

Pattern recognition pathways leading to a Th2 cytokine bias in allergic bronchopulmonary aspergillosis patients

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Summary

Background Allergic bronchopulmonary aspergillosis (ABPA) is characterised by an exaggerated Th2 response to *Aspergillus fumigatus*, but the immunological pathways responsible for this effect are unknown.

Objective The aim of this study was to decipher the pattern recognition receptors (PRRs) and cytokines involved in the *Aspergillus*-specific Th2 response and to study *Aspergillus*-induced responses in healthy controls and ABPA patients.

Methods Peripheral blood mononuclear cells (PBMCs) were stimulated with heat-killed *Aspergillus* conidia, various other pathogens, or PRR ligands. PRRs and cytokine pathways were blocked with PRR-blocking reagents, anti-TNF (Etanercept or Adalimumab), IL-1Ra (Anakinra) or IFN γ (IFN-gamma). ELISA and FACS were used to analyse cytokine responses.

Results *Aspergillus* was the only pathogen that stimulated the Th2 cytokines IL-5 and IL-13, while Gram-negative bacteria, Gram-positive bacteria, *Candida albicans*, chitin, β -glucan or Toll-like receptor (TLR) ligands did not. Depletion of CD4⁺ cells abolished IL-13 production. Blocking complement receptor 3 (CR3) significantly reduced IL-5 and IL-13, while blocking TLR2, TLR4 or dectin-1 had no effect. ABPA patients displayed increased *Aspergillus*-induced IL-5 and IL-13 and decreased IFN γ production compared with healthy controls. All biological agents tested showed the capability to inhibit Th2 responses, but also decreased *Aspergillus*-induced IFN γ .

Conclusions and Clinical Relevance *Aspergillus* conidia are unique in triggering Th2 responses in human PBMCs, through a CR3-dependent pathway. ABPA patients display a significantly increased *Aspergillus*-induced Th2/Th1 ratio that can be modulated by biologicals. These data provide a rationale to explore IFN γ therapy in ABPA as a corticosteroid-sparing treatment option, by dampening Th2 responses and supplementing the IFN γ deficiency at the same time.

Keywords allergic bronchopulmonary aspergillosis, *Aspergillus fumigatus*, interferon- γ , interleukin-13, interleukin-5

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Introduction

Allergic bronchopulmonary aspergillosis (ABPA) is a hypersensitivity reaction against the ubiquitous mould *Aspergillus fumigatus* [1]. Innate immune cells, such as lung epithelia cells or alveolar macrophages, initiate this inflammatory reaction [2]. Fungi are sensed by innate immune cells through pattern recognition

receptors (PRRs), such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs) or nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) [3]. Activation of these receptors by *Aspergillus* leads to induction of cytokines, recruitment of other immune cells, and can eventually trigger adaptive immune responses such as T helper (Th) responses. A robust induction of the Th1 response during *Aspergillus*

infection is associated with the protection and successful clearance, while increased Th2 responses impair fungal clearance [4–6]. Exaggerated Th2 responses are being held responsible for the detrimental inflammatory reaction observed in ABPA, namely eosinophilia, increased mucus production and immunoglobulin class switching to IgE. This inflammatory reaction subsequently results in airway hyper-responsiveness with typical asthmatic symptoms such as bronchial obstruction, coughing and wheezing [7, 8]. The current treatment for ABPA is the administration of oral corticosteroids during exacerbations and control of the underlying asthma with β_2 -agonists. Furthermore, antifungal treatment with azoles in ABPA patients could spare the use of corticosteroids [1, 9]. Due to the potential severe side effects of azoles and the long-term consequences of corticosteroid treatment, new approaches for ABPA therapy are needed.

While most *Aspergillus*-related PRRs have been studied in the context of invasive aspergillosis, limited data exist of the *Aspergillus*-associated molecular patterns and their PRRs responsible for triggering the Th2 response in ABPA. In mouse studies, TLR2 and TLR9 have been described to skew the *Aspergillus*-induced Th response towards a Th2 profile [10, 11], and chitin exposure has been associated with lung eosinophilia [12]. The current study aimed to identify the immunological pathways through which *Aspergillus* induces a Th2 response in humans. Furthermore, the cytokine profile of a cohort of ABPA patients was investigated for assessing the Th1/Th2 balance. The capacity of treatment with biological therapy to modulate *Aspergillus*-induced

Th-responses was tested *in vitro*, with the goal to find innovative corticosteroid-sparing treatment options for ABPA patients.

Methods

Volunteers and patients

Blood was collected from healthy volunteers or patients by venous blood puncture after informed consent. Three dectin-1 deficient patients with a homozygous Y238X mutation in exon 6 of *CLEC7A* gene (the gene encoding dectin-1) donated blood, two of them twice at two different time points. In these patients, diminished expression of dectin-1 and failure to induce a cytokine response to β -glucan was demonstrated previously [13]. Seven asthma patients and nine patients diagnosed with ABPA according to the ABPA diagnostic criteria as described elsewhere [3] were asked for blood donations, two of whom had cystic fibrosis with a homozygous mutation in the *CFTR* gene (Table 1).

Pattern recognition receptor ligands, blockers and other stimuli

Escherichia coli LPS (10 ng/mL) (TLR4 ligand, *E. coli* serotype O55:B5, Sigma-Aldrich, St. Louis, MO, USA), Pam3Cys (10 μ g/mL) (TLR2 ligand, EMC microcollections, Tübingen, Germany), poly I:C (10 μ g/mL) (TLR3 ligand, Cayla-Invivogen, Toulouse, France), flagellin (10 μ g/mL) (TLR5 ligand, Cayla-Invivogen), N-acetylmuramyl-alanyl-D-isoglutamine (MDP) (10 μ g/mL) (NOD-2

Table 1. Demographic table of allergic bronchopulmonary aspergillosis (ABPA) patients

	Age	Gender	Asthma/CF	Total IgE (U/mL)	<i>Aspergillus</i> -specific IgG (mg/mL)	<i>Aspergillus</i> -specific IgE (U/mL)	Eosinophils	Bronchiectasis	Steroids use	Azole use
ABPA1	22	F	F508del/ F508del	60	48.60	1.5	1.06	Yes	Yes (ciclesonide, inhalative 160 mg)	Yes
ABPA2	21	M	F508del/ F508del	229	40	13.9	0.79	Yes	No	Yes
ABPA3	63	M	Asthma	1406	353	25.1	2.44	Yes	Yes (prednisone, 5 mg/day)	No
ABPA4	78	F	Asthma	4515	n.d.	67.7	0.17	n/a	No	Yes
ABPA5	77	F	Asthma	1263	120	46.4	0.88	Yes	No	No
ABPA6	67	M	Asthma	2710	128	29	0.88	Discrete	No	Yes
ABPA7	66	M	Asthma	2508	187	15.1	n/a	Yes	Yes (prednisone, 10 mg/day)	No
ABPA8	70	F	Asthma	3202	196	40.7	0.02	Discrete	Yes (prednisone, 15 mg/day)	No
ABPA9	77	M	Asthma	3343	51	46.4	1.49	Yes	Yes (prednisone, 10 mg/day)	No

Age, gender, underlying disease (asthma or cystic fibrosis), bronchiectasis, eosinophil count, total IgE (U/mL), *Aspergillus*-specific IgE (U/mL), *Aspergillus*-specific IgG (mg/mL) and concomitant medication (corticosteroids and azoles) of the ABPA patients enrolled in this study are listed.

ligand, Sigma-Aldrich), phytohemagglutinin (PHA) (10 µg/mL) (Sigma-Aldrich). *Escherichia coli* ATCC 35218 (*E. coli*) (1×10^7 /mL); *Staphylococcus aureus* clinical isolate (*S. aureus*) (1×10^7 /mL); *Mycobacterium tuberculosis* sonicate H37Rv bub DL 6122005 (*M. tuberculosis*) (10 µg/mL); *Borrelia burgdorferi* ATCC 35210 (*B. burgdorferi*) (1×10^6 /mL); *Candida albicans* ATCC MYA-3573 (UC820) (*C. albicans*) (1×10^6 /mL); *A. fumigatus* clinical isolate V05-27 (*A. fumigatus*), resting conidia and hyphae (1×10^7 /mL), were cultured and isolated as described in a previous study [14].

Fungal cell wall components: β 1,3-(D)-glucan (β -glucan) (10 µg/mL) was kindly provided by Prof. David Williams (Tennessee University), and α 1,3-glucan, α 1,3-1,4-glucan [either dissolved in phosphate-buffered saline (PBS) or DMSO], chitin (*Aspergillus niger*) (all 10 µg/mL) and galactosaminogalactan (GAG) (7.5 µg/mL) were a gift from Prof. Jean-Paul Latgé (Pasteur Institute, Paris).

