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ORIGINAL ARTICLE

Polymorphisms in the Genes Encoding TGF-β1, TNF-α, and IL-6 Show Association with Type 1 Diabetes Mellitus in the Slovak Population

Juraj Javor · Stanislav Ferencik · Maria Bucova · Martina Stuchlikova · Emil Martinka · Lubomir Barak · Lujza Strbova · Hans Grosse-Wilde · Milan Buc

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Abstract Numerous cytokines have been shown to participate in the pathogenesis of type 1 diabetes (T1D). As gene polymorphisms can influence cytokine production or function, they may potentially contribute to genetic predisposition to the disease. The aim of this study was therefore to investigate the role of 22 single nucleotide polymorphisms (SNPs) in 13 cytokine and cytokine receptor genes in genetic susceptibility to T1D. Polymerase chain reaction with sequence-specific primers was used to genotype cytokine SNPs and HLA-DRB1 alleles in 151 diabetics and 140 healthy individuals of Slovak origin. Univariate analysis showed that transforming growth factor (TGF)-β1 codon 10 TT homozygotes were significantly more susceptible to developing T1D than C allele carriers ($P_c = 0.0066$, OR = 2.46). Furthermore, tumor necrosis factor (TNF)-α -308 A allele carriers were also significantly overrepresented among the diabetics ($P_c = 0.0031$, OR = 2.62); however, the association of the -308 A allele with T1D might be due to its strong linkage disequilibrium with the susceptibility allele HLA-DRB1*0301. An association was also found with interleukin (IL)-6 -174 G/C and nt565 G/A SNPs; however, its significance was lost when statistical correction was applied. These data suggest that the TGF-β1 codon 10 SNP is among numerous genetic variations with small individual effects on T1D development. Moreover, a possible role of TNF-α and IL-6 SNPs cannot be ruled out, although their association with T1D was due to strong LD with the HLA class II susceptibility allele or did not withstand statistical correction, respectively.

Keywords Cytokines · Interleukin-6 · Single nucleotide polymorphisms · Transforming growth factor-β1 · Tumor necrosis factor-α · Type 1 diabetes

J. Javor (✉) · M. Bucova · M. Stuchlikova · M. Buc
Department of Immunology, Faculty of Medicine,
Comenius University, Odborarske nam. 14,
813 72 Bratislava 1, Slovakia
e-mail: jurajjavor@gmail.com

S. Ferencik
Institute for Transfusion Medicine, University Hospital of Essen,
Essen, Germany

H. Grosse-Wilde
Institute of Immunology, University Hospital of Essen,
Essen, Germany

E. Martinka
Department of Diabetology,
National Institute of Endocrinology and Diabetology,
Lubochna, Slovakia

L. Barak
1st Department of Pediatrics, Faculty of Medicine,
Comenius University, Bratislava, Slovakia

L. Strbova
2nd Department of Internal Medicine, Faculty of Medicine,
Comenius University, Bratislava, Slovakia

Introduction

Type 1 diabetes (T1D) is one of the most common chronic diseases in children and young adults and usually develops as a result of progressive T cell-mediated autoimmune destruction of pancreatic β cells (American Diabetes Association 2004; Ichinose et al. 2007). To date, several susceptibility loci have been identified on chromosomes 6p21 (HLA class II genes), 11p15 (gene for insulin), 2q33 (*CTLA4*), 1p13 (*PTPN22*), 10p15 (*IL2RA*), 12q24 (with candidate genes *SH2B3*, *TRAFD1*, and *PTPN11*), 12q13 (*ERBB3*), 16p13 (*CLEC16A*), 18p11 (*PTPN2*), 2q24 (*IFIH1*), 18q22 (*CD226*),

and others to confer genetic risk for T1D (Barrett et al. 2009; Smyth et al. 2006; Todd et al. 2007; Wellcome Trust Case Control Consortium 2007). Potential candidates are also genes encoding cytokines, molecules that play an important role in the immunological process and inflammation resulting in the destruction of pancreatic β cells (Ichinose et al. 2007; Yoon and Jun 2005). Several studies have suggested that pro-inflammatory cytokines and chemokines (IL-1, IL-6, IL-8, TNF-α, and others), Th1 (IFN-γ, LT, IL-2), and Th17 cytokines (IL-17) may be responsible for destructive insulitis, whereas Th2 (IL-4), Th3, Tr1, and Treg cytokines (IL-10 and TGF-β) and cytokine antagonists (e.g. IL-1Ra) likely play a protective role via suppressing the synthesis of Th1 and pro-inflammatory cytokines (Rabinovitch 1998; Ichinose et al. 2007). In particular, IL-1β, IFN-γ, and TNF-α are major cytokines capable of inducing the apoptosis of insulin-producing cells and mediating the inflammatory process in Langerhans islets (Mauricio and Mandrup-Poulsen 1998; Rabinovitch 1998). The role of pro-inflammatory cytokine IL-6 is less clear and direct evidence for its deleterious and cytotoxic effects on pancreatic β cells is still lacking (Kristiansen and Mandrup-Poulsen 2005). IL-2 is another attractive candidate for participation in T1D development, as it is involved in the proliferation and activation of effector $CD4^+$ and $CD8^+$ T cells. On the other hand, IL-2 also plays a fundamental role in the development, survival, and function of $Foxp3^+$ $CD4^+$ $CD25^+$ Treg cells. Thus the fine balance between tolerance and autoimmunity may depend on an optimal level of IL-2/IL-2R signaling (Dendrou and Wicker 2008; Wang et al. 2009).

