

# *Staphylococcus aureus* skin colonization in atopic dermatitis children is associated with decreased IFN- $\gamma$ production by peripheral blood CD4<sup>+</sup> and CD8<sup>+</sup> T cells

Machura E, Mazur B, Golemiec E, Pindel M, Halkiewicz F. *Staphylococcus aureus* skin colonization in atopic dermatitis children is associated with decreased IFN- $\gamma$  production by peripheral blood CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

Pediatr Allergy Immunol 2007.

© 2007 The Authors

Journal compilation © 2007 Blackwell Munksgaard

Atopic dermatitis (AD) is a chronic inflammatory skin disorder, which is associated with an increased expression of Th2 cytokines with concomitant decrease in IFN- $\gamma$  production by circulating CD4<sup>+</sup> and CD8<sup>+</sup> T cells. The skin of patients with AD is often colonized by *Staphylococcus aureus*, which may reflect in changes in immunological parameters. The aim of the study was flow cytometric measurement of some peripheral blood lymphocyte subsets expressing naive/memory marker (RA/RO) and activation marker (CD25) as well as intracellular production of IFN- $\gamma$  by peripheral blood CD4<sup>+</sup> and CD8<sup>+</sup> T cells from varied severity AD children and determine the impact of *S. aureus* skin colonization on cytokines profiles. There was a significant increase in the percentage of CD4<sup>+</sup> and CD8<sup>+</sup> T cells producing IL-4 and IL-13 and decrease in the percentage of CD4<sup>+</sup> and CD8<sup>+</sup> T cells producing IFN- $\gamma$  upon *in vitro* stimulation with phorbol 12-myristate 13-acetate and ionomycin in children with AD compared to healthy ones. The absolute number of CD4<sup>+</sup> and CD8<sup>+</sup> T cells expressing memory marker CD45RO was elevated as compared with controls. The severity of AD was positively correlated with the percentage of lymphocyte subsets: CD45RO<sup>+</sup>, CD4<sup>+</sup>CD45RO<sup>+</sup>, and the percentage of CD3<sup>+</sup> and CD4<sup>+</sup> expressing CD25 as well as the number of *S. aureus* on the skin. In conclusion, both CD4<sup>+</sup> and CD8<sup>+</sup> memory T cells are involved in the immunopathogenesis of AD. *S. aureus* skin colonization is related with disease severity and changes in expression of CD45RO and CD25 on T cells. A decrease in the percentage of CD4<sup>+</sup> and CD8<sup>+</sup> T cells producing IFN- $\gamma$  in AD children may explain propensity for skin infection.

**Edyta Machura<sup>1</sup>, Bogdan Mazur<sup>2</sup>, Ewa Golemiec<sup>1</sup>, Mariola Pindel<sup>1</sup> and Franciszek Halkiewicz<sup>1</sup>**

<sup>1</sup>Chair and Department of Pediatric Gastroenterology, Allergology and Developmental Disorders,

<sup>2</sup>Department of Microbiology and Immunology, Medical University of Silesia, Zabrze, Poland

Key words: lymphocyte T; atopic dermatitis; *Staphylococcus aureus*; cytokine; children

Bogdan Mazur PhD, MD, Department of Microbiology and Immunology, Medical University of Silesia, ul. 3-go Maja 13/15, 41-800 Zabrze, Poland  
Tel.: +48 32 3704313  
Fax: +48 32 2736075  
E-mail: bmazur@slam.katowice.pl

Accepted 28 March 2007

Atopic dermatitis (AD) is one of the most common diseases in childhood resulting from both genetic and environmental factors. It is well known that AD is associated with elevated IgE and eosinophilia, the hallmark of Th2 (IL-4, IL-5, IL-13) cytokine response. Increased expressions of Th2 cytokines at the site of allergic inflammation and by circulating allergen-specific

T cells is documented in AD in several studies (1, 2).

Numerous studies pointed to the role of activated CD4<sup>+</sup> T cells in AD and other atopic disease as main producer of Th2 cytokines. Besides CD4<sup>+</sup> T lymphocytes, CD8<sup>+</sup> T cells are likely to play an important role in the pathogenesis of AD. The production of proallergic

cytokines was initially ascribed to CD4<sup>+</sup> cells. There is now broader evidence that CD8<sup>+</sup> T cells are also able to secrete Th2 cytokines (3). The importance of CD8<sup>+</sup> T cells in the pathogenesis and exacerbation of allergic diseases has been recently highlighted by identification of allergen-specific CD8<sup>+</sup> T cells (4).

Patients with AD exhibit defects in innate and acquired immune responses leading to a high susceptibility to certain cutaneous bacterial, fungal and viral infections. In particular, the skin of patients with AD is frequently colonized by *Staphylococcus aureus*, which is found in over 90% of patients (5, 6). A contributing factor for increased *S. aureus* skin infection may be the significantly decreased expression of antimicrobial peptides (AMPs), such as human  $\beta$ -defensin (hBD)-2, LL-37 (a cathelicidin), hBD-3 in AD skin lesion (5–8). Toxins produced by *S. aureus* exacerbate disease activity by both the induction of toxin-specific IgE, augmentation of allergen-specific IgE synthesis and the activation of various cell types including Th-2 cells, eosinophils and keratinocytes (3, 5, 9).

In the study, we wanted to address the question of whether or not differences exist in some immunological parameters between AD and healthy controls particularly in terms of the expression of naïve/memory marker on peripheral blood CD4<sup>+</sup>CD8<sup>+</sup> T cells and cytokine production by those cells. Presuming that any differences exist, we also raised the question whether they are correlated with *S. aureus* skin colonization.

## Materials and methods

Forty children (mean age  $9 \pm 0.8$  range from 2 to 18) suffering from mild to severe AD were enrolled into the study. AD was diagnosed according to the criteria described by Hanifin and Rajka (10). The severity of dermatitis was estimated according to the SCORAD index (ranging from 0 to 103 points) (11). Based on total score, nine children were classified as mild (<25 points), 15 children as moderate (25–50 points) and 16 children as severe AD (>50 points). Thirteen children had concomitant asthma symptoms; nine out of them were taking ICS 100–200  $\mu$ g per day. The control group consisted of 56 healthy children (mean age  $8.4 \pm 0.5$ , range from 2 to 17) with a negative history of allergic disease, the normal level of total serum IgE and negative results of skin prick test.

The study was approved by the Ethics Committee of Medical University of Silesia, Katowice. Written informed consent was obtained from the parents of all patients.

