Cytokine gene expression in the skin and peripheral blood of atopic dermatitis patients and healthy individuals

Elena S. Fedenko,^{1,*} Olga G. Elisyutina,¹ Tatyana M. Filimonova,¹ Margarita N. Boldyreva,¹ Olga V. Burmenskaya,² Olga Yu Rebrova,³ Alexander A. Yarilin¹ and Rakhim M. Khaitov¹

¹National Research Institute of Immunology; ²National Research Center for Obstetrics; Gynecology and Perinatology named after V.I. Kulakov;

³Russian State Medical University; Moscow, Russia

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Objectives: Atopic dermatitis (AD) is an increasingly common, chronic, relapsing, inflammatory skin disease characterized by impaired epidermal barrier function and cutaneous inflammation. The prevalence of AD has steadily increased during the past few decades. The aim of this study was to comparatively investigate cytokine gene expression in the skin and peripheral blood of atopic dermatitis patients and healthy individuals.

Results: In the skin of patients with AD, a significant increase of the level of gene expression was observed for interleukin (IL)-2r ($p < 0.0023$), IL-5 ($p = 0.002$), IL-6 ($p < 0.0023$), IL-8 ($p = 0.01$), IL-12B ($p < 0.0023$), IL-10 ($p < 0.0023$), IL-23 ($p = 0.002$), IL-29 ($p < 0.0023$), and transforming growth factor beta (TGFbeta) ($p < 0.0023$) as compared to healthy individuals. In contrast, no difference between AD patients and healthy donors was detected with respect to cytokine gene expression in the peripheral blood.

Methods: Samples of skin and peripheral blood from 48 severe AD patients (SCORAD = 78.5 [57;89], IGA = 4.2 [3;9;4;7]) at the age of 17 to 45 years and 20 healthy donors aged from 19 to 32 years were analyzed for gene expression of cytokines using real-time reverse transcription polymerase chain reaction (RT-PCR).

Conclusions: Activity of markers of chronic inflammation and Th1 immune response in severe AD, namely IL-2r, IL-8, IL-12B, IL-23, IL-29 and TGFbeta, as well as activity of anti-inflammatory IL-5 were predominant in the skin but not in the blood of AD patients.

Introduction

Atopic dermatitis (AD) is a multifactorial disease based on genetic predisposition to allergy and is characterized by complex immune mechanisms of the development of allergic skin inflammation. Skin is a highly developed organ of the immune system and has a number of immunocompetent cells that interact with each other by means of cell surface receptors and intercellular messengers such as cytokines.¹ Stromal cells (epithelial cells, fibroblasts and skin capillary endotheliocytes) can also be involved in the immune response. The immunopathogenesis of AD is determined by impairment of differentiation of T helper cells as well as their cytokine secretion profiles. The initiation and maintenance of skin inflammation directly involves a number of cytokines.

The AD immune response is considered to be divided into two phases: acute and chronic or early and late.² Acute inflammation is characterized by increased Th2 response activity: expression of interleukin (IL)-4, IL-5 and IL-13, reduced production of interferon (IFN) γ , and increased level of both total and specific IgE.

In contrast, chronic inflammation is characterized by increased Th1 response activity that includes increased production of IL-12 by macrophages and eosinophiles, as well as increased level of markers of chronic skin inflammation such as IL-5, IL-8 and IFN γ . Long-term allergic inflammation is also accompanied by increased level of IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF).

At present, it is known that newly identified interleukins including IL-10, IL-19, IL-20, IL-21, IL-24, IL-26, IL-17, IL-17A, IL-17F, IL-17C, IL-28A, IL-28B and IL-29 that are produced by lymphocytes and other immune cells participate in the AD pathogenesis.³⁻⁷ These cytokines are able to regulate IgE synthesis through both direct and indirect mechanisms with the possibility for positive and negative regulation. Therefore, detailed studies of pathogenic role of cytokines in the development of AD will significantly improve our understanding of the molecular mechanisms underlying the immunopathogenesis of AD.

