigen presenting cells like monocytes, macrophages, DC and B-cells³⁷.

As described above, invading micro-organisms are recognized by antigen presenting cells when a specific molecular motif (PAMP) of the micro-organism is recognized by a TLR or C type lectin receptor. The micro-organism is phagocytosed and an internal vesicle is formed, the so-called phagosome³⁷. By fusion with other structures like the lysosome that contains degrading enzymes, the pathogen is degraded to proteins and subsequently to peptides that can be presented by the HLA class II molecule on the surface of the antigen presenting cell. Peptides bound in the HLA class II molecule are recognized by the T-cell receptor (TCR) on CD4+ (Thelper) cells³⁷, leading to T-cell activation. Depending on the signals the CD4+ T-cell receives from the antigen presenting cells, the Thelper (Th) cell will differentiate into a specific phenotype.

Three main Th phenotypes are recognized: Th1, Th2 and Th17 cells. Th1 cell differentiation is driven by IFN γ , whereas a cytokine milieu with IL-10 and IL-4 impairs the differentiation towards the Th1 phenotype. In the presence of IL-10 and IL-4 the Th2 phenotype is favoured. Th1 cells produce IFN γ , TNF- β and IL-2 and are important for the attraction and activation of monocytes and macrophages to the site of infection. Furthermore they interact with CD8+ cytotoxic T-cells, and IgG-producing B-cells³⁷. Th2-cells produce IL-4, IL-5, IL-10 and IL-13 and interact with mast cells, eosinophil granulocytes, basophil granulocytes and B-cells. B-cells

are stimulated by Th2 cells to produce IgE antibodies, leading to stimulation of mast cells to release the contents of their granules, like histamine and serotonin³⁷. Finally, the combination of TGF- β , IL-1 β and IL-23 drives differentiation of Th17 cells. Th17 cells produce IL-17a, IL-21 and IL-22 and play a role in the recruitment and activation of neutrophil granulocytes to the site of an infection³⁷.

A naïve T-cell can become activated when the TCR binds to a peptide-HLA complex and there is concomitant expression of costimulatory molecules by the antigen presenting cell. The T-cell with high affinity binding for a specific peptide will differentiate into a memory T-cell, and will eventually survive in the resting state. In case of re-encounter with the same peptide, the immune response will be accelerated and more intense than upon first encounter, and no additional danger signal is necessary to activate the memory T-cell. When memory T-cells are activated they will proliferate and exert their effector functions like production of cytokines and killing of infected cells. Cytokine production and proliferation of T cells after antigen stimulation analysed in in-vitro assays can be used to detect memory immune responses in peripheral blood or other tissue samples of individuals.

***Aspergillus*-specific T-cell mediated immune response**

In healthy individuals *Aspergillus*-specific T-cell mediated immunity was studied by stimulating peripheral blood mononuclear cells (PBMC) with crude extracts of *A. fumigatus* or recombinant *A. fumigatus* proteins³⁸⁻⁴⁰. After stimulation with *A. fumigatus* crude extracts or *A. fumigatus* recombinant proteins T-cell proliferation or an increase of cytokine production, mainly IFN γ was observed, indicating the presence of *Aspergillus*-specific T-cells with a Th1 profile in healthy individuals³⁸⁻⁴⁰. Although in other fungal infections the role of IL-17 is well established, in *Aspergillus* infection Th17 cells do not seem to play a role in the defence against *A. fumigatus*⁴¹ or may even be detrimental⁴².

Several studies pointed to the importance of the *A. fumigatus* cell wall glucanase Crf1 in the T-cell mediated immune response against *Aspergillus*^{38;43;44}, and identified HLA class II restricted epitopes for the Crf1 protein^{43;44}. T-cell clones specific for the p41 epitope of the Crf1 protein with a Th1 cytokine profile were shown to be cross-reactive to *Candida albicans*⁴⁴, and in mice vaccination with the Crf1 protein conferred protection against both *Aspergillus* and *Candida* infection⁴⁴.

In ABPA patients other *Aspergillus* antigens were deemed to be important, the so called *Aspergillus* allergens, like Aspf1, Aspf2, Aspf3 and Aspf4⁴⁵⁻⁴⁷. It was hypothesized that the overreaction in ABPA patients is dependent on exposure to these specific *Aspergillus* antigens⁴⁵⁻⁴⁷, and not to other *A. fumigatus* antigens to which T-cell immune responses were frequently detected in healthy humans and in patients with invasive aspergillosis. In ABPA patients T-cells with a Thelper2 phenotype were present specific for the antigens Aspf1 and Aspf2^{45;47;48}, as well as anti-*Aspergillus* IgE, IgA and IgG antibodies directed against Aspf1, Aspf2, Aspf3 and Aspf4^{46;49;50}.

The *Aspergillus*-specific T-cell mediated immunity in patients with invasive aspergillosis, however, showed a similar picture as in healthy individuals with lymphoproliferation and an increase of Th1 cytokine production after stimulation of PBMC with *A. fumigatus* crude extracts and the *A. fumigatus* antigens DPP-V and Catalase⁴⁰. In some patients *Aspergillus*-specific T-cells expressing a Th2 profile were identified; these patients demonstrated a less favourable outcome than patients with *Aspergillus*-specific T-cells expressing a Th1 profile⁴⁰.

Furthermore, several murine studies have investigated the protective role of *Aspergillus*-specific T-cells in aspergillus infection. Studies were performed in mice that were immunosuppressed by injection of cyclophosphamide, or lethally irradiated and transplanted with allogeneic bone marrow. The immunosuppressed mice were vaccinated with *A. fumigatus* recombinant proteins^{38;51} or adoptively transferred with *A. fumigatus* antigen-pulsed dendritic cells (DC)^{38;52} approximately 2 weeks before intratracheal or intravenous infection with *A. fumigatus* conidia. In other studies *Aspergillus*-specific CD4⁺ splenic cells were adoptively transferred the day before or the day after experimental infection of the mice with *A. fumigatus* conidia^{52;53}. In these studies, the survival of mice after aspergillus infection was significantly increased, when they received *A. fumigatus* specific vaccination or were adoptively transferred with *Aspergillus*-specific CD4⁺ splenic cells. In addition, the fungal burden was reduced in these mice.

One study in humans showed that in patients after haploidentical hematopoietic transplantation diagnosed with invasive aspergillosis, the adoptive transfer of *Aspergillus*-specific T-cell lines, generated by stimulation of PBMC with *A. fumigatus* conidia, resulted in a better prognosis with faster clearance of serum *Aspergillus* antigen (Galactomannan) and a trend towards a better survival, compared to patients not receiving adoptive transfer therapy⁵⁴. To further optimize adoptive immunotherapy for aspergillus infections, new methods to generate *Aspergillus*- and multipathogen-specific T-cell lines were studied, showing that isolation based on CD154 expression could be a potential strategy for the generation of T-cell products suitable for adoptive transfer⁵⁵.

It is not yet clear how CD4⁺ T-cells exert their function in aspergillus infections. In general, CD4⁺ T-cells are in the centre of the immune system, interacting with many other immune cells. Depending on the differentiation to a specific Th phenotype, the CD4⁺ T-cell will attract and interact with a particular type of immune cell. As previously mentioned, neutrophil granulocytes as well as monocytes and monocyte-like cells are important players in the defence against *A. fumigatus*. In the light of the knowledge on the immune response against *A. fumigatus*, we speculate that *A. fumigatus*-specific CD4⁺ T cells will improve the recruitment, activation, and function of phagocytic cells at the site of infection.

Aim of the thesis

The immune response against *Aspergillus fumigatus* is initiated by immune cells of the innate immune system, like monocytes, macrophages and neutrophil granulocytes. Although *Aspergillus*-specific T-cells were clearly shown in healthy individuals by stimulation of PBMC with *Aspergillus* crude extracts, many issues deserve further investigation and research. First of all, little information is available on the broadness of the *Aspergillus*-specific T-cell immune response, the epitope-specificities, HLA-restrictions and cytokine secretion profiles. In addition, the role and effectiveness of *Aspergillus*-specific T-cells in the immune response in invasive aspergillosis is unclear. It is known that *Aspergillus fumigatus* can give rise to different Thelper cytokine profiles in healthy individuals, patients with invasive aspergillosis and in patients with allergic bronchopulmonary aspergillosis (ABPA). It is however unclear, what the underlying mechanism is behind the differences in cytokine secretion profiles. Are these triggered by specific *Aspergillus* antigens? And is the cytokine secretion profile in all body compartments similar, or do they differ in this respect? Finally, the way of action and mechanisms through which T-cells exert their function are unknown. Are these immune cells active players in the immune response against *A. fumigatus*? And if so, how can they contribute to the immune defense against *A. fumigatus*?

In **chapter 2** we performed an in-depth analysis of the T-cell mediated immune response to 2 *A. fumigatus* proteins in healthy individuals. We investigated the T-cell responses against Crf1, that is present in both conidia and hyphae, and against Catalase1, that is only present in hyphae. We analyzed CD4+ T-cell cytokine secretion profiles, epitope specificities and HLA restrictions by stimulating PBMC from healthy individuals with synthetic overlapping peptides of Crf1 and Catalase1. *Aspergillus*-reactivity of the activated T-cells was confirmed by stimulation with *Aspergillus* crude extract or *A. fumigatus* recombinant protein.

In murine studies it was shown that adoptive transfer of *Aspergillus*-specific T-cells improve the outcome after experimental aspergillus infection, suggesting a therapeutic or preventive effect of *Aspergillus*-specific T-cell immunity. Few data are available on the *Aspergillus*-specific T-cell response in humans with aspergillus infection. In **chapter 3** we studied the *Aspergillus*-specific T-cell immunity in patients suffering from invasive aspergillosis. In patients after allogeneic stem cell transplantation with invasive aspergillosis we analyzed the frequencies and cytokine profiles of Crf1- and Catalase1-specific T-cells in the period before and after invasive aspergillosis was diagnosed. We correlated these data with the clinical course of invasive aspergillosis.

In peripheral blood of healthy individuals and patients suffering from invasive aspergillosis *Aspergillus*-specific T-cells showed mainly a Thelper1 profile, in contrast to the Thelper17 profile seen in other fungal infections. As *A. fumigatus* infection is primarily located in the lungs, *Aspergillus*-specific T-cells will most likely exert their function in pulmonary tissues and the pulmonary draining lymph nodes. Differences in cytokine profiles of *Aspergillus*-specific T-cells between peripheral

blood and the lungs might be present. In **chapter 4** we investigated the cytokine secretion profiles in lung-derived *Aspergillus*-specific T-cells and compared these with the cytokine secretion profiles in peripheral blood derived *Aspergillus*-specific T-cells in COPD patients.

In one group of patients, *Aspergillus*-specific T-cells do not display a Thelper1 phenotype in peripheral blood, but in contrast show a Thelper2 phenotype: patients with allergic bronchopulmonary aspergillosis (ABPA). The underlying mechanisms of these differential cytokine profiles are unclear. In **chapter 5** we studied whether certain *Aspergillus* antigens are more 'allergenic' than others, by comparing the cytokine secretion profiles of *Aspergillus*-specific T-cells in response to different *A. fumigatus* antigens in healthy individuals and in patients with ABPA.

The way of action and mechanisms by which *Aspergillus*-specific T-cells may prevent the occurrence of aspergillus infection or influence the course of aspergillus infection is not known. Previously it was postulated that *Aspergillus*-specific T-cells may have a direct effect on *A. fumigatus* growth, or interact with other immune cells. In **chapter 6** we describe the results of *in vitro* experiments we performed to get more insight in the mechanisms of action of T-cells in the immune response against *A. fumigatus*. We studied the phagocytosis capacity of monocytes and neutrophil granulocytes in a flow cytometry-based phagocytosis assay.

In **chapter 7** the results of the studies described in this thesis are summarized and discussed, and future directions in this field of research are proposed.

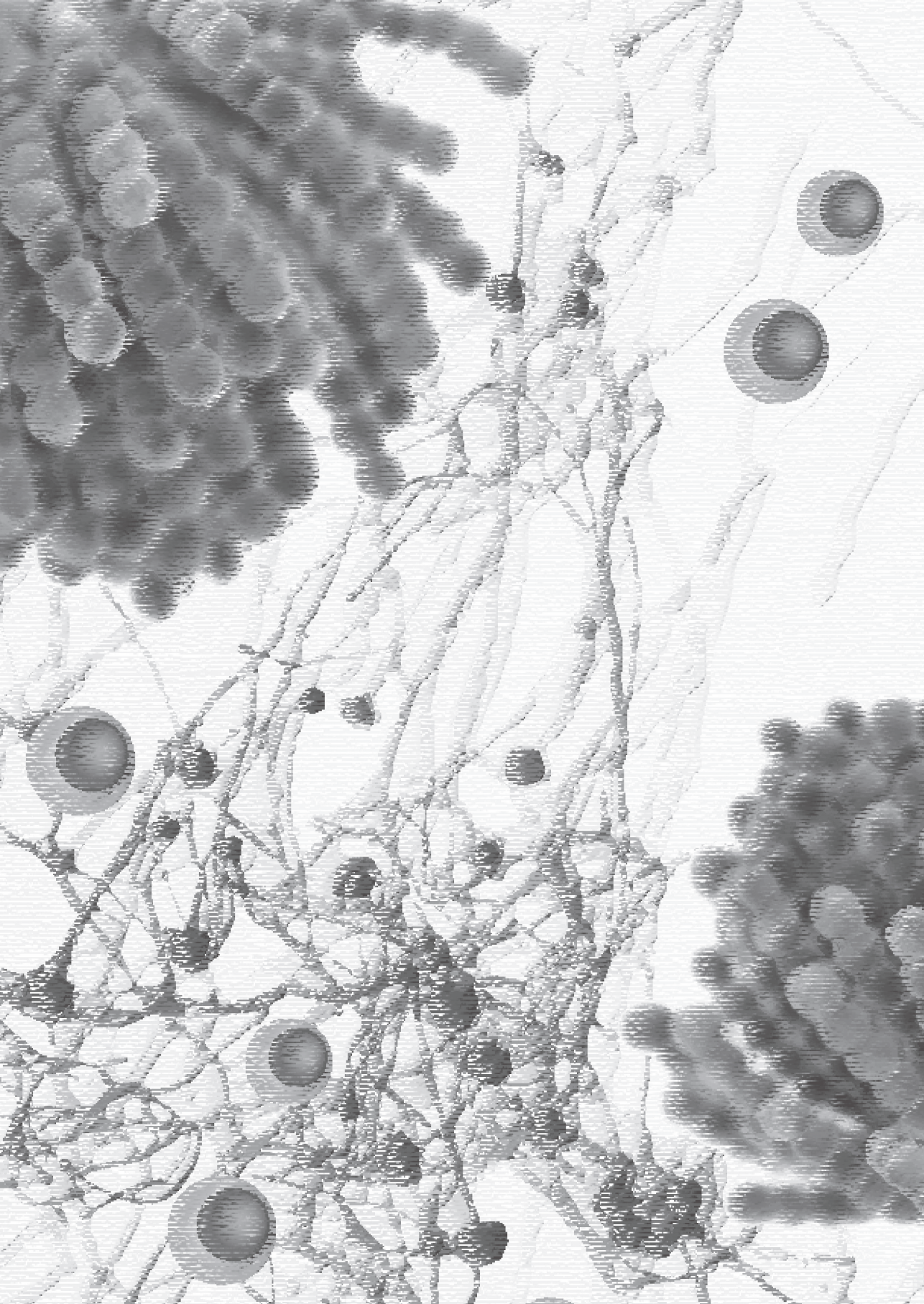
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Chapter 2

Characterization of the T-cell mediated immune response against the *Aspergillus fumigatus* proteins Crf1 and Catalase1 in healthy individuals



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Abstract

Invasive aspergillosis is a serious infectious complication after allogeneic stem cell transplantation. One of the strategies to improve the management of aspergillosis is the adoptive transfer of antigen-specific T-cells, the success of which depends on the development of a broad repertoire of antigen-specific T-cells. In this study we identified CD4⁺ T-cells specific for the *Aspergillus* proteins Crf1 and Catalase1 in 18 of 24 healthy donors by intracellular staining for IFN γ and CD154. Crf1- and Catalase1-specific T-cells were selected on the basis of CD137 expression and single cell expanded. *Aspergillus*-specific T-cell clones mainly exhibited a Th1 phenotype and recognized a broad variety of T-cell epitopes. Five novel and 2 previously described Crf1 epitopes, and 30 novel Catalase1 epitopes were identified. Ultimately, by using overlapping peptides of *A. fumigatus* proteins, *Aspergillus*-specific T-cell lines with a broad specificity and favourable cytokine profile, suitable for adoptive T-cell therapy, can be generated in vitro.

Introduction

Aspergillosis is a common infectious complication in patients with hematological malignancies and in patients after allogeneic stem cell transplantation (SCT) ^{1,2}. There is increasing evidence that, besides the well-established risk factor neutropenia ³, impaired T-cell immune reconstitution is an important risk factor for invasive aspergillosis ⁴⁻⁶.

To improve the outcome of patients after SCT and reduce the morbidity and mortality of aspergillus infections adoptive T-cell therapy for aspergillosis may be beneficial. In murine studies vaccination with *A. fumigatus* recombinant proteins ^{7,8}, the adoptive transfer of antigen pulsed DC ^{8,9} or splenic CD4+ T-cells which were obtained after in vivo stimulation with *A. fumigatus* crude culture antigens ^{5,9}, resulted in a prolonged survival of *Aspergillus*-infected mice and a reduced fungal burden. In humans, it was shown that patients after haploidentical SCT with aspergillosis have a better prognosis when transferred with T-cell lines generated by stimulating PBMC with *A. fumigatus* conidia ¹⁰.

When generating a T-cell product by stimulating with *Aspergillus* crude extract or recombinant protein the specificity of the T-cell line is difficult to establish. Furthermore, a considerable variation between different batches of crude extracts and recombinant proteins can be expected. To avoid these complicating factors, another option for the generation of an *Aspergillus*-specific T-cell product is by stimulating with peptides of *A. fumigatus* proteins. Until now however, few T-cell epitopes of only the Crf1 protein have been published ¹¹⁻¹³.

To get more insight into which *A. fumigatus* peptides should be used to generate a T-cell product with immunological activity against *A. fumigatus*, it is important to study the diversity of the *Aspergillus*-specific T-cell repertoire. Furthermore, it is essential to gain knowledge of the variety of cytokine profiles of *Aspergillus*-specific T-cells before considering infusion of *Aspergillus*-specific T-cells in patients with invasive aspergillosis. We studied the immune response against Crf1 and Catalase1, as in previous studies using recombinant proteins, T-cells directed against Crf1 displayed a Th1 phenotype and T-cells directed against Catalase1 displayed a more Th2 phenotype. Furthermore, Crf1 and Catalase1 are differentially expressed; Crf1 is present in both conidia and hyphae, whereas Catalase1 is only present in *Aspergillus* hyphae.

By stimulating with overlapping peptides of Crf1 and Catalase1 we show that low frequencies of both Crf1- and Catalase1-specific T-cells with a Th1 phenotype are present in healthy individuals, directed against a broad variety of T-cell epitopes. As we identified *Aspergillus*-specific T-cells specific for Crf1 as well as Catalase1, this suggests that the T-cell mediated immune response against *A. fumigatus* is directed against multiple *Aspergillus* proteins.

Materials and 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 (Germany). Peptides were divided in a complete pool, subpools of 8 to 12 peptides, and 96 single peptides for Crf1 and 180 single peptides for Catalase1. To confirm specificity synthetic peptides were made at the Leiden University Medical Center (LUMC).

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 rotated at 250 rpm at 37°C overnight (ON). 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) and CBS 545.65 (Allergon) were used.

Enzyme-linked immunosorbent spot assay (Elispot)

All studies were conducted with approval of the institutional review board of the LUMC. After informed consent was given in accordance with the Declaration of Helsinki, peripheral blood was obtained from healthy donors and PBMC were isolated by Ficoll-Isopaque separation and cryopreserved. PMBC (2×10^6) were cultured for 48 hours in a 24-well plate in the presence or absence of the Crf1 or Catalase1 complete pool (10^{-6} M). After 2 days, 10^5 cells per well were seeded in triplicate in a Multiscreen 96-well plate (Millipore) coated with IFN γ , IL-4 or IL-17 catching antibody (Mabtech AB) and cultured ON. Spots were visualized by adding biotinylated mAb (Mabtech AB), extravidin-ALP (Sigma) and BCIP/NBT ALP substrate (Sigma) and counted with a fully automated computer assisted video-imaging analysis system (BioSys 4000).

Flow cytometry

PBMC from healthy donors (0.5×10^6) were stimulated with the Crf1 or Catalase1 complete pool (10^{-6} M) in a 96-well plate and cultured in IMDM, supplemented with 5% inactivated fetal calf serum (FCS, Gibco), 5% inactivated human serum (ABOS) and 100 IU/ml IL-2 (Chiron) (T-cell medium), and restimulated with non-loaded or peptide-pulsed autologous PBMC (0.5×10^6) after 7 days. 1 hour after restimulation 10 μ g/ml Brefeldin-A (BFA, Sigma-Aldrich) was added to promote intracellular

accumulation of cytokines. 5 hours after restimulation cells were stained with PerCP-labeled anti-CD4 (BD/Pharmingen) for 15 min at 4°C. After washing, cells were fixated with Paraformaldehyde 1% (pharmacy LUMC) and permeabilised with saponin 0.1% (Sigma) and pasteurized plasma proteins 2% (GPO, Sanquin) in Phosphate Buffered Saline (PBS, PAA). FITC-labeled anti-CD154 (BD/Pharmingen) or PE-labeled anti-CD154 (Beckman Coulter) and APC-labeled anti-IFN γ (BD/Pharmingen) were added for 30 min at 4°C for intracellular staining of IFN γ and CD154. Cells were collected and analyzed on the Calibur II using Cellquest pro software (BD). Further analysis was performed with FlowJo software (Tree Star).

Generation of T-cell clones

We selected *Aspergillus*-specific T-cells based on CD137 expression 48 hours after stimulation and restimulation, as described above. Cells were stained with APC-labeled anti-CD137 (BD/Pharmingen) and PerCP-labeled anti-CD4, and sorted by FACS. To generate T-cell clones, cells were sorted 1 cell per well into a U-bottom microtiter plate containing 100 μ l of feeder mixture, consisting of T-cell medium, PHA (800 ng/ml) and irradiated allogeneic PBMC (0.5×10^6 /ml). Fresh T-cell medium was added after 7 days and T-cell clones were restimulated every 3 to 4 weeks with irradiated allogeneic PBMC (10^6 /ml) and PHA (800 ng/ml) in a responder:stimulator (R:S) ratio of 1:5.

V β analysis of T-cell clones

To determine V β usage of the T-cell clones the IOTest® Beta Mark TCR Kit (Beckman Coulter) was used, according to the manufacturer's instructions. For a selection of T-cell clones the TCR V β was analyzed by RT-PCR and sequencing.

Determination of specificity and epitope identification

To determine specificity, proliferating T-cell clones were stimulated ON with autologous PBMC (R:S 1:10) or autologous Epstein-Barr virus-transformed B lymphoblastoid cell lines (EBV-LCL, R:S 1:4) loaded with the Crf1 or Catalase1 complete pool (10^{-6} M). Supernatant was analyzed for production of IFN γ , IL-4, IL-10 or IL-17 by standard ELISA.

For identification of the T-cell epitopes, clones were stimulated with autologous PBMC (R:S 1:10) or EBV-LCL (R:S 1:4) loaded with subpools (10^{-6} M) of the overlapping peptides (JPT). To confirm the identified epitopes the clones were tested with newly produced synthetic peptides (LUMC).

To determine *Aspergillus*-specificity, peptide-specific clones were stimulated ON with autologous EBV-LCL (R:S 1:4) loaded with recombinant protein (100 μ g/ml) or with autologous monocyte derived DC preloaded with *Aspergillus* crude extract (R:S 1:4). Monocyte derived DC were generated as previously described ¹⁴.

Determination of HLA-restriction

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 thorough washing the stimulator cells, mAbs specific for HLA-class II (PdV5.2), HLA-DR (B8.11.2), HLA-DQ (SPVL3), HLA-DP (B7.21) or the HLA-DR antigens DRB3*02 (TL2C9) or DRB1*11 (VR1H5) 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 analyzed using standard ELISA. Further determination of the HLA-restriction was analyzed using an HLA-typed EBV-LCL panel, containing partially matched allogeneic EBV-LCL. T-cell clones and EBV-LCL (R:S 1:4) were incubated ON with the complete overlapping peptide pool (10^{-6} M) and the supernatant was tested in ELISA. To confirm HLA-restriction clones were tested 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, as previously described ¹⁵.

Results

Low frequencies of Crf1- and Catalase1-specific T-cells are present in healthy individuals

To investigate the presence of *Aspergillus*-specific T-cells in healthy individuals PBMC of 10 healthy donors were stimulated with the overlapping peptides of the *A. fumigatus* proteins Crf1 and Catalase1 and analyzed by Elispot for production of IFN γ , IL-4 and IL-17. Low frequencies of IFN γ or IL-17 producing T-cells were observed in 3 of 10 analyzed individuals (figure 2.1).

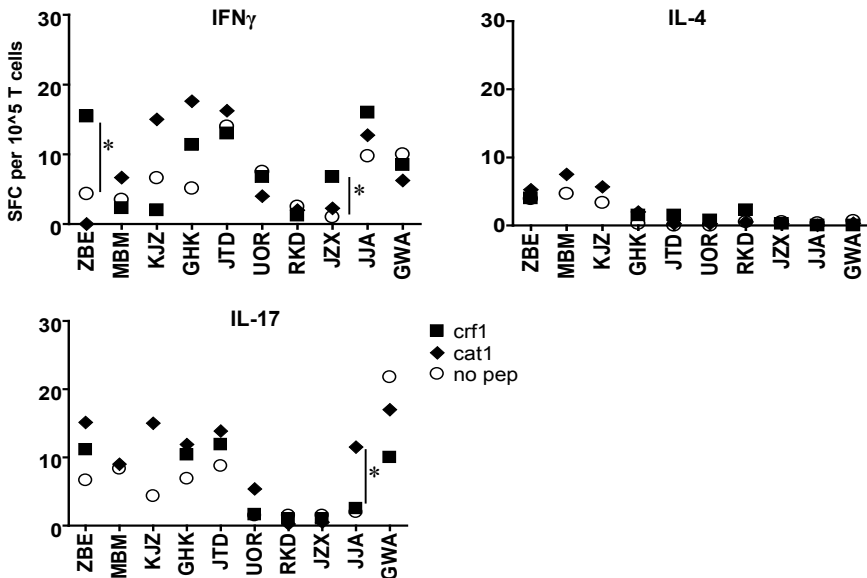


Figure 2.1 Production of IFN γ , IL-4 and IL-17 after stimulation with Crf1 and Catalase1 overlapping peptides in Elispot assay. PBMC of 10 healthy donors were cultured for 48 hours in the presence or absence of overlapping peptides of Crf1 or Catalase1. After washing, cells were cultured ON in three replicate wells in a plate coated with IFN γ , IL-4 or IL-17 catching antibody. The mean antigen-specific SFC of 2 independent experiments are plotted. * Significant difference in spot forming cells (SFC) between Crf1- or Catalase1-stimulated PBMC compared to non-stimulated PBMC ($p < 0.05$, two-tailed unpaired Student T test).

When we analyzed the frequencies of *Aspergillus*-specific T-cells in PBMC of 24 healthy donors by flowcytometric analysis we could not detect *Aspergillus*-specific T-cells directly ex vivo on the basis of IFN γ production or expression of the activation markers CD154 or CD137 (data not shown). To increase the sensitivity, we in vitro expanded the *Aspergillus*-specific T-cells using a restimulation protocol. Frequencies of *Aspergillus*-specific T-cells after restimulation were calculated by subtracting the percentage of IFN γ + CD154+ of CD4+ T-cells after restimulation with unloaded PBMC from the percentage of IFN γ + CD154+ of CD4+ T-cells

Table 2.1 Frequencies of Crf1- and Catalase1-specific T cells in healthy individuals

Donor	%IFN γ +CD154+ of CD4+ T cells ^a		Donor	%IFN γ +CD154+ of CD4+ T cells ^a	
	Crf1	Cat1		Crf1	Cat1
JFB	0.05	0.05	AST	<0.01	0.04
GWA	<0.01	0.02	CSK	0.03	0.04
RSA	0.03	0.06	JTD	0.25	0.12
ZBE	0.35	0.07	UKT	<0.01	<0.01
RKD	0.06	0.05	UZM	0.14	0.11
JJA	0.10	0.17	UOR	0.01	0.09
KSE	0.03	0.05	CMZ	0.11	0.06
WVR	<0.01	0.01	GMZ	0.08	0.03
TKK	0.07	<0.01	JSG	<0.01	0.02
MBM	0.02	0.4	UWH	0.13	0.07
KJZ	<0.01	0.32	GHK	0.02	0.06
JZX	<0.01	0.15	UTL	0.03	0.05

^a Calculated as % IFN γ + CD154+ of CD4+ T cells after restimulation with peptide-pulsed PBMC minus % IFN γ + CD154+ of CD4+ T cells after restimulation with unpulsed PBMC. A frequency of at least 0.05% IFN γ + CD154+ of CD4+ T cells was considered relevant (bold).

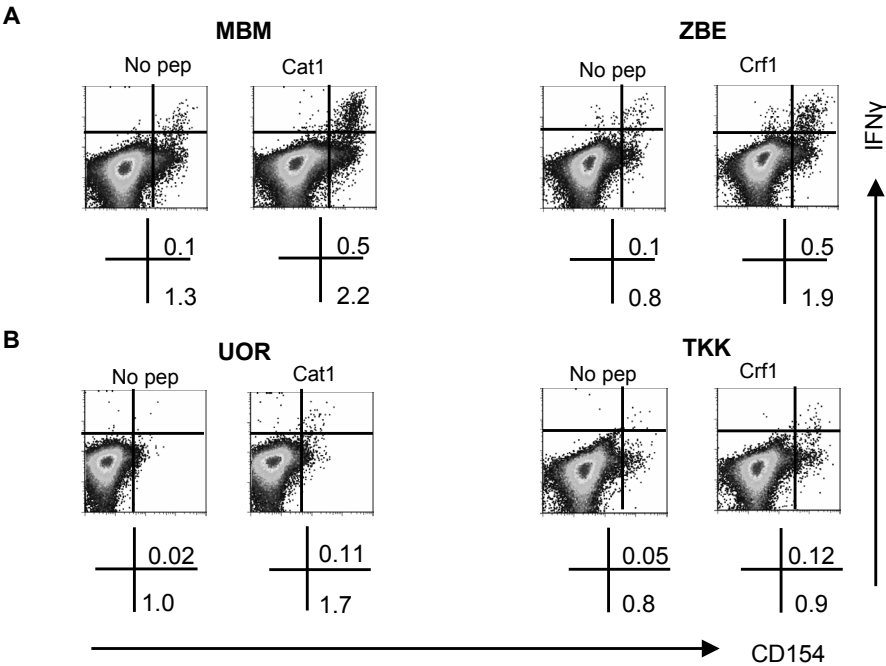


Figure 2.2 Flowcytometric analysis of CD4+ T-cells specific for Crf1 and Catalase1 overlapping peptides. Two representative examples of donors with a high frequency of Aspergillus-specific T-cells (A) and two representative examples of donors with a low frequency of Aspergillus-specific T-cells (B). PBMC of different healthy individuals after 7 days of in vitro stimulation with overlapping peptides of Catalase1 or Crf1 were restimulated with non-pulsed (no pep) or Catalase1 peptide mixture-pulsed (Cat1) or Crf1 peptide mixture-pulsed (Crf1) autologous PBMC. T-cells were analyzed by FACS after 5 hours of stimulation. FACS plots are gated on CD4+ T-cells. Representative results of 3 independent experiments are shown.

after restimulation with PBMC loaded with overlapping peptide-pools, and are presented in table 2.1. A frequency of 0.05 % IFN γ + CD154+ of CD4+ T-cells was considered relevant. In figure 2.2A and 2.2B two representative examples of flowcytometric analyses of donors with high frequencies and low frequencies of *Aspergillus*-specific T-cells are shown. Overall, IFN γ + CD154+ T-cells specific for Crf1 or Catalase1, with a frequency ranging from 0.05% to 0.4% of CD4+ T-cells, were demonstrated in 18 of the 24 healthy donors (table 2.1).