Genome-wide association studies have recently identified an association between T1D and chromosome region 4q27, which harbors a few candidate genes, including *IL2* (Cooper et al. 2008; Todd et al. 2007), providing further support for a role of IL-2 in the pathogenesis of T1D. Polymorphisms in the regulatory and coding regions of cytokine genes have been shown to influence the level of gene expression and in vivo cytokine production or to alter the structure and function of cytokine molecules. In this way they could contribute to genetic susceptibility and development of the disease (Smith and Humphries 2009).

The aim of our study was to investigate the distribution of 22 polymorphisms in 13 cytokine and cytokine receptor genes (for IL-1α, IL-1β, IL-1RI, IL-1Ra, IL-4Rα, IL-12, IFN-γ, TGF-β1, TNF-α, IL-2, IL-4, IL-6, and IL-10) in patients with T1D and healthy controls in order to understand their role in susceptibility to the disease. The selection of candidate genes was based on current knowledge about the role of encoded molecules in the pathogenesis of the disease and the investigated polymorphisms in their sequences have been reported to have impact on cytokine expression or function (Smith and Humphries 2009). Candidate gene association studies have previously indicated possible association between T1D and some of these single nucleotide polymorphisms (SNPs), including IL-1β +3962 T/C, TNF-α −308 G/A, IL-6 −174 G/C, IL-4Rα +1902 G/A, and IL-10 −1082 G/A, −819 C/T, and −592 C/A (reviewed by Bidwell et al. 1999, 2001; Haukim et al. 2002; Hollegaard and Bidwell 2006). Further replication studies and meta-analyses along with functional analyses are, however, required to confirm the role of cytokine SNPs in susceptibility to T1D.

Materials and Methods

Study Population

A total of two hundred and ninety-one unrelated Slovak subjects of Caucasian origin were selected and included in this study. The patient group consisted of 151 T1D cases (median age: 26.0, range: 5–65 years, median age at disease onset: 14.0, range: 0–49 years), of whom 81 were men (median age: 26.0, range: 5–65 years, median age at disease onset: 14.0, range: 0–46 years) and 70 were women (median age: 28.0, range: 7–61 years, median age at disease onset: 14.0, range: 2–49 years). The diagnosis of T1D was established by referring clinicians according to the criteria of the American Diabetes Association (2004). All patients required insulin for glycemic control at the time of diagnosis and had serum C-peptide levels <0.16 nmol/l.

The control group comprised 140 unrelated healthy subjects (66 men and 74 women, median age: 38.0, range: 21–80 years) with neither symptoms nor family history of T1D and other autoimmune disorders. Informed consent from the patients and controls as well as Comenius University Ethics Committee approval were obtained before the study started.

Genotyping

Genomic DNA was extracted from whole blood with a Chemagic Magnetic Separation Module I using the Chemagic DNA Blood2k Kit (Chemagen Biopolymer-Technologie AG, Baesweiler, Germany). Cytokine genotyping was performed by polymerase chain reaction with sequence-specific primers (PCR-SSP) using a commercial kit (Cytokine Genotyping Kit; Invitrogen Corp., Brown Deer, WI, USA) developed for the cytokine polymorphism component of the 13th International Histocompatibility Workshop (http://www.ihwg.org/components/cytokine/cytover.htm). Information on the studied SNPs, including their kit designations, dbSNP-IDs, aliases, and chromosome location, is listed in Table 1. The HLA-DRB1 locus was analyzed in 151 patients and 113 controls by PCR-SSP using a commercially available kit (Dynal AllSet^{+TM} SSP

Table 1 List of the investigated cytokine SNPs (modified from Kleinrath et al. 2007; Gallo et al. 2009)

