

No Indication for a Defect in Toll-Like Receptor Signaling in Patients with Hyper-IgE Syndrome

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Hyper-IgE syndrome is a rare primary immunodeficiency of unknown etiology characterized by recurrent infections of the skin and respiratory system, chronic eczema, elevated total serum IgE, and a variety of associated skeletal symptoms. Recent reports about susceptibility to pyogenic bacterial infections and high IgE levels in patients and animals with defects in toll-like receptor (TLR) signaling pathways prompted us to search for TLR signaling defects as an underlying cause of hyper-IgE syndrome. Blood samples from six patients with hyper-IgE syndrome were analyzed for serum cytokine levels, intracellular cytokine production in T cells after stimulation with PMA/ionomycin, and cytokine production from peripheral blood mononuclear cells stimulated by TLR ligands and bacterial products including LPS (TLR4), peptidoglycan (TLR2), PolyIC (TLR3), R848 (TLR7/8), CpG-A, and CpG-B (TLR9), zymosan and heat killed *Listeria monocytogenes*. All results were compared to data from healthy controls. A reduction in IFN- γ , IL-2, and TNF- α producing T cells after PMA stimulation suggested a reduced inflammatory T cell response in patients with hyper-IgE syndrome. Increased serum levels of IL-5 indicated a concomitant Th2 shift. However, normal production of cytokines (TNF- α , IL-6, IL-10, IFN- α , IP-10) and upregulation of CD86 on B cells and monocytes after TLR stimulation made a defect in TLR signaling pathways highly unlikely. In summary, our data confirmed an imbalance in T cell responses of patients with hyper-IgE syndrome as previously described but showed

no indication for an underlying defect in toll-like receptor signaling.

KEY WORDS: Hyper-IgE syndrome; toll-like receptor signaling; cytokine pattern; interferon- γ ; interleukin-4; innate immunity.

INTRODUCTION

Hyper-IgE syndrome (HIES; OMIM #147060, #243700) is a rare primary immunodeficiency disorder of unknown etiology characterized by recurrent staphylococcal infections of the skin and respiratory tract, mucocutaneous candidiasis, chronic eczema, and elevated total serum IgE (1–4). Associated nonimmunological symptoms including coarse facial features, retained primary dentition, scoliosis, hyperextensible joints, and pathologic fractures define hyper-IgE syndrome as a multisystem disorder (1). Most cases are sporadic but HIES can be inherited in its classical form as a single-locus autosomal dominant trait with variable expressivity. Linkage studies suggested the proximal chromosome 4q21 as a possible disease locus in some families (5, 6).

Early on HIES was thought to be due to a chemotactic defect in neutrophils (7–9). The high polyclonal serum IgE of patients prompted a series of studies in search of a T-helper cell type 1 and type 2 (Th1/Th2) imbalance. Despite the inability to clearly prove that Th1/Th2 dysregulation is involved in the pathogenesis of HIES, these studies provided evidence for abnormalities in cytokine induction (e.g. IFN- γ ; IL-12), chemokine induction (e.g. IL-8, ENA78), and regulation of surface molecules (e.g. ICAM-1) involved in the interaction of antigen presenting

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Abbreviations: PBMC, peripheral blood mononuclear cells; IRAK, IL-1-receptor-associated kinase; MyD88, myeloid differentiation factor 88; IP-10, γ -interferon inducible protein 10; NF κ -B, nuclear factor kappa B; ODN, oligodeoxynucleotide; IRF, interferon regulatory factor; PMA, phorbol 12-myristate 13-acetate.

cells and T cells (10–15). These studies led to the hypothesis that HIES might be less a disease of pathologic IgE production than a disease of impaired inflammatory responses.

