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Th2 and Th9 responses in patients with Chronic Mucocutaneous Candidiasis and Hyper IgE syndrome

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Running title:

CMC and HIES patients who are both Th17 deficient differ in allergic clinical phenotypes.

Abstract:

Background: STAT1 mutations cause Chronic Mucocutaneous Candidiasis (CMC), while STAT3 mutations cause Hyper IgE syndrome (HIES). CMC and HIES patients have T helper(Th)17 defects suffering from mucosal *Candida* infections, but only HIES patients show an allergic phenotype with eczema, eosinophilia and high IgE levels.

Objective: We investigated whether differential Th2 and Th9 responses may explain the clinical differences.

Methods: Peripheral blood mononuclear cells of CMC patients (n=4), HIES patients (n=4), atopic dermatitis patients (n=4) and healthy volunteers (n=13) were stimulated with *Candida* and *Staphylococcus aureus*, with and without IL-4. The cytokines IL-5, IL-13, IL-9, IL-17 and TGF β and regulatory T cells were measured in cell culture supernatants by ELISA or flowcytometry, respectively.

Results: PBMCs of CMC patients showed a significantly impaired production of the Th2 cytokines IL-5 and IL-13, especially in the presence of IL-4. Moreover, IL-9 production was significantly lower in CMC patients compared to healthy controls. In contrast, HIES patients and AD patients showed normal IL-5 and IL-13 production, while IL-9 production was significantly lower in HIES patients compared to healthy controls. Although TGF β was involved in the IL-4-induced IL-9 production, TGF β levels and the frequency of regulatory T cells did not differ between HIES patients and controls. Flowcytometry analysis demonstrated an IL-9⁺IL-17⁺CD4⁺ subset in healthy controls after stimulation with *Candida* which was less present in HIES patients.

Conclusion: CMC patients have a general Th defect including Th2 and Th9, while HIES patients have normal Th2 cytokines. These differences are in line with their clinical presentation. Surprisingly, the allergic cytokine IL-9 was deficient in both HIES and CMC, suggesting a Th-17 derived origin.

Key words:

Chronic mucocutaneous candidiasis, hyper IgE syndrome, fungal allergy, Th2 response, Interleukin-5, Interleukin-13, Interleukin-9, *Aspergillus fumigatus*, *Candida albicans*, *Staphylococcus aureus*

Abbreviations:

<i>Asp con</i>	<i>Aspergillus conidia</i>
<i>Ca yeast</i>	<i>Candida yeast</i>
CMC	Chronic mucocutaneous candidiasis
FOXP3	Forkhead Box Protein 3
GOF	Gain of function
HIES	Hyper IgE syndrome
IMDM	Iscove's modified Dulbecco's Medium
IFN	Interferon
IL	Interleukin
LOF	Loss of function
PMBC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
RPMI	Rosewall Park Memorial Institute
<i>S. aureus</i>	<i>Staphylococcus aureus</i>

TGF Transforming growth factor

Th T helper

T-reg regulatory T cell

Introduction

Atopic diseases such as asthma or atopic dermatitis are classically driven by an elevated T helper (Th) 2 response, characterized by the cytokines Interleukin (IL) -4, IL-5 and IL-13.

These cytokines mediate a type 1 immediate hypersensitivity reaction associated with IgE class switch and eosinophil recruitment that is followed by a late phase allergic reaction caused by infiltrating allergen-specific T cells that lead to persistent allergic symptoms (1, 2).

Next to the Th2 cells, other T helper cell subpopulations have been identified as important players driving pathological atopic reactions. This includes not only an imbalance between Th1 and Th2 cells (3) or between Th2 cells and regulatory T cell (4), but also disturbed immune responses of the more recently identified Th17 and Th9 cells. IL-17A (hereafter named IL-17), produced by Th17 cells, is an important pro-inflammatory cytokine in the host defence against extracellular pathogens, especially against *Candida* (5), but can also drive pathology in allergic airway inflammation, where IL-17 was identified to induce IgE-mediated late-phase asthmatic response (6) and granulocyte influx (7). IL-9, another pro-inflammatory cytokine derived from Th9 cells after differentiation from a naïve T cell in the presence of IL-4 and transforming growth factor (TGF) β , leads to mast cell activation, eosinophil recruitment and IgE class switch during allergic inflammation (8, 9).