In AD, skin is the target organ for allergic inflammation. Thus, advanced study of the local immune response can result in the determination of immune markers of inflammation activity

*Correspondence to: Elena S. Fedenko; Email: efed57@mail.ru
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Table 1. Clinical profile of the group of AD patients

Parameter	Mean value
Age. M (SD)	26.2 (6.5)
Sex (male/female)	28/20 (58/41%)
Disease severity indicators	
SCORAD index. Me [Q1; Q3]	78.5 [57; 89]
IGA score. Me [Q1; Q3]	39 [3.9; 4.7]
AD duration (years) Me [Q1; Q3]	18 [15; 48]
Disease manifestation age	
Under a year (n)	18 (37.5%)
From 2 to 5 years (n)	30 (62.5%)
Allergic examination	
IgE net. ME/ml Me [Q1; Q3]	2,056 [1,300; 5,000]
sensitization to domestic allergens (n)	46 (98.8%)
sensitization to dander (n)	40 (83.3%)
sensitization to pollen allergens (n)	38 (80%)
latent sensitization to pollen allergens (n)	10 (20%)
food allergy (n)	12 (25%)
polyvalent sensitization (n)	16 (33.3%)

at various phases of disease. In this work, using real-time reverse transcription polymerase chain reaction (RT-PCR) we analyzed cytokine gene expression in the skin from 48 severe AD patients (SCORAD = 78.5 [57;89], IGA = 4.2 [3.9;4.7]) at the age of 17 to 45 years and 20 healthy donors aged from 19 to 32 years and found that activity of IL-2r, IL-5, IL-8, IL-12beta, IL-23, IL-29 and transforming growth factor beta (TGFβ) is predominant in the skin but not in the blood of AD patients as compared to healthy donors.

Results and Discussion

Clinical features of the AD patients (Table 1). At the moment of study entry, all 48 AD patients had an acute phase of AD with localization of skin lesions on the face, scalp, neck, back, chest, wrist flexion surface of extremities, elbow and knee joints with severe pruritus especially at night. The AD duration was from 15 to 48 years including 5–10 relapses per year. The manifestation of the disease occurred at early age: 18 patients (37.5%)—at the age under a year, 30 patients (62.5%)—at the age from 2 to 5 years.

All patients underwent clinical, laboratory, allergic and immunologic examination and testing. On the basis of the allergic examination (own and family allergic anamnesis, skin tests for airborne allergens, total and specific IgE level in serum), the sensibilization was evaluated (Table 1). 46 patients (95.8%) were determined to have sensitization to house dust mites, 40 patients (83.3%)—to dander, 38 patients (83.3%)—to pollen allergens resulted in bronchial asthma, rhinitis, conjunctivitis. 10 patients (20%) had latent sensitization to pollen, 12 (25%) patients had food allergy and 16 patients (33.3%) had polyvalent sensitization. All the patients had a high serum IgE level: 2,056 ME/ml [1,300; 1,500] while a normal concentration is up to 130 ME/ml.

Cytokine gene expression level in the skin and peripheral blood of AD patients and healthy individuals. Analysis of the cytokine gene expression level showed no significant difference in skin samples from neck, extremity flexion surfaces and shoulder outer surface of 5 healthy donors. Significantly increased level of IL-2r, IL-5, IL-6, IL-8, IL-10, IL-12B, IL-23, IL-29, TGFβ expression was discovered in AD patients as compared to healthy individuals (Table 2). Also, a strong difference in IFNγ level in skin was revealed between AD patients and healthy donors ($p = 0.0024$). No significant difference in IL-1B, IL-2, IL-4, IL-7, IL-12A, IL-15, IL-15SP, IL-17A, IL-18, IL-28, TNF, FOXP3 levels was observed (Table 2). No statistically significant difference in levels of the cytokine gene expression in the peripheral blood of the healthy donors and AD patients was found (Table 3).

Taken together, these findings suggest that local immune response is predominantly localized in the skin of patients with severe AD and characterizes illness activity. Further, analysis of markers of chronic inflammation such as IL-2, IL-8, IL-12B, IFNγ, IL-23, IL-29 and TGFβ allows to assume a predominant Th1 response. In addition, IL-2, IL-8, IL-12B, (subunit IL-23/p40), IFNγ, IL-23 (subunit IL-23/p19), IL-29 are considered to be the markers of chronic inflammation and predominant Th1/Th17 response under increased expression of IL-5 messenger ribonucleic acid (mRNA).