Cytokine gene	Chromosome location	SNP designation in the kit	dbSNP-ID	Other designations	Function
IL1A	2q13–q21	−889 T/C	rs1800587	IL-1α *NcoI*	5′-UTR
IL1B	2q13–q21	−511 C/T	rs16944	IL-1β *AvaI*	Promoter
IL1B	2q13–q21	+3962 T/C	rs1143634	+3954	Coding/synonymous
IL1R1	2q13–q21	pst1 1970 C/T	rs2234650	IL-1R1 *PstI*	Distal promoter
IL1RN	2q13–q21	mspa1 11100 T/C	rs315952		Coding/synonymous
IL4RA	16p11–p12	+1902 G/A	rs1801275	Gln576Arg	Coding/missense
IL12B	5q31–33	−1188 C/A	rs3212227	1188	3′-UTR
IFNG	12q24	+874 A/T	rs2430561		Intron
TGFB1	19q13	codon 10 T/C	rs1800470	+868	Coding/missense
TGFB1	19q13	codon 25 G/C	rs1800471	+915	Coding/missense
TNFA	6p21	−308 G/A	rs1800629		Promoter
TNFA	6p21	−238 G/A	rs361525		Promoter
IL2	4q27	−330 T/G	rs2069762		Promoter
IL2	4q27	+166 G/T	rs2069763		Coding/synonymous
IL4	5q31–q33	−1098 T/G	rs2243248		Promoter
IL4	5q31–q33	−590 C/T	rs2243250	−589, −524	Promoter
IL4	5q31–q33	−33 C/T	rs2070874		5′-UTR
IL6	7p21	−174 G/C	rs1800795		Promoter
IL6	7p21	nt565 G/A	rs1800797	−597	Promoter
IL10	1q31–q32	−1082 G/A	rs1800896	−1117	Promoter
IL10	1q31–q32	−819 C/T	rs1800871	−854	Promoter
IL10	1q31–q32	−592 C/A	rs1800872	−627	Promoter

Kit; Dynal Biotech, Hamburg, Germany). PCR amplifications were carried out according to the manufacturers' manuals using a PTC-200 Thermal Cycler (MJ Research, Inc., Waltham, MA, USA).

Statistical Analysis

Observed and expected genotype frequencies of all the SNPs were compared using the χ^2 test to test for their fit to Hardy–Weinberg equilibrium. Fisher's exact test was used to compare allele, genotype, and haplotype frequencies between patients and controls. *P* values were corrected by Bonferroni's method for multiple testing and the differences were considered statistically significant when the corrected two-sided *P* value was less than 0.05. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated to estimate the strength of the association. The analyses were performed with InStat statistical software (GraphPad Software, Inc., San Diego, CA, USA). To test for linkage disequilibrium (LD) between HLA-DRB1*0301 and TNF-α −308 A (TNF2) alleles, D, D', χ^2, and P values were calculated using the Arlequin 3.1 software (Excoffier et al. 2005). Power analysis for each investigated SNP was performed according to protocols described elsewhere (Uitenbroek 1997).

Results

The genotype distributions of all the polymorphic cytokine genes followed the Hardy–Weinberg distribution and no deviation from HW equilibrium was observed either in the patient or in the control group. Analysis of the 22 cytokine SNPs revealed marked differences in allele, genotype, and haplotype distribution between patients and controls only in the TGF-β1 codon 10 T/C, TNF-α −308 G/A, IL-6 −174 G/C, and IL-6 nt565 G/A polymorphisms (Tables 2, 3, 4). We found significantly increased frequency of TGF-β1 codon 10 TT homozygotes among the patients (50.67 vs. 29.50%, $P_c = 0.0066$, OR = 2.46) and, similarly, the proportion of TNF-α −308 A allele carriers (AA + AG genotypes) was also higher in the T1D group than in the controls (45.70 vs. 24.28%, $P_c = 0.0031$, OR = 2.62). However, LD testing revealed strong LD between the −308 A allele (also known as TNF2) and the HLA-DRB1*0301 allele in both patients ($D' = 0.746$, $\chi^2 = 149.12$, $P < 0.00001$) and controls ($D' = 0.869$, $\chi^2 = 80.51$, $P < 0.00001$). HLA-DRB1*0301 acts a strong susceptibility allele and its occurrence was significantly increased among the diabetics compared with the healthy controls (27.48 vs. 7.96%, $P_c < 0.01$, OR = 4.38). Finally, the IL-6 −174 C allele and nt565 A allele carriers were also overrepresented in the T1D patients; however, these

Table 2 Allele frequencies of the investigated cytokine polymorphisms in the patients with T1D and the healthy controls

