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Gene Polymorphisms of 22 Cytokines in Macedonian Children with Atopic Dermatitis

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ABSTRACT

Atopic dermatitis (AD) is a common chronically relapsing skin disease associated with abnormal cytokine production, and activation of T-helper 2 cells. The aim if this study was to determine whether cytokine gene polymorphisms might influence the development of AD. Single nucleotide polymorphisms in the genes for *I-L1alpha, IL-1beta, IL-1R, IL-2, IL-4, IL-6, IL-10, IL-12, TGF beta, TNF* and *IFNgamma* were investigated by PCR and sequence specific primers in Macedonian patients with AD (67 children, age of 6 months to 5 years) and 301 normal unrelated individuals. Susceptible cytokine polymorphisms for AD for eleven genotypes (*IL-4 -33/T:T IL-4 -1098/G:G, TGFbeta cdn25C:G, IL-4 -1098/T:T, IL-1alpha -889/C:T, IL-2 +166/T:T, IL-1beta -511/C:T, IL-12 -1188/C:T, IL-10 -1082/A:G, IL-1beta +3962/C:T, IFNgamma +874/A:T*), five diplotypes, six haplotypes, and for alleles were found. Protective cytokine polymorphisms for AD for seven cytokine genotypes (*IL-4 -1098/G:T, TGFbeta cdn25/G:G, IL-4 -33/C:C, IL-1alpha -889/C:C, IFNgamma +874/A:A, IL-10 -1082/A:A, IL-1beta -511/C:C*), one cytokine diplotypes, two cytokine haplotypes, and four cytokine alleles were also found. We concluded that several cytokine polymorphisms are protective, or susceptible associated with AD in population of Macedonians.

Keywords: Atopic dermatitis; Cytokine gene polymorphisms; Macedonian population; SSP genotyping

INTRODUCTION

Atopic dermatitis (AD) is a multifactorial chronic inflammatory skin disease characterized by pruritic, typically distributed eczematous skin lesions. Deficiencies in innate and adaptive immunity based on a genetic predisposition result in skin barrier dysfunction with hyperreactivity to environmental stimuli and susceptibility to skin infections which influence the course and severity of AD.

AD is a biphasic disease where the initial phase is predominated by T helper type 2 (Th2) cytokines that later switches to a more chronic Th1-dominated

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eczematous phase.[1] The effective production of IgE in atopic disease by B cells depends on support by Th2 cells, which produce interleukin-4 (IL-4), IL-5, IL-9 and IL-13. Barrier disruption and exposure to antigen have been shown to induce production of IL-4 and IL-5 in the skin. Whereas mRNA-expression for Th1 cytokines such as IFN-γ and IL-12 were not significantly detectable in acute atopic eczema (AE) skin lesions, increased mRNA- expression of IFN-gamma, IL-5, IL-12 and GM-CSF could be observed in chronic AE.[2]

Several cytokine gene polymorphisms in the cytokine gene regulatory regions correlate with cytokine secretion,[3] and one individual may have a cytokine expression pattern quite different from another.[4] Studies of disease association have been done in order to understand the correlation with immune activation, which may determine the risk or protection from disease expression. Several data have been published about the relationship between cytokine polymorphism and AD. While some authors show positive association of certain cytokine polymorphisms with AD, others report quite opposite results.[5]

Cytokine polymorphism in healthy ethnic Macedonians was published,[6] however there are no data about the associations of cytokine polymorphisms and AD in the Republic of Macedonia. The aim if this study was to determine whether genetic polymorphisms of the cytokine genes might influence the development of AD.

PATIENTS AND METHODS

Groups

The total studied sample consisted of 368 examinees, divided into two different groups as follows: normal individuals, and patients with atopic dermatitis (AD).

Normal Individuals

There were 301 unrelated individuals. They were age and sex non-matched normal individuals who attended the Institute of Immunobiology and Human Genetics for DNA donation between May 1, 2001 and April 25, 2005 and agreed to take part in this study as a control group. Individuals with family history of atopy were excluded from the investigation.

Atopic Dermatitis

67 patients with AD fulfilling the Hanifin and Rajka diagnostic criteria were enrolled in the study. They were 6 months to 5 years old patients who attended the University Children's Clinic, University School of Medicine for outpatient treatment between 2006 and 2010.

All individuals were of Macedonian origin and nationality, and residents of different regions of the Republic of Macedonia. Each individual was interviewed on a one-to-one basis, his/her genealogy was recorded for the past three generations, and a signed consent was obtained. All of the patients and normal individuals included in this study signed a written consent to participate in the study which was approved by the Committee of the Ministry of Education and Science from Republic of Macedonia (No 087405), and Ethical Committee of the Medical Faculty in Skopje.

Genomic DNA Isolation and Storage

Ten millilitres of venous blood was drawn from each donor by the standard venipuncture in a vacutainer with EDTA(K3) after signed written consent. DNA was isolated from peripheral blood leukocytes by phenol-chlorophorm extraction method or with BioRobot EZ1 workstation (QIAGEN).[7] The quality and quantity of DNA were analyzed by GeneQuant (Pharmacia Biotech, Uppsala, Sweden). Isolated DNA samples were stored in the Macedonian Human DNA Bank.[8]

Typing Methods

For cytokine genotyping commercially available PCR-SSP kit (Heidelberg kit, Cytokine genotyping Tray, *Invitrogen,* GmbH, Karlsruhe, Germany) was used. Fourteen cytokine genes with 22 single nucleotide polymorphisms (SNP) were typed: *IL-1alpha -889, IL-1beta -511, IL-1beta +3962, IL-1R psti1970, IL-1RA mspa11100, IL-4Ralpha +1902, IL-12 -1188, IFNgamma utr5644, TGF-beta1 cdn10, TGF-beta1 cdn25, TNF-alpha -308, TNF-alpha -238, IL-2 -330, IL-2 +166, IL-4 -1098, IL-4 -590, IL-4 -33, IL-6 -174, IL-6 565, IL-10 -1082, IL-10 -819,* and *IL-10 -592*. Briefly, PCR-SSP typing Heidelberg kit consists of 48 PCR primer mixes aliquoted in 96-well PCR trays (two typing per tray). Master mix, which was supplied along with the reagents and consisted of $MgCl_2$, buffer, dNTP's, and glycerol was mixed with 1.2-3.0 μg DNA and 20 U Taq polymerase and

dispensed in 48 wells.[9] Agarose gel electrophoresis on a 2% gel revealed a positive or negative signal for specific amplification in each well. Subsequently, the results were analysed according to the interpretation scheme provided with the kit.