Bartonella quintana LPS was prepared and purified as described elsewhere [15] and used as a TLR4 inhibitor (20 ng/mL) [16], isotype control mouse IgG1 (10 µg/mL) (eBioscience, Halle-Zoersel, Belgium), anti-TLR2 (10 µg/mL) (eBioscience), laminarin (50 ng/mL) [17], a Syk kinase inhibitor (50 nM; Merck, Darmstadt, Germany), isotype control goat IgG (10 µg/mL) (R&D Systems, Minneapolis, MN, USA), anti-human β_2 -integrin (anti-CR3) (10 µg/mL) (R&D Systems), anti-human OX40 ligand (anti-OX40L) (10 µg/mL) (R&D Systems), cytochalasin D (1 µg/mL, dissolved in DMSO) (Enzo life sciences, Antwerpen, Belgium), IL-1 receptor antagonist (IL-1Ra) (10 µg/mL) (Amgen, Inc., Thousand Oaks, CA, USA), soluble TNFR2 (Etanercept) (100 µg/mL); human anti-human TNF α (Adalimumab); (100 µg/mL), both kindly provided by Dr. Marije Koenders (Department of Rheumatology Radboud University Nijmegen Medical Centre the Netherlands), Interferon-gamma 1b (Immukine[®]) (50 ng/mL) (Boehringer Ingelheim, Alkmaar, The Netherlands).

Peripheral blood mononuclear cells isolation

Venous blood was drawn in 10-mL EDTA tubes. The blood was diluted 1 : 1 with PBS. Subsequently, Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-paque (GE healthcare, Zeist, The Netherlands) density gradient centrifugation. The PBMC layer was collected and washed twice in cold PBS. Cells were reconstituted in RPMI-1640 culture medium (Dutch modification; Gibco, Invitrogen, Breda, The Netherlands) supplemented with 10 µg/mL gentamicin, 10 mM L-glutamine and 10 mM pyruvate (Gibco). The cells were counted with a particle counter (Beckman Coulter, Woerden, The Netherlands), and the concentration was adjusted to 1×10^7 cells/mL.

Cell depletion

Peripheral blood mononuclear cells were incubated with magnetic anti-CD4⁺, anti-CD56⁺ and anti-CD19⁺ beads and purified using MACS depletion columns (LD, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the instructions supplied by the manufacturer to generate CD4[−] (T cells depleted), CD56[−] (NK cells depleted) or CD19[−] (B cells depleted) PBMC fractions. Next to the labelled cells, also an unlabelled PBMC fraction was run over the depletion column and used as a mock treatment control.

Peripheral blood mononuclear cells stimulation

Peripheral blood mononuclear cells were plated in a 96-well plate (Corning, NY, USA) at a final concentration of 2.5×10^6 /mL in an end volume of 200 µL per well. All stimulations were performed in the presence of 10% human serum. Serum was either complement active if not otherwise indicated or heat-inactivated by incubation for 30 min at 56°C in a water bath according to a commonly used protocol [18]. Mannose-binding lectin (MBL) deficient serum was obtained from a patient with MBL level of 0.09 µg/mL. After 1 h pre-incubation with inhibitor or medium, stimuli or medium was added. Cells were incubated at 37°C with 5% CO₂; after 7 days, supernatants were collected and stored at −20°C.

Cytokines measurements

IL-5, IL-13, IFN γ and IL-10 were measured in the cell culture supernatants using a commercial ELISA kit (IL-5 and IL-13: R&D Systems; IFN γ and IL-10: Sanquin) according to the instructions supplied by the manufacturer.

Intracellular staining and flowcytometric analysis

After 7 days incubation, cells were re-stimulated for 4–6 h with 200 µL of RPMI supplemented with gentamicin, L-glutamine and pyruvate and PMA (50 ng/mL) (Sigma-Aldrich), ionomycin (1 µg/mL) (Sigma-Aldrich), Golgiplug (BD Biosciences, Breda, the Netherlands) and 10% human serum. Cells were stained extracellularly using PE-Cy7-conjugated anti-CD4 (BD Biosciences), PE-Cy7-conjugated anti-CD8 (BioLegend, San Diego, CA, USA), or PE-Cy7-conjugated anti-CD56 (Beckman Coulter) and ECD-conjugated monoclonal anti-CD3 antibody (Beckman Coulter, Krefeld, Germany). Fixation and permeabilisation was performed with Cytofix/Cytoperm solution (eBioscience) according to the instructions supplied by the manufacturer. Cells were stained intracellularly with anti-IL-4 (FITC conjugated), anti-IL-5 (PE conjugated) and anti-IL-13 (APC conjugated) (all

BD Bioscience). Fixed cells were measured with a FC500 flowcytometer (Beckman Coulter), and the data were analysed using CXP analysis software v2.2 (Beckman Coulter).

OX40L expression

RNA was isolated from 1×10^6 PBMCs after stimulation for 48 h with *Aspergillus* conidia either in the presence of medium or Adalimumab or IL-1Ra using TRIzol Reagent (Invitrogen) according to a protocol supplied by the manufacturer. RNA (500 ng) was reverse-transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). Quantitative PCR (qPCR) analysis was performed using SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA, USA) and the Applied Biosystems 7300 real-time PCR system. As PCR protocol, the following conditions were used: 2 min 50°C, 10 min 95°C followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. For the amplification of hOX40L, the primers 5'-TCCCCTCTCTAGGTGCTCA-3' and 5'-GGCTGGTGCATAGCAGAAAT-3' were used. To correct for differences in loading concentrations of RNA between the different conditions, qPCR results were corrected with the housekeeping gene $\beta 2$ microglobulin ($\beta 2$ m) amplified using the primers 5'-ATGAGTATGCCTGCCGTGTG-3' and 5'-CCAAATGCGGCATCTTCAAAC-3'. Primer efficacy was evaluated using a standard curve. OX40L C_t values were compared with the $\beta 2$ m C_t using the formula $2^{-\Delta C_t}$ and the fold change was calculated by setting the *Aspergillus*-stimulated samples as 1 to determine the effect of anti-TNF (Adalimumab) and IL-1Ra on OX40L expression.

Genotyping

Genomic DNA from healthy Caucasian controls was isolated using the Gentra Pure Gene blood kit (Qiagen, Hilden, Germany) according to the instructions supplied by the manufacturer. The specific single nucleotide polymorphism (SNP) identifiers (rs numbers) were extracted from a recent publication describing SNPs associated with ABPA [19]. Genotypes of the different SNPs were obtained from the Illumina immunochip, which has previously been run according to the manufacturers' protocol [20]. Beforehand, a quality filter was applied which automatically excluded SNPs with either a low call rate (< 99%), SNPs that were not in Hardy-Weinberg equilibrium ($P < 0.01$) or that had a reported minor allele frequency of < 0.01.

Statistical analysis

The Mann-Whitney U -test was used to detect differences between healthy controls and patients or between

different genotypes. The Wilcoxon signed rank test was used to determine differences between stimulation with and without inhibitors of PRRs, cytokines or cytokine inhibitors. A P -value of < 0.05 was considered statistically significant (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$). Graphs represent cumulative results of all performed experiments and are presented as mean $\pm$ SEM. Data were analysed with GraphPad Prism v 5.0 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Aspergillus conidia induce a Th2 response in human peripheral blood mononuclear cells, in contrast to other pathogens or pattern recognition receptor ligands

We investigated which PRRs are involved in the initiation of a Th2 response by stimulating PBMCs with different specific ligands for TLR4, TLR2, TLR5, NOD2 or dectin-1 and measuring IL-5 and IL-13 (Fig. 1a). None of these ligands or combinations of TLR2 with ligands for dectin-1 or NOD2 was able to induce IL-5 or IL-13. We thereafter screened a panel of pathogens consisting of extra- and intracellular bacteria, as well as two types of fungi for their capacity to induce Th2 cytokines (Fig. 1b). The whole bacteria *E. coli*, *S. aureus* or *B. burgdorferi*, as well as sonicated *M. tuberculosis*, were unable to induce IL-5 and IL-13, while these pathogens induce other immune responses such as IL-17 and IFN γ in these conditions (data not shown). Among the fungal pathogens, the Th2 response was restricted to the stimulation with *A. fumigatus* conidia, while *C. albicans* yeast did not induce IL-5 and IL-13. Moreover, *Aspergillus* hyphae induced relatively low IL-5 production (76.4 pg/mL hyphae vs. 296 pg/mL conidia) and IL-13 (310 pg/mL hyphae vs. 568 pg/mL conidia). Stimulation with the fungal cell wall components α 1,3-glucan and α 1,3-1,6-glucan, β 1,3-glucan, GAG or chitin did not induce IL-5, while IL-13 production was slightly induced by α 1,3-1,6-glucan or chitin (Fig. 1c). PHA was used as a mitogenic stimulus to detect T-cell memory activation and induced low levels of Th2 cytokines.