## Methods

Venous blood samples were obtained from each subject and serum IgE and lymphocyte populations were assessed. The serum concentration of total IgE was measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Allergopharma; detection threshold – 1.0 IU/ml)

### Lymphocyte subsets in peripheral blood

Lymphocyte populations were analysed by multi-parameter flow cytometry with 3-color analyses. Phenotypes of T-cell population were obtained with PerCP anti-CD3 antibody plus combinations of FITC and PE-labelled antibodies specific for CD4, CD8, CD25, CD45RA and CD45RO. Cells were stained with appropriate isotype control antibodies and non-specific staining was not observed. Antibodies used for flow cytometry were purchased from Becton Dickinson (San Jose, CA, USA). After staining, analysis was performed on a FACScan (Becton Dickinson) equipped with an argon laser tuned to 488 nm. Data were acquired and analysed with Cell Quest software (Becton Dickinson). The proportion of lymphocytes stained with each monoclonal antibody was converted to the absolute per microlitre by multiplying the absolute number of lymphocytes per microliter derived from complete blood count.

### Intracellular cytokine staining

In 19 children with AD (mean age  $8.4 \pm 0.8$ , mean SCORAD index  $50.3 \pm 10.5$ ) and 16 healthy controls (mean age  $9.2 \pm 0.7$ ) intracellular cytokine staining before and after polyclonal stimulation was assessed.

Heparinized whole blood was diluted with RPMI (BioWhittaker, Walkersville, MD, USA) and calf serum and then after addition of 25  $\mu$ l (25 ng/ml) of phorbol 12-myristate 13-acetate (PMA; Sigma, St Louis, MO, USA), 20  $\mu$ l (1  $\mu$ g/ml) of ionomycin (Sigma) and 10  $\mu$ l (2.5  $\mu$ M) of monensin (Sigma) incubated in a water bath for 4 h at 37°C in tightly capped tubes. Stimulated whole blood was distributed in 100  $\mu$ l aliquots into tubes containing 20  $\mu$ l anti-human CD3-FITC and CD4-PerCp (Becton Dickinson), vortexed and incubated for 20 min at room temperature in the dark. To fix cells 100  $\mu$ l of IntraPrep Reagent 1 (DAKO, Glostrup, Denmark) was added to the samples followed by vortexing and incubation for 15 min at room temperature in the dark, and afterwards washing with 2 ml of cell-wash solution. Simultaneous

permeabilization of the white cells and lyses of the red cells was then carried out by the adding of 100  $\mu$ l IntraPrep Reagent 2 (DAKO) and incubation. Intracellular staining procedure included the addition of 20  $\mu$ l of anti-IL-2, IL-4, IL-10, IL-13, IFN- $\gamma$ , IFN- $\alpha$  monoclonal antibodies or isotope control mouse antibodies conjugated with PE (Coulter Immunotech, Fullerton, CA, USA). The flow cytometric assessment with FASCscan flow cytometry (Becton Dickinson) was performed on the same day. Acquisition was gated on CD3<sup>+</sup> CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> cells and then percentage of CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> cells producing IL-2, IL-4, IL-10, IL-13, IFN- $\gamma$  and TNF $\alpha$  was determined on dot plots. Quadrants were set based upon isotope control. The acquisition was performed both on samples stimulated with PMA and ionomycin, and on non-stimulated samples, in order to estimate the residual intracellular expression of the cytokines. Activation of the cells was confirmed by estimation of the CD69 expression, which was over 95% in all samples. Acquisition and analysis were performed with Cell Quest software package (Becton Dickinson).

#### Isolation, identification and quantification of *Staphylococcus aureus*

In all, the children with AD specimens were obtained from lesional and non-lesional areas of the skin, whereas in controls only from non-lesional. Briefly, the sterile glass cylinders were placed on the skin surface and 2 ml of sterile 0.9% NaCl solution was applied, skin surface was rubbed with glass rod and fluid was aspirated. To quantify the number of *S. aureus* 1  $\mu$ l of fluid was plated onto non-selective 5% sheep blood agar plates (Columbia blood agar; Bio-Merieux, Marcy l'Etoile, France) and selective mannitol salt agar and incubated for 24 h at 37°C. A positive identification was confirmed with coagulase activity assay and SLIDEX STAPH plus (BioMerieux), all performing according to the manufacture's recommendations. The number of bacteria was determined by colony forming units (cfu). Nasal, throat smears, using sterile cotton swabs were taken from all the children.

#### Statistical analysis

Statistical analyses were performed using software package (STATISTICA, version 3.0; StatSoft, Tulsa, OK, USA) and data presented as mean value  $\pm$  s.e. Mann-Whitney *U*-test was used for comparisons between groups. Correlations were

Table 1. Demographic and clinical characteristics of patients with atopic dermatitis (AD) and healthy children

|                                                      | AD                     | Healthy               |
|------------------------------------------------------|------------------------|-----------------------|
| No.                                                  | 40                     | 56                    |
| Age (years; mean $\pm$ s.e.)                         | 9 $\pm$ 0.8            | 8.4 $\pm$ 0.5         |
| Sex                                                  | M: 16; F: 24           | M: 22; F: 34          |
| Mean duration of AD (years; mean $\pm$ s.e.)         | 8 $\pm$ 0.8            |                       |
| SCORAD                                               | 40 $\pm$ 3.6           |                       |
| Concomitant disorders                                |                        |                       |
| Asthma                                               | 13 (32.5%)             |                       |
| Rhinitis                                             | 11(27.5%)              |                       |
| Presence of <i>Staphylococcus aureus</i> on the skin | 36 (97.5%)             | 0                     |
| In throat                                            | 16 (40%)               | 11(19.6%)             |
| In nose                                              | 14 (35%)               | 15(26.7)%             |
| Serum total IgE level                                | 719.95 $\pm$ 154 IU/ml | 28.2 $\pm$ 2.23 IU/ml |
| Positive skin prick tests                            | 40 (100%)              |                       |
| Dermatophagoides pteronyssinus                       | 25 (62.5%)             |                       |
| Dermatophagoides farinae                             | 28 (70%)               |                       |
| Grass pollens                                        | 30 (75%)               |                       |
| Tree pollens                                         | 23 (57%)               |                       |
| Weed pollens                                         | 21 (52.5%)             |                       |
| Moulds                                               | 6 (15%)                |                       |
| Feather                                              | 5 (12.5%)              |                       |
| Cat                                                  | 17 (42.5%)             |                       |
| Dog                                                  | 6 (15%)                |                       |
| Milk                                                 | 7 (17.5%)              |                       |
| Eggs                                                 | 8 (20%)                |                       |
| Fish                                                 | 5 (12.5%)              |                       |
| Nuts                                                 | 9 (22.5%)              |                       |

calculated using the Spearman rank test. Values of  $p < 0.05$  were considered as statistically significant.

#### Results

The demographic and background characteristics of study subjects are shown in Table 1.

Difference in mean age between the two groups was not statistically significant. Absolute number of lymphocytes in AD was higher (3208.27  $\pm$  255) than in controls (2643.41  $\pm$  96), but it did not reach a statistical significance.

