

## ORIGINAL ARTICLE

Iran J Allergy Asthma Immunol  
August 2016; 15(4):264-274.

# House Dust Mites Confer a Distinct Immunological Feature among Dermatitis

Safa Baris<sup>1</sup>, Ahmet Ozen<sup>1</sup>, Tuba Akdeniz<sup>2</sup>, Elif Karakoc-Aydiner<sup>1</sup>, Ovgu Aydin<sup>3</sup>, Hulya Ercan<sup>4</sup>, Ismail Ogulur<sup>1</sup>, Yildiz Camcioglu<sup>5</sup>, Reha Cengizlier<sup>4</sup>, Cuyan Demirkesen<sup>3</sup>, Deniz Yucelten<sup>6</sup>, Gulderen Demirel<sup>2</sup>, and Isil B Barlan<sup>1</sup>

<sup>1</sup> Division of Pediatric Allergy and Immunology, Pendik Research and Training Hospital, Marmara University, Istanbul, Turkey

<sup>2</sup> Stem Cell Laboratory, Yeditepe University Hospital, Istanbul, Turkey

<sup>3</sup> Department of Pathology, Cerrahpasa Medical Faculty, Istanbul University, Istanbul, Turkey

<sup>4</sup> Division of Pediatric Allergy, Yeditepe University Hospital, Istanbul, Turkey

<sup>5</sup> Division of Pediatric Infectious Diseases, Immunology and Allergy, Department of Pediatrics, Cerrahpasa Medical Faculty, Istanbul University, Istanbul, Turkey

<sup>6</sup> Department of Dermatology, Pendik Research and Training Hospital, Marmara University, Istanbul, Turkey

Received: 18 November 2015; Received in revised form: 7 March 2016; Accepted: 3 April 2016

## ABSTRACT

Atopic dermatitis (AD) is a heterogeneous disease with regard to clinical phenotype and natural history. We investigated T cell subtypes and cytokine responses in peripheral blood and skin lesions of AD patients with various sensitivities.

Immunological studies were performed in 27 subjects: 9 house dust mite (HDM)-sensitized; 6 subjects with sensitizations other than HDM; 7 non-allergic AD patients and 5 healthy controls. Among those, skin biopsy samples of 13 subjects were evaluated for immunohistochemical analyses, as well.

The mean age was  $8.93 \pm 5.17$  years. HDM-allergic AD emerged as a distinct immunologic phenotype, with higher production of interleukin (IL)-4, -5, -2 both at rest and when stimulated by Der p1 or SEB along with higher Th17. As for Th17 cell percentage, it was increased in all AD groups compared to healthy controls, while HDM-allergic group was distinguished with a significantly lower production of IL-17. Patients with sensitizations other than HDM were mostly similar to non-allergic AD, with increased Th17 and CD4<sup>+</sup>CD69<sup>+</sup>interferon-gamma (IFN- $\gamma$ )<sup>+</sup> T cells percentage. The biopsy of lesional skin showed that HDM-allergic AD had lower IFN- $\gamma$  and IFN- $\gamma$  co-expressing CD8<sup>+</sup> T cells compared to patients with other sensitizations ( $p=0.03$  and  $p=0.04$ , respectively). Among the HDM allergic patients, pairwise comparison of lesional versus non-lesional skin revealed higher CD4<sup>+</sup> T cells numbers, expression of forkhead box P3 (Foxp3) and T-cell-specific transcription factor (T-bet) ( $p=0.018$ ,  $p=0.018$ ,  $p=0.018$ , respectively).

HDM-allergic AD is a distinct subtype with a predominant skewing in Th2 and higher Th17 cell percentage along with a blunted Th1 response in the skin, all of which may have therapeutic implications.

**Keywords:** Atopic dermatitis; House dust mite; Interferon-gamma; Skin lesion; Th17 Cells

**Corresponding Author:** Safa Baris, MD;  
Turkish Republic Ministry of Health, Marmara University, Research and Training Hospital, Division of Pediatric Allergy and Immunology,

Mimar Sinan Cade. No: 41, 34890, Istanbul, Turkey. Tel: (+90 0216) 657 06 06-7811, Fax: (+90 0216) 657 06 95, E-mail: safabaris@hotmail.com

### INTRODUCTION

Atopic dermatitis (AD) is a chronic recurrent inflammatory skin disease that affects both pediatric and adult population.<sup>1-3</sup> The pathogenesis of AD has been attributed to alternate immune mechanisms, including innate and adaptive immune system. A defective formation of the epidermal barrier with mutations in filaggrin and abnormal regulation of immune cytokines, such as interleukin (IL)-4, IL-5, IL-13, and IL-31 can initiate and maintain the cardinal features of AD.<sup>4,5</sup>

Among the environmental factors, house dust mite (HDM) allergens are important for the development of AD which are known to contribute to the immunopathogenesis through the induction of IgE binding.<sup>3</sup> Although mite allergens have been previously shown to activate toll like receptors, a recent study has provided evidence for direct activation of the inflammasome in epidermal keratinocytes.<sup>6</sup> While early models of immune activation in patients with AD focused on the differential roles for T helper 2 (Th2) and T helper 1 (Th1) in disease initiation and maintenance, recently, some roles for T helper 17 (Th17) and T helper 22 (Th22) cells have also been proposed.<sup>5,7,8</sup> It was shown that an increased percentage of Th17 cells in the peripheral blood correlates with disease severity.<sup>9</sup> However, the exact mechanisms related to AD still warrant further investigation.

Considering the diverse heterogeneity of AD in terms of both clinical phenotypes and underlying mechanisms, various allergens may exhibit immunologically distinct features. Therefore, we aimed to investigate T cell subtypes and cytokine responses in peripheral blood and skin lesions of AD patients with various sensitivities.

### MATERIALS AND METHODS

#### Subjects

AD was diagnosed according to the criteria of Hanifin and Rajka.<sup>10</sup> 38 AD patients and 5 healthy controls were enrolled in this study based on the results of skin prick testing, serum IgE levels and specific IgE (Immulate 2000 analyzer; Euro/DPC, Llanberis, UK). AD patients were first grouped in two populations: 28 allergic and 10 non-allergic patients. Then allergic patients were further divided into two groups; 19 with HDM sensitization and 9 with sensitivities other than

HDM. Only moderate and severe AD patients were included in the study. Immunological studies were performed in 27 subjects: 9 HDM - sensitized; 6 other sensitizations; 7 non-allergic AD and 5 healthy controls. Of these patients, immunohistochemistry analyses of skin biopsy samples were evaluated only in 13 subjects.

The study protocol was approved by the local ethics committee of Marmara University (IRB number: IRB00009067) and a written informed consent was obtained from all patients and parents of the children. Due to the young age of our patients, a simple oral description of the study was given to participating children in the presence of their parent(s) and a verbal assent was requested.