Aspergillus-induced IL-5 and IL-13 are dose and time dependent and predominantly produced by CD4⁺ T cells

To study the kinetics and dose dependency of IL-5 and IL-13 induced by *Aspergillus* conidia, we performed a time course of 7 days with three different concentrations ranging from 1×10^5 to 1×10^7 conidia/mL (Fig. 2a). IL-4 was undetectable in all stimulations (data not shown). In contrast, IL-5 and IL-13 were detectable from day 4 onwards and increased steadily. Only the highest dosage of 1×10^7 conidia/mL induced IL-5 and IL-13, while dosages below this threshold induced

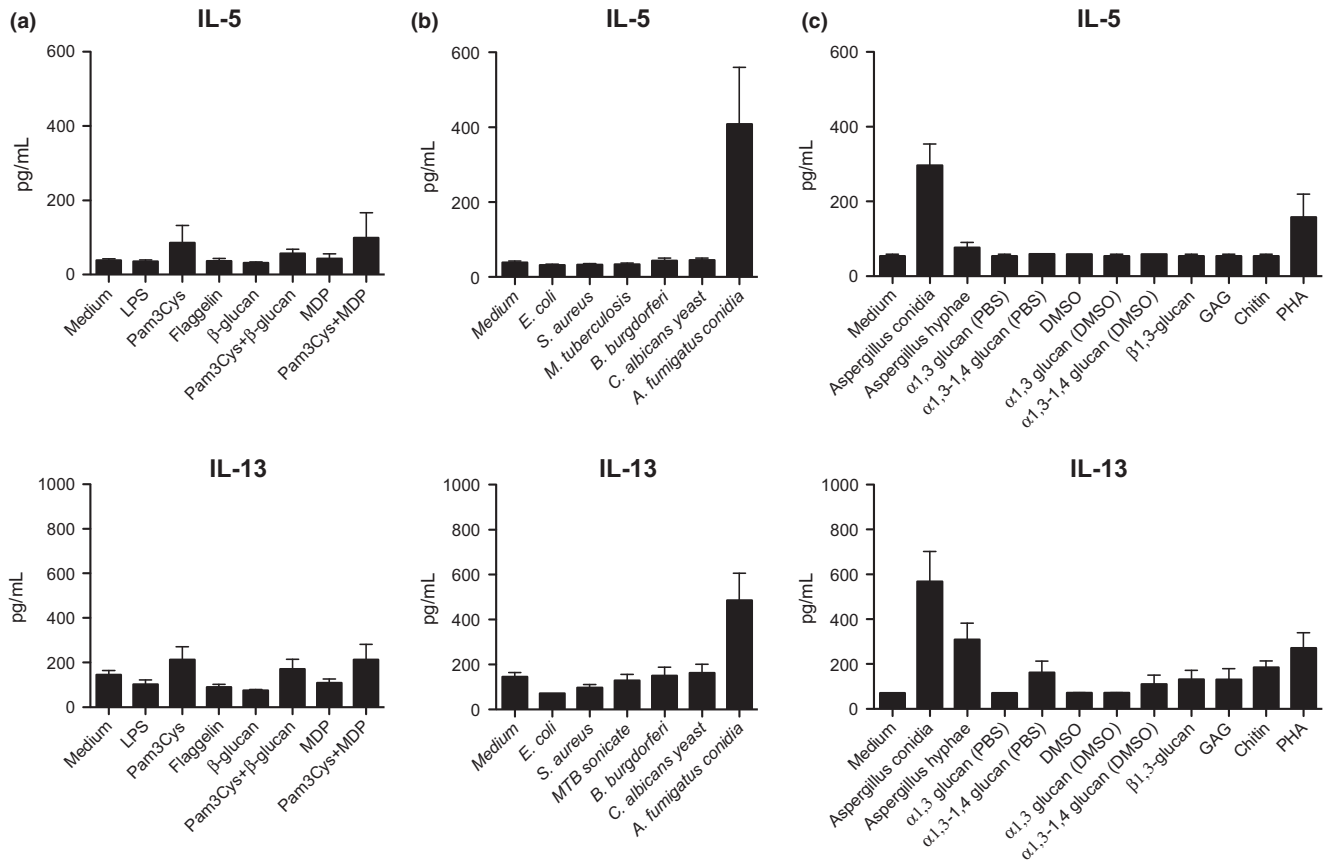


Fig. 1. *Aspergillus* conidia induce a Th2 response in human peripheral blood mononuclear cells (PBMCs) in contrast to other pathogens or pattern recognition receptor ligands. IL-5 and IL-13 concentrations were measured in cell culture supernatants of PBMCs of healthy controls (2.5×10^6 / mL, $n = 6$ donors) by ELISA (IL-5 detection level 24 pg/mL, IL-13 detection level 71 pg/mL) after stimulation with (a) TLR4 ligand (LPS), TLR2 ligand (Pam3Cys), TLR3 ligand (Poly I:C), TLR 5 ligand (flagellin), NOD2 ligand (MDP) or dectin-1 ligand (β -glucan) (b) Various pathogens such as *Escherichia coli*, *Staphylococcus aureus*, *Mycobacterium tuberculosis* sonicate (H37Rv), *Borrelia burgdorferi*, *Candida albicans* yeast and *Aspergillus fumigatus* conidia (c) *A. fumigatus* conidia and hyphae as well as different cell wall components such as α 1,3 glucan, α 1,3-1,4 glucan, β 1,3 glucan, galactosaminogalactan, chitin and phytohemagglutinin.

cytokine values barely detectable above the detection limit.

To investigate which cells are responsible for the production of IL-4, IL-5 and IL-13 induced by *Aspergillus*, flowcytometric analyses with intracellular staining for IL-4, IL-5 and IL-13 were performed on PBMCs that were cultured for 7 days in the presence of *Aspergillus* conidia. Within the Th2 cytokine-positive population, CD4⁺ cells were the main representatives (72.9% of IL-4⁺, 61.7% of IL-5⁺ and 68.5% of IL-13⁺ cells; Fig. 2b). Surface staining for CD8, CD56 and CD19 revealed the CD8⁺ cells as the second biggest subpopulation contributing to the IL-5 and IL-13 production (Fig. 2c). To determine which cell populations within PBMCs contribute to the IL-5 and IL-13 secretion induced by *Aspergillus* stimulation, PBMCs were depleted of CD4⁺, CD56⁺ or CD19⁺ cells (Fig. 2d). Mock-treated PBMCs lost their capacity to produce IL-5, which made it impossible to evaluate the effect of depleting a

special subpopulation on IL-5 production (Figure S1). Depletion of the CD4⁺ cells led to a reduction in *Aspergillus*-induced IL-13 production to undetectable levels, while depleting CD56⁺ or CD19⁺ cells did not affect IL-13 production. These data suggest that CD4⁺ T-lymphocytes are the main producers of Th2 cytokines induced by *Aspergillus* conidia.

Aspergillus-induced IL-5 and IL-13 response is dependent on complement receptor 3 and phagocytosis

As most PRRs have been studied in the context of invasive aspergillosis [3], only little is known about the PRRs and pathways involved in the pathogenesis of ABPA. We investigated the role of TLR2, TLR4, dectin-1 and complement receptor 3 (CR3) pathways in *Aspergillus*-induced Th2 responses in human PBMCs. PBMCs were stimulated with *Aspergillus* conidia in the absence or presence of specific inhibitors for the PRR pathways.

