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CLINICAL STUDY

Distribution of the 22 Cytokine Gene Polymorphisms in Healthy Macedonian Population

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Abstract: *Background:* Distribution of cytokine gene polymorphisms may vary significantly among different ethnic groups, and eventually contribute to observed differences in disease frequencies.
Objectives: To genotype 22 cytokine polymorphisms in the Macedonian population. The Macedonian population consists of 301 healthy unrelated individuals.
Methods: Blood samples were collected after written consent, DNA was isolated from peripheral blood, and 22 polymorphisms were typed: *IL-1α -889, IL-1β -511, IL-1β +3962, IL-1R psti1970, IL-1RN mspa11100, IL-4Rα +1902, IL-12 -1188, IFNγ utr5644, TGF-β1 cdn10, TGF-β1 cdn25, TNF-α -308, TNF-α -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.* Cytokine genotyping was performed by PCR-SSP (Heidelberg kit). The population genetics analysis package, PyPop, was used for analysis of the cytokine data.
Results: Test of neutrality (Fnd) showed negative value, but was significantly different from 0 for *TGF-β1 cdn10* and *IFNγ utr5644* (p of F = 0.001, and 0.012 respectively). Several SNPs *(IL-1α -889, IL-1β +3962, IL-2 +166, IL-4 -1098, IL-4 -590, IL-4 -33,* and *IL-10 -592)* were not in HWP (p 0.005). Test of neutrality for cytokine haplotypes *(TGF-β1, TNF-α, IL-2, IL-4, IL-6,* and *IL-10)* showed significantly difference from 0 only for *IL-2* haplotypes (p=0.020).
Conclusion: The results of cytokine polymorphisms in Macedonian population can be used for anthropological comparisons, as well as for association studies with different diseases *(Tab. 6, Ref. 34).* Full Text (Free, PDF) *www.bmj.sk.*
Key words: cytokine gene polymorphisms, SSP genotyping, Macedonian population.

Cytokines are soluble proteins or glycoproteins that modulate the activities of target cells via binding to specific receptor ligands. These in turn initiate the cytokine-specific signal transduction and second messenger pathways. The release of cytokines is fundamentally important in the immune regulation. Cytokines are produced by a wide range of immune cell and display a high degree of pleiotropism. Interaction between different cytokines leads to the formation of complex networks that initiate gene activation and suppression. Cytokines modulate both the cellular (Th1) and humoral (Th2) forms of the immune response. Th1 cell produced cytokines include the tumour necrosis factor alpha (TNFα), interleukin-2 (IL-2), and interferon gamma. These are in general pro-inflammatory cytokines that initiate T cell proliferation during the immune response. Th2 cell produced cytokines include IL-4, IL-6 and IL-10. These cytokines display a predominantly anti-inflammatory profile and play a key role in the initiation of the B-cell mediated immune response. Maintenance of the Th1/Th2 balance is crucial for the efficient functioning of the immune system. Any disruption of this balance could have major implications for the clinical course of many immune and infectious diseases. A differential response to cytokine stimulation can exert a strong control on the Th1/Th2 balance and hence affect the immune disease etiology (1).

It has been known for some time that cytokines and their receptors are often encoded by highly polymorphic genes. This polymorphism may be responsible for the observed inter-individual differences in cytokine production and may be one possible mechanism for the perturbation of the Th1/Th2 balance. In recent years the number of studies analyzing genes encoding polymorphism in cytokines and their receptors has increased greatly (2–4).

Distribution of cytokine gene polymorphisms may vary significantly among different ethnic groups, what could eventually contribute to the observed differences in disease incidence (5).

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Numerous SNPs in cytokine genes have been identified, and some of them are associated with qualitative or quantitative changes in protein production and susceptibility to various diseases, including autoimmune, infectious, allergic or cardiovascular diseases (for review see: 6–8). This fact has to be taken into consideration when using an association analysis for detecting gene polymorphisms that contribute to susceptibility or resistance to multifactorial diseases (9).

We published preliminary results of cytokine gene polymorphisms in 125 healthy Macedonians, but the number of participants was not sufficient for a precise analysis of haplotypes and diplotypes (haplotype zygosity), especially for the association analyses (10). The aim of this study was to present the data of the 22 cytokines gene polymorphism in 301 healthy Macedonians which can be used as a part of n anthropology report, and as a base for the studies of disease association.

Methods

Population

The Macedonian population included in this study consists of 301 healthy unrelated individuals, aged 20–35 years. All in-

Tab. 1. Frequencies of polymorphic alleles, test of neutrality with Fnd statistic (Ewens-Watterson test of neutrality (EWN)), and Slatkin's Exact P. Value (SEPV) with p of F statistics in Macedonian population.

Cytokine Polymorphism	Alleles			Test of Neutrality	
				EWN	SEPV
	Allele	Number	Frequency	Fnd	p of F
IL-1α -889	C	482	0.814	-0.967	0.214
	T	110	0.186		
IL-1β -511	C	404	0.671	-1.809	0.103
	T	198	0.329		
IL-1β +3962	C	439	0.729	-1.527	0.141
	T	163	0.271		
IL-1R psti1970	C	399	0.663	-1.843	0.096
	T	203	0.337		
IL-1RA mspa11100	T	420	0.698	-1.691	0.119
	C	182	0.302		
IL-4Rα +1902	A	502	0.834	-0.814	0.230
	G	100	0.166		
IL-12 -1188	A	433	0.744	-1.438	0.154
	C	149	0.256		
IFNγ utr5644	T	259	0.520	-2.127	0.012*
	A	239	0.480		
TGF-β1 cdn10	T	282	0.502	-2.152	0.001*
	C	280	0.498		
TGF-β1 cdn25	G	532	0.947	0.258	0.414
	C	30	0.053		
TNF-α -308	A	74	0.123	-0.443	0.282
	G	528	0.877		
TNF-α -238	A	27	0.045	0.343	0.437
	G	575	0.955		
IL-2 -330	G	191	0.332	-1.817	0.100
	T	383	0.667		
IL-2 +166	G	422	0.735	-1.487	0.147
	T	152	0.265		
IL-4 -1098	G	176	0.308	-1.708	0.117
	T	396	0.692		
IL-4 -590	C	377	0.659	-1.849	0.095
	T	195	0.341		
IL-4 -33	C	479	0.837	-0.779	0.235
	T	93	0.163		
IL-6 -174	C	182	0.302	-1.691	0.119
	G	420	0.698		
IL-6 565	A	173	0.287	-1.616	0.129
	G	429	0.713		
IL-10 -1082	A	352	0.589	-2.067	0.053
	G	246	0.411		
IL-10 -819	C	435	0.727	-1.537	0.139
	T	163	0.273		
IL-10 -592	A	173	0.289	-1.625	0.128
	C	425	0.711		