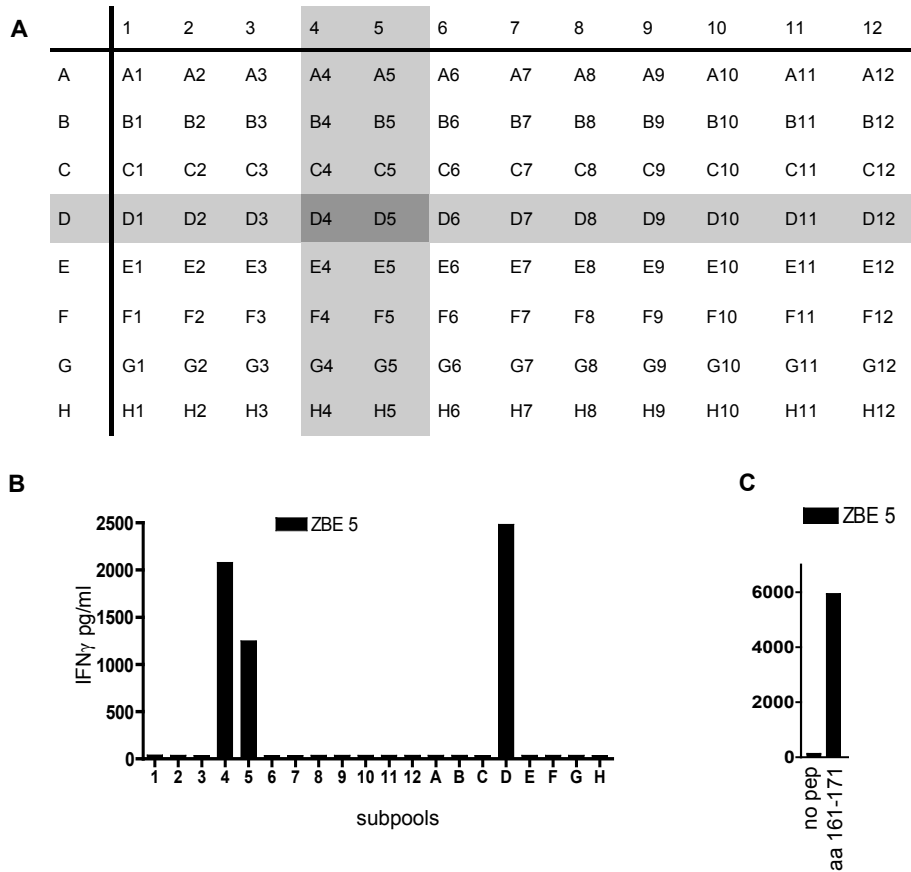


Figure 2.3 Identification of the recognized epitopes by subpool analysis. (A) Composition of the peptide subpools. Every peptide is only present in 2 subpools, to be able to identify the target peptide directly from the subpool analysis. (B) IFN γ production of T-cell clone 5 from individual ZBE after ON stimulation with the subpools of Crf1 loaded onto autologous PBMC. The target peptides for clone ZBE5 are peptide D4 (aa 157-171, QETFHTYTIDWTKDA) and D5 (aa 161-175, HTYTIDWTKDAVTWS). The T-cell epitope recognized by this clone is therefore most likely the 11mer peptide consisting of the overlap between the two recognized peptides. (C) For clone ZBE5 the peptide aa 161-171 was newly synthesized and tested in a stimulation assay.

Table 2.2 Identified *T* cell epitopes of the *A. fumigatus* proteins *Crf1* and *Catalase1***A**

Crf1 epitope	aa sequence	Clone	Vβ^{a,b}	HLA-restriction	<i>Aspergillus</i> extract	<i>Catalase1</i> rec protein^c
aa 101-111	APGTGVVSSIV	GHK 101	2	DQB1*0301	yes	n/a
aa 161-171	HTYTIDWTKDA	ZBE 5, JTD 104, 105	12,12,u.k.	DRB3*0101	yes	n/a
aa 165-179	IDWTKDAVTSIDGA	ZBE 12	9	DPB1*0101	yes	n/a
aa 233-243	YTMVVKSVRIE	GHK 116	13.2	DRB1*0701	yes	n/a
aa 233-247	YTMVVKSVRIENANP	GHK 107	u.k.	DRB1*1101	yes	n/a
aa 237-247	VKSVRIENANP	CMZ 106	20	DQB1*0603	yes	n/a
aa 381-395	ACALVFGAAVAVLAF	ZBE 2	2	DRB1*0401	no	n/a

B

Cat1 epitope	aa sequence	Clone	Vβ^{a,b}	HLA-restriction	<i>Aspergillus</i> extract	<i>Catalase1</i> rec protein
aa 13-27	ANAVCPYMTGELNRR	UTL 19	22	DRB1*1501	no	no
aa 77-91	TLLEDIFRQKIQR	UTL 29	17	DPB1*0501	no	yes
aa 81-91	DFIFRQKIQR	CMZ 1	12	DRB1*0401	no	no
aa 89-103	QRFDHERVPERAVHA	UOR 25	17	DRB5*0101	no	yes
aa 109-123	HGVFTSYGDFSINITA	KJZ 1	9	DRB1*1501	no	yes
aa 117-131	DFSNITAASFLAKEG	GHK 1	13.6	DRB1*0701	no	no
aa 129-143	KEGKQTPVFVRFSTV	UTL 20	17	DQB1*0602	no	yes
aa 145-159	GSRGSSDLARDVHGF	UOR 13	u.k.	DRB1*1501	no	no
aa 177-187	IPVFFIQDAIL	UTL 13	17	DRB5*01	no	yes
aa 177-191	IPVFFIQDAILFPDL	CMZ 5	u.k.	DRB3*0101	no	no
aa 249-259	HTFRFVTDDGA	CMZ 7	5.1	DPB1*0201	no	yes
aa 257-271	DGASKLVKFHWKSLQ	GHK 4	13.2	DPB1*0501	no	yes
aa 261-271	KLVKFHWKSLQ	MBM 5	5.1	DRB1*1102/ DRB1*1301	yes	yes
aa 333-347	IVPEEFVPITKLGM	GHK 6, 20	u.k., 4	DRB1*1101	no	yes
aa 345-359	GKMQLNRNPNRYFAE	GHK 3, 8, 14, 17	7, 15, 18, 24 ^d	DRB3*0202	variable	variable
aa 453-467	DGFFTAPGRITSGKL	UOR 8	2	DRB1*1501	no	yes
aa 465-475	GKLVRAVSSSF	UOR 3	17	DRB5*01	no	yes
aa 465-479	GKLVRAVSSSFEDVW	JTD 7	u.k.	DPB1*0401	no	yes
aa 497-511	VIDAIRFENANVKSP	UOR 1, 2, 4	17, u.k., 9	DRB1*1501	no	variable
aa 509-523	KSPVVKNNVVIQLNR	MBM 1	1	DRB1*1102/ DRB1*1301	no	yes
aa 513-527	VKNNVIIQLNRIDND	GHK 2, 24	12, 5.1	DRB1*1101	no	yes
aa 533-543	ARAIGVAEPEP	UOR 7	u.k.	DPB1*0401	no	no
aa 541-555	PEPDPTFYHNNKTAD	UTL 1, 2	18,6 ^d	DRB1*1501	no	variable
aa 589-603	LRDLRKDDGVDVVLV	UTL 28	u.k.	DPB1*0401	no	no
aa 620-630	IQFDAVVAAG	UTL 3	5.3	DRB1*1501	no	yes
aa 629-643	AAGAESLFAASSFTG	UTL 7	5.2	DRB1*1501	no	no
aa 669-679	LIDGFRFGKTVGALG	GHK 25	18	DRB1*0701	no	yes
aa 705-719	DDFANDLKEGLRTEK	UOR 14	17	DRB1*1501	no	no
aa 705-719	DDFANDLKEGLRTEK	JTD 3, 8, 9	2, 5.1, 21.3	DRB1*0301	no	variable

^a Vβ analysis by TCR Vβ monoclonal antibodies^b u.k.: Vβ unknown when not present in TCR Vβ kit^c n/a: not applicable^d Vβ analysis by RT-PCR and sequencing*Cat1/Catalase1*

Crf1- and Catalase1-specific T-cells recognize a broad variety of *Aspergillus* epitopes and are predominantly of the Th1 phenotype

To identify the recognized T-cell epitopes and determine the cytokine profiles of the Crf1- and Catalase1-specific T-cells, *Aspergillus*-specific CD4⁺ T-cells of 9 donors were single cell sorted on the basis of CD137 expression using the restimulation protocol. Twenty-five Crf1- and 52 Catalase1-specific T-cell clones were isolated from 8 of the 9 donors. Twenty-one Crf1-specific T-cell clones produced IFN γ , of which 16 (76%) also produced low levels of IL-4, IL-10 or IL-17. The other 4 Crf1-specific clones only produced IL-4 and no other cytokines. Forty-nine of the 52 Catalase1-specific T-cell clones produced IFN γ , of which 32 (65%) also produced low levels of IL-4, IL-10 or IL-17. The other 3 Catalase1-specific clones only produced IL-4 and no other cytokines. There were no *Aspergillus*-specific T-cell clones that produced IL-10 or IL-17 without IFN γ production (data not shown). A selection of clones was tested for the production of other cytokines in the Bio-plex Pro 17-plex luminex assay (Bio-Rad). Production of IL-5 and IL-13 was detected, which was restricted to clones that produced IL-4 (data not shown).

The recognized epitopes were identified by measuring IFN γ or IL-4 production after stimulating the T-cell clones with the subpools of overlapping Crf1- or Catalase1 peptides, which have been composed in a matrix (figure 2.3A). Every peptide was only present in 2 subpools, which enabled us to identify the target peptide directly from the subpool analysis. An example is shown in figure 2.3B. T-cell clone ZBE5 recognized the Crf1 subpools 4, 5 and D, and therefore recognized the peptides D4 (aa 157-171) and D5 (aa 161-175), as can be derived from the matrix in figure 2.3A. The T-cell epitope is the 11 amino acid overlap between these two peptides, aa 161-171 (HTYTIDWTKDA), which was confirmed by stimulating the clone with newly synthesized peptide (figure 2.3C). All identified T-cell epitopes were confirmed in this way, and amino acid sequences of the epitopes and their position in the Crf1 or Catalase1 protein are listed in table 2.2. TCR V β usage was analyzed by flowcytometry using the TCR V β kit and in some cases by RT-PCR and sequencing, and results are also presented in table 2.2.

HLA-restrictions of the T-cell clones were characterized using blocking mAbs, an HLA-typed EBV-LCL panel and HLA class II-transduced Hela cell-lines and the results of two representative examples are presented in figure 2.4. IFN γ production by clone ZBE5 was reduced with HLA class II- and HLA-DR antibodies and by clone UTL20 after blocking with HLA-DQ antibodies (figure 2.4A). For further determination of the HLA-restriction, the clones were stimulated with a peptide-loaded HLA-typed EBV-LCL panel. Clone ZBE5 recognized the Crf1 peptide aa 161-171 (HTY) when loaded on a DRB3*01 EBV-LCL and clone UTL20 recognized the Catalase1 peptide aa 129-143 (KEG) when loaded on a DQB1*0602 EBV-LCL (figure 2.4B). HLA-restriction was confirmed by stimulating the clones with peptide-loaded Hela cells transduced with different HLA-DR, -DQ or -DP molecules (figure 2.4C). The HLA-restrictions of all identified epitopes are summarized in table 2.2.

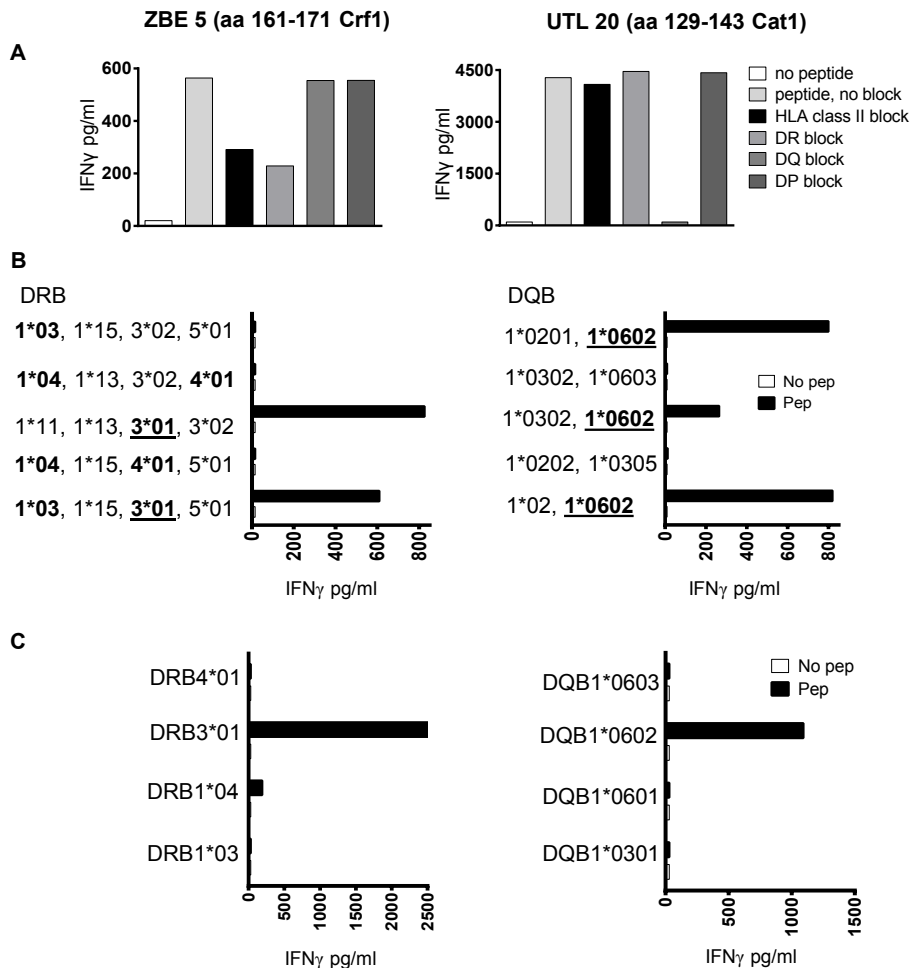


Figure 2.4 Determination of the HLA-restriction of Crf1 and Catalase1 clones. (A) Clone ZBE5 and UTL20 were stimulated with Crf1 or Catalase1 peptide-loaded autologous EBV-LCL incubated with saturating concentrations of different HLA class II blocking mAbs. IFN γ production was measured after ON incubation. (B) Clone ZBE5 and UTL20 were stimulated with Crf1- or Catalase1-peptide mixture loaded EBV-LCL expressing one or more HLA-DR or HLA-DQ molecules corresponding to the HLA type of the individual the T-cell clone was derived from, as indicated in bold. The HLA-restriction molecule used for presentation of these epitopes is underlined. (C) HLA-restriction of Crf1 and Catalase1-specific clones was confirmed by testing with peptide-loaded HLA class II transduced Hela cells. T-cell clone ZBE5 was isolated from an individual with HLA-DR type DRB1*0301, DRB1*04, DRB3*01 and DRB4*01. T-cell clone UTL20 was isolated from an individual homozygous for DQB1*0602.

We considered T-cells of the same clonal origin, when T-cell clones were generated from one individual, had similar TCR V β usage based on TCR V β monoclonal antibody analysis, and recognized one epitope presented by the same HLA-molecule. Taken all these data together, from the 25 Crf1-specific T-cell clones 9 were unique clones, originating from 4 different donors, recognizing 7 different Crf1 epitopes and from the 52 Catalase1-specific T-cell clones 36 were unique clones, originating from 7 different donors, recognizing 30 different Catalase1 epitopes. The majority of the epitopes are presented by HLA-DR, although some epitopes are HLA-DQ or HLA-DP restricted (table 2.2).

Reactivity of Crf1- and Catalase1-specific T-cells to *Aspergillus* protein extract and recombinant protein is dependent on the avidity of the T-cell clone

To prove *Aspergillus*-specificity, clones were stimulated with DC loaded with *Aspergillus* protein extract or EBV-LCL loaded with *Aspergillus* recombinant protein and cytokine production was analyzed. Of the 9 Crf1-specific T-cell clones with 7 different peptide specificities, 8 clones recognizing 6 different epitopes were reactive to autologous DC loaded with protein extract (figure 2.5A and table 2.2). Of the 36 Catalase1-specific T-cell clones with 30 different peptide specificities, only two clones recognizing two different epitopes were reactive to autologous DC loaded with *Aspergillus* protein extract. However, when we tested the Catalase1-specific T-cell clones against autologous EBV-LCL loaded with Catalase1 recombinant protein, IFN γ production was seen in 22 of the 36 Catalase1-specific T-cell clones covering 20 different epitopes. The reactivity to *Aspergillus* protein extract and Catalase1 recombinant protein of a representative selection of Catalase1-specific clones is presented in figure 2.5B and 2.5C, respectively. The results of all clones are presented in table 2.2.

Only the Catalase1-specific T-cell clones with the highest avidity recognize the *Aspergillus* crude extract, as is demonstrated by the differences in recognition between clones 3, 8, 14 and 17, derived from donor GHK. These clones all recognize Catalase1 peptide aa 345-359 (GKM) presented by HLA-DRB3*0202. TCR V β analysis revealed that GHK clones 3, 8, 14 and 17 all used a different V β TCR (table 2.2). Titration of peptide and recombinant protein, as shown in figure 2.5D and 2.5E, demonstrated that Catalase1 clone 8 from donor GHK that is reactive to the *Aspergillus* crude extract has the highest avidity in contrast to the non-reactive clones 3 and 17. Clone 14 from donor GHK showed very low or no recognition of the protein extract (figure 2.5B), but showed IFN γ production after stimulation with EBV-LCL loaded with Catalase1 recombinant protein (figure 2.5C). This clone is also a high avidity T-cell clone, but needs more peptide for stimulation compared to GHK clone 8, as is demonstrated by loading EBV-LCL with titrated concentrations of peptide (figure 2.5D) and Catalase1 recombinant protein (figure 2.5E).

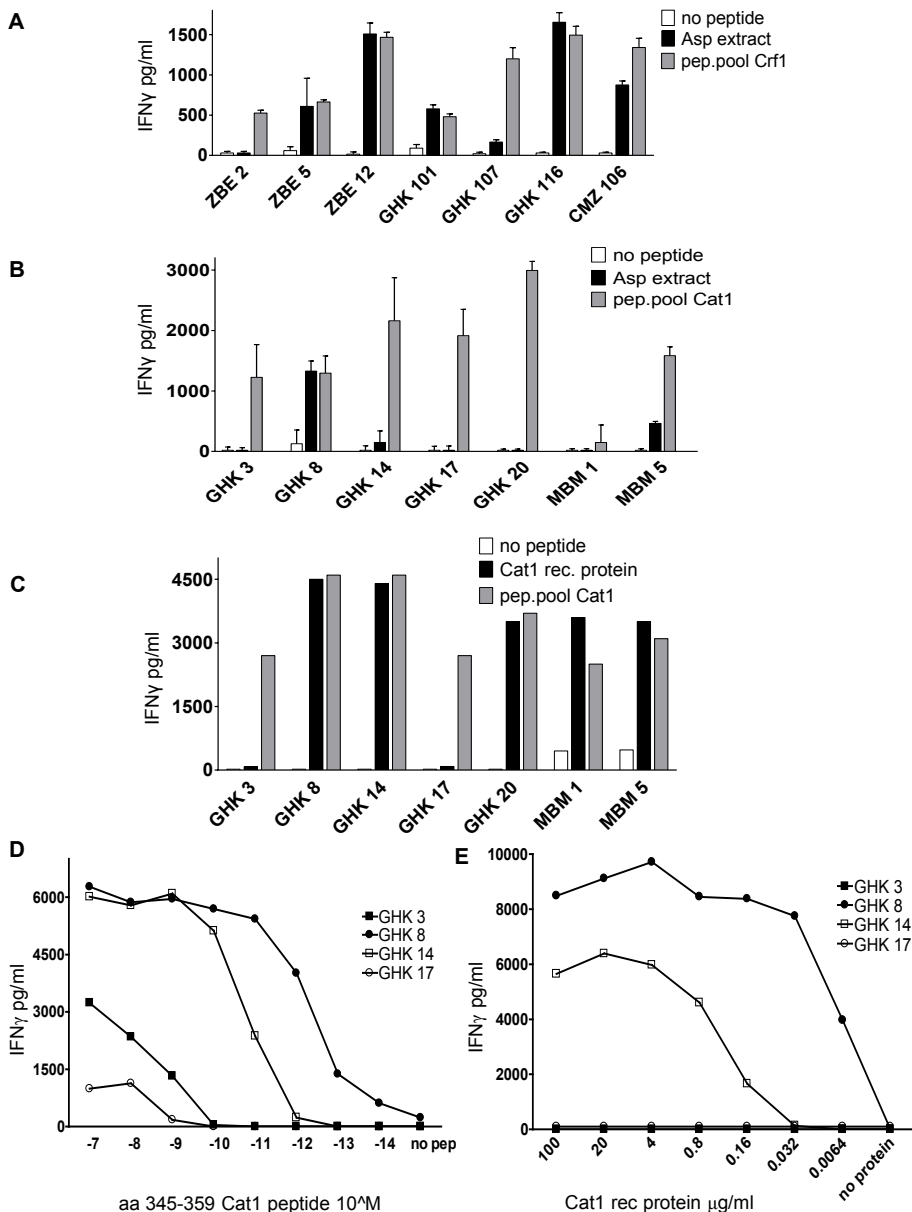


Figure 2.5 Determination of *Aspergillus* specificity of T-cell clones recognizing Crf1 and Catalase1 epitopes. Crf1- (A) and Catalase1-specific (B) T-cell clones were stimulated with autologous monocyte-derived DC that were preloaded with *Aspergillus* protein extract for 4 hours and then matured for 3 days. IFN γ production was measured after ON stimulation of T-cells with DC. (C) Catalase1-specific T-cell clones were stimulated ON with autologous EBV-LCL loaded with Catalase1 recombinant protein. The Catalase1-specific clones from donor GHK reactive to the *Aspergillus* crude extract are the highest avidity T-cell clones, as shown by loading EBV-LCL with titrated concentrations of the Catalase1 peptide (D) and Catalase1 recombinant protein (E). Results of 3 independent experiments are shown.

Discussion

In this study we demonstrate the presence of low frequencies of *Aspergillus*-specific T-cells directed against the *A. fumigatus* proteins Crf1 and Catalase1 in healthy individuals. These T-cells recognize a diverse repertoire of T-cell epitopes presented in different HLA-class II molecules. The *Aspergillus*-specific T-cell clones isolated on the basis of CD137 expression are predominantly of the Th1 phenotype, producing high amounts of IFN γ and no or low amounts of the cytokines IL-4, IL-10 and IL-17. *Aspergillus*-reactivity was confirmed for the majority of the Crf1-specific T-cell clones by stimulating the T-cells with DC loaded with *Aspergillus* protein extract and for the majority of Catalase1-specific T-cells by stimulating with EBV-LCL loaded with recombinant protein.

In previous studies the presence of *Aspergillus*-specific T-cells in healthy individuals was shown by stimulating PBMC with *Aspergillus* protein extracts^{6,16,17}, conidia or hyphae⁶ or with *A. fumigatus* recombinant proteins^{6,8,18}. Depending on the *Aspergillus* antigens used for stimulation, a lymphoproliferative response was identified in up to 87% of healthy individuals. The frequencies of *Aspergillus*-specific T-cells varied between 0.24% and 0.91% of CD4+ T-cells measured directly ex vivo by intracellular staining for IFN γ ¹⁶. These rather high percentages of *Aspergillus*-specific T-cells are in contrast with the results found in this study, in which no *Aspergillus*-specific T-cells could be identified directly ex vivo after stimulating with synthetic peptides of the *A. fumigatus* proteins Crf1 and Catalase1, when tested by intracellular staining for CD154 and IFN γ . Furthermore, we found no or very low frequencies of *Aspergillus*-specific T-cells by Elispot, in accordance with the results of Topp and colleagues who did not detect an IFN γ response directly ex vivo by Elispot after stimulation with overlapping peptides of Crf1^{12,19}. However, after stimulation and restimulation with overlapping peptides of Crf1 and Catalase1, we identified low frequencies of *Aspergillus*-specific T-cells, similar to the results in previous studies using Crf1 synthetic peptides^{12,19}. Frequencies of *Aspergillus*-specific T-cells were stable in an individual at different time points. Furthermore, there were no differences in frequencies, levels of cytokine production and cytokine profiles between individuals with different HLA types.

The marked difference in frequencies of *Aspergillus*-specific T-cells after stimulation with crude extracts compared to synthetic peptides can be explained by the number of T-cell target proteins present in the crude extract, whereas with stimulation with synthetic peptides, the T-cell response against only one *A. fumigatus* protein is evaluated. Furthermore, T-cells can become activated by other components in the crude extract or recombinant proteins than *Aspergillus*-derived antigens, and therefore higher frequencies of activated and cytokine-producing T-cells will be found^{6,8,16-18}. Considering the large variety of T-cell epitopes identified after stimulation with the overlapping peptides of Crf1 and Catalase1, these two *A. fumigatus* proteins are important target proteins for the T-cell immune response against *Aspergillus*. Furthermore, it indicates that the *Aspergillus*-specific T-cell

response is most likely directed against various epitopes of multiple *A. fumigatus* proteins without one dominant peptide-specificity.

Several studies have assessed the presence of *Aspergillus*-specific T-cells after stimulation with overlapping peptides of Crf1. Until now 15 *Aspergillus*-specific T-cell epitopes have been described, all from the Crf1 protein¹¹⁻¹³. In the present study 7 Crf1-specific T-cell epitopes were identified, among which the 2 previously described epitopes aa 161-171 (HTY) and aa 233-247 (YTM)^{12,13}, although we determined that the HLA-restriction of Crf1 epitope aa 161-171 (HTY) was HLA-DRB3*01, in contrast to what was described before¹².

Besides Crf1-specific T-cells in 10 individuals, we found Catalase1-specific T-cells in 16 of 24 healthy individuals, recognizing 30 different Catalase1 epitopes. *Aspergillus*-specificity was confirmed for 20 of the epitopes by stimulating with EBV-LCL loaded with Catalase1 recombinant protein. Only the two Catalase1-specific T-cell clones with the highest avidity also recognized DC loaded with *A. fumigatus* crude extract. These results may indicate that the amount of Catalase1 protein present in the crude extract is very low. However, in the in vivo situation the Catalase1 protein is expressed, processed and presented, leading to a Catalase1-specific T-cell immune response, as can be concluded from the isolation of a large variety of Catalase1-specific T-cells from several individuals.

By isolating *Aspergillus*-specific T-cells on the basis of CD137 predominantly IFN γ -producing T-cells were obtained. This is in line with previous studies showing that stimulation with *Aspergillus* conidia resulted in IFN γ and almost no IL-17 production²⁰, and stimulation with Crf1 recombinant protein resulted in IFN γ , no IL-4 and low IL-17 production¹⁸. In our study the T-cell phenotype did not differ between Crf1- and Catalase1-specific T-cells, in contrast to Bozza et al who found that Catalase1-specific T-cells produced IL-4 next to IFN γ , IL-10 and IL-17, whereas no IL-4 production was seen in Crf1-specific T-cells. A predominant Th1 inflammatory response against *A. fumigatus* might reflect the actual in vivo situation, or might be a consequence of the selection method by sorting on the basis of CD137 expression. For the generation of an *Aspergillus*-specific T-cell product suitable for infusion in aspergillosis patients it can be an advantage that predominantly IFN γ producing T-cells are selected, while several studies have found that Th1 cells result in a favourable outcome^{5,7-9}, in contrast to Th2⁴ and Th17 cells^{21,22}.

In summary, in this study in healthy individuals we demonstrate the presence of *Aspergillus*-specific T-cells directed against a large variety of epitopes of the Crf1 and Catalase1 protein. This broad T-cell repertoire against *A. fumigatus* will make it difficult to select the appropriate T-cells for adoptive T-cell therapy on the basis of one single peptide specificity. However, by stimulating with overlapping peptides of several *A. fumigatus* proteins and selecting on the basis of CD137 expression, a T-cell product with the desired broad repertoire, known specificity and favourable cytokine profile, suitable for adoptive T-cell therapy, can be generated.

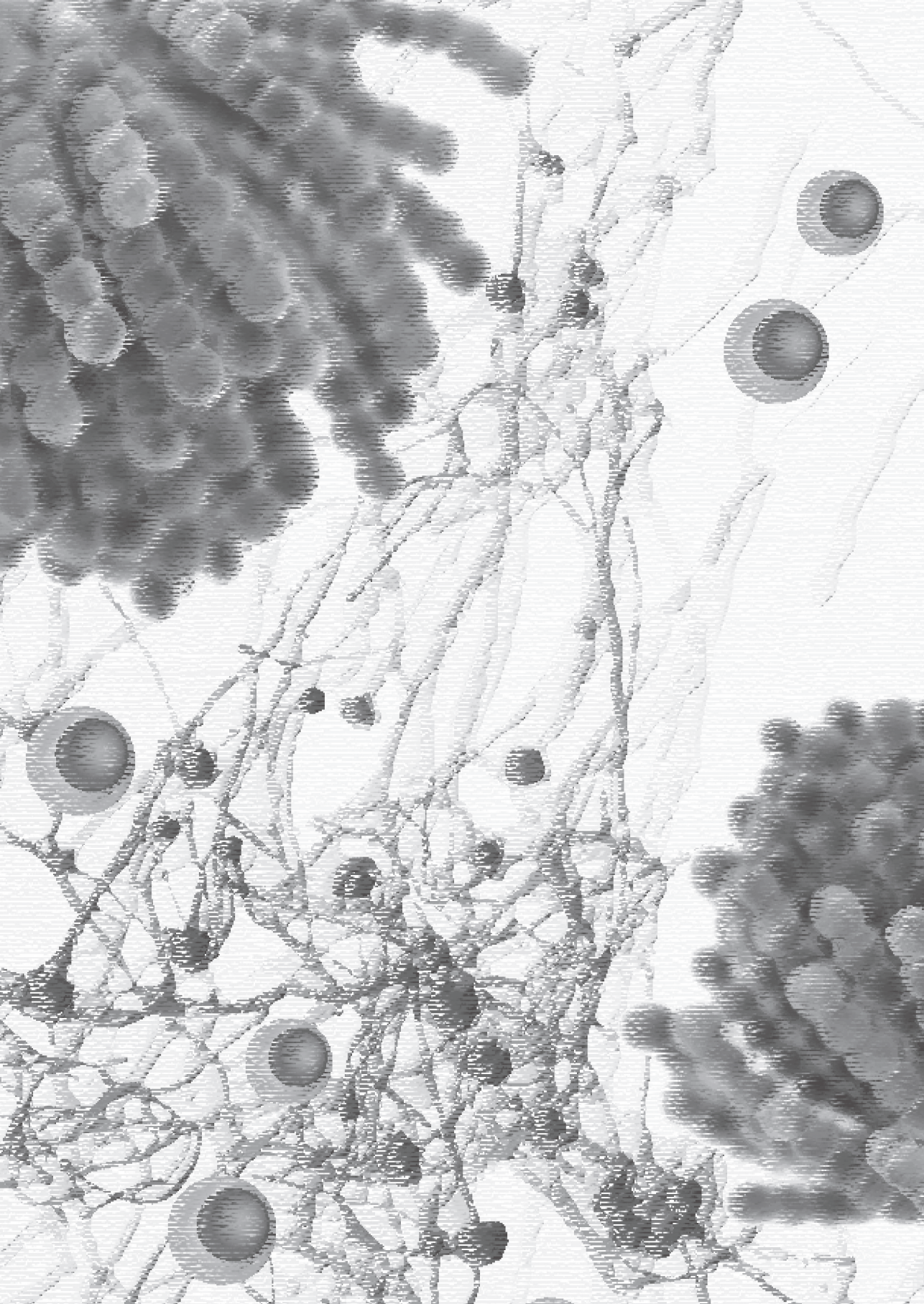
Acknowledgements

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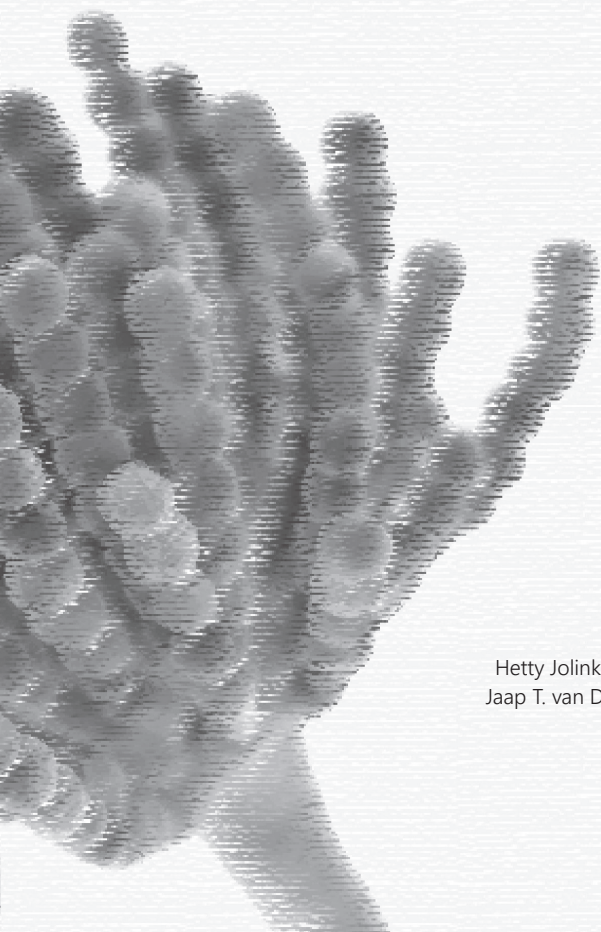
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Chapter 3

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



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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 IFN γ 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.¹ 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.²⁻⁵ 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.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 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.¹⁴ 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.⁵ 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, Ångelholm, 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 (0.5×10^6) were stimulated with the overlapping peptide pools (10^{-6} M) in 96-well plates, cultured for 7 days in 150 μ l 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 (0.5×10^6). 1 hour after restimulation 10 μ g/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-IFN γ (BD/Pharmingen) were added for intracellular staining of IFN γ production and the activation marker CD154. Cells were collected and analyzed on the Calibur II (BD, Breda, Netherlands).