Neutralization of IL-9 in an ovalbumin-induced asthma model was shown to significantly reduce allergic symptoms (10). Interestingly, next to the distinct Th lineage of Th9 cells, Th17 have also been described to be a source for IL-9. TGF β and IL-1 β were thereby

identified as the main inducers of IL-9 and IL-17 co-expressing cells and mainly driving pathology in auto-immune diseases (11, 12).

A Th17 deficient immunological phenotype does not implicitly lead to altered allergic immune responses. The recently identified *gain-of-function* (GOF) mutation in STAT1 leads to deficient IL-17 production (13, 14). Patients with STAT1 GOF-mutation display next to recurrent infections and a higher incidence of auto-immune diseases or oesophageal cancer also chronic mucocutaneous candidiasis (CMC), manifesting as fungal infections on skin, nails and mucous membranes (14), but an atopic constitution is not a feature of the clinical presentation in these patients. In contrast, patients with the autosomal dominant *loss-of-function* (LOF) STAT3 mutation, also leading to a deficient IL-17 production, present with an atopic phenotype with eczema, eosinophilia and high IgE levels, as well as recurrent staphylococcal skin, pulmonary abscesses accompanied by bronchiectasis and also CMC (15, 16). In the present study we wanted to elucidate whether the clinical allergic phenotype in HIES correlates with alterations in Th2 and Th9 immune responses, and compare this with patients without allergic symptoms that share many features of HIES including a Th17 deficiency, namely CMC.

Methods:

Volunteers and patients

Blood was collected from healthy volunteers or patients by venous blood puncture after informed consent. Healthy volunteers between 20 and 40 years of age with no chronic disease or allergic disease in their history as well as without any medication were asked for blood donation. Four CMC patients with an autosomal dominant mutation in the STAT1 gene donated blood, two of them twice at two different time points. Four patients diagnosed with HIES with an autosomal dominant mutation in the STAT3 gene were asked for blood

donations; two of them donated blood twice at different time points (demographic characteristics, Table I). Four patients with atopic dermatitis (AD) recruited from the department of dermatology and diagnosed according to their clinical presentation were included into the study (demographic characteristics, Table II).

All experiments were performed and conducted in accordance to Good Clinical practice, the Declaration of Helsinki, and the approval of the Arnhem-Nijmegen Ethical Committee (nr.2010/104).

Stimuli, recombinant cytokines and blocking antibodies

Staphylococcus aureus isolated from a clinical case was cultured by the department of microbiology, heat killed and provided to us (*S. aureus*) (1×10^7 /ml).; *Candida albicans* ATCC MYA-3573 (UC820) yeast (*Ca yeast*) (1×10^6 /ml) were grown overnight in Sabouraud broth at 37°C, cells were harvested by centrifugation, washed twice, and resuspended in culture medium. *Ca yeast* was heat-killed for one hour at 95 °C. The lack of viability was tested by culturing heat-killed yeast over night at 37°C and used, when no growth was observed (17). *Aspergillus fumigatus* clinical isolate V05-27 (*Asp con*), resting conidia (1×10^7 /ml) were cultured and isolated as described previously (18). Briefly, *A. fumigatus* was cultured on Sabouraud glucose agar supplemented with chloramphenicol for 7 days at 37°C. Conidia were harvested by gently scraping the surface of the slants and suspending them in PBS with 0.05% Tween 80. To remove hyphae and debris, the conidial suspension was filtered through 4 layers of sterile gauze. The Tween of the mixture of conidia and PBS was removed by washing 3 times with PBS, resuspended into cell culture medium and adjusted to a concentration of 4×10^8 /ml. Conidia were heat-killed at 95°C for 30 minutes. The lack of viability was checked by culture in Sabouraud glucose broth. Conidia were stored at -80°C until used for the experiment.