Statistical Analysis

The population genetics analysis package, PyPop, developed by the Biostatistics Core for the Workshop,[10] was used for analysis of the cytokine data for this study. Allele frequencies and expected Hardy Weinberg proportions (HWP) for each single nucleotide polymorphism (SNP) were determined.[11] The exact test for genotype frequency deviation from HWP was calculated using the Arlequin implementation accessed via PyPop.[12] Those SNPs that did not fit HWP were evaluated to determine whether there was an excess of homozygotes or heterozygotes, or if any particular genotypes significantly differed from the expected frequencies by the chi square test.[13] Comparisons of frequencies for two groups were tested by the χ^2 test. Crude odds ratios (OR) (as estimates of the relative risk) were calculated with 95% confidence interval (CI).

RESULTS

Cytokine Alleles

Cytokine allele frequency, Pearson's *p*-value, Odds ratio and Wald's 95% confidence interval in patients with atopic dermatitis and normal Macedonian population are shown in Table 1. Positive (susceptible) odds ratio was found for *IL-4 -33/T* ($p<0.001$, OR=6.438, Wald's 95% CI between 3.602 and 11.509), meaning that people with *IL-4 -33/T* allele have 6.438 times higher risk to develop AD in comparison to others with *IL-4 -33/C* allele. Positive (susceptible) odds ratio was also found for *IL-4 -1098/T*, *TGF-beta1 cdn25/C,* and *IL-1alpha -889/T*. Negative (protective) association for AD was found for the following alleles: *IL-4 -33/C, IL-4 -1098/G*, *TGF- beta1 cdn25/G,* and *IL-1alpha -889/C* (Table 1).

Cytokine Genotypes

Cytokine genotype frequency, Pearson's *p*-value, Odds ratio and Wald's 95% confidence interval in patients with AD and normal Macedonian population are shown in table 2. We found positive (susceptible) association between patients with atopic dermatitis and following genotypes (according to the level of susceptibility): *IL-4 -33/T:T, IL-4 -1098/G:G, TG-beta1 cdn25/C:G, IL-4 -1098/T:T, IL-1alpha -889/C:T, IL-2 +166T:T, IL-1beta -511/C:T, IL-12 -1188/A:A, IL-10 -1082/A:G, IL-1 beta +3962/C:T,* and *IFN-gamma +874/A:T* (Table 2).

Negative (protective) association between patients with AD and following genotypes (according to the protectively level) was found for: *IL-4 -1098/G:T, TGFbeta1 cdn25/G:G, IL-4 -33/C:C, IL-1alpha -889/C:C, IFN-gamma +874/A:A, IL-10 -1082/A:A,* and *IL-1beta -511/C:C* (Table 2).

Genotypes *IL-1alpha -889/T: T, IL-1beta +3962/T:T, TNF-alpha -238/A:A* and *IL-10 -819/T:T* were present only in normal Macedonian population, while only patients with AD had *TGFbeta1 cdn25/C:C* genotypes (Table 2).

Cytokine Haplotypes

Cytokine haplotypes frequency, Pearson's *p*-value, crude odds ratio and Wald's 95% confidence interval in the patients with atopic dermatitis and normal Macedonian population are presented in table 3. With the Heidelberg kit it was possible to analyse haplotypes for *TGF-beta1, TNF-alpha, IL-2, IL-4, IL-6* and *IL-10*. Positive (susceptible) association between the patients with AD and following haplotypes was found (according the level of susceptibility): *IL-4/TCT*, *IL-4/GTT*, *IL-4/GCT*, *TGF-beta1/CC*, *IL-10/ACA*, and *IL-4/TTT*. Negative (protective) association was found between the patients with AD and haplotypes for: *IL-4/GCC* and *TGF-beta1/TG*. Haplotypes *IL-4/GCT and IL- 4/TTC,* were present only in normal Macedonian population, while only patients with atopic dermatitis had *TGF-beta1/TC* and *TNF-alpha/AA* haplotype (Table 3).

Cytokine Diplotypes (Haplotype Zygosity)

Cytokine diplotypes (haplotype zygosity), Pearson's *p*-value, crude odds ratio and Wald's 95% confidence interval for each SNP in the patients with AD and normal Macedonian population are shown in table 4.

Positive (susceptible) association between the patients with AD and following diplotypes was found (according to the level of susceptibility): *IL-4/TCT:TTT*, *IL-10/ACA:GCC*, *TGF-beta1/CC:CG*, *IL-4/GCT:TTT*, and *IL-2/TT:TT* (Table 4).

Table 1. Cytokine allele frequency, Pearson's *p*-value, Odds ratio and Wald's 95% confidence interval in patients with atopic dermatitis and normal Macedonian population