Generation of T-cell clones

PBMC (0.5×10^6) were stimulated with the overlapping peptide pools (10^{-6} M) in a 96-well plate, cultured for 7 days in 150 μ l T-cell medium and restimulated with peptide-pulsed autologous PBMC (0.5×10^6). Anti-CD40 antibody (1 μ g/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).¹⁴

Epitope identification and determination of *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 *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 *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 molecules¹⁴ (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 group¹⁵. 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 *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 *A. fumigatus* culture and Galactomannan in serum or broncho-alveolar lavage fluid were used; *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 *A. fumigatus*. All patients had typical CT-findings. 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

Patient	Age	Gender	Hem dis	SCT	Syst Immunosup at time of IA	EORTC category IA	EORTC criteria IA		Antifungal medication ^{††}	Occurrence of Asp-specific T-cells		Clinical course IA
							A. fum culture	Galacto- mannan		Crp ^{††}	Cat ^{††}	
MLF	41	female	MM	NMA MSD	no	probable	BAL, sputum	BAL	noduli	yes	yes	clearance
FBV	40	male	CML	MA MUD	ciclo, pred	proven	biopsy, sputum	neg	noduli, cavitation	yes	no	clearance
NKB	43	male	MM	NMA MUD	no	probable	BAL	BAL, serum	noduli	yes	yes	clearance
CSS	45	female	NHL	MA MUD	ciclo, pred	probable	neg	BAL	noduli, cavitation	yes	yes	clearance
MPK	62	female	MM	NMA MUD	ciclo	probable	sputum	BAL, serum	noduli	yes	yes	clearance
TPA	62	female	NHL	NMA MUD	pred	probable	BAL	BAL	noduli	yes	yes	clearance
ACD	55	female	MM	NMA MUD	pred	probable	sputum	serum	noduli	yes	yes	regression
JKJ	73	male	CLL	NMA MUD	pred	probable	neg	BAL	noduli, halo	no	yes	regression
JHF	48	female	MM	NMA MSD	no	probable	neg	serum	noduli, halo	yes	no	stable
MST	37	male	NHL	NMA MSD	ciclo, pred	probable	sputum	serum	noduli	no	no	progressive
ESF	42	male	CML	MA MSD	ciclo, pred, MMF	probable	sputum	neg	noduli, halo	no	no	progressive

^{††} consecutively prescribed antifungal medication, starting from day of diagnosis

Hemdis 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 mycophenolate-mofetil, BAL bronchoalveolar lavage, Vori voriconazole, Vori* voriconazole dose adjusted according to serum levels, Itra 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).

Crfl- 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. IFN γ -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 IFN γ -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 IFN γ -production (figure 3.1E and G), coinciding with the regression of aspergillus lesions (figure 3.1H).

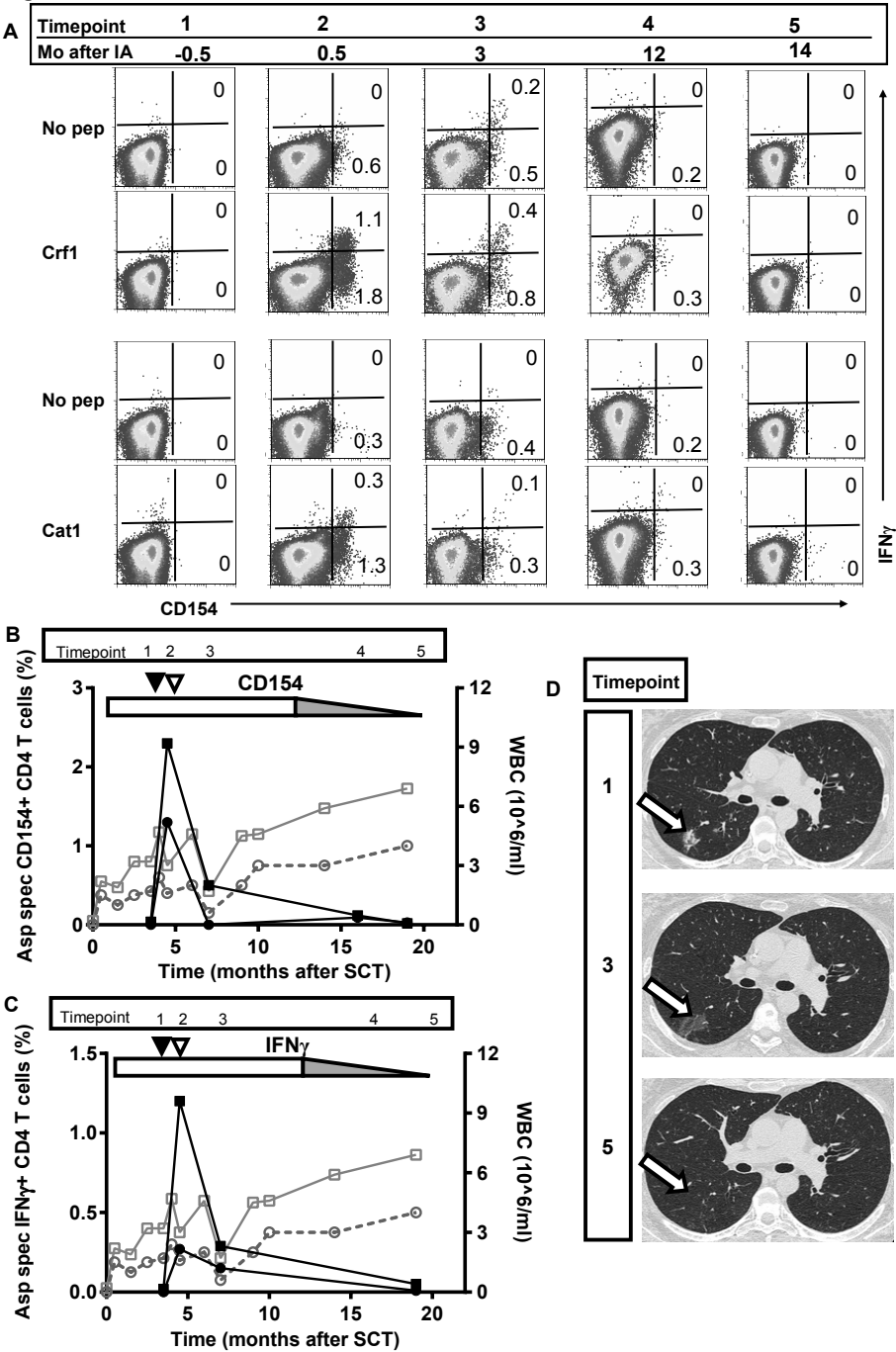


Figure 3.1 Aspergillus-specific T-cell responses and imaging studies in patients with improvement of invasive aspergillosis.

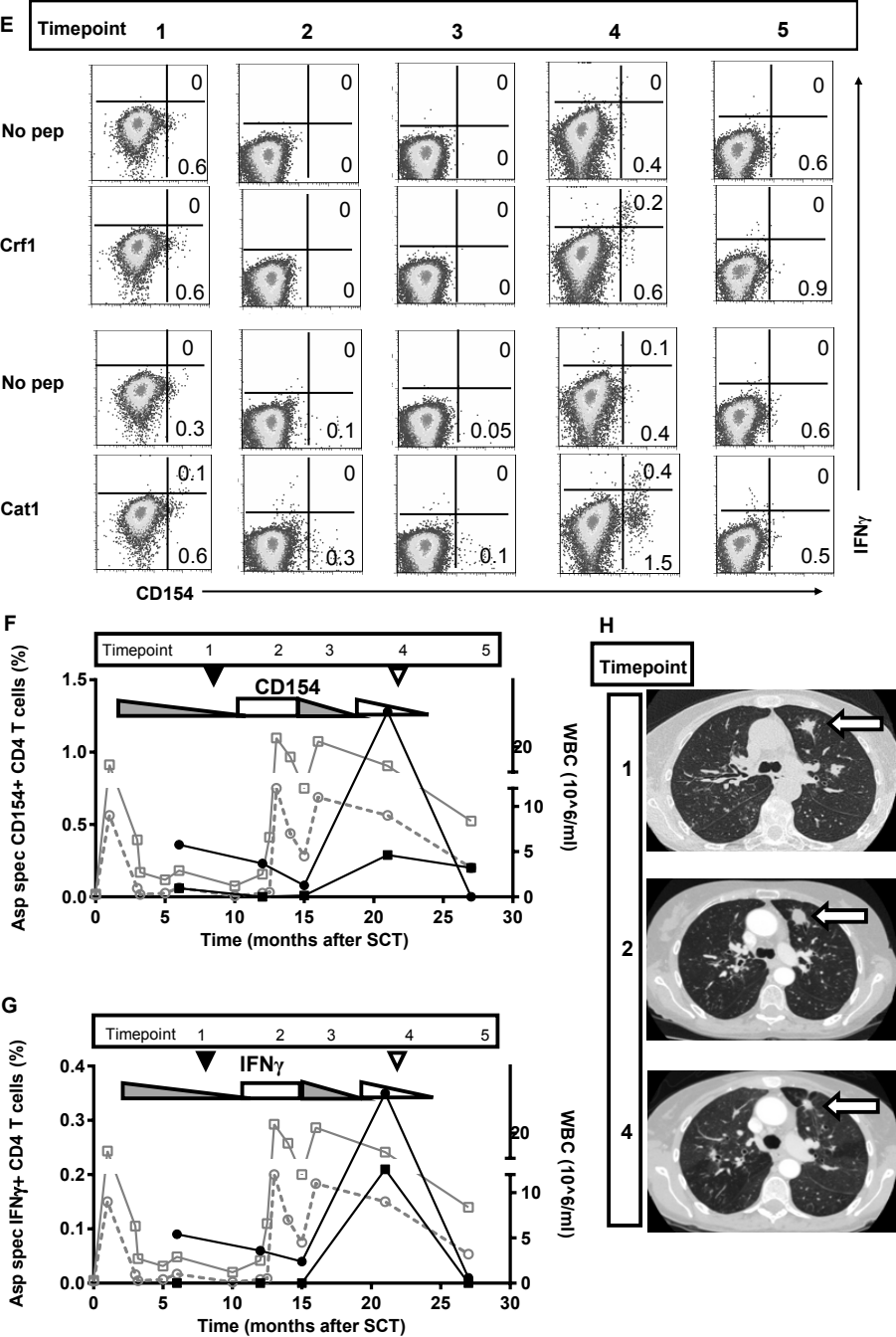


Figure 3.1 Continued

Figure 3.1 Continued

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 hours 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 IFN γ + 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. For IFN γ + CD4+ T-cells the same calculation method was used. Pulmonary CT-scans of patient MLF (D) and patient TPA (H) at different timepoints after diagnosis show the changes in 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 figure B and C and in figure F and G correspond to the timepoints of FACS-analyses and CT-scans in respectively figure A and D, and figure E and H. In figure A and E the corresponding timeperiod in months after invasive aspergillosis (Mo after IA) is given for every timepoint.

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 IFN γ -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 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 *A. fumigatus* and *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 IFN γ -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 *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

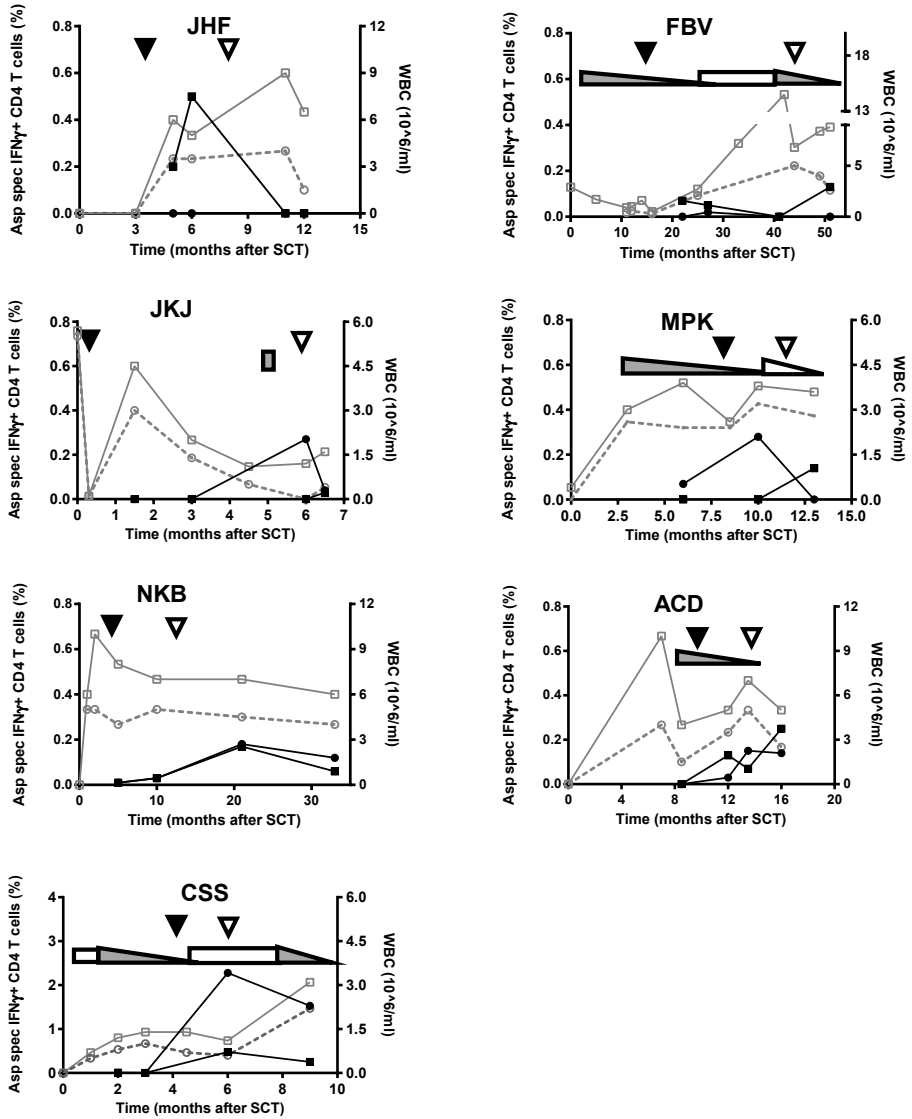


Figure 3.2 Kinetics of Aspergillus-specific T-cells in patients with improvement of aspergillus infection. Percentages of Crf1- (■) and Catalase1-specific (●) IFN γ + CD4+ T-cells (left y-axis) and absolute leukocyte (□) and granulocyte counts (○) (right y-axis) in 7 patients before and after diagnosis (▼) and improvement (▽) of 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 IFN γ + CD4+ T-cells were calculated as % IFN γ + of CD4+ T-cells after restimulation with peptide-pulsed PBMC minus % IFN γ + of CD4+ T-cells after restimulation with unpulsed PBMC.

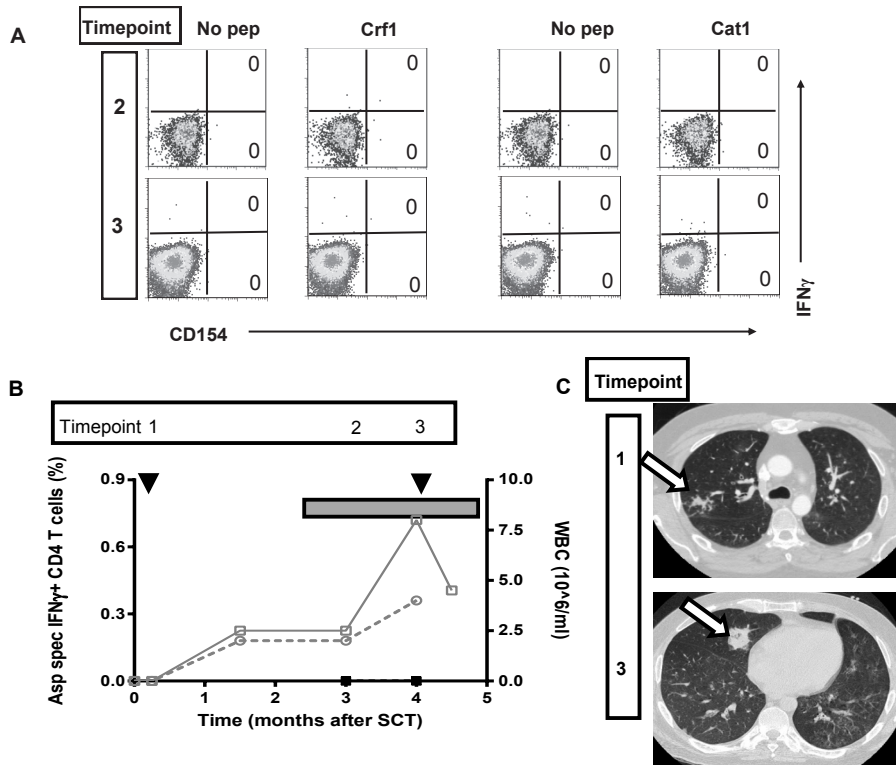


Figure 3.3 Aspergillus-specific T-cell responses and imaging studies in patients with progressive invasive aspergillosis. FACS-analyses, imaging studies and kinetics of the Aspergillus-specific T-cell responses of patient MST (A-C) and ESF (D-F).

(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 (●) IFN γ + 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 IFN γ + CD4+ T-cells were calculated as % IFN γ + of CD4+ T-cells after restimulation with peptide-pulsed PBMC minus % IFN γ + 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.

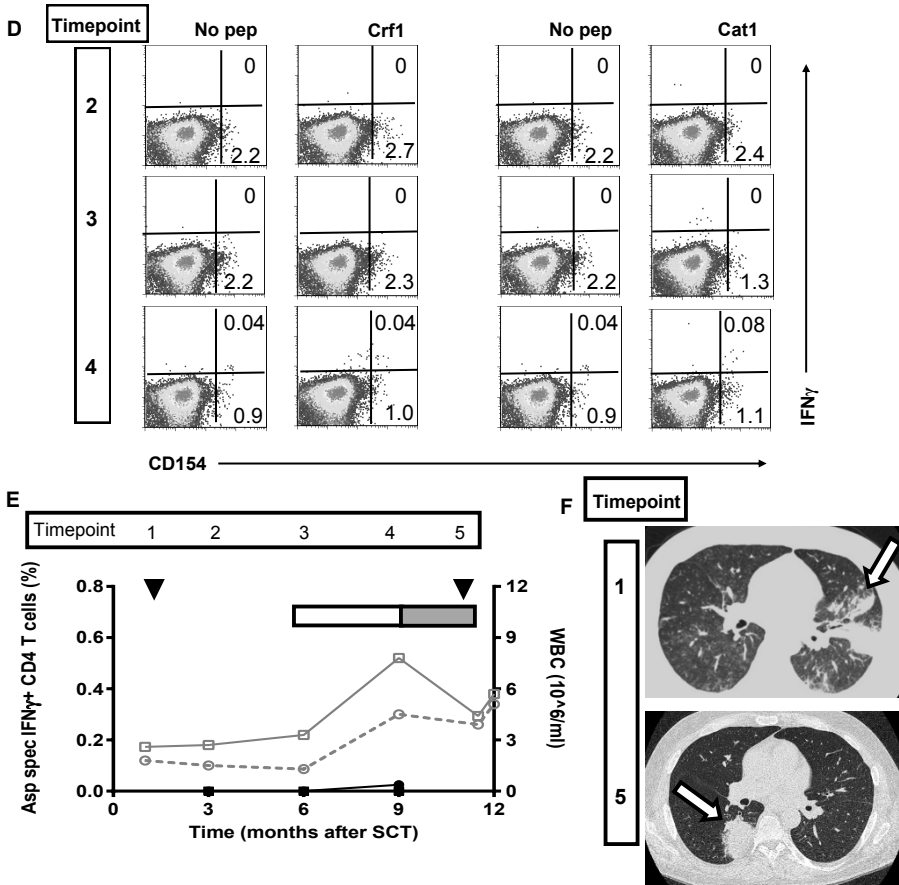


Figure 3.3 Continued

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 IFN γ -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 Asp1, Asp2, Asp3 and Asp4 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 IFN γ -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.

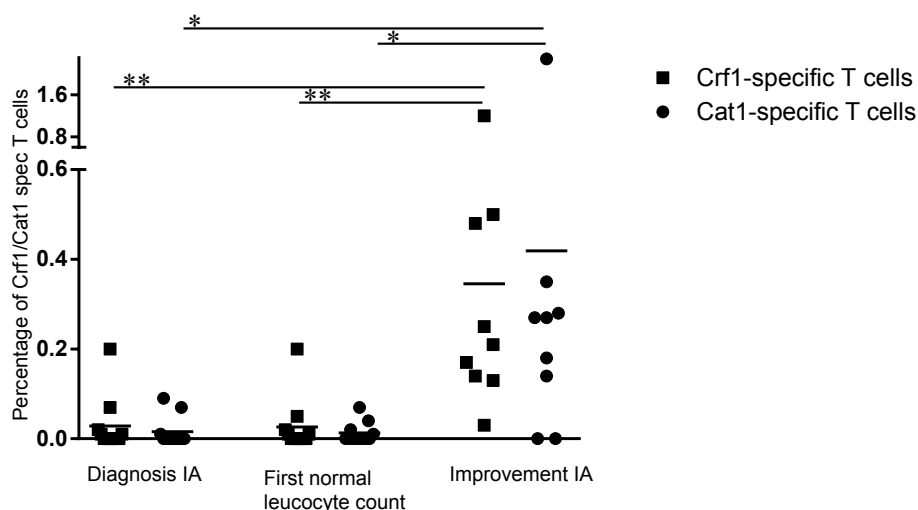


Figure 3.4 Increases of Crf1- and Catalase1-specific T-cells at the moment of improvement of aspergillus infection. Percentages of Crf1- (■) and Catalase1-specific (●) IFN γ + CD4+ T-cells around diagnosis of aspergillus infection (11 patients), at the first timepoint with normal leukocyte counts (11 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 V β 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 IFN γ , IL-4, IL-10 and IL-17 was measured using standard Elisa. Of the 19 T-cell clones, 16 produced high amounts of IFN γ and a minority of the IFN γ -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 IFN γ producing T-cell clones and 1 IL-17 producing T-cell clone and in another patient (FBV) we identified 2 IFN γ 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 IFN γ , 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 Asp1, Asp2, Asp3 and Asp4 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. IFN γ -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*,^{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 conidia⁷ or crude extracts,⁵ and we

have previously demonstrated that T-cells directed against *A. fumigatus* can be identified by the use of overlapping peptides of Crf1 and Catalase1.¹⁴ 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.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 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 IFN γ /IL-10 ratio,⁵ whereas high amounts 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 the present study, similar 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 Th1 phenotype, producing high amounts of IFN γ . 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,^{2-5;7;17} whereas Thelper2 and Thelper17 cells are associated with increased fungal growth.^{5;6;16;17} 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- IFN γ 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.¹⁸ 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.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, 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^{2-4;7} and a human study⁶ 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 IFN γ -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.

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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 (Miltenyi Biotec, Bergisch Gladbach, Germany) and CD14+ cells (1×10^6) 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

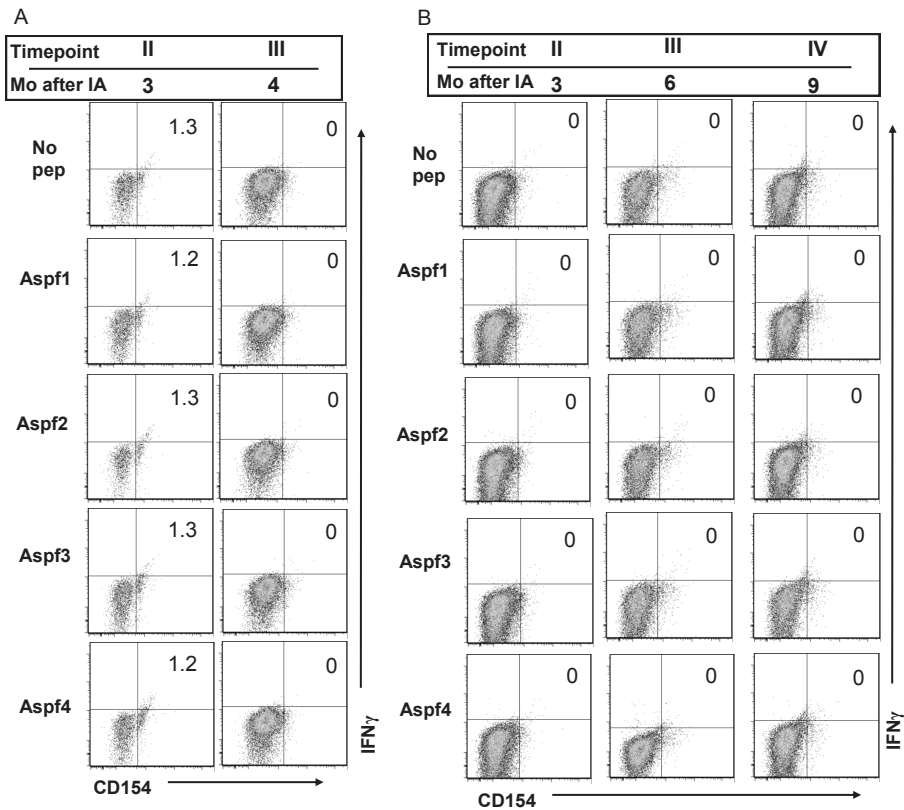
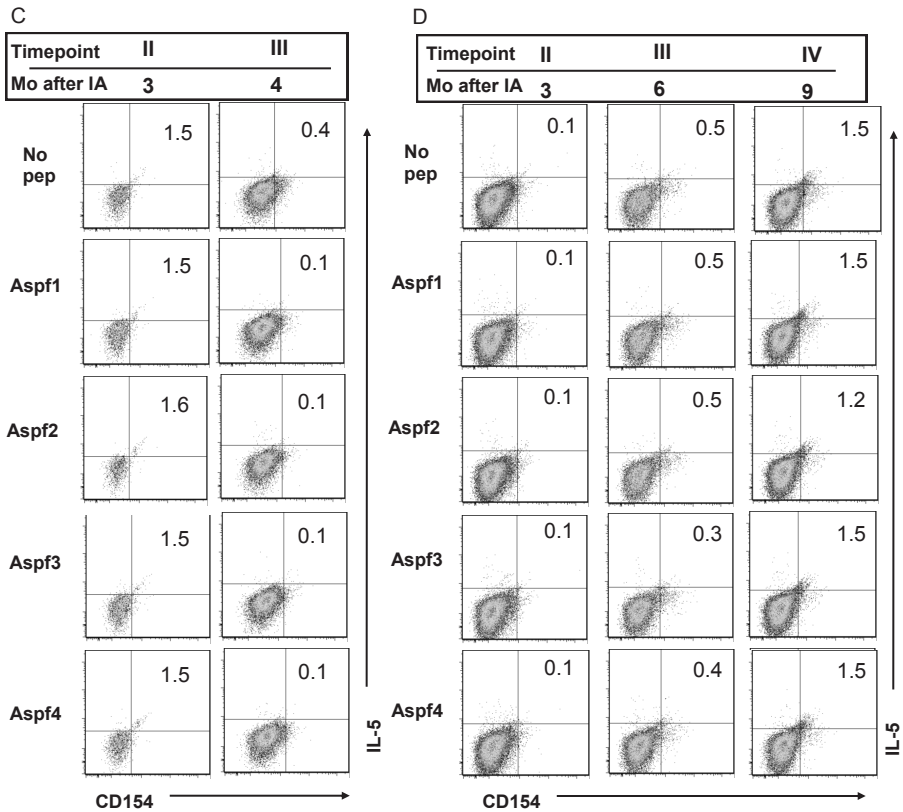


Figure S3.1 FACS-analyses of T-cell responses specific for Aspf1, Aspf2, Aspf3 and Aspf4 in patients with progressive invasive aspergillosis.

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 IFN γ -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



Supplemental figure S3.2

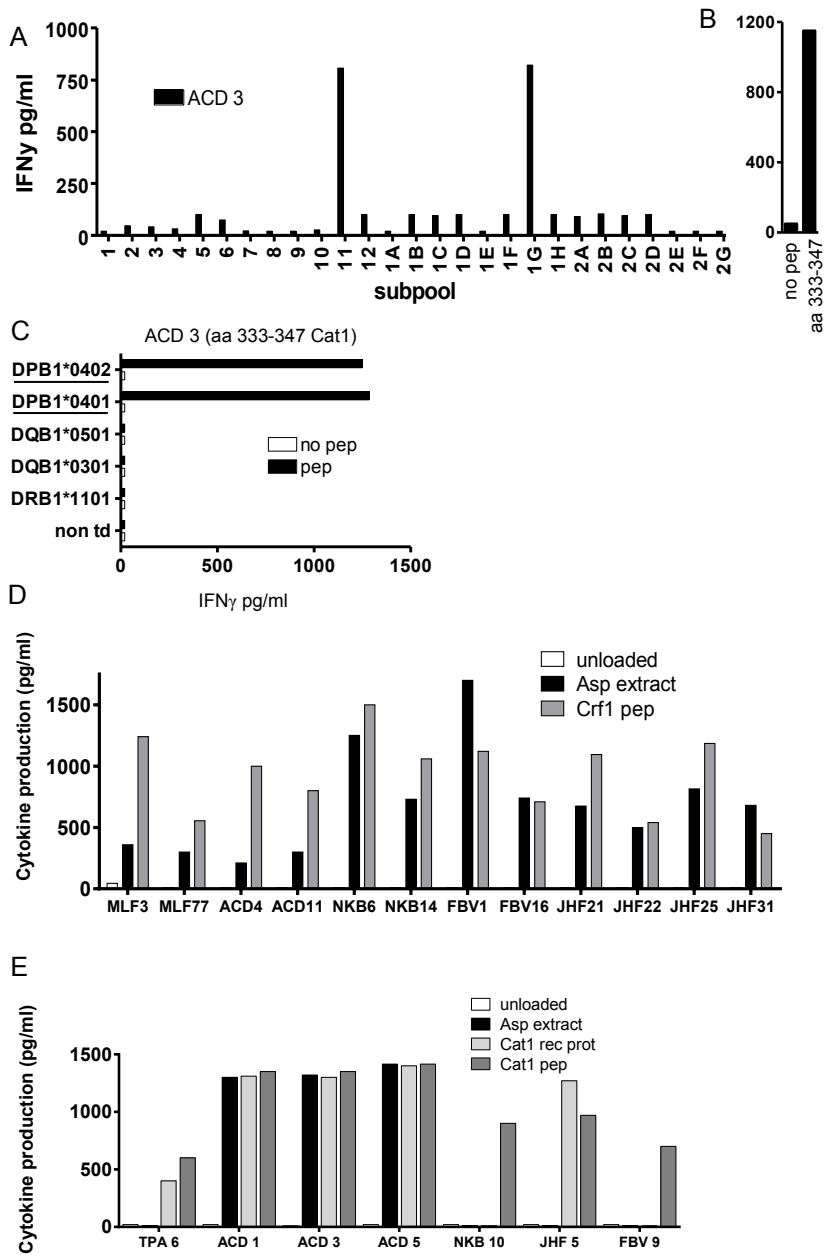


Figure S3.2 Determination of epitope-specificity, HLA-restriction and Aspergillus reactivity.