Pattern recognition receptors (PRR) of the innate immune system play a key role in sensing the presence of microbial infection and driving inflammatory responses. PRRs are also essential for initiating and shaping adaptive immune responses according to the invading pathogen thereby “bridging” the innate and adaptive immune system (16). The toll-like receptor (TLR) family is the best-characterized class of PRRs in mammals. In humans the 10 known family members recognize conserved molecular patterns of microbes including lipopolysaccharide (LPS) (detected by TLR4), bacterial and fungal lipoproteins and lipoteichoic acids (detected by TLR2), flagellin (detected by TLR5), unmethylated CpG DNA of bacteria and viruses (detected by TLR9), double stranded RNA (detected by TLR3), and single-stranded viral RNA (detected by TLR7/TLR8) that are unique to the microbial world and specific for entire classes of pathogens (16). Signaling pathways of TLRs and the IL-1 receptor that lead to the induction of proinflammatory genes share many of their components. For instance the adaptor MyD88 and the kinase IRAK-4 are used by the IL-1 receptor and all TLRs except for TLR3. However, the expression in different cell types and the use of additional non-overlapping signaling molecules enables individual TLRs to induce specific immune responses adequate to microbes containing its ligand (17). Defects in TLR pathways have been described in mice and humans to be associated with variable immune phenotypes and susceptibility to infectious agents (reviewed in (18)). MyD88 knockout mice, that are susceptible to a broad range of microbes including Gram positive and Gram negative bacteria, mycobacteria and viruses, fail to induce Th1 responses, but show normal Th2 responses and over time spontaneously develop high levels of serum IgE (19). Human carriers of an allelic variant of TLR4 have been shown to have a higher risk for sepsis with Gram negative bacteria (20), while TLR2 knockout mice are susceptible to staphylococcal infections (21). In addition a polymorphism in TLR2 has been associated with a predisposition to staphylococcal infections in humans (22). Recently, three children with IRAK-4 deficiency were described to have recurrent infections caused by the Gram positive bacteria *Staphylococcus aureus* and *Streptococcus pneumoniae*, and two of the patients had high serum levels of IgE (23).

These findings prompted us to test the hypothesis that cytokine imbalance, high levels of serum IgE, and susceptibility to infections with *Staphylococcus aureus* and *Candida albicans* in HIES are all caused by an underlying

defect in TLR pathways. In the present study we therefore examine the response to a broad panel of TLR ligands in cells from six patients, that have clinically established HIES.

METHODS

Patients

Six patients, two females and four males, ages 3–37 years, who are treated at the University Children’s Hospital in Munich, were included in this study. All patients presented with classical hyper-IgE syndrome, including chronic eczema, elevated serum-IgE (>2942 IU/mL), eosinophilia (>950 cells/ μ L), recurrent skin abscesses, respiratory tract infections, and candidiasis of mucosal sites and nails. In four of the six patients eczema was present before 6 weeks of life. More than one episode of pneumonia has been diagnosed in each patient. Two developed pneumatozysts as a complication of pneumonia. Two of six patients had a history of severe infections like meningitis or mastoiditis. Characteristic facial features and at least one other associated skeletal symptom like pathologic fractures (in two), hyperextensible joints (in 50%) or persistence of primary teeth (in four patients), were present in each of the six patients. None of the patients had scoliosis. The HIES scoring system developed at the National Institutes of Health (NIH), that accounts the manifestation and severity of 20 clinical and laboratory findings corrected for age, was used for patient evaluation. The mean NIH score for our patients was 60 points, ranging from 55 to 75, at the time of investigation.

To exclude other underlying immunodeficiencies a standard panel of laboratory tests was performed using standard protocols as previously described (24). These tests gave normal results and included quantification of immunoglobulin subclasses, complement components, antibody titers to viral and vaccine antigens, lymphocyte subpopulations, the oxidative burst of granulocytes, and the assessment of proliferative responses of lymphocytes to mitogens. At the time of this study the patients were free of active infection and none was treated with immunomodulators or oral steroids. PBMC samples from HIES patients were compared to control samples taken and stimulated in parallel at the same time from age and sex matched healthy volunteers free of active infection or medication. For serum cytokines and intracellular cytokine analysis samples from HIES patients were compared to samples collected from 65 normal subjects with an equal age distribution between 6 months and 35 years of age that had no known immune defect, no active infection, and did not take immunomodulatory medication.