#### Atopic markers in AD patients and control group

The percentage of eosinophils (AD: 8.7  $\pm$  1.14, controls: 2.24  $\pm$  0.18) and the counts of eosinophils (AD: 305  $\pm$  13.15, controls: 135  $\pm$  6.16) were significantly higher in AD than in the healthy control group ( $p < 0.00002$ ). Total serum IgE levels was significantly higher in AD patients (719.9  $\pm$  154 UI/ml) than in controls (28.2  $\pm$  2.23 UI/ml;  $p < 0.00002$ ).

Table 2. Mean percentage and numbers of lymphocyte subsets in the peripheral blood in atopic dermatitis (AD) children and healthy controls

| T-cells phenotype                    | AD (% , no.)<br>(n = 40)                   | Healthy (% , no.)<br>(n = 56) |
|--------------------------------------|--------------------------------------------|-------------------------------|
| CD3 <sup>+</sup>                     | 67 ± 0.9<br>2156.1 ± 175                   | 66.8 ± 0.89<br>1768 ± 68.1    |
| CD4 <sup>+</sup>                     | 40.7 ± 1.1<br>1287 ± 93.9*                 | 38.9 ± 0.99<br>1028 ± 46.85   |
| CD8 <sup>+</sup>                     | 23.2 ± 0.8**<br>758 ± 84.2.                | 27.3 ± 0.84<br>720 ± 31.5     |
| CD45RA <sup>+</sup>                  | 72 ± 1.8<br>2233.7 ± 215                   | 74 ± 1.2<br>1842.86 ± 114.9   |
| CD4 <sup>+</sup> CD45RA <sup>+</sup> | 27.8 ± 1.3<br>870.2 ± 81.1                 | 27.48 ± 0.99<br>737.7 ± 43.2  |
| CD8 <sup>+</sup> CD45RA <sup>+</sup> | 19 ± 0.78**<br>621 ± 75.8                  | 21.6 ± 0.64<br>572 ± 26.5     |
| CD45RO <sup>+</sup>                  | 38.5 ± 1.85#<br>1161.9 ± 76.1##            | 32.8 ± 1.23<br>803 ± 51.9     |
| CD4 <sup>+</sup> CD45RO <sup>+</sup> | 21.18 ± 1.0<br>638.8 ± 48.53 <sup>Φ</sup>  | 20. ± 0.53<br>522 ± 21.9      |
| CD8 <sup>+</sup> CD45RO <sup>+</sup> | 9.8 ± 0.83<br>315.12 ± 31.55 <sup>ΦΦ</sup> | 7.8 ± 0.33<br>200.4 ± 11.9    |
| CD3 <sup>+</sup> CD25 <sup>+</sup>   | 5.28 ± 0.5<br>158 ± 15.4                   | 7.27 ± 0.44<br>196.53 ± 33.45 |
| CD4 <sup>+</sup> CD25 <sup>+</sup>   | 4.9 ± 0.3<br>148 ± 9.8 <sup>§</sup>        | 4.5 ± 0.44<br>110 ± 12.52     |
| CD4 <sup>+</sup> /CD8 <sup>+</sup>   | 1.89 ± 0.12 <sup>§</sup>                   | 1.51 ± 0.08                   |

Data as shown as mean ± s.e.

\*p < 0.03, \*\*p < 0.008, #p < 0.005, ##p < 0.006, <sup>Φ</sup>p < 0.05, <sup>ΦΦ</sup>p < 0.002, <sup>§</sup>p < 0.01, <sup>§§</sup>p < 0.004.

Comparison of lymphocyte subsets between AD patients and control group

The mean percentage ± s.e. for all examined subpopulations are shown in Table 2. The percentage of CD3<sup>+</sup> and CD4<sup>+</sup> did not differ between two groups but the number of CD4<sup>+</sup> was significantly higher than in the healthy controls (p < 0.03). The percentage but not the number of CD8<sup>+</sup> T cells was significantly lower in AD children than in the healthy controls (p < 0.008). Also the CD4<sup>+</sup>/CD8<sup>+</sup> ratio was significantly higher in AD compared with controls (p < 0.004).

Comparison of expression of naive/memory (RA/RO) marker on lymphocyte subsets between AD patients and control group

The percentage but not the number of CD8<sup>+</sup>CD45RA<sup>+</sup> was significantly lower in AD than in the healthy controls (p < 0.008). Moreover, the percentage of CD45RO<sup>+</sup> (p < 0.005) and the number of CD45RO<sup>+</sup> lymphocytes (p < 0.006) was significantly higher in the children with AD than in the healthy controls. The percentage of CD4<sup>+</sup>CD45RO<sup>+</sup> (p = 0.5) and CD8<sup>+</sup>CD45RO<sup>+</sup> T cells (p < 0.07) in AD did not differ compared with healthy children.

The number of CD4<sup>+</sup>CD45RO<sup>+</sup> (p < 0.05) and CD8<sup>+</sup>CD45RO<sup>+</sup> (p < 0.002) T cells was significantly higher in AD children than in healthy children.

Comparison of expression of CD25 on CD3<sup>+</sup> and CD4<sup>+</sup> T cell between AD patients and control group

The percentage of CD3<sup>+</sup> and CD4<sup>+</sup> T cells expressing activation markers CD25 did not differ between AD and controls, but the absolute number of CD4<sup>+</sup>CD25<sup>+</sup> was significantly higher than in controls (p < 0.01).

Frequencies of CD4<sup>+</sup> and CD8<sup>+</sup> T cell spontaneously producing IL-2, IL-4, IL-10, IL-13, IFN-γ and TNF-α in AD patients and control group

Frequencies of CD4<sup>+</sup> T cell staining positively for IL-2, IL-4, IL-10, IL-13, IFN-γ and TNF-α in unstimulated whole blood were similar in atopic children and in healthy controls. The mean cytokine production values of IL-2, IL-4, IL-10, IL-13 IFN-γ and TNF-α in the CD4<sup>+</sup> subpopulation in patients with AD were: 2.74 ± 0.26, 3.0 ± 0.33, 3.3 ± 0.28, 3.68 ± 0.28, 2.42 ± 0.22, 2.58 ± 0.34 respectively; in the control group, they were: 3.11 ± 0.31, 2.83 ± 0.23, 4.58 ± 0.71, 3.33 ± 0.22, 2.50 ± 0.26, 3.0 ± 0.35. Frequencies of CD8<sup>+</sup> T cell spontaneously producing IL-4 tended to be higher than in the healthy controls (p < 0.051). The mean cytokine production values of IL-2, IL-4, IFN-γ, IL-10, IL-13 and TNF-α in the CD8<sup>+</sup> subpopulation in patients with AD were: 3.95 ± 0.48, 3.63 ± 0.31, 3.42 ± 0.43, 4.05 ± 0.38, 3.79 ± 0.25, 3.32 ± 0.32, while in controls, they were: 3.58 ± 0.36, 2.75 ± 0.13, 4.0 ± 0.48, 3.42 ± 0.19, 3.08 ± 0.4, 3.0 ± 0.28.