(A) IFN γ 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. IFN γ production was measured after ON stimulation.

Supplemental table S3.1

Identified Crf1 and Catalase1 epitopes in patients with invasive aspergillosis

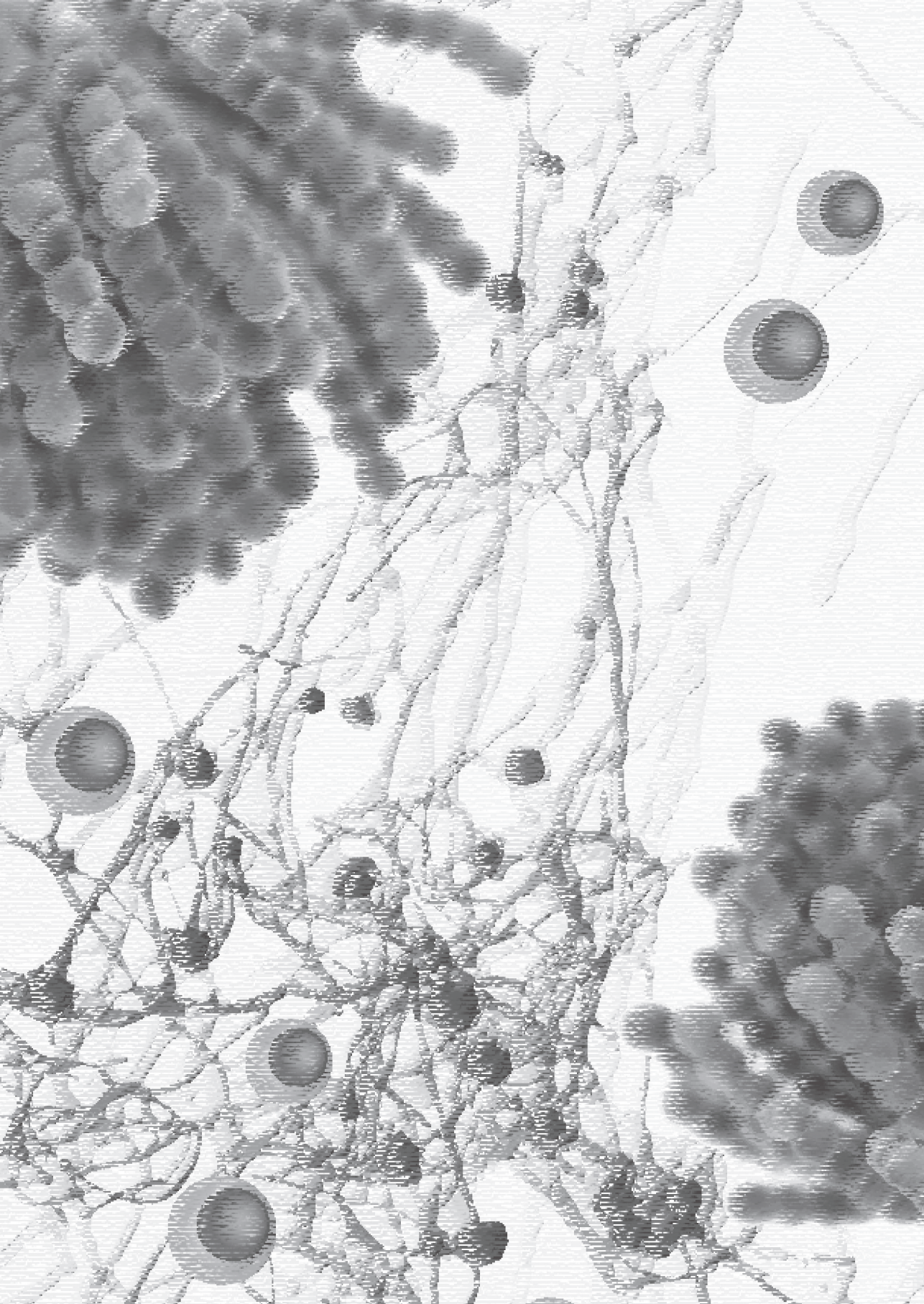
Epitope	aa sequence	Clone	Cytokine production	Vβ‡§	HLA-restriction	<i>Aspergillus</i> extract	Catalase1 rec protein*
Crf1							
aa 57-67	EVTAGKVPVGP	MLF 77	IFNγ	7.2	DRB1*11	yes	n/a
aa 57-67	EVTAGKVPVGP	ACD 11, JHF 31	IL-17, IFNγ	17, 13.2	DQB1*0301	yes	n/a
aa 89-99	FFFGKAEVVMK	ACD 4	IFNγ	u.k.	DRB1*0101	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	YTMVYKSVRIE	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





Chapter 4

**Pulmonary immune
responses against
Aspergillus fumigatus
are characterized by
high frequencies of IL-17
producing T-cells**



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Abstract

In healthy individuals and in patients with invasive aspergillosis, *Aspergillus*-specific T-cells in peripheral blood display mainly a Thelper1 phenotype. Although in other fungal infections Thelper17 immunity is important, it was suggested that in aspergillus infection Thelper17 cells do not play a role or may even be detrimental.

Objectives

To compare the cytokine profiles of *Aspergillus*-specific CD4+ T-cells in peripheral blood and in the lung. To investigate the Thelper phenotype at the primary location of *A. fumigatus* exposure.

Methods

Lung-derived T-cells and peripheral blood T-cells from COPD-patients were stimulated with overlapping peptides of 6 *A. fumigatus* proteins. *Aspergillus*-specific T-cells were identified on the basis of the activation marker CD154 and production of TNF α . In addition, production of the cytokines IFN γ , IL-17, IL-4 and IL-5 by the *Aspergillus*-specific T-cells was measured.

Results

The majority of lung-derived *Aspergillus*-specific T-cells displayed a Thelper17 phenotype, and only low percentages of cells produced IFN γ . In contrast, in the peripheral blood of COPD patients *Aspergillus*-specific T-cells displayed a Thelper1 phenotype, similar as peripheral blood-derived *Aspergillus*-specific T-cells from healthy individuals.

Conclusions

These data demonstrate that in *A. fumigatus* infection, similar as in other fungal infections, Thelper17 cells may play a more important role in the immune response than was appreciated until now.

Introduction

Aspergillus fumigatus is a mold that can lead to serious infectious complications in immunocompromised patients. Neutropenia and impaired neutrophil granulocyte function are known to be risk factors for invasive aspergillosis ¹. In addition, other parts of the innate immune system, like monocytes and macrophages, play an important role in the defense against *Aspergillus fumigatus* ², and there is mounting evidence that the adaptive immune system, especially T-cells, are also involved in the immune response against *A. fumigatus* ³⁻⁹.

Although in fungal infections like candidiasis the role of IL-17 is well established ¹⁰⁻¹², in *Aspergillus fumigatus* a role for Thelper17 (Th17) cells was so far not demonstrated. In peripheral blood from healthy individuals ⁴⁻⁶ and from patients with invasive aspergillosis ^{5,7,8}, *Aspergillus*-specific T-cells were detected that primarily displayed a Thelper1 (Th1) phenotype with production of IFN γ . Several papers suggest that Th17 cells do not play a role in the defense against *Aspergillus* ¹³ or may even have a detrimental effect ¹⁴.

However, because it is well known that neutrophil granulocytes are important in the immune defense against *A. fumigatus* and Th17 cells are involved in recruitment, activation and migration of neutrophil granulocytes to the site of a fungal infection, a role for Th17 cells would also be expected in the immune response against *A. fumigatus*. Since the primary location of an aspergillus infection is the lung, we hypothesized that in contrast to *Aspergillus*-specific CD4+ T-cells in peripheral blood, pulmonary *Aspergillus*-specific CD4+ T-cells may have a Th17 phenotype.

In this study, we demonstrate that the frequencies of *Aspergillus*-specific CD4+ T-cells in peripheral blood and lung material are similar, but that the cytokine secretion profiles of these T-cells are different. Peripheral blood derived *Aspergillus*-specific T-cells displayed a Th1 phenotype with production of mainly IFN γ , identical to previous studies on *Aspergillus*-specific T-cell immunity in peripheral blood. However, the majority of lung-derived *Aspergillus*-specific T-cells displayed a Th17 phenotype with primarily production of IL-17, in accordance with the phenotype of Thelper cells pivotal for the protection against other fungal infections.

Material and Methods

Cell collection and preparation

The study was performed according to the Declaration of Helsinki and approved by the local medical ethics committees. After informed consent, peripheral blood samples and pulmonary tissue were obtained from chronic obstructive pulmonary disease (COPD) patients who either underwent a lung transplantation because of COPD Gold stage IV, or a lobectomy because of a peripherally located lung tumor. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Isopaque separation and cryopreserved. Lung-derived mononuclear cells (LMC) were isolated by enzymatic

digestion from a tissue specimen obtained directly after lobectomy or lung explantation as described previously¹⁵. Briefly, tissue specimens (1 x 1 cm) were sliced into pieces of 1 mm and incubated for 20 minutes in RPMI with 20mM Hepes, 15% fetal calf serum (FCS), 50 U/ml DNase type I (Sigma-Aldrich, Zwijndrecht, Netherlands) while shaking at 37°C. Tissue pieces were carefully dried with sterile gauzes and incubated for 60 minutes in medium supplemented with collagenase type I 300 U/ml (Worthington, Lakewood, NJ) while shaking at 37°C. A cell suspension was obtained by grinding the tissue through a flow-through chamber. Mononuclear cells were isolated from the cell suspension by standard density gradient techniques and cryopreserved for later analysis.

***Aspergillus* antigens**

Overlapping peptides of the *A. fumigatus* proteins Aspf1, Aspf2, Aspf3, Aspf4, Crf1 and Catalase1, consisting of 15-mer peptides with an 11-amino acid overlap, were synthesized by JPT Peptide Technologies (Berlin, Germany) and dissolved in DMSO.

Flow cytometry

PBMC or LMC (0.5×10^6) were incubated with the combination of overlapping *A. fumigatus* peptide pools (10^{-6} M) in 96-well plates for 2 hours at room temperature, and then washed to remove DMSO and peptides. Subsequently, the cells were cultured in 150 μ l 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). After 2 weeks the T-cells were harvested and restimulated with peptide-pulsed or non-pulsed autologous PBMC or LMC (0.5×10^6). Depending on the expansion of T-cells after this 2-week culture period, cells were split and restimulated with the separate antigens. 2×10^6 or more cells were split in 7 wells and separately restimulated with overlapping peptides (10^{-6} M) of the 6 different *A. fumigatus* proteins or with non-pulsed PBMC or LMC. 1×10^6 cells were split in 3 wells and restimulated with a combination of Aspf1, Aspf2, Aspf3 and Aspf4 in 1 well, a combination of Crf1 and Catalase1 in the 2nd well or with non-pulsed PBMC or LMC in the 3rd well. When the T-cells had minimally expanded ($\leq 0.6 \times 10^6$ cells), cells were split in 2 wells and restimulated with the combination of all proteins or with non-pulsed PBMC or LMC. 1 hour after restimulation 10 μ g/ml Brefeldin-A (BFA, Sigma-Aldrich) was added to promote intracellular accumulation of cytokines. 5 hours after restimulation, cells were stained with Pacific Blue-labeled anti-CD4 (BD, Breda, Netherlands), fixated with paraformaldehyde 1% (pharmacy LUMC, Leiden, Netherlands) and permeabilized with saponin 0.1% (Sigma-Aldrich). FITC-labeled anti-CD154 (BD/Pharmingen, Breda, Netherlands), PE-Cy7-labeled anti-TNF α (BD/Pharmingen), APC-labeled anti-IFN γ (BD/Pharmingen), Brilliant Violet 605-labeled anti-IL-17 (IKT/Biolegend, Uithoorn, Netherlands), PerCP-Cy5.5-labeled anti-IL-4 (BD/Pharmingen) and PE-labeled anti-IL-5 (BD/Pharmingen) were added for intracellular staining of the activation marker CD154 and cytokine production. Cells were collected and analyzed on the LSR-II (BD, Breda, Netherlands).

Results

Clinical characteristics of the patients

In this study, we included 10 COPD patients who underwent a lung transplantation or lobectomy, of whom lung material was available for analysis of lung-derived *Aspergillus*-specific T-cells. From 3 of these 10 patients, we also had peripheral blood samples to compare frequencies and cytokine profiles of lung-derived *Aspergillus*-specific T-cells with frequencies and cytokine profiles of peripheral blood derived *Aspergillus*-specific T-cells. Because of limited availability of peripheral blood samples from the included patients with lung material, we included 7 additional COPD patients who underwent a lobectomy, of whom only peripheral blood samples were available for analysis of *Aspergillus*-specific T-cells. Characteristics of included patients are summarized in table 4.1.

All patients had a history of smoking; 12 patients were former smokers, 5 patients were current smokers and tobacco exposure ranged from 25 to 57 pack years. 12 of 17 patients were male. The mean age of included patients was 64 years (range 49-76). Six patients had COPD Gold grade I, 2 patients had COPD Gold grade II and 9 patients had COPD Gold grade IV. There was no clinical or microbiological evidence for aspergillus infection in these patients.

Table 4.1 Characteristics of patients with COPD

Patient	Gender	Age	Gold stage	Surgery	FACS LMC*	FACS P BMC#
C1	Male	60	IV	Lung transplant	pos	n/a
C2	Male	60	IV	Lung transplant	pos	n/a
C3	Female	58	IV	Lung transplant	pos	n/a
C4	Female	56	IV	Lung transplant	pos	n/a
C5	Female	49	IV	Lung transplant	pos	n/a
C6	Male	60	IV	Lung transplant	neg	n/a
C7	Male	60	IV	Lung transplant	pos	n/a
C8	Female	54	IV	Lung transplant	pos	pos
C9	Male	57	IV	Lung transplant	pos	pos
C10	Male	73	I	Lobectomy	neg	neg
C11	Male	73	I	Lobectomy	n/a	pos
C12	Male	73	II	Lobectomy	n/a	neg
C13	Male	66	I	Lobectomy	n/a	pos
C14	Male	72	I	Lobectomy	n/a	pos
C15	Male	72	I	Lobectomy	n/a	pos
C16	Female	76	I	Lobectomy	n/a	pos
C17	Male	72	II	Lobectomy	n/a	pos

*# n/a not available, pos detectable *Aspergillus*-specific immune response, neg non-detectable *Aspergillus*-specific immune response

***Aspergillus*-specific T-cells with a Th17 profile are present in lung-derived mononuclear cells**

Because the primary focus of infection in aspergillosis is located in the lung, we aimed to study the frequencies and cytokine profiles of *Aspergillus*-specific T-cells in pulmonary material. In previous studies *Aspergillus*-specific T-cells in peripheral blood could not be detected directly ex vivo^{6,7}, probably due to low frequencies or inability to produce cytokines in the short detection period used in the intracellular staining protocol. We hypothesized that frequencies of *Aspergillus*-specific T-cells in lung material might be higher than those in peripheral blood and therefore might be detectable directly ex vivo. We stimulated lung-derived mononuclear cells (LMC) with a mixture of overlapping peptides of 6 *A. fumigatus* proteins and analyzed cytokine production and activation based on the marker CD154 directly ex vivo by FACS. However, in the direct analysis we did not detect *Aspergillus*-specific T-cells (data not shown).

Therefore, we used a restimulation protocol, in which LMC were stimulated with a combination of overlapping peptides of 6 *A. fumigatus* proteins, cultured for 2 weeks and subsequently restimulated with peptide-pulsed or non-pulsed autologous LMC. In 8 of 10 analyzed COPD patients we detected *Aspergillus*-specific T-cells in the lung material based on expression of the activation marker CD154 and production of TNF α after *Aspergillus*-specific stimulation. In 2 of 10 analyzed COPD patients no *Aspergillus*-specific T-cells were detected, most likely due to the limited viability of the T-cells in the lung samples. Frequencies of *Aspergillus*-specific T-cells were calculated as percentage CD4+TNF α + T-cells after restimulation with autologous LMC pulsed with overlapping peptides minus percentage CD4+TNF α + T-cells after restimulation with non-pulsed autologous LMC, and ranged from 0.5% to 9.8% of total CD4+ T-cells (figure 4.1A). In 2 patients (C1 and C9) we were able to investigate the specificity of the *Aspergillus*-specific T-cells by restimulating the T cells after the first *Aspergillus*-specific stimulation with the separate peptide pools. The results demonstrate that the *Aspergillus*-specific T-cells in the lungs are directed against a wide range of antigens. In the lungs of patient C1 and C9 CD4+ T-cells were detected against the *Aspergillus* proteins Aspf2, Crf1 and Catalase1 (figure 4.1B).

Subsequently, we analyzed which cytokines were produced by the lung-derived *Aspergillus*-specific T-cells. In 4 of 8 patients (C1, C2, C7, C8) with a high frequency of lung-derived *Aspergillus*-specific T-cells after restimulation with *A. fumigatus* proteins, ranging between 2% and 9.8% of total CD4+ T cells, we detected *Aspergillus*-specific T-cells with a Th17 cytokine profile, producing mainly IL-17 and low amounts of IFN γ (figure 4.2A). This Th17 cytokine profile is illustrated in figure 4.2B, in which the cytokine production of the *Aspergillus*-specific T-cells of patient C2 after restimulation with the overlapping peptides is shown. In patient C1 we were able to analyze the cytokine production after restimulation with the individual *Aspergillus* proteins. The results demonstrate that the lung-derived *Aspergillus*-specific T-cells of this patient directed against Aspf2, Crf1, and Catalase all exhibited a Th17 phenotype (figure 4.2C). In 1 of the 8 patients (C9)

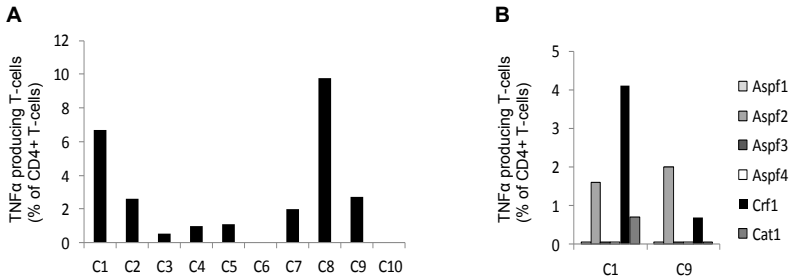


Figure 4.1 *Aspergillus*-specific T-cells in lung-derived mononuclear cells of COPD patients. (A) LMC were stimulated with overlapping peptides of the combination of 6 *A. fumigatus* antigens and after a 2-week culture period restimulated with the overlapping peptides of the antigens Aspf1, Aspf2, Aspf3, Aspf4, Crf1 and Catalase1. (B) For patient C1 and C9, cells were split in 7 wells and restimulated with the separate antigens or with non-pulsed LMC. Percentages of TNFα producing CD4+ T-cells were calculated as % TNFα+ T-cells of CD4+ T-cells after restimulation with peptide-pulsed LMC minus % TNFα+ T-cells of CD4+ T-cells after restimulation with non-pulsed LMC.

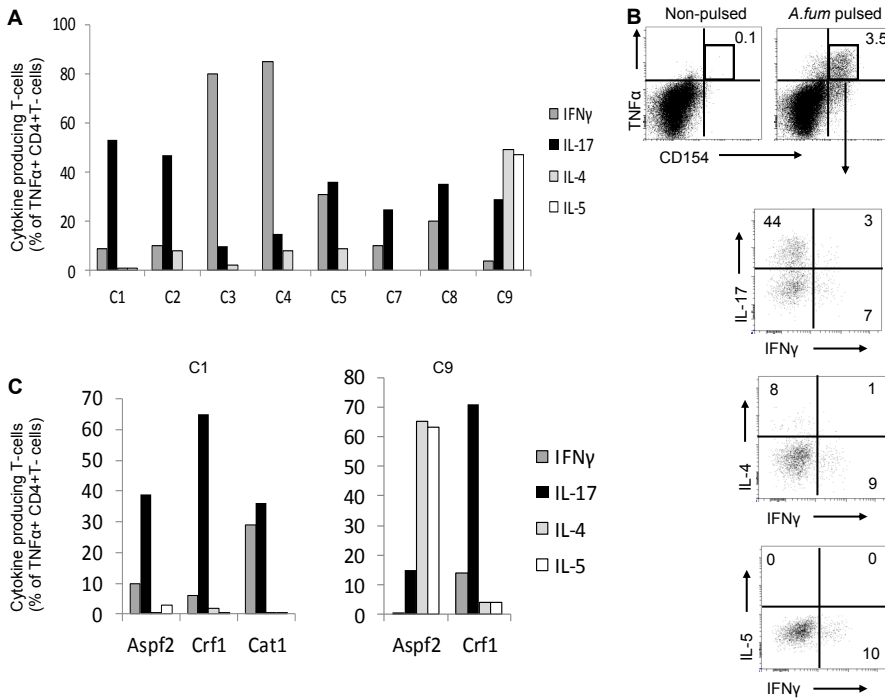


Figure 4.2 *Cytokine profiles of lung-derived Aspergillus*-specific T-cells. (A) Production of the cytokines IFNγ, IL-17, IL-4 and IL-5 by activated lung-derived CD4+ T-cells after restimulation with overlapping peptides of *A. fumigatus* antigens depicted as % cytokine-producing T-cells of TNFα+CD4+ T-cells after restimulation with peptide-pulsed LMC. (B) Representative example of FACS-plot of patient C2 after restimulation with unpulsed LMC and after restimulation with LMC pulsed with combination of overlapping peptides of *A. fumigatus* antigens. CD154+ TNFα+ CD4+ T-cells after peptide-pulsed LMC restimulation were gated and IFNγ, IL-17, IL-4 and IL-5 production of the T-cells is shown. (C) Cytokine profiles of *Aspergillus*-specific T-cells in patient C1 and C9 after restimulation with the separate *A. fumigatus* antigens. T-cells were analyzed by FACS after 5 hours of restimulation. Only results of patients in which TNFα expression was induced after stimulation with *Aspergillus* antigens are shown.

the lung-derived *Aspergillus*-specific T-cells demonstrated a mixed response with production of IL-17, IL-4 and IL-5. By restimulating with the individual *Aspergillus* proteins we were able to demonstrate that the *Aspergillus* specific T-cells directed against Asp2 primarily produced IL-4 and IL-5, whereas the T-cells directed against Crf1 produced mainly IL-17 (figure 4.2C). In 3 of 8 patients (C3, C4, and C5) with a low number of *Aspergillus*-specific T-cells (0.5-1%) after restimulation in vitro, the lung derived *Aspergillus* specific T-cells mainly produced IFN γ (C3 and C4), or both IFN γ and IL-17 (C5) (figure 4.2A). These results demonstrate that in COPD patients with a strong immune response against *Aspergillus*, the lung-derived *Aspergillus*-specific T-cells mainly exhibited a Th17 phenotype.

***Aspergillus*-specific T-cells in peripheral blood of COPD patients exhibit a Th1 phenotype rather than a Th17 phenotype.**

In previous studies that showed no or low frequencies of IL-17 producing *Aspergillus*-specific T-cells in peripheral blood, mainly healthy individuals were analyzed ^{6,13}. A repeated analysis in healthy individuals in this study showed identical results with varying frequencies of *Aspergillus*-specific T-cells directed to a broad variety of *A. fumigatus* antigens, displaying a Th1 phenotype (supplemental figure 4.1A-D). In one healthy donor a mixed phenotype was present with production of IFN γ , IL-17 and IL-4 (supplemental figure 4.1B and D).

The analyzed pulmonary samples in the present study were derived from tissue samples from COPD patients who had undergone a lobectomy or lung transplantation. Therefore the high frequencies of IL-17 producing *Aspergillus*-specific T-cells identified in the lung material might be a representation of a generalized Th17 response in COPD patients. To investigate whether *Aspergillus*-specific T-cells in peripheral blood of COPD patients also exhibited a Th17 phenotype like the lung-derived *Aspergillus*-specific T-cells, or whether the *Aspergillus*-specific T-cells exhibited a Th1 phenotype similar to healthy individuals, we analyzed the cytokine profiles of *Aspergillus*-specific T-cells in peripheral blood from COPD patients. From 3 of the 10 patients (C8, C9, C10) of whom we had pulmonary material, we also had peripheral blood samples to directly compare cytokine profiles of *Aspergillus*-specific T-cells in both compartments. In patient C10 no *Aspergillus*-specific T-cells were detected in either lung or peripheral blood after stimulation with *A. fumigatus* antigens most likely due to the impaired viability of the samples (data not shown). In patient C8 and C9 *Aspergillus*-specific T-cells were present in peripheral blood, based on TNF α production (figure 4.3). These *Aspergillus*-specific T-cells were directed against a wide range of *Aspergillus* antigens, as shown in figure 4.3B. *Aspergillus*-specific T-cells in peripheral blood of both patients displayed a Th1 phenotype, mainly producing IFN γ , with low percentages of cells producing IL-17 (figure 4.4A and C), whereas the *Aspergillus*-specific T-cells present in the lung material of these patients mainly exhibited a Th17 phenotype (figure 4.2A and 4.2C).

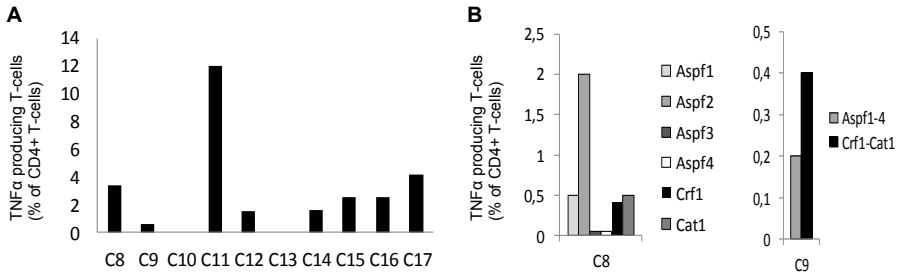


Figure 4.3 *Aspergillus*-specific T-cells in peripheral blood of COPD patients. (A) PBMC were stimulated with overlapping peptides of the combination of 6 *A. fumigatus* antigens and after a 2-week culture period restimulated with the overlapping peptides of the antigens Aspf1, Aspf2, Aspf3, Aspf4, Crf1 and Catalase1. (B) For patient C8 and C9, cells were split in 7 wells and 3 wells respectively, and restimulated with separate antigens or with non-pulsed PBMC. Percentages of TNFα producing CD4+ T-cells were calculated as % TNFα+ T-cells after restimulation with peptide-pulsed PBMC minus % TNFα+ T-cells of CD4+ T-cells after restimulation with non-pulsed PBMC.

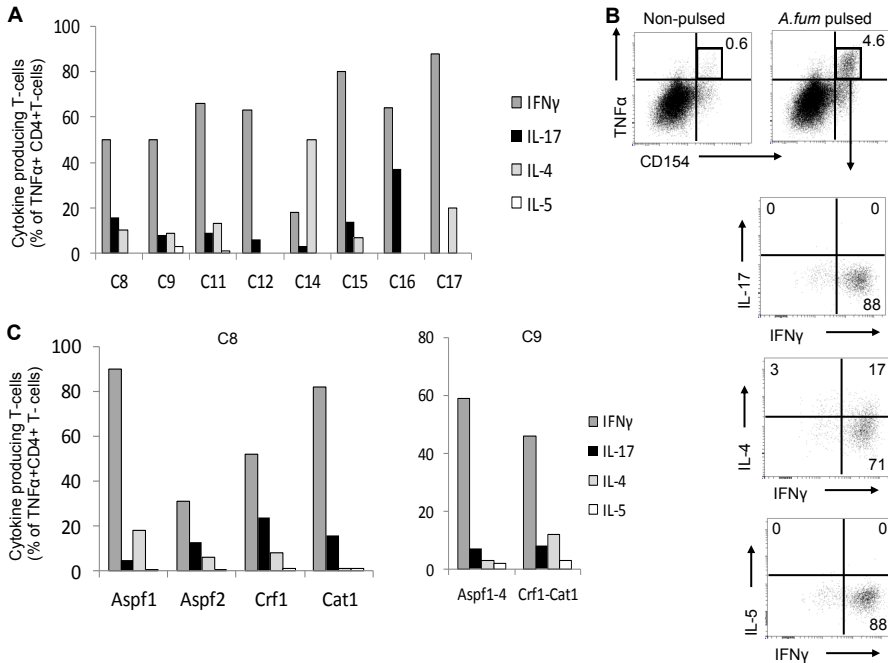


Figure 4.4 *Cytokine profiles of peripheral blood-derived Aspergillus*-specific T-cells. (A) Production of the cytokines IFNγ, IL-17, IL-4 and IL-5 by activated peripheral blood-derived CD4+ T-cells after restimulation with overlapping peptides of *A. fumigatus* antigens depicted as % cytokine-producing T-cells of TNFα+CD4+ T-cells after restimulation with peptide-pulsed PBMC. (B) Representative example of FACS-plot of patient C17 after restimulation with unpulsed PBMC and after restimulation with PBMC pulsed with combination of overlapping peptides of *Aspergillus* antigens. CD154+ TNFα+ CD4+ T-cells after peptide-pulsed PBMC restimulation were gated and IFNγ, IL-17, IL-4 and IL-5 production of the T-cells is shown. (C) Cytokine profiles of *Aspergillus*-specific T-cells in patient C8 and C9 after restimulation with the separate *A. fumigatus* antigens and with 2 combinations of *A. fumigatus* antigens, respectively. T-cells were analyzed by FACS after 5 hours of restimulation. Only results of patients in which TNFα expression was induced after stimulation with *Aspergillus* antigens are shown.

These data indicate that it is most likely the location of the T-cells that determines the phenotype of the *Aspergillus*-specific CD4⁺ T-cells and not the type of disease. To verify these data we studied the Thelper phenotype of *Aspergillus*-specific T-cells in peripheral blood from 7 additional COPD patients (C11-C17). In 6 of 7 additional peripheral blood samples *Aspergillus*-specific CD4⁺ T-cells were present after restimulation with overlapping peptides of *A. fumigatus* proteins (figure 4.3A). Next, we analyzed the cytokine secretion profiles of the peripheral blood-derived *Aspergillus*-specific CD4⁺ T-cells. In peripheral blood samples of 5 of 6 COPD patients the *Aspergillus*-specific T-cells produced primarily IFN γ and only low percentages of the cells produced IL-17 (figure 4.4A). In figure 4.4B the FACS-analysis of peripheral blood-derived *Aspergillus*-specific T-cells from patient C17 is shown, with IFN γ producing T-cells and lower percentages of IL-4 producing T-cells after restimulation with the overlapping peptides.

Thus, *Aspergillus*-specific T-cells in these COPD patients showed a Th1 cytokine profile in peripheral blood (figure 4.4A and C), and not a Th17 phenotype like

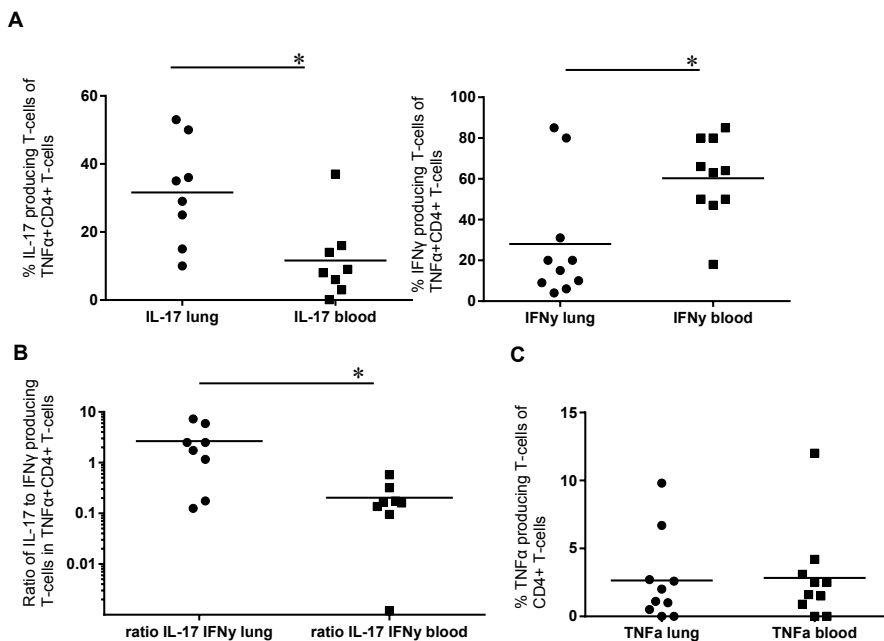


Figure 4.5 Differences in cytokine production between *Aspergillus*-specific T-cells derived from lung and peripheral blood. (A) Percentages of IL-17- or IFN γ -producing TNF α +CD4⁺ T-cells after restimulation with *A. fumigatus* overlapping peptides in lung and peripheral blood. (B) Ratio of IL-17 to IFN γ producing TNF α +CD4⁺ T-cells after restimulation with *A. fumigatus* overlapping peptides in lung and peripheral blood. (C) Percentages of TNF α producing CD4⁺ T-cells after restimulation with *A. fumigatus* overlapping peptides in lung and peripheral blood. * Significant difference in the mean percentage of IL-17 or IFN γ producing TNF α +CD4⁺ T-cells and in the ratio of IL-17 to IFN γ producing TNF α +CD4⁺ T-cells after restimulation with *A. fumigatus* overlapping peptides in lung compared to peripheral blood ($p < 0.05$, two-tailed unpaired Student T test).

in lung material (figure 4.2A and C). The cytokine profiles of *Aspergillus*-specific T-cells in peripheral blood in COPD patients were similar as in healthy individuals (figure 4.4 and supplemental figure 4.1).

