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Th17 Differentiation in Hyper-IgE Syndrome; IL-17 Secretion and RORγt Expression

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ÖZET

Hiper-IgE sendromunda Th17 farklılaşması; IL-17 salınımı ve RORγt gösterimi

Amaç: Hiper-IgE sendromu (HIES), enfeksiyona duyarlılık ve düşük sayıdaki Th17 hücreleri ile karakterize edilir. Th17 hücreleri fungal ve hücre dışı bakteriyel enfeksiyonların eliminasyonunda önemli rol oynarlar. Bu çalışmada amacımız, HIES hastaları ve sağlıklı kontroller arasında interlökin 17 (IL-17) salgılanması ve RAR-bağımlı orphan reseptör gama t (RORγt) ekspresyonunun ölçülmesi ile Th17 hücrelerinin farklılaşmasını araştırmaktır.

Yöntem: Çalışmaya 3 adet HIES tanısı almış çocuk ve 4 adet sağlıklı kontrol alındı. HIES skorları değerlendirildi ve hastaların klinik verileri hastane kayıtlarından toplandı. Th17 farklılaşması, ELİSA yöntemi ile IL-17 üretiminin ve gerçek zamanlı PZR yöntemi ile ROR-γt ekpresyonunun ölçülmesiyle değerlendirildi.

Bulgular: HIES hastalarında, peripheral kan mononükleer hücreler (PKMH) ve CD45+RA naif T hücreler Th17 farklılaştırma koşullarında kültüre edildiğinde, IL-17 sitokin seviyesinde sağlıklı bireylere göre önemli derecede azalma gözlendi. Buna ek olarak, sağlıklı kontrollerin IL-17 seviyeleri forbol 12-miristat 13-asetat (FMA) ve iyonomisin uyarımlı durumda uyarımsız duruma göre daha yüksek bulundu. Ayrıca PKMH kültürlerinde IL-17 seviyesi FMA ve iyonomisin uyarımlı durumda, HIES hastaları ve sağlıklı kontroller için uyarımsız koşullarla karşılaştırıldığı zaman önemli derecede yüksek gözlendi. HIES hastasında uyarılmış PKMH'lerdeki RORγt ekspresyon seviyesi, sağlıklı kontrolün yarı düzeyinde olarak tespit edildi.

Sonuç: IL-17 salgılanması ve ROR-γt ifadesinin değerlendirilmesi mutasyon analizleri için aday olan hastaları belirlemek için yapılmalıdır. Ülkemizde bu adımların uygulanması ve bilinen mutasyonları olmayan HIES hastalarının seçimi, yeni genetik bozuklukların keşfedilmesine ve böylece yeni tedavi yaklaşımlarına olanak sağlayacaktır.
Anahtar sözcükler: Th17, interlökin 17 (IL-17), RAR-bağımlı orphan reseptör gama t (ROR-γt), Hiper-IgE sendromu (HIES)

ABSTRACT

Th17 Differentiation in Hyper-IgE Syndrome; IL-17 Secretion and RORγt Expression

Objective: Hyper-IgE syndrome (HIES) is characterized by susceptibility to infection and low number of Th17 cells. Th17 is believed to be critical in the clearance of fungal and extracellular bacterial infections. Present study investigates the differentiation of Th17 cells by evaluation of interleukin 17 (IL-17) secretion and RAR-related orphan receptor gamma t (RORγt) expression in HIES compared with healthy subjects.

Method: Three children diagnosed with HIES and 4 healthy subjects were enrolled in the study. HIES scores were evaluated and clinical data of patients were collected from their hospital records. At Th17 polarizing conditions, Th17 differentiation was assessed by the secretion of IL-17 with ELISA and the expression of ROR-γt with real time PCR.

Results: HIES (n=3) patients showed significantly lower levels of IL-17 secretion compared to the healthy subjects (n=4) regarding the peripheral blood mononuclear cells (PBMCs) and CD45+RA naive T cells cultured in Th17 differentiating conditions. In addition, phorbol 12-myristate 13-acetate (PMA) and ionomycin stimulated IL-17 levels of healthy group were significantly higher than unstimulated conditions. Moreover, PMA and ionomycin stimulated IL-17 levels of PBMC cultures were significantly higher when compared to unstimulated conditions for both HIES patients and healthy subjects. ROR-γt expression level of stimulated PBMCs for HIES patient was detected nearly half of that of the healthy subject.