Statistically significant Fnd < 0 indicates a balancing selection; significant Fnd > 0 indicates a directional selection; *, Statistically significant.

Tab. 2. The observed vs. the expected cytokine genotypes for each SNP, Hardy Weinberg proportions (HWP), and Guo and Thompson Hardy Weinberg Output (GTHWO) in Macedonian population.

Polymorphism	Genotype	Observed number	Observed frequency	Expected number	p-value	HWP p-value	GTHWO p-value
IL-1α -889	C:C	204	0.689	196.2	0.579	0.003*	0.006**
	C:T	74	0.250	89.6	0.100		
	T:T	18	0.061	10.2	0.015*		
IL-1β -511	C:C	143	0.475	135.6	0.523	0.052	0.052
	C:T	118	0.392	132.9	0.197		
	T:T	40	0.133	32.6	0.192		
IL-1β +3962	C:C	174	0.578	160.0	0.271	<0.001*	<0.001*
	C:T	91	0.302	118.9	0.011*		
	T:T	36	0.120	22.1	0.003*		
IL-1R psti1970	C:C	133	0.442	132.2	0.946	0.84	0.898
	C:T	133	0.442	134.5	0.894		
	T:T	35	0.116	34.2	0.895		
IL-1RA mspa11100	C:C	30	0.100	27.5	0.635	0.497	0.499
	C:T	122	0.405	127.0	0.659		
	T:T	149	0.495	146.5	0.837		
IL-4Rα +1902	A:A	212	0.704	209.3	0.852	0.262	0.297
	A:G	78	0.259	83.4	0.555		
	G:G	11	0.037	8.3	0.350		
IL-12 -1188	A:A	160	0.550	161.1	0.932	0.741	0.879
	A:C	113	0.388	110.9	0.838		
	C:C	18	0.062	19.1	0.805		
IFNγ utr5644	A:A	64	0.257	57.4	0.380	0.091	0.100
	A:T	111	0.446	124.3	0.233		
	T:T	74	0.297	67.4	0.418		
TGF-β1 cdn10	C:C	65	0.231	69.8	0.570	0.257	0.288
	C:T	150	0.534	140.5	0.423		
	T:T	66	0.235	70.8	0.572		
TGF-β1 cdn25	C:G	30	0.107	28.4	0.764	0.761	1.000
	G:G	251	0.893	251.8	0.960		
	C:C	0	0	0.8	§		
TNF-α -308	A:G	66	0.219	64.9	0.892	0.888	1.000
	G:G	231	0.768	231.5	0.971		
	A:A	4	0.013	4.5	§		
TNF-α -238	A:G	23	0.076	25.8	0.583	0.579	0.111
	G:G	276	0.917	274.6	0.933		
	A:A	2	0.007	0.6	§		
IL-2 -330	G:G	27	0.094	31.8	0.397	0.204	0.236
	G:T	137	0.477	127.4	0.397		
	T:T	123	0.429	127.8	0.672		
IL-2 +166	G:G	162	0.565	155.1	0.581	0.037*	0.048*
	G:T	98	0.341	111.7	0.193		
	T:T	27	0.094	20.1	0.125		
IL-4 -1098	G:T	174	0.608	121.8	<0.001*	<0.001*	<0.001*
	T:T	111	0.388	137.1	<0.001*		
	G:G	1	0.004	27.1	§		
IL-4 -590	C:C	95	0.332	124.2	0.009**	<0.001*	<0.001*
	C:T	187	0.654	128.5	<0.001*		
	T:T	4	0.014	33.2	§		
IL-4 -33	C:C	209	0.731	200.6	0.551	<0.001*	<0.001*
	C:T	61	0.213	77.9	0.558		
	T:T	16	0.056	7.6	0.002**		
IL-6 -174	C:C	25	0.083	27.5	0.632	0.493	0.588
	C:G	132	0.439	127.0	0.656		
	G:G	144	0.478	146.5	0.836		
IL-6 565	A:A	25	0.083	24.9	0.977	0.968	1.000
	A:G	123	0.409	123.3	0.980		
	G:G	153	0.508	152.9	0.990		
IL-10 -1082	A:A	70	0.234	103.6	0.001*	<0.001*	<0.001*
	A:G	212	0.709	144.8	<0.001*		
	G:G	17	0.057	50.6	<0.001*		
IL-10 -819	C:C	155	0.518	158.2	0.798	0.348	0.391
	C:T	125	0.418	118.6	0.555		
	T:T	19	0.064	22.2	0.495		
IL-10 -592	A:A	28	0.094	25.0	0.552	0.403	0.404
	A:C	117	0.391	123.0	0.592		
	C:C	154	0.515	151.0	0.809		

§, Cannot be calculated because expected <5, $\chi 2$ test; *, Statistically significant.