When we compared the percentages of IL-17 and IFN γ producing *Aspergillus*-specific T-cells in lung with those in peripheral blood from COPD patients we found significant differences ($p < 0.05$), with mainly IL-17 producing T-cells in lung and IFN γ producing T-cells in peripheral blood (figure 4.5A). This is also reflected in the ratio of IL-17 to IFN γ producing T-cells, as depicted in figure 4.5B. In contrast, when we compared percentages of TNF α producing *Aspergillus*-specific T-cells in lung and peripheral blood, no differences were identified (figure 4.5C), indicating that the percentages of activated T-cells in both compartments were similar.

Discussion

In this study, we have demonstrated the presence of *Aspergillus*-specific T-cells in the lungs of COPD patients. In the majority of patients, these *Aspergillus*-specific T-cells exhibited a Th17 phenotype producing besides TNF α primarily IL-17, whereas only low percentages of T-cells produced IFN γ . In contrast, in peripheral blood of COPD patients as well as healthy individuals *Aspergillus*-specific T-cells showed a Th1 phenotype producing TNF α and IFN γ , and only few peripheral blood-derived *Aspergillus*-specific T-cells produced IL-17.

In the COPD patients with a strong pulmonary *Aspergillus*-specific T-cell response ($> 2\%$ TNF α + CD4+ T cells), the *Aspergillus*-specific T-cells primarily exhibited a Th17 phenotype. In contrast in the two patients with a low pulmonary *Aspergillus*-specific T-cell response ($< 1\%$ TNF α + CD4+ T cells) the *Aspergillus*-specific T-cells mainly produced IFN γ . This may indicate that in those low reactive patients no recent exposure to *Aspergillus* has occurred.

Previous studies on T-cell mediated immunity against *Aspergillus fumigatus* investigated the presence of *Aspergillus*-specific T-cells in peripheral blood from healthy individuals^{3-6;9;13}. In these studies low frequencies of *Aspergillus*-specific T-cells could be detected with a Th1 cytokine secretion profile, producing primarily IFN γ ^{4-6;9;13;16}. Isolation of *Aspergillus*-specific T cells from peripheral blood of healthy individuals by CD154 based selection also demonstrated that the peripheral blood derived *Aspergillus*-specific T-cells primarily exhibited a Th1 phenotype whereas *Candida albicans*-specific T-cells selected by the same method exhibited a Th17 phenotype¹⁷. In addition, analyses of the Thelper phenotype of *Aspergillus*-specific T-cells in peripheral blood from patients with invasive aspergillosis established no evidence for a role for IL-17 in the immune defense against *A. fumigatus*^{5;7;13;16}. In one study, IL-17 levels in bronchoalveolar lavage fluid from patients diagnosed with invasive aspergillosis were measured and only low levels were found¹³. Some authors suggested that the Th17 pathway even increases susceptibility to aspergillus infections¹⁴.

However, already for a long time it is known that neutrophil granulocytes are major players in the immune response to fight *Aspergillus*^{1,2}, similar as in other fungal infections like *Candida albicans*¹⁸. Because Th17 cells are involved in the recruitment of neutrophil granulocytes to the site of inflammation¹², it is to be expected that Th17 cells have a role in these infections as well. In the host defense against *Candida* infections the role of IL-17 is well established¹¹: IL-17 deficiency leads to impaired neutrophil recruitment and overt oropharyngeal candidiasis¹⁰.

The results of the present study demonstrating *Aspergillus*-specific T-cells with a Th17 phenotype in lung material from COPD patients, confirm the data by Mirkov et al. on pulmonary aspergillus infections in a study in rats. That study, evaluating the immunological differences at the time of pulmonary aspergillus infection in 2 different immunocompetent rat strains, revealed a difference in IL-17 production. Highest IL-17 levels were identified in the rat strain that demonstrated the fastest clearance of aspergillus infection¹⁹. Our study and the study of Mirkov et al. are in conflict with an earlier human study, in which low levels of IL-17 were measured in bronchoalveolar lavage fluid of immunocompromised patients at the time of invasive aspergillosis¹³. However, since this analysis was performed at the time of diagnosis of invasive aspergillosis, an explanation for this discrepancy could be that no *Aspergillus*-specific T-cells were present yet. We and others have demonstrated that at the time of diagnosis no *Aspergillus*-specific T-cells can be detected in peripheral blood, but that *Aspergillus*-specific T-cells are induced in the course of aspergillus infection. Appearance of *Aspergillus*-specific T-cells is correlated with regression of aspergillus lesions^{7,8}.

Two studies of T-cell responses in humans²⁰ and in mice²¹ with corneal infections caused by *Aspergillus*, showing induction of both IFN γ and IL-17 producing T-cells at the site of infection, supporting the hypothesis that besides Th1 cells, at the site of infection Th17 cells play an important role, most likely to attract neutrophil granulocytes to the site of aspergillus infection. A recent publication on *Aspergillus* skull base osteomyelitis also implies a role for Th17 in the immune response against *A. fumigatus* and suggests that a defect in the Th17 response in these patients may have caused or contributed to the development of this disease²².

Whether *Aspergillus*-specific T-cells in the lung and *Aspergillus*-specific T-cells in peripheral blood are separate populations is not clear. Differences in cytokine profiles could point to different effector patterns, that may be caused by different precursor cells, as was previously suggested²³. Alternatively, the IL-17 producing *Aspergillus*-specific T-cells in the lung belong to the resident memory T-cell (T_{RM}) population, and are of common clonal origin with peripheral blood-derived *Aspergillus*-specific T-cells, as described by Gaide et al.²⁴. Differences in cytokine profiles between the two populations may be explained by differences in the local milieu, that can influence the inflammatory response, corresponding with an effective response at the site of infection.

In other infections differences in the immune response between peripheral blood and the site of infection have also been described. In influenza infection, influenza-specific T_{RM} have been shown to have a different phenotype compared to T-cells in peripheral blood ²⁵, and discrepancy in disease related biomarker signature between peripheral blood samples and pulmonary samples was recently also described for tuberculosis infection ²⁶.

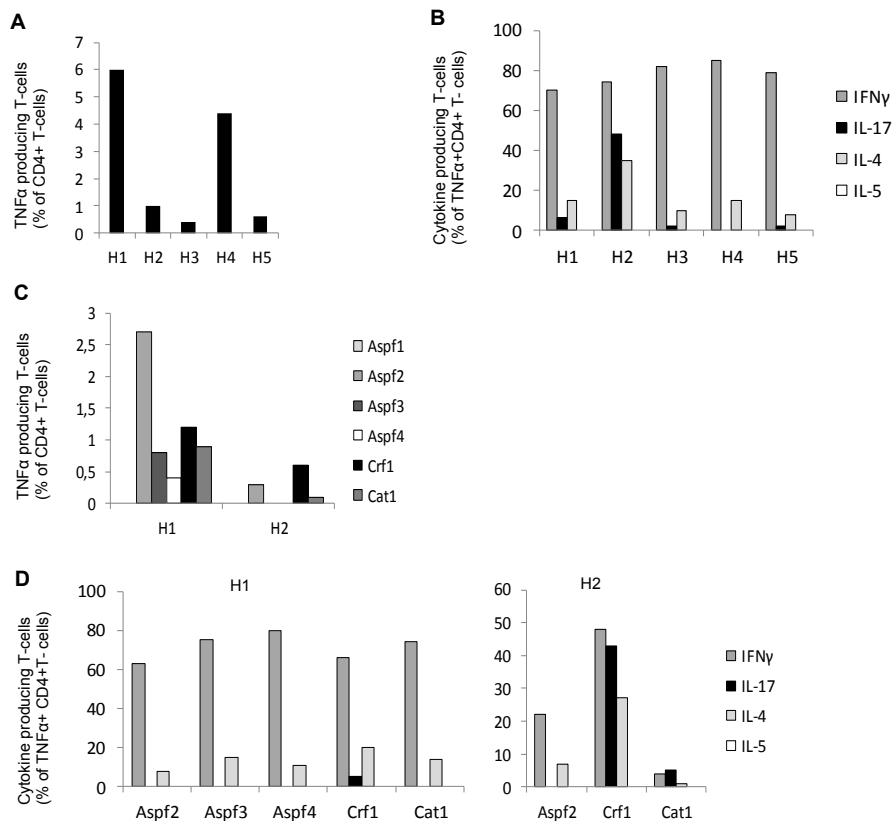
In conclusion, this is the first study in humans that investigates the *Aspergillus*-specific T-cell response in the lung, the site of first exposure to *A. fumigatus*. Here we demonstrate that *Aspergillus*-specific T-cells in the lung primarily display a Th17 cytokine secretion profile, whereas *Aspergillus*-specific T-cells in peripheral blood display a Th1 phenotype. These data indicate that, like in other fungal infections, also in the immunity against *A. fumigatus* Th17 cells play a role at the site of infection.

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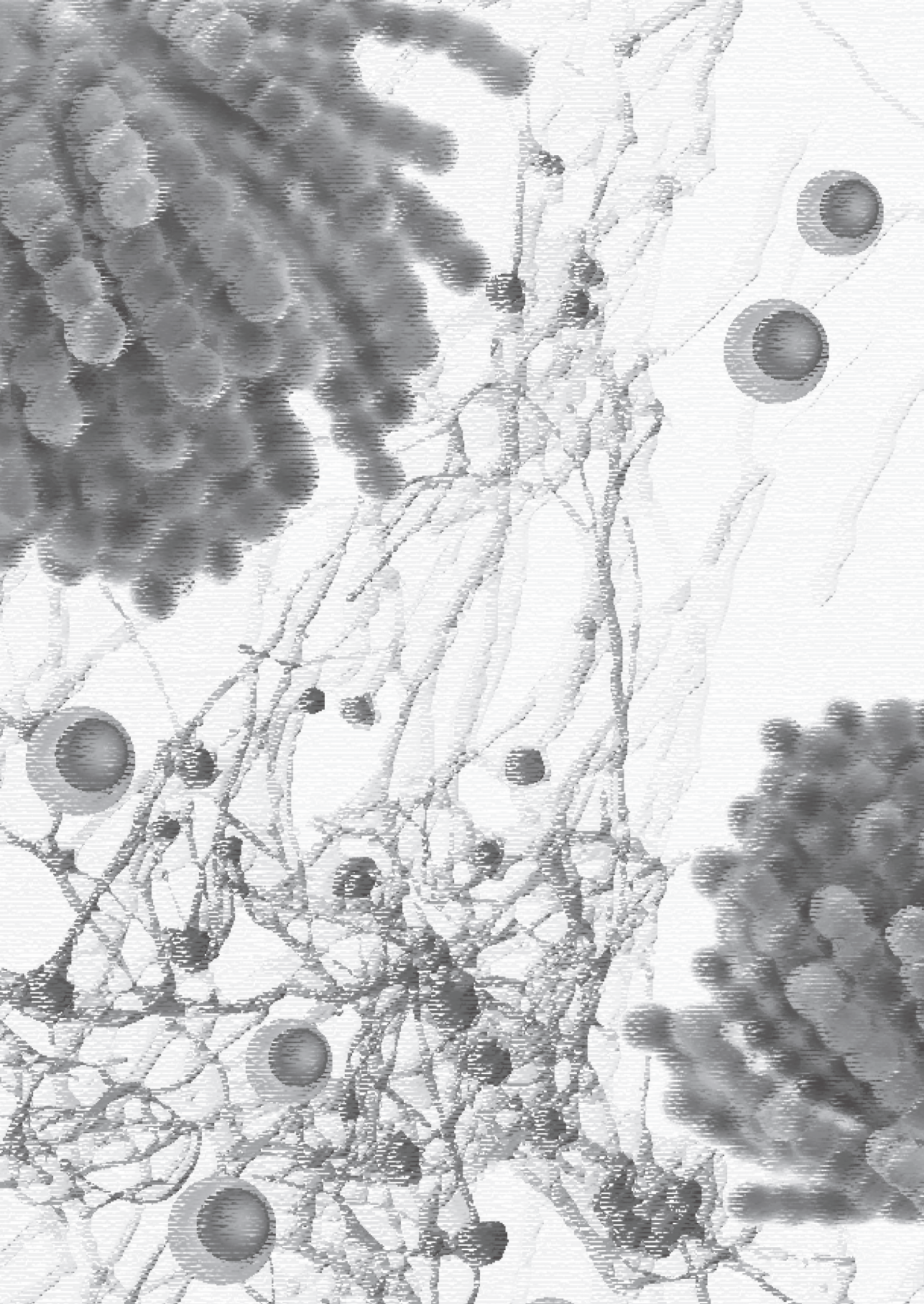
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Supplemental figure S4.1



Supplemental figure S4.1

Aspergillus-specific T-cells in peripheral blood mononuclear cells from healthy individuals. (A) PBMC were stimulated with overlapping peptides of the combination of 6 *A. fumigatus* antigens and after a 2-week culture period restimulated with overlapping peptides of the antigens Asp1, Asp2, Asp3, Asp4, Crf1 and Catalase1. (B) Production of the cytokines IFN γ , IL-17, IL-4 and IL-5 by activated peripheral blood-derived CD4+ T-cells after restimulation with overlapping peptides of *A. fumigatus* antigens depicted as % cytokine-producing T-cells of TNF α +CD4+ T-cells after restimulation with peptide-pulsed PBMC. (C) For individual H1 and H2, cells were split in 7 wells and restimulated with separate antigens or with non-pulsed PBMC. (D) Cytokine profiles of *Aspergillus*-specific T-cells in individual H1 and H2 after restimulation with the separate *A. fumigatus* antigens. Percentages of TNF α producing T-cells were calculated as % TNF α + T-cells of CD4+ T-cells after restimulation with peptide-pulsed PBMC minus % TNF α + T-cells of CD4+ T-cells after restimulation with non-pulsed PBMC. T-cells were analyzed by FACS after 5 hours of restimulation.





Chapter 5

Thelper2 response in allergic bronchopulmonary aspergillosis is not driven by specific *Aspergillus* antigens



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Abstract

Allergic bronchopulmonary aspergillosis (ABPA) is characterized by an allergic immunological response to *A. fumigatus*. In this study we investigated whether certain *Aspergillus* antigens are more allergenic than others, as was postulated previously. We stimulated PBMC from patients with ABPA with the classically described *A. fumigatus* allergens Asp1, Asp2, Asp3 and Asp4, as well as 2 other *Aspergillus* antigens, Crf1 and Catalase1. Activated CD4⁺ T-cells displayed a Thelper2 phenotype with production of IL-4 in response to stimulation with several of these different antigens. Immune responses were not limited to the classically described *A. fumigatus* allergens. In healthy individuals we demonstrated a similar recognition profile to the different antigens, but in contrast the activated CD4⁺ T-cells exerted a Thelper1 phenotype and mainly produced IFN γ after stimulation with *A. fumigatus* antigens. In conclusion, irrespective of the *A. fumigatus* antigen the T-cell immune response in ABPA patients is skewed to a Thelper2 cytokine secretion profile.

Introduction

Allergic bronchopulmonary aspergillosis (ABPA) is a hypersensitivity pulmonary disease that occurs almost exclusively in patients with asthma or cystic fibrosis ^{1,2}, and is characterized by an allergic immunological response to *A. fumigatus*. In patients with ABPA IgE, IgA and IgG anti-*A. fumigatus* antibodies are present ³⁻⁶, as well as *Aspergillus*-specific T-cells with a Th2 phenotype ⁷⁻⁹. The pathogenesis of ABPA is however not completely understood. Previously, it was postulated that certain *A. fumigatus* antigens, like Aspf1 or Aspf2 are more allergenic than others ^{6,8,9}, and it was suggested this might play a role in the development of ABPA. In this study we studied T-cell responses against 4 classically described *A. fumigatus* allergens and 2 other *A. fumigatus* antigens in patients with ABPA, and compared these with healthy controls.

Materials and methods

Patients

This study was conducted with approval of the institutional review board of the Leiden University Medical Center. After informed consent was given, peripheral blood was obtained from patients with ABPA and from healthy controls, and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Isopaque separation and cryopreserved. We included ABPA-patients in chronic phase, as well as patients shortly after diagnosis. ABPA-patients fulfilled at least 6 of the criteria as described in the supplementary data.

Aspergillus antigens

Overlapping peptides of the *A. fumigatus* proteins Aspf1, Aspf2, Aspf3, Aspf4, Crf1 and Catalase1, consisting of 15-mer peptides with an 11-amino acid overlap, were synthesized by JPT Peptide Technologies (Berlin, Germany) and dissolved in DMSO.

Flow cytometry

To be able to identify *Aspergillus*-specific T-cells, a restimulation protocol after MACS-enrichment of *Aspergillus*-specific T-cells based on CD154-expression was used ¹⁰, as described in the supplementary methods. Five hours after restimulation, cells were stained according to the staining protocol for intracellular staining, as described previously ¹¹. The following monoclonal antibodies were used: anti-CD4 Pacific Blue, anti-CD154 FITC, anti-IFN γ APC, anti-TNF α PE-Cy7, anti-IL-4 PerCP-Cy5.5, anti-IL-5 PE and anti-IL-17 Brilliant Violet 605. Cells were analyzed on the LSR-II (BD, Breda, Netherlands).

Results and discussion

To investigate whether T-cell responses in patients with ABPA are triggered by specific *A. fumigatus* antigens, we stimulated PBMC from 6 ABPA patients with overlapping peptides of 4 classically described *Aspergillus* allergens Asp1, Asp2, Asp3 and Asp4, and with 2 other *Aspergillus* antigens, Crf1 and Catalase1, which we previously used for the analysis of immune responses in healthy individuals and patients with invasive aspergillosis^{11,12}. After overnight stimulation, we selected *Aspergillus*-specific T-cells on the basis of CD154-expression by MACS and cultured the T-cells for 3 weeks. After restimulation with overlapping peptides of the different *Aspergillus* antigens, we identified in 4 of the 6 patients detectable frequencies of *Aspergillus*-specific T-cells based on an increase of CD154-expression after restimulation with *A. fumigatus* antigens, compared to non-peptide restimulated cells. The increase of CD154-expression after antigen stimulation varied between 0.5% and 12.6%, and was directed against Asp2 and Asp4, as well as Crf1 and Catalase1 (figure 5.1A). Activation of CD4+ T-cells was detected after restimulation with Asp2 in 3 patients, Asp4 in 1 patient, Crf1 in 3 patients and Catalase1 in 3 patients (figure 5.1A). In two patients almost no expansion of cells was observed after 3 weeks of culture and percentages of CD154-expressing T-cells in peptide specific stimulated and non-peptide stimulated samples were almost comparable (data not shown). We concluded that the frequencies of *Aspergillus*-specific T-cells in these patients were too low to exceed the background of intrinsically activated T-cells expressing CD154. These patients were excluded from further analysis. There was no difference in clinical stage of ABPA or use of systemic corticosteroids between patients with and without increase of CD154+ T-cells.

In patients with increased CD154-expression, we analyzed whether the different *A. fumigatus* antigens induced differences in cytokine profiles. In figure 5.1B the cytokine secretion profile of the *Aspergillus*-specific T-cells in patient HCA after restimulation with Asp4 is shown. The *Aspergillus*-specific T cells produced mainly TNF α , IL-4 and IL-5. Overall, the *Aspergillus*-specific T-cell immune responses in ABPA patients showed a mixed cytokine profile, although predominantly a Th2 phenotype was present (figure 5.1C). Activated CD4+ T-cells produced TNF α , with frequencies ranging from 19% to 95% of CD154+ cells. The majority of *Aspergillus*-specific T-cells produced besides TNF α mainly IL-4, and this was similar for all *A. fumigatus* antigens (figure 5.1C). Patient MMT showed mixed responses with TNF α and IL-4 production in response to Asp2 and Crf1, and TNF α , IFN γ and low IL-4 in response to Catalase1. *Aspergillus*-specific T-cells in patient JMR had a predominantly Th1 phenotype, although a high percentage of T-cells produced IL-4 upon Asp2 restimulation (figure 5.1C).

When we analyzed the immune responses against these *A. fumigatus* antigens in 4 healthy donors with this technique, in only 1 donor *Aspergillus*-specific T-cells were present, based on CD154-expression (figure 5.1D). Activated CD4+ T-cells in this individual produced TNF α and IFN γ (figure 5.1E). To further analyze the

Aspergillus antigen specificities and cytokine profiles in healthy donors, another restimulation protocol was used in which no MACS selection was performed ¹¹. The cytokine secretion profile of healthy donor HVM after restimulation with Crf1 is shown in figure 5.1F, and displays a Th1 phenotype with production of mainly TNF α and IFN γ . With this technique, used in 5 healthy donors, *Aspergillus*-specific T-cells were detected in response to Asp1, Asp2, Crf1 and Catalase1, and these T-cells produced primarily TNF α and IFN γ (figure 5.1G).

When we compared the cytokine profiles of *Aspergillus*-specific T-cells in ABPA patients ¹ and healthy donors, we observed a Th2 phenotype in ABPA patients with production of mainly IL-4 ($p=0.03$) and a Th1 phenotype in healthy donors with production of IFN γ ($p=0.01$) (figure 5.2).

In conclusion, in this study in which we analyzed T-cell responses against 6 different *A. fumigatus* antigens instead of focusing on one *Aspergillus* antigen as in previous studies ⁷⁻⁹, we demonstrate that in all individuals *Aspergillus*-specific immune responses were directed to a broad variety of *Aspergillus* antigens, and this was independent of the underlying health condition. Furthermore, in contrast to the previously raised hypothesis that the Th2 skewing of the *Aspergillus*-specific T-cell immune response in ABPA patients is dependent on specific *A. fumigatus* allergens ^{6,8,9}, we demonstrate that the Th2 phenotype of the *Aspergillus* T-cells was irrespective of the *A. fumigatus* antigen recognized.

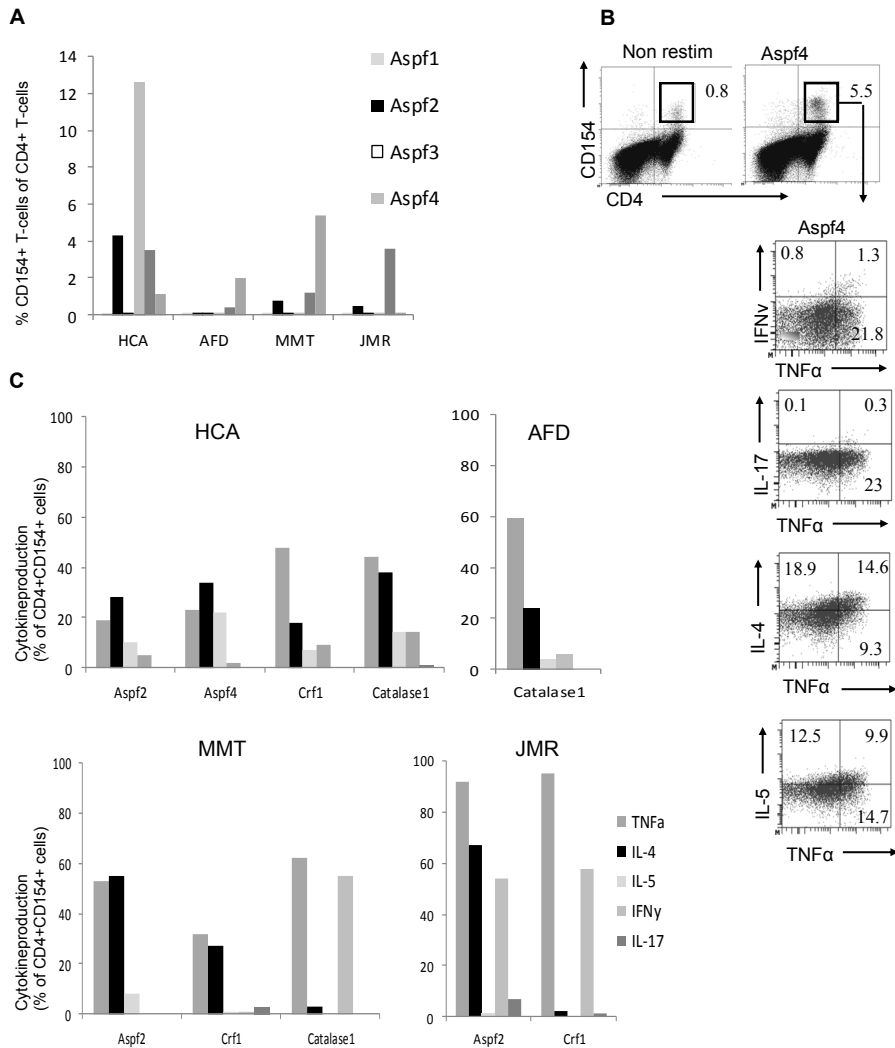


Figure 5.1 T-cell activation and cytokine production in response to different *A. fumigatus* antigens in ABPA patients and in healthy donors. PBMC from 4 ABPA patients and 1 healthy donor were stimulated with the mixture of 6 *A. fumigatus* antigens Aspf1, Aspf2, Aspf3, Aspf4, Crf1 and Catalase1, and activated CD4⁺ T-cells were selected by MACS based on CD154-expression and cultured for 3 weeks. Expanded T-cells were restimulated with the separate *A. fumigatus* antigens. Percentages of Aspergillus-specific CD4⁺ T-cells after restimulation in 4 ABPA patients (A) and 1 healthy donor (D), calculated as % CD154⁺ of CD4⁺ T-cells after restimulation with peptide-pulsed PBMC minus % CD154⁺ of CD4⁺ T-cells after restimulation with unpulsed PBMC. (B) Representative example of FACS-plot of ABPA patient HCA after restimulation with Aspf4-pulsed PBMC. Percentages of cytokine-producing T-cells of CD154⁺CD4⁺ T-cells in 4 ABPA patients (C) and 1 healthy donor (E). PBMC from 5 healthy donors were stimulated with the mixture of *A. fumigatus* antigens, cultured for 2 weeks and restimulated with the separate *A. fumigatus* antigens. (F) Representative example of FACS-plot of healthy donor HVM after restimulation with Crf1-pulsed PBMC. (G) Percentages of cytokine-producing T-cells of CD154⁺CD4⁺ T-cells in 5 healthy donors. T-cells were analyzed by FACS after 5 hours of restimulation.

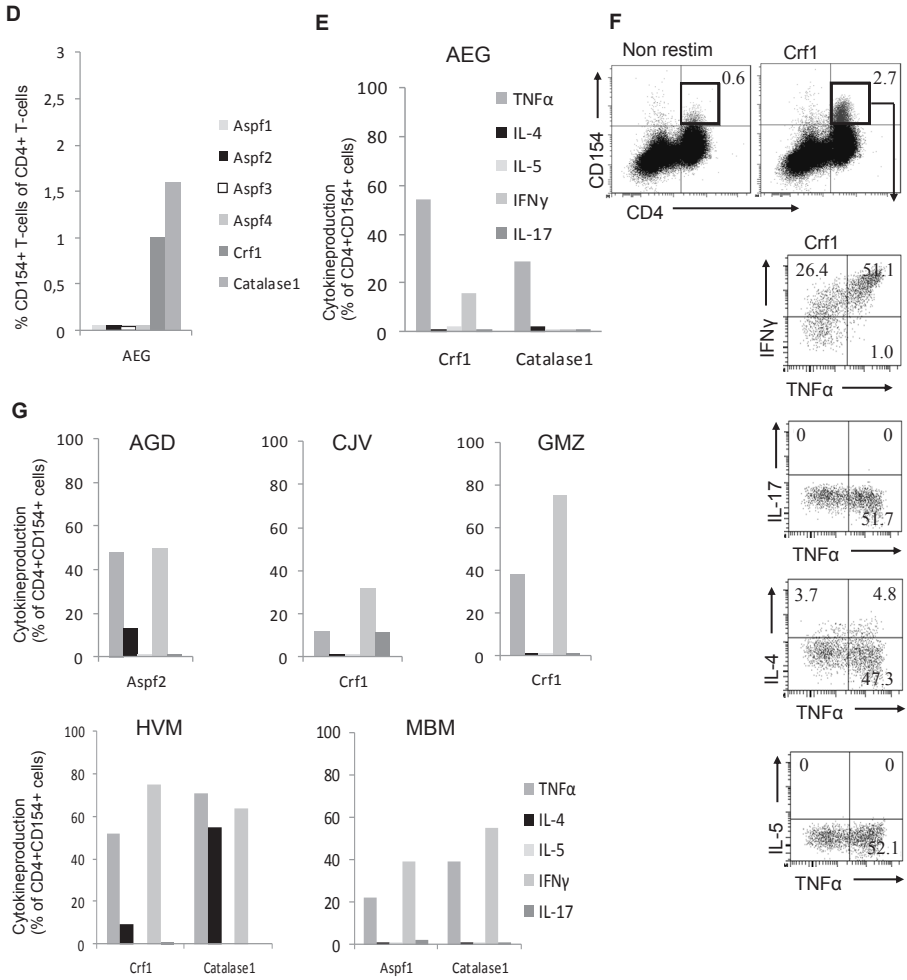


Figure 5.1 Continued

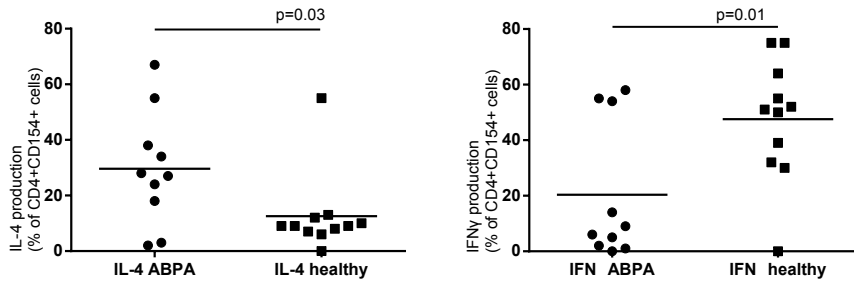
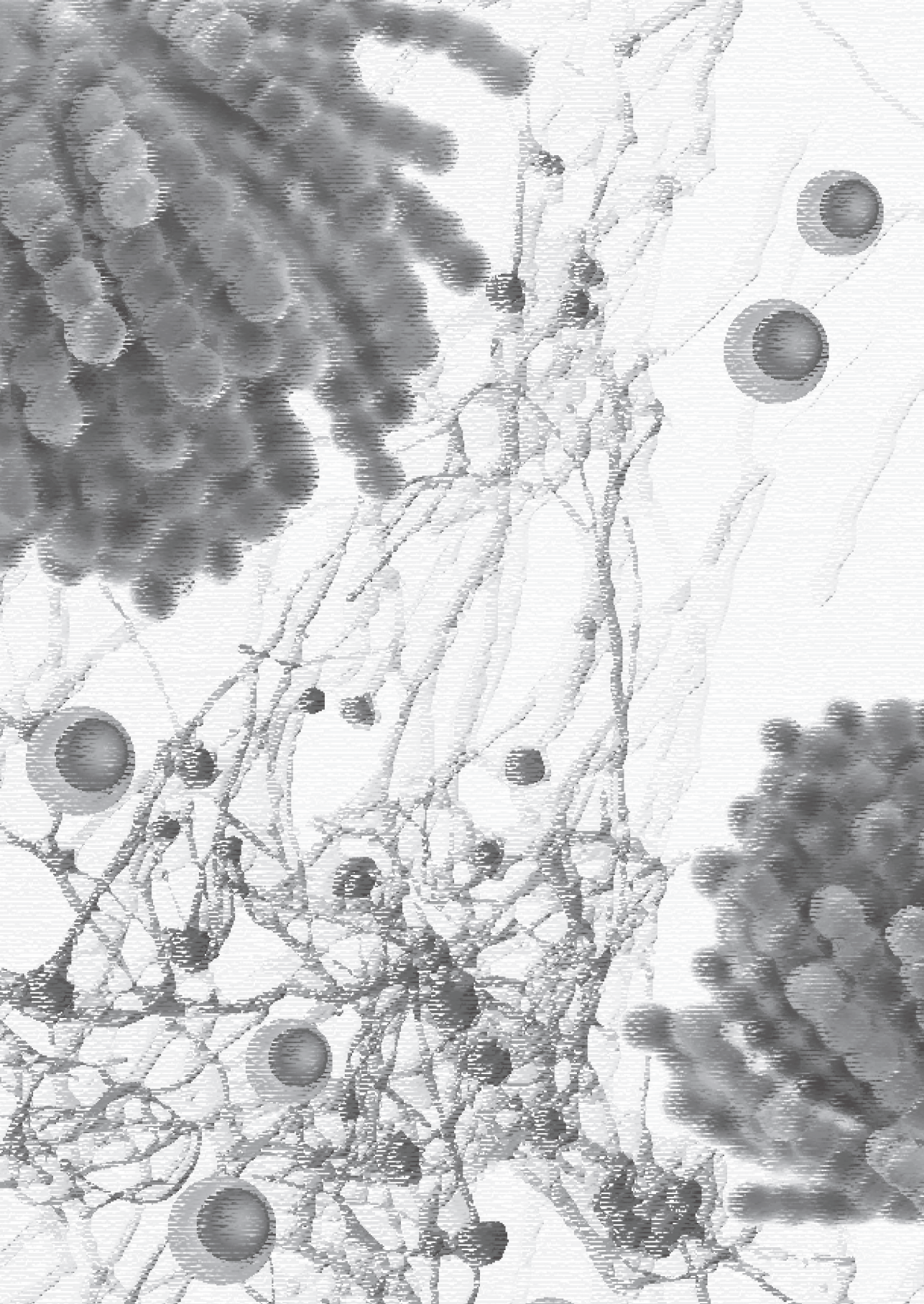


Figure 5.2 Cytokine profiles in ABPA patients and in healthy donors. (A) Percentages of IL-4-producing T-cells of CD154+CD4+ T-cells in ABPA patients compared to percentages of IL-4-producing T-cells of CD154+CD4+ T-cells in healthy donors. (B) Percentages of IFN γ -producing T-cells of CD154+CD4+ T-cells in ABPA patients compared to percentages of IFN γ -producing T-cells of CD154+CD4+ T-cells in healthy donors. Significance of difference between groups was tested by two-tailed unpaired Student T test.