Recombinant human IL-4 (10ng/ml), IL-1 β (10ng/ml) and IL-23 (50ng/ml) were all purchased from R&D Systems. The anti-TGF β I neutralizing antibody (10 μ g/ml) and its isotype control monoclonal IgG1 mouse anti-human antibody (10 μ g/ml) were purchased from R&D Systems.

Peripheral blood mononuclear cell (PBMC) isolation

Venous blood was drawn in 10 ml EDTA tubes. The blood was diluted 1:1 with Phosphate Buffered Saline (PBS). Subsequently PBMCs were isolated using Ficoll-paque (GE healthcare, Zeist, The Netherlands) density gradient centrifugation, as described previously (19). The PBMCs layer was collected and washed twice in cold PBS. Cells were reconstituted in Iscove's modified Dulbecco's Medium (IMDM) (life technologies) supplemented with 10 μ g/ml gentamicin, and 10 mM pyruvate (Gibco, life technologies). The cells were counted with a particle counter (Beckmann Coulter) and the concentration was adjusted to 1×10^7 cells/ml.

PBMCs stimulation

PBMCs were plated in a 96-well plate (Corning) at a final concentration of 2.5×10^6 /ml in an end-volume of 200 μ l per well. All stimulations were performed in the presence of 10% human pooled serum. Cells were incubated at 37°C with 5% CO₂, after 7 days supernatants were collected and stored at -20°C.

Cytokine measurements

IL-5, IL-9, IL-13, IL-17, IL-22 and TGF β were measured in the cell culture supernatants using commercial ELISA kits (IL-5, IL-13, IL-17, IL-22 and TGF β : R&D Systems; IL-9: Biolegend) according to the instructions supplied by the manufacturer.

Intracellular staining and flowcytometry

After 7 days incubation in the presence of the pathogen with or without additional IL-4, cells were re-stimulated for 4-6 hours with PMA (50 ng/ml) (Sigma-Aldrich) ionomycin (1 μ g/ml) (Sigma-Aldrich), in 200 μ l of IMDM medium supplemented with 10 μ g/ml gentamicin, and 10 mM pyruvate (Gibco) and 10% human serum in the presence of Golgiplug (BD Biosciences, Breda, the Netherlands). Cells were stained extracellular using PE-Cy7–conjugated anti-CD4 (ITK Diagnostics BV) and ECD–conjugated anti-CD45 (Beckman Coulter). Fixation and permeabilization was performed with Cytofix/Cytoperm solution (eBioscience) according to the instructions supplied by the manufacturer. Cells were stained intracellular with anti-IL-4 (PE conjugated, BD Pharmingen), anti-IL-17A (FITC conjugated, ITK Diagnostics BV) and anti-IL-9 (Alexa 647 conjugated, ITK Diagnostics BV). Regulatory T cells (T-regs) were stained with anti-CD25 (PE-Cy7 conjugated, eBioscience) and anti-Forkhead Box Protein (FOX) P3 (APC conjugated, eBioscience). Fixated cells were measured with a FC500 flowcytometer (Beckman Coulter) and the data were analyzed using CXP analysis software v2.2 (Beckman Coulter).

Statistical analysis

The Mann-Whitney-U test was used to detect differences between healthy controls and patients. The Wilcoxon-Signed Rank test was used to detect differences between isotype control and blocking antibody. 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 $\pm$ standard error of the mean. Data were analyzed with GraphPad Prism v 5.0.

Results

CMC and HIES patients have reduced IL-17A and IL-22 responses

Stimulation of PBMCs from CMC and HIES patients showed a significant defect in IL-17 and IL-22 production (Figure 1). CMC patients demonstrated significantly lower IL-17 and IL-22 production after stimulation with *Aspergillus*, *Candida* and *S. aureus*, while HIES patients still produced significant amounts of IL-17, especially after *S. aureus* stimulation. HIES patients produced significantly less IL-22 after stimulation with all different pathogens. To further validate this, flowcytometry analysis was performed and in both patient groups a lower frequency of IL-17+ CD4+ cells was observed.