It is known that endogenic IL-2 is mainly produced by activated Th1 lymphocytes (90% of endogenic IL-2 production). T-lymphocytes are the only cells that are able to express IL-2 gene under strict control by T-lymphocytes antigen recognition complex.⁸ IL-8 plays an important role in the local inflammation response and is produced by Th1-lymphocytes, fibroblasts and macrophages. Recent studies reveal that IL-12 produced by macrophages, dendritic cells and eosinophils stimulates Th1-response.⁹ IL-12B receptor is known to be expressed in Th1 rather than Th2 cells.

Increased expression of IL-23 and IL-29 genes in the skin of AD patients revealed in this study is in line with the literature data.^{10,11} At present, a recently discovered cytokine IL-29 that is mainly produced in skin by virus-infected epithelial cells, keratinocytes, myeloid DCs, plasmacytoid DCs and regulatory T-cells has become known to participate in the AD pathogenesis by increasing the IFNγ production. IL-29 (also known as type III interferon) exhibits anti-inflammatory activity and enhances proliferation of T-lymphocytes. Thus, in the AD patients' skin, IL-23 and IL29 regulate immune response that is mediated by Th1-lymphocytes.^{10,11}

Increase of TGFβ in the skin of patients with AD as compared to healthy donors also illustrates predominance of Th1-response. It is known that TGFβ is able to change IL-4 effect and lead to predominance of the Th1-response via activation of Th0 lymphocytes.¹² At the early phase, IL-6 influence on maturation of Th17-cells while IL-23 and TGFβ are key regulatory cytokines at later phase. Lack of one of these cytokines is known to result in poor production of Th17 cells. It is also known that Th17 cells produce IFNγ and IL17 in response to IL23.

Table 2. Cytokine gene expression levels in the skin of patients with severe AD and healthy donors (medians and quartiles)

Indicator	Healthy donors n = 20	AD patients n = 48	Mann-Whitney U-test. p
IL-1B	0.196 [0.096; 0.337]	0.061 [0.030; 0.117]	0.0056
IL-2	0.002 [0.001; 0.006]	0.005 [0.003; 0.009]	0.0135
IL-2r	0.003 [0.001; 0.006]	0.011 [0.007; 0.026]	0.000064*
IL-4	0.00004 [0; 0.00025]	0.0003 [0.00005; 0.0009]	0.0270
IL-5	0.0003 [0.00009; 0.0008]	0.001 [0.0005; 0.003]	0.0021*
IL-6	0.0004 [0; 0.000059]	0.0005 [0.0001; 0.002]	0.000098*
IL-7	0.218 [0.139; 0.303]	0.139 [0.085; 0.362]	0.3324
IL-8	0.005 [0.003; 0.011]	0.066 [0.029; 0.141]	0*
IL-10	0.0008 [0.0004; 0.0019]	0.005 [0.003; 0.013]	0.000004*
IL-12A	0.004 [0.002; 0.006]	0.003 [0.001; 0.005]	0.1033
IL-12B	0.001 [0.0002; 0.0032]	0.004 [0.003; 0.009]	0.00058*
IL-15	0.0043 [0.032; 0.064]	0.057 [0.037; 0.075]	0.1333
IL-15SP	0.009 [0.007; 0.014]	0.009 [0.002; 0.016]	0.4924
IL-17A	0 [0; 0.000412]	0 [0; 0.0008]	0.6303
IL-18	2.107 [1.444; 2.462]	1.711 [1.319; 3.732]	0.9302
IL-23	0.003 [0.002; 0.007]	0.007 [0.005; 0.012]	0.00205*
IL-28	0 [0; 0.0002]	0 [0; 0.004]	0.2514
IL-29	0 [0; 0]	0.0005 [0; 0.001]	0.000252*
IFNγ	0.0009 [0.0005; 0.004]	0.003 [0.002; 0.006]	0.0024
TNF	0.057 [0.036; 0.121]	0.088 [0.045; 0.165]	0.2610
TGFβ	1.018 [0.616; 1.444]	1.933 [1.366; 3.310]	0.00001*
FOXP3	0.064 [0.051; 0.088]	0.072 [0.054; 0.105]	0.4078

*p < 0.0023.