Frequencies of CD4<sup>+</sup> and CD8<sup>+</sup> T cell producing IL-2, IL-4, IL-10, IL-13, IFN-γ and TNF-α after *in vitro* stimulation with PMA + ionomycin in AD patients and control group

Mean value of frequencies of CD4<sup>+</sup> and CD8<sup>+</sup> T cell producing IL-2, IL-4, IL-10, IL-13, IFN-γ and TNF-α after *in vitro* stimulation with PMA + ionomycin was shown in Table 3. Frequencies of CD4<sup>+</sup> and CD8<sup>+</sup> T cells producing IL-4, IL-13 after *in vitro* stimulation with PMA + ionomycin were significantly higher in AD patients than in the control group (IL-4: p < 0.00001, p < 0.0003, for CD4<sup>+</sup> and CD8<sup>+</sup> T cells, respectively, IL-13: p < 0.00001 for CD4<sup>+</sup> and CD8<sup>+</sup> T cells). In contrast, frequencies of CD4<sup>+</sup> and CD8<sup>+</sup> T cells producing IFN-γ in AD children were significantly

Table 3. Percentage of CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells producing IL-2, IL-4, IL-10, IL-13, INF- $\gamma$  and TNF- $\alpha$  after *in vitro* activation with phorbol 12-myristate-13-acetate and ionomycin. Differences between children with atopic dermatitis (AD) and control subjects were found

|                 | CD4 <sup>+</sup> /IL-2 <sup>+</sup> | CD4 <sup>+</sup> /IL-4 <sup>+</sup> | CD4 <sup>+</sup> /IL-10 <sup>+</sup> | CD4 <sup>+</sup> /IL-13 <sup>+</sup> | CD4 <sup>+</sup> /INF- $\gamma$ <sup>+</sup> | CD4 <sup>+</sup> /TNF- $\alpha$ <sup>+</sup> |
|-----------------|-------------------------------------|-------------------------------------|--------------------------------------|--------------------------------------|----------------------------------------------|----------------------------------------------|
| AD n = 19       | 8.05 $\pm$ 0.6                      | 8.89 $\pm$ 0.21*                    | 7.42 $\pm$ 0.55                      | 12.89 $\pm$ 0.74 *                   | 6.84 $\pm$ 0.41*                             | 7.21 $\pm$ 0.61                              |
| Controls n = 16 | 9.11 $\pm$ 0.56                     | 6.16 $\pm$ 0.39                     | 9.25 $\pm$ 0.69                      | 7.83 $\pm$ 0.49                      | 11.67 $\pm$ 0.58                             | 7.22 $\pm$ 0.42                              |
|                 | CD8 <sup>+</sup> /IL-2 <sup>+</sup> | CD8 <sup>+</sup> /IL-4 <sup>+</sup> | CD8 <sup>+</sup> /IL-10 <sup>+</sup> | CD8 <sup>+</sup> /IL-13 <sup>+</sup> | CD8 <sup>+</sup> /INF- $\gamma$ <sup>+</sup> | CD8 <sup>+</sup> /TNF- $\alpha$ <sup>+</sup> |
| AD n = 19       | 8.21 $\pm$ 0.46                     | 8.68 $\pm$ 0.34**                   | 7.95 $\pm$ 0.49                      | 12.68 $\pm$ 0.33*                    | 6.63 $\pm$ 0.32 <sup>s</sup>                 | 6.58 $\pm$ 0.58                              |
| Controls n = 16 | 8.08 $\pm$ 0.47                     | 6.08 $\pm$ 0.4                      | 8.67 $\pm$ 0.63                      | 7.58 $\pm$ 0.53                      | 8.5 $\pm$ 0.38                               | 6.83 $\pm$ 0.44                              |

Data as shown as mean  $\pm$  s.e.

\*p < 0.00001, \*\*p < 0.0003, <sup>s</sup>p < 0.002.