Cytokine Polymorphism	Allele	AD (n=66)		Control (n=301)		Pearson's *p*-value	Odds ratio	Wald' 95% CI
		N	F	N	F			
IL-1alpha -889	*C*	90	0.682	482	0.814	**<0.001**[a]	0.489	0.321-0.745
	T	42	0.318	110	0.186		2.045	1.343-3.115
IL-1beta -511	*C*	80	0.606	404	0.671	0.153	0.754	0.513-1.112
	T	52	0.394	198	0.329		1.326	0.899-1.956
IL-1beta +3962	*C*	102	0.785	439	0.729	0.192	1.353	0.858-2.132
	T	28	0.215	163	0.270		0.739	0.469-1.166
IL-1R pstl1970	*C*	84	0.646	399	0.662	0.717	0.929	0.625-1.382
	T	46	0.354	203	0.337		1.076	0.724-1.601
IL-1RA mspa11100	*T*	94	0.712	420	0.698	0.743	1.072	0.708-1.623
	C	38	0.288	182	0.302		0.933	0.616-1.413
IL-4R alpha +1902	*A*	111	0.841	502	0.834	0.844	1.053	0.630-1.760
	G	21	0.159	100	0.166		0.950	0.568-1.587
IL-12 -1188	*A*	77	0.837	433	0.744	0.053	1.766	0.985-3.167
	C	15	0.163	149	0.256		0.566	0.316-1.015
IFN gamma +874	*T*	76	0.585	259	0.520	0.189	1.299	0.879-1.919
	A	54	0.415	239	0.480		0.770	0.521-1.138
TGF-beta1 cdn10	*T*	55	0.444	282	0.502	0.240	0.791	0.535-1.170
	C	69	0.556	280	0.498		1.264	0.855-1.868
TGF-beta1 cdn25	*G*	97	0.782	532	0.947	**<0.001**[a]	0.203	0.115-0.356
	C	27	0.218	30	0.053		4.936	2.811-8.669
TNF-alpha -308	*A*	16	0.123	74	0.123	0.996	1.001	0.562-1.783
	G	114	0.877	528	0.877		0.999	0.561-1.778
TNF-alpha -238	*A*	3	0.023	27	0.045	0.256	0.503	0.150-1.684
	G	127	0.977	575	0.955		1.988	0.594-6.654
IL-2 -330	*G*	41	0.315	191	0.332	0.704	0.924	0.614-1.390
	T	89	0.685	383	0.667		1.083	0.719-1.629
IL-2 +166	*G*	88	0.677	422	0.735	0.179	0.755	0.500-1.139
	T	42	0.323	152	0.264		1.325	0.878-2.000
IL-4 -1098	*G*	7	0.130	176	0.308	**<0.001**[a]	0.192	0.068-0.540
	T	47	0.870	396	0.692		5.222	1.853-14.718
IL-4 -590	*C*	37	0.685	377	0.659	0.699	1.126	0.618-2.051
	T	17	0.315	195	0.341		0.888	0.488-1.618
IL-4 -33	*C*	24	0.444	479	0.837	**<0.001**[a]	0.155	0.087-0.278
	T	30	0.556	93	0.163		6.438	3.602-11.509
IL-6 -174	*C*	41	0.315	182	0.302	0.769	1.063	0.706-1.600
	G	89	0.685	420	0.698		0.941	0.625-1.416
IL-6 nt565	*A*	41	0.315	173	0.287	0.524	1.142	0.758-1.721
	G	89	0.685	429	0.713		0.875	0.581-1.319
IL-10 -1082	*A*	71	0.538	352	0.589	0.285	0.813	0.557-1.188
	G	61	0.462	246	0.411		1.299	0.842-1.796
IL-10 -819	*C*	105	0.795	435	0.727	0.107	1.457	0.920-2.308
	T	27	0.205	163	0.272		0.686	0.433-1.087
IL-10 -592	*A*	33	0.250	173	0.289	0.365	0.819	0.532-1.261
	C	99	0.750	425	0.710		1.221	0.793-1.881

N= Absolute number; F=Frequency; CI=Confidence Interval; [a] Statistically Significant.

Table 2. Cytokine genotype frequency, Pearson's *p*-value, Odds ratio and Wald's 95% confidence interval in patients with atopic dermatitis and normal Macedonian population

Cytokine polymorphism	Genotype	AD (n=66)		Control (n=301)		Pearson's *p*-value	Odds ratio	Wald' 95% CI
		N	F	N	F			
IL-1 alpha-889	*C:C*	24	0.364	204	0.689	**<0.001** [a]	0.258	0.147-0.451
	C:T	42	0.636	74	0.250	**<0.001** [a]	5.250	2.980-9.250
	T:T	0	/	18	0.061	§	§	§
IL-1 beta -511	*C:C*	20	0.303	143	0.475	**0.011** [a]	0.480	0.271-0.851
	C:T	40	0.606	118	0.392	**0.001** [a]	2.386	1.383-4.116
	T:T	6	0.091	40	0.133	0.351	0.653	0.265-1.609
IL-1 beta +3962	*C:C*	37	0.569	174	0.578	0.896	0.965	0.561-1.658
	C:T	28	0.431	91	0.302	**0.045** [a]	1.764	1.008-3.024
	T:T	0	/	36	0.120	§	§	§
IL-1R pstl1970	*C:C*	25	0.385	133	0.442	0.398	0.790	0.456-1.367
	C:T	34	0.523	133	0.442	0.233	1.385	0.810-2.371
	T:T	6	0.092	35	0.116	0.578	0.773	0.311-1.922
IL-1RA mspa11100	*C:C*	4	0.061	30	0.100	0.322	0.583	0.198-1.715
	C:T	30	0.454	122	0.405	0.462	1.223	0.715-2.091
	T:T	32	0.485	149	0.495	0.881	0.960	0.563-1.636
IL-4R alpha +1902	*A:A*	48	0.727	212	0.704	0.710	1.120	0.617-2.031
	A:G	15	0.227	78	0.259	0.590	0.841	0.448-1.580
	G:G	3	0.046	11	0.037	0.732	1.255	0.340-4.631
IL-12 -1188	*A:A*	33	0.717	160	0.550	**0.033** [a]	2.078	1.051-4.111
	A:C	11	0.239	113	0.388	0.051	0.495	0.242-1.014
	C:C	2	0.044	18	0.062	0.624	0.689	0.155-3.075
IFN gamma +874	*A:A*	8	0.123	64	0.257	**0.022** [a]	0.406	0.184-0.896
	A:T	38	0.585	111	0.446	**0.046** [a]	1.750	1.007-3.042
	T:T	19	0.292	74	0.297	0.934	0.977	0.536-1.779
TGF-beta1 cdn10	*C:C*	21	0.339	65	0.231	0.077	1.702	0.939-3.085
	C:T	27	0.435	150	0.534	0.161	0.674	0.387-1.723
	T:T	14	0.226	66	0.235	0.879	0.970	0.493-1.831
TGF-beta1 cdn25	*C:G*	25	0.403	30	0.107	**<0.001** [a]	5.653	3.001-10.648
	G:G	36	0.581	251	0.893	**<0.001** [a]	0.166	0.088-0.311
	C:C	1	0.016	0	/	§	§	§
TNF-alpha -308	*A:G*	14	0.216	66	0.219	0.945	0.977	0.510-1.875
	G:G	50	0.769	231	0.768	0.975	1.010	0.535-1.908
	A:A	1	0.015	4	0.013	0.895	1.160	0.128-10.554
TNF-alpha -238	*A:G*	3	0.046	23	0.076	0.389	0.585	0.177-2.009
	G:G	62	0.954	276	0.917	0.310	1.872	0.548-6.397
	A:A	0	/	2	0.007	§	§	§
IL-2 -330	*G:G*	5	0.077	27	0.094	0.664	0.803	0.297-2.170
	G:T	31	0.477	137	0.477	0.995	0.998	0.582-1.711
	T:T	29	0.446	123	0.429	0.796	1.074	0.625-1.845
IL-2 +166	*G:G*	36	0.554	162	0.565	0.876	0.958	0.557-1.647
	G:T	16	0.246	98	0.341	0.138	0.630	0.341-1.165
	T:T	13	0.200	27	0.094	**0.015** [a]	2.407	1.165-4.973
IL-4 -1098	*G:T*	5	0.185	174	0.608	**<0.001** [a]	0.146	0.054-0.398
	T:T	21	0.778	111	0.388	**<0.001** [a]	5.518	2.160-14.097
	G:G	1	0.037	1	0.004	**0.037** [a]	10.962	0.666-180.401
IL-4 -590	*C:C*	11	0.407	95	0.332	0.430	1.382	0.617-3.095
	C:T	15	0.556	187	0.654	0.307	0.661	0.298-1.469
	T:T	1	0.037	4	0.014	0.361	2.712	0.292-25.163
IL-4 -33	*C:C*	11	0.407	209	0.731	**<0.001** [a]	0.253	0.113-0.570