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Chapter 6

Phagocytosis of *A. fumigatus* spores by neutrophil granulocytes is improved by activated CD4+ T-cells



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Abstract

Neutrophil granulocytes, monocytes and T-cells play important roles in the immune defense against *Aspergillus fumigatus*. However, the essential interactions between these cells to maximise the immune response are unknown. In flow cytometry-based phagocytosis assays the phagocytosis capacity of *A. fumigatus* spores by neutrophil granulocytes and monocytes and influence of activated CD4+ T-cells on this process was studied. After 30 minutes of phagocytosis time 45-76% of monocytes had phagocytosed *A. fumigatus* conidia, increasing to 85-100% after 3 hours. Activated CD4+ T-cells did not improve this. Phagocytosis capacity of neutrophils was less efficient with 5-26% having phagocytosed spores after 30 minutes and 11-31% after 3 hours. Activated CD4+ T-cells, however, improved phagocytosis capacity of these cells. Based on the much larger pool of neutrophils present in the body compared to monocytes and their short turn-over we anticipate that this interaction between T-cells and neutrophil granulocytes might be essential for increasing the defense against *A. fumigatus*.

Introduction

Although humans inhale hundreds of *Aspergillus fumigatus* spores every day, this only rarely leads to clinical symptoms. In the normal human host the immune system prevents the development of invasive aspergillus infection. Three different immune cells play a role in the defense against *Aspergillus fumigatus*: neutrophil granulocytes, monocytes and T-cells. Clinical observations have shown an increased risk of invasive aspergillosis in patients with prolonged neutropenia¹⁻³, and *in vitro* experiments confirmed that neutrophil granulocytes can phagocytose *Aspergillus* conidia⁴ and lead to damage of *Aspergillus* hyphae due to degranulation and the formation of neutrophil granulocyte traps (NETs)⁵⁻⁷. Monocytes and monocyte-derived cells like macrophages and dendritic cells are also capable of phagocytosing *Aspergillus* conidia. These phagocytic cells recognize *Aspergillus fumigatus* by one of their pathogen recognition receptors (PRRs) including the toll-like receptors (TLR) 2 and 4 and C-type lectin receptors Dectin-1 and DC-SIGN⁸⁻¹². Polymorphisms in the genes encoding these PRRs are associated with an increased risk of invasive aspergillosis^{13,14}. Finally, in recent years *Aspergillus*-specific T-cells have been identified in healthy individuals¹⁵⁻¹⁸ and in patients recovering from invasive aspergillosis^{17,19,20} at the moment of regression of aspergillus lesions. In mice and humans, the adoptive transfer of T-cell lines generated by stimulating with *Aspergillus* crude extracts or *A. fumigatus* conidia, improved the prognosis of invasive aspergillosis^{21,22}. However, it is unclear what the underlying mechanism is by which CD4+ T-cells exert their function in the immune response against *A. fumigatus*. In a previous study, direct killing of *Aspergillus* conidia and *Aspergillus* hyphae by *Aspergillus*-specific T-cells was observed, when tested in a FUN-1 conidiacidal assay or an XTT dye reduction assay²³. However, the mechanism of action remains unclear, because no antigen presenting cells were present in this assay, and thus T-cells cannot have been activated through a TCR interaction with *Aspergillus* peptide presented in the context of HLA. *Aspergillus*-specific lung-derived T-cells display similar to *Candida*-specific T-cells a Th17 cytokine profile, suggesting that T-cells *in vivo* may play a role in attraction of neutrophil granulocytes to the site of infection.

Thus, although there is evidence that neutrophil granulocytes, monocytes and T-cells are involved in the immune defense against *Aspergillus fumigatus*, little is known about the interplay between these immune cells and how they interact to maximise the immune response against *A. fumigatus*.

In this study we analyzed in an *in vitro* system the phagocytosis capacity of *A. fumigatus* conidia by monocytes and neutrophil granulocytes, and studied the influence of CD4+ T-cells on their phagocytosis capacity. The percentage of monocytes that had phagocytosed *A. fumigatus* spores was high, as well as the amount of spores phagocytosed per monocyte, and neither was increased in the presence of activated CD4+ T-cells. In contrast, the percentage of neutrophil granulocytes that had phagocytosed *A. fumigatus* conidia was low, and per

neutrophil granulocyte only one or few spores were engulfed. In the presence of activated CD4⁺ T-cells the percentage of neutrophil granulocytes that had phagocytosed spores increased, whereas no clear difference was observed in the amount of spores phagocytosed per neutrophil granulocyte.

Material and methods

Preparation of cells and *A. fumigatus* antigens

All studies were conducted with approval of the institutional review board of the Leiden University Medical Center. After informed consent was given in accordance with the Declaration of Helsinki, peripheral blood was obtained from hemochromatosis patients undergoing therapeutic flebotomy. To obtain a pure fraction of monocytes, peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Isopaque separation and subsequently labelled with anti-CD14 magnetic beads (Miltenyi Biotec, Leiden, Netherlands) for 15 minutes at 4°C. CD14⁺ cells were enriched by a LS MACS column (Miltenyi Biotec). To obtain a mixture of neutrophil granulocytes, monocytes and lymphocytes, we performed erythrocyte lysis with ice-cold NH₄Cl solution on freshly drawn peripheral blood. We did not perform any further procedures to purify the neutrophil granulocytes, because the phagocytosis capacity will be negatively influenced by such procedures.²⁴ Resting T-cells were isolated from erythrocyte lysed peripheral blood after labelling with anti-CD4 magnetic beads (Miltenyi Biotec) and enriched by an LS MACS column. For non-specific activation of T-cells CD3/28 beads were added to the resting T-cells in a bead : T-cell ratio of 1:5. *Aspergillus*-specific T-cell clones were previously obtained from healthy individuals and from patients with invasive aspergillosis by single cell sorting on the basis of upregulation of CD137- or CD154-expression in response to Crf1 or Catalase1 overlapping peptides.^{18;19}

Crf1 and Catalase1 overlapping peptides, consisting of 15mer peptides with an 11mer overlap, were synthesized by JPT Peptide Technologies (Berlin, Germany).

Flow cytometry-based phagocytosis assay

Spores of *A. fumigatus* were heat-inactivated for 30 minutes at 90°C and subsequently labelled with CellVue (Sigma-Aldrich, Zwijndrecht, Netherlands). For the labelling, 3x10⁷ spores were suspended in 250 µl Diluent C and subsequently 250 µl CellVue solution (2 µM in Diluent C) was added for 5 minutes at room temperature. Then, 500 µl fetal calf serum was added for 2 minutes to block the labelling. After labelling, spores were washed extensively and resuspended in PBS and stored at 4°C. Erythrocyte-lysed peripheral blood cells containing 1x10⁴ monocytes and 5x10⁴ granulocytes were incubated in 100 µl X-vivo (Lonza, Wijnchen, Netherlands) in a 96-well polypropylene plate at 4°C or 37°C overnight, or used directly. Cells were stained with anti-CD4 PerCP (BD/Pharmingen, Breda, Netherlands), anti-CD14 APC (BD/Pharmingen), anti-CD14 PE (BD/Pharmingen) or

anti-CD15 FITC (BD/Pharmingen) for 15 minutes at 4°C. Cells were washed and subsequently incubated at 37°C for 30 minutes or 3 hours with CellVue-labelled spores (2×10^5) in a 96-well polypropylene plate to allow phagocytosis of spores, making use of gravity to establish contact between spores and immune cells. At the end of the phagocytosis time samples were washed twice with PBS supplemented with EDTA to prevent aspecific binding of spores and cells. The percentages of CellVue+CD14+ cells of CD14+ cells or CellVue+CD15+ cells of CD15+ cells were analysed by flow cytometry on the Calibur II (BD).

To study the influence of T-cells on the phagocytosis capacity of monocytes and neutrophil granulocytes, the erythrocyte-lysed blood cell mixture was coincubated for 18 hours with MACS-enriched autologous CD4+ T-cells (3×10^4), either non stimulated (resting T cells) or activated with CD3/28 beads (activated T-cells) or an *Aspergillus*-specific T-cell clone (3×10^4) with or without pre-incubation with Crf1- or Catalase1-peptides. The peptide concentrations varied between 10^{-6} M and 10^{-14} M, depending on the Crf1- or Catalase1-specific T-cell clone used in the experiment; these concentrations corresponded to levels of IFN γ -production at IC90, IC50 and IC10 of the used T-cell clone. Whether the phagocytosis capacity of neutrophil granulocytes was influenced by cytokines was studied by adding IL-2 (100 IU/ml) or GM-CSF (100 ng/ml) to the erythrocyte-lysed peripheral blood cells, but no external T-cells. The percentages of CellVue+CD14+ cells of CD14+ cells or CellVue+CD15+ cells of CD15+ cells were analysed by flow cytometry on the Calibur II (BD).

Flow cytometry monocyte markers

To study activation of monocytes, MACS-enriched CD14+ cells (1×10^5) were incubated O/N with or without an HLA-matched *Aspergillus*-specific T-cell clone (3×10^4), with or without *Aspergillus* peptides (10^{-6} M) to activate the T-cell clone. The cells were stained with anti-CD3 APC (BD/Pharmingen), to be able to distinguish the monocytes from the *Aspergillus*-specific T-cell clone, and with the monocyte markers anti-CD14 FITC (BD/Pharmingen), anti-CD40 FITC (BD/Pharmingen), anti-CD86 (B7-2) PE (BD/Pharmingen) and anti-HLA-DR PE (BD/Pharmingen). Mean fluorescence intensity of the monocyte markers was analysed by flow cytometry on the Calibur II (BD).

Confocal fluorescence microscopy

For confocal fluorescence microscopy experiments mixtures of monocytes, neutrophil granulocytes and lymphocytes obtained from peripheral blood after erythrocyte lysis with ice-cold NH_4Cl solution were used. CellVue-labelled heat-inactivated spores were added to these mixtures for 30 minutes at 37°C. Subsequently cells were kept on ice and labelled with either anti-CD14 FITC to stain monocytes or anti-CD15 FITC to stain neutrophil granulocytes. Wells were visualized on a Leica SP5 microscope.

Results

Monocytes are more efficient in phagocytosing spores than neutrophil granulocytes

To study the phagocytosis capacity of monocytes and neutrophil granulocytes we set up a flow cytometry-based phagocytosis assay. We incubated erythrocyte-lysed peripheral blood cells, composed of approximately 12% monocytes, 25% lymphocytes and 63% neutrophil granulocytes with heat-inactivated CellVue-stained *A. fumigatus* spores, for 30 minutes or 3 hours at 37 degrees. Repeated phagocytosis experiments showed considerable variation in phagocytosis of *A. fumigatus* spores. We observed that already after 30 minutes of phagocytosis time 45% to 76% of the monocytes had phagocytosed *A. fumigatus* spores, which increased to 85% to 100% after 3 hours of phagocytosis time. An example is shown in figure 6.1A. Besides an increase in the percentage of monocytes that had phagocytosed spores, we also found an approximately 10-fold increase of mean fluorescence intensity (MFI) of CellVue-positive monocytes from MFI 330 at 30 minutes to MFI 4250 at 3 hours (figure 6.1A), indicating also an increase of CellVue-stained spores phagocytosed per monocyte. In contrast, only 5% to 26% of neutrophil granulocytes had phagocytosed spores after 30 minutes of phagocytosis time, with a minor increase to 11% to 32% after 3 hours and with only a 2-fold increase in MFI (133 to 280) (figure 6.1B). Control phagocytosis experiments at 4°C to correct for adhesion of spores showed a range of 2% to 20% CellVue-positive cells for monocytes, and 1% to 13% CellVue-positive cells for neutrophil granulocytes. Examples are shown in figures 6.1A and B.

To confirm the differences in phagocytosis between monocytes and neutrophil granulocytes observed in the flow cytometry experiments, we performed confocal fluorescence microscopy after 30 minutes of phagocytosis time at 37 degrees. Indeed, already after 30 minutes of phagocytosis time, nearly all monocytes (CD14-FITC positive) had phagocytosed spores and per monocyte approximately 8-10 spores were phagocytosed (figure 6.1C), whereas only part of the neutrophil granulocytes stained with CD15 FITC had phagocytosed spores and per neutrophil granulocyte only 1 spore was phagocytosed (figure 6.1D).

In conclusion, both the flow cytometry-based phagocytosis assay and the confocal fluorescence microscopy showed differences in phagocytosis of *A. fumigatus* spores by monocytes and neutrophil granulocytes, with almost 100% of monocytes phagocytosing spores after 3 hours of phagocytosis time compared to 32% of neutrophil granulocytes. In addition, the amount of spores phagocytosed per cell was almost 10-fold higher for monocytes compared to neutrophil granulocytes.

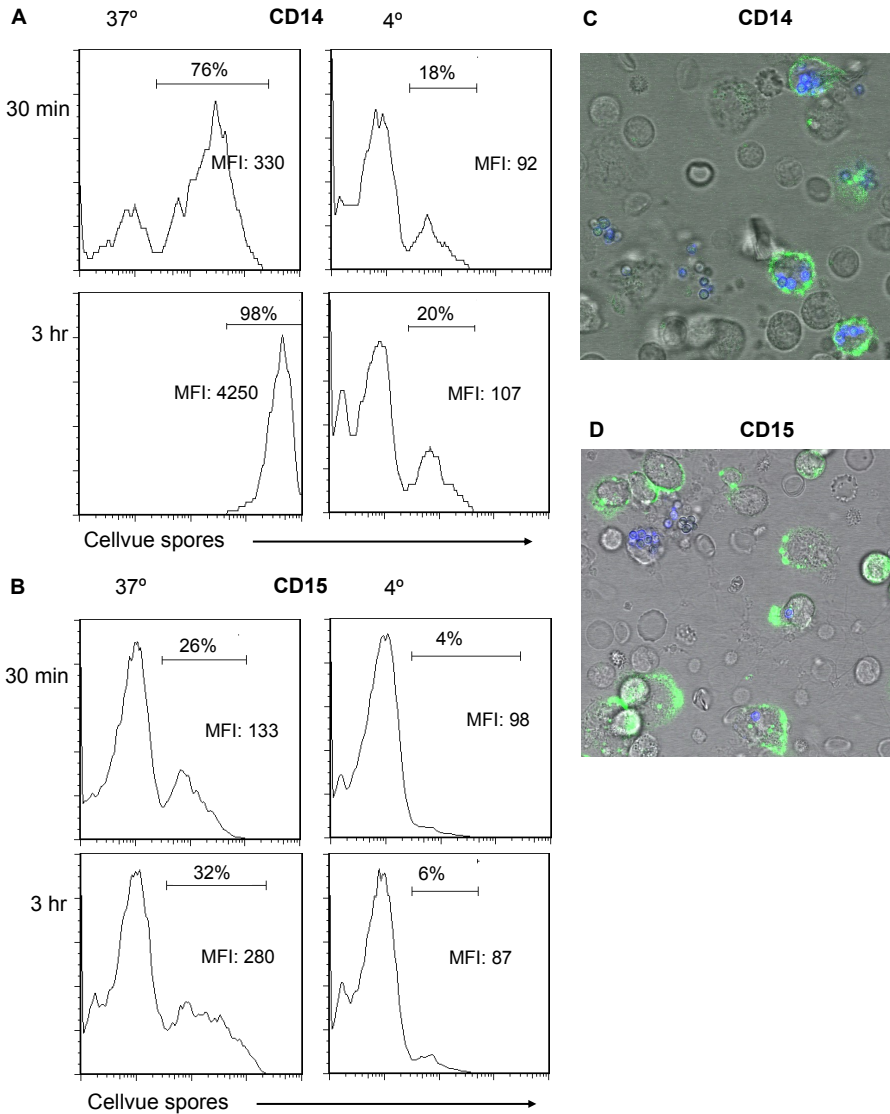


Figure 6.1 Phagocytosis of spores by monocytes is more efficient than by neutrophil granulocytes. Erythrocyte-lysed peripheral blood cells containing 1×10^4 monocytes and 5×10^4 neutrophil granulocytes were incubated with 2×10^5 CellVue-labelled spores for 30 minutes or 3 hours at 37°C. After incubation, cells were stained with anti-CD14 PE and anti-CD15 FITC and analyzed by FACS. To correct for aspecific binding of spores to blood cells, control experiments were performed at 4 °C. Cells were gated on CD14+ cells (A) or CD15+ cells (B). The % CellVue+ cells of CD14+ or CD15+ cells, and the mean fluorescence intensity (MFI) of the CellVue positive population is indicated in the figures. For confocal fluorescence microscopy, peripheral blood cells were coincubated with CellVue-labelled spores for 30 minutes at 37°C, and stained with either anti-CD14 FITC (C) or anti-CD15 FITC (D) and visualized on a Leica SP5 microscope.

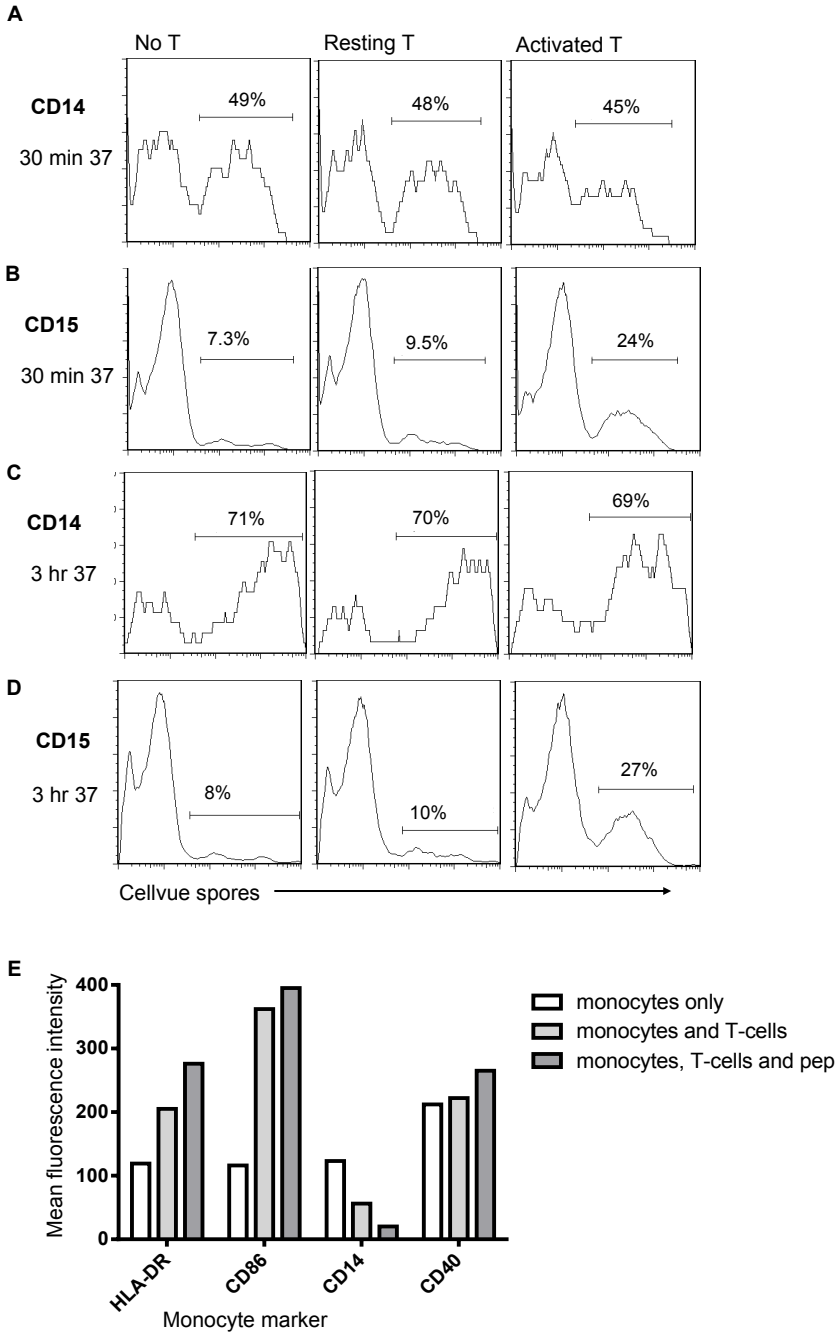
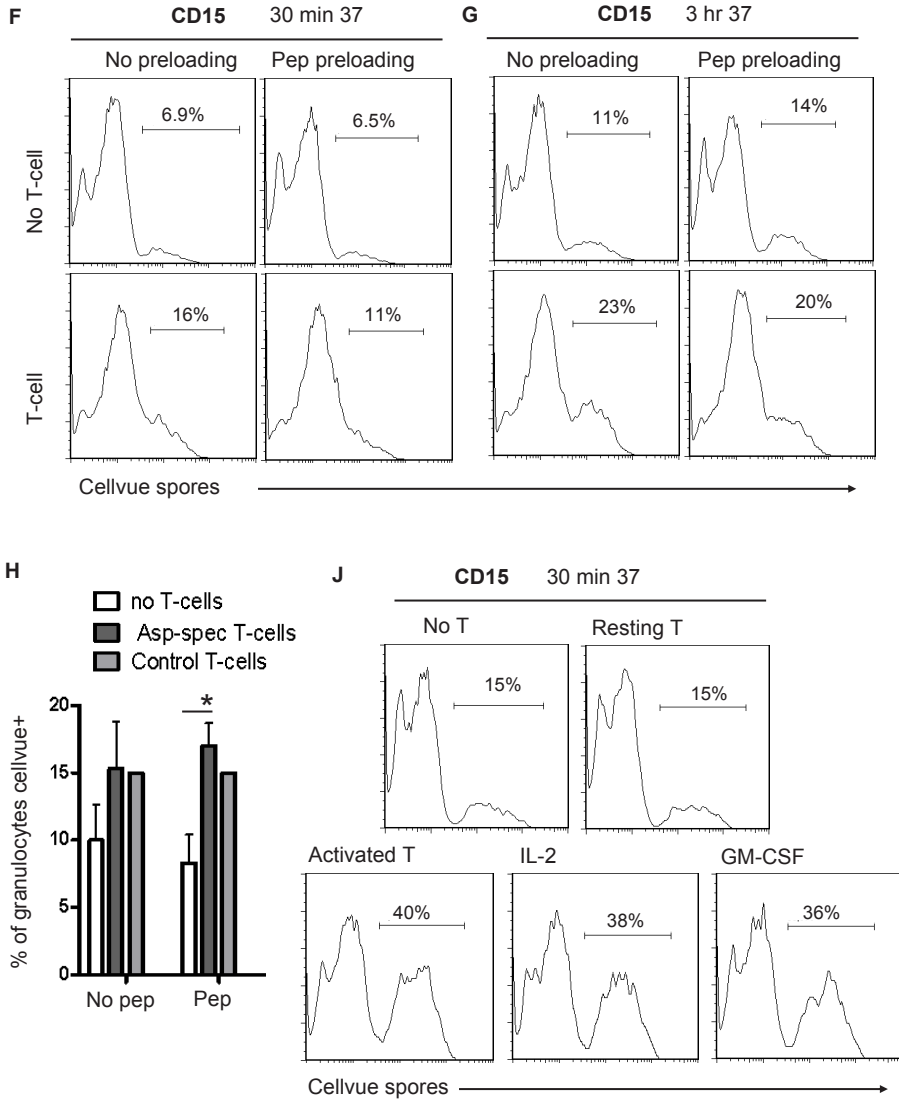


Figure 6.2 Influence of CD4⁺ T-cells on phagocytosis capacity of monocytes and neutrophil granulocytes.

**Figure 6.2 Continued**

(A-D) Erythrocyte-lysed peripheral blood cells containing 1×10^4 monocytes and 5×10^4 neutrophil granulocytes were coincubated overnight (ON) with 3×10^4 resting CD4⁺ T-cells or CD3/28-bead activated T-cells. After ON incubation, 2×10^5 CellVue-labelled spores were added for 30 minutes or 3 hours at 37 °C, cells were stained with anti-CD14 PE and anti-CD15 FITC, analyzed by FACS and gated on CD14⁺ cells (A+C) or CD15⁺ cells (B+D).

(E) 1×10^5 monocytes were coincubated ON with 3×10^4 Aspergillus-specific T-cells with or without preloading with *A. fumigatus* peptides. After ON incubation, cells were stained with several monocyte markers and anti-CD3 APC. FACS-plots were gated on the CD3-negative population.

(F-H) Erythrocyte-lysed peripheral blood cells containing 1×10^4 monocytes and 5×10^4 neutrophil granulocytes were coincubated ON with Aspergillus-specific T-cells with or without preloading with

Figure 6.2 Continued

A. *fumigatus* peptides. After ON incubation, CellVue-labelled spores were added for 30 minutes (F) or 3 hours (G) at 37 °C, cells were stained with anti-CD15 FITC, analyzed by FACS and gated on the CD15+ population. (H) Percentages of CellVue+ neutrophil granulocytes in the presence or absence of *Aspergillus*-specific T-cells. Results of 3 independent experiments. In one experiment activated T-cells with a different specificity were used as control T-cells. (I) CellVue-stained spores were added for 30 minutes at 37 °C to erythrocyte-lysed peripheral blood after ON incubation with IL-2 or GM-CSF, cells were stained with anti-CD15 FITC, analyzed by FACS and gated on CD15+ population. Percentages indicated are the percentages CD15+CellVue+ cells of the CD15+ cells.

* Significant difference between groups, tested by two-tailed unpaired student T test ($p=0.005$)

Activated T-cells increase the percentage of neutrophil granulocytes that phagocytose *Aspergillus* spores

Next, we studied the effect of CD4+ T-cells on the phagocytosis capacity of monocytes and neutrophil granulocytes. Peripheral blood cells after erythrocyte lysis were coincubated overnight with MACS enriched autologous CD4+ T-cells without further treatment (resting T-cells) or after CD3/28-bead activation (activated T-cells). The next day, the cell mixture was incubated with CellVue-stained spores for 30 minutes or 3 hours and stained for the markers CD14, CD15 and CD3 and analyzed by flow cytometry.

The percentage of monocytes that had phagocytosed spores after 30 minutes was around 50% and did not increase after adding resting or activated T-cells (figure 6.2A). The percentage of neutrophil granulocytes that had phagocytosed spores was below 10% and did not increase by adding resting T-cells. However, an increase to 24% was seen after coincubation with CD3/28-bead activated T-cells (figure 6.2B). A similar pattern was seen after 3 hours of phagocytosis time (figure 6.2C and D).

Subsequently, we studied the effect of *Aspergillus*-specific T-cells on the phagocytosis capacity of monocytes and neutrophil granulocytes. Peripheral blood cells after erythrocyte lysis were incubated overnight with an HLA-matched *Aspergillus*-specific T-cell clone with or without *Aspergillus* peptides to activate the T-cell clone. The next day, after incubation with CellVue-stained spores for 30 minutes or 3 hours, monocytes and neutrophil granulocytes were stained for the markers CD14 and CD15 respectively and analyzed by flow cytometry.

Similarly as with aspecifically activated CD4+ T-cells, no increase in the percentage of monocytes that had phagocytosed spores was observed, when coincubated with *Aspergillus*-specific T-cells (data not shown), although activation of the monocytes by the T-cell clone was observed (figure 6.2E). In contrast, an increase in the percentage of neutrophil granulocytes that had phagocytosed spores was observed when *Aspergillus*-specific T-cells were added, which was not dependent on preloading of PBMC with *Aspergillus* peptides to facilitate activation of the T-cell clones (figure 6.2F-H). This suggested an effect of the T-cell clones that was independent of peptide-specific activation. In addition, when we added a T-cell clone with a different specificity a similar increase in the percentage of

neutrophil granulocytes that had phagocytosed spores was seen, comparable to the effect of adding *Aspergillus*-specific T-cells (figure 6.2H). Although, by performing IFN γ ELISA, we could not demonstrate activation of the *Aspergillus*-specific T-cell clones in the absence of preloading of PBMC with peptides (data not shown), we hypothesize that T-cell clones in culture are nevertheless in an activated state compared to T-cells isolated directly from peripheral blood, and therefore can influence phagocytosis capacity of neutrophil granulocytes. When we added IL-2 or GM-CSF to the wells but no T-cells, the percentage of neutrophil granulocytes that phagocytosed spores also increased. (figure 6.2J).

These data show that activated CD4 $^{+}$ T-cells can improve the phagocytosis capacity of neutrophil granulocytes, presumably by secretion of cytokines like IL-2 and GM-CSF. In contrast, the phagocytosis capacity of monocytes is already quite efficient, and is not influenced by the addition of activated CD4 $^{+}$ T-cells.

Discussion

We studied in an *in vitro* system the phagocytosis of *A. fumigatus* by monocytes and neutrophil granulocytes and the influence of CD4 $^{+}$ T-cells on their phagocytosis capacity. Our experiments showed that monocytes can phagocytose spores very efficiently, with no clear additional effect of adding CD4 $^{+}$ T-cells, although cocubation of monocytes with CD4 $^{+}$ T-cells did change the expression of several monocyte markers. In contrast, the phagocytosis capacity of neutrophil granulocytes was improved by *Aspergillus*-specific T-cell clones or aspecifically activated CD4 $^{+}$ T-cells, whereas resting CD4 $^{+}$ T-cells only had minimal influence on phagocytosis by neutrophil granulocytes.

Humans are exposed to *A. fumigatus* by inhalation of *Aspergillus* spores. The first way to prevent further spread of aspergillus infection is to phagocytose and kill these spores. It was shown previously that monocytes ⁴ and macrophages ²⁵ can phagocytose *Aspergillus* spores very efficiently. Analyzing the capacity of monocytes to phagocytose *A. fumigatus* spores demonstrated that 45-76% of monocytes had phagocytosed *A. fumigatus* spores after 30 minutes, and 85-100% of monocytes had phagocytosed *A. fumigatus* spores after 3 hours of phagocytosis time.

Although we could show that adding T-cells to the monocytes resulted in monocyte activation, demonstrated by upregulation of CD86 and HLA-DR and downregulation of CD14, we did not observe an increase in phagocytosis capacity of the monocytes, probably due to already very efficient phagocytosis of *Aspergillus* spores by monocytes. However, we cannot exclude that CD4 $^{+}$ T-cells may influence the phagocytosis capacity of monocytes *in vivo*, in case monocytes are less activated in the *in vivo* situation. To minimize activation of monocytes by components present in medium or attachment to the plates, we tried to eliminate these factors as much as possible. For this purpose, we cultured monocytes in

X-vivo medium without any serum components, and used polypropylene plates instead of polystyrene plates to prevent activation. Under these circumstances, phagocytosis capacity of monocytes was still high and not improved by T-cells.

