Clin Rheumatol (2009) 28:1291–1300
DOI 10.1007/s10067-009-1238-4

ORIGINAL ARTICLE

Association of 22 cytokine gene polymorphisms with rheumatoid arthritis in population of ethnic Macedonians

Dejan Trajkov · Snezhana Mishevska-Perchinkova · Anzelika Karadzova-Stojanoska · Aleksandar Petlichkovski · Ana Strezova · Mirko Spiroski

Received: 7 February 2009 / Revised: 28 June 2009 / Accepted: 17 July 2009 / Published online: 7 August 2009

Abstract To examine the possible role of 22 cytokine gene polymorphisms in host susceptibility to or protection against RA in Macedonians. In this study, 301 healthy unrelated individuals and 85 patients with RA were studied. Cytokine genotyping was performed by PCR with sequence-specific priming (PCR–SSP) (Heidelberg kit). Results showed susceptible association for four cytokine alleles, six cytokine genotypes, one haplotype, and four combinations of haplotypes, while protective associations were found for four cytokine alleles, three cytokine genotypes, three haplotypes, and only one combination of haplotypes. These results suggest that *IL-4 −1098*, *IL-4 −590*, *IL-10 −1082*, *IL-10 −819*, *IL-2 −330*, *IL-6 −174*, and *TNF-α −238* cytokine gene polymorphisms might be significantly associated and affect host susceptibility and/or resistance to RA in Macedonians.

Keywords Cytokine polymorphism · Macedonians · Rheumatoid arthritis

D. Trajkov · A. Petlichkovski · A. Strezova · M. Spiroski (✉)
Institute of Immunobiology and Human Genetics,
Faculty of Medicine, University "Ss. Kiril and Metodij",
1109 Skopje, P.O. Box 60,
Republic of Macedonia
e-mail: mspiroski@yahoo.com
URL: http://www.iibhg.ukim.edu.mk/

S. Mishevska-Perchinkova · A. Karadzova-Stojanoska
Clinic for Rheumatology, Clinical Center, Faculty of Medicine,
University "Ss Kiril and Metodij",
Skopje, Republic of Macedonia

Introduction

Rheumatoid arthritis (RA) is a complex heterogeneous chronic autoimmune disease, whereby both environmental and genetic factors contribute to the etiology and/or clinical severity that affects approximately 1% of the world's population, with the prevalence significantly higher in women than in men (in a 3:1 ratio). Genetics of RA, which contribution to etiology is estimated to be over 30%, is very complex involving many genes each with small individual effects [1]. RA is characterized by abnormal immune responses and inflammation in the lining of the joint capsule (synovium) and the lining of tendons. Although the exact immunopathogenesis of arthritic inflammation is still unknown, it has been postulated that clinical symptoms may reflect an imbalance in pro- and anti-inflammatory cytokines. RA represented typical T-helper 1-mediated disease in which T cells infiltrate synovial inflammatory lesions, stimulating monocytes, macrophages, and synovial fibroblasts to produce a large amount of cytokines [2].

It is already known that the number of cytokine gene polymorphisms in the cytokine gene regulatory regions correlate with their secretion [3], and that their inheritance is influenced by ethnicity [4]. These polymorphisms may result in inter-individual variation in cytokine production, which may have significant influence on disease susceptibility, severity, and outcome.

Until now, we have only published data for the cytokine polymorphisms in healthy Macedonian population [5, 6] and for the possible association of cytokine polymorphism with bronchial asthma [7] and chronic obstructive pulmonary disease [8]. The aim of this study was to investigate

the existence of possible associations between 22 cytokine genes polymorphisms and RA in Macedonians, in order to add knowledge about the genetic background of this disease, and to provide data for meta-analysis.

Materials and methods

Population

The sample of the population of subjects from the Republic of Macedonia included in this study comprised 301 healthy unrelated individuals and 85 patients with RA, fulfilling the 1987 American College of Rheumatology criteria for RA [9]. All of the patients and healthy individuals included in this study participated in the study which was approved by the Committee of the Ministry of Education and Science from Republic of Macedonia (No. 087405) after signing a written consent. Blood samples were collected, DNA was isolated from peripheral blood leukocytes by the phenol–chloroform extraction method [10], and DNA samples were stored in the Macedonian Human DNA Bank (hDNAMKD) [11].

Typing methods

Fourteen cytokine genes (22 SNP alleles) were identified as the candidates for the cytokine polymorphism component (CPC) at the 13th International Histocompatibility Workshop and Congress, and cytokine genotyping was performed by PCR with sequence-specific priming (PCR–SSP; Heidelberg kit): *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 Heidelberg kit consists of 48 PCR primer mixes aliquoted in 96-well PCR trays (two typings per tray). Master mix, which was supplied along with the reagents and consisted of $MgCl_2$, buffer, dNTPs, and glycerol, was mixed with 1.2–3.0 μg DNA and 20 U Taq polymerase and dispensed in the 48 wells [12]. Agarose gel electrophoresis on a 2% gel revealed a positive or negative signal for specific amplification in each well. Subsequently, the results were analyzed according to the interpretation scheme provided with the kit [13].

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 in this study. Allele frequencies and expected Hardy Weinberg proportions (HWP) for each 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 genotype frequencies were significantly different from the expected frequencies. Comparisons of different genotypes for two groups were tested by the χ^2 test. Crude odds ratios (OR), as estimates of the relative risk, were calculated within 95% CI and $p < 0.05$ was considered statistically significant.