Table I. Serum Cytokines in HIES Patients and Normal Controls in the Absence of Infection

	IFN- γ (\pm SEM)	TNF- α (\pm SEM)	IL-10 (\pm SEM)	IL-5 (\pm SEM)	IL-4 (\pm SEM)	IL-2 (\pm SEM)
HIES patients ($n = 6$) (pg/mL)	13.9 (\pm 2.1)	5.2 (\pm 0.5)	8.8 (\pm 0.6)	4.4 (\pm 0.3)	11.8 (\pm 1.9)	3.2 (\pm 0.8)
Normal controls (pg/mL)	13.1 (\pm 1.1)	6.6 (\pm 1.6)	7.4 (\pm 0.7)	2.4 (\pm 0.1)	11.9 (\pm 1.0)	2.1 (\pm 0.5)
<i>p</i> value	0.73	0.52	0.24	<0.001	0.98	0.26

This control group was recruited from hospital staff and patients admitted to the hospital for elective surgical procedures. The patients and controls or their parents gave informed consent to participate in the study.

Cell Stimulation and Cytokine Analysis

Heparinized whole blood obtained from patients and controls was divided and either directly processed for intracellular cytokine staining or used to isolate PBMC by Ficoll-Hypaque density gradient centrifugation (Biochrom, Berlin, Germany).

For *ex vivo* intracellular cytokine staining in T cells whole blood was diluted 1:10 with RPMI 1640 supplemented with 10% heat inactivated FCS and stimulated for 6 h at 37°C with 10 ng/mL PMA and 0.75 μ g/mL ionomycin in the presence of 4 μ g/mL monensin (all from Sigma, St. Louis, MO). For the determination of cytokine production in monocytes 1 mL aliquots of whole blood were stimulated for 4 h with 1 μ g/mL LPS in the presence of 10 μ g/mL Brefeldin A. Cells were then washed, stained with surface markers (CD3 PerCP (BD Pharmingen, Heidelberg, Germany) and CD8 FITC (Coulter Immunotech, Krefeld, Germany) or CD14 FITC (BD Pharmingen) respectively), fixed with 4% paraformaldehyde and permeabilized with 0.5% saponin (Sigma). T cells were stained with PE-labeled antibodies against TNF- α , IFN- γ , IL-2, IL-4 (Coulter Immunotech, Krefeld, Germany), and IL-10 (BD Pharmingen) and monocytes were marked for TNF- α , IL-1b, IL-6, IL-8 (Coulter Immunotech), and IL-10. Expression of cytokines in CD8⁻CD3⁺ T cells, CD8⁺CD3⁺ T cells, and CD14⁺ monocytes was measured by flow cytometry and analyzed using CellQuest software (BD Pharmingen).

Isolated PBMC were seeded in 96 well plates at 3×10^5 cells/200 μ L/well in RPMI supplemented with 10% FCS and stimulated as follows: lipopolysaccharide (LPS) from *E. coli* (1 μ g/mL) (Sigma), zymosan from yeast cell wall extracts (10 μ g/mL), petidoglycan from *S. aureus* (1 μ g/mL), R848 (10 μ M) (all three from InvivoGen, CA, USA), CpG ODN (small letters: phosphorothioate linkage; capital letters: phosphodiester linkage 3' of the base; CpG-B ODN 2006: 5' tcgtcgttttgcgttttgcgtt 3'; CpG-A ODN 2216: 5' ggGGGACGATCGTCgggggG 3', both provided by Coley Pharmaceuticals Wellesley, MA,

USA), poly IC (25 μ g/mL) (Amersham Biosciences, NJ, USA) and IL-1b (50 ng/mL) (R&D Systems). After 36 h the secretion of IL-6, TNF- α , IL-12, IL-10, and IFN- α was measured in serial dilutions of culture supernatants using IL-12p40, IL-6, IL-10, TNF- α (all from BD Pharmingen), IP-10 (R&D Systems), and IFN- α (Bender MedSystems, Graz, Austria) ELISA kits according to the manufacturer's instructions. At the same time point the PBMC were stained with antibodies against CD19^{APC}, CD14^{FITC}, and CD86^{PE} (all from BD Pharmingen) and the expression of CD86 on CD19⁺ B cells and CD14⁺ monocytes was analyzed by flow cytometry as previously described (25).

Serum levels of IFN- γ , TNF- α , IL-10, IL-5, IL-4, and IL-2 in HIES patients and controls were measured using the Th1/Th2-cytometric bead assay according to the manufacturer's instructions (BD Pharmingen). Statistical significance of differences was determined using the Student's *t* test. Differences were considered statistically significant for $p < 0.05$.