Polymorphism	Allele	T1D ($n = 151$)	Controls ($n = 140$)	P	P_c	OR	95% CI
IL-1α −889	C	216 (71.52)	204 (74.45)	n.s.	n.s.	0.862	0.60–1.25
	T	86 (28.48)	70 (25.55)			1.160	0.80–1.68
IL-1β −511	C	205 (67.88)	195 (69.64)	n.s.	n.s.	0.921	0.65–1.31
	T	97 (32.12)	85 (30.36)			1.086	0.76–1.54
IL-1β +3962	C	227 (75.17)	209 (74.64)	n.s.	n.s.	1.028	0.71–1.50
	T	75 (24.83)	71 (25.36)			0.973	0.67–1.42
IL-1R1 pst1 1970	C	194 (64.24)	174 (62.14)	n.s.	n.s.	1.094	0.78–1.53
	T	108 (35.76)	106 (37.86)			0.914	0.65–1.28
IL-1Ra mspa1 11100	C	104 (34.44)	97 (34.46)	n.s.	n.s.	0.991	0.71–1.39
	T	198 (65.56)	183 (65.36)			1.009	0.72–1.42
IL-4Rα +1902	A	233 (77.15)	211 (76.45)	n.s.	n.s.	1.040	0.71–1.53
	G	69 (22.85)	65 (23.55)			0.961	0.65–1.42
IL-12 −1188	A	237 (78.48)	215 (77.34)	n.s.	n.s.	1.068	0.72–1.58
	C	65 (21.52)	63 (22.66)			0.936	0.63–1.39
IFN-γ +874	A	163 (54.33)	149 (53.21)	n.s.	n.s.	1.046	0.76–1.45
	T	137 (45.67)	131 (46.79)			0.956	0.69–1.33
TGF-β1 codon 10	C	90 (30.41)	125 (44.96)	0.0004	0.0087	0.535	0.38–0.75
	T	206 (69.59)	153 (55.04)			1.870	1.33–2.63
TGF-β1 codon 25	C	11 (3.72)	22 (7.91)	0.0326	n.s.	0.449	0.21–0.95
	G	285 (96.28)	256 (92.09)			2.227	1.06–4.68
TNF-α −308	A	76 (25.17)	37 (13.21)	0.0003	0.0074	2.209	1.43–3.40
	G	226 (74.83)	243 (86.79)			0.453	0.29–0.70
TNF-α −238	A	12 (3.97)	11 (3.93)	n.s.	n.s.	1.012	0.44–2.33
	G	290 (96.03)	269 (96.07)			0.988	0.43–2.28
IL-2 −330	G	102 (33.77)	87 (31.52)	n.s.	n.s.	1.108	0.78–1.57
	T	200 (66.23)	189 (68.48)			0.903	0.64–1.28
IL-2 +166	G	198 (65.56)	174 (63.04)	n.s.	n.s.	1.116	0.79–1.57
	T	104 (34.44)	102 (36.96)			0.896	0.64–1.26
IL-4 −1098	G	14 (4.64)	23 (8.21)	n.s.	n.s.	0.543	0.27–1.08
	T	288 (95.36)	257 (91.79)			1.841	0.93–3.65
IL-4 −590	C	248 (82.12)	229 (81.79)	n.s.	n.s.	1.023	0.67–1.56
	T	54 (17.88)	51 (18.21)			0.978	0.64–1.49
IL-4 −33	C	248 (82.12)	229 (81.79)	n.s.	n.s.	1.023	0.67–1.56
	T	54 (17.88)	51 (18.21)			0.978	0.64–1.49
IL-6 −174	C	147 (48.68)	108 (38.57)	0.0154	n.s.	1.510	1.09–2.10
	G	155 (51.32)	172 (61.43)			0.662	0.48–0.92
IL-6 nt565	A	145 (48.01)	103 (36.79)	0.0072	n.s.	1.587	1.14–2.21
	G	157 (51.99)	177 (63.21)			0.630	0.45–0.88
IL-10 −1082	A	166 (54.97)	159 (56.79)	n.s.	n.s.	0.929	0.67–1.29
	G	136 (45.03)	121 (43.21)			1.077	0.78–1.49
IL-10 −819	C	238 (78.81)	205 (72.21)	n.s.	n.s.	1.361	0.93–1.99
	T	64 (21.19)	75 (26.79)			0.735	0.50–1.08
IL-10 −592	A	64 (21.19)	75 (26.79)	n.s.	n.s.	0.735	0.50–1.08
	C	238 (78.81)	205 (72.21)			1.361	0.93–1.99

Allelic frequencies are presented as absolute numbers with percentages in parentheses

P_c corrected P value, *OR* odds ratio, *CI* confidence interval, *n.s.* not significant