Results

Cytokine alleles

In Table 1, frequencies of polymorphic cytokine alleles, Pearson's p value, odds ratio, and Wald's 95% confidence interval in RA patients and healthy Macedonians are shown.

For the members of anti-inflammatory cytokines, we found positive association for *IL-4 −1098/T* allele ($p<0.001$, OR=3.153, Wald's 95% CI between 1.932 and 5.148) and *IL-4 −590/C* allele ($p=0.006$, OR=1.737, Wald's 95% CI between 1.168 and 2.585), while *IL-4 −1098/G* and *IL-4 −590/T* alleles showed negative (protective) association ($p<0.001$, OR=0.317, Wald's 95% CI between 0.194 and 0.518 and $p=0.006$; OR=0.576, Wald's 95% CI between 0.387 and 0.856, respectively) for RA. In the group of proinflammatory cytokines, protective association was obtained for the *TNFα −238/A* allele ($p=0.047$, OR=0.257, Wald's 95% CI between 0.060 and 1.090) and *IL-2 −330/T* allele ($p=0.032$, OR=0.681, Wald's 95% CI between 0.479 and 0.969). Results showed that people with *TNF-α −238/G* allele have a 3.897-fold risk to develop RA ($p=0.047$, Wald's 95% CI between 0.917 and 16.560) in comparison to others with *TNF-α −238/A* allele. Analysis of the IL-2 polymorphisms showed that *IL-2 −330/G* allele was susceptibly associated with RA ($p=0.032$, OR=1.468, Wald's 95% CI between 1.032 and 2.087) (Table 1).

Cytokine genotypes

Table 2 contains summarized results for different cytokine genotypes found in our study.

We found positive (susceptible) association between patients with RA and following genotypes (according the level of susceptibility): *IL4 −1098/T:T* ($p<0.001$), OR 51.71 (Wald's 95% CI between 23.492 and 113.801); *IL-2 −330/G:G* ($p=0.001$), OR 2.815 (1.474–5.374); *IL-10 −819/T:T* ($p=0.008$), OR 2.661 (1.255–5.642); *IL-6 −174/C:C* ($p=0.005$), OR 2.598 (1.314–5.134); *IL-4 −590/C:C* ($p<0.001$), OR

Table 1 Cytokine allele frequency, Pearson's *p* value, odds ratio, and Wald's 95% confidence interval in RA patients and healthy Macedonian population

Cytokine polymorphism	Allele	RA (*n*=85)		Control (*n*=301)		Pearson's *p* value	Odds ratio	Wald' 95% CI
		N	*F*	*N*	*F*			
IL-1α −889	C	134	0.848	482	0.814	0.323	1.274	0.787–2.062
	T	24	0.152	110	0.186		0.785	0.485–1.270
IL-1β −511	C	118	0.702	404	0.671	0.443	1.157	0.797–1.678
	T	50	0.298	198	0.329		0.865	0.596–1.254
IL-1β +3962	C	121	0.720	439	0.729	0.817	0.956	0.653–1.400
	T	47	0.280	163	0.270		1.046	0.715–1.533
IL-1R psti1970	C	115	0.676	399	0.662	0.738	1.064	0.740–1.529
	T	55	0.324	203	0.337		0.940	0.654–1.352
IL-1RA mspa11100	T	122	0.726	420	0.698	0.474	1.149	0.785–1.683
	C	46	0.274	182	0.302		0.870	0.594–1.274
IL-4Rα +1902	A	142	0.835	502	0.834	0.965	1.010	0.639–1.598
	G	28	0.165	100	0.166		0.990	0.626–1.566
IL-12 −1188	A	125	0.735	433	0.744	0.820	0.956	0.648–1.409
	C	45	0.265	149	0.256		1.046	0.710–1.543
IFNγ utr5644	T	57	0.467	259	0.520	0.295	0.809	0.544–1.203
	A	65	0.533	239	0.480		1.236	0.831–1.836
TGF-β1 cdn10	T	94	0.553	282	0.502	0.242	1.228	0.870–1.733
	C	76	0.447	280	0.498		0.814	0.577–1.149
TGF-β1 cdn25	G	160	0.941	532	0.947	0.784	0.902	0.432–1.886
	C	10	0.059	30	0.053		1.108	0.530–2.317
TNF-α −308	A	19	0.113	74	0.123	0.730	0.910	0.532–1.555
	G	149	0.887	528	0.877		1.099	0.643–1.878
TNF-α −238	A	2	0.012	27	0.045	0.047[b]	0.257	0.060–1.090
	G	166	0.988	575	0.955		3.897	0.917–16.560
IL-2 −330	G	71	0.423	191	0.332	0.032[b]	1.468	1.032–2.087
	T	97	0.577	383	0.667		0.681	0.479–0.969
IL-2 +166	G	122	0.726	422	0.735	0.817	0.955	0.649–1.406
	T	46	0.274	152	0.264		1.047	0.711–1.541
IL-4 −1098	G	21	0.124	176	0.308	<0.001[b]	0.317	0.194–0.518
	T	149	0.876	396	0.692		3.153	1.932–5.148
IL-4 −590	C	131	0.771	377	0.659	0.006[b]	1.737	1.168–2.585
	T	39	0.229	195	0.341		0.576	0.387–0.856
IL-4 −33	C	148	0.581	479	0.837	0.294	1.306	0.792–2.153
	T	22	0.129	93	0.163		0.766	0.464–1.262
IL-6 −174	C	62	0.369	182	0.302	0.100	1.350	0.943–1.932
	G	106	0.631	420	0.698		0.741	0.518–1.060
IL-6 nt565	A	59	0.352	173	0.287	0.111	1.342	0.934–1.929
	G	109	0.649	429	0.713		0.745	0.518–1.071
IL-10 −1082	A	105	0.618	352	0.589	0.496	1.129	0.796–1.601
	G	65	0.382	246	0.411		0.886	0.625–1.257
IL-10 −819	C	114	0.671	435	0.727	0.148	0.763	0.529–1.101
	T	56	0.329	163	0.272		1.311	0.908–1.892
IL-10 −592	A	56	0.329	173	0.289	0.313	1.207	0.837–1.739
	C	114	0.671	425	0.710		0.313	0.575–1.194