	C:T	2	0.074	61	0.213	0.085	0.295	0.068-1.281
	T:T	14	0.519	16	0.056	**<0.001** [a]	18.173	7.331-55.052
IL-6 -174	*C:C*	9	0.138	25	0.083	0.138	1.840	0.814-4.160
	C:G	23	0.354	132	0.439	0.210	0.701	0.402-1.224
	G:G	33	0.508	144	0.478	0.668	1.124	0.658-1.922
IL-6 nt565	*A:A*	9	0.138	25	0.083	0.163	1.774	0.786-4.006
	A:G	23	0.354	123	0.409	0.413	0.793	0.454-1.385
	G:G	33	0.508	153	0.508	0.993	0.996	0.584-1.705
IL-10 -1082	*A:A*	8	0.121	70	0.234	**0.043** [a]	0.451	0.206-0.991
	A:G	55	0.833	212	0.709	**0.039**[a]	2.052	1.025-4.107
	G:G	3	0.046	17	0.057	0.713	0.790	0.225-2.778
IL-10 -819	*C:C*	39	0.591	155	0.518	0.285	1.342	0.782-2.304
	C:T	27	0.409	125	0.418	0.894	0.964	0.561-1.657
	T:T	0	/	19	0.064	§	§	§
IL-10 -592	*A:A*	3	0.046	28	0.094	0.204	0.461	0.136-1.564
	A:C	27	0.409	117	0.391	0.789	1.077	0.626-1.853
	C:C	36	0.545	154	0.515	0.655	1.130	0.662-1.929

N= Absolute number; F=Frequency; CI=Confidence Interval; &, cannot be calculated because expected <5, $\chi 2$ test; [a], Statistically Significant

Table 3. Haplotype frequency of cytokine polymorphism, Pearson's *p*-value, Odds ratio and Wald's 95% confidence interval in patients with atopic dermatitis and normal Macedonian population

Cytokine polymorphism	Haplotype	AD (n=66)		Control (n=301)		Pearson's *p*-value	Odds ratio	Wald' 95% CI
		N	F	N	F			
TGF-beta1	*CC*	20	0.161	30	0.053	**<0.001** [a]	3.410	1.865-6.236
	CG	49	0.395	250	0.445	0.313	0.815	0.548-1.212
	TG	48	0.387	282	0.502	**0.021** [a]	0.627	0.422-0.933
	TC	7	0.057	/	/	§	§	§
TNF-alpha	*AG*	14	0.108	74	0.123	0.628	0.861	0.470-1.578
	GA	1	0.008	26	0.043	0.051	0.172	0.023-1.277
	GG	113	0.869	502	0.834	0.319	1.324	0.762-2.303
	AA	2	0.015	/	/	§	§	§
IL-2	*GG*	39	0.300	178	0.310	0.822	0.954	0.630-1.444
	GT	2	0.015	14	0.024	0.534	0.625	0.140-2.784
	TG	49	0.377	244	0.425	0.314	0.818	0.553-1.210
	TT	40	0.308	138	0.240	0.111	1.404	0.924-2.135
IL-4	*GCC*	2	0.037	163	0.285	**<0.001** [a]	0.093	0.022-0.386
	GCT	4	0.074	8	0.014	**0.003** [a]	5.423	1.580-18.617
	GTC	/	/	4	0.007	§	§	§
	GTT	1	0.019	1	0.002	**0.037** [a]	10.744	0.664-174.724
	TCC	22	0.407	202	0.353	0.427	1.259	0.713-2.225
	TCT	9	0.167	4	0.007	**<0.001** [a]	28.400	8.416-95.841
	TTC	/	/	110	0.192	§	§	§
	TTT	16	0.296	80	0.140	**0.002** [a]	2.590	1.379-4.863
IL-6	*CA*	40	0.307	172	0.286	0.616	1.111	0.736-1.678
	CG	1	0.008	9	0.150	0.518	0.511	0.064-4.067
	GG	88	0.677	420	0.698	0.641	0.908	0.605-1.364
	GA	1	0.008	1	0.002	0.232	4.659	0.290-74.975
IL-10	*ACA*	7	0.053	12	0.020	**0.031** [a]	2.735	1.056-7.085
	ACC	35	0.265	177	0.296	0.480	0.858	0.561-1.312
	ATA	27	0.205	161	0.269	0.124	0.698	0.441-1.106
	ATC	1	0.007	2	0.003	0.492	2.275	0.205-25.276
	GCC	62	0.470	246	0.412	0.219	1.267	0.868-1.851

N= Absolute number; F=Frequency; CI=Confidence Interval; &, cannot be calculated because expected <5, $\chi 2$ test; [a], Statistically Significant.