Table 3 Genotype frequencies of the investigated cytokine polymorphisms in the patients with T1D and the healthy controls

Polymorphism	Genotype	T1D ($n = 151$)	Controls ($n = 140$)	P	P_c	OR	95% CI
IL-1α −889	CC	77 (50.99)	76 (55.47)	n.s.	n.s.	0.835	0.52–1.33
	CT	62 (41.06)	52 (37.96)	n.s.	n.s.	1.139	0.71–1.83
	TT	12 (7.95)	9 (6.57)	n.s.	n.s.	1.228	0.51–3.01
IL-1β −511	CC	72 (47.68)	69 (49.29)	n.s.	n.s.	0.938	0.59–1.49
	CT	61 (40.40)	57 (40.71)	n.s.	n.s.	0.987	0.62–1.58
	TT	18 (11.92)	14 (10.00)	n.s.	n.s.	1.218	0.58–2.55
IL-1β +3962	CC	86 (56.95)	81 (57.86)	n.s.	n.s.	0.964	0.61–1.54
	CT	55 (36.42)	47 (33.57)	n.s.	n.s.	1.134	0.70–1.84
	TT	10 (6.62)	12 (8.57)	n.s.	n.s.	0.757	0.32–1.81
IL-1R1 pst1 1970	CC	64 (42.38)	53 (37.86)	n.s.	n.s.	1.208	0.76–1.93
	CT	66 (43.71)	68 (48.57)	n.s.	n.s.	0.822	0.52–1.31
	TT	21 (13.91)	19 (13.57)	n.s.	n.s.	1.029	0.53–2.01
IL-1Ra mspa1 11100	CC	19 (12.58)	21 (15.00)	n.s.	n.s.	0.816	0.42–1.59
	CT	66 (43.71)	55 (39.29)	n.s.	n.s.	1.200	0.75–1.92
	TT	66 (43.71)	64 (45.71)	n.s.	n.s.	0.922	0.58–1.47
IL-4Rα +1902	AA	91 (60.27)	82 (59.42)	n.s.	n.s.	1.036	0.65–1.66
	AG	51 (33.77)	47 (34.06)	n.s.	n.s.	0.987	0.61–1.61
	GG	9 (5.96)	9 (6.52)	n.s.	n.s.	0.909	0.35–2.36
IL-12 −1188	AA	91 (60.27)	85 (61.15)	n.s.	n.s.	0.964	0.60–1.55
	AC	55 (36.42)	45 (32.37)	n.s.	n.s.	1.197	0.74–1.95
	CC	5 (3.31)	9 (6.48)	n.s.	n.s.	0.495	0.16–1.51
IFN-γ +874	AA	46 (30.67)	41 (29.28)	n.s.	n.s.	1.068	0.65–1.77
	AT	71 (47.33)	67 (47.86)	n.s.	n.s.	0.979	0.62–1.55
	TT	33 (22.00)	32 (22.86)	n.s.	n.s.	0.952	0.55–1.65
TGF-β1 codon 10	CC	17 (11.49)	27 (19.42)	n.s.	n.s.	0.538	0.28–1.04
	CT	56 (37.84)	71 (51.08)	0.0321	n.s.	0.583	0.36–0.93
	TT	75 (50.67)	41 (29.50)	0.0003	0.0066	2.456	1.51–3.99
TGF-β1 codon 25	CC	0 (0.00)	0 (0.00)	–	–	–	–
	CG	11 (7.43)	22 (15.83)	0.0276	n.s.	0.427	0.20–0.92
	GG	137 (92.57)	117 (84.17)	0.0276	n.s.	2.342	1.09–5.03
TNF-α −308	AA	7 (4.64)	3 (2.14)	n.s.	n.s.	2.220	0.56–8.76
	AG	62 (41.06)	31 (22.14)	0.0007	0.0144	2.449	1.47–4.10
	GG	82 (54.30)	106 (75.72)	0.0001	0.0031	0.381	0.23–0.63
TNF-α −238	AA	0 (0.00)	0 (0.00)	–	–	–	–
	AG	12 (7.95)	11 (7.86)	n.s.	n.s.	1.012	0.43–2.38
	GG	139 (92.05)	129 (92.14)	n.s.	n.s.	0.988	0.42–2.32
IL-2 −330	GG	16 (10.60)	16 (11.59)	n.s.	n.s.	0.904	0.43–1.89
	GT	70 (46.36)	55 (39.86)	n.s.	n.s.	1.304	0.82–2.08
	TT	65 (43.04)	67 (48.55)	n.s.	n.s.	0.801	0.51–1.27
IL-2 +166	GG	64 (42.38)	56 (40.58)	n.s.	n.s.	1.077	0.67–1.72
	GT	70 (46.36)	62 (44.93)	n.s.	n.s.	1.059	0.67–1.68
	TT	17 (11.26)	20 (14.49)	n.s.	n.s.	0.749	0.38–1.50
IL-4 −1098	GG	0 (0.00)	1 (0.71)	n.s.	n.s.	0.307	0.01–7.60
	GT	14 (9.27)	21 (15.00)	n.s.	n.s.	0.579	0.28–1.19
	TT	137 (90.73)	118 (84.29)	n.s.	n.s.	1.824	0.89–3.73
IL-4 −590	CC	103 (68.21)	94 (67.14)	n.s.	n.s.	1.050	0.64–1.72
	CT	42 (27.82)	41 (29.29)	n.s.	n.s.	0.930	0.56–1.55
	TT	6 (3.97)	5 (3.57)	n.s.	n.s.	1.117	0.33–3.75