Th2 response is deficient in CMC but not in HIES and AD patients

Next, we investigated whether the Th2 responses can be induced in STAT1 GOF or STAT3 LOF patients which both lack optimal Th17 responses. Stimulation with diseases-associated pathogens *Aspergillus*, *Candida* or *S. aureus* showed decreased IL-5 and IL-13 production in CMC patients (Figure 2A and B), but not in HIES patients. We did not explore classical mitogenic stimulation with aCD3/aCD28 because it did not induce IL-5 and IL-13 in healthy volunteers (data not shown). Since the induction of IL-5 and IL-13 was relatively low, stimulation in the presence of IL-4 was performed to shift the Th responses towards an allergic phenotype. CMC patients demonstrated a significant defect in IL-5 and IL-13 production, while cells isolated from HIES patients responded normally when stimulated with different pathogens in the presence of IL-4 (Figure 2 C and D). To explore Th2 responses in another patients group with allergic phenotype, we included another control group of patients with AD. Except for one outlier in the AD patients group, comparable IL-5 and IL-13 levels were measured in the cell culture supernatants of PBMCs of AD patients and healthy controls using the same stimulation assay (Figure 3).

IL-9 is induced by Candida and increased in the presence of IL-4 and IL-1/IL-23 in healthy controls

IL-9 has been described to be produced by Th17 cells under certain conditions; therefore we wanted to investigate IL-9 responses in patients with a defective Th17 response. Stimulation of PBMCs with different fungal pathogens or *S. aureus* demonstrated that *Candida* was the most potent inducer of IL-9 production (Figure 3A). The production increased steadily during stimulation for 7 days (Figure 3B). In the presence of the Th2-polarizing cytokine IL-4 and *Candida*, IL-9 production was further increased, while IL-4 stimulation alone did not induce detectable IL-9 production (Figure 3C). The IL-17-inducing cytokine cocktail IL-1 β and IL-23 also increased *Candida*-induced IL-9 but to a lesser extent than IL-4 (Figure 3C). We next wanted to identify which T helper subset displayed IL-9⁺ cells. *Candida* stimulation induced IL-9⁺ cells, which were either single positive or present in the IL-17⁺ subset. No IL-9⁺ cells were detected in the IL-4⁺ subset (Figure 3D). Since the frequency of IL-9⁺ cells was very low, additional analysis was performed in the presence of IL-4 (Figure 3E). The percentage of IL-9⁺ cells remained low even after IL-4 stimulation, and again IL-9 was almost absent in IL-4⁺ cells, but present in the Th17 subset and IL-9 single positive cells.

HIES patients have a striking defect in IL-4 induced IL-9 production

Stimulation of PBMCs isolated from CMC and HIES patients resulted in significant lower *Candida*-specific IL-9 concentrations in CMC, while HIES cells produced IL-9, albeit lower (Figure 4). Interestingly, we observed that the addition of IL-4 to *Candida* stimulation resulted in significant lower IL-9 production in cells isolated from patients with HIES (Figure 4). Next, we measured IL-9 in the serum of HIES patients, AD patients and healthy controls; no IL-9 was detectable (data not shown).

This made us explore the underlying mechanism. Since TGF β contributes to the induction of IL-9, and TGF β expression of circulating activated T cells of HIES patients has been described to be low (20), we investigated whether TGF β was responsible for this observed deficiency of IL-9 in HIES. The total IL-9 production was partially dependent on TGF β in PBMCs of healthy controls. However, the synergistic effect of IL-4 on *Candida*-induced IL-9 was still present, when TGF β was blocked in PBMCs of healthy controls (Figure 5A). In addition, TGF β concentrations in cell culture supernatants of PBMCs isolated from HIES patients were comparable to healthy controls (Figure 5B). TGF β induces regulatory T cells (Tregs), which are able to modulate Th2 responses, therefore we measured the frequency of Tregs in HIES patients (21). The percentage of FOXP3⁺ CD25⁺ T-reg cells of total CD4⁺ cells was not different between healthy controls and HIES patients (Figure 5C). In contrast, intracellular staining with flowcytometry analysis of cells from HIES patients demonstrated a decreased percentage of overall CD4⁺ IL-9⁺ cells that was mainly due to a decrease in the IL-17⁺/IL-9⁺ cells compared to controls (Figure 5D).