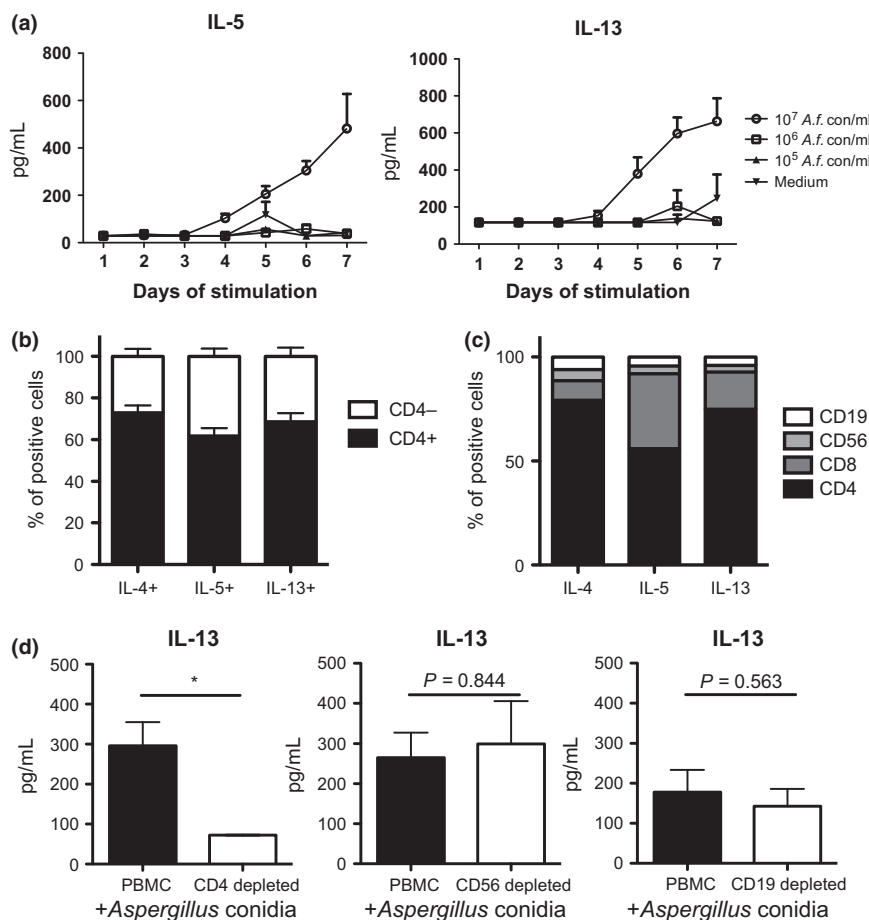


Fig. 2. *Aspergillus*-induced Th2 cytokines are dose and time dependent and predominantly produced by CD4⁺ cells. (a) Peripheral blood mononuclear cells (PBMCs) (2.5×10^6 /mL, $n = 4$ donors) were stimulated with increasing concentrations of *Aspergillus* conidia (1×10^5 /mL, 1×10^6 /mL, 1×10^7 /mL) for 1–7 days, and IL-5 and IL-13 were measured in the cell culture supernatant by ELISA. (b, c) Cells were stained extracellular for CD4⁺, CD8⁺, CD56⁺ and CD19⁺ and intracellular for IL-4, IL-5 and IL-13. (b) CD4⁺ and CD4[−] cells ($n = 10$) within the IL-4⁺, IL-5⁺ or IL-13⁺ positive cells. (c) CD4⁺, CD8⁺, CD56⁺ and CD19⁺ cells within the *Aspergillus*-specific IL-4⁺, IL-5⁺ and IL-13⁺ populations (normalised to 100%, one representative donor) (d) PBMCs were depleted for either CD4⁺, CD56⁺ or CD19⁺ cells (white bars) or run over the depletion columns unlabelled (black bars) and stimulated with *Aspergillus* conidia. IL-13 was measured in the cell culture supernatant by ELISA ($n = 6$ donors). The Wilcoxon signed rank test was used to determine, whether the means were significantly different (* $=P < 0.05$, ** $=P < 0.01$ and *** $=P < 0.001$).

Inhibition of TLR4 did not alter IL-5 production, while IL-13 was slightly reduced (mean 15.7% reduction, Fig. 3a). Although TLR2 has been described to polarise *Aspergillus*-specific T-cell responses towards Th2 [21], neither IL-5 nor IL-13 was affected when TLR2 was blocked (Fig. 3b).

Dectin-1 is known to recognise the fungal cell wall component β -glucan [22] and is known to promote immunopathology in the lung during fungal allergy [23]. However, blocking dectin-1 with laminarin (Fig. 3c), or inhibition of Syk kinase, the downstream signalling kinase of the dectin-1 receptor, did not alter the Th2 response induced by *Aspergillus* (Fig. 3d). Additionally, PBMCs isolated from three dectin-1 deficient patients produced IL-5 and IL-13 levels comparable to healthy controls (Fig. 3e). Subsequently, we investigated CR3 (CD11b/CD18), a β_2 -integrin expressed

on neutrophils and monocytes, because it has been described that CR3 is also able to recognise β -glucan [24]. Blockade of CR3 inhibited the *Aspergillus*-induced Th2 response completely (Fig. 3f). We did not observe different cytokine productions when the serum was de-complemented by heat inactivation (Fig. 3g).

As CR3 is involved in phagocytosis [25], we blocked phagocytosis by inhibiting actin polymerisation by cytochalasin D, which significantly reduced IL-5 and IL-13 production (Fig. 3h). High plasma levels of MBL are associated with bronchial asthma with allergic rhinitis and ABPA [26]; therefore, we wanted to investigate whether the recognition of *Aspergillus* conidia with subsequent Th2 induction via CR3 is MBL dependent. *Aspergillus*-specific Th2 cytokines in the presence of MBL-deficient serum were significantly decreased after blocking CR3, suggesting that signalling via CR3

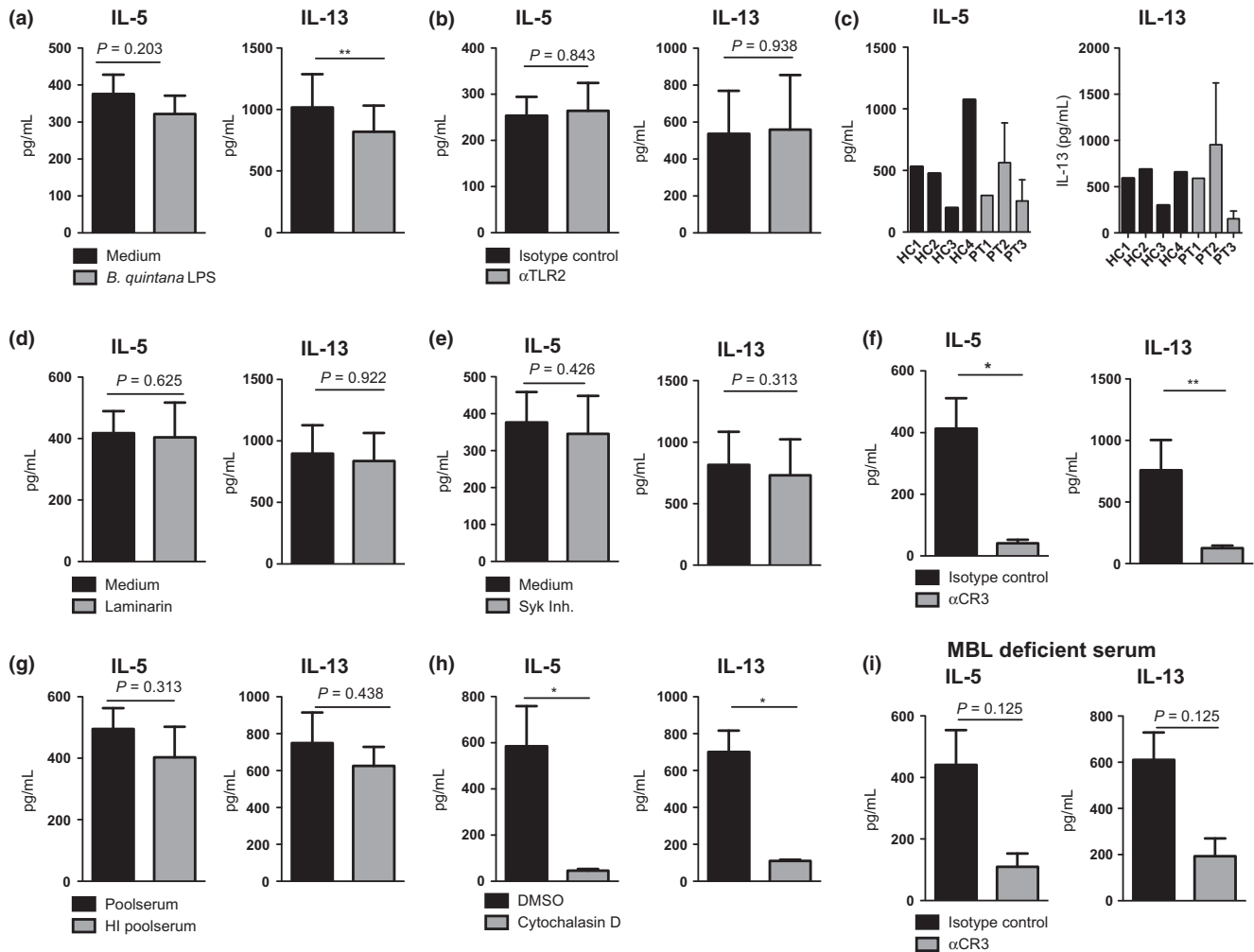


Fig. 3. *Aspergillus*-induced IL-5 and IL-13 response is dependent on complement receptor 3 (CR3) and phagocytosis. Peripheral blood mononuclear cells (PBMCs) were pre-incubated for 1 h with different blockers and stimulated with *Aspergillus* conidia. IL-5 and IL-13 were measured in the cell culture supernatant (black bars: *Aspergillus* conidia stimulation with medium or control, grey bar: *Aspergillus* conidia stimulation after blocking). (a) TLR4 blocking with *Bartonella quintana* LPS (IL-5 $n = 9$ donors, IL-13 $n = 8$ donors). (b) TLR2 blocking with α TLR2 antibody (IL-5 $n = 6$ donors, IL-13 $n = 7$ donors). (c) PBMCs of three dectin-1 deficient patients compared with four healthy controls. (d) Dectin-1 blocking with laminarin ($n = 10$ donors). (e) Syk inhibition (IL-5 $n = 9$ donors, IL-13 $n = 7$ donors). (f) CR3 blocking with α CR3 antibody (IL-5 $n = 7$ donors, IL-13 $n = 9$ donors). (g) PBMC stimulation with 10% active human serum compared with 10% heat-inactivated human serum (IL-5 $n = 5$ donors, IL-13 $n = 6$ donors). (h) Blocking phagocytosis with cytochalasin D ($n = 6$ donors). (i) CR3 blocking with α CR3 antibody (IL-5 and IL-13 $n = 4$ donors) in the presence of mannose-binding lectin-deficient serum. The Wilcoxon signed rank test was used to determine, whether the means were significantly different (* = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$).

leading to Th2 responses is independent of MBL (Fig. 3i). These data demonstrate that the IL-13 response induced by *Aspergillus* in human PBMCs is partially dependent on TLR4, while both IL-5 and IL-13 are dependent on CR3 and phagocytosis.