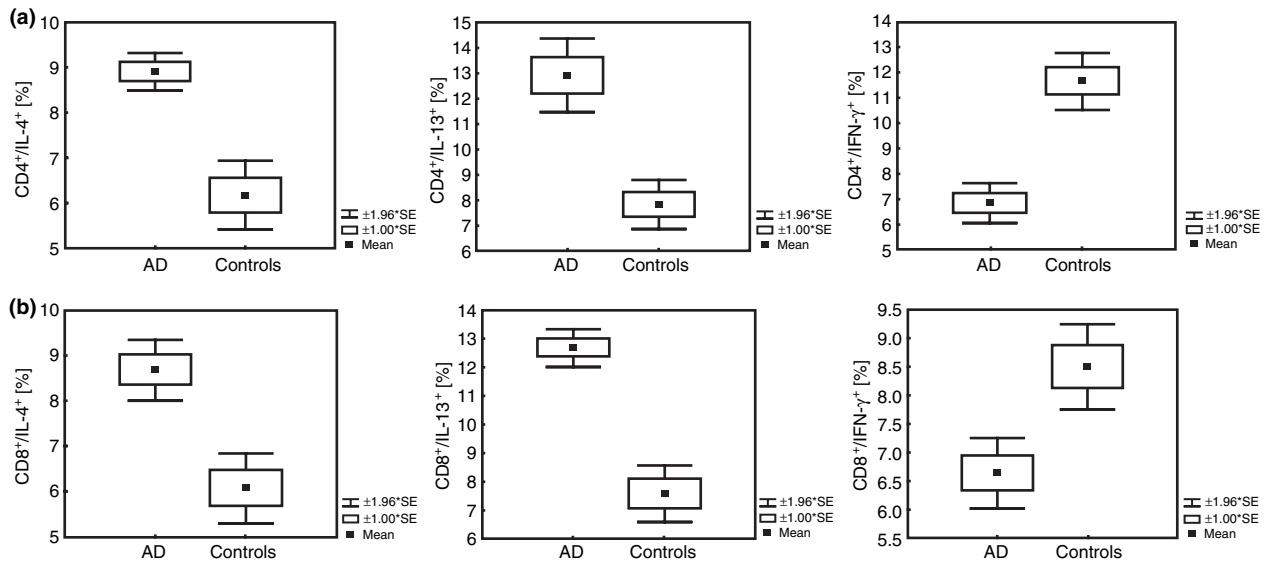


Fig. 1. Percentage of CD4<sup>+</sup>IL-4<sup>+</sup>, CD4<sup>+</sup>IL-13<sup>+</sup>, CD4<sup>+</sup>INF- $\gamma$ <sup>+</sup> and CD8<sup>+</sup>IL-4<sup>+</sup>, CD8<sup>+</sup>IL-13<sup>+</sup>, CD8<sup>+</sup>INF- $\gamma$ <sup>+</sup> in PMA and ionomycin stimulated peripheral blood from children with AD (n = 19) and healthy children (n = 16). (a) The percentage of CD4<sup>+</sup>IL-4<sup>+</sup>, CD4<sup>+</sup>IL-13<sup>+</sup> in AD children was significantly higher (p < 0.00001) and the percentage of CD4<sup>+</sup>INF- $\gamma$ <sup>+</sup> was significantly lower than in healthy children (p < 0.00001). (b) The percentage of CD8<sup>+</sup>IL-4<sup>+</sup>, CD8<sup>+</sup>IL-13<sup>+</sup> was significantly higher in AD children (p < 0.0003, p < 0.00001 respectively) and the percentage of CD8<sup>+</sup>INF- $\gamma$ <sup>+</sup> was significantly lower than in healthy children (p < 0.002). Other cytokines (IL-2, IL-10, TNF- $\alpha$ ) were similar in the two groups. The Mann-Whitney U-test was used to determine significant differences between two groups.

reduced compared to normal subjects (p < 0.00001, p < 0.002 for CD4<sup>+</sup> and CD8<sup>+</sup> T cells respectively) (Fig. 1). Frequencies of CD4<sup>+</sup> and CD8<sup>+</sup> T cells staining positively for IL-2, IL-10, TNF- $\alpha$  and IFN- $\gamma$  after stimulation with PMA and ionomycin were similar in both groups.

Relationships between serum IgE levels and immunological parameters

Significant positive correlations were observed between the percentage of CD4<sup>+</sup>CD45RO<sup>+</sup> (R = 0.32, p < 0.04) and frequencies of unstimulated CD4<sup>+</sup> staining positively for IL-13 and stimulated CD4<sup>+</sup> staining positively for IL-10 (R = 0.57; p < 0.01, R = 0.49; p < 0.03 respectively) and serum IgE. We

found that there was a trend towards a correlation between serum IgE and stimulated CD4<sup>+</sup> staining positively for IL-13, but it did not reach a statistical significance (R = 0.45; p < 0.06). In addition, there was a significant inverse correlation between total serum IgE levels and the percentage of CD45RA<sup>+</sup> lymphocytes and absolute number of CD4<sup>+</sup> T cell expressing naive marker RA (R = -0.34; p < 0.03, R = -0.33; p < 0.04 respectively).

Relationship between *Staphylococcus aureus* skin colonization, severity of AD and immunological parameters

*Staphylococcus aureus* was present on the skin in 36 (97.5%) out of 40 children. None of the children in the control subjects showed the presence of *S. aureus* on the skin. The number

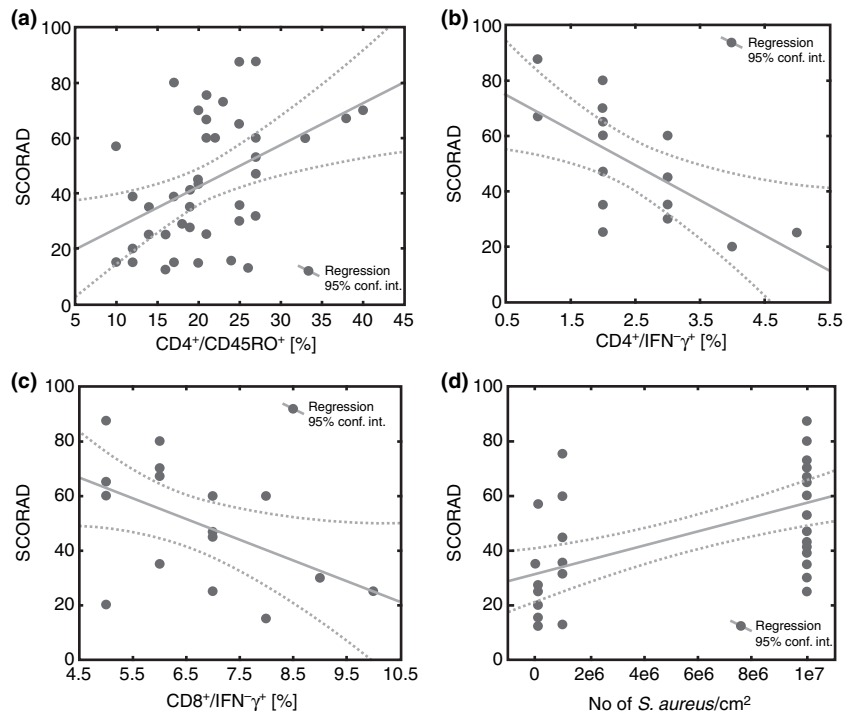


Fig. 2. (a) Correlation between severity of AD measured by the *SCORAD* index and the percentage of circulating  $CD4^+CD45RO^+$  T cells ( $R = 0.46$ ;  $p < 0.002$ ). (b) Correlation between severity of AD and the percentage of  $CD4^+$  spontaneously expressing  $IFN-\gamma^+$  ( $R = -0.57$ ;  $p < 0.009$ ). (c) Correlation between severity of AD and the percentage of  $CD8^+/IFN-\gamma^+$  in PMA and ionomycin stimulated peripheral blood ( $R = -0.47$ ;  $p < 0.03$ ). (d) Correlation between severity of AD and density of *Staphylococcus aureus* ( $R = 0.6$ ;  $p < 0.00009$ ).

of *S. aureus* on the skin was significantly correlated with serum IgE levels ( $R = 0.37$ ,  $p = 0.02$ ) and the percentage of lymphocyte subsets expressing following markers  $CD45RO^+$  ( $R = 0.48$ ,  $p = 0.002$ ),  $CD4^+CD45RO^+$  ( $R = 0.39$ ,  $p = 0.01$ ),  $CD3^+CD25^+$  ( $R = 0.54$ ,  $p = 0.0005$ ) and  $CD4^+CD25^+$  ( $R = 0.53$ ,  $p = 0.0007$ ). There was an inverse correlation between the number of *S. aureus* on the skin and the percentage of lymphocytes expressing naive marker, e.g.  $CD45RA^+$  ( $R = -0.35$ ,  $p < 0.03$ ) and frequencies of unstimulated  $CD4^+$  staining positively for  $IFN-\gamma$  ( $R = -0.76$ ,  $p < 0.0002$ ). On the other hand, *SCORAD* index was positively correlated with serum IgE level ( $R = 0.52$ ;  $p < 0.0005$ ) as well with the percentage of eosinophils ( $R = 0.35$ ;  $p < 0.047$ ) and the percentage of  $CD45RO^+$  ( $R = 0.47$ ;  $p < 0.001$ ),  $CD4^+CD45RO^+$  ( $R = 0.46$ ;  $p < 0.002$ ),  $CD3^+CD25^+$  ( $R = 0.5$ ;  $p < 0.0009$ ),  $CD4^+CD25^+$  T cells ( $R = 0.49$ ;  $p < 0.001$ ) and the number of *S. aureus* on the skin ( $R = 0.6$ ,  $p < 0.00009$ ). There was a significant inverse correlation between *SCORAD* and the frequencies of  $CD4^+$  spontaneously producing  $IFN-\gamma$  and  $CD8^+$  T cells positively staining for  $IFN-\gamma$  in response to PMA + ionomycin ( $R = -0.57$ ;

$p < 0.009$ ,  $R = -0.47$ ;  $p < 0.03$  respectively) (Fig. 2).