Table 3 continued

Polymorphism	Genotype	T1D ($n = 151$)	Controls ($n = 140$)	P	P_c	OR	95% CI
IL-4 −33	CC	103 (68.21)	94 (67.14)	n.s.	n.s.	1.050	0.64–1.72
	CT	42 (27.82)	41 (29.29)	n.s.	n.s.	0.930	0.56–1.55
	TT	6 (3.97)	5 (3.57)	n.s.	n.s.	1.117	0.33–3.75
IL-6 −174	CC	31 (20.53)	21 (15.00)	n.s.	n.s.	1.464	0.80–2.69
	CG	85 (56.29)	66 (47.14)	n.s.	n.s.	1.444	0.91–2.29
	GG	35 (23.18)	53 (37.86)	0.0074	n.s.	0.495	0.30–0.82
IL-6 nt565	AA	31 (20.53)	18 (12.86)	n.s.	n.s.	1.751	0.93–3.30
	AG	83 (54.97)	67 (47.86)	n.s.	n.s.	1.330	0.84–2.11
	GG	37 (24.50)	55 (39.28)	0.0080	n.s.	0.502	0.30–0.83
IL-10 −1082	AA	51 (33.78)	49 (35.00)	n.s.	n.s.	0.947	0.58–1.54
	AG	64 (42.38)	61 (43.57)	n.s.	n.s.	0.953	0.60–1.52
	GG	36 (23.84)	30 (21.43)	n.s.	n.s.	1.148	0.66–1.99
IL-10 −819	CC	92 (60.93)	77 (55.00)	n.s.	n.s.	1.276	0.80–2.04
	CT	54 (35.76)	51 (36.43)	n.s.	n.s.	0.972	0.60–1.57
	TT	5 (3.31)	12 (8.57)	n.s.	n.s.	0.365	0.13–1.07
IL-10 −592	AA	5 (3.31)	12 (8.57)	n.s.	n.s.	0.365	0.13–1.07
	AC	54 (35.76)	51 (36.43)	n.s.	n.s.	0.972	0.60–1.57
	CC	92 (60.93)	77 (55.00)	n.s.	n.s.	1.276	0.80–2.04

Genotype frequencies are presented as absolute numbers with percentage in parentheses

P_c corrected P value, *OR* odds ratio, *CI* confidence interval, *n.s.* not significant

Table 4 Haplotype frequencies of the TGF-β, TNF-α, IL-2, IL-4, IL-6, and IL-10 polymorphisms in the patients with T1D and the healthy controls

Gene	Haplotype	T1D ($n = 151$)	Controls ($n = 140$)	P	P_c	OR	95% CI
TGF-β1	TG	206 (69.59)	153 (55.04)	0.0004	0.0087	1.870	1.33–2.63
	CG	79 (26.69)	103 (37.05)	0.0092	n.s.	0.619	0.43–0.88
	CC	11 (3.72)	22 (7.91)	0.0326	n.s.	0.449	0.21–0.95
TNF-α	GG	214 (70.86)	232 (82.86)	0.0008	0.0181	0.503	0.39–0.75
	AG	76 (25.17)	37 (13.21)	0.0003	0.0074	2.209	1.43–3.40
	GA	12 (3.97)	11 (3.93)	n.s.	n.s.	1.012	0.44–2.33
IL-2	TT	104 (34.44)	102 (36.96)	n.s.	n.s.	0.896	0.64–1.26
	TG	96 (31.79)	87 (31.52)	n.s.	n.s.	1.012	0.71–1.44
	GG	102 (33.77)	87 (31.52)	n.s.	n.s.	1.108	0.78–1.57
IL-4	GCC	14 (4.64)	23 (8.21)	n.s.	n.s.	0.543	0.27–1.08
	TCC	234 (77.48)	206 (73.58)	n.s.	n.s.	1.236	0.85–1.81
	TTT	54 (17.88)	51 (18.21)	n.s.	n.s.	0.978	0.64–1.49
IL-6	CA	145 (48.01)	103 (36.79)	0.0072	n.s.	1.587	1.14–2.21
	CG	2 (0.66)	5 (1.79)	n.s.	n.s.	0.367	0.07–1.91
	GG	155 (51.32)	172 (61.43)	0.0154	n.s.	0.662	0.48–0.92
IL-10	GCC	136 (45.03)	121 (43.21)	n.s.	n.s.	1.077	0.78–1.49
	ACC	102 (33.78)	84 (30.00)	n.s.	n.s.	1.190	0.84–1.69
	ATA	64 (21.19)	75 (26.79)	n.s.	n.s.	0.735	0.50–1.08