Materials and Methods

68 individuals were involved in the research including 31 women aged from 17 to 45 years as well as 37 men aged from 20 to 32 years. This includes 48 patients with severe AD and 20 healthy individuals. The AD diagnosis was made in accordance with J.M. Hanifin and G. Rajka's criteria.¹³ The Scoring Atopic Dermatitis (SCORAD) index⁹ and Investigators' Global Assessment (IGA) score were applied for the assessment of AD severity according to 5 point scale including the following parameters:

0-clear-no inflammatory signs of AD

1-almost clear-just perceptible erythema, just perceptible papulation/infiltration

2-mild AD-mild erythema, mild papulation/infiltration

3-moderate AD-moderate erythema, moderate papulation/infiltration

4-severe AD-severe erythema, severe papulation/infiltration

5-extremely severe AD-severe erythema, severe papulation/infiltration with oozing/crusting.

The AD group consisted of 48 patients with severe AD: the SCORAD index median and quartiles are 78.5 [57; 89], IGA score was 4.2 [3.9; 4.7]. Among them, there were 20 women and 28 men aged from 17 to 45 years. The mean age and standard deviation (SD) were 26.2 and 6.5 years, respectively. The reference group included 20 healthy individuals. Among them, there

were 12 women and 8 men aged from 19 to 32. The mean age (SD) was 22.2 (4.3) years.

The AD and reference groups were similar in gender (p = 0.192, Fisher's exact test) but not in age (p = 0.014, t-test).

Samples of skin and peripheral blood of patients with AD and healthy donors were used in this study. Skin biopsies 3 mm in diameter were taken with a single-use sterile puncher (Stumpp Medizintechnik GmbH, Germany) under aseptic conditions using 2% hydrochloride lidocaine as a local analgesia. Skin samples were taken from neck back surface, elbow and knee joint flexion surfaces lesion of 48 patients with AD. Simultaneous punch biopsy of 3 different skin localizations in 5 out of 20 healthy donors (neck back surface, elbow and knee joint flexion surfaces and shoulder outer surface) was performed to determine possible differences of cytokine gene expression in skin in various body areas. No significant difference in cytokine gene expression was observed between biopsies taken from various skin areas. Thus, in the remaining 15 healthy donors, biopsy was performed only in 1 skin localization (shoulder outer surface). RT-PCR was used for testing samples of skin and peripheral blood of AD patients and healthy donors. Cytokine gene expression was measured on mRNA cytokine genes.

To avoid RNA destruction, the skin biopsy samples were placed in thiocyanate guanidine solution immediately after taking. For the same reason, the blood samples were processed immediately after taking. Then, the blood was treated with a

Table 3. Cytokine gene expression levels in the peripheral blood of the AD patients and healthy donors (medians and quartiles)

Indicator	Healthy donors n = 20	AD patients n = 48	Mann-Whitney U-test. p
IL-1B	5.282 [3.427; 9.212]	5.756 [3.797; 7.337]	0.924
IL-2	0.0005 [0.003; 0.007]	0.004 [0.003; 0.007]	0.818
IL-2r	0.0366 [0.023; 0.049]	0.037 [0.026; 0.059]	0.403
IL-4	0.0053 [0.0006; 0.0105]	0.006 [0.004; 0.012]	0.086
IL-5	0.00029 [0.00009; 0.00029]	0.00031 [0.00018; 0.0006]	0.034
IL-6	0.0016 [0.0012; 0.0025]	0.0013 [0.00098; 0.002]	0.168
IL-7	0.038 [0.0296; 0.057]	0.045 [0.026; 0.058]	0.594
IL-8	46.108 [38.054; 80.761]	48.091 [21.484; 60.767]	0.244
IL-10	0.0043 [0.0027; 0.0052]	0.0049 [0.003; 0.008]	0.385
IL-12A	0.015 [0.0122; 0.024]	0.0175 [0.012; 0.025]	0.686
IL-12B	0.00063 [0.00045; 0.00119]	0.0011 [0.0007; 0.0018]	0.022
IL-15	0.225 [0.157; 0.308]	0.233 [0.189; 0.308]	0.590
IL-15SP	0.067 [0.036; 0.095]	0.055 [0.0384; 0.075]	0.686
IL-17A	0.00016 [0; 0.0002]	0.00013 [0; 0.00043]	0.362
IL-18	0.107 [0.069; 0.162]	0.133 [0.101; 0.203]	0.092
IL-23	0.074 [0.037; 0.115]	0.053 [0.029; 0.082]	0.174
IL-28	0.00009 [0; 0.00029]	0.00011 [0; 0.00029]	0.731
IL-29	0 [0; 0]	0 [0; 0]	0.006
IFN γ	0.0055 [0.0226; 0.0743]	0.057 [0.0384; 0.0964]	0.247
TNF	0.341 [0.2107; 0.5645]	0.420 [0.329; 0.671]	0.087
TGF β	13.516 [10.022; 19.073]	15.727 [13.225; 18.379]	0.118
FOXP3	0.160 [0.086; 0.165]	0.131 [0.110; 0.203]	0.118