#### Sample Preparation

Two peripheral blood samples from each patient and healthy controls were collected into EDTA Vacutainer (BD BioSciences) collection tubes. One of them was used for surface and intracellular staining and cell culture studies were performed on the other. Mononuclear cells were separated using Ficoll 1.077 density gradient (Sigma-Aldrich, USA). Cultured cells were stimulated with Staphylococcal Enterotoxin B (SEB) (1µg/mL) (Sigma-Aldrich, USA) and Der p1 (1µg/mL) (Indoor Biotechnologies Inc., USA). Cell concentration was adjusted to  $0.5-3 \times 10^3$ /mL cell before cell culturing. Supernatants obtained from cell culture were analyzed for Th1/Th2 cytokines, IL-9, IL-13, IL-17. Cell pellet obtained from stimulated cells in cell culture was used for Th17/Treg and CD4 intracellular cytokine assays.

#### Surface Immunophenotyping

Antibody panels used for surface immunophenotyping were CD4-FITC/CD25-PE-CY5, CD8-FITC/CD25-PE-CY5 (BD BioSciences, USA). Whole blood lysis method was used for preparation of samples. Then, the samples were run on a daily standardized flow cytometry (Beckman Coulter FC 500 Cytomics Flow Cytometry with CXP software v2.2).

#### Serum Cytokine Measurements

The BD Cytometric Bead Array (CBA Human) Th1/Th2 Cytokine Kit (BD BioSciences, USA) was used to quantitatively detect human Interleukine-2 (IL-2), Interleukine-4 (IL-4), Interleukine-5 (IL-5), Interleukine-10 (IL-10), Tumor Necrosis Factor (TNF) and Interferon- $\gamma$  (IFN- $\gamma$ ) by flow cytometry (FC500,

Beckman Coulter, USA). Cytokine measurements were performed on culture supernatants stimulated by SEB and Der p1. FL2 and FL4 scattergrams were employed for analysis of cytokines. Results were obtained by assessing X mean values across standard values obtained from standard beads. The values were expressed as pg/mL.

IL-9, IL-13, IL-17A were assessed in culture supernatants using BD Human Flex Set. Preparation was completed in accordance with the manufacturer instructions. Samples were analysed by flow cytometry (Beckman Coulter FC 500 Cytomics Flow Cytometry with CXP software v2.2). FL2 and FL3 scattergrams were used for analysis of cytokines. Results were assessed by using X mean values obtained from standard beads. The values were expressed as pcg/mL.

### **Intracellular Cytometry Measurements**

For Treg analysis, immunophenotyping protocol was performed with CD4-FITC/Foxp3-PE/CD25-PE-CY5, CD8-FITC/Foxp3-PE/CD25-PE-CY5 antibodies. CD4-FITC/CD25-PE-CY5, CD8-FITC/CD25-PE-CY5 tubes were used as internal controls. Intracellular staining for Foxp3-PE, (Becton Dickinson, USA) was performed in accordance with the manufacturer instructions. Permeabilization of cells was obtained by solutions (A and B solutions containing detergents) provided in the kit. Samples were run on flow cytometer for two hours (Beckman Coulter FC500 Cytomics CXP Analyses software v2.2).

### **Human Th17/Treg Phenotyping**

Human Th17/Treg Phenotyping Kit (BD BioSciences) was used for detection of Th17 cells. Cells were incubated for 2 hours with stimulants (SEB and Der p1) and 1 $\mu$ L Golgi Stop was added on cultured cells. After 2 hours of incubation at 37°C, the cells were centrifuged at 250 g for 5 minutes. Supernatant was discarded, intracellular staining protocols was performed in accordance with the manufacturer's instructions. Samples were analysed by flow cytometry (Beckman Coulter FC500 Cytomics CXP Analyses software v2.2).

### **CD4 T cell Intracellular Cytokine Detection**

BD FastImmune CD4 Intracellular Cytokine Detection Kit (BD BioSciences, USA) was used for measurement of intracellular IFN- $\gamma$  concentrations in CD4<sup>+</sup>T helper cells. Cells in culture were stimulated

with CD28/CD49d prior to activation with SEB and Der p1. Intracellular staining process was performed according to the manufacturer's instruction and run on flow cytometry (Beckman Coulter FC500 Cytomics CXP Analyses software v2.2). CD69 values on CD4<sup>+</sup> cells were also obtained using the kit. Activated CD4<sup>+</sup>CD69<sup>+</sup> cells were detected in the same run.

### **Immunohistochemical Assessment of Skin Biopsies:**

Histological examination of skin biopsy samples were performed in 13 children with allergic sensitivities: 7 with HDM and 6 with sensitivities other than HDM. Meanwhile, biopsies from non-lesional sites were obtained in those 7 patients with HDM sensitivity to compare with accompanying lesional skin. The study samples were immediately placed in 10% buffered formalin within 12-24 hours. After the fixation, each block was immediately processed in to paraffin. Four  $\mu$ m-thick sections of the paraffin-embedded tissues were prepared and used for immunohistochemical analysis. The immunohistochemical analysis for all the antibodies except IL-17 was performed using a multimer-based detection system with monoclonal antibodies according to the manufacturer's instructions. Sections were deparaffinized and rehydrated with deionized water. All slides were loaded onto an automated system (Autostainer; Ventana BenchMark XT). The automated system includes pretreatment with EDTA for 60 minutes; incubation with inhibitor (included in detection system); incubation with primary antibody for 32 minutes at 37°C; incubation with secondary antibody (ready to use included in detection system) for 8 minutes and incubation with DAB chromogen for 8 minutes.

For IL-17, manual immunostaining was carried out using cell and tissue staining kit of R&D Systems. After deparaffinization, antigen retrieval was done with citrate buffer. Sections were incubated with normal blocking serum. Then the tissue sections were incubated with primary antibody overnight at room temperature. Biotinylated secondary antibody, HSS-HRP and AEC chromogen were applied. Finally the sections were stained with Hematoxylin. Double immunostaining was performed for IL-9/CD4, IL-9/CD8, IFN- $\gamma$ /CD4, IFN- $\gamma$ /CD8; double staining was also performed using Ventana BenchMark XT processor (Ventana, Tucson, AZ). Both negative and positive controls were used for each antibody in all staining procedures. Nuclear and/or cytoplasmic positivity were counted in lymphocytes in 5 HPF in the

## HDM Sensitivity in Atopic Dermatitis

areas with highest expression for FOXP-3, ROR- $\gamma$ , T-bet and IL-17, IL-9, IL-10, IFN- $\gamma$ , CD4, and CD8 respectively. The median of 5 HPFs was accepted as final score for the related antibody staining.