Discussion

The present study investigated Th2 and Th9 responses in patients with CMC and HIES. CMC patients had deficient Th2 response, while HIES patients were capable to produce IL-5 and IL-13, such as AD patients. Since both CMC and HIES patients are deficient in Th17 responses, this suggests that Th2 responses are initiated and regulated independently from IL-17. In addition, CMC patients were completely deficient on *Candida*-induced IL-9 production, with only a partial defect in HIES patients.

An interesting observation was a defect in IL-4-induced IL-9 production after stimulation with *Candida* in the HIES patients. IL-9 was co-expressed in a Th17 subset in healthy controls, and the percentages of this subset was lower in HIES patients, suggesting that a robust IL-9 response is dependent on an intact Th17 subset that can produce IL-9. The intact Th2 response in HIES and a completely deficient Th2 response in CMC correlate with the clinical symptoms seen in CMC and HIES, because allergic responses are not a key feature of CMC, while atopic constitution with eczema, eosinophilia and high IgE levels are classical symptoms of HIES. Since we have not assessed the expression levels of IL-4R in CMC and HIES we cannot rule out that the IL-4R expression is significantly lower in CMC compared to HIES, which might explain our observations.

Th2 responses in HIES and CMC patients have been little studied until now. Here we describe a significant defect of IL-5 and IL-13 only in CMC patients, but not only after stimulation with Th2 inducing pathogens, but also after skewing these responses towards Th2 by the addition of IL-4. This is in line with a study investigating cytokine responses of eight CMC patients from Brazil, in whom no elevated Th2 responses were observed after mitogenic stimulation (22); it is important to note that in these patients the presence of STAT1 GOF mutations has not been tested. Khosravi *et al.* speculate about increased Th2 responses without having measured IL-4, IL-5 or IL-13 (23). However, Eyerich *et al.* had observed low interferon (IFN) γ and higher IL-10 in CMC patients and attributed the deficient Th responses to the elevated IL-10 response. One hypothesis is that STAT1 hyperphosphorylation (13) results in increased IFN γ signaling, subsequently leading to a strong inhibition of the Th17 differentiation resulting in an IL-17 production defect (24). This mechanism could also underlie the deficient IL-5, IL-13 and IL-9 production, since they are all inhibited by IFN γ (3, 25). One other explanation why all T helper subset are deficient in

CMC would be a general defect in T helper cell proliferation. However, we observed that the T cell proliferation capacity of CMC patients was intact (Supplemental Figure 1), which is in line with previously published data (26). Nevertheless, a lower percentage of CD4⁺ on CD45⁺ cells as seen in CMC patients could also partially contribute to the low production of T helper derived cytokines (Supplemental Figure 2). In contrast, HIES patients displayed a selective IL-9 deficiency, while the Th2 cytokines IL-5 and IL-13 were produced in normal amounts. Intact Th2 responses were anticipated since HIES patients can present with an atopic constitution. AD patients presented with a robust Th2 response comparable to a non-allergic control group, suggesting that the Th2 assay used in this study is rather a qualitative assay than a quantitative assay for allergic responses. On the one hand, Boos *et al.* had described higher frequencies of Th2 cells in AD patients compared to healthy controls. On the other hand, they described comparable Th2 responses of HIES patients and healthy controls (27). Furthermore, these data are in line with previous reports describing normal frequencies of CD4⁺ IL-4⁺ cells, but elevated CD4⁺ IL-13⁺ cells (28), and normal Th2 responses (27, 29) in STAT3-HIES patients. Collectively these data suggests that a disturbed immune balance causes the atopic pathology rather than a strictly isolated increased Th2 response.