## Discussion

In this study, we demonstrated, similarly to other reports, that elevated serum IgE levels and the percentage of eosinophils in AD children are correlated with disease severity (12). Characterizing T-cell subsets, we could show some significant differences with respect to the general proportion of examined  $CD3^+$ ,  $CD4^+$ ,  $CD8^+$  T cells as well and the naive/memory compartment in the peripheral blood between the group with AD and the healthy non-atopic children. We showed the predominance of  $CD4^+$  T lymphocyte in peripheral blood in AD children as patients with AD had decreased percentage of  $CD8^+$  T-cell subset and significantly increased  $CD4^+/CD8^+$  ratio and number of circulating  $CD4^+$  T cells as compared with healthy controls. Despite decreased percentage of  $CD8^+$  T cells, the absolute number of this lymphocyte population did not differ between AD and controls. The predominance of  $CD4^+$  T lymphocytes in peripheral blood as well as in the cellular dermal infiltrate in AD was shown in several studies (3, 13).

Histology of skin lesions, as studied by Picker et al. (14) revealed that, the great majority of T cell in cellular infiltrate of AD skin lesions is of the CD45RO<sup>+</sup> (memory) phenotype along with CLA (cutaneous leukocyte antigen) expression. Imbalance of memory CD45RO<sup>+</sup> T cells in peripheral blood of patients with allergic disease has been reported, but results are inconsistent (13, 15, 16). In the present study, the percentage and the number of circulating CD45RO<sup>+</sup> T cells were significantly higher in AD children, but the proportion of CD4<sup>+</sup>CD45RO<sup>+</sup> cells did not differ between AD patients and controls, similarly to other reports (17). In addition, the percentage of CD8<sup>+</sup> cell expressing naive marker (RA) was significantly decreased, in contrast – expressing memory marker (RO) tended to be higher in AD children. Moreover, the percentage of CD4<sup>+</sup>CD45RO<sup>+</sup> in AD children correlated with disease activity. In one study, increased percentage of CD4<sup>+</sup>CD45RO<sup>+</sup> in children and young people with severe AD, but not with mild AD as compared to healthy controls was evidenced (16). In contrast, in adult with severe AD, decreased percentage of memory CD4<sup>+</sup>CD45RO<sup>+</sup> T cells in peripheral blood in AD compared with that of healthy controls was found (15). Interestingly, in our study, the number of memory CD4<sup>+</sup>CD45RO<sup>+</sup> and CD8<sup>+</sup>CD45RO<sup>+</sup> T cells were significantly higher in children with AD than in healthy ones.

Our findings suggest that both compartment of memory CD4<sup>+</sup> and CD8<sup>+</sup> T cells are implicated in the immunopathogenesis of AD and this may be also due to *S. aureus* skin colonization. In our study, almost all children were affected *S. aureus* skin colonization. Nasal and throat *S. aureus* was also more frequent in AD children than healthy controls. The higher frequency of *S. aureus* carriage in AD patients was reported in other study (18). Increased colonization of AD skin by *S. aureus* is associated with exacerbation of the inflammatory immune response, which is largely mediated by bacterial superantigen, such as staphylococcus enterotoxin (SE), SEA, SEB, and toxic shock syndrome toxin (TSSS)-1. The potent immunostimulatory properties of superantigen result from simultaneous interaction with V $\beta$  domain of the T-cell receptor on lymphocytes and the MHC class II molecule on surface of antigen presenting cells (APC) (5, 9). In the peripheral blood of AD patients whose skin is colonized by *S. aureus*, a relevant skewing of superantigen reactive V $\beta$  families was observed in CD4<sup>+</sup> and CD8<sup>+</sup> T cells co-expressing CLA (19). Moreover, the CLA<sup>+</sup>CD8<sup>+</sup> T cells isolated from skin or peripheral blood of AD patients

respond to superantigenic stimulation to the same extent as CD4<sup>+</sup> T cells (20).

We found similar proportions of CD25 expression on circulating T cells in general and on CD4<sup>+</sup> T cells when comparing patients with AD and control group, which is in accord with other studies (13, 16). However, both the percentage of CD3<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> lymphocytes were correlated with disease activity and density of *S. aureus* on the skin. It is known that, CD25 is upregulated upon activation of T cells. However, among CD4<sup>+</sup> expressing CD25<sup>+</sup> is a distinct subset with regulatory activity (CD4<sup>+</sup>CD25<sup>+</sup> T<sup>reg</sup>). Regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells normally inhibit Th2 cytokine expression and proliferation of PBMC from non-atopic donors in response to allergen. Some recent reports have suggested the existence of defects in the number/or function of CD4<sup>+</sup>CD25<sup>+</sup> T<sup>reg</sup> in atopic individuals (21). In recent study, it was demonstrated that CD4<sup>+</sup>CD25<sup>+</sup> T<sup>reg</sup> cells are substantially increased in AD, but lose their immunosuppressive activity in response to stimulation by superantigen (22). In AD patients with skin colonization by superantigen producing *S. aureus* because of an inappropriate function of CD4<sup>+</sup>CD25<sup>+</sup> T<sup>reg</sup> instead of suppression may increase T-cell activation and the inflammatory reactions.

Using flow cytometric assay on whole blood from children with AD, we demonstrated significantly higher frequencies of CD4<sup>+</sup>/IL-4<sup>+</sup>, CD4<sup>+</sup>/IL-13<sup>+</sup>, CD8<sup>+</sup>/IL-4<sup>+</sup> and CD8<sup>+</sup>/IL-13<sup>+</sup> T cells compared with blood obtained from healthy, non-atopic children. The excessive Th2 production by polyclonally stimulated T cells was accompanied with significantly decreased frequencies of both CD4<sup>+</sup> and CD8<sup>+</sup> cells producing IFN- $\gamma$  in the patients with AD as compared with controls. Our results are in accord with other works, which has reported increased production/mRNA of IL-4 and IL-13 with concomitant decreased IFN- $\gamma$  expression by polyclonally stimulated PBMC from AD patients (17, 23).