Tab. 3. Linkage disequilibrium (LD) measures for the each pair of loci of cytokine polymorphisms.

		IL-1α -889	IL-1β -511	IL-1β +3962	IL-1R psti 1970	IL-1RA mspa 11100	IL-4 Rα +1902	IL-12 -1188	IFNγ utr5644	TGF-β1 cdn10	TGF-β1 cdn25	TNF-α -308	TNF-α -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	IL-10 -592
IL-1α -889																							
IL-1β -511	D'	0.028																					
	Wn	0.457																					
	S	8.56																					
	p	0.002*																					
IL-1β +3962	D'	0.080	0.036																				
	Wn	0.587	0.410																				
	S	72.25	12.99																				
	p	<0.001*	0.001*																				
IL-1R psti 1970	D'	0.011	0.017	0.014																			
	Wn	0.182	0.080	0.154																			
	S	1.44	2.21	1.77																			
	p	0.225	0.150	0.189																			
IL-IRA mspa 11100	D'	0.025	0.032	0.035	0.099																		
	Wn	0.197	0.321	0.184	0.091																		
	S	7.23	8.81	10.90	2.36																		
	p	0.009*	0.004*	0.001*	0.153																		
IL-4 Rα +1902	D'	0.187	0.003	0.114	0.081	0.008																	
	Wn	0.040	0.002	0.084	0.050	0.005																	
	S	0.59	0.00	2.67	0.79	0.01																	
	p	0.427	0.975	0.121	0.375	0.874																	
IL-12 -1188	D'	0.463	0.132	0.207	0.022	0.080	0.052																
	Wn	0.131	0.054	0.072	0.018	0.031	0.013																
	S	6.42	0.92	1.99	0.10	0.28	0.07																
	p	0.011*	0.363	0.141	0.738	0.606	0.817																
IFNγ utr5644	D'	0.031	0.077	0.002	0.101	0.001	0.139	0.132															
	Wn	0.017	0.055	0.001	0.075	0.001	0.064	0.073															
	S	0.08	0.88	0.00	1.54	0.00	1.09	1.35															
	p	0.778	0.355	0.954	0.223	0.989	0.305	0.239															
TGF-β1 cdn10	D'	0.049	0.143	0.123	0.091	0.087	0.050	0.126	0.000														
	Wn	0.024	0.110	0.075	0.066	0.056	0.023	0.072	0.000														
	S	0.20	2.93	2.10	1.21	0.92	0.14	1.35	0.00														
	p	0.636	0.091	0.139	0.284	0.330	0.725	0.239	0.999														
TGF-β1 cdn25	D'	0.380	0.085	0.610	0.318	0.003	0.018	0.171	0.208	1.000													
	Wn	0.044	0.029	0.088	0.054	0.001	0.009	0.072	0.054	0.238													
	S	0.60	0.20	3.71	0.93	0.00	0.03	1.20	0.79	24.10													
	p	0.459	0.626	0.060	0.349	0.967	0.858	0.254	0.404	<0.001*													
TNF-α -308	D'	0.158	0.100	0.117	0.409	0.212	0.007	0.604	0.091	0.243	0.468												
	Wn	0.028	0.054	0.027	0.109	0.052	0.006	0.134	0.033	0.091	0.042												
	S	0.36	0.91	0.28	4.06	0.84	0.01	7.62	0.25	1.48	0.40												
	p	0.581	0.344	0.603	0.041*	0.360	0.930	0.010*	0.592	0.225	0.529												
TNF-α -238	D'	0.048	0.084	0.056	0.018	0.249	0.038	0.078	0.145	0.153	0.08	1.000											
	Wn	0.005	0.013	0.020	0.003	0.082	0.018	0.029	0.028	0.032	0.077	0.081											
	S	0.01	0.05	0.15	0.00	2.34	0.12	0.26	0.26	0.34	1.65	7.26											
	p	0.944	0.772	0.732	0.961	0.129	0.761	0.599	0.586	0.540	0.141	0.004*											

