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Toll-Like Receptor Stimulation Induces Higher TNF- α Secretion in Peripheral Blood Mononuclear Cells from Patients with Hyper IgE Syndrome

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Key Words

Hyper IgE syndrome · Toll-like receptor · TNF- α

Abstract

Hyper IgE syndromes (HIES) are primary immunodeficiency disorders of unknown pathogenesis. Patients are typically affected with 'cold' abscesses of the skin, recurrent cyst-forming pneumonia, chronic mucocutaneous candidiasis and other less frequent features such as progressive skeletal abnormalities. Defective signaling in the Toll-like receptor (TLR) pathways has been suggested as a responsible pathologic mechanism, however, in previous reports, 10 patients revealed no defect in inflammatory cytokine responses to different TLR ligands. Here, we report the increase in pro-inflammatory cytokines TNF- α and IL-8, following TLR2 and TLR4 stimulation in a larger cohort of 25 additional patients with HIES, and provide a meta-analysis of the TLR data in HIES.

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Introduction

Hyper IgE syndromes (HIES) are rare primary immunodeficiency disorders characterized by the clinical triad of recurrent cutaneous abscesses, recurrent pneumonia and elevated levels of serum IgE [1]. HIES can be inherited as an autosomal dominant or recessive trait, however, most patients are sporadic. In addition to the aforementioned classical triad, HIES patients suffer of several other symptoms including progressive skeletal deformities and pathologic fractures, delayed secondary dentition, eczema, neonatal rash and autoimmune manifestations [1, 2]. The underlying pathogenesis of HIES still remains elusive, despite 4 decades of research since HIES was first described [3]. Recently, the gene encoding *Tyk2*,

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a member of the JAK tyrosine kinase family, was shown to be mutated in a single patient with an autosomal recessive HIES-like phenotype [4, 5].

Toll-like receptors (TLRs) have been shown to play an important role in innate immunity. The TLRs are pattern-recognition receptors that collectively recognize lipid, carbohydrate, peptide and nucleic acid structures that are broadly expressed by different groups of microorganisms [6]. A defective TLR signaling pathway has been associated with primary vulnerability to a broad range of bacteria and viruses in animal models. In contrast, human IRAK-4 deficiency [7] and MyD88 deficiency [pers. commun.] present with a relatively narrow susceptibility to infections with pyogenic bacteria, most prominently *Streptococcus pneumoniae* and *Staphylococcus aureus*. Deficiency in UNC-93B, which interacts with the nucleotide receptors TLR3, 7, 8 and 9, causes particular susceptibility to *Herpes simplex virus 1* infections [8]. Interestingly, *S. aureus* is the major responsible pathogen for skin abscesses and pneumonia in HIES, prompting us to investigate TLR signaling in patients with a HIES phenotype.

To date, 2 reports on a limited number of patients have failed to reveal defects in TLR signaling in HIES patients [9, 10]. In addition, both studies showed that in HIES patients pro-inflammatory cytokines are secreted at least in similar amounts compared to normal controls, a finding somewhat surprising given that the hallmark of HIES is the absence of inflammation (lack of warmth of abscesses and fevers in severely ill patients). However, given the heterogeneity of the syndrome and the fact that the 2 previous studies were limited to a total of 10 patients, the published data could not sufficiently rule out defects in TLR signaling in HIES. Accordingly, we here report on TLR responses in a larger group of 25 HIES patients, who were collected through an international network. Furthermore, we provide a meta-analysis summarizing the data of all 3 reports on the inflammatory response to TLR stimulation in HIES.

Methods

Patients

Patients with the diagnosis of autosomal dominant or sporadic HIES were evaluated according to the National Institute of Health scoring system [11]. Patients with scores between 19 and 65 points were included into the study to cover most possible phenotypes observed in this heterogeneous disease. Patients were enrolled at their respective clinical center. Ethical approval was obtained from the Ethics Committee of the Freiburg University Hospital (protocol No. 239/99 to B.G.).