Conclusion: Evaluation of IL-17 secretion and ROR-γt expression should be performed to determine the patients who are candidates for mutation analyses. Performing these steps and selection of HIES patients without known mutations in our country would provide an opportunity to discover new genetic defects and so new therapeutic approaches in HIES.
Key words: Th17, interleukin 17 (IL-17), RAR-related orphan receptor gamma t (ROR-γt), Hyper-IgE syndrome (HIES)

INTRODUCTION

Th17 cells constitute a recently discovered subset of T helper cells characterized by expression of the transcription factor RORγt and secretion of IL-17 and IL-22 (1). They are instrumental in mucosal immunity by orchestrating antimicrobial peptides expression in epithelial cells as well as by recruiting neutrophils to mucosal tissues (2). Th17 cells

are generally considered to be pro-inflammatory and have been shown to mediate autoimmunity in both rodents and humans (3). Furthermore, studies using animal models have demonstrated a role for Th17 cells in mediating protection against several different bacteria and fungi, suggesting dual and context-dependent roles for Th17 cells. Recently, a number of human disorders characterized by susceptibility to infection and low number of Th17 cells have been described, including Hyper-IgE syndrome (HIES) (4-7).

HIES is a complex primary immunodeficiency disorder (PID) characterized by dermatitis associated with extremely high serum IgE levels and susceptibility to staphylococcal skin abscesses and pneumonia (8,9). There are two forms of HIES: A dominant form caused by mutations in the signal transducer and activator of transcription 3 (STAT3) (10), and a recessive form caused by mutations in DOCK8 (11).

Autosomal dominant (AD) HIES results from dominant negative mutations in STAT3 gene and associated with facial, dental, skeletal, and connective tissue abnormalities (9,12). On the other hand, most cases of autosomal recessive (AR) HIES are caused by mutations in the gene encoding the DOCK8 protein, which maps to the chromosomal locus 9p24.3 (11,13). DOCK8 disruption is associated with a phenotype of severe cellular immunodeficiency characterized by susceptibility to viral infections, atopic eczema, defective T-cell activation and Th17 cell differentiation, and impaired eosinophil homeostasis and dysregulation of IgE (11).

In the light of these data, present study aimed to elucidate the competency of Th17 cell differentiation by evaluation of IL-17 secretion and RORγt expression in children with HIES in comparison to healthy subjects.

MATERIAL AND METHODS

Study Design And Subjects

Children diagnosed with HIES at the Marmara University Pediatric Allergy-Immunology Pediatric Allergy-Immunology Clinics (Clinics/Department?) (n=3) and unrelated healthy subjects (n=4; 10yrs-F, 12yrs F, 9yrs-M, 13yrs-M) were enrolled in the study. Diagnosis of HIES was given on the basis of elevated serum IgE levels, blood eosinophil count, eczematous rashes, and unusual, severe, recurrent infections including recurrent pneumonias, skin and deep-seated staphylococcal abscesses, candidiasis, and other fungal infections. HIES scores were evaluated as previously described by Grimbacher et al (8). Clinical data of the patients were collected from their hospital records including the parameters needed for HIES scoring, serum immunoglobulin levels and lymphocyte subset analyses. Healthy subjects were asked for 10 warning signs of primary immune deficiencies (http://www.info4pi.org/) (14) and chronic diseases or medication use. All subjects were evaluated for Th17 differentiation; IL-17 secretion by ELISA and ROR-γt expression by real time PCR. STAT3 mutations were kindly provided by Talal Chatila from UCLA. Written informed consents were obtained from the parents. Study protocol was approved by the local ethical committee of Marmara University (date and number; 19-12-2008, MAR-YC-2008-0216; respectively).

Th17 differentiation and evaluation of IL-17

Venous blood samples (5 ml) were drawn from HIES patients and healthy subjects. PBMCs and naive CD45RA+ cells were obtained as previously described by Akkoc et al (15). PBMCs and naive CD45RA+ cells were stimulated with phytohemagglutinin (2 μg/ml) (Sigma-Aldrich, Germany) and IL-2 (20 ng/ml) (BD Bioscience, Belgium) for 3 days. For 4 days, stimulation by anti-CD2/3/28 (0.5 μg/ml) (BD Bioscience, Belgium), TGFβ (5 ng/ml), anti-IFNγ (10 μg/ml) (BD Bioscience, Belgium), IL-1β (20 ng/ml) (BD Bioscience, Belgium), IL-6 (20 ng/ml) (BD Bioscience, Belgium), IL-21 (20 ng/ml) (BD Bioscience, Belgium) and IL-23 (20 ng/ml) (BD Bioscience, Belgium) was ended with polarized T cells. These T cells were stimulated with 20 ng/ml phorbol 12-myristate 13-acetate and 1 μg/ml ionomycin (Sigma-Aldrich, Germany) for the following 3 days. Culture supernatants were collected and assayed for IL-17 with Human IL-17 ELISA KIT (Invitrogen, UK) by following the manufacturer's instructions.