RESULTS

Serum Levels of Cytokines in HIES

To evaluate the *in vivo* production of cytokines in HIES patients in the absence of external stimulation we measured the serum levels of a panel of cytokines during a routine visit when no clinical evidence for a current infection was present (see Table I). HIES patients showed an increased level of IL-5 compared to normal controls, while no significant difference was seen for IFN- γ , IL-4, TNF- α , IL-10, or IL-2.

Cytokine Profiles of T Cells in HIES Patients

To assess the ability to produce cytokines upon activation, T cells of HIES patients were stimulated immediately *ex vivo* in whole blood cultures with PMA and ionomycin. Intracellular cytokine staining and single cell analysis by flow cytometry showed that a reduced percentage of CD4⁺ and CD8⁺ T cells expressed IFN- γ , TNF- α , and IL-2 in HIES patients compared to normal controls (see Fig. 1; $p = 0.07$ for differences in CD8⁺ T cells expressing IL-2). The percentage of IL-4 producing cells was significantly

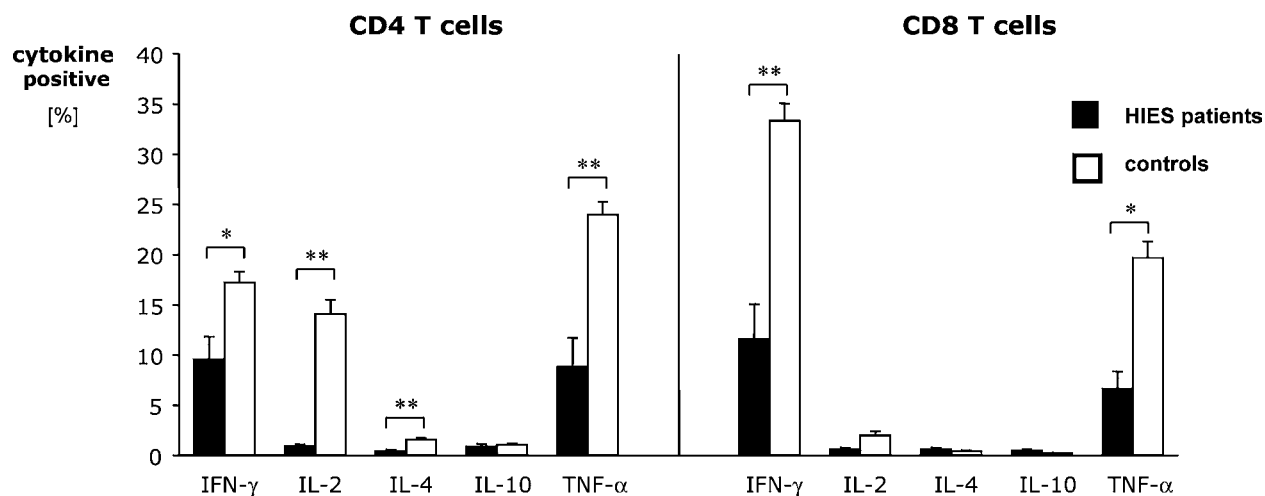


Fig. 1. Cytokine production in T cells of HIES patients after stimulation with PMA. Whole blood samples of HIES patients and normal controls were stimulated with PMA/ionomycin for 6 h. After intracellular staining the production of the depicted cytokines was analyzed by flow cytometry in CD4 and CD8 T cells on a single cell level. The percentages of cytokine positive cells out of all CD4⁺ or CD8⁺ cells respectively are shown as the means \pm SEM of six patients and the control group. * and ** indicate a *p* value of <0.05 and <0.01, respectively, for differences between HIES patients and the controls.

reduced within CD4⁺ T cells (1.6% (controls); 0.4% (patients); *p* = 0.004). IL-4 appeared to be slightly increased within CD8⁺ T cells (0.6% (patients); 0.4% (controls); *p* = 0.58), but this difference did not reach statistical significance. No significant differences were seen for IL-10 between the two groups (CD4: 1.1% (controls); 0.9% (patients); *p* = 0.5; CD8: 0.23% (controls); 0.48% (patients); *p* = 0.1). Within the T cell populations positive for IFN- γ , TNF- α , or IL-2 the distribution and maxima of the fluorescence intensities did not differ between HIES patients and controls (data not shown). Individual T cells from HIES patients therefore can produce these cytokines in normal amounts, arguing against a T cell inherent defect to produce these cytokines.