	D'	0.245	0.067	0.041	0.034	0.060	0.020	0.030	0.022	0.065	0.264	0.124	0.429									
IL-2	Wn	0.083	0.066	0.035	0.017	0.056	0.006	0.025	0.016	0.045	0.091	0.032	0.067									
-330	S	1.92	1.18	0.41	0.08	0.85	0.01	0.14	0.05	0.48	2.13	0.24	1.42									
	p	0.162	0.256	0.527	0.772	0.332	0.884	0.726	0.772	0.487	0.157	0.631	0.229									
	D'	0.060	0.117	0.168	0.045	0.033	0.171	0.136	0.055	0.209	0.390	0.578	0.031	0.934								
IL-2	Wn	0.048	0.049	0.061	0.039	0.013	0.044	0.129	0.036	0.126	0.056	0.127	0.011	0.396								
+166	S	0.77	0.91	1.45	0.54	0.06	0.59	4.87	0.33	4.62	0.76	6.55	0.04	62.66								
	p	0.360	0.331	0.225	0.469	0.776	0.437	0.024*	0.530	0.023*	0.373	0.010*	0.809	<0.001*								
	D'	0.387	0.244	0.333	0.023	0.080	0.027	0.367	0.005	0.016	0.424	0.339	1.000	0.586	0.153							
IL-4	Wn	0.279	0.113	0.134	0.021	0.080	0.018	0.146	0.004	0.011	0.142	0.081	0.145	0.278	0.135							
-1098	S	16.01	2.34	3.77	0.07	1.38	0.06	2.11	0.00	0.01	3.03	1.11	5.80	8.77	3.40							
	p	<0.001*	0.100	0.020*	0.790	0.238	0.762	0.149	0.931	0.882	0.098	0.281	0.008*	0.008*	0.038*							
	D'	0.270	0.024	0.062	0.004	0.094	0.020	0.070	0.019	0.141	0.289	0.061	1.000	0.379	0.207	0.716						
IL-4	Wn	0.179	0.023	0.027	0.004	0.087	0.007	0.058	0.014	0.103	0.090	0.030	0.157	0.193	0.170	0.663						
-590	S	5.61	0.10	0.15	0.00	1.35	0.01	0.42	0.02	1.36	0.97	0.17	12.61	3.48	6.03	94.71						
	p	0.015*	0.729	0.650	0.995	0.237	0.975	0.525	0.845	0.291	0.303	0.710	<0.001*	0.093	0.006*	<0.001*						
	D'	0.073	0.022	0.025	0.017	0.026	0.211	0.045	0.143	0.155	0.352	0.073	1.000	0.134	0.054	0.305	0.742					
IL-4	Wn	0.016	0.014	0.019	0.010	0.017	0.043	0.033	0.066	0.068	0.036	0.060	0.096	0.085	0.040	0.090	0.454					
-33	S	0.10	0.08	0.13	0.04	0.11	0.66	0.36	1.18	1.60	0.44	1.18	3.92	2.18	0.57	2.24	58.77					
	p	0.764	0.808	0.720	0.870	0.712	0.392	0.568	0.307	0.200	0.510	0.274	0.066	0.117	0.437	0.091	<0.001*					
	D'	0.027	0.179	0.071	0.209	0.066	0.020	0.055	0.044	0.153	0.187	0.004	0.147	0.005	0.013	0.083	0.1054	0.020				
IL-6	Wn	0.020	0.083	0.066	0.098	0.066	0.014	0.050	0.027	0.102	0.067	0.002	0.048	0.005	0.005	0.081	0.096	0.014				
-174	S	0.12	2.28	1.20	2.93	1.24	0.06	0.67	0.19	3.03	1.19	0.00	0.90	0.01	0.01	0.93	1.01	0.06				
	p	0.716	0.124	0.257	0.092	0.278	0.816	0.399	0.626	0.071	0.250	0.987	0.356	0.913	0.934	0.363	0.361	0.772				
	D'	0.097	0.138	0.037	0.197	0.055	0.019	0.038	0.078	0.144	0.120	0.025	0.130	0.048	0.017	0.043	0.063	0.042	1.000			
IL6	Wn	0.029	0.061	0.036	0.089	0.053	0.014	0.036	0.047	0.092	0.045	0.015	0.044	0.043	0.007	0.041	0.055	0.029	0.965			
565	S	0.29	1.28	0.38	2.47	0.81	0.06	0.36	0.56	2.58	0.58	0.07	0.79	0.46	0.02	0.26	0.37	0.26	479.96			
	p	0.628	0.238	0.514	0.131	0.369	0.788	0.560	0.441	0.108	0.469	0.817	0.363	0.486	0.922	0.603	0.555	0.596	<0.001*			
	D'	0.080	0.103	0.101	0.012	0.044	0.091	0.093	0.026	0.207	0.212	0.306	0.027	0.115	0.059	0.724	0.613	0.030	0.203	0.247		
IL-10	Wn	0.046	0.060	0.051	0.007	0.034	0.034	0.064	0.022	0.173	0.060	0.138	0.005	0.069	0.042	0.584	0.535	0.016	0.112	0.132		
-1082	S	0.37	0.71	0.64	0.01	0.21	0.23	0.53	0.07	3.80	0.36	3.60	0.01	0.45	0.38	54.47	36.04	0.06	1.70	2.82		
	p	0.520	0.371	0.376	0.912	0.609	0.622	0.471	0.724	0.078	0.557	0.064	0.904	0.505	0.499	<0.001*	<0.001*	0.806	0.193	0.084		
	D'	0.108	0.006	0.112	0.154	0.059	0.102	0.010	0.173	0.009	0.307	0.258	0.013	0.147	0.061	0.377	0.097	0.095	0.073	0.066	1.000	
IL-10	Wn	0.032	0.005	0.111	0.067	0.056	0.074	0.003	0.106	0.005	0.120	0.059	0.004	0.130	0.022	0.154	0.082	0.026	0.067	0.063	0.512	
-819	S	0.30	0.01	3.97	1.46	0.87	1.77	0.00	2.39	0.01	3.44	0.90	0.01	4.28	0.16	2.06	0.72	0.22	1.28	1.20	82.87	
	p	0.597	0.930	0.047*	0.232	0.375	0.208	0.974	0.121	0.886	0.075	0.322	0.949	0.035*	0.681	0.197	0.447	0.639	0.264	0.255	<0.001*	
	D'	0.137	0.022	0.087	0.083	0.035	0.092	0.101	0.128	0.022	0.348	0.284	0.070	0.088	0.019	0.073	0.126	0.051	0.052	0.050	1.000	0.982
IL-10	Wn	0.042	0.020	0.083	0.038	0.034	0.064	0.038	0.083	0.014	0.130	0.068	0.010	0.081	0.007	0.031	0.111	0.014	0.051	0.050	0.533	0.942
-592	S	0.60	0.15	2.41	0.51	0.36	1.44	0.43	1.71	0.05	4.25	1.12	0.03	1.73	0.02	0.15	1.95	0.07	0.81	0.83	98.46	430.05
	p	0.436	0.693	0.110	0.470	0.551	0.211	0.536	0.182	0.758	0.052	0.302	0.830	0.197	0.917	0.688	0.134	0.790	0.388	0.364	<0.001*	<0.001*

D' weights the contribution to LD of specific allele pairs by the product of their allele frequencies (19); W_n is a re-expression of the chi-square statistic for deviations between the observed and expected haplotype frequencies; *S* is defined as twice the difference between log-likelihood of obtaining the observed data given the inferred haplotype frequencies [*ln(L_1)*], and the likelihood of the data under the null hypothesis of linkage equilibrium [*ln(L_0)*]); *p*-value is the fraction of permutations that results in values of *S* greater or equal to that observed. A *p*-value <0.05 is indicative of overall significant LD. *, statistically significant.