Allergic bronchopulmonary aspergillosis patients have an Aspergillus-specific increased Th2/Th1 ratio

Peripheral blood mononuclear cells isolated from nine ABPA patients and healthy controls were stimulated with *Aspergillus* conidia, and IL-5, IL-13 and IFN γ production was measured. Compared with non-allergic

healthy controls, *ex vivo* induction of Th2 cytokines by *Aspergillus* stimulation was stronger in ABPA patients, which was statistically significant for IL-5, but not for IL-13 (Fig. 4a). In contrast, *Aspergillus*-induced IFN γ production by PBMCs was significantly lower in ABPA patients when compared to healthy controls (Fig. 4a). Interestingly, IFN γ was significantly decreased after stimulation with both *Aspergillus* conidia and hyphae (data only shown for conidia). When we compared the IL-5/IFN γ and IL-13/IFN γ ratios in ABPA patients and controls, we observed that they were significantly different and discriminated ABPA patients from healthy controls (Fig. 4b,c).

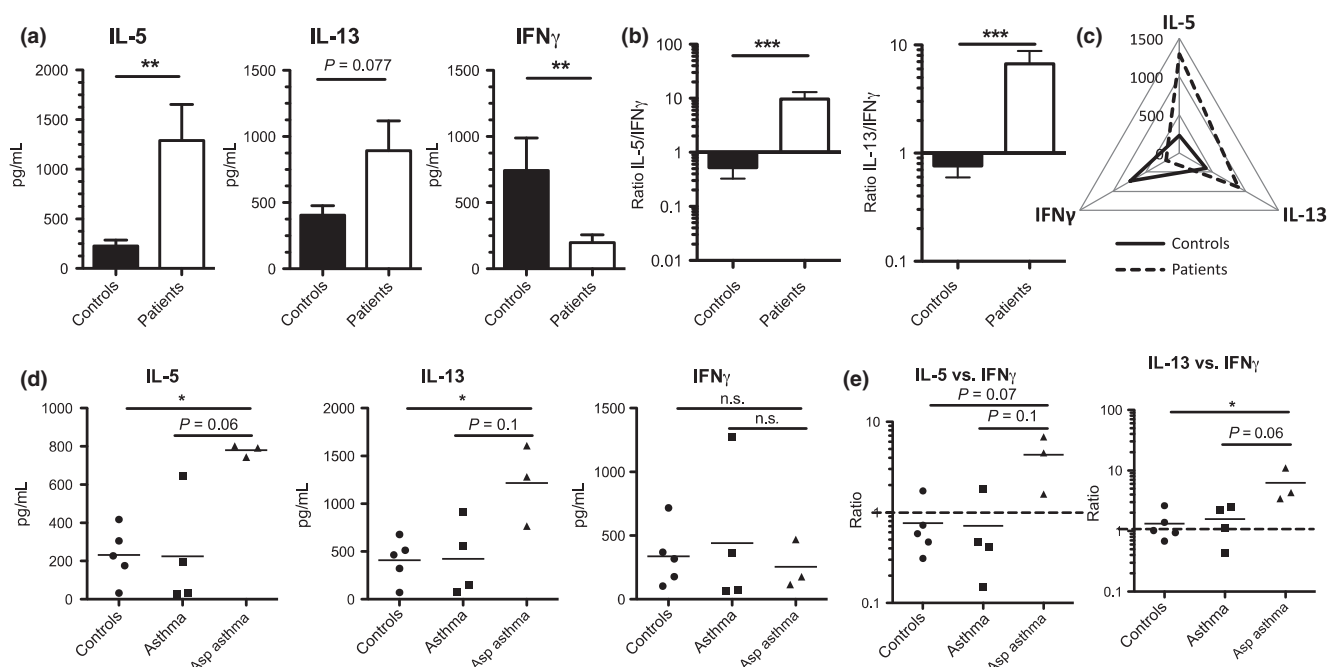


Fig. 4. The Th2/Th1 balance in allergic bronchopulmonary aspergillosis (ABPA) patients is shifted towards Th2. (a) Peripheral blood mononuclear cells (PBMCs) of 9 ABPA patients were stimulated with *Aspergillus* conidia and compared with PBMCs of healthy controls. IL-5, IL-13 and IFN γ levels were measured in the cell culture supernatant (black bars controls and white bars patients). (b) The Th2/Th1-ratio between IL-5 vs. IFN γ and IL-13 vs. IFN γ was generated by dividing the individual IL-5 or IL-13 level by the individual IFN γ level. (c) The triangle diagram shows the mean of IL-5, IL-13 or IFN γ of every group on the corresponding cytokine axis (continuous line: controls, dotted line: patients). (d) PBMCs isolated from 3 asthma patients with an elevated *Aspergillus*-specific IgE (Asp asthma) and 4 without elevated *Aspergillus*-specific IgE (asthma) were stimulated with *Aspergillus* conidia and compared with healthy controls. IL-5, IL-13 and IFN γ levels were measured in the cell culture supernatant. (e) The Th2/Th1 ratio between IL-5 vs. IFN γ and IL-13 vs. IFN γ was generated by dividing the individual IL-5 or IL-13 level by the individual IFN γ level. The Mann-Whitney U -test was used to determine, whether the means were significantly different (* = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$).

To determine whether the increased Th2/Th1 ratio in ABPA is due to the underlying asthma itself or due to *Aspergillus* sensitisation, we investigated *Aspergillus*-specific ratios in asthma patients with and without an increased *Aspergillus*-specific IgE (Table S1). PBMCs isolated from asthma patients with *Aspergillus* sensitisation that were stimulated with *Aspergillus* conidia showed a higher *Aspergillus*-specific Th2 response than the asthma group without *Aspergillus* sensitisation and the control group (Fig. 4d). This resulted in an increased Th2/Th1 ratio comparable to ABPA patients (Fig. 4e). In addition, we investigated whether the anti-inflammatory cytokine IL-10 is involved in the increased ratios observed in ABPA. Neither in the control nor in the patient group did we detect IL-10 concentrations (Figure S2).

Etanercept, Adalimumab, Anakinra and IFN-gamma have the capability to modulate the Aspergillus-induced Th2/Th1 ratio in human peripheral blood mononuclear cells

As we observed significantly different Th2/Th1 ratios in ABPA patients, we wanted to investigate whether

available biologicals for treatment of inflammatory disease are capable of modulating the *Aspergillus*-specific Th2/Th1 ratio. We used Etanercept (soluble TNF α receptor II-Fc), Adalimumab (human anti-human TNF α), Anakinra (IL-1 receptor antagonist) and Immukine (recombinant human IFN γ) and stimulated PBMCs either with *Aspergillus* conidia in the presence of medium or one of the biologicals. All biologicals significantly suppressed *Aspergillus*-induced IL-5 and IL-13 production in human PBMCs. However, Etanercept, Adalimumab and Anakinra also suppressed IFN γ production in response to *Aspergillus* and hence did not correct the IL-5/IFN γ ratios (Medium: 0.65, Etanercept 1: 0.61, Adalimumab: 0.92, Anakinra: 1.82) and IL-13/IFN γ -ratios (Medium: 1.49, Etanercept: 1.41, Adalimumab: 1.89, Anakinra: 2.77). Therefore, it was only recombinant IFN γ that was able to restore the increased Th1/Th2 ratio by down-regulating IL-5 and IL-13, but not IFN γ .

The co-stimulatory molecule OX40L is known to be involved in inflammatory Th2 responses triggered by thymic stromal lymphopoietin (TSLP)-activated dendritic cells in ABPA patients [27]. We therefore investigated whether OX40L is also involved in the Th2 responses induced by *Aspergillus* in human PBMCs.