Response of HIES Patients to TLR Stimulation

To screen for a defect in TLR signaling pathways we assessed the response of our patients to a broad panel of TLR ligands. PBMC were stimulated with LPS (recognized by TLR-4), peptidoglycan, the fungal product zymosan, heat killed listeria (all recognized by TLR-2 in cooperation with TLR-6 and TLR-1), poly IC (recognized by TLR-3), R848 (recognized via TLR-7 and TLR-8), CpG-A (induces IFN- α in plasmacytoid dendritic cells via TLR-9), CpG-B (activates B cells via TLR-9), and IL-1b. The production of different cytokines (IL-6, TNF- α , IL-10, IL-12, IFN- α) was analyzed. The CXCR3 chemokine IP-10, which was thought to be a candidate gene due to its location on 4q21, was measured in one patient. We found a considerable interindividual variability

within HIES patients as well as within the controls. However all HIES patients responded to each of the TLR ligands and there was no significant difference in the pattern of cytokines produced between HIES patients and controls. The responses seemed sometimes even higher in HIES patients but there was no statistically significant difference between HIES patients and controls for any of the TLR-ligands in any of the measured cytokines (see Fig. 2A). The evaluation of the cytokine production into the cell supernatant of unseparated PBMC was supplemented by the analysis of monocytes and B cells on a single cell level by flow cytometry. Upregulation of the costimulatory molecule CD86 on B cells (Fig. 2B) with strong responses especially to the TLR-7/8/9 ligands was seen in both HIES patients and controls. Similarly, no difference was seen in the expression of CD86 on monocytes in response to the TLR panel (data not shown). The analysis of early cytokine production of monocytes in response to LPS by intracellular cytokine staining and flow cytometry showed similar amounts of cells positive for TNF- α , IL1- β , IL-6, IL-8, and IL-10 in HIES patients and healthy controls (Fig. 2C).

DISCUSSION

In this study we found increased serum levels of IL-5, reduced numbers of T cells producing IFN- γ , TNF- α , IL-2, and IL-4 upon activation with PMA/ionomycin and a normal response to TLR ligands in six patients with hyper-IgE syndrome. IL-5 acts specifically on

eosinophils and increased production results in accelerated eosinophilopoiesis, chemotaxis, cell activation, and delayed apoptosis (26). Overproduction of IL-5 therefore seems consistent with the blood-eosinophilia and high numbers of infiltrating eosinophils that are a histopathologic hallmark of the eczematous skin lesions in HIES (27) and might contribute to the pathogenesis of the skin lesions in HIES. IL-5 is mainly produced by Th2 cells. However our analysis of cytokine production by activated T cells did not indicate overproduction of Th2 cytokines but a decreased number of T cells able to produce IFN- γ , TNF- α , IL-2, or IL-4. Similar results have been found by other groups that described reduced numbers of T cells producing IFN- γ , TNF- α , and TGF- β while the number of IL-4 or IL-13 producing T cells in HIES varied in different studies from normal or reduced to mildly increased (12–14, 28, 29). These findings suggest that decreased production of inflammatory cytokines, especially IFN- γ , might be the upstream event responsible for high serum IgE and eosinophilia. There is convincing evidence that IFN- γ inhibits the development of IL-5 and IL-4 producing Th2 cells and acts directly on B cells to repress the

class switch to IgE and IgG1 isotypes (reviewed in (30)). Underproduction of IFN- γ as observed in our patients would diminish this inhibition and therefore could account for increased IgE levels and via IL-5 increased numbers of eosinophils. The observation in our FACS analysis that T cells from HIES patients can produce as high amounts of IFN- γ and TNF- α as cells from healthy controls—even though fewer cells do so—suggests that there is no intrinsic defect in the cytokine production of T cells. Therefore we hypothesize that this distorted cytokine production in T cells reflects alterations in innate immune mechanisms that—besides the initiation of indispensable early defense mechanism—control and shape evolving T and B cell responses.