The most striking finding in our study was a decrease in IFN- $\gamma$  production by both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, which was inversely correlated with *SCORAD* index and the number of *S. aureus* on the skin. Both parameters *SCORAD* and the number of bacteria were correlated with CD4<sup>+</sup> T expressing memory marker RO and the percentage of CD3<sup>+</sup> and CD4<sup>+</sup> T cells expressing activation markers CD25 as well serum IgE levels. There was recently demonstrated, that impaired IFN- $\gamma$  production may account for the abnormal immunopathogenesis

of severe, intractable AD (24). IFN- $\gamma$  is considered a key cytokine of innate and adaptive immunity playing pivotal roles in host defence against microbial pathogens thorough activation of monocytes, macrophages, natural killer (NK) cells, enhancing antigen processing and presentation and modulation of humoral immune response, therefore, its deficiency results in an inappropriate pathogen elimination and in part may explain propensity to skin infections in AD patients (25). On the other hand, increased total IgE levels may lead to the inhibition of skin defence as IgE inhibits neutrophil adhesion, phagocytosis as well respiratory burst (26). Also Th2 cytokines IL-4 and IL-13 may increase propensity to *S. aureus* skin colonisation due the fact, that they can increase expression of fibronectin and fibrinogen, receptors that mediate the adhesion *S. aureus* to stratum corneum (27). Besides this, IL-4 and IL-13 suppress the TNF- $\alpha$  and IFN- $\gamma$  induced upregulation of HD-2 and HD-3 mRNA in keratinocytes and normal skin (28). Our study findings support notion, that the severity of AD is correlated with density of *S. aureus* on the skin and that the number of *S. aureus* is correlated with total IgE levels (29).

It is believed that defect in IFN- $\gamma$  secretion following polyclonal stimulation is a generalized feature of atopic disease (30). Comparing atopic children with healthy subjects, in the culture supernatants of PBMC with SEB stimulation, AD/or atopic children produced more Th2 cytokines, such as IL-4 (30), IL-5 (31), IL-13 (32), and less Th1 cytokines, such as IFN- $\gamma$  (30).

Concerning the serum IgE levels, we have also found that it was positively correlated with IL-13 and IL-10 producing CD4<sup>+</sup> T cells. Akdis et al. (3) in a previous study demonstrated that in AD patients IgE production by B cells is induced mainly by IL-13 (not IL-4) derived from both memory/effector CD4<sup>+</sup> and CD8<sup>+</sup> expressing CLA. If the key role of IL-13 in allergic diseases including its impact on IgE production is well documented, the contribution of IL-10 in human atopic disease seems to be more complex. IL-10 inhibits Th2 and Th1 cytokine synthesis, and eosinophil survival and decreases epsilon transcript expression and IgE synthesis induced by IL-4, but actually increases IgE production from B cells that have already switched (33). Most studies indicate an association between increased IL-10 production and decreased allergic reaction. It was shown that severe AD is associated with reduced frequency of IL-10 producing allergen-specific CD4<sup>+</sup> T cells (34). Also impaired IL-10 responses to broad range of environmental

stimuli including foods, intestinal flora, *S. aureus* and vaccines in young children with AD were found (35). In contrast, some studies demonstrated increased frequency of intracellular IL-10 expressing peripheral CD4<sup>+</sup> and CD8<sup>+</sup> T cells as well serum IL-10 levels in AD patients (36). Moreover, skin lesions in AD exhibited elevated IL-10 gene expression, which downregulates HBD-2 expression in the skin (8). In our study, we could not find any differences according IL-10 production by T cells between AD children and healthy controls, which is in line with other studies (13).

In this study, serum IgE level was positively correlated with CD4<sup>+</sup> T cells expressing memory markers (RO) but negatively with the percentage of CD45RA lymphocytes and the number of CD4<sup>+</sup> T expressing naive marker (RA). Prior reports suggest that both CD45 (RA/RO) subsets may play significant and distinct roles in the induction of IgE synthesis (37).

In conclusion, this study demonstrated immunological differences in peripheral blood between AD and healthy controls, in particular in terms of number of CD4<sup>+</sup> and CD8<sup>+</sup> T cell expressing memory marker cell and impaired type 1 and dominant type 2-cytokine expressions in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. An increase in memory CD4<sup>+</sup> and CD8<sup>+</sup> T cells indicate that both subsets may be decisive in the immunopathogenesis of AD and this could be related to the *S. aureus* skin colonization. Since diminished IFN- $\gamma$  expression was correlated with disease severity and density of *S. aureus* on the skin, it is possible that deficiency of IFN- $\gamma$  in AD may at least partially explain propensity to skin infection in AD children.

### Acknowledgments

This study was supported by grant no NN- 2/153/05 from the State Committee for Research in Poland.

### References

1. LEUNG DY, BOGUNIEWICZ M, HOWELL MD, NOMURA I, HAMID QA. New insights into atopic dermatitis. *J Clin Invest* 2004; 113: 651–7.
2. VAN REIJSEN FC, BRUIJNZEEL-KOOMEN CA, KALTHO FS, et al. Skin derived aeroallergen-specific T-cells clones of Th2 phenotype in patients with atopic dermatitis. *J Allergy Clin Immunol* 1992; 90: 184–93.
3. AKDIS M, SIMON HU, WEIGL L, KREYDEN O, BLASER K, AKDIS CA. Skin homing (cutaneous lymphocyte-associated antigen-positive) CD8<sup>+</sup> T cells respond to superantigen and contribute to eosinophilia and IgE production in atopic dermatitis. *J Immunol* 1999; 163: 466–75.
4. SENEVIRATNE SL, JONES L, KING AS, et al. Allergen-specific CD8(+) T cells and atopic disease. *J Clin Invest* 2002; 110: 1283–91.