Table 4. Cytokine diplotypes (haplotype zygotes), Pearson's *p*-value, Odds ratio and Wald's 95% confidence interval in patients with atopic dermatitis and normal Macedonian population

Citokine Polymorphism	Genotype	AD (n=75)		Control (n=301)		Pearson's *p*-value	Odds ratio	Wald' 95% CI
		N	F	N	F			
TGF- beta1	*CC:CG*	15	0.242	16	0.057	**<0.001** [a]	5.286	2.448-11.413
	CC:TG	3	0.048	14	0.050	0.962	0.970	0.270-3.482
	CG:CG	5	0.081	49	0.174	0.067	0.415	0.158-1.090
	CG:TG	17	0.274	136	0.484	**0.002** [a]	0.403	0.220-0.738
	TG:TG	14	0.226	66	0.235	0.879	0.950	0.493-1.831
	CG:TC	7	0.113	0	/	§	§	§
	CC:CC	1	0.016	/	/	§	§	§
TNF-alpha	*AG:GG*	12	0.185	66	0.219	0.536	0.806	0.407-1.597
	GA:GG	1	0.015	24	0.080	0.062	0.180	0.024-1.358
	GG:GG	49	0.754	206	0.684	0.269	1.412	0.764-2.611
	AG:AG	1	0.015	4	0.013	0.895	1.160	0.128-10.554
	GG:AA	2	0.031	0	/	§	§	§
	GA:GA	/	/	1	0.004	§	§	§
IL-2	*GG:GG*	5	0.077	27	0.094	0.664	0.803	0.297-2.170
	GG:TG	21	0.324	85	0.296	0.669	1.134	0.636-2.022
	GG:TT	8	0.123	38	0.133	0.840	0.920	0.407-2.078
	GT:TG	1	0.015	11	0.058	0.357	0.392	0.050-3.092
	TG:TG	10	0.154	50	0.174	0.693	0.862	0.411-1.806
	TG:TT	7	0.108	48	0.168	0.232	0.601	0.259-1.397
	TT:TT	12	0.185	25	0.087	**0.021** [a]	2.373	1.122-5.018
	GT:GG	/	§	1	0.003	§	§	§
	GT:TT	1	0.015	2	0.007	0.505	2.227	0.199-24.394
IL-4	*GCC:GCC*	/	/	1	0.003	§	§	§
	GCC:TCC	1	0.037	26	0.091	0.341	0.385	0.050-2.951
	GCC:TTC	/	/	103	0.360	§	§	§
	GCC:TTT	1	0.037	32	0.112	0.226	0.305	0.040-2.327
	TCC:TCC	10	0.370	68	0.238	0.128	1.886	0.825-4.313
	TCC:TTC	/	/	7	0.025	§	§	§
	TCC:TTT	1	0.037	28	0.098	0.297	0.354	0.046-2.712
	TTT:TTT	1	0.037	4	0.014	0.361	2.712	0.292-25.163
	GCT:TTT	3	0.111	8	0.028	**0.025** [a]	4.343	1.081-17.455
	GTC:TTC	/	/	4	0.014	§	§	§
	TCT:TTT	9	0.334	4	0.014	**<0.001** [a]	35.25	9.894-125.589
	GTT:TTC	/	/	1	0.003	§	§	§
	GTT:GCT	1	0.037	0	/	§	§	§
IL-6	*CA:CA*	9	0.139	25	0.083	0.163	1.774	0.786-4.006
	CA:GG	22	0.339	122	0.405	0.317	0.751	0.428-1.318
	CG:GG	1	0.015	9	0.030	0.515	0.507	0.063-4.073
	GG:GG	32	0.492	144	0.479	0.839	1.057	0.618-1.808
	GA:GG	1	0.015	1	0.003	0.232	4.688	0.289-75.93
IL-10	*ACC:ACC*	1	0.015	21	0.070	0.089	0.204	0.027-1.542
	ACC:ATA	4	0.061	21	0.070	0.779	0.854	0.283-2.576
	ACC:GCC	29	0.439	114	0.381	0.381	1.272	0.742-2.181
	ATA:ATA	/	/	19	0.064	§	§	§
	ATA:GCC	20	0.303	93	0.311	0.897	0.963	0.540-1.719
	GCC:GCC	4	0.061	17	0.057	0.906	1.070	0.348-3.291
	ACA :GCC	4	0.061	3	0.010	**0.007** [a]	6.366	1.390-29.157
	ACA :ATA	3	0.045	9	0.030	0.527	1.534	0.404-5.829
	ATC :GCC	1	0.015	2	0.007	0.491	2.285	0.204-25.576

N= Absolute number; F=Frequency; CI=Confidence Interval; &, cannot be calculated because expected <5, $\chi 2$ test; [a], Statistically Significant

Negative (protective) association between patients with AD and following diplotype (according to the protective level) was found for *TGF-beta1/CG:TG* (Table 4).

Diplotypes *TNF-alpha/GA:GA, IL-2/GT:GG, IL-4/GCC:GCC, IL-4/GCC:TCC, IL-4/TCC:TTC, IL-4/GTC:TTC, IL-4/GTT:TTC* and *IL-10/ATA:ATA* were present only in normal Macedonian population, while diplotypes *TGF-beta1/CG:TC, TGF-beta1/CC:CC, TNF-alpha/GG:AA,* and *IL-4/GTT:GCT* had only patients with AD (Table 4).

Summary of all susceptible and protective cytokine polymorphisms for atopic dermatitis in Macedonian population are presented in table 5. If odds ratio showed a significant value above 1.000 we indicate that positive or susceptible association exists, and if the odds ratio showed a significant value below 1.000 then negative or protective association exists.

From the table 5 we can see that the highest number of cytokine genotypes (11 of them) are susceptible for atopic dermatitis with biggest odds ratio of 18.173 for *IL-4 -33/T:T*, and more than three times bigger risk (p<0.001) for *IL-4 -1098/G:G* (OR=10.962), *TGF-beta1 cdn25/C:G* (OR=5.653); *IL-4 -1089/T:T* (OR=5.518), and *IL-1alfa -889/C:T* (OR=5.250). Five cytokine diplotypes, six cytokine haplotypes, and four cytokine alleles were found to be positively (susceptible) associated with AD (Table 5).