Cytokine levels in 25 patients' samples, including 12 males and 13 females, were compared to control samples from 15 sex- and age-matched healthy volunteers.

TLR Stimulation and Cytokine Assay

Whole blood EDTA samples were sent from participating centers to Freiburg with an overnight carrier. Peripheral blood mononuclear cells (PBMCs) were then immediately prepared without delay and specifically without freezing the cells. Human PBMCs were isolated by gradient centrifugation on Histopaque 1077 (Sigma-Aldrich) according to the manufacturer's protocol. The cells were resuspended in RPMI 1640 medium containing 10% FBS and plated at a density of 2×10^6 /ml in a 96-well dish. PBMCs were stimulated with the following reagents. Lipopolysaccharide (LPS) derived from *Escherichia coli* strain 0111:B4 (Sigma-Aldrich) was twice re-extracted by phenol chloroform. A clinical isolate of *S. aureus* was grown on blood agar plates (Remel). Bacterial colonies were removed from the plates after overnight culture, washed 3 times in PBS, and then used to inoculate RPMI and grown to midlog phase ($ABS_{650} = 0.27-0.30$). Subsequently, bacteria were harvested by centrifugation and ethanol inactivated (70% ETOH, 45 min, on ice), washed and resuspended in pyrogen-free water at a concentration of 20 mg/ml (corresponding to approximately 1×10^{10} bacteria/ml as determined by CFU/ml before inactivation). Peptidoglycan from *S. aureus* was purchased from Sigma. Peptidoglycan was essentially free of LPS, since it did not activate TLR2^{-/-} mouse macrophages that respond to LPS concentrations as low as 100 pg/ml. After addition of the indicated preparations, incubation proceeded for an additional 16 h at 37°C and 5% CO₂. Supernatants were collected and stored at -80°C until assayed with a commercial ELISA for human TNF-α and IL-8 (R&D Systems).

Wilcoxon W and Mann-Whitney U statistical tests were used to examine the results. p values less than 0.05 were considered significant.

Results

PBMC from 25 patients with the diagnosis of HIES were isolated and stimulated with *S. aureus* extracts, LPS derived from *E. coli* and peptidoglycan. After stimulation, culture supernatants were assayed for human TNF-α and IL-8 production by ELISA.

None of the patients had a loss of TNF-α or IL-8 production, indicating that in all patients tested, the TLR signaling pathway was not grossly defective. In fact, TNF-α production by patient cells was increased compared to controls in response to LPS, *S. aureus* and peptidoglycan stimulation (table 1). However, the rise in TNF-α level was significant only at higher concentrations of 10 ng/ml for LPS and at 1×10^8 CFU/ml for *S. aureus* (fig. 1; $p < 0.05$). IL-8 production after stimulation did not statistically differ between patients and controls (table 1; fig. 2).

