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

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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 Aspf1, Aspf2, Aspf3 and Aspf4, 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 IFNγ 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-IFNγ APC, anti-TNFα 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 Aspf1, Aspf2, Aspf3 and Aspf4, 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 Aspf2 and Aspf4, as well as Crf1 and Catalase1 (figure 5.1A). Activation of CD4+ T-cells was detected after restimulation with Aspf2 in 3 patients, Aspf4 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 Aspf4 is shown. The *Aspergillus*-specific T cells produced mainly TNFα, 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 TNFα, with frequencies ranging from 19% to 95% of CD154+ cells. The majority of *Aspergillus*-specific T-cells produced besides TNFα mainly IL-4, and this was similar for all *A. fumigatus* antigens (figure 5.1C). Patient MMT showed mixed responses with TNFα and IL-4 production in response to Aspf2 and Crf1, and TNFα, IFNγ 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 Aspf2 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 TNFα and IFNγ (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 TNFα and IFNγ. With this technique, used in 5 healthy donors, *Aspergillus*-specific T-cells were detected in response to Aspf1, Aspf2, Crf1 and Catalase1, and these T-cells produced primarily TNFα and IFNγ (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 IFNγ (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.

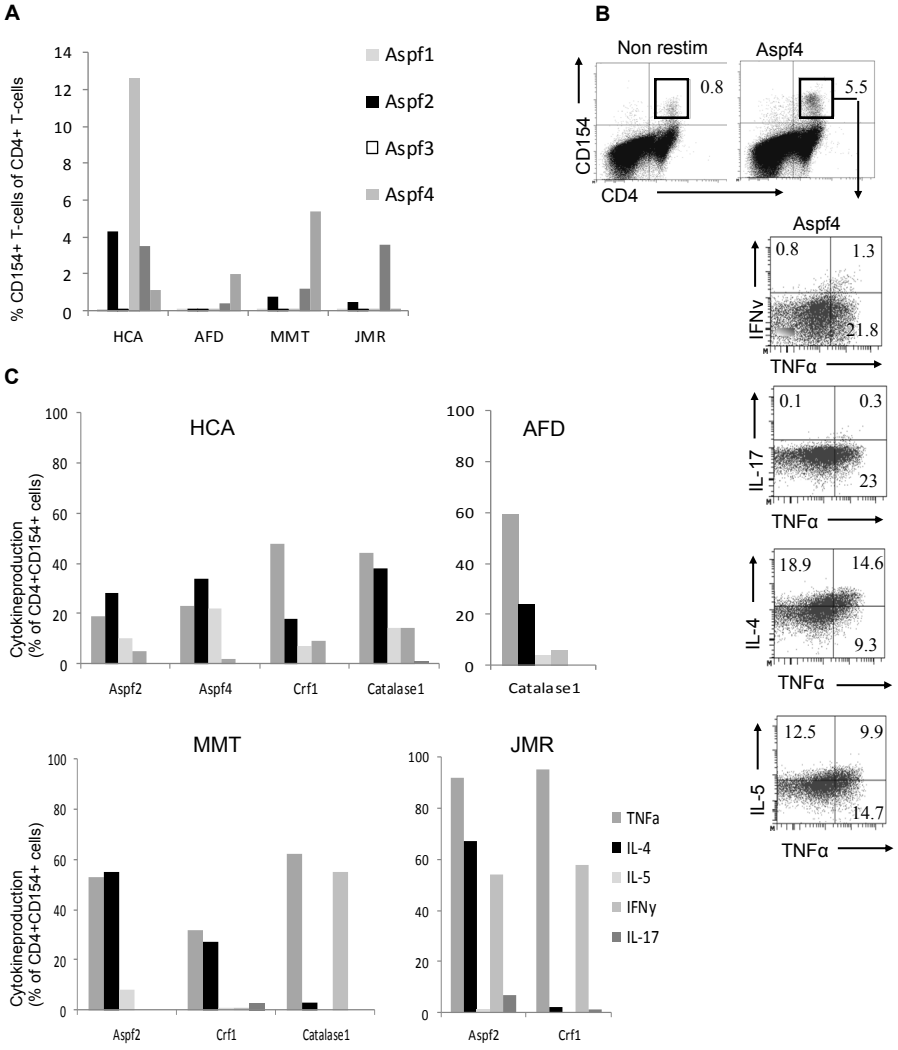

Figure 5.1 T-cell activation and cytokine production in response to different A. fumigatus antigens in ABPA patients and in healthy donors. *PBMC from 4 ABPA patients and 1 healthy donor were stimulated with the mixture of 6 A. fumigatus antigens Aspf1,Aspf2, Aspf3, Aspf4, Crf1 and Catalase1, and activated 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.*