N absolute number, *F* frequency, *CI* confidence interval

[b] Statistically significant

Table 2 Cytokine genotype frequency, Pearson's *p* value, odds ratio, and Wald's 95% confidence interval in RA patients and healthy Macedonians

Polymorphism	Genotype	RA (*n*=85)		Controls (*n*=301)		Pearson's *p* value	Odds ratio	Wald' 95% CI
		N	*F*	*N*	*F*			
IL-1α −889	C:C	61	0.772	204	0.689	0.150	1.528	0.855–2.731
	C:T	12	0.152	74	0.250	0.065	0.537	0.275–1.048
	T:T	6	0.076	18	0.061	0.625	1.269	0.486–3.313
IL-1β −511	C:C	45	0.536	143	0.475	0.326	1.275	0.785–2.070
	C:T	28	0.333	118	0.392	0.327	0.775	0.466–1.290
	T:T	11	0.131	40	0.133	0.963	0.983	0.481–2.012
IL-1β +3962	C:C	51	0.607	174	0.578	0.633	1.128	0.688–1.849
	C:T	19	0.226	91	0.302	0.172	0.675	0.383–1.190
	T:T	14	0.167	36	0.120	0.257	1.472	0.753–2.881
IL-1R psti1970	C:C	38	0.447	133	0.442	0.932	1.021	0.629–1.658
	C:T	39	0.459	133	0.442	0.781	1.071	0.660–1.737
	T:T	8	0.094	35	0.116	0.566	0.790	0.352–1.773
IL-1RA mspa11100	C:C	6	0.071	30	0.100	0.432	0.695	0.279–1.730
	C:T	34	0.405	122	0.405	0.933	0.998	0.610–1.633
	T:T	44	0.524	149	0.495	0.641	1.122	0.692–1.821
IL-4Rα +1902	A:A	59	0.694	212	0.704	0.856	0.953	0.564–1.608
	A:G	24	0.282	78	0.259	0.668	1.125	0.657–1.927
	G:G	2	0.024	11	0.037	0.557	0.635	0.138–2.923
IL-12 −1188	A:A	46	0.541	160	0.550	0.888	0.966	0.595–1.569
	A:C	33	0.388	113	0.388	0.999	1.000	0.609–1.641
	C:C	6	0.071	18	0.062	0.772	1.152	0.442–3.000
IFNγ utr5644	A:A	23	0.376	64	0.257	0.061	1.750	0.969–3.158
	A:T	19	0.312	111	0.446	0.057	0.562	0.310–1.022
	T:T	19	0.312	74	0.297	0.827	1.070	0.584–1.962
TGF-β1 cdn10	C:C	18	0.212	65	0.231	0.706	0.893	0.495–1.610
	C:T	40	0.470	150	0.534	0.307	0.776	0.477–1.262
	T:T	27	0.318	66	0.235	0.125	1.517	0.890–2.585
TGF-β1 cdn25	C:G	8	0.094	30	0.107	0.737	0.869	0.383–1.975
	G:G	76	0.894	251	0.893	0.982	1.009	0.459–2.219
	C:C	1	0.012	0	0	[a]	[a]	[a]
TNF-α −308	A:G	15	0.178	66	0.219	0.537	0.826	0.449–1.517
	G:G	67	0.798	231	0.768	0.559	1.194	0.658–2.167
	A:A	2	0.024	4	0.013	0.491	1.811	0.326–10.062
TNF-α −238	A:G	2	0.024	23	0.076	0.083	0.295	0.068–1.277
	G:G	82	0.976	276	0.917	0.060	3.714	0.861–16.011
	A:A	0	0	2	0.007	[a]	[a]	[a]
IL-2 −330	G:G	19	0.226	27	0.094	0.001[b]	2.815	1.474–5.374
	G:T	33	0.393	137	0.477	0.172	0.709	0.432–1.163
	T:T	32	0.381	123	0.429	0.436	0.821	0.498–1.351
IL-2 +166	G:G	45	0.536	162	0.565	0.641	0.890	0.546–1.451
	G:T	32	0.381	98	0.341	0.505	1.187	0.717–1.964
	T:T	7	0.083	27	0.094	0.764	0.875	0.367–2.088
IL-4 −1098	G:T	19	0.223	174	0.608	<0.001[b]	0.185	0.106–0.325
	T:T	65	0.765	111	0.388	<0.001[b]	51.705	23.492–113.801
	G:G	1	0.012	1	0.004	0.361	3.393	0.210–54.828
IL-4 −590	C:C	47	0.553	95	0.332	<0.001[b]	2.487	1.518–4.073

Table 2 (continued)