At the same time protective cytokine polymorphisms regarding atopic dermatitis for seven cytokine genotypes, one cytokine diplotype, two cytokine haplotypes, and four cytokine alleles were found. Most of the negative (protective) associations with AD were at very high protective levels (p<0.001) (Table 5).

Table 5. Summary of all susceptible and protective cytokine polymorphisms for atopic dermatitis in Macedonian population

	Susceptible			**Protective**		
	Polymorphism	***p***	**Odds ratio**	**Polymorphism**	***p***	**Odds ratio**
Cytokine	*IL-4 -33/T*	<0.001	6.438	*IL-4 -33/C*	<0.001	0.155
Alleles	*IL-4 -1098/T*	<0.001	5.222	*IL-4 -1098/G*	<0.001	0.192
	TGF-beta1 cdn25/C	<0.001	4.936	*TGF-beta1 cdn25/G*	<0.001	0.203
	IL-1 alpha -889/T	<0.001	2.045	*IL-1 alpha -889/C*	<0.001	0.489
Cytokine	*IL-4 -33/T:T*	<0.001	18.173	*IL-4 1098/G:T*	<0.001	0.146
Genotypes	*IL-4 -1098/G:G*	0.037	10.962	*TGF-beta1 cdn25/G:G*	<0.001	0.166
	TGF-beta1 cdn25/C:G	<0.001	5.653	*IL-4 -33/C:C*	<0.001	0.253
	IL-4 -1098/T:T	<0.001	5.518	*IL-1alpha -889/C:C*	<0.001	0.258
	IL-1 alpha -889/C:T	<0.001	5.250	*IFN-gamma +874/A:A*	0.022	0.406
	IL-2 +166T:T	0.015	2.407	*IL-10 -1082/A:A*	0.043	0.451
	IL-1 beta -511/C:T	<0.001	2.386	*IL-1 beta -511/C:C*	0.011	0.480
	IL-12 -1188/A:A	0.033	2.078			
	IL-10 -1082/A:G	0.039	2.052			
	IL-1 beta +3962/C:T	0.045	1.764			
	IFNgamma +874/A:T	0.046	1.750			
Cytokine	*IL-4/TCT*	<0.001	28.400	*IL-4/GCC*	<0.001	0.093
Haplotypes	*IL-4/GTT*	0.037	10.774	*TGF-beta1/TG*	0.021	0.627
	IL-4/GCT	0.003	5.423			
	TGF-beta1/CC	<0.001	3.410			
	IL-10/ACA	0.031	2.735			
	IL-4/TTT	0.002	2.590			
Cytokine	*IL-4/TCT:TTT*	<0.001	35.25	*TGF-beta1/CG:TG*	0.002	0.403
Diplotypes	*IL-10/ACA:GCC*	0.007	6.366			
(Haplotype	*TGF-beta1/CC:CG*	<0.001	5.286			
Zygosity)	*IL-4/GCT:TTT*	0.025	4.343			
	IL-2/TT:TT	0.021	2.373			

DISCUSSION

Our results of 22 cytokine polymorphisms in patients with AD and in normal Macedonian population are presented in this paper.

The *IL-1alpha*, *IL-1beta* and *IL-1RA* (IL-1 receptor antagonist) genes are clustered within a 430-kb interval on chromosome 2q14.[14] IL-1 is known to be one of the key inflammatory mediators in primary response to microbes. The genetic variability within the *IL-1* gene complex could therefore affect this agonist/antagonist balance directly or via stimulation of other inflammatory cytokines. *IL-1alpha* and *IL-1beta* map on chromosome 2q14.

In the present study, we have tested AD for association with five different markers within the IL-1 cluster: *IL-1alpha -889, IL-1beta -511, IL-1beta +3962, IL-1R pstl1970* and *IL-1Ra mspa11100*. The *-899T>C* SNP of the *IL-1alpha* gene was not associated with AD risk.[15] No association was found between either the *-511C>T, 3953T>C, 3953T>C, -1418T>C* or the *315T>C* SNPs of the *IL-1beta* gene and AD.[16,17] Cindy showed protective role of *T* allele of 889 SNP of *IL-1alpha* gene in patients with contact iritative dermatitis. The reduced cutaneous level of the pro-inflammatory cytokine *IL-1alpha* associated with the *IL-1alpha -889T* allele explains a protective effect of this allele towards hand dermatitis.[18]

However, AD is associated with elevated skin production of Th2 cytokines and low levels of proinflammatory cytokines such as TNF-alpha, IFN-gamma, and IL-1beta. Decreased expression of antimicrobial genes occurs as the result of local up-regulation of Th2 cytokines and the lack of elevated amounts of TNF-alpha and IFN-gamma under inflammatory conditions in AD skin could explain the increased susceptibility of AD skin to microorganisms and severe score.[19]

We found the protective role of *C* allele of *-889* SPN *IL-1alpha* gene which is confirmed by the presence of *C:C* genotype. Genotype *IL-1alfa -889/T:T* is present only in normal Macedonian population with very small frequency. In this study we could not demonstrate association between *IL-1beta* alleles and AD. Analyzing the genotypes, we found the protective association between *IL-1beta-511/C:C* and AD while *IL-1beta -511/C:T* and *IL-1beta +3962/C:T* genotypes showed positive association. *IL-1beta +3962/T:T* genotype was present only in normal Macedonian population. Our investigation did not show any association between AD and *IL-1R psti1970* and *IL-1RA mspa1110* polymorphisms (alleles or genotypes). The dominance of *C* allele of *IL-1alpha* and *IL-1beta* gene and its protective role can explains the low incidence and no so severe AD in Macedonian population.