Toll-like receptors seemed to be ideal candidates to be involved in the pathogenesis of HIES for several reasons: (a) they have been shown to be important in the defense against the microbes that cause disease in HIES including *Staphylococcus aureus*, (b) a defect in only one of the TLRs or its signaling components has the theoretical potential to cause susceptibility to a circumscribed range of pathogens leaving responses to other pathogens intact,

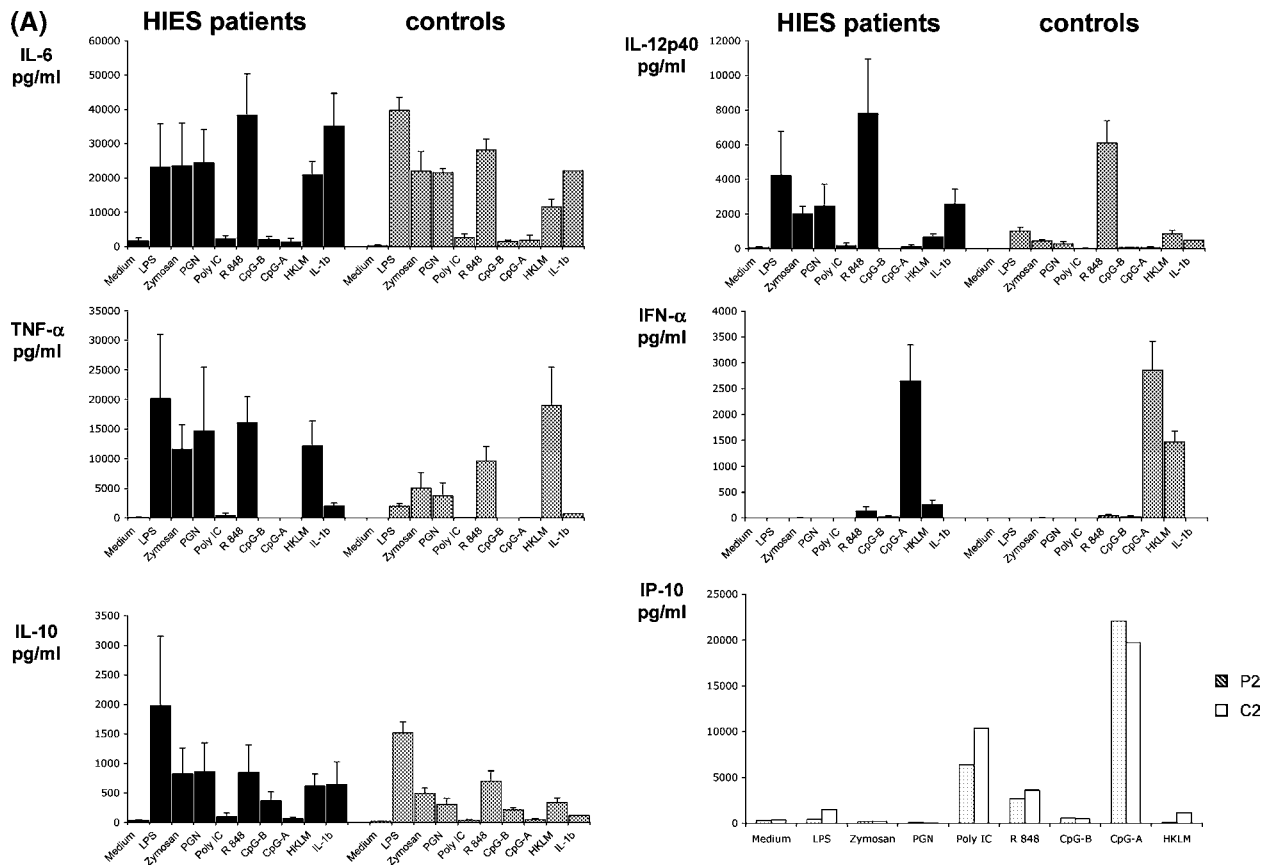


Fig. 2. Continued

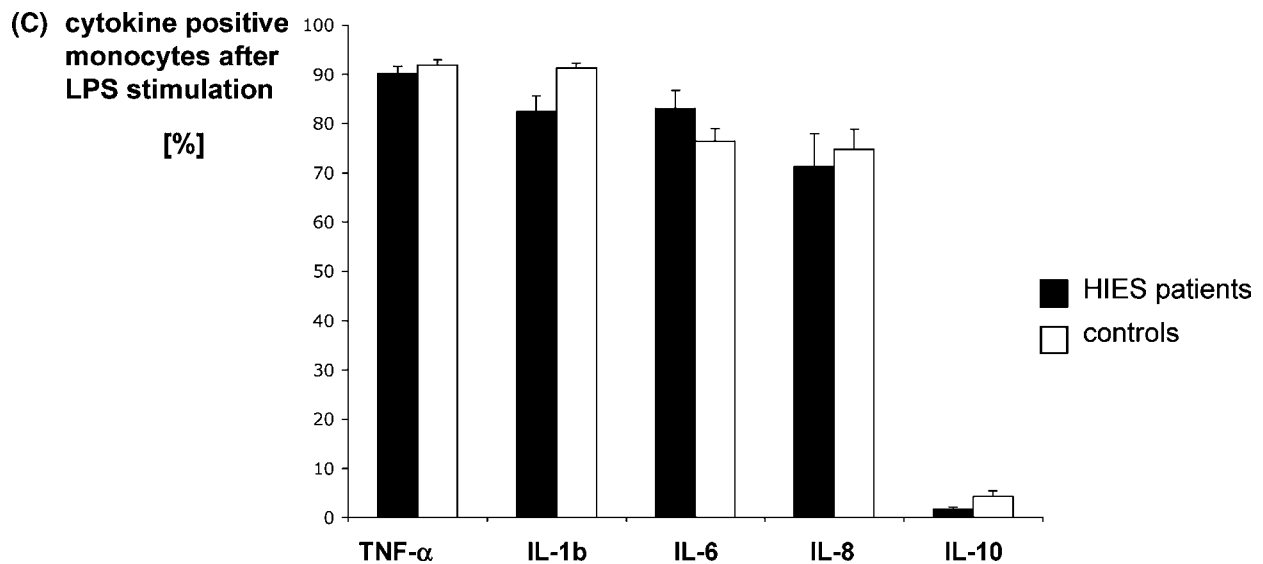
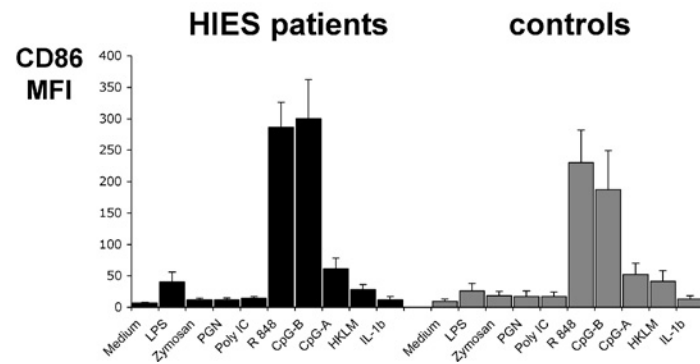
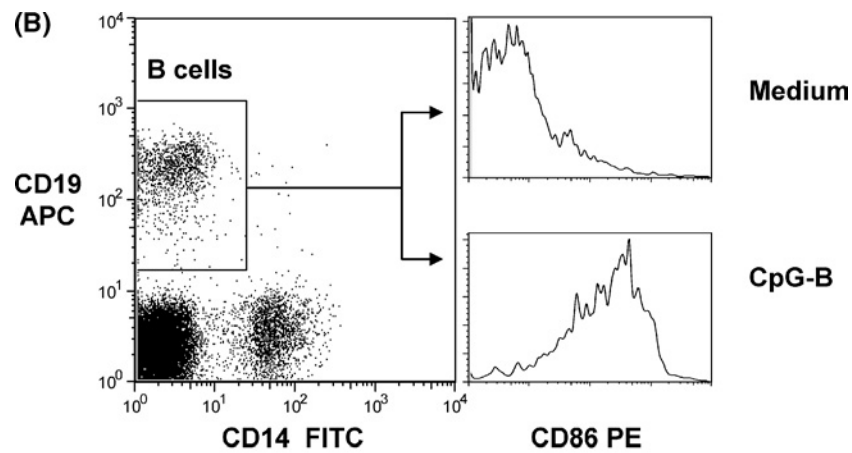


Fig. 2. Normal responses to TLR ligands in HIES patients. (A and B) PBMC from patients and normal controls were stimulated with the indicated TLR ligands. After 36 h the secreted cytokines were measured in the supernatants by ELISA (A) and the expression of CD86 was determined on CD19 positive B cells by flow cytometry as exemplified (B). Data from six HIES patients and their controls are shown as means \pm SEM. (C) Whole blood cultures were stimulated for 4 h with LPS. The cytokine production of CD14⁺ monocytes was measured after intracellular staining by flow cytometry. The percentages of cytokine positive CD14⁺ cells are presented as mean \pm SEM of six HIES patients and the control group.