Tab. 4. The pairs of cytokine polymorphisms that displayed a significant (p<0.05) LD in the Macedonian population.

Cytokine polymorphism	Significant LD
IL-1α -889	*IL-1β -511, IL-1β +3962, IL-1RA mspa11100, IL-12 -1188, IL-4 -1098, IL-4 -590*
IL-1β -511	*IL-1α -889, IL-1β +3962, IL-1RA mspa11100*
IL-1β +3962	*IL-1α -889, IL-1β -511, IL-1RA mspa11100, IL-4 -1098, IL-10 -819*
IL-1R psti1970	*TNF-α -308*
IL-1RA mspa11100	*IL-1α -889, IL-1β -511, IL-1β +3962*
IL-4Rα +1902	-
IL-12 -1188	*IL-1α -889, TNF-α -308, IL-2 +166*
IFNγ utr5644	-
TGF-β1 cdn10	*TGF-β1 cdn25, IL-2 +166*
TGF-β1 cdn25	*TGF-β1 cdn10*
TNF-α -308	*IL-1R psti1970, IL-12 -1188, TNF-α -238, IL-2 +166*
TNF-α -238	*TNF-α -308, IL-4 -1098, IL-4 -590*
IL-2 -330	*IL-2 +166, IL-4 -1098, IL-10 -819*
IL-2 +166	*IL-12 -1188, TGF-β1 cdn10, TNF-α -308, IL-2 -330, IL-4 -1098, IL-10 -819*
IL-4 -1098	*IL-1α -889, IL-1β +3962, TNF-α -238, IL-2 -330, IL-2 +166, IL-4 -590, IL-10 -1082*
IL-4 -590	*IL-1α -889, TNF-α -238, IL-2 +166, IL-4 -590, IL-4 -33, IL-10 -1082*
IL-4 -33	*IL-4 -590*
IL-6 -174	*IL-6 565*
IL-6 565	*IL-6 -174*
IL-10 -1082	*IL-4 -1098, IL-4 -590, IL-10 -819, IL-10 -592*
IL-10 -819	*IL-1β +3962, IL-2 -330, IL-10 -819, IL-10 -592*

dividuals are of Macedonian origin and nationality, Christian Orthodox religion, and residents of the geographical areas of different regions of the Republic of Macedonia. The spoken language is Macedonian. Each individual was interviewed on the one-to-one basis, his/her genealogy was recorded for the last three generations, and a written consent was obtained. Admixture, if any, was recorded for each individual. Individuals with only one Macedonian parent were excluded from the study. Blood samples were collected, DNA was isolated from peripheral blood leukocytes by the phenol-chlorophorm extraction method (11) and samples were stored in the Anthropology field of the Macedonian Human DNA Bank (hDNAMKD) (12).

Typing Methods

The cytokine genotyping for the anthropology samples was performed by PCR-SSP (Heidelberg kit). Fourteen cytokine genes with 22 single nucleotide polymorphisms (SNP) were typed: *IL-1α -889, IL-1β -511, IL-1β +3962, IL-1R psti1970, IL-1RA mspa11100, IL-4Rα +1902, IL-12 -1188, IFNγ utr5644, TGF-β1 cdn10, TGF-β1 cdn25, TNF-α -308, TNF-α -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 by the Heidelberg kit consisted of 48 PCR primer mixes aliquotted in 96 well PCR trays (two typings 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 the 48 wells. Agarose gel electrophoresis on a 2 % agarose gel revealed a positive or a negative specific amplification for each well (13). Subsequently, the results were analyzed according to the interpretation scheme provided along with the kit.

Statistical Methods

The population genetics analysis package, PyPop, developed by the Biostatistics Core for the Workshop (14, 15), was used for analysis of the cytokine data. Allele frequencies and expected Hardy Weinberg proportions (HWP) for each single nucleotide polymorphisms (SNP) were determined (16). The exact test for genotype frequency deviation from HWP was calculated using the Arlequin implementation accessed via PyPop (17). 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 were significantly different from the expected frequencies by the chi square test. The Ewens-Watterson homozygozity test of neutrality (18) with Slatkins' p-values (19) were used to indicate any deviations from the hypothesis of neutral selection for each locus. Linkage disequilibrium was calculated, where *D'* weights the contribution to LD of specific allele pairs by the product of their allele frequencies, *W*n is a re-expression of the chi-square statistic for deviations between observed and expected haplotype frequencies, and *S* is defined as twice the difference between log-likelihood of obtaining the observed data given the inferred haplotype frequencies [*ln(L_1)*], and the likelihood of the data under the null hypothesis of linkage equilibrium [*ln(L_0)*]) (20). The Heidelberg kit allowed SNP haplotypes in *TGF-β1, TNF-α, IL-2, IL-4, IL-6*, and *IL-10* to be detected.

Results

Cytokine Alleles

Frequencies of polymorphic alleles, test of neutrality with Fnd statistic [Ewens-Watterson test of neutrality (EWN)], and Slatkin's Exact P. Value (SEPV) with p of F statistics in Macedonian population are shown on the Table 1. The frequency of alleles for some single nucleotide polymorphisms (SNPs) varies from 0.955 for *TNF-α 238/G*, 0.947 for *TGF-β1 cdn25/G*, 0.877 for *TNF-α 308/*G, followed by 0.837 for *IL-4 -33/C*, 0.834 for *IL-4Rα +1902/A*, and 0.814 for *IL-1α -889/C* indicating common "wild type" allele in those cytokines. The frequency ranges spanned 50 % for each allele of *IFNγ utr5644, TGF-β1 cdn10*, and *IL-10 -1082*, indicating no common "wild type" allele in those cytokines (Tab. 1).