### Statistical Analysis

Statistical analyses were carried out with SPSS program (version 16.0; SPSS, Inc, Chicago, USA). The normality of the distribution was assessed by Kolmogorov Smirnov test. Descriptive statistics were expressed as frequency, percentage, mean $\pm$ SD (standard deviation) and median with interquartile range (IQR). Median values were compared by Mann-Whitney U or Wilcoxon matched - pairs signed rank test where appropriate. Spearman rank correlation coefficient test was used to assess correlations between continuous variables. A *p*-value less than 0.05 was considered statistically significant.

## RESULTS

A total of 38 patients (25 male, 13 female) and 5 healthy controls were enrolled. The mean age of the

patients was 8.93 $\pm$ 5.17 years. Characteristics of study participants are presented in Table 1. The percentage of atopy in family history (*p*=0.040), serum eosinophil counts (*p*=0.030) and IgE levels (*p*=0.01) were found to be significantly higher in patients with sensitivities other than HDM when compared to non-allergic ones.

### AD with HDM Sensitivity

#### Versus Healthy Controls

HDM-allergic AD patients had higher IL-4 at rest and in response to SEB and Der p1 (*p*=0.008, *p*=0.028 and *p*=0.039, respectively) (Figure 1); higher IL-5 at rest and in response to Der p1 (*p*=0.009 and *p*=0.023, respectively); higher IL-2 at rest and in response to SEB and Der p1 stimulation (*p*=0.032, *p*=0.05 and *p*=0.009, respectively) (Figure E1); higher number of CD4<sup>+</sup> T cells (*p*=0.04) and CD4<sup>+</sup>Th17 cells at rest and in response to CD28, SEB and Der p1 (*p*=0.014, *p*=0.006, *p*=0.017 and *p*=0.006, respectively). Interestingly, despite high numbers of CD4<sup>+</sup>IL-17 producing Th17 cells, production of IL-17 was found to be lower in this group (Figure 2). The cytokines and T cells responses were demonstrated in Table 2.

**Table 1. Characteristics of study population with atopic dermatitis.**

Topics	Patients with HDM sensitivity (n=19)	Patients with sensitivities other than HDM (n=9)	Non-allergic (n=10)	<i>p</i> * values	<i>p</i> ** values	<i>p</i> *** values
Gender (n;%)						
Male	13 (68.4%)	5 (55.5%)	7 (70.0%)	>0.05	>0.05	>0.05
Female	6 (31.6%)	4 (45.5%)	3 (30.0%)	>0.05	>0.05	>0.05
Age (years)	9 $\pm$ 5	10 $\pm$ 6	5 $\pm$ 5	>0.05	>0.05	>0.05
Mean $\pm$ SD, median (IQR)	8 (6-12)	12 (7-17)	4 (2-6)			
SCORAD	48 $\pm$ 16	58 $\pm$ 17	47 $\pm$ 14	>0.05	>0.05	>0.05
Mean $\pm$ SD, median (IQR)	50 (34-60)	50 (4 -74)	48 (46-50)			
Eosinophil counts (cell/mm <sup>3</sup> )	979 $\pm$ 1018	733 $\pm$ 115	420 $\pm$ 148	>0.05	>0.05	<b>0.03</b>
Mean $\pm$ SD, median (IQR)	700 (400-1100)	800 (600-800)	430 (400-500)			
IgE (IU/ml)	3313 $\pm$ 5656	11843 $\pm$ 17131	386 $\pm$ 643	>0.05	<b>0.02</b>	<b>0.01</b>
Mean $\pm$ SD, median (IQR)	1250 (612-4753)	4083 (2260-5330)	177 (8-214)			
Asthma (n;%)	12 (70.6%)	4 (57.1%)	2 (40.0%)	>0.05	>0.05	>0.05
Rhinitis (n;%)	11 (64.7%)	4 (57.1%)	2 (40.0%)	>0.05	>0.05	>0.05
Family history of atopy (n;%)	12 (70.6%)	6 (85.7%)	2 (25.0%)	>0.05	>0.05	<b>0.04</b>

\* Comparison between HDM and patients with other sensitivities.

\*\* Comparison between HDM and non-allergic patients.

\*\*\* Comparison between other sensitivities and non-allergic patients.

Data were expressed as mean $\pm$ SD and median with interquartile range (IQR).

† The significance was determined by Mann Whitney U test and Chi-square test. Statistically significant *p*-values were presented in bold. HDM: House dust mite.

**Table 2. The cytokines and T cells responses of the patients with atopic dermatitis.**

Parameters	Patients with HDM sensitivity (n=9)	Patients with sensitivities other than HDM (n=6)	Non-allergic (n=7)	Healthy control (n=5)
US-IL-4 (pg/ml)	17.6±9*	9.5±0.9	10.6±1.2**	10.2±0.9***
Mean±SD, median (IQR)	15 (11.7-18.8)	9.9 (9.2-10.8)	10.2 (9.9±10.8)	10.1 (9.8-10.2)
SEB induced IL-4 (pg/ml)	17.4±8.9*	10.1±1.2	11.3±2.6	9.6±1.3
Mean±SD, median (IQR)	16.3 (11.8-18.6)	9.9 (9.2-10.8)	10.7 (9.4-12.5)	9.4 (9-10)
Der p1-induced IL-4 (pg/ml)	13.9±3.7	10.6±3	11.8±3.3	10.1±1.5***
Mean±SD, median (IQR)	13.9 (11.7-15.9)	10.1 (8.6-12.4)	11.2 (9.3-16)	10.2 (8.6-11.4)
US-IL-5 (pg/ml)	6.9±2.8*	4.2±0.3	4.7±1.2**	4.6±0.5***
Mean±SD, median (IQR)	5.9 (4.9-7.3)	4.2 (4-4.3)	4.1 (4-4.7)	4.5 (4.3-4.6)
SEB induced IL-5 (pg/ml)	7±3.2*	4.5±0.6	4.6±0.5**	4.4±0.4
Mean±SD, median (IQR)	6.2 (4.8-8.2)	4.3 (4-5.1)	4.6 (4.1-5)	4.4 (4.37-4.7)
Der p1-induced IL-5 (pg/ml)	6.1±1.7*	4.4±0.7	4.4±0.5**	4±0.8***
Mean±SD, median (IQR)	5.8 (5-7)	4.7 (3.8-4.8)	4.5 (3.8-4.8)	4.2 (4-4.5)
US-CD4 <sup>+</sup> Th17 (%)	0.43±0.48	0.22±0.23 <sup>†</sup>	0.1±0.07 <sup>††</sup>	0***
Mean±SD, median (IQR)	0.4(0-0.6)	0.2 (0.1-0.2)	0.1 (0.1-0.1)	0
SEB-induced CD4 <sup>+</sup> Th17 (%)	0.6±0.4	0.7±1	0.44±0.43 <sup>††</sup>	0.1±0.09***
Mean±SD, median (IQR)	0.4 (0-0.6)	0.3 (0.1-1.3)	0.2 (0.2-0.4)	0.1 (0-0.1)
Der p1-induced CD4 <sup>+</sup> Th17 (%)	0.5±0.2	0.32±0.26	0.24±0.25	0.1±0.09***
Mean±SD, median (IQR)	0.5 (0.4-0.5)	0.4 (0.1-0.5)	0.2 (0-0.5)	0.1 (0-0.2)
US-CD4 <sup>+</sup> CD69 <sup>+</sup> IFN-γ <sup>+</sup> T cells (%)	0.13±0.2	0.18±0.11 <sup>†</sup>	0.12±0.12 <sup>††</sup>	0
Mean±SD, median (IQR)	0 (0-0.3)	0.2 (0.2-0.2)	0.1(0-0.2)	0