Several studies suggest that T-regs, IL-10, and TGF β are main players in regulating Th1/Th2 balance (30, 31). Interestingly, IL-10-treated dendritic cells derived from HIES patients have an impaired capability to induce FOXP3⁺ T-regs (31). However, we observed a normal frequency of peripheral T-regs in patients with HIES, which is in line with previously reported data (31). TGF β is a cytokine of great interest in the context of our findings, since it is on the one hand a T-reg inducing cytokine (32), but in the presence of IL-4 it is a significant inducer of IL-9, and initiates the differentiation of naïve CD4⁺ T cells towards

Th9 cells (33). Since we observed a deficient *Candida*-induced IL-9 response in the presence of IL-4 in HIES patients, we hypothesized that lower TGF β concentration in HIES patients might be a possible mechanism. TGF β concentrations were however not significantly different after stimulation in the patients. This is in line with the study of Saito *et al.*, who also showed an intact TGF β response in HIES patients (31), but in contrast with a study of Ohga *et al.*, who described low TGF β expression of circulating activated T cells of HIES patients (20). Interestingly, *Candida* induced more IL-9⁺IL-17⁺ co-expressing CD4⁺ cells than IL-9⁺ single positive cells in healthy volunteers. Secondary signals such as the IL-1 family cytokines IL-1 α and IL-1 β (34) have been described to potentiate IL-9 production. *Candida* is a strong IL-1 and IL-17 inducer (35), which is reflected by the relatively high frequency of IL-9⁺IL-17⁺ co-expressing CD4⁺ cells in response to *Candida* compared to other stimuli. Here we show that HIES patients, most likely due to their deficient Th17 response due to their *STAT3* LOF mutation, also have a lower Th17 subset that is able to produce IL-9. Therefore it is likely that the lower IL-9 response in response to IL-4 in HIES patients is due to a deficiency to induce a subset of Th17 cells that are able to produce IL-9.

Since HIES patients suffer from cold staphylococcal abscesses, chronic mucocutaneous candida infection as well as from pulmonary aspergillosis, mainly in preformed bronchiectatic cavities causing aspergilloma or even invasive pulmonary aspergillosis, specific immune responses against these three pathogens have been investigated and compared to CMC patients (36). Interestingly, both fungal pathogens *Candida* and *Aspergillus* did not cause different immune responses, although *Candida* had a stronger cytokine inducing capacity. This matches with their clinical presentation, since it has recently been published that also CMC patients can suffer from *Aspergillus* and *S. aureus* infections

(37) making HIES and CMC syndromes very similar in terms of susceptibility to fungal infections due to their Th17 deficiency.

In conclusion, CMC and HIES patients share several immunological defects, but differ in their capability to induce Th2 and Th9 responses. CMC patients have a completely abolished Th2 and IL-9 response, while HIES patients have normal Th2 responses and only lack an IL-4-induced IL-9 response. These data might explain both the similarities and differences in clinical presentation seen between HIES and CMC patients; sharing an increased susceptibility to mucocutaneous candidiasis due to a Th17 deficiencies on the one hand, but displaying clear differences in allergic disease due to difference in Th2 and Th9 responses on the other hand. Why CMC patients present with a more general defect in Th subsets, and how the lack of IL-9 further contributes to the immunopathology of HIES patients remains to be elucidated.

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Authors contribution:

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

Disclosures: The authors declare no conflicts of interest.