Polymorphism	Genotype	RA (n=85)		Controls (n=301)		Pearson's p value	Odds ratio	Wald' 95% CI
		N	*F*	*N*	*F*			
	C:T	37	0.435	187	0.654	<0.001[b]	0.408	0.249–0.668
	T:T	1	0.012	4	0.014	0.876	0.839	0.093–7.611
IL-4 −33	C:C	64	0.753	209	0.731	0.684	1.123	0.643–1.962
	C:T	20	0.235	61	0.213	0.666	1.135	0.638–2.018
	T:T	1	0.012	16	0.056	0.876	0.839	0.093–7.611
IL-6 −174	C:C	16	0.191	25	0.083	0.005[b]	2.598	1.314–5.134
	C:G	30	0.357	132	0.439	0.182	0.711	0.431–1.174
	G:G	38	0.452	144	0.478	0.673	0.901	0.554–1.464
IL-6 nt565	A:A	13	0.155	25	0.083	0.051	2.021	0.985–4.149
	A:G	33	0.393	123	0.409	0.794	0.936	0.571–1.535
	G:G	38	0.452	153	0.508	0.365	0.799	0.492–1.299
IL-10 −1082	A:A	29	0.341	70	0.234	0.046[b]	1.694	1.005–2.856
	A:G	47	0.553	212	0.709	0.007[b]	0.508	0.309–0.833
	G:G	9	0.106	17	0.057	0.112	1.964	0.842–4.581
IL-10 −819	C:C	42	0.494	155	0.518	0.693	0.907	0.560–1.469
	C:T	30	0.353	125	0.418	0.280	0.759	0.460–1.253
	T:T	13	0.153	19	0.064	0.008[b]	2.661	1.255–5.624
IL-10 −592	A:A	13	0.153	28	0.094	0.118	1.748	0.862–3.541
	A:C	30	0.353	117	0.391	0.521	0.849	0.514–1.402
	C:C	42	0.494	154	0.515	0.733	0.920	0.568–1.489

N absolute number, *F* frequency, *CI* confidence interval

[a] Cannot be calculated because expected <5, χ^2 test

[b] Statistically significant

2.487 (1.518–4.073); and *IL-10 −1082/A:A* (p=0.046), OR 1.694 (1.005–2.856) (Table 2).

Negative (protective) association between patients with RA and following genotypes (according to the protective level) was found for: *IL-4 −1098/G:T* (p<0.001), OR 0.185 (Wald's 95% CI between 0.106 and 0.325); *IL-4 −590/C:T* (p<0.001), OR 0.408 (0.249–0.668); and *IL-10 −1082/A:G* (p=0.007), OR 0.508 (0.309–0.833) (Table 2).

Genotype *TNF-a/G:G* was present only in healthy Macedonian population (Table 2).

Cytokine haplotypes

For several genes with multiple SNPs per gene (*TGF-β1*, *TNF-α*, *IL-2*, *IL-4*, *IL-6*, *IL-10*), using the Heidelberg PCR–SSP kit, we were able to detect true haplotypes. Cytokine haplotype frequency in the RA patients and healthy Macedonians, together with the Pearson's p value, OR, and Wald's 95% CI, is shown in Table 3.

Significant association with RA was observed in three *IL-4* haplotypes and one *IL-2* haplotype. Positive association was shown only for *IL-4/TCC* haplotype (p<0.001), OR 3.446 (2.406–4.936). Negative (protective) association (according to the protective level) was found for *IL-4/GCC* (p<0.001), OR 0.297 (0.176–0.500); *IL-4/TTC* (p=0.009), OR 0.497 (0.293–0.846); and *IL-2/TG* haplotype (p=0.016), OR 0.641 (0.445–0.922). Haplotypes *IL-4/GTT* and *IL-4/TCT* were present only in healthy Macedonian population (Table 3).

Cytokine diplotypes (haplotype zygosity)

Cytokine diplotypes (or haplotype zygosity) are combinations of haplotypes from both parents. Table 4 comprises results from cytokine diplotypes analysis.

Obtained results reveal that *IL-4/TCC:TTC* (p<0.001, OR=7.859, Wald's 95% CI between 3.058 and 20.199), *IL2/GG:GG* (p<0.001, OR=2.815, Wald's 95% CI between 1.474 and 5.375), *IL-10/ATA:ATA* (p=0.020, OR=2.423, Wald's 95% CI between 1.125 and 5.218), and *IL-4/TCC:TCC* (p<0.001, OR=2.355, Wald's 95% CI between 1.416 and 3.919) combination of haplotypes have susceptible association, while only *IL-4/GCC:TTC* (p<0.0001, OR=0.065, Wald's 95% CI between 0.20 and 0.211) has strong protective association with RA (Table 4).

In Table 5, we can see the summary of all susceptible and protective cytokine polymorphisms obtained in our study.