Data were expressed as mean±SD and median with interquartile range (IQR).

\*Statistically different between HDM and other than HDM patients

\*\*Statistically different between HDM and non-allergic AD patients

\*\*\*Statistically different between HDM patients and healthy controls

<sup>†</sup> Statistically different between non-HDM patients and healthy controls

<sup>††</sup> Statistically different between non-allergic patients and healthy controls

Us= Unstimulated

<sup>†††</sup> The significance was determined by Mann Whitney U test. A  $p$ -value<0.05 was considered statistically significant. Significant values were presented in bold. The exact  $p$ -values were mentioned in the results. US: Unstimulated, HDM: House dust mite, SEB: Staphylococcal Enterotoxin B.

### Versus other sensitivities

Mono-sensitivity to HDM was associated with higher IL-5 (at rest, SEB- and Der p1- stimulated condition;  $p=0.002$ ,  $p=0.039$ ,  $p=0.018$ , respectively) and IL-4 (at rest and SEB induced condition;  $p=0.001$ ,  $p=0.025$ , respectively) (Figure 1).

### Versus non-allergic subjects

HDM sensitive subjects had higher levels of serum IgE ( $p=0.021$ ) (Table 1), IL-5 at rest, SEB- and Der p1- stimulated condition ( $p=0.011$ ,  $p=0.05$ ,  $p=0.017$ , respectively) and unstimulated IL-4 ( $p=0.009$ ) (Figure 1).

### Patients with Sensitivities Other Than HDM

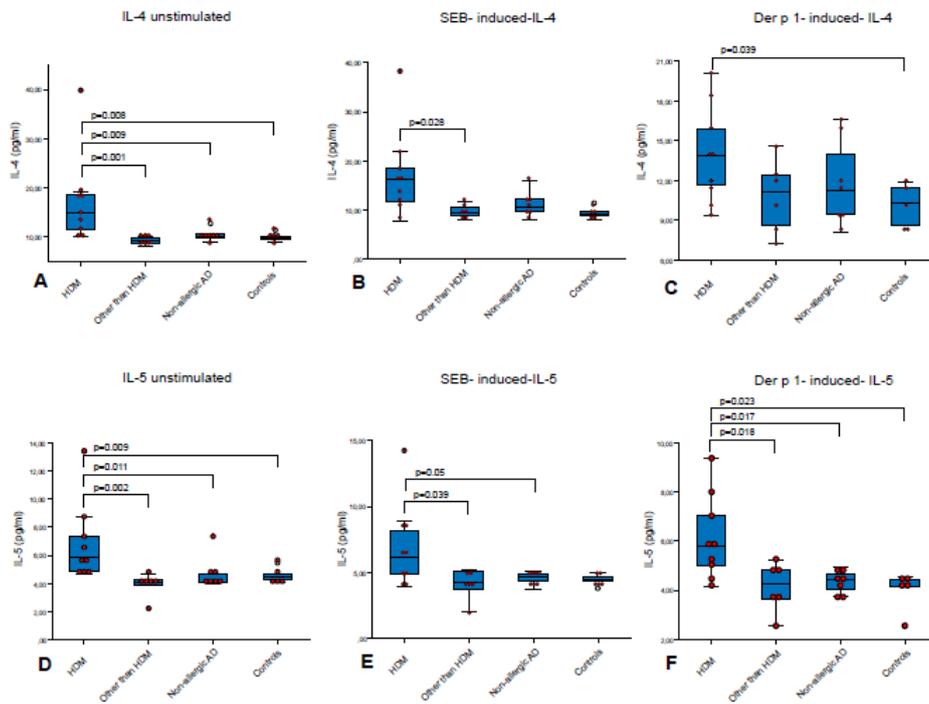
**Versus Healthy Controls;** patients with sensitivities other than HDM had higher levels of IL-17 producing CD4<sup>+</sup> T cells, IFN-γ producing CD4<sup>+</sup>CD69<sup>+</sup>IFN-γ<sup>+</sup> T cells in unstimulated condition ( $p=0.022$  and  $p=0.032$ )

and IL-2 production with SEB- and Der p1- induction ( $p=0.045$  and  $p=0.022$ , respectively) (Figure 2, Supplementary Figure 1).

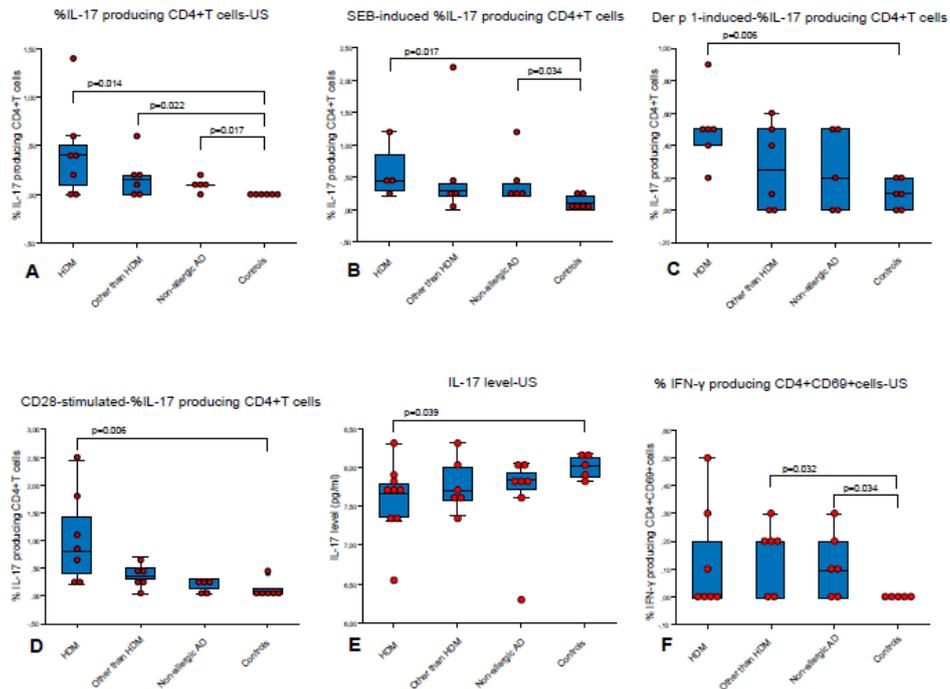
### Similar T cell immune responses in allergic (both HDM and other sensitizations) and non-allergic AD patients