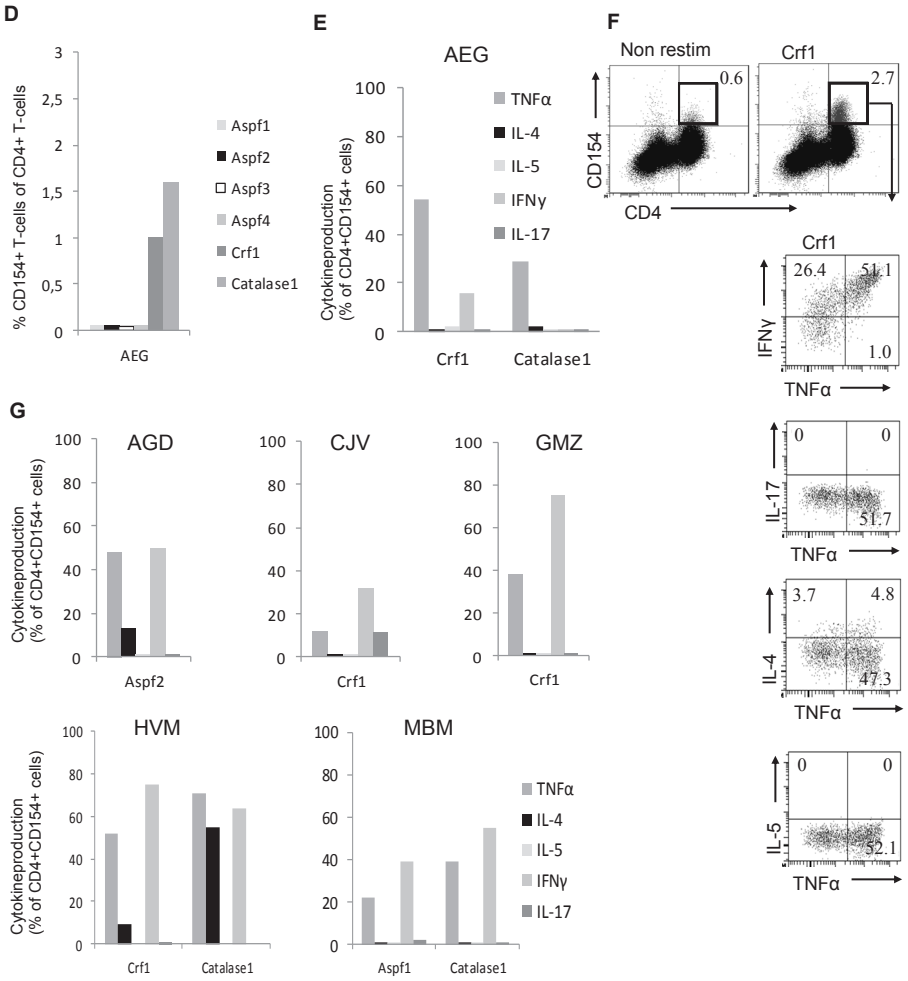

Figure 5.1 Continued

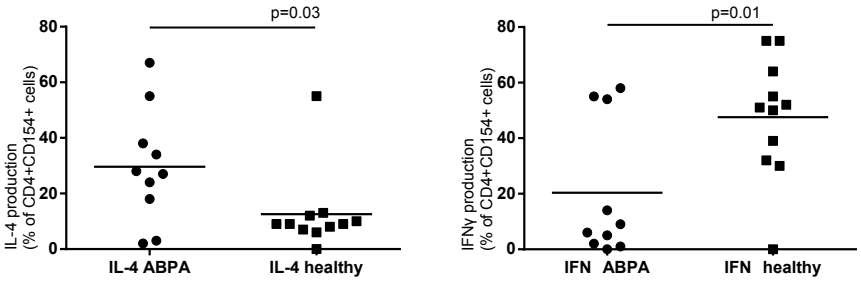

Figure 5.2 Cytokine profiles in ABPA patients and in healthy donors. *(A) Percentages of IL-4-producing T-cells of CD154+CD4+ T-cells in ABPA patients compared to percentages of IL-4-producing T-cells of CD154+CD4+ T-cells in healthy donors. (B) Percentages of IFNγ-producing T-cells of CD154+CD4+ T-cells in ABPA patients compared to percentages of IFNγ-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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