Table 3 Haplotype frequency of cytokine polymorphism, Pearson's *p* value, odds ratio, and Wald's 95% confidence interval in RA patients and healthy Macedonians

Polymorphism	Haplotype	RA (*n*=85)		Control (*n*=301)		Pearson's *p* value	Odds ratio	Wald's 95% CI
		N	*F*	*N*	*F*			
TGF-β1	CC	10	0.059	30	0.053	0.784	1.108	0.530–2.317
	CG	66	0.388	250	0.445	0.192	0.792	0.558–1.124
	TG	94	0.553	282	0.502	0.242	1.228	0.870–1.733
TNF-α	AG	19	0.113	74	0.123	0.730	0.910	0.532–1.555
	GA	2	0.012	26	0.043	0.055	0.267	0.063–1.136
	GG	147	0.875	502	0.834	0.195	1.394	0.841–2.311
IL-2	GG	68	0.405	178	0.310	0.058	1.410	0.987–2.013
	GT	3	0.018	14	0.024	0.619	0.727	0.207–2.561
	TG	54	0.321	244	0.425	0.016[b]	0.641	0.445–0.922
	TT	43	0.256	138	0.240	0.680	1.087	0.732–1.615
IL-4	GCC	18	0.106	163	0.285	<0.001[b]	0.297	0.176–0.500
	GCT	2	0.012	8	0.014	0.825	0.839	0.177–3.990
	GTC	1	0.006	4	0.007	0.876	0.840	0.093–7.569
	GTT	0	0	1	0.002	[a]	[a]	[a]
	TCC	111	0.653	202	0.353	<0.001[b]	3.446	2.406–4.936
	TCT	0	0	4	0.007	[a]	[a]	[a]
	TTC	18	0.106	110	0.192	0.009[b]	0.497	0.293–0.846
	TTT	20	0.117	80	0.140	0.456	0.820	0.486–1.383
IL-6	CA	58	0.345	172	0.286	0.136	1.318	0.916–1.897
	CG	4	0.024	9	0.150	0.431	1.607	0.489–5.285
	GG	105	0.625	420	0.698	0.074	0.722	0.505–1.033
	GA	1	0.006	1	0.002	0.334	3.599	0.224–57.84
IL-10	ACA	1	0.006	12	0.020	0.206	0.289	0.037–2.238
	ACC	48	0.282	177	0.296	0.730	0.936	0.642–1.365
	ATA	55	0.324	161	0.269	0.165	1.298	0.898–1.877
	ATC	1	0.006	2	0.003	0.640	1.763	0.159–19.57
	GCC	65	0.382	246	0.411	0.496	0.886	0.625–1.257

N absolute number, *F* frequency, *CI* confidence interval

[a] Cannot be calculated because expected <5, χ^2 test

[b] Statistically significant

Discussion

IL-4 plays an important modifying role in the pathogenesis of RA, shifting the Th1/Th2 balance toward Th2. Gene for IL-4, together with some other Th2 cytokine genes and other genes related with immune response, is located in the long arm of the 5 chromosome. Today, several SNPs in the promoter and the coding region of the IL-4 gene is reported, some of them with functional alterations [18]. In this study, we investigated alleles and genotypes of three polymorphisms of *IL-4* (at positions *−1098*, *−590*, and *−33*) mapped in the promoter region, as well as haplotypes and diplotypes of investigated polymorphisms. Our results showed that subjects with *IL-4 −1098/T* allele are three times more susceptible for RA, while in *IL-4 −1098/T:T* homozygous genotype bearers this risk rises to 51 times. We found susceptible association for *IL-4 −590/C* allele (OR=1.737) and *IL-4 −590/C:C* homozygous genotype also. From the haplotype and diplotype analysis, we found susceptible association for *IL-4/TCC* haplotype and *IL-4/TCC:TTC* and *IL-4/TCC:TCC* diplotypes. On the other hand, protective associations for RA were found for the *IL-4 −1098/G* and *IL-4 −590/T* allele, *IL-4 −1098/G:T* and *IL-4/C:T* genotypes, *IL-4/GCC* and *IL-4/TTC* haplotypes, and *IL-4/GCC:TTC* combination of haplotypes. Results obtained from the studies about the association of *IL-4* polymorphisms and RA is still controversial reflecting the influence of the genetic background [19, 20]. It remains questionable whether this observed association is solely the effect of *IL-4* polymorphisms or association of other genes

Table 4 Cytokine diplotypes (haplotype zygotes), Pearson's *p* value, odds ratio, and Wald's 95% confidence interval in RA patients and healthy Macedonians