Blocking OX40L resulted in significantly decreased *Aspergillus*-induced IL-13 production (Fig. 5d). As TNF and IL-1 can modulate the expression of OX40L [28, 29], we analysed whether blocking TNF or IL-1 would result in a different expression of OX40L in PBMCs. We observed no significant modulation of the *Aspergillus*-induced OX40L expression in the presence of Adalimumab or Anakinra (Fig. 5e).

Polymorphisms influencing *Aspergillus*-specific Th2/Th1 ratios

Single nucleotide polymorphisms in the *IL10*, *IL4R α* , *MBL* and *SFTPA2* genes have been associated with an increased susceptibility to ABPA [19]. We took a systematic approach to investigate whether reported polymorphisms that are associated with ABPA could influence *Aspergillus*-specific Th2/Th1 ratios (Table 2). PBMCs isolated from 35 healthy controls were stimulated with *Aspergillus* conidia and IL-5, and IFN γ was measured. The homozygote GG genotype of IL-4R α (rs1805010) showed a trend towards higher IL-5 production and an increased IL-5/IFN γ ratio (Fig. 6a). The GG genotype in the IL-10 SNP (rs1800896) showed a trend towards higher IFN γ production; however, no

association was observed with *Aspergillus*-specific IL-5 or IL-5/IFN γ ratio (Fig. 6e). The other polymorphisms in SFTPA2 (rs17886395 and rs17880349) and MBL (rs5030737) did not show an association with IL-5 or IFN γ production (Fig. 6b–d).

Discussion

In the present study, we investigated the general and *Aspergillus*-specific features of the Th2 response in primary human PBMCs of healthy controls and ABPA patients. Th2 cytokine responses *in vitro* could only be detected when PBMCs were stimulated with *A. fumigatus*, while *Candida*, specific PRR ligands, Gram-positive and Gram-negative bacteria did not induce IL-5 or IL-13 production. Furthermore, no single fungal component induced IL-5 or IL-13 in the same amount as *Aspergillus* conidia, suggesting that a complex of fungal PAMPs present on the cell wall of *Aspergillus* conidia is needed to induce a Th2 response in human PBMCs. The *Aspergillus*-specific IL-5 and IL-13 production was dependent on CD4⁺ cells, the pattern recognition receptor CR3 and phagocytosis. Moreover, we identified that ABPA patients, in contrast to non-allergic controls, had a significantly increased Th2/Th1 ratio

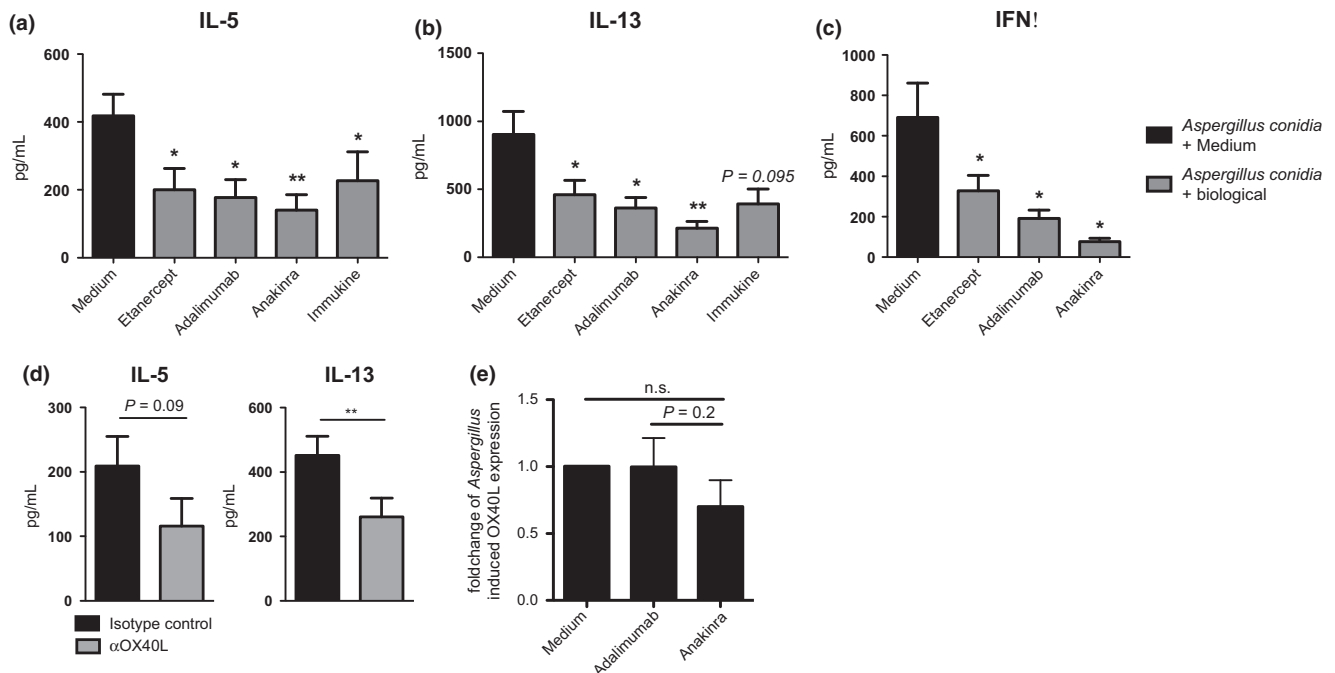


Fig. 5. Etanercept, Adalimumab, Anakinra and IFN γ have the capability to modulate the *Aspergillus*-induced Th2/Th1 ratio in human peripheral blood mononuclear cells (PBMCs). PBMCs of healthy controls were stimulated with *Aspergillus* conidia either in the presence of medium (black bars) or one of the biological drugs (grey bars) such as Etanercept (soluble TNF α receptor), Adalimumab (human TNF α antibody), Anakinra (IL-1Ra) or recombinant IFN γ . (a) IL-5, (b) IL-13 and (c) IFN γ were measured with ELISA in the cell culture supernatant. (d) IL-5 and IL-13 measurement after blocking OX40L with α OX40L antibody (IL-5 and IL-13, $n = 9$ donors). (e) OX40L expression in PBMCs of healthy controls stimulated with *Aspergillus* conidia either in the presence of adalimumab or anakinra ($n = 6$). The Wilcoxon signed rank test was used to determine, whether the means were significantly different (* = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$).

Table 2. Single nucleotide polymorphisms (SNPs) associated with allergic bronchopulmonary aspergillosis (ABPA)

Gene	rs number	SNP position	MAF	Function
IL-4R α (16p12.1-p11.2)	rs1805010	4679 A/G	0.466	Missense
SFTPA2 (10q22.3)	rs17886395	1649 C/G	0.206	Missense
SFTPA2	rs17880349	1492 C/T	0.461	intron variant
IL-10 (1q31-q32)	rs1800896	min 1082 A/G	0.303	upstream variant
MBL (10q11.2-q21)	rs5030737	868 C/T	0.028	Missense

Gene, rs number, SNP position, minor allele frequency (MAF) and resulting defect of gene function of SNPs associated with ABPA are listed.

induced by *Aspergillus*. Interestingly, not the asthma itself but the *Aspergillus* sensitisation in asthma patients appears to be responsible for the increased Th2/Th1 ratios observed in ABPA. We suggest that treatment strategies restoring this Th2/Th1 imbalance triggered by *Aspergillus* might be beneficial in the treatment of ABPA. Although anti-TNF and IL-1Ra had the capacity to decrease *Aspergillus*-induced IL-5 and IL-13 production, they also lowered IFN γ production. Therefore, the capacity of recombinant IFN γ to lower

IL-5 and IL-13 and to supplement the IFN γ deficiency in ABPA provides a rationale to use IFN γ as a corticosteroid-sparing treatment option in ABPA.

The observation that the induction of Th2 cytokines in human PBMCs was restricted to *Aspergillus* was striking. In the context of diseases caused by bacteria such as *E. coli*, *S. aureus*, *M. tuberculosis* and *B. burgdorferi*, it can be anticipated that they do not induce strong Th2 responses in human PBMCs. However, the observation that no single TLR ligand, NOD2 ligand or fungal cell wall component, specifically chitin, did not induce IL-5 and IL-13 was unexpected. Chitin has been associated with allergic inflammation in the lung [30, 31]. Therefore, it is most likely that chitin in the setting of an inflammatory milieu is able to polarise towards Th2 responses, while as a single ligand, it is not able to induce a Th2 response.