RNA isolation and cDNA synthesis

Samples were stored at -80°C after homogenization in RLT buffer supplied by the manufacturer. Total RNA was isolated by Qiagen RNeasy Plus Mini Kit (Qiagen, GmbH, Germany) to eliminate the genomic DNA prior to RNA isolation. RNA quality and quantity was checked with Nanodrop 1000

(Thermo Fisher Scientific, Germany) and cDNA was synthesized by random hexamers and MMLV reverse transcriptase, from 1µg of total RNA according to manufacturer procedures (MBI Fermentas Life Sciences, Lithuania).

RORγt expression by real-time quantitative PCR (RQ-PCR) analysis

RQ-PCR was carried out on the Light Cycler 480 Instrument (Roche Applied Sciences, Manheim, Germany). The specific primer-probe sets were designed using human universal probe library tool as described by the manufacturer. The real time amplification was performed with a final reaction mixture of 20µl containing, 5µM of each primer, 0.5 µM of each probe, Light Cycler 480 Probe Master Mix and 100ng/µl of cDNA. Each sample was studied in duplicates and all runs were repeated twice. The PCR protocol was as followed: Initial denaturation at 95˚C for 7 min, amplification segment is 5 sec at 95˚C, 10 sec at 60˚C 10 sec at 72˚C for 45 cycles. For normalization, the most stable three genes (CYCLOPHILIN and ABL) were selected by GeNorm (V3.4, Belgium) software. The underlying principles and formulas were described by Vandesompele et al. (16). Relative expressions were calculated according to the delta-delta Ct method, based on the mathematical model described by Livak et al. (17).

RESULTS

Clinical And Laboratory Data

P1 was a 10-year-old boy with an HIES score of 70 and STAT3 mutation. Clinical history revealed neonatal eryhtrodermic rash, recurrent skin abscesses and varicella infection. Physical examination showed severe eczema, retained primary teeth, prominent forehead, wide nasal bridge and high palatal arch. Absolute neutrophil and lymphocyte counts were within normal range in comparison to age-matched references. Immunoglobulin and lymphocyte subset values were normal except low IgM, high IgE levels and high eosinophil counts. P1 was receiving regular intravenous immunoglobulin, anticandidal and antibacterial prophylaxis.

P2 was a 13-year-old girl born to consanguineous parents with an HIES score of 61 and DOCK-8 mutation. P2 also had recurrent skin abscess. In addition, she had recurrent pneumonias, Heck's disease at the oral cavity and carcinoma in situ at vulvar region due to HPV infection. Physical examination revealed retained primary teeth, wide nasal bridge and high palatal arch. Immunological evaluation was normal except low IgM and IgA, high IgE levels and high eosinophil counts. P2 was also receiving regular intravenous immunoglobulin and IFN-α for HPV infection.

P3 was a 9-year-old girl with an HIES score of 78 and STAT3 mutation. She had recurrent skin and lung abscesses, and fractures. Physical examination showed facial features, scoliosis, retained primary teeth and eczema. Low IgA levels, multiple pneumatoceles and cerebral infarcts were detected at laboratory analyses. She was receiving intravenous immunoglobulin, antifungal and antibacterial prophylaxis, and subcutaneous IFN-γ and inhaled α-1 antitrypsin for pneumatoceles.

Demographic and clinical data were summarized in Table 1 and laboratory data were shown in Table 2.

Table 1: Clinical and demographic data of HIES patients

Patients	Consanguinity	Age (years)	Sex	STAT-3 mutation	HIES Score	Eosinophil count	IgE (IU/ml)	Abscess	Pneumonia	Eczema	Characteristic face*	Candidal/Viral infections	Skeletal abnormality
P1	-	10	M	+	70	1270	3246	+	+	+	+	-	+
P2	+	13	F	-	61	1000	19100	+	+	+	+	+	+
P3	-	9	F	+	78	900	25852	+	+	+	+	+	+

*wide nasal bridge, high palatal arch, coarse face

Table 2: Laboratory data of HIES patients

Patients	ANC	ALC	IgG (mg/dl)	IgM (mg/dl)	IgA (mg/dl)	CD3 (%)	CD4(%)	CD8(%)	CD19/20 (%)	CD16-56 (%)
P1	4100	4400	1400	46	165	85	58	19	9/9	2
P2	3750	1500	1568	26	20	54	24	27	20/22	15
P3	4700	1600	1910	150	44	71	55	19	25/24	3

ANC: absolute neutrophil count, ALC: absolute lymphocyte count, Ig: immunoglobulin, CD: cluster of differentiation.