Patients with allergic AD had higher percentage of IL-17 producing CD4<sup>+</sup> T cells both in unstimulated and SEB-, Der p1-, CD28-stimulated conditions when compared to healthy subjects ( $p=0.017$ ,  $p=0.025$ ,  $p=0.037$  and  $p=0.013$ , respectively, data not shown). Similarly, non-allergic AD group also had significantly higher percentage of IL-17 producing CD4<sup>+</sup> T cells both unstimulated and with SEB stimulation ( $p=0.017$ ,  $p=0.034$ , respectively) and higher IFN-γ producing CD4<sup>+</sup>CD69<sup>+</sup> T cells at rest ( $p=0.034$ ) when compared to controls (Figure 2).

## HDM Sensitivity in Atopic Dermatitis



**Figure 1. Median levels of the secreted cytokines; IL-4 secretion: A) Unstimulated, B) SEB- induced, C) Der p1-induced; and IL-5 secretion: D) Unstimulated, E) SEB-induced, and F) Der p1-induced. US: Unstimulated, HDM: House dust mite, SEB: Staphylococcal Enterotoxin B.**



**Figure 2. Median percentages of the IL-17 producing CD4<sup>+</sup> T cells in A) Unstimulated condition, and when stimulated with B) SEB, C) Der p1, D) anti-CD28, E) Unstimulated IL-17 secretion, and F) % IFN- $\gamma$  producing CD4<sup>+</sup>CD69<sup>+</sup> cells. US: Unstimulated, HDM: House dust mite, SEB: Staphylococcal Enterotoxin B.**

### Impact of Disease Severity

When the disease severity was assessed with respect to the parameters we analyzed, there was no significant difference between the moderate versus severe AD except for higher CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cell percentage in the moderate group ( $p=0.026$ ).

### Correlations

There were strong correlations of serum IgE levels with the percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells ( $p<0.0001$ ,  $r=0.953$ ) and CD8<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells ( $p<0.0001$ ,  $r=0.918$ ); IL-10 with TNF- $\alpha$  ( $p<0.0001$ ,  $r=0.945$ ); and TNF- $\alpha$  with IFN- $\gamma$  ( $p<0.0001$ ,  $r=0.957$ ).

### Histopathological Studies

#### HDM Sensitive Patients Versus Those with Other Sensitivities

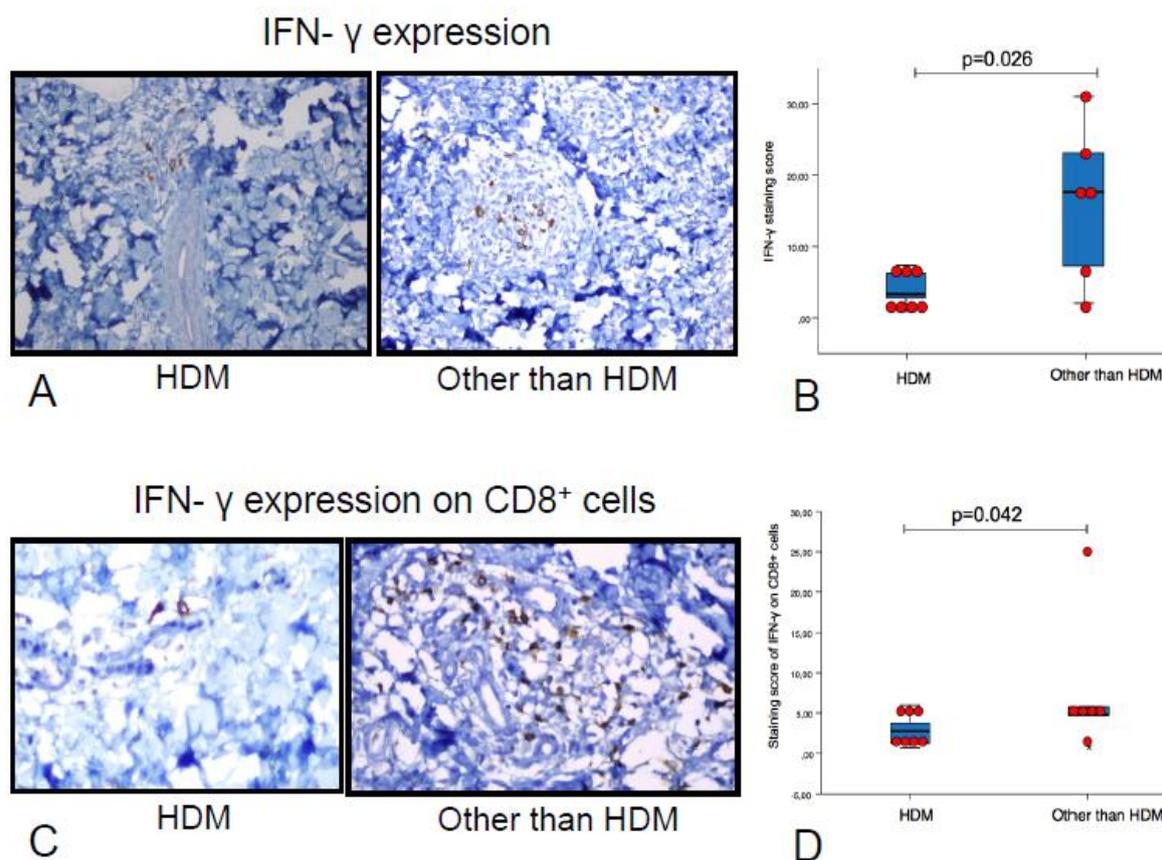
A remarkable feature of lesional sites in HDM

sensitized subjects was the lower expression of IFN- $\gamma$  ( $p=0.026$ ) as well as, CD8<sup>+</sup> T cells co-expressing IFN- $\gamma$  when compared to ones with other sensitivities ( $p=0.042$ ) (Figure 3).

In accordance with our findings from peripheral blood, disease severity did not show any significant impact on skin biopsy findings.