5. BAKER BS. The role of microorganisms in atopic dermatitis. *Clin Exp Immunol* 2006; 144: 1–9.
6. HOEGER PH. Antimicrobial susceptibility of skin-colonizing *S. aureus* strain in children with atopic dermatitis. *Pediatr Allergy Immunol* 2004; 15: 474–7.
7. BOGUNIEWICZ M, LEUNG DY. Atopic dermatitis. *J Allergy Clin Immunol* 2006; 117(2 Suppl. Mini-Primer): S475–80. Review.
8. HOWELL MD, NOVAK N, BIEBER T, et al. Interleukin-10 downregulates anti-microbial peptide expression in atopic dermatitis. *J Invest Dermatol* 2005; 125: 738–45.
9. HOFER MF, HARBECK RJ, SCHLIEVERT PM, LEUNG DY. Staphylococcal toxin augment specific IgE responses by atopic patients exposed to allergen. *J Invest Dermatol* 1999; 112: 171–6.
10. HANIFIN JM, RAJKA G. Diagnostic features of atopic dermatitis. *Acta Derm Venereol Suppl (Stockh)* 1980; 92: 44–7.
11. European Task Force on Atopic Dermatitis. Severity scoring of atopic dermatitis: the SCORAD index. *Dermatology* 1993; 186: 23–31.
12. YOSHIZAWA Y, NOMAGUCHI H, IZAKI S, KITAMURA K. Serum cytokine levels in atopic dermatitis. *Clin Exp Dermatol* 2002; 27: 225–9.
13. ANTUNEZ C, TORRES MJ, MAYORGA C, et al. Cytokine production, activation marker, and skin homing receptor in children with atopic dermatitis and bronchial asthma. *Pediatr Allergy Immunol* 2006; 7: 66–74.
14. PICKER LJ, TREER JR, FERGUSON-DARNELL B, COLLINS PA, BERGSTRESSER PR, TERSTAPPEN LW. Control of lymphocyte recirculation in man. II. Differential regulation of the cutaneous lymphocyte-associated antigen, a tissue-selective homing receptor for skin-homing T cells. *J Immunol* 1993; 150: 1122–36.
15. MATSUYAMA T, URANO K, OHKIDO M, et al. The quantitative and qualitative defect of CD4+ CD45RO+ memory-type T cells are involved in the abnormality of Th1 immunity in atopic dermatitis patients. *Clin Exp Allergy* 1999; 29: 687–94.
16. DWORZAK MN, FROSCHL G, PRINTZ D, et al. Skin-associated lymphocytes in the peripheral blood of patients with atopic dermatitis: signs of subset expansion and stimulation. *J Allergy Clin Immunol* 1999; 103: 901–6.
17. OKAZAKI H, KAKURAI M, HIRATA D, et al. Characterization of chemokine receptor expression and cytokine production in circulating CD4+ T cells from patients with atopic dermatitis: up-regulation of C-C chemokine receptor 4 in atopic dermatitis. *Clin Exp Allergy* 2002; 32: 1236–42.
18. GILANI SJ, GONZALEZ M, HUSSAIN I, FINLAY AY, PATEL GK. Staphylococcus aureus re-colonization in atopic dermatitis: beyond the skin. *Clin Exp Dermatol* 2005; 30: 10–3.
19. SKOV L, OLSEN JV, GIORNO R, SCHLIEVERT PM, BAADSGAARD O, LEUNG DY. Application of Staphylococcal enterotoxin B on normal and atopic skin induces up-regulation of T cells by a superantigen-mediated mechanism. *J Allergy Clin Immunol* 2000; 105: 820–6.
20. STRICKLAND I, HAUKE PJ, TRUMBLE AE, PICKER LJ, LEUNG DY. Evidence for superantigen involvement in skin homing of T cells in atopic dermatitis. *J Invest Dermatol* 1999; 112: 249–53.
21. ROMAGNANI S. Regulatory T cells: which role in the pathogenesis and treatment of allergic disorders? *Allergy* 2006; 1: 3–14.
22. OU LS, GOLEVA E, HALL C, LEUNG DY. T regulatory cells in atopic dermatitis and subversion of their activity by superantigens. *J Allergy Clin Immunol* 2004; 113: 756–63.
23. TERAKI Y, HOTTA T, SHIOHARA T. Increased circulating skin-homing cutaneous lymphocyte-associated antigen (CLA)+ type 2 cytokine-producing cells, and decreased CLA+ type 1 cytokine-producing cells in atopic dermatitis. *Br J Dermatol* 2000; 143: 373–8.
24. KATSUNUMA T, KAWAHARA H, YUKI K, AKASAWA A. Impaired interferon- $\gamma$  production in subset population of severe atopic dermatitis. *Int Arch Allergy Immunol* 2004; 134: 240–7.
25. GRASSEGER A, HOPFL R. Significance of the cytokine interferon gamma in clinical dermatology. *Clin Exp Dermatol* 2004; 29: 584–8.
26. AL-MOHANNA F, PARHAR R, KAWAASI A, et al. Inhibition of neutrophil functions by human immunoglobulin E. *J Allergy Clin Immunol* 1993; 92: 757–66.
27. CHO SH, STRICKLAND I, BOGUNIEWICZ M, LEUNG DY. Fibronectin and fibrinogen contribute to the enhanced binding of Staphylococcus aureus to atopic skin. *J Allergy Clin Immunol* 2001; 108: 269–74.
28. NOMURA I, GOLEVA E, HOWELL MD, et al. Cytokine milieu of atopic dermatitis, as compared to psoriasis, skin prevents induction of innate immune response genes. *J Immunol* 2003; 171: 3262–9.
29. GUZIK TJ, BZOWSKA M, KASPROWICZ A, et al. Persistent skin colonization with Staphylococcus aureus in atopic dermatitis: relationship to clinical and immunological parameters. *Clin Exp Allergy* 2005; 35: 448–55.
30. SMART JM, KEMP AS. Increased Th1 and Th2 allergen-induced cytokine response in children with atopic disease. *Clin Exp Allergy* 2002; 32: 796–802.
31. HEATON T, MALLON D, VENAILLE T, HOLT P. Staphylococcal enterotoxin induced IL-5 stimulation as a cofactor in the pathogenesis of atopic disease: the hygiene hypothesis in reverse. *Allergy* 2003; 58: 252–6.
32. LEHMANN HS, HEATON T, MALLON D, HOLT PG. Staphylococcal enterotoxin-B-mediated stimulation of interleukin-13 as a potential etiologic factor in eczema in infants. *Int Arch Allergy Immunol* 2004; 135: 306–12.
33. JEANNIN P, LECOANET S, DELNESTE Y, GAUCHAT JF, BONNEFOY JY. IgE vs. IgG4 production can be differentially regulated by IL-10. *J Immunol* 1998; 160: 3555–61.
34. SENEVIRATNE SL, JONES L, BAILEY AS, BLACK AP, OGG GS. Severe atopic dermatitis is associated with a reduced frequency of IL-10-producing allergen-specific CD4+ T cells. *Clin Exp Dermatol* 2006; 31: 689–94.
35. DUNSTAN JA, HALE J, BRECKLER L, et al. Atopic dermatitis in young children is associated with impaired interleukin-10 and interferon-gamma responses to allergens, vaccines and colonizing skin and gut bacteria. *Clin Exp Allergy* 2005; 35: 1309–17.
36. ALEKSZA M, IRYNYI B, LUKACS A, ANTAL-SZALMAS P, HUNYADI J, SZEGEDI A. Increased frequency of intracellular interleukin (IL)-13 and IL-10, but not IL-4, expressing CD4+ and CD8+ peripheral T cells of patients with atopic dermatitis. *Br J Dermatol* 2002; 147: 135–41.
37. PATEL HR, OSHIBA A, JEPSON JD, GELFAND EW. Differential expression of CD40 ligand on T cell subsets. Implications for different roles of CD45RA+ and CD45RO+ cells in IgE production. *J Immunol* 1996; 156: 1781–7.