Besides monocytes and macrophages, also neutrophil granulocytes are capable of phagocytosing and killing spores²⁶, albeit to a lesser extent. In our experiments phagocytosis by neutrophil granulocytes varied considerably, 5-26% of neutrophil granulocytes phagocytosed spores the first 30 minutes, increasing to 11-32% after 3 hours of phagocytosis time. This is significantly lower than the percentages of monocytes that had phagocytosed spores after 30 minutes and 3 hours of phagocytosis time. Percentages of neutrophil granulocytes that phagocytosed spores increased when we added activated T-cells, but not when we added resting T-cells. Similar results were seen by adding *A. fumigatus*-specific T-cell clones, this was however irrespective of activation of the T-cell clones by peptide-preloaded monocytes. Furthermore, a non-*Aspergillus*-specific T-cell clone could elicit a similar increase in phagocytosis by the neutrophil granulocytes. Probably, the specificity and recent TCR triggering of the T-cell is not important, because T-cell clones are due to *in vitro* culture in an activated state. The effect of activated T-cells on neutrophil granulocyte function is probably mediated by the secretion of cytokines, since addition of the cytokines IL-2 and GM-CSF increased the phagocytosis capacity of neutrophil granulocytes. Others have shown a favourable effect of G-CSF and IFN γ on neutrophil granulocyte function.²⁷

The difference in phagocytosis capacity between macrophages, monocytes and neutrophil granulocytes may be explained by the difference in cell surface of these phagocytosing cells. In an *in vitro* phagocytosis assay different rate-limiting steps are measured: probability of contact between spore and immune cell, attachment to the cell surface, ingestion, intracellular transport and killing. Because the probability of contact for cells with a large cell surface, like macrophages and monocytes, and spores is higher than for cells with a smaller cell surface like neutrophil granulocytes, this can affect the phagocytosis capacity that is measured. However, in our *in vitro* system the amount of neutrophil granulocytes in the system was approximately 5 times higher than the amount of monocytes, increasing the probability of contact of spores with neutrophil granulocytes. This did however, not lead to a higher percentage of neutrophil granulocytes that had phagocytosed spores compared to the percentage of monocytes. Therefore we think it is unlikely that the high percentage of monocytes that have phagocytosed spores, nor the high amount of spores phagocytosed per monocyte is explained by a higher probability of contact between spores and monocytes compared to neutrophil granulocytes.

The first line of defense in the lungs are the alveolar macrophages, phagocytosing inhaled *Aspergillus* conidia. The population of alveolar macrophages is limited, and when the phagocytosis capacity of these cells is saturated, additional phagocytosis capacity is needed to prevent outgrowth of *aspergillus* leading to overt infection. We hypothesize that T-cells play an important role in the expansion and activation

of phagocytosing cells in the lungs. Although we and others have shown that neutrophil granulocytes are less efficient in phagocytosing *Aspergillus* spores than monocytes and macrophages, neutrophil granulocytes may have an important role in preventing outgrowth of hyphae after exposure to *A. fumigatus*. There is a large pool of neutrophil granulocytes present in the body, approximately 5 times larger than the monocyte population, and due to the short circulating half-life of neutrophil granulocytes which is only 6-8 hours²⁸ the neutrophil granulocyte pool is constantly renewed²⁸. When additional phagocytosing cells are needed, the potential of neutrophil granulocytes is therefore much larger than that of the monocyte population. We have recently shown that similar to *Candida*-specific T-cells also lung-derived *Aspergillus*-specific T-cells have a Th17 cytokine profile, which supports the hypothesis that *Aspergillus*-specific T-cells in the lungs will mainly attract neutrophil granulocytes to expand the population of phagocytosing cells in case of exposure to *A. fumigatus*. Furthermore, as we have shown in this study, activated T-cells can help to increase the phagocytosis capacity of neutrophil granulocytes.

In conclusion, we studied the effect of CD4+ T-cells in the early immune response against *A. fumigatus* by measuring the influence of CD4+ T-cells on the phagocytosis capacity of monocytes, macrophages and neutrophil granulocytes. Activated CD4+ T-cells improved the phagocytosis capacity of neutrophil granulocytes, but had no effect on monocytes and macrophages, which were already very efficient in phagocytosing *A. fumigatus* spores.

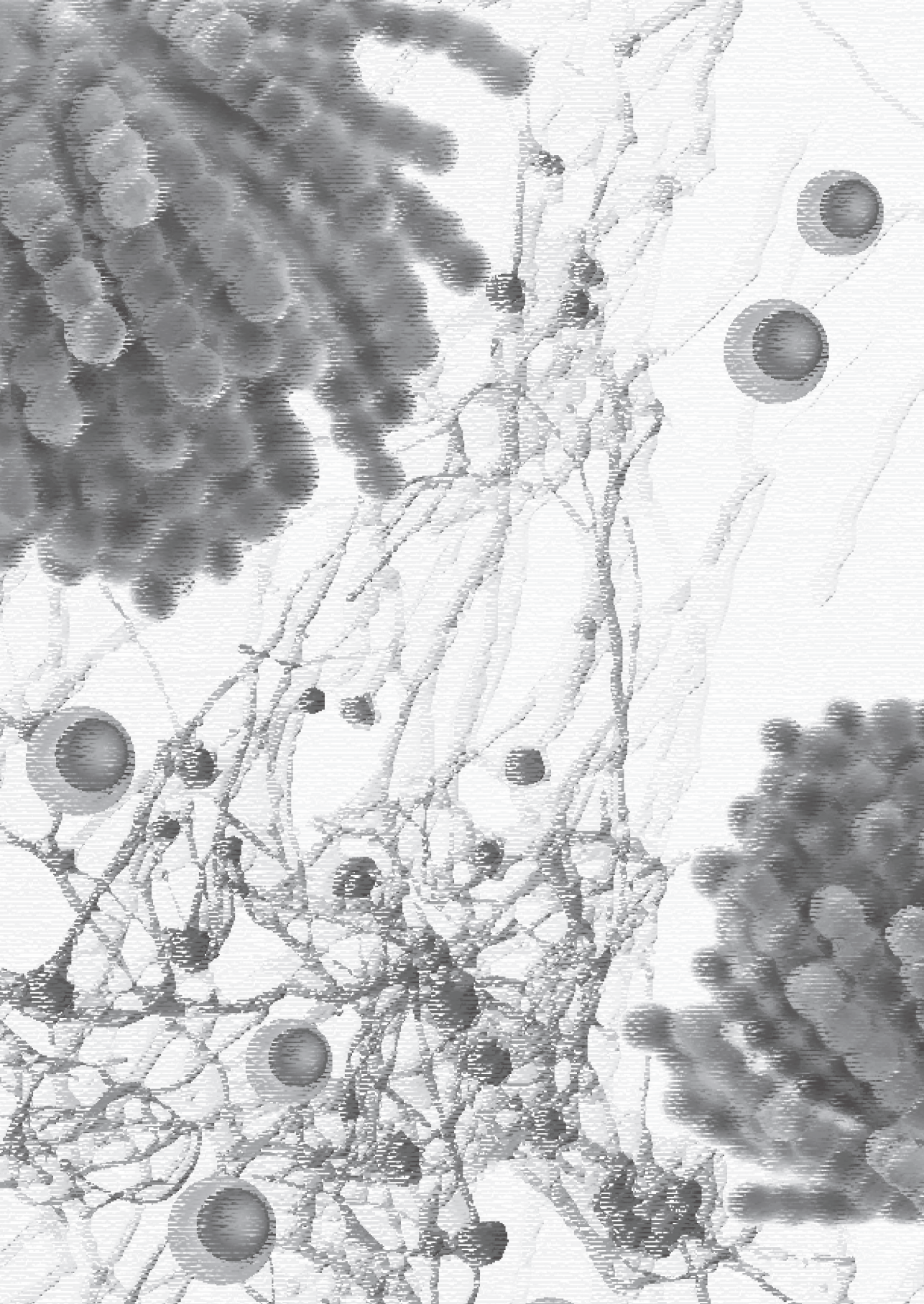
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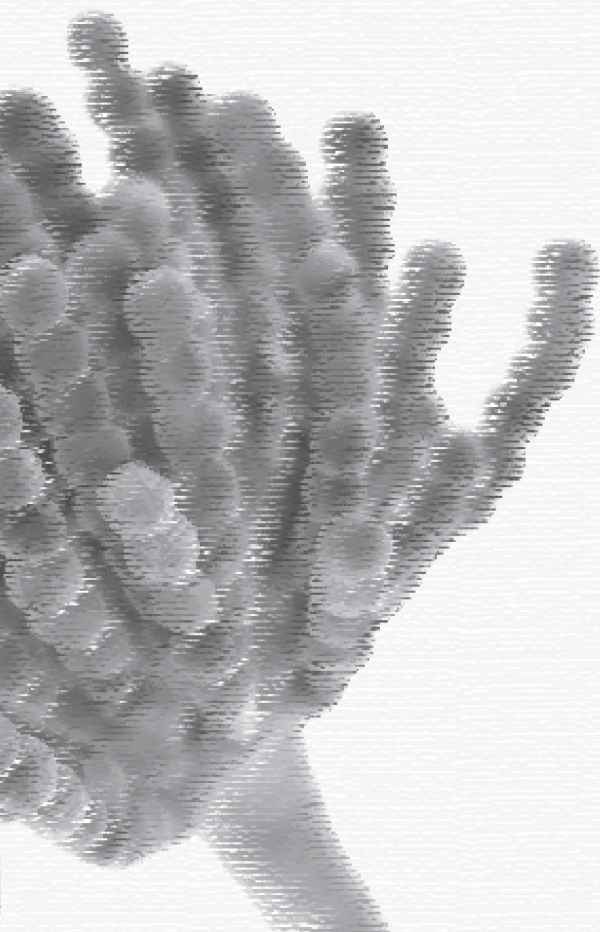
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Chapter 7

Summary



Summary

Aspergillus fumigatus is a mould that is ubiquitously present and hundreds of spores of *A. fumigatus* are inhaled by humans every day. In humans with a normal immune system this has no clinical consequences. However, patients who are severely immunocompromised are at risk for invasive aspergillosis. On the other hand, patients with asthma or cystic fibrosis can overreact upon exposure to *A. fumigatus* spores and develop allergic bronchopulmonary aspergillosis (ABPA).

Known risk factors for invasive aspergillosis are neutropenia, prolonged use of corticosteroids and allogeneic stem cell transplantation (alloSCT). The role of neutrophil granulocytes in the immune response against *Aspergillus fumigatus* has already been studied in the 1970s by Lehrer et al, and showed a role for these cells in response to *A. fumigatus* conidia as well as *A. fumigatus* hyphae. Other phagocytosing cells like monocytes, macrophages and dendritic cells (DC) have been shown to play a role as well, both in *in vitro* studies, as well as in genetic studies showing an increased risk of invasive aspergillosis in patients with polymorphisms in genes encoding toll-like receptors (TLRs) expressed by these immune cells and other genes involved in innate immunity. Because the T-cell mediated immunity after allogeneic stem cell transplantation is impaired for a prolonged period, also after recovery of neutrophil counts, the increased risk of invasive aspergillosis after this therapy suggests that T-cell mediated immunity might be important in the immune response against *A. fumigatus* as well.

Aspergillus-specific T-cell mediated immunity has been studied in healthy individuals, in whom *Aspergillus*-specific T-cells with a Thelper1 phenotype were identified by stimulating peripheral blood mononuclear cells (PBMC) with crude *Aspergillus* extracts or *A. fumigatus* recombinant proteins. In one study, patients with invasive aspergillosis were studied, showing *Aspergillus*-specific T-cells in peripheral blood with a Thelper1 cytokine profile in patients with invasive aspergillosis with a favourable outcome, similar as in healthy individuals. To further characterize the T-cell immune response against *Aspergillus fumigatus* and study the broadness of the immune response, epitope specificities and human leukocyte antigen (HLA)-restrictions, we used overlapping peptides of *Aspergillus* proteins for stimulation of peripheral blood-derived T-cells instead of crude *Aspergillus* extracts or *A. fumigatus* recombinant proteins. Furthermore we studied frequencies and cytokine profiles of *Aspergillus*-specific T-cells in lung, the primary location of *Aspergillus* exposure. To get insight in the possible role of *Aspergillus*-specific T-cells in the immune response against *Aspergillus fumigatus*, we studied the T-cell immune response against *A. fumigatus* in peripheral blood cells of patients with invasive aspergillosis at different time points during the course of aspergillus infection, complementary to an earlier study by Hebart et al. in patients with invasive aspergillosis that reported a single-time point analysis.

Studies on *Aspergillus*-specific adaptive immunity in healthy individuals and patients with invasive aspergillosis were limited, whereas patients with ABPA,

another *Aspergillus*-related disease, have been studied more extensively. Patients with ABPA exhibit an allergic reaction upon exposure to *A. fumigatus*. In these patients IgE and IgG antibodies against *A. fumigatus* have been shown to be present, as well as *Aspergillus*-specific T-cells that display a Thelper2 phenotype. This is not surprising, since allergic diseases are associated with Thelper2 CD4+ T-cells. However, it is unclear why *Aspergillus*-specific T-cells in these patients exhibit a Thelper2 phenotype. Authors of previous studies suggested that certain *Aspergillus* antigens were more allergenic than others in patients with ABPA. In our study we investigated the hypothesis postulated by these authors.

Finally, we studied the interactions between *Aspergillus*-specific T-cells and phagocytic cells – monocytes and neutrophil granulocytes- in an *in vitro* model to further elucidate the role of *Aspergillus*-specific T-cells in the immune response against *A. fumigatus*.

In **chapter 2** the *Aspergillus*-specific T-cell immune responses in healthy individuals were analyzed. PBMC from 24 healthy individuals were stimulated with overlapping peptides of the 2 *A. fumigatus* proteins Crf1 and Catalase1. Directly ex vivo no *Aspergillus*-specific T-cells could be identified. To increase the sensitivity we used a restimulation protocol: PBMC were stimulated with the overlapping peptides, cultured for 1 week and subsequently restimulated with the overlapping peptides. On the basis of CD154 expression and IFN γ production *Aspergillus*-specific T-cells were identified in 18 of 24 healthy individuals. We selected *Aspergillus*-specific T-cells in 9 donors on the basis of CD137 expression using the restimulation protocol and clonally expanded these T-cells. Crf1- and Catalase1-specific clones were isolated in 8 of 9 donors. Epitope specificity was diverse and the majority of clones had a Thelper1 phenotype with mainly production of IFN γ . *Aspergillus*-reactivity was confirmed by stimulating the clones with *Aspergillus* crude extract or with Catalase1 recombinant protein. In conclusion, in healthy individuals the *Aspergillus*-specific T-cell immune response in peripheral blood has a broad recognition pattern and shows a Thelper1 phenotype.

In **chapter 3** we describe the analysis of *Aspergillus*-specific T-cell responses at different times during the course of invasive aspergillosis. Thirty-three patients who suffered from invasive aspergillosis after allogeneic stem cell transplantation were screened for inclusion in the study. In 22 patients the median survival time was only 4 weeks, and therefore no peripheral blood samples were available, or no T-cells could be identified in the available material. The other 11 patients were included in the study, their median survival time was 9 months. Peripheral blood samples before and at several times after the diagnosis of invasive aspergillosis were stimulated with overlapping peptides of Crf1 and Catalase1. In 9 patients with a favorable course of the aspergillus infection *Aspergillus*-specific T-cells were identified around the time of improvement of aspergillus lesions. These T-cells mainly produced IFN γ and recognized a broad variety of T-cell epitopes. In 2 patients with progressive aspergillus infection, no *Aspergillus*-specific T-cells were identified. These data show a correlation between the occurrence of *Aspergillus*-

specific T-cells and improvement of invasive aspergillosis, suggesting that *Aspergillus*-specific T-cells may help in the clearance of an aspergillus infection.

In peripheral blood of healthy individuals and patients with invasive aspergillosis *Aspergillus*-specific T-cells display a Thelper1 phenotype. However, in other fungal infections T-cells mainly have a Thelper17 cytokine profile. We hypothesized that the cytokine profiles in the lung, the primary location of *A. fumigatus* exposure, might be different than in peripheral blood. In **chapter 4** the frequencies and cytokine profiles of *Aspergillus*-specific T-cells in lung and in peripheral blood of chronic obstructive pulmonary disease (COPD) patients were analyzed. Mononuclear cells were isolated from lung material derived from 10 COPD patients who underwent a lung transplantation or a partial lung resection because of a peripherally located lung tumor. From 3 of these patients also peripheral blood samples were available. Furthermore, 7 additional COPD patients were included of whom only peripheral blood samples were available. Mononuclear cells from lung or peripheral blood were stimulated and restimulated with overlapping peptides of the 6 *A. fumigatus* proteins Crf1, Catalase1, Aspf1, Aspf2, Aspf3 and Aspf4. The activation marker CD154 and cytokine production of TNF α , IFN γ , IL-4, IL-5 and IL-17 were analyzed by flow cytometry with an intracellular staining protocol. Frequencies of *Aspergillus*-specific T-cells in lung and peripheral blood of COPD patients were comparable. However, cytokine profiles of *Aspergillus*-specific T-cells in both compartments were significantly different: *Aspergillus*-specific T-cells in the lung showed a Thelper17 cytokine profile with mainly production of IL-17, whereas *Aspergillus*-specific T-cells in peripheral blood displayed a Thelper1 phenotype with mainly production of IFN γ , similar as was shown in previous studies in healthy individuals and patients with invasive aspergillosis. No differences in TNF α production were identified, indicating that the activation and ability to produce cytokines were not different in both compartments. In conclusion, *Aspergillus*-specific T-cells in the lung have a Thelper17 phenotype, similar as the T-cell cytokine profiles in other fungal infections like *Candida albicans*.

In one patient group, patients with asthma or cystic fibrosis who suffer from ABPA, *Aspergillus*-specific T-cells in peripheral blood have been reported to be of a Thelper2 phenotype. In **chapter 5** we investigated whether certain *A. fumigatus* antigens are more allergenic, i.e., inducing this Thelper2 phenotype, than others. PBMC derived from ABPA patients or from healthy individuals were stimulated with overlapping peptides of 6 *A. fumigatus* antigens, activated CD4 $^{+}$ T-cells were subsequently isolated by magnetic-activated cell sorting (MACS) on the basis of CD154 expression. After a 3-week culture period, cells were restimulated with overlapping peptides of the separate *A. fumigatus* antigens and cytokine production was analyzed by flow cytometry. In both populations T-cells directed to different *A. fumigatus* proteins were identified and there were no differences in the antigen-specificities of *Aspergillus*-specific T-cells between ABPA patients and healthy individuals. In ABPA patients *Aspergillus*-specific T-cells had a Thelper2 phenotype, whereas *Aspergillus*-specific T-cells in healthy individuals had a

Thelper1 phenotype. Cytokine profiles were irrespective of the *Aspergillus* antigens used for stimulation, but dependent on the underlying health condition.

In **chapter 6** we set up an *in vitro* model to investigate the interplay between the different immune cells known to be involved in the immune response against *A. fumigatus*. Cell subsets were isolated from peripheral blood by MACS selection on the basis of cell surface markers. The different immune cells were labelled with fluorochromes coupled to cell surface markers and *A. fumigatus* conidia were heat-inactivated and stained with Cellvue. Phagocytosis capacity of monocytes and neutrophil granulocytes were analyzed by a flow cytometry based assay. The percentage of monocytes that had phagocytosed spores after 30 minutes and 3 hours was high, up to 95%, and per monocyte 8-10 spores were phagocytosed. Addition of T-cells did not improve phagocytosis capacity of the monocytes. In contrast, phagocytosis capacity of neutrophil granulocytes was much lower, maximum 30% of neutrophils had phagocytosed spores at 3 hours, and only one or few spores were phagocytosed per neutrophil granulocyte. Addition of *Aspergillus*-specific T-cell clones, or nonspecifically activated T-cells resulted in an increased percentage of neutrophil granulocytes that had phagocytosed spores. This effect was probably mediated by cytokine secretion by the T-cells, like IL-2 or GM-CSF. In conclusion, the phagocytosis capacity of neutrophil granulocytes was increased by activated T-cells. Because the pool of neutrophil granulocytes is much larger compared to monocytes, we hypothesize that the interaction between T-cells and neutrophil granulocytes may add importantly to the immune response against *A. fumigatus*.

General discussion

The results described in this thesis demonstrate that the *Aspergillus*-specific T-cell mediated immune response is directed to a broad range of *A. fumigatus* proteins. *Aspergillus*-specific CD4+ T-cells present in different human populations, varying from healthy individuals, patients suffering from invasive aspergillosis, patients diagnosed with ABPA to COPD patients, recognize a diversity of T-cell epitopes from these *A. fumigatus* proteins, which are presented by different HLA-class II molecules. In previous studies it was suggested that certain *A. fumigatus* proteins, like Crf1, Pep1 and Catalase1 have a more protective effect in the immune response against *A. fumigatus*¹⁻³, whereas other *A. fumigatus* proteins, like Aspf1, Aspf2, Aspf3 and Aspf4, would be more allergenic⁴⁻⁶. The findings in these studies however were not consistent.

Differences may be explained by the fact that in previous human studies often only the response against one *A. fumigatus* antigen was studied and different antigens were not compared. In our studies we used several *A. fumigatus* antigens for stimulation. Our results demonstrate that in different human populations, *Aspergillus*-specific T-cells are present that are directed to all studied *A. fumigatus*

proteins, Crf1, Catalase1, Asp1, Aspf2, Aspf3 and Aspf4, although not every specificity is present in every individual. Another explanation for the different findings may be the study set-up. In previous murine studies different *A. fumigatus* antigens were compared in the setting of experimental *Aspergillus* infection. In these studies the protective effect of vaccination with different *A. fumigatus* antigens or pre-emptive administration of T-cells specific for *A. fumigatus* antigens were evaluated, whereas in human studies the naturally occurring *Aspergillus*-specific T-cell immune responses are studied in the immunocompetent and immunocompromised host.

In the studies presented in this thesis we tested 2 to 6 different *A. fumigatus* antigens, and identified T-cell immune responses to a variety of these antigens in all patients. Therefore we hypothesize that the T-cell mediated immune response against *A. fumigatus* is polyclonal and directed to a broad range of *A. fumigatus* proteins, extending beyond the here tested antigens, similar as was previously demonstrated in viral infections such as cytomegalovirus (CMV)^{7,8}. Bacher et al. recently also identified a broad, diverse *Aspergillus*-specific T-cell response when PBMC were stimulated with different morphogenic stages of *A. fumigatus*, different *A. fumigatus* recombinant proteins, as well as synthetic overlapping peptide pools of different *A. fumigatus* proteins, supporting the notion that the immune response against *A. fumigatus* is not triggered by one or few immunodominant *A. fumigatus* antigens⁹.

As described in chapter 5, the T-cell mediated immune response in patients with ABPA is directed against a broad diversity of *Aspergillus* antigens, not different from the immune reactivity in healthy individuals or patients with invasive aspergillosis. Thus, the differences in cytokine profiles between these groups cannot be explained by differences in allergenic potential of certain *Aspergillus* antigens. However, other explanations for the Th2 response in ABPA patients can be postulated. First, the Th2 cytokine profile of *Aspergillus*-specific T-cells in patients with ABPA may be explained by a general Th2 immune response in these patients.

Another possible explanation may be a different pulmonary milieu in ABPA patients, who have an underlying diagnosis of asthma or cystic fibrosis (CF). The clearance of the lower airways is often impaired with a longer exposure to *A. fumigatus* conidia and more likely outgrowth of *A. fumigatus* hyphae, compared to healthy individuals. It is unclear whether the prolonged exposure and outgrowth of *A. fumigatus* gives rise to the reversed ratio of *Aspergillus*-specific regulatory T-cells (Treg) to conventional T-cells (Tcon), which was observed in peripheral blood of CF patients suffering from allergic reactions to *A. fumigatus*¹⁰. The reversed ratio was caused by an increase of Tcon, which exhibited a Th2 profile, whereas the Treg population was comparable to healthy individuals¹⁰. The expansion of *Aspergillus*-specific T-cells differentiated to a Th2 cytokine profile was therefore not caused by insufficient Treg numbers. However, Treg were apparently not capable of limiting the expansion of *Aspergillus*-specific Th2 cells in these patients.

Furthermore, in mice it was shown that repeated exposure to *A. fumigatus* conidia resulted in the development of Th2 cells besides Th1 and Th17 cells, whereas after 1 or 2 challenges with *Aspergillus* conidia only Th1 cells were present¹¹. Another study showed that pulmonary DC display a different cytokine production pattern after exposure to *Aspergillus* conidia compared to hyphae, with production of IL-12 after exposure to conidia and production of IL-4 and IL-10 upon exposure to hyphae¹². As it is known that IL-12 is involved in the differentiation of T-cells into Th1 cells, whereas IL-4 and IL-10 downregulate the Th1 pathway, the impaired clearance of the lower airways in patients with ABPA resulting in *A. fumigatus* hyphae outgrowth in the lungs, may be an explanation for the occurrence of *Aspergillus*-specific T-cells with a Th2 profile in ABPA patients.

To detect *Aspergillus*-specific T-cells we stimulated mononuclear cells with overlapping peptide pools of *A. fumigatus* proteins. Previously, the majority of studies used *A. fumigatus* crude extracts or *A. fumigatus* recombinant proteins^{1,13-19}. Frequencies of *Aspergillus*-specific T-cells in these studies were almost 10 times higher than frequencies we observed in our experiments. This discrepancy is likely caused by differences between the number of antigens present in the stimulation assay. *A. fumigatus* crude extracts consist of a variety of different proteins and other substances like polysaccharides and glycolipids from *Aspergillus fumigatus*. In addition, the crude extract will contain contaminating compounds derived from culturing the fungus. The detected T-cell immune responses will therefore most likely be composed of immune responses directed against multiple *A. fumigatus* proteins, but also contain nonspecific immune reactivity. Similarly, analysis of the *A. fumigatus* specific immune responses using *A. fumigatus* recombinant proteins as stimulation will contain both the *A. fumigatus* specific immune reactivity but also immune reactivity directed against bacterial contaminating material, incorrectly leading to higher immune responses compared to synthetic overlapping peptide pools. A pure product to stimulate mononuclear cells is especially important when it is used to isolate antigen-specific T-cells for adoptive T-cell therapy. A strategy with overlapping peptide pools will give a better guarantee that the infused antigen-specific T-cells indeed have the desired specificity. Furthermore, there is a considerable variation between different badges from *A. fumigatus* crude extracts and *A. fumigatus* recombinant proteins leading to less reproducible products, a major drawback for use in adoptive T-cell therapy. To answer the question whether adoptive T-cell therapy in invasive aspergillosis is a good therapeutic strategy several issues need consideration.

In the study presented in chapter 3 we demonstrated in patients suffering from invasive aspergillosis the induction of *Aspergillus*-specific T-cells at the time of improvement of aspergillus lesions. Similar results were published in two recent studies in patients after allogeneic stem cell transplantation^{20,21} and in a study concerning immunocompromised patients due to hematological malignancies²². This correlation suggests that *Aspergillus*-specific T-cells may be involved in the clearance of *A. fumigatus*. Several murine studies and one human study support

the hypothesis that *Aspergillus*-specific T-cells are involved in the clearance or prevention of aspergillus infections. In these studies the effects of vaccination with *A. fumigatus* antigens or the adoptive transfer of *Aspergillus*-specific T-cells on the prognosis of invasive aspergillosis were studied. These studies showed a significant longer survival and lower fungal burden in mice after vaccination or adoptive transfer therapy compared to non-treated animals^{1,2;23;24}. The study in humans showed a faster clearance of serum *Aspergillus* antigen and a trend towards a better survival rate in patients with invasive aspergillosis after adoptive transfer of *Aspergillus*-specific T-cells compared to a control group receiving only antifungal medication without cellular therapy²⁵. In this study T-cell lines were infused that were generated after stimulation with *A. fumigatus* conidia, which hampers the determination of the exact specificities of the infused T-cells. Information on the phenotype of the *Aspergillus*-specific T-cells was limited, because evaluation of the cytokine production of the T-cell lines was restricted to IFN γ and IL-10.

It is unclear what would be the preferable Thelper phenotype of the T-cells to be infused. Initially it was thought that Thelper1 phenotype *Aspergillus*-specific T-cells are most important in the immune response against *A. fumigatus*, however, the results of the study described in chapter 4 show that *Aspergillus*-specific T-cells in the lung display a Thelper17 profile. This is in line with the Thelper phenotype most prominent in other fungal infections, like *Candida albicans*, although in contrast to aspergillus infection, in candida infection Th17 cells are also dominant in peripheral blood. The fact that *Aspergillus*-specific T-cells in peripheral blood do not display a Th17 phenotype, in contrast to *Candida*-specific T-cells, may be explained by the location of aspergillus exposure and infection. Exposure to *Aspergillus fumigatus* takes place through repeated inhalation of conidia, whereas *Candida albicans* colonizes the human gastrointestinal tract. Aspergillosis is primarily a pulmonary infection, with only in a minority of cases dissemination throughout the body. On the other hand, most infections with *Candida albicans* are bloodstream infections. The location of colonization and invasiveness of both micro-organisms may be an explanation for the differences of the cytokine profiles in peripheral blood in these infections.

Other studies also have shown the presence of IL-17 producing *Aspergillus*-specific T-cells at the site of aspergillus infection in pulmonary and corneal infections²⁶⁻²⁸. In the light of these findings it may be preferable to infuse *Aspergillus*-specific T-cells with a Thelper17 cytokine profile in adoptive T-cell therapy for aspergillosis. However, *Aspergillus*-specific T-cells may quickly adapt to a different phenotype at the site of infection under the influence of local factors. In that case the cytokine profile of the infused T-cells would be less important. Recently, a new subset of memory T-cells, the tissue resident memory T-cells (T_{RM}) have been described²⁹⁻³¹. These memory T-cells have a distinct phenotype and reside at the organ or tissues of infection and exposure to the pathogen. Locally, they can protect the host to rechallenges with the same pathogen in a rapid and more effective way^{29;30}. Gaide et al. showed that T_{RM} can originate from circulating central memory T-cells²⁹.

This latter observation underlines the hypothesis that differences in phenotype between peripheral blood-derived T-cells and lung-derived T-cells may arise under the influence of local factors in the lung.

Another uncertainty concerning adoptive T-cell therapy for invasive aspergillosis is the influence of *Aspergillus*-specific Treg infusion on the prognosis of aspergillus infection, since Treg have been shown to be present in *Aspergillus*-specific T-cell lines¹⁰. In healthy humans, the frequencies of *Aspergillus*-specific Treg are as high as those of *Aspergillus*-specific Tcon¹⁰. Bacher et al. showed that Treg and Tcon are directed to the same *Aspergillus* antigens⁹, therefore it is not possible to influence the phenotype of *Aspergillus*-specific T-cells in the T-cell product by using specific *Aspergillus* antigens for stimulation. However, most T-cell clones^{32,33} and T-cell lines²⁰ generated by isolation and *in vitro* expansion of CD154+ and/or CD137+ T-cells after stimulation with overlapping peptide pools of *A. fumigatus* proteins, do not contain large amounts of Treg (chapter 2). Furthermore, it is possible to purify the end product by depleting unwanted cells using the MACS technology.

