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

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Summary 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 TNFα. In addition, production of the cytokines IFNγ, 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 IFNγ. In contrast, in the peripheral blood of

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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 IFNγ. 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 IFNγ, 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 previously.[15] Briefly, tissue specimens (1 $\times$ 1 cm) were sliced into pieces of 1 mm and incubated for 20 min in RPMI with 20 mM 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 min 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 h at room temperature, and then washed to remove DMSO and peptides. Subsequently, the cells were cultured in 150 μl T-cell medium consisting of Iscove's Modified Dulbecco's Medium (IMDM, Lonza, Breda, Netherlands), supplemented with 5% fetal calf serum (Gibco, Invitrogen, Bleiswijk, Netherlands), 5% human serum and 100 IU/ml IL-2 (Novartis, Emeryville, CA). 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