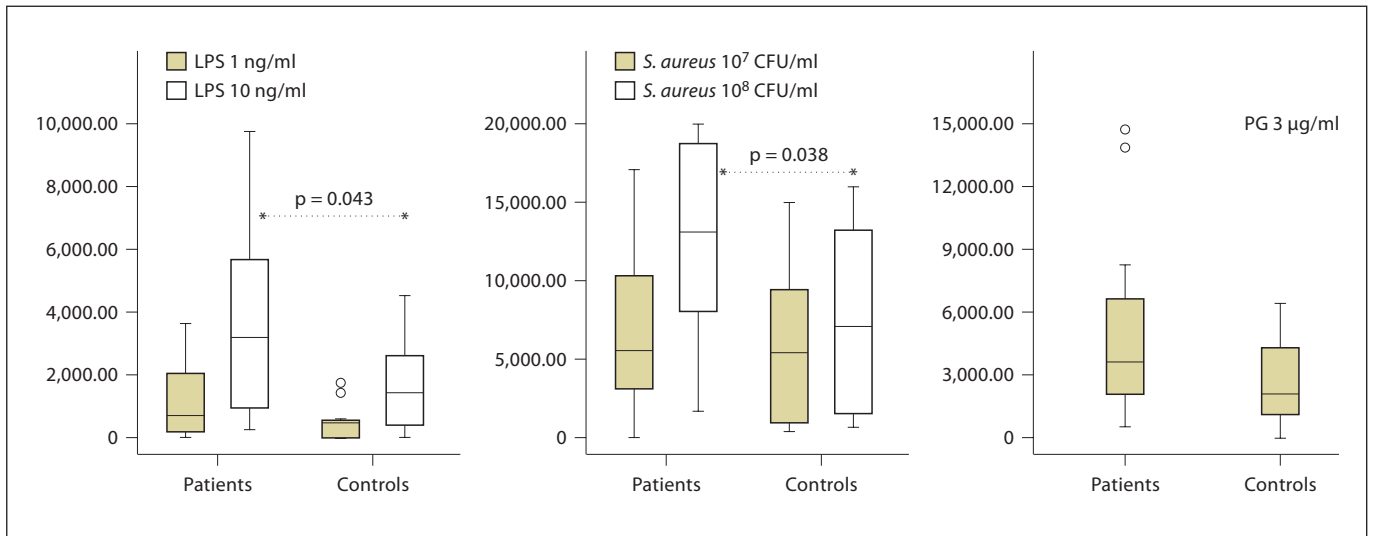


Fig. 1. TNF- α response to various TLR ligands at different concentrations: LPS at 1 and 10 ng/ml, *S. aureus* at 10⁷ and 10⁸ CFU/ml and peptidoglycan (PG) at 3 μ g/ml. $p < 0.05$ for LPS at 10 ng/ml and for *S. aureus* at 10⁸ CFU/ml. Boxes represent values between the 25th and 75th percentiles. The horizontal lines correspond to the median, minimum and maximum.