To further support the use of adoptive T-cell transfer as a complementary therapeutic strategy to antifungal medication in invasive aspergillosis, more insight in the working mechanisms of *Aspergillus*-specific T-cells should become available. In chapter 6 we describe the results of an *in vitro* model which was set up to study the influence of *Aspergillus*-specific T-cells on phagocytosis capacity of monocytes, macrophages and neutrophil granulocytes. *Aspergillus*-specific T-cells do not have an effect on the phagocytosis capacity of monocytes and macrophages, which is already excellent by itself. However, in the *in vitro* model an increase in phagocytosis capacity of neutrophil granulocytes was observed after adding *Aspergillus*-specific T-cells or nonspecifically activated CD4+ T-cells, which was probably mediated by secretion of cytokines by the CD4+ T-cells. It is known that neutrophil granulocytes are difficult to isolate and to culture³⁴, so this might have had effect on the phagocytosis capacity of the neutrophil granulocytes in the *in vitro* model. We cannot exclude that the phagocytosis capacity of neutrophil granulocytes *in vivo* is significantly better. In that case the effect of CD4+ T-cells may be less substantial or even not present in the *in vivo* situation. On the other hand, it was recently shown that although 30 days after allogeneic stem cell transplantation the neutrophil granulocytes have recovered to normal levels, the functionality of neutrophil granulocytes is impaired up to one year after alloSCT. Both reactive oxygen species (ROS) production, as well as oxygen-independent killing of *A. fumigatus* were reduced for a long period after SCT²¹. In this clinical situation, the presence of activated CD4+ T-cells might contribute to a better functionality of neutrophil granulocytes. In summary, we observed an increase of phagocytosis capacity of *A. fumigatus* conidia by neutrophil granulocytes in the presence of activated CD4+ T-cells or when cytokines like IL-2 and GM-CSF were added. Furthermore, we hypothesize that activated CD4+ T-cells are also important for attraction of phagocytic cells, mainly neutrophil granulocytes to the site of infection, however, it was not possible to test this hypothesis in this *in*

vitro model. Neutrophil granulocytes are a large and constantly renewing pool of phagocytic cells. Therefore neutrophil granulocytes may be more advantageous than monocytes in helping to prevent outgrowth of *Aspergillus* hyphae in the lungs in case the alveolar macrophages are saturated upon exposure to *A. fumigatus*. In chapter 4 we have shown that *Aspergillus*-specific T-cells in the lungs have a Th17 cytokine profile, also supporting the hypothesis that activated CD4+ T-cells in the lungs will mainly attract neutrophil granulocytes to prevent invasive pulmonary aspergillosis.

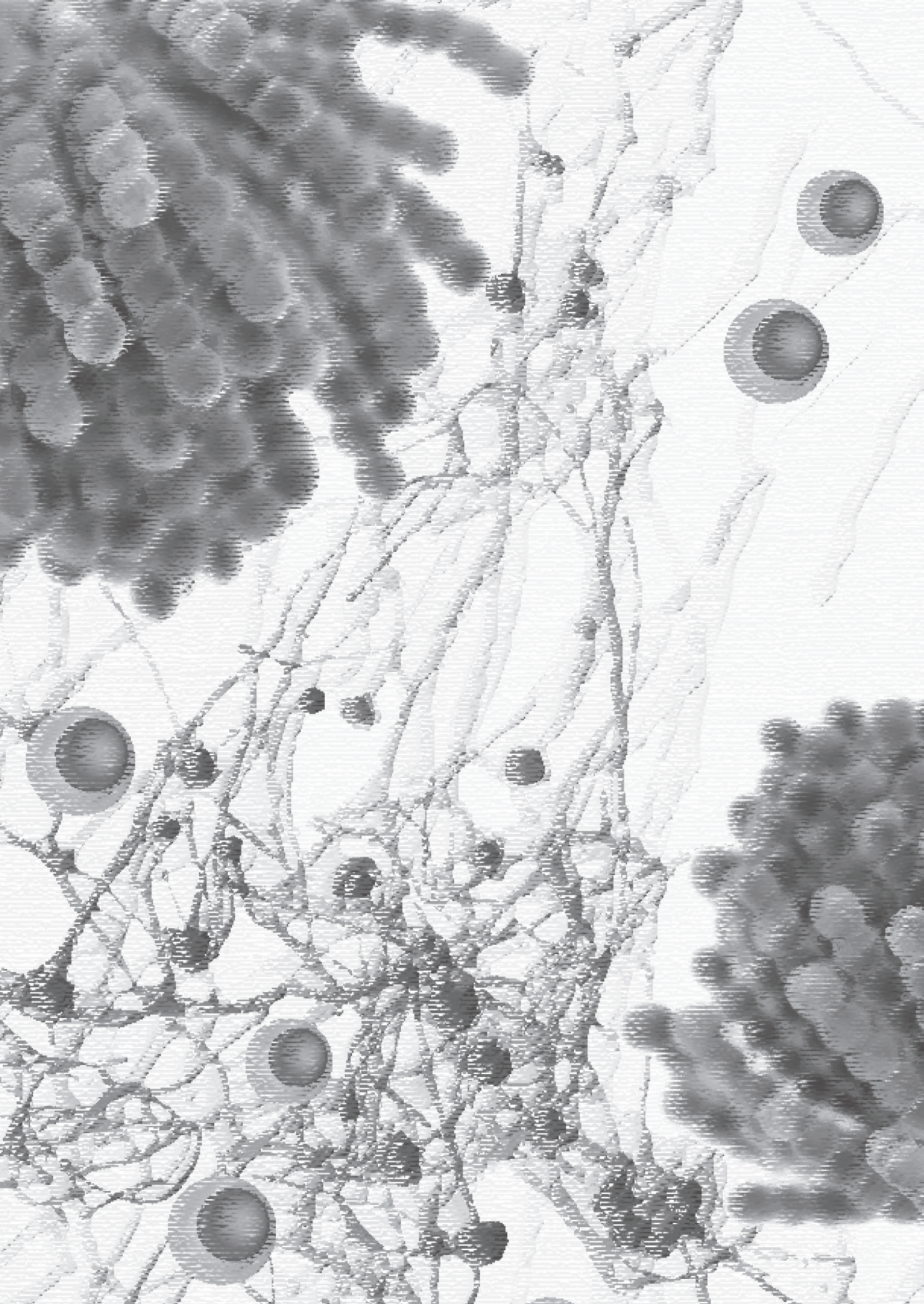
In conclusion, the results described in this thesis expand and deepen the knowledge on the role of T-cells in the immune response against *Aspergillus fumigatus*, in healthy humans as well as in several different patient groups. The data presented here can be a first step towards adoptive transfer of *Aspergillus*-specific T-cells as an adjunctive therapy to cure or prevent invasive aspergillosis. Recently, a rapid isolation method was described for the generation of *Aspergillus*-specific T-cell lines using CD137- and CD154-selection after stimulation with overlapping peptide pools of *A. fumigatus* proteins, with good specificity and viability²⁰. When using CD154-selection approximately 80% of selected cells were CD4+, whereas CD137-selection resulted in a mixed cell population of CD4+, CD8+ and CD4⁻CD8⁻ cells, including $\gamma\delta$ T-cells and natural killer cells²⁰. *A. fumigatus*-specific T-cell lines were shown to be cross-reactive to other *Aspergillus* species, as well as to *Mucorales* species and *Candida albicans*²⁰. The feasibility of clinical-grade isolation of *A. fumigatus*-reactive T-cells suitable for adoptive transfer was shown using CD137-selection³⁵. A protocol fulfilling Good Manufacturing Practice (GMP)-criteria was developed to isolate *A. fumigatus*-specific CD4+ T-cells by depleting Treg and CD8+ T-cells from a mononuclear cell fraction, subsequent stimulation with GMP *A. fumigatus* lysate and selection of *A. fumigatus*-specific CD4+ T-cells on the basis of CD137 expression³⁵. Since end of 2014 a clinical trial is enrolling patients with invasive aspergillosis to assess the safety and tolerability of adoptive transfer of donor-derived *Aspergillus*-specific T-cells (EudraCT 2013-002914-11). Ultimately, a randomized therapeutic trial would be needed to reliably answer the question whether adoptive T-cell therapy can help to reduce the high morbidity and mortality rates for patients suffering from invasive aspergillosis.

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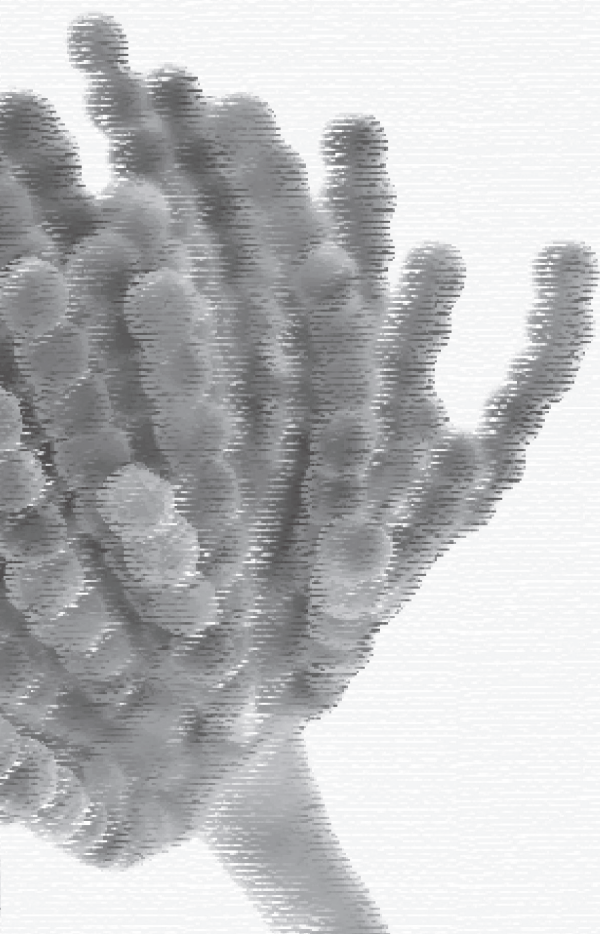
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Addendum



Nederlandse samenvatting

Achtergrond

Aspergillus fumigatus is een schimmel die alom aanwezig is in de omgeving zoals in grond en compost, maar die ook in pandig aangetoond kan worden, met name in het geval van bouwwerkzaamheden. Sporen van deze schimmel bevinden zich in de lucht, waardoor mensen iedere dag honderden *Aspergillus fumigatus* sporen inademen. In mensen met een normaal functionerend afweersysteem geeft dit geen aanleiding tot symptomen.

Bij patiënten met een ernstige afweerstoornis kan *Aspergillus fumigatus* echter leiden tot invasieve aspergillose, een ernstige infectie die zich manifesteert met koorts en longklachten, zoals hoesten, kortademigheid en pijn bij ademen. De infectie kan zich soms verspreiden naar andere organen, waaronder de huid en de hersenen. In het geval van verspreiding naar de hersenen heeft de patiënt meestal neurologische uitvalsverschijnselen zoals krachtsverlies. De mortaliteit in patiënten met invasieve aspergillose is hoog, 50-80%, waarbij dit deels ook verklaard wordt door het ernstige onderliggende lijden.

Aspergillus fumigatus kan aan de andere kant een allergische aandoening veroorzaken in patiënten met astma of cystic fibrose (CF) als onderliggend lijden. Deze aandoening, allergische bronchopulmonale aspergillose (ABPA), wordt gekenmerkt door toename van astma symptomen, zoals hoesten, piepen, kortademigheid en toename van sputumproductie. Dit sputum is vaak dik en taai, en moeilijk op te hoesten. Algemene symptomen zoals gewichtsverlies, malaise en lichte temperatuursverhoging kunnen ook voorkomen. ABPA wordt veroorzaakt door een afwijkende reactie van het afweersysteem na blootstelling aan *Aspergillus fumigatus*, het is echter niet geheel duidelijk waardoor deze afwijkende reactie uitgelokt wordt.

Het afweersysteem speelt dus een belangrijke rol in het ontstaan van *Aspergillus* gerelateerde ziektes. Het afweersysteem bestaat uit verschillende typen witte bloedcellen, die een rol spelen in de afweer tegen ziekteverwekkers, waaronder *Aspergillus fumigatus*. Het immuunsysteem wordt onderverdeeld in 2 delen, het aangeboren of innate immuunsysteem en het verworven of adaptieve immuunsysteem. Tot het aangeboren immuunsysteem behoren de neutrofiële granulocyten, monocyt, macrofagen en dendritische cellen. Al deze afweercellen zijn goed in staat om vreemde deeltjes te herkennen en in de cel op te nemen, een proces dat fagocytose wordt genoemd. De monocyt, macrofagen en dendritische cellen kunnen vervolgens de deeltjes afbreken tot kleine stukjes eiwit – peptiden – die ze aan het oppervlak van de cel kunnen presenteren in speciale moleculen, de human leukocyt antigen (HLA) moleculen. Daarom worden deze cellen ook wel antigeen presenterende cellen genoemd. Dit proces is van belang om T-cellen te activeren, die behoren tot het tweede deel van het afweersysteem, het adaptieve immuunsysteem.

De T-cel wordt geactiveerd als een peptide in een HLA molecuul herkend wordt door de T-cel. Hiervoor heeft de T-cel een receptor op het oppervlak. T-cellen die ziekteverwekkers herkennen die zich in de cel bevinden zoals virussen, hebben een CD8 receptor, en herkennen peptiden in HLA klasse I moleculen. T-cellen die ziekteverwekkers herkennen die zich buiten de cel bevinden zoals een groot deel van de bacteriën en schimmels, hebben een CD4 receptor, en herkennen peptiden in HLA klasse II moleculen. CD8+ T-cellen worden ook wel cytotoxische T-cellen genoemd en CD4+ T-cellen worden ook wel Thelper cellen genoemd. Elke T-cel heeft een unieke T-celreceptor en herkent in principe maar één bepaalde peptide-HLA combinatie. Afhankelijk van signalen van de antigeen presenterende cel en bepaalde signaalstoffen (cytokines) in de omgeving van de CD4+ T-cel, zal de T-cel een bepaald fenotype tot expressie brengen. Er zijn 3 belangrijke Thelper cel fenotypes: Thelper1, Thelper2 en Thelper17. T-cellen produceren cytokines, waarbij Thelper1 cellen voornamelijk IFN γ , TNF- β en IL-2 produceren, Thelper2 cellen IL-4, IL-5, IL-10 en IL-13, en Thelper17 cellen IL-17, IL-21 en IL-22. De verschillende Thelper fenotypes gaan met verschillende andere cellen van het afweersysteem een interactie aan. Zo activeren Thelper1 cellen monocyt en macrofagen, en gaan ze een interactie aan met CD8+ cytotoxische T-cellen en met IgG-producerende B-cellen. Thelper2 cellen gaan een interactie aan met mestcellen, eosinofiele granulocyten en met B-cellen, zodat B-cellen IgE gaan produceren. Tot slot zorgen Thelper17 cellen voor de activatie en aantrekking van neutrofiele granulocyten.

Als een T-cel een peptide herkent dat nog niet eerder in het lichaam aanwezig is geweest, dan is er een extra signaal nodig om de T-cel te activeren. Deze naïeve T-cel zal zich delen en uitgroeien tot een grote populatie van T-cellen met dezelfde herkenning. In de loop van dit proces zullen T-cellen die het peptide het beste binden (hoog-avide T-cellen) overblijven. Als de infectie bestreden is, dooft de immuunrespons uit, waarbij een deel van de T-cellen in lage aantallen in het lichaam aanwezig blijft. Dit zijn de geheugen of memory T-cellen. Bij een tweede infectie met dezelfde verwekker, zal de immuunrespons sneller en krachtiger op kunnen treden door de aanwezigheid van deze memory T-cellen. Van dit gegeven kan men ook gebruik maken om de aanwezigheid van een T-celrespons tegen een bepaalde ziekteverwekker te meten.

In de afweerreactie tegen *Aspergillus fumigatus* zijn verschillende afweercellen van belang. Al heel lang is bekend dat neutrofiele granulocyten van groot belang zijn om een aspergillus infectie te voorkomen en om deze te kunnen klaren. Neutrofiele granulocyten kunnen *Aspergillus fumigatus* sporen fagocyteren en zo uitgroei tot schimmeldraden (hyfen) voorkomen. Als hyfen al gevormd zijn, zorgen neutrofiele granulocyten door uitstoot van verschillende stoffen zoals proteases en door de vorming van neutrophil extracellular traps (NETs) voor schade aan en afgenomen groei van hyfen. Andere fagocyterende cellen zijn ook van belang: Alveolaire macrofagen, aanwezig in de long, komen als eerste in aanraking met geïnhalede sporen van *Aspergillus fumigatus*. Subtiële genetische afwijkingen in de herkenning- en signaalsystemen van macrofagen, monocyt en dendritische

cellen geven een verhoogd risico op het optreden van invasieve aspergillose bij patiënten die een verminderde afweer hebben na stamceltransplantatie. Dit wijst er op dat deze cellen zeker een rol hebben in de afweer tegen *Aspergillus fumigatus*.

Recent is er meer aandacht gekomen voor de rol van T-cellen in de immuunrespons tegen *Aspergillus fumigatus*. In het bloed van gezonde mensen zijn *Aspergillus*-specifieke T-cellen aangetoond door witte bloedcellen afkomstig uit het bloed te stimuleren met extract van *Aspergillus fumigatus* eiwitten of met recombinant *Aspergillus* eiwitten. Een enkele studie heeft daarnaast de aanwezigheid van *Aspergillus*-specifieke T-cellen in patiënten met invasieve aspergillose aangetoond. In muizenstudies waarin muizen met een verminderde afweer werden geïnfecteerd met *Aspergillus fumigatus* sporen, zorgde vaccinatie met *Aspergillus* antigenen of de toediening van *Aspergillus*-specifieke T-cellen voor een betere prognose. In één studie bij patiënten na haploidentieke stamceltransplantatie zijn ook aanwijzingen gevonden dat de toediening van *Aspergillus*-specifieke T-cellijnen tot een verbeterde prognose zou leiden.

Dit proefschrift

Hoewel er de laatste tijd aanwijzingen zijn dat *Aspergillus*-specifieke T-cellen een rol spelen in de afweer tegen *Aspergillus fumigatus*, zijn veel aspecten nog onduidelijk. Hoe is de afweerrespons tegen *Aspergillus fumigatus* opgebouwd? Welke rol hebben *Aspergillus*-specifieke T-cellen in het voorkomen of klaren van een aspergillus infectie in patiënten met een verminderde afweer? Welk type helper T-cellen zijn belangrijk in de afweer tegen *Aspergillus fumigatus*? En zijn er verschillen in de cytokine profielen in verschillende compartimenten van het lichaam? Hoe komt het dat sommige patiënten allergisch reageren op blootstelling aan *Aspergillus* sporen? Zorgen bepaalde *Aspergillus* eiwitten voor zo'n allergische reactie? Tot slot, als er *Aspergillus*-specifieke T-cellen zijn, betekent dit ook dat zij een actieve rol spelen in het voorkomen of klaren van aspergillus infecties, en op welke manier doen ze dat dan? De studies beschreven in dit proefschrift proberen een antwoord te geven op deze vragen.

Karakterisering van de immuunrespons tegen *Aspergillus fumigatus* in gezonde mensen

In **hoofdstuk 2** is de afweerrespons tegen *Aspergillus fumigatus* in gezonde mensen in kaart gebracht. Witte bloedcellen (mononucleaire cellen) uit het bloed van 24 gezonde donoren werden gestimuleerd met overlappende peptiden van 2 verschillende eiwitten van *Aspergillus fumigatus*: Crf1 en Catalase1. In 18 van de 24 donoren werden Crf1- en Catalase1-specifieke T-cellen aangetoond in lage frequenties. Deze T-cellen waren gericht tegen een groot aantal verschillende epitopen met een diverse HLA-restrictie en produceerden voornamelijk IFN γ , wijzend op een Thelper1 profiel. Door de Crf1- en Catalase1-specifieke T-cellen 1 cel per well te selecteren op basis van expressie van activatiemarkers en vervolgens klonen op te kweken, kon de *Aspergillus*-reactiviteit worden getest. Het merendeel van de klonen produceerde IFN γ en kleinere hoeveelheden IL-4 of IL-17 na

stimulatie met *Aspergillus* eiwit extract, waarmee de specificiteit van de T-cellen voor *Aspergillus* werd aangetoond. Concluderend was er sprake van een brede T-celrespons tegen *Aspergillus fumigatus* met een Thelper1 cytokine profiel.

Kinetiek van *Aspergillus*-specifieke T-cellen in patiënten met invasieve aspergillose

In **hoofdstuk 3** wordt een studie beschreven, waarin bij 11 patiënten met invasieve aspergillose de aanwezigheid en het cytokineprofiel van Crf1- en Catalase1-specifieke T-cellen wordt bestudeerd gedurende het beloop van de aspergillus infectie. Er werden 33 patiënten met invasieve aspergillose geselecteerd voor deelname aan deze studie; de overlevingsperiode van 22 van de 33 patiënten was gemiddeld slechts 4 weken, waardoor er geen perifeer bloed materiaal beschikbaar was of er geen T-cellen aangetoond konden worden in het bloed. De overige 11 patiënten werden geïncubeerd in de studie. Mononucleaire cellen uit het bloed van deze patiënten, afgenomen op verschillende tijdstippen voor en na de diagnose invasieve aspergillose, werden gestimuleerd met overlappende peptiden van de eiwitten Crf1 en Catalase1. Op basis van expressie van de activatiemarker CD154 en de productie van IFN γ werden *Aspergillus*-specifieke T-cellen aangetoond in 9 patiënten, die een gunstig beloop van de aspergillus infectie hadden. Deze *Aspergillus*-specifieke T-cellen namen toe rond het moment dat er sprake was van afname van aspergillus afwijkingen. De *Aspergillus*-specifieke T-cellen hadden een Thelper1 cytokine profiel en de herkenning van T-cel epitopen door deze T-cellen was gevarieerd. In 2 patiënten met een ongunstig beloop van de aspergillus infectie konden geen *Aspergillus*-specifieke T-cellen worden aangetoond. De resultaten van deze studie wijzen op een correlatie tussen de opkomst van *Aspergillus*-specifieke T-cellen en het herstel van invasieve aspergillose.

Cytokineprofiel van *Aspergillus*-specifieke T-cellen in verschillende lichaamscompartimenten

Veel informatie over *Aspergillus*-specifieke T-cellen is afkomstig van onderzoek naar *Aspergillus*-specifieke T-cellen in perifeer bloed. De blootstelling aan *Aspergillus fumigatus* vindt echter primair plaats in de long, en slechts bij disseminatie van de infectie zal er blootstelling aan *Aspergillus fumigatus* zijn in het bloed. In **hoofdstuk 4** worden daarom frequenties en cytokineprofielen van *Aspergillus*-specifieke T-cellen afkomstig uit longweefsel vergeleken met *Aspergillus*-specifieke T-cellen uit perifeer bloed. Het longweefsel was afkomstig van 10 COPD-patiënten die een longtransplantatie of een partiële longresectie vanwege een longtumor hadden ondergaan. Van 3 van hen was ook perifeer bloed beschikbaar. Ook werden nog 7 andere COPD-patiënten geïncubeerd die een partiële longresectie hadden ondergaan, van wie alleen perifeer bloed aanwezig was. Mononucleaire cellen werden uit bloed of longweefsel geïsoleerd en gestimuleerd met overlappende peptiden van 6 verschillende *Aspergillus fumigatus* eiwitten: Crf1, Catalase1, Asp1, Asp2, Asp3 en Asp4. *Aspergillus*-specifieke T-cellen werden geïdentificeerd op

basis van CD154-expressie; de frequenties in longmateriaal en perifeer bloed bleken niet verschillend. Zoals in eerdere studies naar *Aspergillus*-specifieke T-cel immuniteit, hadden *Aspergillus*-specifieke T-cellen in perifeer bloed voornamelijk een Thelper1 profiel met productie van IFN γ en waren er slechts lage frequenties van IL-17 producerende *Aspergillus*-specifieke T-cellen. *Aspergillus*-specifieke T-cellen in longmateriaal daarentegen hadden voornamelijk een Thelper17 cytokineprofiel met productie van IL-17 en slechts weinig *Aspergillus*-specifieke T-cellen afkomstig uit de long produceerden IFN γ . Het cytokineprofiel van *Aspergillus*-specifieke T-cellen in de long was dus vergelijkbaar met het Thelper fenotype zoals in andere schimmel- en gistinfecties, waar sprake is van een Thelper17 cytokineprofiel. Dit zou men ook verwachten, aangezien Thelper17 cellen van belang zijn voor de activatie en aantrekking van neutrofiele granulocyten, waarvan al langer bekend is dat ze een belangrijke rol spelen in de afweer tegen schimmelinfecties, waaronder *Aspergillus fumigatus*.

Rol van *Aspergillus* eiwitten in het ontstaan van een Thelper2 respons in ABPA

Aspergillus-specifieke T-cellen van patiënten met allergische bronchopulmonale aspergillose (ABPA) hebben een Thelper2 cytokineprofiel, met productie van IL-4, IL-5 en IL-13, in tegenstelling tot gezonde mensen en patiënten met invasieve aspergillose waar *Aspergillus*-specifieke T-cellen in perifeer bloed een Thelper1 respons laten zien. In **hoofdstuk 5** werd de rol die bepaalde *Aspergillus* eiwitten spelen in het ontstaan van deze Thelper2 respons bestudeerd. Mononucleaire cellen uit perifeer bloed van patiënten met ABPA en van gezonde mensen werden gestimuleerd met overlappende peptiden van de 6 *Aspergillus fumigatus* eiwitten Crf1, Catalase1, Asp1, Asp2, Asp3 en Asp4. Er was geen verschil in de T-celrespons in patiënten met ABPA en gezonde mensen; in beide groepen werd een brede T-celrespons geïdentificeerd, gericht tegen verschillende *Aspergillus* eiwitten. De cytokineprofielen van de *Aspergillus*-specifieke T-cellen in beide groepen waren wel verschillend. In patiënten met ABPA werd een overwegend Thelper2 respons gevonden, terwijl in gezonde mensen sprake was van een Thelper1 respons. Dit was niet afhankelijk van het eiwit dat was gebruikt voor stimulatie. Concluderend, hadden *Aspergillus*-specifieke T-cellen in ABPA patiënten een Thelper2 fenotype, onafhankelijk van het *Aspergillus* antigeen waarmee gestimuleerd was.

Werkingsmechanisme van *Aspergillus*-specifieke T-cellen in de afweer tegen *Aspergillus fumigatus*

In **hoofdstuk 6** wordt een *in vitro* model beschreven, waarin werd bestudeerd wat het werkingsmechanisme van *Aspergillus*-specifieke T-cellen is in de afweer tegen *Aspergillus fumigatus*. Uit perifeer bloed van gezonde mensen werden op basis van specifieke oppervlaktecelmarkeringen de verschillende typen afweercellen geïsoleerd met behulp van MACS (Magnetic-Activated Cell Sorting). Monocyten, neutrofiele granulocyten en T-cellen werden gelabeld met fluorochromen gekoppeld

aan specifieke oppervlaktecelmarkers en geïnactiveerde *Aspergillus fumigatus* sporen werden gekleurd met Cellvue, waardoor de verschillende populaties in flow cytometry experimenten zichtbaar gemaakt konden worden. Het effect van inactieve, aspecifiek geactiveerde T-cellen en *Aspergillus*-specifieke T-cellen op de fagocytose capaciteit van monocyt en neutrofiele granulocyten werd geanalyseerd. De fagocytose capaciteit van monocyt was hoog en veranderde niet door de toevoeging van T-cellen. De fagocytose capaciteit van neutrofiele granulocyten was veel lager en nam toe bij toevoeging van geactiveerde T-cellen, waarbij het niet van belang was of deze cellen *Aspergillus*-specifiek waren of aspecifiek geactiveerd. Rustende T-cellen hadden geen effect op de fagocytose capaciteit van neutrofiele granulocyten. Waarschijnlijk wordt het effect gemedieerd door cytokines, waaronder IL-2, GM-CSF en IFN γ . Mogelijk hebben T-cellen ook een rol in de latere fase van de afweer tegen *Aspergillus fumigatus* of in de aantrekking van afweercellen naar de locatie van de infectie. Er werd een assay opgezet om het effect van T-cellen in een later stadium van aspergillus infectie te bestuderen. Deze assay gaf helaas geen betrouwbare, reproduceerbare resultaten en is daarom niet geschikt voor het beantwoorden van deze onderzoeksvraag.

De resultaten beschreven in dit proefschrift verbreden en verdiepen de kennis over *Aspergillus*-specifieke T-cellen in gezonde mensen en verschillende patiëntengroepen. In alle populaties zijn *Aspergillus*-specifieke T-cellen aanwezig met een breed herkenningsprofiel. Afhankelijk van de onderliggende ziekte hebben de *Aspergillus*-specifieke T-cellen een ander cytokineprofiel, dit is echter niet afhankelijk van specifieke *Aspergillus* antigenen. Het opkomen van *Aspergillus*-specifieke T-cellen in patiënten met invasieve aspergillose ten tijde van verbetering van de infectie, suggereert een rol van *Aspergillus*-specifieke T-cellen in het klaren van *Aspergillus fumigatus*, hoewel deze aanname slechts gebaseerd kan worden op een correlatie in de tijd tussen beide observaties. Verdere studies zijn nodig om vast te kunnen stellen of bijvoorbeeld het toedienen van *Aspergillus*-specifieke T-cellen (adoptieve T-cel therapie) aan patiënten met invasieve aspergillose een goede therapeutische strategie is. Daarin zouden dan ook de verschillen in effectiviteit van *Aspergillus*-specifieke T-cellen met verschillende Thelper profielen kunnen worden bestudeerd, aangezien er een verschillend cytokineprofiel werd gevonden in *Aspergillus*-specifieke T-cellen afkomstig uit perifeer bloed en uit longweefsel.

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Lieve Simon, met jouw lach en knuffels maak je me altijd blij.

Curriculum Vitae

Hetty Jolink werd op 9 april 1977 geboren te Dalfsen. Na het behalen van haar VWO diploma aan het Meander College in Zwolle in 1995, begon zij datzelfde jaar met de studie Geneeskunde aan de Rijksuniversiteit Groningen. In 2002 behaalde zij het artsexamen met een afsluitend keuze-coschap bij de afdeling Interne Geneeskunde van de Isala Klinieken in Zwolle onder begeleiding van dr. B.E. Schenk en dr. M. Oudkerk Pool. Vanaf 2002 werkte zij als assistent-geneeskundige niet in opleiding in het Groene Hart Ziekenhuis in Gouda. In 2003 begon zij in dit ziekenhuis aan de opleiding tot internist (opleider dr. J.T.M. van der Heyden). De opleiding werd vanaf 2006 vervolgd in het Leids Universitair Medisch Centrum (opleider prof. dr. J.A. Romijn), waarbij zij zich specialiseerde in het aandachtsgebied Infectieziekten (opleider prof. dr. J.T. van Dissel). Op 1 september 2009 vond de registratie als internist-infectioloog plaats.

In oktober 2009 werd gestart met een onderzoeksproject bij het laboratorium voor Experimentele Hematologie naar de rol van T-cellen in de afweer tegen *Aspergillus fumigatus*, uiteindelijk uitmondend in een promotietraject onder begeleiding van dr. M.H.M. Heemskerk, prof. dr. J.T. van Dissel en prof. dr. J.H.F. Falkenburg. In de periode oktober 2009 tot januari 2014 combineerde zij het onderzoek op het laboratorium voor Experimentele Hematologie met klinische werkzaamheden bij de afdeling Infectieziekten van het LUMC. Sinds januari 2014 is zij werkzaam als stafid op de afdeling Infectieziekten.

List of abbreviations

ABOS	inactivated human serum
ABPA	allergic bronchopulmonary aspergillosis
alloSCT	allogeneic stem cell transplantation
APC	allophycocyanin
CD3, CD4, etc	cluster of differentiation 3, cluster of differentiation 4, etc
CF	cystic fibrosis
CMV	cytomegalovirus
COPD	chronic obstructive pulmonary disease
CT-scan	computed tomography-scan
DC	dendritic cell
DMSO	dimethylsulfoxide
EBV-LCL	Epstein-Barr virus-transformed B lymphoblastoid cell lines
EDTA	ethyleendiaminetetra-azijnzuur
ELISA	enzyme-linked immunosorbent assay
EORTC/MSG	European Organization for Research and Treatment of Cancer/ Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte macrophage colony stimulating factor
GMP	good manufacturing practice
GvHD	graft-versus-host disease
HLA	human leukocyte antigen
IC90	concentration that leads to 90% of maximal response
IFN γ	interferon γ
IgA	immunoglobulin A
IgE	immunoglobulin E
IgG	immunoglobulin G
IL-2, IL-4, etc	interleukin-2, interleukin-4, etc
IMDM	Iscove's Modified Dulbecco's Media
LMC	lung-derived mononuclear cells
MACS	magnetic-activated cell sorting
MFI	mean fluorescence intensity
MSD	matched sibling donor
MUD	matched unrelated donor
NET	neutrophil extracellular trap
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PE	phycoerythrin
PerCP	peridinin chlorophyll protein complex
PHA	phytohaemagglutinin
PRR	pathogen recognition receptor
ROS	reactive oxygen species
R:S	responder stimulation ratio
RT-PCR	real time-polymerase chain reaction
SCT	stem cell transplantation
Tcon	conventional T-cells
TCR	T-cell receptor
TGF β	transforming growth factor β
Th cell	Thelper cell
TLR	toll-like receptor
TNF α	tumor necrosis factor α
Treg	regulatory T-cells
T _{RM}	resident memory T-cells

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