Polymorphism	Genotype	RA (*n*=85)		Control (*n*=301)		Pearson's *p* value	Odds ratio	Wald's 95% CI
		N	*F*	*N*	*F*			
TGF-β1	CC:CG	4	0.047	16	0.057	0.725	0.818	0.266–2.516
	CC:TG	4	0.047	14	0.050	0.918	0.942	0.302–2.941
	CG:CG	13	0.153	49	0.174	0.644	0.855	0.439–1.664
	CG:TG	36	0.423	136	0.484	0.328	0.783	0.480–1.278
	TG:TG	27	0.318	66	0.235	0.125	1.517	0.890–2.585
	CC:CC	1	0.012	0	0	[a]	[a]	[a]
TNF-α	AG:GG	15	0.179	66	0.219	0.418	0.774	0.416–1.441
	GA:GG	2	0.024	24	0.080	0.071	0.282	0.065–1.216
	GG:GG	65	0.774	206	0.684	0.056	1.763	0.981–3.170
	AG:AG	2	0.024	4	0.013	0.491	1.811	0.326–10.062
	GA:GA	0	0	1	0.004	[a]	[a]	[a]
IL-2	GG:GG	19	0.226	27	0.094	<0.001[b]	2.815	1.474–5.375
	GG:TG	17	0.202	85	0.296	0.090	0.603	0.334–1.087
	GG:TT	13	0.155	38	0.133	0.629	1.183	0.598–2.340
	GT:TG	3	0.036	11	0.058	0.912	0.929	0.253–3.411
	TG:TG	9	0.107	50	0.174	0.139	0.569	0.267–1.211
	TG:TT	16	0.191	48	0.168	0.620	1.172	0.626–2.192
	TT:TT	7	0.083	25	0.087	0.914	0.953	0.397–2.287
	GT:GG	0	0	1	0.003	[a]	[a]	[a]
	GT:TT	0	0	2	0.007	[a]	[a]	[a]
IL-4	GCC:GCC	0	0	1	0.003	[a]	[a]	[a]
	GCC:TCC	11	0.129	26	0.091	0.298	1.487	0.702–3.149
	GCC:TTC	3	0.035	103	0.360	<0.001[b]	0.065	0.020–0.211
	GCC:TTT	4	0.047	32	0.112	0.076	0.392	0.135–1.142
	TCC:TCC	36	0.423	68	0.238	<0.001[b]	2.355	1.416–3.919
	TCC:TTC	14	0.165	7	0.025	<0.001[b]	7.859	3.058–20.199
	TCC:TTT	14	0.165	28	0.098	0.088	1.817	0.908–3.634
	TTT:TTT	1	0.012	4	0.014	0.876	0.839	0.093–7.611
	GCT:TTT	0	0	8	0.028	[a]	[a]	[a]
	GTC:TTC	0	0	4	0.014	[a]	[a]	[a]
	TCT:TTT	0	0	4	0.014	[a]	[a]	[a]
	GTT:TTC	0	0	1	0.003	[a]	[a]	[a]
	TTC:GCT	1	0.012	0	0	[a]	[a]	[a]
	GTC:GCT	1	0.012	0	0	[a]	[a]	[a]
IL-6	CA:CA	13	0.155	25	0.083	0.051	2.021	0.985–4.149
	CA:GG	29	0.345	122	0.405	0.319	0.774	0.467–1.282
	CG:GG	1	0.012	9	0.030	0.359	0.391	0.049–3.130
	GG:GG	37	0.440	144	0.479	0.538	0.858	0.528–1.396
	GA:GG	1	0.012	1	0.003	0.333	3.615	0.224–58.407
	CG:CA	3	0.036	0	0	[a]	[a]	[a]
IL-10	ACC:ACC	8	0.094	21	0.070	0.462	1.375	0.586–3.226
	ACC:ATA	7	0.082	21	0.070	0.705	1.188	0.487–2.897
	ACC:GCC	25	0.294	114	0.381	0.140	0.676	0.401–1.139
	ATA:ATA	12	0.141	19	0.064	0.020[b]	2.423	1.125–5.218
	ATA:GCC	22	0.259	93	0.311	0.354	0.774	0.449–1.332
	GCC:GCC	9	0.106	17	0.057	0.112	1.964	0.842–4.581

Table 4 (continued)

Polymorphism	Genotype	RA (n=85)		Control (n=301)		Pearson's p value	Odds ratio	Wald's 95% CI
		N	*F*	*N*	*F*			
	ACA:GCC	0	0	3	0.010	[a]	[a]	[a]
	ACA:ATA	1	0.012	9	0.030	0.349	0.384	0.048–3.071
	ATC:GCC	0	0	2	0.007	[a]	[a]	[a]
	ATC:ATA	1	0.012	0	0	[a]	[a]	[a]

N absolute number, *F* frequency, *CI* confidence interval

[a] Cannot be calculated because expected <5, χ^2 test

[b] Statistically significant

located in the long arm of the 5 chromosome may play a significant role in pathogenesis of RA.

Another powerful anti-inflammatory cytokine that regulates the balance of Th1–Th2 responses is IL-10. It also plays an important role in the pathogenesis of RA. Today, several polymorphisms in the *IL-10* gene have been described [21]. Some of them have been associated with low, some with intermediate, and some with high production of IL-10. We examined the association of three SNPs located in the promoter region of the gene with RA. Studied independently, there was no significant association between all three investigated *IL-10* alleles and RA presumably. However, analysis of genotypes showed that only *A:A* genotype (homozygous *A* allele) for the position −1082 and *T:T* genotype (homozygous *T* allele) for the position −819 were susceptible for RA. Patients with these genotypes have approximately 2.6 times bigger risk to develop the disease. This risk stays the same for *IL-10/ATA:ATA* diplotype. On the other hand, from all examined *IL-10* polymorphisms, only −1082 *A:G* genotype showed protective association with RA (OR=0.5). Several studies deal with the role of *IL-10* polymorphisms and RA. Results are still contradictory [22, 23]. One presumable explanation is that, in different populations, different alleles/haplotypes may be important in regulating the expression of IL-10.

Only a few studies about the potential role of IL-2 exist. Its role in the pathogenesis of RA remains obscure. Our result showed that only *IL-2 −330* polymorphism was associated with RA. *IL-2 −330/G* allele showed susceptible association. The strength of this association rises in *IL-2 −330/G:G* genotype, but stayed the same in *IL-2/GG:GG* diplotype. Protective associations for RA were found for *IL-2 −330/T*

Table 5 Summary of all susceptible (positive) and protective (negative) cytokine polymorphisms for RA in Macedonians