This study revealed a non-predominant role of TLRs in the induction of *Aspergillus*-specific Th2 cytokines. Blocking of TLR2 did not have a significant impact on IL-5 and IL-13 production induced by *Aspergillus*. In contrast, it has been shown that there is an association between increased TLR2 expression in fatal asthma [32], and polymorphisms in TLR2 are associated with a higher prevalence of asthma [33]. Furthermore, TLR2 $^{-/-}$ mice show a deficient Th2 production in a chronic

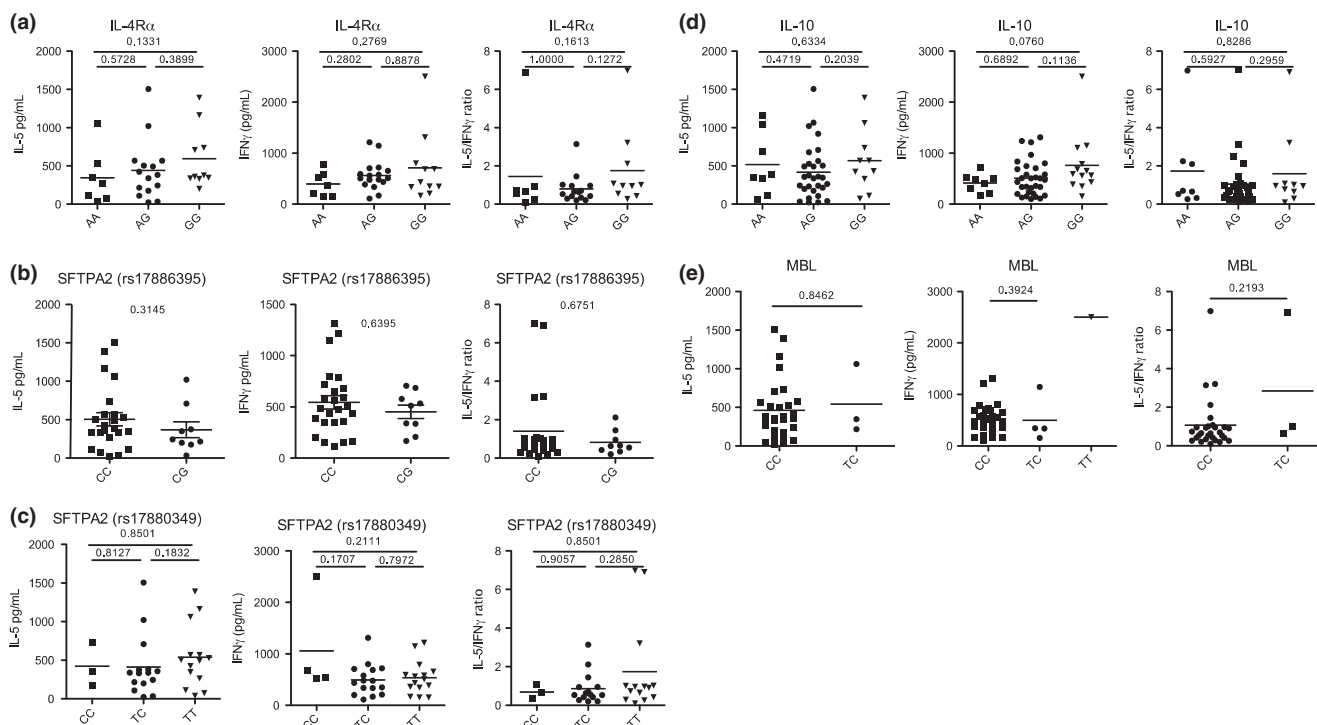


Fig. 6. The role of SNPs associated with allergic bronchopulmonary aspergillosis on *Aspergillus*-specific Th2/Th1 ratios. Peripheral blood mononuclear cells isolated from healthy volunteers ($n = 35$) were stimulated with *Aspergillus* conidia, and IL-5 and IFN γ were measured in the cell culture supernatant. Single nucleotide polymorphisms were analysed for association with cytokine production. Statistic analysis was performed with the Mann–Whitney U -test.

fungal asthma model [11]. The minor but significant contribution of TLR4 in the *Aspergillus*-induced IL-13 induction found in the present study is in line with former studies describing TLR4 in allergic responses against house dust mite in which an endotoxin containing extract signalling via TLR4 [34]. In addition, we identified a redundant role of dectin-1 in the *Aspergillus*-induced Th2 response. Blocking dectin-1 or Syk did not alter IL-5 and IL-13 production, and dectin-1 deficient patients produced Th2 cytokine responses comparable to healthy controls. The dectin-1 ligand β -glucan [17], however, has recently been described to play an important role in *Aspergillus*-triggered asthma immunopathology in mice [23]. The role of dectin-1 in the immunopathology of asthma is not clear. It has been suggested that dectin-1 promotes the immunopathology in fungal asthma [23], while another study demonstrated that dectin-1 plays a role in suppressing asthmatic inflammation [35]. Our findings suggest that TLR2 and dectin-1 are not the main pathways driving the *Aspergillus*-specific Th2 response in human mononuclear cells. Whether TLR2 and dectin-1 play a role in *Aspergillus*-induced Th2 responses that are dependent on epithelial cells remains to be elucidated.

Th2 induction by *Aspergillus* was dependent on CR3 (CD11b/CD18). CR3 is a β_2 -integrin that contains a lectin-like domain and is expressed on neutrophils and monocytes [25]. CR3 contributes to antifungal host defence by interacting with pentraxin-3 opsonised particles and by recognising β -glucan [24]. Furthermore, CR3 is involved in complement-driven host responses [36]; however, the induction of IL-5 and IL-13 was still present in de-complemented serum, suggesting a complement-independent contribution of CR3 to the Th2 response. Activation of the complement via the MBL pathway is an alternative route of the complement pathway, and increased MBL serum concentrations have been associated with ABPA [26]. However, Th2 induction by *Aspergillus* via CR3 signalling was independent of serum MBL, suggesting that MBL is not crucial for CR3-mediated Th2 responses induced by *Aspergillus*. Phagocytosis of non-opsonised particles via signalling together with FcR is also dependent on CR3 [25]. In line with blocking CR3, blocking phagocytosis led to a complete abolishment of Th2 production induced by *Aspergillus*. These data collectively suggest that CR3 is involved in phagocytosis of *Aspergillus* conidia and that this process is essential for Th2 cytokine production by human PBMCs. Recently, CR3 was described in the induction of the *Aspergillus*-specific pro-inflammatory T helper cytokines [16], suggesting a more general role for CR3 in modulating T helper responses.

An important issue to address was the cellular source of the Th2 cytokine in human mononuclear cells induced by *Aspergillus*. We could demonstrate

that CD4⁺, but not CD19⁺ and CD56⁺, cells were the predominant source for the *Aspergillus*-induced IL-13 in human PBMCs. This is in line with previous studies showing that CD4⁺ cells are responsible effector cells in allergic reactions [10] and that CD19⁺ and CD56⁺ play a redundant role in an asthmatic mouse model [37]. Although increased levels of CD8⁺ and IL-13⁺ cells have been associated with asthma and bronchial obstruction [38], IL-13 production in human PBMCs was completely dependent on CD4⁺ cells, suggesting that CD8⁺ T cells are not involved in the production of *Aspergillus*-induced IL-13 in human mononuclear cells. We could not demonstrate the cellular source of IL-5 in our setting because IL-5 was not measurable in depletion experiments. As FACS analysis demonstrated a significant CD8⁺ population that stained positive for IL-5, we cannot exclude that CD8⁺ T cells play a role in *Aspergillus*-induced IL-5 production in human PBMCs.

Several studies have shown an exaggerated Th2 response in ABPA [39–41]. We investigated the *Aspergillus*-specific Th2 response in the context of *Aspergillus*-induced T helper responses in human PBMCs isolated from ABPA patients. Interestingly, ABPA patients not only show an increased IL-5 and IL-13 production after stimulation with *Aspergillus* conidia, which is in line with previous studies [42], but also they have an *Aspergillus*-specific decreased IFN γ production. As we also observed an increased Th2/Th1 ratio in asthma patients with *Aspergillus* sensitisation, but not in patients with asthma that did not have *Aspergillus* sensitisation, it is most likely that the increased Th2/Th1 ratios in ABPA are due to an *Aspergillus*-specific host response and not due to the underlying host response associated with asthma itself. These observations strengthen the hypothesis that ABPA and asthma with *Aspergillus* sensitisation share underlying pathophysiological mechanisms that are distinct from asthma itself and support the concept that similar treatment approaches could be beneficial in both asthma-sensitized patients and ABPA, such as the treatment with azoles [9].

Aspergillus hyphae have been found in tissue biopsies in ABPA and have been suggested to play a significant role in inflammation in ABPA [43, 44]. Furthermore, IgE antibodies directed against Asp4 and Asp6 can be observed in ABPA in patients with CF [45, 46]. Asp6 has been shown to be a hyphae-specific protein. Therefore, ABPA patients are exposed to *Aspergillus* hyphae, which contributes to a Th2 response *in vivo*. We observed, however, that *Aspergillus* hyphae induced much lower amounts of Th2 cytokines in ABPA patients compared with *Aspergillus* conidia, suggesting that fungal cell wall structures specific for *Aspergillus* conidia, or phagocytosis might play a more prominent

role in the induction of Th2 responses in ABPA than previously thought.