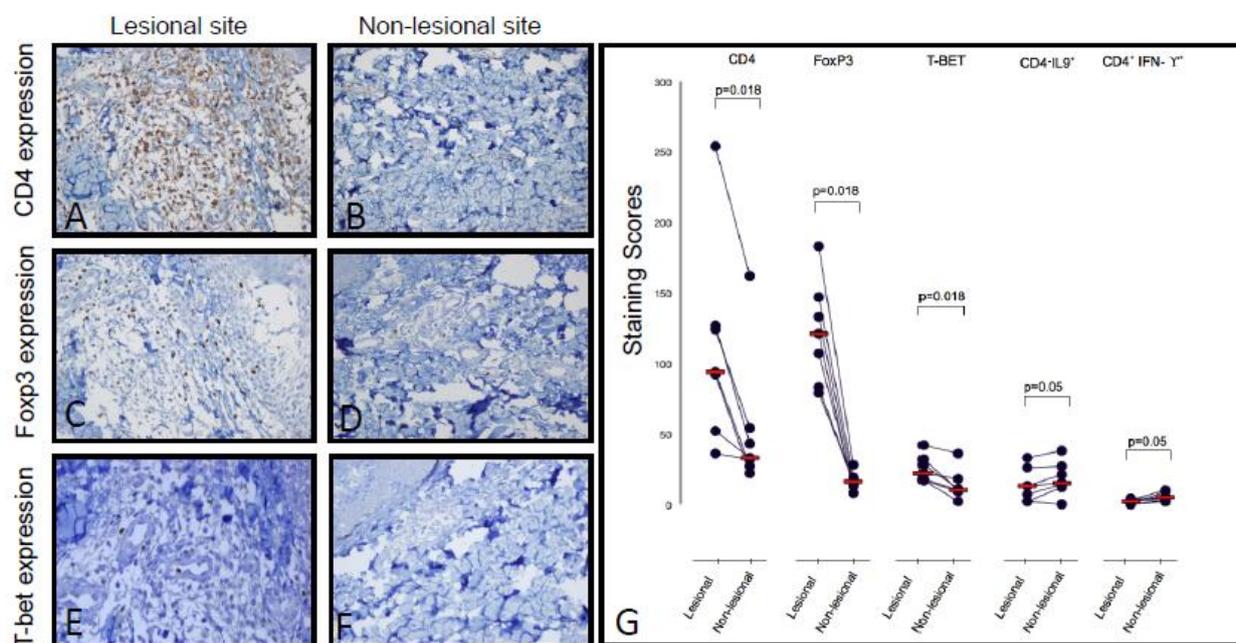
#### HDM Sensitive Patients: Pairwise Analysis

Biopsies from both the lesional and non-lesional skin were examined in 7 patients with HDM-allergic AD; in the lesional sites expression of CD4<sup>+</sup> T cells, Foxp3 and T-bet were increased ( $p=0.018$ ,  $p = 0.018$ ,  $p=0.018$ , respectively); CD4<sup>+</sup>IL-9<sup>+</sup> and CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> co-expressing cells were decreased ( $p=0.05$  and  $p=0.05$ , respectively) (Figure 4).



**Figure 3.** Expression of cytokines in lesional skin biopsies of HDM sensitized patients and other sensitizations; A) Representative expression of IFN- $\gamma$ , B) Median IFN- $\gamma$  staining scores, C) Representative expression of IFN- $\gamma$  on CD8<sup>+</sup> cells and D) Median IFN- $\gamma$  staining scores on CD8<sup>+</sup> cells. HDM: House dust mite.

## HDM Sensitivity in Atopic Dermatitis



**Figure 4.** Expressions in the lesional and non-lesional skin biopsies of HDM sensitized patients of A,B) CD4; C,D) Foxp3; E,F) T-Bet; and G) Median staining scores. HDM: House dust mite.

### DISCUSSION

The results of our study demonstrated that HDM sensitivity emerges as a critical factor in the subtyping of chronic AD. Interestingly, with regard to immunologic responses, patients with sensitivities other than HDM behaved similarly as the non-allergic ones. Although a characteristic feature of all AD patients was a high Th17 percentage, those with HDM sensitivity were distinguished with a dominant Th2 type cytokine production. Moreover, in the skin, the expression of IFN- $\gamma$  was found to be depressed in HDM sensitive patients when compared to ones with other sensitivities. Among the group with HDM sensitivity, comparison of lesional versus non-lesional sites revealed higher CD4<sup>+</sup>T cells and increased expression of Foxp3 and T bet while lower CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> co-expressing T cells were seen in the affected skin.

Atopic dermatitis is a highly heterogeneous disorder with regard to the clinical phenotype and natural history. While common mechanisms are shared by a majority of patients, distinct pathogenic pathways might be dominant in particular subgroups.<sup>11</sup> Two models have been proposed to explain the pathogenesis.<sup>12</sup> The “inside-out” (or immune-driven)

hypothesis suggests that underlying immune activation triggers epithelial barrier dysfunction, whereas the “outside-in” (or barrier-driven) hypothesis favors an intrinsic epidermal abnormality allowing the penetration of large immunogenic proteins and subsequent immune activation.<sup>12-15</sup> Supporting the roles of both models, several AD-associated genes have been identified including, those encoding factors of adaptive and innate immune system and genes for terminal differentiation of keratinocytes.<sup>16</sup> Thus, detailed knowledge about the pathophysiologic pathways underlying each subgroup is essential for therapeutic approaches.<sup>11</sup>

According to the findings of our study, the presence of unique allergen sensitivity, house dust mite, emerged as a fate-determining factor in AD. Only HDM sensitized group came out to be Th2 dominant which argues against the clinical dogma that defines AD as a uniform disease with respect to the Th1/Th2 and Th17 paradigm. Previous studies which shed light on the role of HDM in AD demonstrated that cysteine protease activity of Der f1 and Der p1 reduces the functions of tissue barriers, cleaves and/or interacts with various molecules including cell-surface molecules and endogenous protease inhibitors, modulates the

functions of various cells, and induces Th2-skewed sensitization.<sup>17-22</sup> Mite-derived serine proteases also have biological activities including the ability to disrupt tight junctions, stimulate airway and ocular conjunctival epithelial cells and cleave an endogenous protease inhibitor.<sup>23-25</sup> HDM-induced activation of NLRP3 inflammasome in epidermal keratinocytes and consequent release of IL-1 family proteins may be important in sustained inflammation.<sup>6</sup> T-cell activation induced cell death (AICD) characterizes the death of a fraction of antigen-activated T lymphocytes to avoid accumulation of harmful cytokine-releasing cells. Previous studies on asthma<sup>26</sup> and AD<sup>27</sup> suggested that the Th2 imbalance in atopy results from apoptosis of allergen-activated IFN- $\gamma$ -producing cells. Thus, the selective AICD of high IFN- $\gamma$ -secreting Th1 cells in peripheral blood skews the immune response toward Th2 cells in patients with AD.<sup>28</sup> The IgE-facilitated allergen uptake along with year-round exposure to the HDM antigen may partially explain the unique specificity of this allergen. It is intriguing that AD patients with sensitizations other than HDM showed closer immunologic responses to non-allergic ones rather than HDM-sensitized subjects.