For the majority of SNPs, test of neutrality showed a negative value for F_{nd} statistic (Ewens-Watterson test of neutrality), which indicates a balancing selection operating on the alleles at that locus (except for the *TGF-β1 cdn25* and *TNF-α -238*). F_{nd} was negative and significantly different from 0 for *TGF-β1 cdn10* and *IFNγ utr5644* (p of F = 0.001, and 0.012 respectively) (Tab. 1).

Cytokine genotypes

The observed versus the expected cytokine genotypes for each SNP, Hardy Weinberg proportion (HWP), and Guo and Thompson Hardy Weinberg Output (GTHWO) in Macedonian population is given in the Table 2. Several observed frequencies of cytokine genotypes were significantly different from the expectations: *IL-1α -889/T:T* (p=0.015), *IL-1β +3962/C:T* (p=0.011), *IL-1β +3962/T:T* (p=0.003), *IL-4 -1098/GT* (p<0.001), *IL-4 -1098/TT* (p<0.001), *IL-4 -590/CC* (p=0.009), *IL-4 -590/CT* (p<0.001), *IL-4 -33/TT* (p=0.002), *IL-10 -1082/AA* (p=0.001), *IL-10 -1082/AG* (p<0.001), and *IL-10 -1082/GG* (p<0.001). In some instances, $\chi 2$ test cannot be calculated because the expected frequency was smaller than 5 *(TGF-β1 cdn25/C:C, TNF-α -308/A:A, TNF-α -238/A:A, IL-4 -1098/G:G,* and *IL-4 -590/T:T).* Most of SNPs showed a good fit with HWP expectations. A few SNPs (*IL-1β +3962, IL-2 +166, IL-4 -1098, IL-4 -590, IL-4 -33*, and *IL-10 -819*) were not in HWP (p<0.050), and Guo and Thompson Hardy Weinberg Output (GTHWO) was significant (p< 0.050) (Tab. 2).

Linkage Disequilibrium (LD)

The linkage disequilibrium (LD) measures for each pair of loci for cytokine polymorphisms are presented in Table 3. The pairs of cytokine polymorphisms that displayed a significant (p<0.05) LD in Macedonian population are presented in Table 4.

We can see from the Table 3 and Table 4 that all pairs of loci for *IL-1* gene cluster, except for *IL-1R psti1970*, are in linkage disequilibrium, with D' les than 0.5 and p<0.05. *IL-4Rα +1902* and *IFNγ utr5644* are not in LD with any cytokine polymorphism. Other pairs of loci for cytokine genes are in linkage disequilibrium with different number of cytokine gene polymorphisms (Tabs 3 and 4).

Cytokine Haplotypes

The cytokine haplotypes frequency and test of neutrality with Fnd statistic (Ewens-Watterson test of neutrality (EWN)), and Slatkin's Exact P. Value (SEPV) with p of F statistics in Macedonian population is shown in the Table 5.

For several genes with multiple SNPs per gene (*TGF-β1, TNF-α, IL-2, IL-4, IL-6, IL-10*), the Heidelberg PCR-SSP kit was able to detect true haplotypes. The most frequent haplotypes for *TGF-β1* are */TG* (0.502), and */CG* (0.445) with the absence of *TGF-β1/ /TC* haplotype in Macedonian population. The most frequent *TNF-α* haplotype was */GG* (0.834), following with */AG* (0.123), and/ *GA* (0.043). *IL-2* haplotypes in Macedonian population are nearly equally distributed between the */TG* (0.425), */GG* (0.310), and/ *TT* (0.240), with the smallest frequency of */GT* (0.024) haplotype. The most frequent *IL-4* haplotype is */TCC* (0.353), followed with */GCC* (0.285), */TTC* (0.192), and */TTT* (0.140) haplotypes. The rest of *IL-4* haplotypes have a very small frequency (*/GCT, /GTC, /TCT*, and */GTT*). The most frequent *IL-6* haplotype is */GG* (0.698), followed with */CA* (0.286), */CG* (0.150), and */GA* (0.002). Several *IL-10* haplotypes are equally

Tab. 5. The haplotypes frequency and the test of neutrality with Fnd statistic (Ewens-Watterson test of neutrality (EWN)), and Slatkin's Exact P. Value (SEPV) with p of F statistics in Macedonian population.

Polymorphism	Haplotypes			Test of Neutrality	
	Haplotype	Number	Frequency	EWN Fnd	SEWN p of F
TGF-β1	CC	30	0.053	-1.520	0.063
	CG	250	0.445		
	TG	282	0.502		
TNF-α	AG	74	0.123	-0.182	0.424
	GA	26	0.043		
	GG	502	0.834		
IL-2	GG	178	0.310	-1.650	0.020*
	GT	14	0.024		
	TG	244	0.425		
	TT	138	0.240		
IL-4	GCC	163	0.285	-1.086	0.106
	GCT	8	0.014		
	GTC	4	0.007		
	GTT	1	0.002		
	TCC	202	0.353		
	TCT	4	0.007		
	TTC	110	0.192		
	TTT	80	0.140		
IL-6	CA	172	0.286	-0.468	0.397
	CG	9	0.150		
	GG	420	0.698		
	GA	1	0.002		
IL-10	ACA	12	0.020	-1.355	0.062
	ACC	177	0.296		
	ATA	161	0.269		
	ATC	2	0.003		
	GCC	246	0.411		

Statistically significant Fnd <0 indicates a balancing selection; significant Fnd >0 indicates a directional selection; *, Statistically significant.

distributed in Macedonian population [*/GCC* (0.411), */ACC* (0.296), and */ATA* (0.269)], and two of them with a very small frequency [*/ACA* (0.020), and */ATC* (0.003)]. Test of neutrality showed a negative value for Fnd statistic (Ewens-Watterson test of neutrality), which indicates a balancing selection operating on the haplotypes at that locus. Fnd was negative and significantly different from 0 for *IL-2* haplotypes (p=0.020) (Tab. 5).