TGF-β1 haplotypes were formed by alleles of codon 10 and 25 SNPs; TNF-α haplotypes by −308 and −238 SNPs; IL-2 haplotypes by −330 and +166 SNPs; IL-4 haplotypes by −1098, −590 and −33 SNPs; IL-6 haplotypes by −174 and nt565 SNPs; IL-10 haplotypes by −1082, −819 and −592 SNPs

Haplotype frequencies are presented as absolute numbers with percentages in parentheses

P_c corrected P value, *OR* odds ratio, *CI* confidence interval, *n.s.* not significant

differences lost their significance when statistical correction was applied.

Discussion

This case–control study aimed to investigate the role of specific cytokine and cytokine receptor gene polymorphisms in genetic susceptibility to T1D. Our results suggest that SNPs in genes for the anti-inflammatory cytokine TGF-β1 and pro-inflammatory TNF-α may be associated with the development of the disease. Moreover, evidence of a weak association was also found with two polymorphisms in IL-6 gene, although it lost its significance after statistical correction.

The first SNP significantly associated with T1D in this study was a T/C substitution at position +869 (codon 10, rs1800470 a.k.a. rs1982073) in exon 1 of the gene encoding TGF-β1 located on chromosome 19q13 (Cambien et al. 1996). Homozygotes for the T allele were at significantly increased risk of developing T1D compared with those carrying codon 10 C allele. The C allele was repeatedly associated with increased TGF-β1 production, resulting from a leucine-to-proline substitution in the signal amino-acid sequence of the protein (Cambien et al. 1996; Dunning et al. 2003; Yamada et al. 1998; Yokota et al. 2000). As an immunosuppressive and regulatory cytokine produced by numerous cells, including Th3 and Treg subsets, TGF-β1 could possibly prevent or slow down the autoimmune-mediated destruction of pancreatic Langerhans islets, leading to an absolute lack of insulin production (Ichinose et al. 2007; Richer et al. 2008). We speculate that carriage of two copies of the T allele may contribute to susceptibility to T1D via reduced production of the anti-inflammatory TGF-β1. Until now, only one study has indicated a possible role of the codon 10 SNP in susceptibility to T1D (Jahromi et al. 2010). In contrast to our study, the authors found significant association of TC genotype, but not TT, with the disease. Although both studies suggest a role of this SNP in the predisposition to developing T1D, further investigations are necessary to replicate or question these results.

A SNP in the gene encoding tumor necrosis factor-α was another polymorphism associated with T1D. TNF-α is a cytokine with distinct cytostatic effects on pancreatic β cells, including inhibition of insulin synthesis and secretion. In cooperation with IL-1β and IFN-γ, TNF-α also participates in the destruction of insulin-producing cells by inducing their apoptosis and mediating the inflammatory process in Langerhans islets (Mauricio and Mandrup-Poulsen 1998; Rabinovitch 1998). We found that A allele carriers of the *TNFA* −308 G/A SNP (rs1800629) were significantly more prone to develop T1D than GG homozygotes. As the variant A allele (TNF2) was repeatedly associated with higher transcriptional activity and TNF-α production (Kroeger et al. 1997; Wilson et al. 1997), this effect on TNF-α production may be a possible explanation for a mechanism by which this SNP contributes to T1D development. Our result is in line with the outcomes of several studies reporting significantly increased frequencies of TNF2 in T1D patients in different European populations, including the neighboring Polish and Hungarian populations (Cox et al. 1994; Deja et al. 2006; Krikovszky et al. 2002; Noble et al. 2006; Pociot et al. 1993). In addition, this association was also confirmed by a meta-analysis in both Caucasian and Asian populations (Feng et al. 2009). Strong LD between TNF2 and HLA-DRB1*0301 alleles was also found in the present study, which indicates that the association of this TNF-α variation may actually not be independent. Moreover, several other polymorphisms within the *TNFA* gene may also confer/modify the risk for T1D, including TNFa microsatellite (Törn et al. 2006) or −1031 T/C, −863 C/A, and −857 C/T SNPs (Hamaguchi et al. 2000).