Regulatory T cells (Tregs) are important for controlling allergic immune responses [47]. A recent study has shown that *Aspergillus*-sensitised asthma patients with high *Aspergillus*-specific Th2 induction had significant decreased levels of *Aspergillus*-specific Tregs, while they were strongly induced in the healthy control group [48]. Also in CF patients with ABPA, Tregs were less frequent than in non-ABPA CF patients [27] suggesting that a deficient Treg response could contribute to the excessive and unbalanced inflammatory response observed in ABPA patients. It is tempting to speculate that the increased *Aspergillus*-specific Th2/Th1 response that we observed in the ABPA patients is due to a deficient Treg response and that a deficient Treg response, accompanied by a high Th2 and low Th1 response, plays a significant role in the pathophysiology of ABPA.

The characterisation of this immunological profile of ABPA patients with an increased Th2/Th1 ratio provides a read-out for testing potential immunomodulatory treatment options in ABPA. TNF and IL-1 are interesting targets. TNF has been shown to be the key cytokine inducing increased IL-17 levels and neutrophilia in the lung in a murine ABPA model [49]. The involvement of the inflammasome and epithelial-derived IL-1 cytokines is also described in bronchial asthma in several studies [50, 51]. Therefore, we tested the TNF neutralising biologicals, Etanercept and Adalimumab, and the IL-1 pathway blocking biological IL-1Ra (Anakinra). All biologicals decreased IL-5 and IL-13 production, but also IFN γ production, therefore resulting in unaltered IL-13/IFN γ and IL-5/IFN γ ratios. In addition, we investigated recombinant IFN γ , which decreased *Aspergillus*-specific IL-5 and IL-13 production.

The co-stimulatory molecule OX40L is involved in the initiation and maintenance of allergic responses, mainly driven by TSLP-activated dendritic cells [52]. Clinical studies that have been performed to elucidate the role of OX40L as a possible therapeutic target in asthma have shown that blocking OX40L results in a decrease in serum IgE and eosinophils in asthma [53]. OX40L was also involved in the *Aspergillus*-induced Th2 response, which is in line with former studies showing OX40L expressed on a variety of cells such as activated T cells, NK cells, B cells and monocytes [54]. We observed a low expression of OX40L in PBMCs that was not significantly modulated by TNF or IL-1. Although it is reported that TNF can increase OX40L expression in smooth muscle cells, significantly higher in asthmatic than non-asthmatic patients [28] and TNF can up-regulate OX40L promoter-activity [29], this mechanism appears to play a more prominent role in DC-driven Th2 responses [55].

Taking the shifted *Aspergillus*-induced Th1/Th2 ratio into account, restoring the increased Th1/Th2-ratio might be an interesting alternative for target-specific immunomodulatory therapy in ABPA. Previous studies have shown that neutralising IL-13 has beneficial effects in an asthmatic mouse model [56] by dampening the IL-13-driven allergic airway inflammation. Similarly, blocking IL-4 has been tried to attenuate allergic airway responsiveness; however, the effect was not as strong and long-lasting as blocking IL-13 [56]. Biologicals targeting IL-4, IL-5 and IL-13 have been shown to have some efficacy in patients with high Th2 cytokine levels; however, optimal biomarkers to identify responders and non-responders are still lacking [57]. There is only little clinical evidence for immunomodulatory treatment with biologicals in ABPA. Omalizumab has been successfully used in a few cases of ABPA with and without CF resulting in a corticosteroid-sparing treatment [58–60]. The data of this study suggest that IFN γ might be an adequate immunomodulatory therapeutic option for ABPA, because it has the capacity to restore the increased Th1/Th2 ratio. Next to lowering the Th2 response, it also supplements the relative *Aspergillus*-specific IFN γ deficiency. Recombinant IFN γ has been used with success in patients with chronic granulomatous disease to prevent *Aspergillus* infection [61], and there are several case reports that describe the beneficial effect of IFN γ treatment in *Aspergillus* infection [62]. Thus, IFN γ might contribute to disease control in ABPA by having a beneficial effect on clearing the fungal burden, thereby decreasing the trigger of the allergic inflammatory reaction and by restoring the increased Th2/Th1 ratio.

Single nucleotide polymorphisms in the *IL10*, *IL4R α* , *MBL* and *SFTPA2* genes have been associated with an increased susceptibility to ABPA [19]. We therefore investigated whether these genetic variants could modulate the *Aspergillus*-induced Th1 or Th2 response. Variants in the MBL [63] and SFTPA2 [64] genes did not lead to different *Aspergillus*-specific Th2 and Th1 responses. In contrast, we could identify a trend between the IL-4R α [65] GG genotype with higher *Aspergillus*-specific IL-5 production and *Aspergillus*-specific Th2/Th1 ratio. We observed a trend in the IL-10 (rs1800896) GG genotype towards higher IFN γ production. Previous studies have identified this genotype to be associated with *Aspergillus* colonisation and ABPA in CF patients [66].

In conclusion, *Aspergillus* conidia are unique in triggering Th2 responses in human PBMCs, and we provide evidence that CR3 and phagocytosis play a predominant role in *Aspergillus*-induced Th2 responses. Moreover, we identified that patients with ABPA have an increased *Aspergillus*-specific Th2/Th1 ratio, and the

data in this study together with the clinical experience and safety of IFN γ treatment provide a rationale for exploring IFN γ in a clinical trial in patients with ABPA.

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Author contribution

K.L.B., F.L. vd V., M.S.G. and S.P.S. designed the experiments; K.L.B., M.S.G., S.P.S., R.L. and M.O. performed the experiments; K.L.B., M.S.G., C.J., M.J. and X.W. analysed the experiments; K.L.B., F.L. vd V., M.G.N., L.A.B.J. and M.H.R. wrote the manuscript; F.L. vd V., C.M.-E. and M.H.R. included the patients.

Conflict of interest

The authors declare no conflict of interests.

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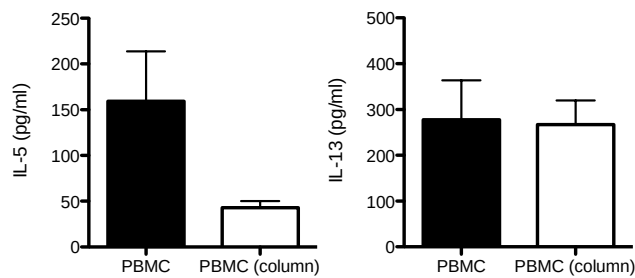
Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The Th2 response against *Aspergillus* conidia of PBMCs before and after running over a cell depletion column.

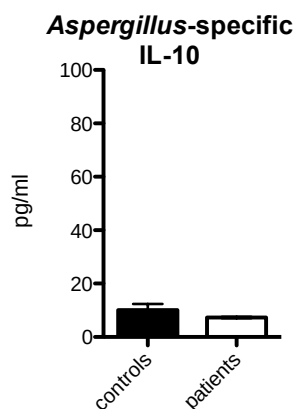
Figure S2. IL-10 induction by *Aspergillus* conidia of PBMCs in ABPA patients and healthy controls.

Table S1. Demographic table of asthma patients.



Supplemental Figure 1: The Th2 response against *Aspergillus* conidia of PBMCs before and after running over a cell depletion column.

PBMCs were either directly stimulated or mock treated by running unlabeled cells of a cell depletion column. PBMCs (2.5×10^6 /ml, n=4 donors) were stimulated with *Aspergillus* conidia and IL-5 and IL-13 were measured in the cell culture supernatant by ELISA.



Supplemental Figure 2: IL-10 induction by *Aspergillus* conidia of PBMCs in ABPA patients and healthy controls. PBMCs (n=9 ABPA patients or healthy controls) were stimulated with *Aspergillus* conidia for 7 days and IL-10 was measured in the cell culture supernatant by ELISA.

	age	gender	Asp. Spec. IgE (U/ml)	Asp. Spec. IgG (mg/ ml)	total IgE (U/ml)
Asp-asthma #1	61	m	>5	neg	1913
Asp-asthma #2	76	m	11,2	62,1	570
Asp-asthma #3	44	m	>5	3,76	52
asthma #1	44	f	<0,35	n.a.	79
asthma #2	23	f	<0,35	36,2	212
asthma #3	47	f	<0,35	4,11	182
asthma #4	47	f	<0,35	43,1	95

Supplemental table 1: Demographic table of asthma patients

Age, gender, total IgE (U/ml), Asp-specific IgE (U/ml) and Asp-specific Ig G (mg/ml) of the asthmatic control groups enrolled in this study are listed.