Cytokine diplotypes

The cytokine diplotypes, observed vs. expected genotype frequency for each SNP, Hardy Weinberg proportions (HWP), Guo and Thompson Hardy Weinberg output (GTHWO), and Linkage Disequilibrium in Macedonian population is shown in the Table 6.

The cytokine diplotypes (or haplotype zygozity) are combinations of haplotypes from both parents. They showed a good fit with HWP expectations for *TGF-β1, TNF-α*, and *Il-6*. Several observed frequencies of cytokine diplotypes were significantly different from the expectations: *IL-2/GT:TG* (p=0.038), *IL-2/TT:TT* (p=0.039), *IL-4/GCC:GCC* (p<0.001), *IL-4/GCC:TCC* (p<0.001), *IL-4/GCC:TTC* (p<0.001), *IL-4/TCC:TCC* (p<0.001), *IL-4/TCC:TTC* (p<0.001), *IL-4/TTC:TTC* (p=0.001), *IL-4/TTC:TTT* (p<0.001), *IL-10/ACC:ATA* (p<0.001), *IL-10/ACC:GCC* (p<0.001), *IL-10/ATA:GCC* (p=0.001), and *IL-10/GCC:GCC* (p<0.001). Several frequencies of diplotypes were less than 5 and chi-square test was not calculated. Hardy-Weinberg proportion and Guo and Thompson Hardy Weinberg output were statistically significant for *IL-2* (p=0.016 and p=0.019), *IL-4* (p<0.001), and *IL-10* (p<0.001) (Table 6).

Discussion

This report summarizes 22 cytokine polymorphisms and variation that exists in alleles, genotypes, haplotypes and diplotypes in Macedonian population.

We found a negative Fnd and significantly different from 0 for *IFNγ UTR5644* and *TGF-β1 cdn10*, which indicates a balancing selection operating on the alleles at that locus. Only 2 SNPs (*TGF-β1 cdn25* and *TNF-α -238*) showed a positive value for F_{nd} statistic, but without significant p of F statistics. We found also that majority of the SNPs showed a good fit with HWP expectations. A few SNPs (*IL-1α -889, IL-1β +3962, IL-2 +166, IL-4 -1098, IL-4 -590, IL-4 -33,* and *IL-10 -1082*) were not in HWP, and Guo and Thompson Hardy Weinberg Output was significant. The haplotype frequencies were not significantly different

Tab. 6. The observed vs. the expected diplotype frequency, Hardy Weinberg proportions (HWP), and Guo and Thompson Hardy Weinberg output (GTHWO), and Linkage Disequilibrium in Macedonian population.

Polymorphism	Diplotype	Observed	Observed frequency	Expected	p-value	HWP p-value	GTHWO p-value
TGF-β1	CC:CG	16	0.057	13.3	0.467	0.459	0.423
	CC:TG	14	0.050	15.1	0.786		
	CG:CG	49	0.174	55.6	0.376		
	CG:TG	136	0.484	125.4	0.346		
	TG:TG	66	0.235	70.8	0.572		
	CC:CC	0	0	0.8	§		
TNF-α	AG:GG	66	0.219	61.7	0.585	0.439	0.134
	GA:GG	24	0.080	21.7	0.61		
	GG:GG	206	0.684	209.3	0.819		
	AG:AG	4	0.013	4.5	§		
	GA:AG	0	0	3.2	§		
	GA:GA	1	0.004	0.6	§		
IL-2	GG:GG	27	0.094	27.6	0.909	0.016*	0.019*
	GG:TG	85	0.296	75.7	0.283		
	GG:TT	38	0.133	42.8	0.464		
	GT:TG	11	0.058	6.00	0.038*		
	TG:TG	50	0.174	51.9	0.796		
	TG:TT	48	0.168	58.7	0.164		
	TT:TT	25	0.087	16.6	0.039*		
	GT:GG	1	0.003	4.3	§		
	GT:TT	2	0.007	3.4	§		
	GT:GT	0	0	0.2	§		
IL-4	GCC:GCC	1	0.003	23.2	<0.001*	<0.001*	<0.001*
	GCC:TCC	26	0.091	57.6	<0.001*		
	GCC:TTC	103	0.360	31.3	<0.001*		
	GCC:TTT	32	0.112	22.8	0.054		
	TCC:TCC	68	0.238	35.7	<0.001*		
	TCC:TTC	7	0.025	38.8	<0.001*		
	TCC:TTT	28	0.098	28.3	0.962		
	TTC:TTC	0	0	10.6	0.001*		
	TTC:TTT	0	0	15.4	<0.001*		
	TTT:TTT	4	0.014	5.6	0.500		
	GCT:TCC	0	0	2.8	§		
	GCT:GCC	0	0	2.3	§		
	GCT:TTC	0	0	1.5	§		
	GCT:TTT	8	0.028	1.1	§		
	GCT:GCT	0	0	0.1	§		
	GTC:TTC	4	0.014	1.4	§		
	GTC:GCC	0	0	1.1	§		
	GTC:TTC	0	0	0.8	§		
	GTC:TTT	0	0	0.6	§		
	GTC:GCT	0	0	0.1	§		
	TCT:TCC	0	0	1.4	§		
	TCT:GCC	0	0	1.1	§		
	TCT:TTC	0	0	0.8	§		
	TCT:TTT	4	0.014	0.6	§		
	TCT:GCT	0	0	0.1	§		
	GTT:TCC	1	0.003	0.4	§		
	GTT:GCC	0	0	0.3	§		
	GTT:TTC	0	0	0.2	§		
	GTT:TTT	0	0	0.1	§		
IL-6	CA:CA	25	0.083	24.6	0.931	0.261	0.261
	CA:GG	122	0.405	120.0	0.855		
	CG:GG	9	0.030	6.3	0.278		
	GG:GG	144	0.479	146.5	0.836		
	CG:CA	0	0	2.6	§		
	CG:CG	0	0	0.1	§		
	GA:GG	1	0.003	0.7	§		
	GA:CA	0	0	0.3	§		
IL-10	ACC:ACC	21		26.2	0.310	<0.001*	<0.001*
	ACC:ATA	21	0.070	47.7	<0.001*		
	ACC:GCC	114	0.070	72.8	<0.001*		
	ATA:ATA	19	0.381	21.7	0.566		
	ATA:GCC	93	0.064	66.2	0.001*		
	GCC:GCC	17	0.311	50.6	<0.001*		
	ACA :GCC	3	0.057	4.9	§		