The *IL12B* gene maps on chromosome 5q31-33 encodes the p40 subunit of interleukin 12, an immunomodulatory cytokine that is the primary inducer of the development of T-helper 1 (Th1) cells, with down regulation of T-helper2 (Th2) cytokines. Several SNP of *IL12B* gene are evaluated. The *4237G>A, 4496A>G* and *4510G>A* SNPs of the *IL12B* gene did not contribute to the development of AD.[20] Takahashi showed that in individuals with the *-111T:T* genotype, reduced *IL-12R beta1* expression may lead to increased Th2 cytokine production in the skin and contribute to the development of AD and other subsequent allergic diseases.[21] The rs582504 (*IVS-798A/T*) SNP and the haplotype *TA* (rs582054 and rs2243151) in the *IL12A* gene, were significantly associated with the AD phenotype in Koreans population.[22]

Our data showed no associations between AD and *IL-12-1188* allele polymorphisms but *A: A* genotype was associated with increased risk of AD. Contrary to our data, Tsunemi showed that the *A:A* genotype of *IL12B -1188A>C* SNP was associated with decreased risk of AD in a Japanese population.[23] Although the *IL-12 -1188/A* allele is predominant in our population, *A:A* genotype is also with increased frequency in Macedonian patients with asthma but it is not significant.[24]

IFN-gamma maps on chromosome 12q14. Patients with atopic dermatitis have reduced production of IFN-gamma which correlates with the severity of the disease. Although there is no association between AD and IFN-gamma alleles, the analysis of genotypes showed significant susceptible association of *IFN-gamma +874/A:T* genotype and protective role of *A:A* genotype, contrary to Hussein who showed significant association between genotype and the frequency of the *A* allele of the *+874T/A* polymorphism in atopic patients.[25] Pravica *et al.*[26] reported that or the *+874T/A* SPN of human IFN-gamma gene directly affects the level of IFN-gamma production and correlates with the presence of the *A874* allele and low production of IFN-

gamma. Ohly[27] revealed that there was no significant association between the *+874T/A* polymorphism and IgE level in atopic German newborns. In a Chinese population, there was no association between short tandem repeats at the first intron of IFN-gamma gene and AD.[20] We found the protective role of *IFN-gamma +874/A:A* genotype in our patients with AD, contrary to findings of Hussein and Pravica.[25,26] Further investigations of gene-gene and gene-environment are needed.

TGF-beta1 map on chromosome 19q13.2 is a T regulatory cytokine that regulate Th1 and Th2 immune response. A significant expression of Tr1, their suppressive cytokines IL-10 and TGF-beta as well as their receptors have been observed in lesional and eczematous lesions of atopy patch tests, whereas nT regs were not detectable.[28] The secreted IFN-gamma induces apoptosis of keratinocytes, leading eventually to the eczematous lesions characteristic of AD.[29] Patients with AD have decreased level of TGF-beta. Several studies have analyzed the association between polymorphisms of transforming growth factor-beta1 (*TGFbeta1*) gene and AD. No association between AD and the *-590C>T* SNP was observed.[15] We did not find any significant association in *TGFbeta1 codon 10* frequencies of alleles and genotypes. Our results demonstrated susceptible effect of *TGFbeta1 codon 25/C* allele, *TGFbeta1 cdn25/C: G* genotype, *TGFbeta1/CC* haplotype and *TGFbeta1/CC:CG* diplotype. Protective effect was found for *TGFbeta1 cdn25/G* allele, *TGFbeta1 cdn25/G:G* genotype, *TGFbeta1/TG* haplotype and *TGFbeta1/CG:TG* diplotype. *TGFbeta1 cdn25/C:C*, genotype is not present in normal Macedonian population. The frequency of *TGFbeta1 cdn25/G* allele is very high (97%) in healthy Macedonian population which indicate common "wild type" allele of this cytokine. All these data support that *C* allele of *TGFbeta1 codon 25* polymorphism is associated with an increased risk of AD. Similar data had Arkwright in his study.[30] The *C* allele (a low *TGFbeta1* producer allele) of the *TGFbeta1 915G>C* SNP of codon 25 was associated with an increased risk of AD (OR = 4.8, 95% CI = 2.4-9.7) while there was no statistical significant difference in the frequencies of the *869T>C* genotypes (codon 10).[30]

Many authors investigated the role of TNF-alpha in the pathogenesis of AD. Our results did not demonstrated association of *TNF-alpha -238* as well as *TNF-alpha -308* SPN with AD. TNF-alpha and TNF-beta share a common receptor on tumour cells whose expression is upregulated by gamma-interferon. *TNF* maps on chromosome 6p21.3. No significant association was found between *-308G>A* SNP of the *TNF-alpha* gene and AD.[17] Neither *-1031T>C, -863C>A, -857C>T, -308G>A,* nor *-238G>A* SNPs of the *TNF-alpha* gene was associated with AD in a Chinese population.[20] No association was found between AD and *-238G>A* and *-308G>A* SNPs of the *TNF-beta* gene in a German population.[16] Also, the two SNPs were not linked to AD risk in Americans.[15]

Th1 cytokines such as interferon (IFN-gamma), IL-12, and IL-2 are thought to have an inhibitory effect on Th2 cells and decrease the amount of IL-4, IL-5, IL-13 and IgE production. We found susceptible association between AD and *IL-2 +166/T:T* genotype, and *IL-2/TT:TT* diplotype. It supports our previous findings in patients with asthma.[24] We can conclude that patients homozygous for *T* allele are more susceptible for allergic disease than those homozygous and/or heterozygous for *G* allele.

IL-4 induces the differentiation of Th2 cells from naïve CD4+ precursors, IgE isotype switching in B cells together with IL-13 [31] , alters the homeostasis of the skin and makes Langerhans cells more efficient in taking up and processing naïve proteins [32], upregulates the expression of FcεRI and skin homing molecules on DCs and suppresses interferon-dependent macrophage functions. *IL-4* maps on chromosome 5q31.1. In this study, we investigated alleles and genotypes of three polymorphisms of *IL-4* (at the positions *-1098, - 590,* and -33) and one of *IL-4RA* (at position *-1908*), as well as haplotypes and diplotypes of investigated polymorphisms. The results showed protective association of DA with *IL-4 -1098/G* allele and *IL-4 -33/C* allele. We found protective association between DA and two heterozygous genotype polymorphisms of *IL-4 (IL-4 - 1098/G: G* and *IL-4 -33/C:C*). *IL-4 -590* SPN did not show association with AD. We also found protective association between AD and *IL-4/GCC* haplotypes, as well as susceptible association with *IL-4/TCT, IL-4/GTT, IL-4/GCT, IL-4/TTT* haplotype. From the diplotypes analysis, we can see that combination of *IL- 4* haplotypes *IL-4/TCT:TTT* and *IL-4/GCT:TTT* has the susceptible association with DA.