	Susceptible (positive)			Protective (negative)		
	Polymorphism	*p*	Odds ratio	Polymorphism	*p*	Odds ratio
Cytokine alleles	*TNF-α −238/G*	0.047	3.897	*TNF-α −238/A*	0.047	0.257
	IL-4 −1098/T	<0.001	3.153	*IL-4 −1098/G*	<0.001	0.317
	IL-4 −590/C	0.006	1.737	*IL-4 −590/T*	0.006	0.576
	IL-2 −330/G	0.032	1.468	*IL-2 −330/T*	0.032	0.681
Cytokine genotypes	*IL-4 −1098/T:T*	<0.001	51.71			
	IL-2 −330/G:G	0.001	2.815			
	IL-10 −819/T:T	0.008	2.661	*IL-4 −1098/G:T*	<0.001	0.185
	IL-6 −174/C:C	0.005	2.598	*IL-4 −590/C:T*	<0.001	0.408
	IL-4 −590/C:C	<0.001	2.487	*IL-10 −1082/A:G*	0.007	0.508
	IL-10 −1082/A:A	0.046	1.694			
Cytokine haplotypes	*IL-4/TCC*	<0.001	3.446	*IL-4/GCC*	<0.001	0.297
				IL-4/TTC	0.009	0.497
				IL-2/TG	0.016	0.641
Cytokine diplotypes (haplotype zygosity)	*IL-4/TCC:TTC*	<0.001	7.859	*IL-4/GCC:TTC*	<0.001	0.065
	IL-2/GG:GG	<0.001	2.815			
	IL-10/ATA:ATA	0.020	2.423			
	IL-4/TCC:TCC	<0.001	2.355			

allele and *IL-2/TG* haplotype. Our results for *IL-2/G:G* genotype correlate with those obtained for Polish population [24], but are in disagreement with others [25]

Having in mind the fact that RA represents Th1 inflammatory autoimmune disease, one can presume that polymorphisms in the proinflammatory cytokine genes might play an important role in the pathogenesis. Although IL-6 has wide range of proinflammatory activities and induces secretion of acute phase proteins, stimulate T and B cells, and stimulate synoviocytes and osteoclasts, with resultant damage to cartilage and bone, only a few studies have analyzed the possible relationship between IL-6 polymorphisms and RA. High level of IL-6 in serum and synovial tissue of patient with RA was found [26]. We found susceptible association only for *IL-6 −174/C:C* genotype.

Another member of the proinflammatory cytokines that might play a key role in the development of RA is *TNF-α*. There are studies that showed no association between the *TNF-a* polymorphism and clinical manifestations or severity of RA [27], although some meta-analysis of 14 studies from different ethnic groups showed that the *TNF-alpha −308 A/G* polymorphism may represent a significant risk factor for RA in Latin Americans but not in Europeans [28]. Our results showed protective association for *TNF-alpha −238/A* allele. This contradictory finding implies that the *TNF-α* allele may not play or may have a limited role in the predisposition to RA, or maybe these differences reflect not only the genetic background but also the differences in climate, food, and parasite exposure.

Several SNPs (*IL-1α −889*, *IL-1β +3962*, *IL-2 +166*, *IL-4 −1098*, *IL-4 −590*, *IL-4 −33*, and *IL-10 −592*) in the control group were not in HWP ($p<0.005$) [12] and we should be very careful about their associations with RA in our population. The number of patients in our study is very small. In the association studies, there are possibilities that some positive results might be spurious and some negative findings might be a consequence of low statistical power. It is necessary to investigate cytokine gene polymorphisms in our population in well-defined subgroups of phenotypes with bigger number of participants in order to have more precise conclusions for genetic background of development of RA in Macedonians. Multicentric studies and/or meta-analysis of the patients with RA and association with cytokine polymorphisms should be very useful.

It can be concluded that, at least in Macedonian patients with RA, some cytokine polymorphisms contribute to susceptibility/protection to disease. Ethnic factors might play a role in the variability of results in different populations. Therefore, additional studies are needed to clarify this issue.

Acknowledgements This research is part of the project "Molecular analysis of cytokine gene polymorphisms in the Republic of Macedonia" supported by the Ministry of Education and Science from Republic of Macedonia (Project No. 13-874/3-05). We would like to gratefully acknowledge Prof. G. Opelz and Dr. J. Mytilineos from the Institute of Immunology, Department of Transplantation Immunology, University of Heidelberg, Heidelberg, Germany for kindly supplying the Heidelberg PCR–SSP kit reagents in this project. For sample collection, technical support, and laboratory direction, we thank Elena Zaharieva.