§, Cannot be calculated because expected <5, $\chi 2$ test; *, Statistically significant.

in Macedonian population. The Fnd was negative and significantly different from 0 for *IL-2* haplotypes.

We can analyze and/or use our data in two different aspects: population differences, and cytokine concentration.

Population differences: The most comprehensive database for frequencies of cytokines (as a part of Allele Frequencies in Worldwide Populations – www.allelefrequencies.net) contains genotype frequencies of 104 different populations (February, 2008) and can be used for the inter-population comparisons (21). Unfortunately, the database does not contain data for cytokine alleles, haplotypes and diplotypes, which can be obtained from the literature only.

The inter-population discrepancies in allele frequencies, particularly between Caucasian and non-Caucasian cohorts, are often large (22). Significant differences in allelic frequencies among ethnic groups were reported (23). The *IL-1RN* allele 2 was very rare in Koreans (frequency, 0.060). In addition, a significant difference was also found for the *IL-1*α (-889) and *IL-1*β (+3953) polymorphisms in Koreans compared with Caucasians (24). The frequency of *IL-1RN* 2-repeat allele was significantly lower in Taiwanese compared to Caucasians. In contrast, the frequencies of the pro-inflammatory *IL-1*β *-511T* allele and *+3954C* allele were significantly higher among Taiwanese compared to Caucasians (25).

These observations are important for two reasons. First, many of the established cytokine allele frequencies are Caucasian allele frequencies, because of the work is done in European and North American laboratories. Second, these loci are two of the most frequently analyzed in disease association studies and have been positively associated with a number of immune diseases. In some countries, particularly the United States, where large ethnic populations exist in dense clusters, these observations should be taken into account when designing a high impact association study. Here it is particularly important to achieve an accurate matched population profiling in both case and control cohorts, as small differences can provide positive associations brought about by ethnic mismatch rather than disease susceptibility.

Even within the Caucasian populations in Europe, there are striking differences in polymorphic cytokine allele frequencies (26). Evaluation of the allele frequencies of the Dutch, Italian and Czech populations showed that five SNPs were significantly different between the Dutch and the Italians, while these SNPs did not vary between the Dutch and the Czechs (27). The allelic distribution of all polymorphisms in the Slovak population was very close to the geographically and historically closest populations in Central Europe – the Czech and the Polish. However, several differences were found between the Slovak and four populations from Southern Europe: Italian (28), Macedonian (10), Bulgarian (29), and Greek-Cypriot (30).

Cytokine concentration: There is the evidence that cytokine production level is under a genetic control. It was demonstrated that nucleic acid sequence polymorphism within promoter regions of the gene may affect the transcriptional activation of the gene, causing variation in the amounts of cytokine production (31). Such variation provides a unique profile of high and low cytokine response for each individual (32). In general, the high and low cytokine producer status is defined by the zygosity, being homozygote high/high, or low/low and heterozygote high/low. Thus, the zygosity status determines the inheritance of the cytokine production profile in patients, and that might have an impact on the outcome of certain disease.

The influence of *IL-1alpha -889, IL-1beta -511* and *IL-1Ra* VNTR genotypes and *IL-10* genotypes/haplotypes (*ACC, GCC* and *ATA*) on IL-10 plasma levels, and a putative correlation between IL-10 and IL-1alpha plasma levels were analyzed. The *IL-10, IL-1alpha, IL-1beta* and *IL-1Ra* gene polymorphisms were analyzed using PCR. IL-1beta and IL-10 plasma levels were measured using an ELISA method. The results indicated that increased IL-10 plasma levels were associated with the *ATA* haplotype ($p=0.03$) and, surprisingly, with the *IL-1alpha* allele 2 carrier status ($p=0.02$) in healthy individuals. The findings suggest that the genotype combination of *IL-1alpha 2+/ATA+* has a regulatory effect on basal IL-10 levels and that among individuals with measurable IL-10 plasma levels, IL-1beta and IL-10 basal levels correlate (33).

Published results indicated that after the exposure to LPS, whole blood leukocytes from subjects with the homozygous haplotype *IL-1*α *-1470/G, -511/C*, and *-31/T* (*/GCT*) produced more IL-1 in vitro than those from subjects with haplotype *IL-1*α *-1470/C, -511/T*, and *-31/C* (*/CTC*) and that the transcriptional activity of the haplotype */GCT* was also higher than that of the haplotype */CTC*. It is suggested that the haplotypes of the *IL-1* promoter influence the expression and transcriptional activity of the *IL-1* gene and that the upregulation of IL-1α gene expression after LPS exposure in subjects with haplotype */GCT* may be due to an increased transcriptional activity of the haplotype (34).

In summary, the results of cytokine alleles, genotypes, haplotypes, diplotypes and linkage disequilibrium in Macedonian population are in accord with the published data for cytokine polymorphism, and can be used for the anthropological comparisons, as well as for the association studies with different diseases.

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