Our results support the findings that are confirmed in several studies. No association was found between

AD and *-33, +166* and *-590* SPN of *IL-4* gene in Americans, Chinese, Australians and Czech population.[16,20,33,34] However, the *T* allele of *-590T>C* SNP was associated with an increased risk of AD in a Japanese population.[35] In Caucasians the *T* allele of *IL-4 -589C>T* SNP was significantly associated with the development of AD at 24 months of age.[36] A case-control comparison suggested a genotypic association of the *T:T* genotype with AD.[37]

A significantly preferential transmission to AD offspring of the *T* allele of the *IL-4 –590* gene polymorphism was reported. Because the *T* allele is associated with increased *IL-4* gene promoter activity compared with the *C* allele, the data suggest that genetic differences in transcriptional activity of the *IL-4* gene influence AD predisposition.[37] In severe AD, an allele for the *IL-4Ra* subunit that segregates with atopy was found; the *R576* allele of *IL-4R*a being strongly associated with AD may predispose persons to allergic diseases by enhancing *IL-4R* signalling function.[38] On the whole, the data suggest that *IL-4* gene expression plays a crucial role in AD pathogenesis.

Our results showed no significant differences of *IL-4Ra +1902* frequency at allele and genotype level. These results are in agreement with others. No association between SNPs (*1199C>A, 1242T>G, 1507C>T* and *1727G>A*) of *IL-4R* and AD was found in a Chinese population.[20]

However *IL-4* and the *IL-4 receptor alpha* chain genotypes (*IL-4 -589/C:T*, *Ile50Val, Ala375Glu* and *Arg551Gln* of *IL-4* receptor alpha chain) were not significantly associated with either total patients with atopic eczema or atopic eczema patients who had normal IgE productivity.[39]

Receptors for IL-4 and IL-13 shared common alpha chain that is coded with the gene of IL-4RA. *IL-4R* maps on chromosome 16p11.2-12.1. Many SNPs *(-3112C>T, -1803T>C, -327C>A, -326A>C* and *-186G>A*) or haplotypes (alpha) of the *IL-4R* gene are associated with AD.[40] Seven SNPs (*223C>G, 223T>A, 1199C>A, 1291C>T, 1307T>C, 1727G>A, 2356C>T*) and a silent *1242T>G* have been demonstrated to have functional significance. Caucasian children with the rare homozygous *1727G>A* polymorphism had a higher prevalence of flexural eczema in the first 6 months compared with the heterozygote and the wild type homozygote genotypes combined.[41] It has been demonstrated that the *1727G>A* SNP was significantly associated with AD in another Japanese population.[42]

IL6 maps on chromosome 7p21. No association was found between the *-174C>G* SNP of the *IL6* gene and AD in German population.[16] Similarly, the *-174C>G* and *-922A>G* SNPs were not linked to AD.[15] We investigated the association for two polymorphisms of *IL-6 -174* and *IL-6 nt565*. Our data did not show any association between those polymorphisms and AD.

Interleukin-10 (IL-10) is anti-inflammatory cytokine that reduces production of pro-inflammatory cytokines during inflammatory responses. We did not find any association between the *IL-10* alleles (at the positions *-1082, -819,* and *-592*) and AD. Analysis of genotypes showed significant protective associated between AD and *IL-10 -1082/A:A* genotype, and positive (susceptible) association with *IL-10 -1082/A:G* genotype. *IL-10* genotypes at the locations *-819,* and *-592* were not significantly association with AD. Neither *IL-10* haplotypes showed associated with BA. One haplotype combination of *IL-10* (diplotype or haplotype zygozity) was negatively associated with AD IL-*10/ACC:GCC*. Some data suggested that there is no association between *IL-10 -1082 SPN* polymorphisms and AD,[16,20,30,43] while other data claimed the opposite for *IL-10 -819 and -33 SPN*.[43] Significant risk of AD with *IL-10 -1082/A:G* and *IL-4 -819/C:C* genotype was found in Czech population.[33]

IL-10 and transforming growth factor beta1 (*TGF-beta1*) are immunosuppressive cytokines that inhibit the activity of both Th cell types in human subjects. Human skin keratinocytes can be stimulated to produce *TGF-beta1* but not IL-10. It is possible that a relative deficiency of *TGF-beta1* in the skin results in the immune response characteristic of AD. The inability of skin keratinocytes to produce IL-10 provides one explanation for the lack of a correlation between polymorphisms in this cytokine gene and atopic eczema.[30]

Inconsistent results obtained from various authors highlight the genetic role among different ethnic groups. Because the complex-trait diseases, like AD, are influenced not only by genetic factor but by gene-gene and gene-environment interaction as well, it is possible that different ethnic groups will show association with different cytokine polymorphisms.

Our data showed that the Macedonian healthy population have protective alleles and genotypes of cytokine genes that are involved in initiate of initiation immune response (IL-1alpha and IL-1beta) as well Th2 immune response (IL-4) and T regulatory

cytokines (TGF- beta). All those protective alleles have frequency higher than 70% as well the protective genotypes (frequency higher than 65%). Although *A* allele of *IL-10 -1089* gene is in balanced selection, the protective *AA* genotype is less frequent in our healthy population. The polymorphisms of Th1 cytokines genes show higher frequency of protective allele and genotype of *IL-2 -166*, and lower frequency of protective genotypes of *IL-12* and *INF-gamma* genes.

CONCLUSION

Susceptible cytokine polymorphisms for AD for eleven genotypes *(IL-4-33/T:T IL-4-1098/G:G, TGF-beta cdn25C:G, IL-4 -1098/T:T, IL-1alpha -889/C:T, IL-2 +166/T:T, IL-1beta -511/C:T, IL-12 -1188/C:T, IL-10 -1082/A:G, IL-1beta +3962/C:T, IFN-gamma +874/A:T),* five diplotypes, six haplotypes, and for alleles were found. Protective cytokine polymorphisms for AD for seven cytokine genotypes *(IL-4 -1098/G:T, TGF-beta cdn25/G:G, IL-4 -33/C:C, IL-1alpha -889/C:C, IFN-gamma +874/A:A, IL-10 -1082/A:A, IL-1beta -511/C:C),* one cytokine diplotypes, two cytokine haplotypes, and four cytokine alleles were found.

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