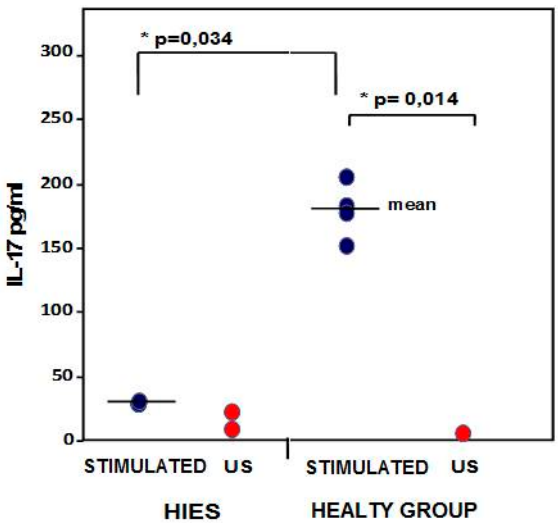

Figure 1: IL-17 levels in CD45RA+ naive T cells at Th17 differentiating conditions of HIES and healthy groups.

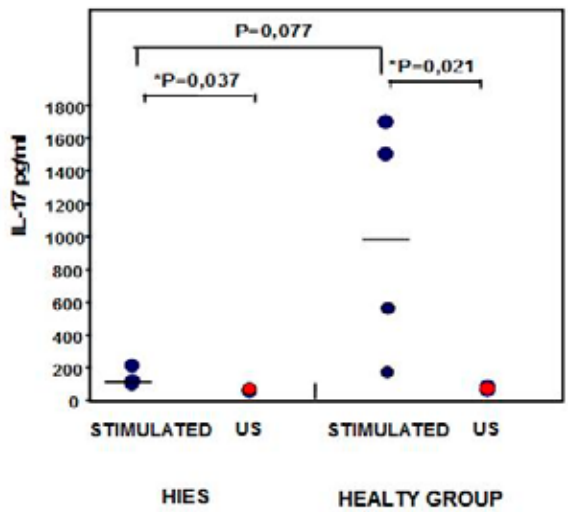

Figure 2: IL-17 levels in peripheral blood mononuclear cells at Th17 differentiating conditions of HIES and healthy groups.

Th17 differentiation and evaluation of IL-17

HIES (n=3) patients showed significantly lower levels of IL-17 secretion compared to healthy subjects (n=4) regarding the CD45+RA naive T cells cultured in Th17 differentiating conditions (p=0.034). In addition, stimulated IL-17 levels of healthy group were significantly higher than unstimulated conditions (p=0.014). Moreover, stimulated IL-17 levels of PBMC cultures were significantly higher when compared to unstimulated conditions for both HIES patients and healthy subjects (p=0.037, p=0.021; respectively). HIES patients showed remarkably lower IL-17 secretion of PBMCs at Th17 differentiating conditions in comparison to healthy controls (p=0.0077). These data were presented at Figure 1 and 2. No significant differences were detected for unstimulated conditions of PBMC and CD45+RA naive T cells of HIES patients compared to healthy controls (data not shown).

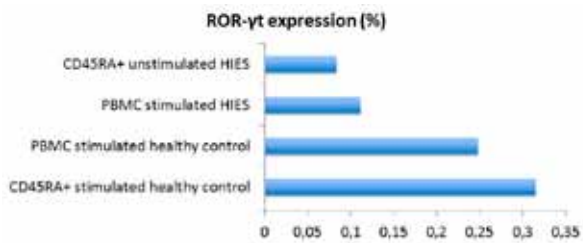

Figure 3: ROR-γt expression in peripheral blood mononuclear cells at Th17 differentiating conditions of HIES and healthy groups.