In the current study, HDM allergic AD was distinguished from all other groups in that, IL-4 and IL-5 production was remarkably higher both in response to Der p1 or SEB, and had higher Th17 cell percentage. Another interesting finding in HDM allergic subjects was the low IL17 levels despite the high number of Th17 cells. The pathogenesis of AD has long been explained by the concept of altered Th1/Th2 driven immune responses, which was recently revised and extended to include Th17 cells.<sup>11,29</sup> Previous studies demonstrated increased numbers of Th17 cells in the peripheral blood and the skin lesions of patients with acute AD.<sup>9,30</sup> Furthermore, an increased percentage of 17 cells in the peripheral blood correlated with disease severity.<sup>9</sup> Meanwhile, in the chronic phase of AD, IL17 levels are usually low in the skin and IL-22 producing Th22 cells might play a role, too. In the current study, Th17 cells were increased in all AD groups compared to healthy controls. Previous studies suggested that although Th17 cells might exist in AD skin lesions, they might not be activated or might be inhibited by Th2 cells secreting IL-4 and IL-13.<sup>5,31</sup> This may partially explain why IL-17 levels were low despite the high numbers of Th17 cells in the HDM allergic AD group. In the skin biopsy samples of lesional sites, this

group had lower expression of IFN- $\gamma$  and fewer CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> co-expressing cells.

Previous studies demonstrated higher expression of Foxp3 in both acute and chronic AD lesions.<sup>32-34</sup> Although it may also be expressed by regulatory T cells, those Foxp3 transcription factors are not immunosuppressive, but display functions of effector Th2 cells after stimulation with *S. aureus* enterotoxins in vitro.<sup>35</sup> In our study, pairwise comparison of the lesional versus non-lesional skin of HDM-allergic patients revealed overexpression of FoxP3 and T-bet and infiltration of CD4<sup>+</sup> T cells along with paradoxically reduced IL-9 or IFN- co-expressing CD4<sup>+</sup> cells in the lesional sites. Moreover, HDM-allergic AD was distinguished from other sensitizations by their lower expression of IFN- $\gamma$  and sparse CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells in the skin. This finding is in line with the previous suggestion that impaired IFN- $\gamma$  signaling together with attenuated IFN- $\gamma$  responses in dendritic cells and their precursors might contribute to the Th2 bias in AD.<sup>36</sup>

The current classification of AD in terms of allergic versus non-allergic reactions appears suboptimal in terms of strategies for prevention and therapeutic implications. We, here, present the data suggesting to consider AD with HDM sensitivity as a distinct phenotype which in turn may enhance the response rate to targeted therapy such as allergen specific immunotherapy.<sup>37</sup> In HDM-allergic AD patients, at least in selected ones, it might be conceivable to balance Th1 and Th2 responses by use of recombinant IFN- $\gamma$ . Although studies including such patients have been carried out so far, a firm conclusion could not be drawn in this respect since the type of allergen sensitization was not always specified.<sup>38</sup> On the other hand, a limitation in our study is that these immunological differences between HDM-allergic patients and other AD patients could be associated with the duration of allergen exposure. While the burden of HDM allergen is throughout the whole year, other allergens may affect the skin transiently and allergen avoidance can probably alleviate AD patients' symptoms. Therefore, evaluation of the immunological findings at different time points with more patients could provide more precise information regarding the differences of sensitizations.

In conclusion, in the light of our findings, we strongly suggest to consider HDM sensitization as a distinct group in designing future studies related to AD.

## HDM Sensitivity in Atopic Dermatitis

Future studies investigating the differences in response to therapeutic agents including IFN- $\gamma$  in HDM sensitized and other AD patients may be helpful in the management of these patients.

### ACKNOWLEDGEMENTS

This study was supported by The Marmara University Scientific Research Committee (BAPKO no. TIP/SAG-A-080410-0065 and SAG-A-041213-0444) and Yeditepe University Scientific Research Committee (no. 2010 08 001).

### REFERENCES

1. Palmer CN, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee Sp, et al. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat Genet* 2006; 38(4):441-6.
2. Rodríguez-Orozco AR, Kanán-Cedeño EG, Guillén Martínez E, Campos Garibay MJ. Family functioning and illness perception of parents of children with atopic dermatitis, living without skin symptoms, but with psychosomatic symptoms. *Iran J Allergy Asthma Immunol* 2011; 10(1):61-5.
3. Leung DY, Boguniewicz M, Howell MD, Nomura I, Hamid QA. New insights into atopic dermatitis. *J Clin Invest* 2004; 113(5):651-7.
4. Boniface K, Bernard FX, Garcia M, Gurney AL, Lecron JC, Morel F. IL-22 inhibits epidermal differentiation and induces proinflammatory gene expression and migration of human keratinocytes. *J Immunol* 2005; 174(6):3695-702.
5. Guttman-Yassky E, Nograles KE, Krueger JG. Contrasting pathogenesis of atopic dermatitis and psoriasis--part II: immune cell subsets and therapeutic concepts. *J Allergy Clin Immunol* 2011; 127(6):1420-32.
6. Dai X, Sayama K, Tohyama M, Shirakata Y, Hanakawa Y, Tokumaru S, et al. Mite allergen is a danger signal for the skin via activation of inflammasome in keratinocytes. *J Allergy Clin Immunol* 2011; 127(3):806-14.
7. Nograles KE, Zaba LC, Shemer A, Fuentes-Duculan J, Cardinale I, Kikuchi T, et al. IL-22-producing "T22" T cells account for upregulated IL-22 in atopic dermatitis despite reduced IL-17-producing TH17 T cells. *J Allergy Clin Immunol* 2009; 123(6):1244-52.
8. Guttman-Yassky E, Lowes MA, Fuentes-Duculan J, Zaba LC, Cardinale I, Nograles KE, et al. Low expression of the IL-23/Th17 pathway in atopic dermatitis compared to psoriasis. *J Immunol* 2008; 181(10):7420-27.
9. Koga C, Kabashima K, Shiraiishi N, Kobayashi M, Tokura Y. Possible pathogenic role of Th17 cells for atopic dermatitis. *J Invest Dermatol* 2008; 128(11):2625-30.
10. Hanifin JM, Chan SC, Cheng JB, Tofte SJ, Henderson WR Jr, Kirby DS, Weiner ES. Type 4 phosphodiesterase inhibitors have clinical and in vitro anti-inflammatory effects in atopic dermatitis. *J Invest Dermatol* 1996;107(1):51-6.
11. Novak N, Leung DY. Advances in atopic dermatitis. *Curr Opin Immunol* 2011; 23(6):778-83.
12. Bieber T. Atopic dermatitis. *N Engl J Med* 2008; 358(14):1483-94.
13. Leung DY, Bieber T. Atopic dermatitis. *Lancet* 2003; 361(9352):151-60.
14. Elias PM, Schmuth M. Abnormal skin barrier in the etiopathogenesis of atopic dermatitis. *Curr Opin Allergy Clin Immunol* 2009; 9(5):437-46.
15. Elias PM, Steinhoff M. "Outside-to-inside" (and now back to "outside") pathogenic mechanisms in atopic dermatitis. *J Invest Dermatol* 2008; 128(5):1067-70.
16. Guttman-Yassky E, Nograles KE, Krueger JG. Contrasting pathogenesis of atopic dermatitis and psoriasis--part I: clinical and pathologic concepts. *J Allergy Clin Immunol* 2011; 127(5):1110-18.
17. Chapman MD, Wunschmann S, Pomes A. Proteases as Th2 adjuvants. *Curr Allergy Asthma Rep* 2007; 7(5):363-7.
18. Kikuchi Y, Takai T, Kuhara T, Ota M, Kato T, Hatanaka H, et al. Crucial commitment of proteolytic activity of a purified recombinant major house dust mite allergen Der p 1 to sensitization toward IgE and IgG responses. *J Immunol* 2006; 177(3):1609-17.
19. Gough L, Schulz O, Sewell HF, Shakib F. The cysteine protease activity of the major dust mite allergen Der p 1 selectively enhances the immunoglobulin E antibody response. *J Exp Med* 1999; 190(12):1897-902.
20. Wan H, Winton HL, Soeller C, Tovey ER, Gruenert DC, Thompson PJ, et al. Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J Clin Invest* 1999; 104(1):123-33.
21. Nakamura T, Hirasawa Y, Takai T, Mitsuishi K, Okuda M, Kato T, et al. Reduction of skin barrier function by proteolytic activity of a recombinant house dust mite major allergen Der p 1. *J Invest Dermatol* 2006; 126(12):2719-23.
22. Shakib F, Schulz O, Sewell H. A mite subversive:

- cleavage of CD23 and CD25 by Der p 1 enhances allergenicity. *Immunol Today* 1998; 19(7):313-6.
23. Wan H, Winton HL, Soeller C, Taylor GW, Gruenert DC, Thompson PJ, et al. The transmembrane protein occluding of epithelial tight junctions is a functional target for serine peptidases from faecal pellets of *Dermatophagoides pteronyssinus*. *Clin Exp Allergy* 2001; 31(2):279-94.
24. Sun G, Stacey MA, Schmidt M, Mori L, Mattoli S. Interaction of mite allergens Derp3 and Derp9 with protease-activated receptor-2 expressed by lung epithelial cells. *J Immunol* 2001; 167(2):1014-21.
25. Seto T, Takai T, Ebihara N, Matsuoka H, Wang XL, Ishii A, et al. SLPI prevents cytokine release in mite protease exposed conjunctival epithelial cells. *Biochem Biophys Res Commun* 2009; 379(3):681-5.
26. Cormican L, O'Sullivan S, Burke CM, Poulter LW. IFN-gamma but not IL-4 T cells of the asthmatic bronchial wall show increased incidence of apoptosis. *Clin Exp Allergy* 2001; 31(5):731-9.
27. Akkoc T, de Koning PJ, Ruckert B, Barlan I, Akdis M, Akdis CA. Increased activation-induced cell death of high IFN-gamma-producing T(H)1 cells as a mechanism of T(H)2 predominance in atopic diseases. *J Allergy Clin Immunol* 2008; 121(3):652-8.
28. Akdis M, Trautmann A, Klunker S, Daigle I, Kucuksezer UC, Deglmann W, et al. T helper (Th) 2 predominance in atopic diseases is due to preferential apoptosis of circulating memory/effector Th1 cells. *FASEB J* 2003; 17(9):1026-35.
29. Weidinger S, Novak N. Atopic dermatitis. *Lancet* 2015; 387(10023):1109-22.
30. Toda M, Leung DY, Molet S, Boguniewicz M, Taha R, Christodoulopoulos P, et al. Polarized in vivo expression of IL-11 and IL-17 between acute and chronic skin lesions. *J Allergy Clin Immunol* 2003; 111(4):875-81.
31. Eyerich K, Pennino D, Scarponi C, Foerster S, Nasorri F, Behrendt H, et al. IL-17 in atopic eczema: linking allergen-specific adaptive and microbial-triggered innate immune response. *J Allergy Clin Immunol* 2009; 123(1):59-66.
32. Caproni M, Antiga E, Torchia D, Volpi W, Barletta E, Gitti G, et al. FoxP3-expressing T regulatory cells in atopic dermatitis lesions. *Allergy Asthma Proc* 2007; 28(5):525-8.
33. Szegeedi A, Barath S, Nagy G, Szodoray P, Gal M, Sipka S, et al. Regulatory T cells in atopic dermatitis: epidermal dendritic cell clusters may contribute to their local expansion. *Br J Dermatol* 2009; 160(5):984-93.
34. Schnopp C, Rad R, Weidinger A, Weidinger S, Ring J, Eberlein B, et al. Fox-P3-positive regulatory T cells are present in the skin of generalized atopic eczema patients and are not particularly affected by medium-dose UVA1 therapy. *Photodermatol Photoimmunol Photomed* 2007; 23(2-3):81-5.
35. Lin YT, Wang CT, Chao PS, Lee JH, Wang LC, Yu HH, et al. Skin-homing CD4+ Foxp3+ T cells exert Th2-like function after staphylococcal superantigen stimulation in atopic dermatitis patients. *Clin Exp Allergy* 2011; 41(4):516-25.
36. Gros E, Petzold S, Maintz L, Bieber T, Novak N. Reduced IFN- $\gamma$  receptor expression and attenuated IFN- $\gamma$  response by dendritic cells in patients with atopic dermatitis. *J Allergy Clin Immunol* 2011; 128(5):1015-21.
37. Novak N, Bieber T, Hoffmann M, Fölster-Holst R, Homey B, Werfel T, et al. Efficacy and safety of subcutaneous allergen-specific immunotherapy with depigmented polymerized mite extract in atopic dermatitis. *J Allergy Clin Immunol* 2012; 130(4):925-31.
38. Boguniewicz M, Jaffe HS, Izu A, Sullivan MJ, York D, Geha RS, et al. Recombinant gamma interferon in treatment of patients with atopic dermatitis and elevated IgE levels. *Am J Med* 1990; 88(4):365-70.