as seen in HIES, and (c) they have been well proven to influence the costimulatory potential of antigen presenting cells especially dendritic cells thereby determining the cytokine profile, migration pattern, and kinetics in developing T cell responses and are essential for the establishment of memory in T and B cells (16, 17). In this study we assessed the cellular responses of HIES patients to a panel of different TLR ligands, but found no significant difference between patients and healthy controls. Each group responded to all TLR ligands with the same cytokine pattern and if anything the HIES patients responded even better. Due to its documented role for staphylococcal and fungal infections (22, 31) TLR-2 and its pathways seemed the most likely to be affected in HIES. However, normal responses were seen to three stimuli recognized via TLR-2, zymosan (a fungal cell wall product), peptidoglycan (from *Staphylococcus aureus*; recognized in cooperation with TLR-6) and heat killed listeria. The tested TLR ligands and readouts were chosen to cover the various known signaling pathways described for TLRs. Our results therefore suggest that there are no gross functional defects in the tested TLRs itself nor in the adaptors MyD88, TRIF, MAL, or TRAM or in the signaling molecules (e.g. IRAK-4, NEMO, and $\text{I}\kappa\text{B}-\alpha$) involved in the pathways leading to $\text{NF}\kappa\text{-B}$ activation. The activation of MAP kinases (e.g. p38) and the IRF/STAT1/STAT2 pathway seem to be intact as well, as these would be required for normal responses in our readouts covering inflammatory cytokines, $\text{IFN-}\alpha$ production and the induction of costimulatory molecules (17). However one must consider that our readouts might only exclude major defects in the TLR pathways. Mutations causing partial loss or gain of function might only be detectable using more sensitive or specific methods. Further, the causes for HIES may be diverse and even though normal responses in six patients can exclude a defect in the TLR response as a common reason for HIES, the screening of larger numbers of patients would be necessary to assure that TLRs do not play a role in certain subgroups of HIES like the recently described autosomal recessive form (24).

A striking feature of HIES is the association with a narrow spectrum of infections that consist mainly of mucocutaneous candidiasis and skin and respiratory infections with *Staphylococcus aureus*, that lead to the formation of abscesses. Besides pneumonias, deep-seated infections are unusual and bacteremia and sepsis are rare. Patients with HIES seem to be perfectly able to fight off most other Gram positive and Gram negative bacteria, intracellular bacteria, mycobacteria, and viruses. This spectrum does not conform to a general defect in the Th1 response. A defect in the IL-12/ $\text{IFN-}\gamma$ axis has been suggested in HIES (10, 12) but infections with mycobacteria and salmonella

characteristic of patients with proven genetic defects in this axis are uncommon in HIES (32). Patients with mutations in NEMO, an essential molecule in most pathways leading to activation of $\text{NF}\kappa\text{-B}$, and patients with deletion-mutations in IRAK-4, an early kinase/adaptor in the pathway of most TLRs to $\text{NF}\kappa\text{B}$ activation (reviewed in (32, 33)), have infections with pyogenic bacteria like HIES patients. However these patients show an additional susceptibility to microbes not commonly seen in HIES (e.g. mycobacterial infections due to NEMO mutations; *Streptococcus pneumoniae* in IRAK-4 deficiency). Together with our results of normal responses to TLR ligands this suggest that the reason for susceptibility to *Staphylococcus aureus* and candida in HIES lies upstream of the $\text{NF}\kappa\text{-B}$ activation and still upstream or parallel to the recognition by TLRs in antigen presenting cells.

The observation that bone marrow transplantation did not cure HIES in the only patient reported with long-term follow-up (34) stresses the point that all changes observed in the haematopoietic system are probably only secondary, to a pathology in non-haematopoietic cells. Recently successful linkage studies opened up unexpected associations of atopic diseases with genes that center around the barrier and innate immune functions of epithelial and mesenchymal cells (reviewed in (35)). These findings and the location of pathology in HIES should put keratinocytes, respiratory epithelial cells, and fibroblasts in the focus of future research to define the underlying defect.

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