RORγt expression by real-time quantitative PCR analysis

ROR-γt expressions of stimulated PBMCs were available for HIES (n=1) patients and healthy subjects (n=1). Due to low number of samples, statistical analysis could not be performed regarding these data. ROR-γt expressions for each case are presented in Figure 3. ROR-γt expression of

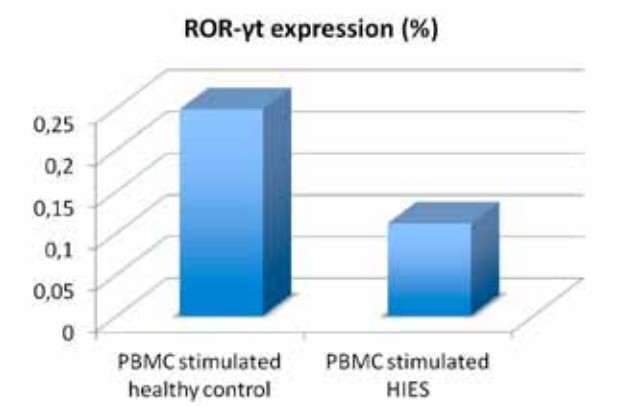

Figure 4: ROR-γt expression of HIES patients and healthy subjects

stimulated PBMCs for the HIES patient was detected as 0,11% and as 0,25% for the healthy subject. ROR-γt expressions of stimulated CD45+RA naive T cells and PBMCs derived from healthy subjects were available for 3 and 1 subjects, respectively. In addition, only 1 HIES patient could be evaluated for RORγt expression of naive T cells at unstimulated conditions. Data of each condition are presented in Figure 4.

DISCUSSION

In the present study, 3 HIES patients with NIH score >40 were searched for STAT3 mutation and evaluated for differentiation of Th17 cells via both IL-17 secretion and ROR-γt expression as transcription factor. To our knowledge, this is the first national multicentre study evaluating all these steps by the efforts of local laboratories. Although the main limitation of the study was low number of subjects due to the rarity of this disease in addition to high cost of analyses, this paper presents preliminary data and more patients are being collected by now.

HIES is a rare disease and characterized by high titers of serum IgE, chronic eczema, recurrent staphylococcal infections, and pneumatoceles (8,9). AD HIES or Job's syndrome is caused by dominant negative mutations in STAT3 (10). Clinically, AD HIES is associated with facial, dental, skeletal, and connective tissue abnormalities (9,12). Three HIES patients in our unit were suggestive for AD HIES. All 3 patients were analysed for STAT3 mutations and 2 of them found to have mutations. On the other hand, AR HIES is characterized by the absence of skeletal and dental findings and the presence of susceptibility to severe viral infections, eczema and higher levels of eosinophil counts (18). Recently, mutations in the gene encoding the DOCK8 protein were reported to cause most cases of AR HIES (11,13).

IL-17-producing (Th17) T helper cells have emerged as an important subset of helper T cells that are believed to be critical in the clearance of fungal and extracellular bacterial infections (4). Milner et al. demonstrated impaired Th17 responses in HIES (4). Purified naive T cells were shown to be unable to differentiate into Th17 cells in vitro and had lower expression of ROR-γt, which is consistent with a crucial role for STAT3 signaling in the generation of Th17 cells (4). In addition, Khatib et al. showed severely reduced IL-17 levels in STAT3 mutant HIES patients proposing a defect in early steps of Th17 differentiation and moderate decrease with normal STAT3 indicated a defect in more distal steps (18). In accordance with previous studies, purified naive T cells and PBMCs at Th17-polarizing conditions resulted with significantly lower levels of IL-17 secretion in HIES compared to healthy subjects in the present study. Milner et al. demonstrated that ROR-γt mRNA expression in HIES T cells after 48 h under Th17-polarizing conditions was significantly lower in T cells from subjects with HIES than in healthy controls (4). ROR-γt mRNA expression of PBMCs at Th17 differentiating conditions was available for 1 HIES case and 1 healthy subject. Although the data is limited, the HIES patient showed lower level of ROR-γt mRNA expression. In vitro experiments on naive T cells at Th17-polarizing conditions and the determination of ROR-γt mRNA expression are being continued to expand the number of HIES subjects and data.

In conclusion, HIES is a rare disease in European countries although it is more common in our country due to high consanguinity. Determination of clinical status by a detailed history and NIH score are the first steps in selection of patients for further analyses. Low IL-17 secretion and ROR-γt expression are other clues to determine the patients who are candidate for STAT3 or DOCK8 mutation analyses. Performing these steps and selection of HIES patients without known mutations in our country would provide an opportunity to discover new genetic defects and so new therapeutic approaches in HIES.

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