Finally, distinct differences in allele, genotype, and haplotype distribution between patients and controls were also found for the −174 G/C (rs1800795) and nt565 G/A (rs1800797) polymorphisms in the gene for IL-6. IL-6 is a pleiotropic cytokine with important proinflammatory and regulatory roles that participates in the pathogenesis of several autoimmune diseases, such as rheumatoid arthritis and inflammatory bowel disease (Ishihara and Hirano 2002; Kristiansen and Mandrup-Poulsen 2005; Naugler and Karin 2008). Its role in the pathogenesis of T1D remains, however, unclear and direct evidence for its deleterious and cytotoxic effects on pancreatic β cells is lacking (Kristiansen and Mandrup-Poulsen 2005). Moreover, some studies have indicated a protective effect of IL-6 on cytokine-induced β-cell death and function impairment in NOD mice (DiCosmo et al. 1994; Choi et al. 2004). In the present study, carriers of the IL-6 −174 C and nt565 A alleles were at increased risk of developing T1D, although the significance of this association disappeared after conservative statistical correction. Both alleles were in almost complete LD, generating the susceptibility haplotype −174 C/nt565 A. Genetic studies on IL-6 SNPs have so far delivered inconsistent evidence of their association with T1D (for reviews, see Kristiansen and Mandrup-Poulsen 2005; Cooper et al. 2007). Our results are in contrast with one of the first case–control studies on the IL-6 −174 G/C SNP, which identified GG homozygotes as those at increased risk of T1D (Jahromi et al. 2000). On the other hand, they are in line with the results of a recent study in the neighboring Polish population (Myśliwiec et al. 2008) as well as a large-scale UK case–control study (Cooper et al. 2007), both showing a weak positive association of the −174 C allele with T1D. Hence we suggest that IL-6

−174 G/C polymorphism may be among numerous genetic variations with modest effects on T1D development. In light of our insufficient knowledge about the role of IL-6 in the pathogenesis of T1D as well as the functional effect of the −174 G/C SNP, the exact mechanism by which this promoter polymorphism participates in the genetic determination of T1D has yet to be elucidated.

Limitations of the Study

Although the number of subjects in both the patient and control groups was comparable to those in similar studies in neighboring populations, it may still be argued that our study suffers from relatively small sample sizes. The statistical power to detect association at the 5% type I error rate with the given numbers of patients and controls was 84% for the TGF-β1 codon 10 T/C SNP, 85% for TNF-α −308 G/A, 57% for IL-6 −174 C/G, and 64% for IL-6 nt565 G/A SNP. Hence suboptimal sample sizes must be considered as the major limitation of the present study.

We are also aware that population stratification (PS) and cryptic relatedness (CR) are among the important sources of genetic confounding. Therefore we carefully excluded all first-degree relatives as well as any ethnic and racial minorities from our study. Previous comparisons of the allele frequencies of all the 22 SNPs in the Slovak population with those found in other Central and Western European populations revealed no significant differences (Javor et al. 2007; Kubistova et al. 2006), which indicates that the cytokine SNP distribution is homogenous in this region. Therefore, any unintentional sporadic occurrence of individuals from other European Caucasoid populations in our samples should have negligible effect on the outcome. However, as no further analyses were performed to address the problem of PS and CR, we cannot fully exclude their impact on the outcome of this study. This can therefore also be considered as one of the shortcomings of the study.

In our work we only focused on selected SNPs within cytokine genes and did not perform a complete assessment of all genetic variations at each locus. Therefore a possible role of polymorphisms other than those identified in our study cannot be ruled out. Future complex studies on candidate cytokine genes with haplotype analyses are required to fully understand the role of genetic variations and their interactions in genetic predisposition to T1D.

In conclusion, our results suggest that a T/C polymorphism at position +869 (codon 10) in the gene encoding TGF-β1 may contribute to the complex genetic predisposition to T1D. We have also confirmed a previously reported association of TNF-α −308 G/A with T1D; however, this may reflect the existence of a strong LD pattern within the HLA region rather than its actual role in susceptibility to T1D. Finally, despite the loss of significance after strict statistical correction, a possible role of the IL-6 −174 G/C and nt565 SNPs also cannot be ruled out, especially in consideration of the results of other studies reported previously. Advances in understanding the genetic factors underlying immunological processes that lead to the development of the auto-destructive process in pancreatic Langerhans islets promise future improvements in the prediction, prevention, and therapy of T1D.

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