Table I: Demographic characteristics of CMC and HIES patients

Table	Age at exp.	Gender	Serum IgE (IU/ml)	Eosinophil count ($\times 10^9/l$)	Clinical presentation – allergic phenotype	Treatment	Mutation
CMC 1	70	male	< 2	0.02	Chronic mucocutaneous candidiasis, recurrent pulmonary infections, COPD, squamous cell carcinoma, autoimmune hemolytic anemia Allergies : no atopic eczema	Colistin, Anidulafungin, Azithromycin, Citalopram, Ciclesonid, Calciumcarbonat, Amphotericin B, Prednisolon (10mg/d)	R274W
CMC 2	43	male	N/A	0.04	Chronic mucocutaneous candidiasis, recurrent pulmonary infections, bronchiectasis Allergies: no atopic eczema	Fluconazole, Amoxiclav	QT267R
CMC 3	31	male	< 2	0.20	Chronic mucocutaneous candidiasis, type I diabetes, hypothyroidism, recurrent pulmonary infections, SLE Allergies : no atopic eczema	Prednisolone (stop 5 month before testing), Itraconazole, Furosemid, Doxycycline, L-Thyroxin, Insulin	D23V
CMC 4	40	male	-	0.18	Chronic mucocutaneous candidiasis, recurrent pulmonary infections with abscess, recurrent sinusitis Allergies: none, no atopic eczema	Anidulafungin, Flucocaxillin, Ibuprofen	R274W
HIES 1	43	female	2702	0.21	Hyper IgE syndrome, recurrent staphylococcal skin abscesses Allergies: none, no atopic eczema	Miconazol, Flucloxacillin, Valciclovir	S560del
HIES 2	45	male	3942	0.14	Hyper IgE syndrome, recurrent pneumonia with <i>S. aureus</i> , bronchiectasis, pulmonary	Moxifloxacin, Bacitracin, Ciprofloxacin,	R382W

					aspergilloma Allergies: hayfever, no atopic eczema	Amoxicillin, Miconazol, Doxicyclin	
HIES 3	49	female	4524	0.13	Hyper IgE syndrome, recurrent staphylococcal and streptococcal upper airways infections, recurrent mucomycosis and skin abscesses, chronic mucocutaneous candidiasis, kidney abscess Allergies: none, no atopic eczema	Amoxiclav	R382Q
HIES 4	33	female	7493	0.33	Hyper IgE syndrome Allergies: none, atopic eczema	Flucloxacillin, Itraconazol	V473del

Table II: Demographic characteristics of AD patients

Table	Age at exp.	Gender	Serum IgE (IU/ml)	Eosinophil count ($\times 10^9/l$)	Clinical presentation – allergic phenotype	Treatment
AD 1	30	Female	899	0.86	Atopic eczema	Oral: Promethazin, Local: cetomacrogol salve, clobetason salve, coal tar, vaseline/paraffin
AD 2	28	Female	<4,7	N/A	Atopic eczema	Local: Vaseline/paraffin
AD 3	28	Female	302	N/A	Atopic eczema	Local: Vaseline/paraffin
AD 4	38	Female	69	N/A	Atopic eczema	Local: vaseline/paraffin, Betamethason, Clobetasol, Tacrolimus, coal tar

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Additional material:

CFSE proliferation assay

Proliferation of PBMCs was measured using the CFSE kit (Biolegend) according to the instructions described by the manufacturer. Briefly, PBMCs were re-suspended to a density of 10×10^6 cells/ml in PBS and labeled by adding CFSE in a 1:1 ratio to a final concentration of 1.25uM. The suspension was mixed gently and incubated for 5 minutes at 37°C. An equal volume of 100% human pooled serum was added and incubated for 3 minutes at room temperature. Cells were washed two times in RPMI+ (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) supplemented with 10%

human pooled serum and re-suspended to a concentration of 5×10^6 /ml in RPMI+. An unlabeled cell fraction and a labeled cell fraction were measured by flowcytometry on day 0 to control for the staining efficacy. Labeled cells were stimulated for 6 days with IL-2 (75 U/ml, R&D systems) and different pathogens in the presence or absence of IL-4. On day 6 cells were stained with anti-CD3 (PE-Cy5 conjugated, ITK Diagnostics BV) and measured by flowcytometry. The percentage of proliferated CD3+ cells were calculated as percentage of all CD3+ cells.