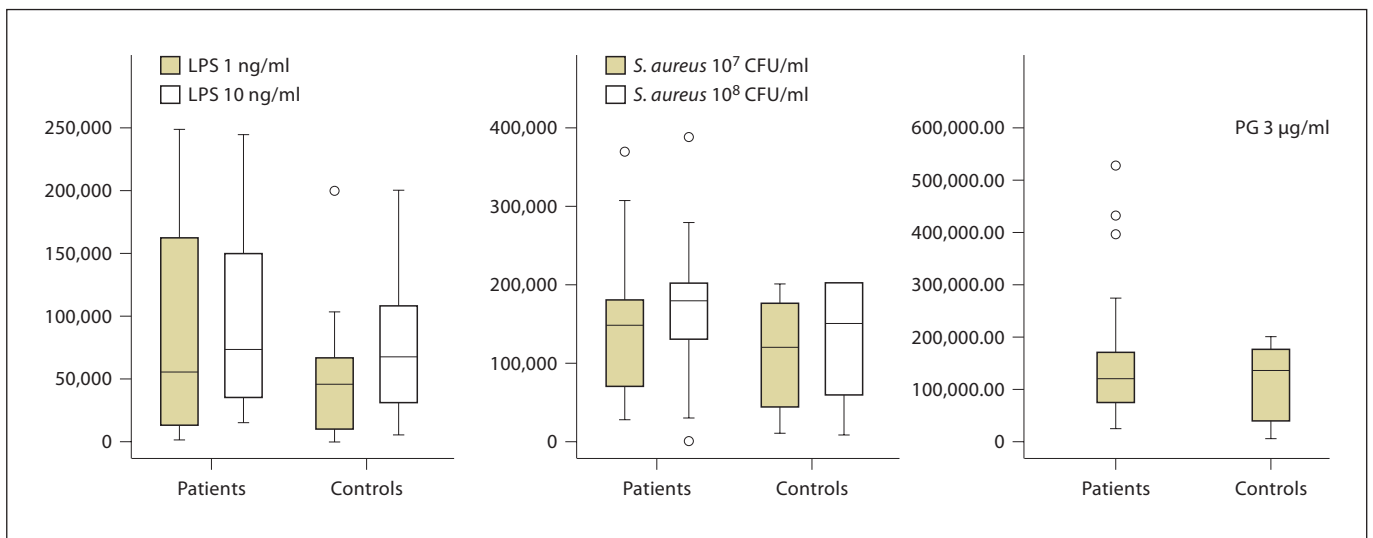


Fig. 2. IL-8 response to various TLR ligands at different concentrations: LPS at 1 and 10 ng/ml, *S. aureus* at 10⁷ and 10⁸ CFU/ml and peptidoglycan (PG) at 3 μ g/ml. Boxes represent values between the 25th and 75th percentiles. The horizontal lines correspond to the median, minimum and maximum.

We then attempted to perform a meta-analysis on TNF- α response with the data from previous studies [9, 10]. However, due to differences in the study design, only data from the study by Hawn et al. [9] could be compared with our data. In addition, it may be worth mentioning

that the data by Hawn et al. [9] were obtained by a whole blood assay, whereas our data were collected by studying PBMCs. The combined analysis confirmed that TNF- α levels were considerably higher in patients than controls when stimulated with LPS 10 ng/ml (3,257 vs. 1,240 pg/

Table 1. Median of TNF- α and IL-8 production levels in response to TLR stimulation

Stimulants	TNF- α , pg/ml			IL-8, pg/ml		
	patients	controls	p value	patients	controls	p value
LPS 1 ng/ml	691	446	0.175	55,400	45,600	0.357
LPS 10 ng/ml	3,257	1,418.5	0.043	79,800	67,500	0.292
<i>S. aureus</i> 1×10^7 CFU/ml	5,434.5	5,372	0.726	140,800	119,000	0.299
<i>S. aureus</i> 1×10^8 CFU/ml	11,520	5,806	0.038	174,741.5	107,500	0.252
Peptidoglycan 3 μ g/ml	3,550.5	2,023.5	0.085	120,900	135,090	0.557

p values were calculated by nonparametric Mann-Whitney U tests.

ml, $p = 0.006$), *S. aureus* 1×10^8 CFU/ml (9,024 vs. 2,430 pg/ml, $p = 0.001$) and peptidoglycan 3 μ g/ml (3,270 vs. 1,718 pg/ml, $p = 0.004$).

Discussion

We have evaluated the TNF- α and IL-8 response to various TLR ligands, and found it not to be deficient in cells from HIES patients. In contrast, we found that production of TNF- α was significantly increased following TLR2 and TLR4 stimulation.

Classical HIES is characterized by 'cold' skin abscesses and a lack of inflammation in patients. At the same time, the disorder has features which indicate a generalized inflammatory process. In addition, retained primary teeth and progressive skeletal malformations suggest defective osteohomeostasis.

TNF- α is a pro-inflammatory cytokine that acts as a key molecule in different inflammatory diseases [12]. In addition, it has been shown that TNF- α induces apoptosis in osteoblast and inhibits their differentiation [13, 14].

Furthermore, it has previously been shown that over-expression of *Btk*, a member of Tec family tyrosine kinases, enhances TNF- α and not IL-8 production in response to TLR2 and TLR4 ligands [15]. Given the different requirements of TLR signaling for cytokine production, the higher levels of pro-inflammatory cytokines in HIES might be due to an accessory pathway, as previously suggested [10].

In conclusion, we show that patients with HIES do not have a deficient TLR signaling pathway. In contrast, we found that mononuclear cells respond with increased TNF- α formation to TLR stimulation. Hence, an imbalanced inflammatory response to microbial stimuli might account in part for the pathology associated with HIES.

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