Disclosures None

References

1. MacGregor AJ, Snieder H, Rigby AS, Koskenvuo M, Kaprio J, Aho K et al (2000) Characterizing the quantitative genetic contribution to rheumatoid arthritis using data from twins. Arthritis Rheum 43:30–37
2. Miossec P (2004) An update on the cytokine network in rheumatoid arthritis. Curr Opin Rheumatol 16:218–222
3. Warle MC, Farhan A, Metselaar HJ, Hop WC, Perrey C, Zondervan PE, Kap M, Kwekkeboom J, Ijzermans JN, Tilanus HW, Pravica V, Hutchinson IV, Bouma GJ (2003) Are cytokine gene polymorphisms related to in vitro cytokine production profiles? Liver Transplant 9(2):170–181
4. Hoffmann SC, Stanley EM, Cox ED et al (2002) Ethnicity greatly influences cytokine gene polymorphism distribution. Am J Transplant 2:560–567
5. Trajkov D, Atanasovska-Stojanovska A, Petlichkovski A, Strezova A, Gogusev J, Hristomanova S, Djulejic E, Petrov J, Spiroski M (2008) IL-1 gene cluster polymorphisms in the Macedonian population. Maced J Med Sci 1(1):21–28
6. Trajkov D, Arsov T, Petlichkovski A, Strezova A, Efinska-Mladenovska O, Gogusev J, Spiroski M (2009) Distribution of the 22 cytokine gene polymorphisms in healthy Macedonian population. Bratisl Lek Listy 110(1):7–17
7. Trajkov D, Stojkovikj JM, Arsov T, Petlichkovski A, Strezova A, Mladenovska OE, Sandevska E, Gogusev J, Spiroski M (2008) Association of cytokine gene polymorphisms with bronchial asthma in Macedonians. Iran J Allergy Asthma Immunol 7 (3):143–156
8. Trajkov D, Mirkovska-Stojkovikj J, Petlichkovski A, Strezova A, Efinska-Mladenovska O, Sandevska E, Sibinovska O, Hristomanova S, Djulejic E, Petrov J, Gogusev J, Spiroski M (2009) Association of cytokine gene polymorphisms with chronic obstructive pulmonary disease in Macedonians. Iran J Allergy Asthma Immunol 8(1):31–42
9. Arnett FC, Edworthy SM, Bloch DA et al (1998) The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 31:315–324
10. Towner P (1995) Purification of DNA. In: Brown TA (ed) Essential molecular biology. Oxford University Press, Oxford, pp 47–54
11. Spiroski M, Arsov T, Petlichkovski A, Strezova A, Trajkov D, Efinska-Mladenovska O, Zaharieva E (2005) Case study: Macedonian Human DNA Bank (hDNAMKD) as a source for public health genetics. In: Georgieva L, Burazeri G (eds) Health determinants in the scope of new public health. Hans Jacobs, Sofia, pp 33–44
12. Tseng LH, Chen PJ, Lin MT, Singleton K, Martin EG, Yen AH, Martin PJ, Hansen JA (2002) Simultaneous genotyping of single nucleotide polymorphisms in the IL-1 gene complex by multiplex polymerase chain reaction–restriction fragment length polymorphism. J Immunol Methods 267:151–156
13. Helmberg W, Lanzer G, Zahn R, Weinmayr B, Wagner T, Albert E (1998) Virtual DNA analysis—a new tool for combination and standardised evaluation of SSO, SSP and sequencing-based typing results. Tissue Antigens 51:587–592

14. Lancaster AK, Single RM, Solberg OD, Nelson MP, Thomson G (2007) PyPop update—a software pipeline for large-scale multi-locus population genomics. Tissue Antigens 69(Suppl 1):192–197
15. Single RM, Meyer D, Mack SJ, Lancaster A, Erlich HA, Thomson G (2007) 14th International HLA and Immunogenetics Workshop: report of progress in methodology, data collection, and analyses. Tissue Antigens 69(Suppl 1):185–187
16. Guo S, Thomson E (1992) Performing the exact test of Hardy Weinberg proportion for multiple alleles. Biometrics 48:361
17. Schneider S, Roessli D, Excoffier L (2000) Arlequin version 2.000: a software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Geneva
18. Kruse S, Japha T, Tedner M, Sparholt SH, Forster J, Kuehr J et al (1999) The polymorphisms S503P and Q576R in the interleukin-4 receptor α gene are associated with atopy and influence the signal transduction. Immunology 96:365–371
19. Moreno O, Gonzalez CI, Saaibi DL, Otero W, Badillo R, Martin J, Ramirez G (2007) Polymorphisms in the IL4 and IL4RA genes in Colombian patients with rheumatoid arthritis. J Rheumatol 34:36–42
20. Huang CM, Wu MC, Wu JY, Tsai FJ (2002) No association of interleukin-4 gene polymorphisms in Chinese patients with rheumatoid arthritis in Taiwan. Clin Exp Rheumatol 20:871–872
21. Turner DM, Williams DM, Sankaran D, Lazarus M, Sinnott PJ, Hutchinson IV (1997) An investigation of polymorphism in the interleukin-10 gene promoter. Eur J Immunogenet 24(1):1–8
22. Ates O, Hatemi G, Hamuryudan V, Topal-Sarikaya A (2008) Tumor necrosis factor-alpha and interleukin-10 gene promoter polymorphisms in Turkish rheumatoid arthritis patients. Clin Rheumatol 27:1243–1248. doi:10.1007/s10067-008-0893-1
23. Martinez A, Pascual M, Pascual-Salcedo D, Balsa A, Martin J, de la Concha EG (2003) Genetic polymorphisms in Spanish rheumatoid arthritis patients: an association and linkage study. Genes Immun 4:117–121. doi:10.1038/sj.gene.6363931
24. Pawlik A, Kurzawski M, Florczak M, Gawronska Szklarz B, Herczynska M (2005) IL1beta+3953 exon 5 and IL-2–330 promoter polymorphisms in patients with rheumatoid arthritis. Clin Exp Rheumatol 23(2):159–164
25. Fedetz M, Matesanz F, Caliz R, Ferrer MA, Collado MD, Alcina A, Martin J (2003) Lack of association between −384 and 114 IL-2 gene polymorphisms and rheumatoid arthritis. J Rheumatol 30 (3):435–437
26. Wood NC, Symons JA, Dickens E, Duff GW (1992) In situ hybridization of IL-6 in rheumatoid arthritis. Clin Exp Immunol 87:183–189
27. Wilson AG, de Vries N, van de Putte LB, Duff GW (1995) A tumour necrosis factor alpha polymorphism is not associated with rheumatoid arthritis. Ann Rheum Dis 54:601–603
28. Lee YH, Ji JD, Song GG (2007) Tumor necrosis factor-alpha promoter −308 A/G polymorphism and rheumatoid arthritis susceptibility: a metaanalysis. J Rheumatol 34:43–49