buffer for lysis of erythrocytes. RNA was isolated from the blood and skin biopsy samples using the standard method of phenol extraction of Homczyński.¹⁵ Commercially available reagents (DNK-Technologie, Russia) were used in this research. The manufacturer guarantees no amplification of genome DNA of cytokines and the reference gene. This allowed to avoid the treatment of nucleic acids with DNA-ase.

The reverse transcription test for cDNA was conducted with specific primers for each cytokine in 40 μ l volume (including 33 μ l of the studied sample). The reverse transcription test was carried out for 30 min at 40°C. Inactivation of the reverse transcriptase enzyme was performed for 5 minutes at 95 after the reaction. Then, the sample of cDNA was diluted 5-fold in TE buffer. Real time RT-PCR was performed using the “DT-96” instrument (DNK-Technologie, Russia) in 35 μ l volume using the following amplification program:

1 cycle-80°C for 30 seconds, then 94°C for 1.5 min; 50 cycles-94°C for 20 seconds, then 62°C for 20 seconds.

Fluorescence intensities on each cycle real-time PCR were measured at each cycle through the channel FAM. The test was conducted twice for each point. Normalization of real-time RT-PCR values of cytokine genes was carried out with respect to the reference gene HPRT1.

The data received were processed in MS Excel. Method Δ Cq was used for comparative analysis of the threshold cycles. Level of expression of cytokine mRNA was calculated in relative units using the method based on determining the threshold cycles for cytokine genes and reference gene HPRT1 calculated by

mathematical analysis of the curve shape of amplification of cytokine cDNA (Cq) and HPRT1. The following formula (Formula 1) was used for calculations of cytokine gene expression:

$[IL]/[HPRT1] = E \times Cp1_{HPRT1} / E \times Cp2_{IL}$, where E is the efficiency of amplification, Cp1 is the threshold cycle for HPRT1 cDNA and Cp2 is the threshold cycle for cDNA of the analyzed cytokine.

The expression of the following genes was studied: IL 1B, IL 2, IL 2r, IL 4, IL 5, IL 6, IL 7, IL 8, IL 10, IL 12A, IL 12B, IL 15, IL 15 (long peptide izoform), IL 17A, IL 18, IL 23, IL 28, IL 29, IFNG, TNF, TGF β 1, FOXP3.

Statistical analysis was carried out using “Statistica 9.1” software (StatSoft, Inc., USA).

Descriptive statistics for quantitative data was presented by medians and quartiles as Me [Q₁; Q₃], in case of normal distributions as mean and standard deviations M(s). Nonparametric Mann-Whitney U-test and Fisher’s exact test were used for comparison of groups. Bonferroni correction was used to address the problem of multiple comparisons. Thus, null hypothesis was rejected at p < 0.0023.

Conclusion

In the result of this study, predominant activity of IL-2r, IL-5, IL-8, IL-12B, IL-23, IL-29, IFN γ and TGF β is revealed in the skin of patients with AD. With exception of IL-5, these cytokines represent markers of chronic inflammation and predominant Th1 immune response that is characteristic for severe AD. IL-5 is anti-inflammatory cytokine that stimulates IgE production and

migration of eosinophils in inflammation focus. In contrast, no significant difference in gene expression of the studied cytokines was revealed in the blood of patients with severe AD and healthy donors. This suggests need for studies of the local, rather than the systemic immune response in these patients.

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