IL10 promoter polymorphisms are associated with systemic onset juvenile idiopathic arthritis (SoJIA)

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Abstract

Objective

*Juvenile idiopathic arthritis (*JIA*) is a rare, but severe cause of childhood disability. Systemic onset* JIA *(*SoJIA*) accounts for approximately 5.8% of all* JIA *cases and is associated with cytokine dysregulation, including interleukin (IL-)1, IL-6 and tumour necrosis factor (TNF-)*α. IL-10 *is an immuno-regulatory cytokine, which in part regulates inflammation by controlling inflammatory cytokine expression. Dysregulation in* IL-10 *expression and certain single nucleotide polymorphisms (*SNPs*) in the* IL-10 *promoter were shown to be associated with autoimmune and infectious diseases.*

Methods

Genomic DNA-samples from SoJIA *patients from two German Paediatric Rheumatology centres, and healthy controls were analysed for three well defined* IL-10 *promoter* SNPs *(-1082G>A, -819C>T, and -592C>A). These* SNPs *are in tight linkage disequilibrium, and result in three predominant (or "classical") haplotypes: ATA, ACC, and GCC. ATA and ACC are associated with low and medium, GCC is associated with high* IL-10 *expression.*

Results

Here, we show a strong association of IL-10 *promoter polymorphisms with* SoJIA. *We demonstrate a significantly increased frequency of low* IL-10 *expressing -1082A/A alleles, the medium* IL-10 *expressing ACC haplotype (p=0.01), and an enrichment of the rare GTC haplotype (p<0.001) in patients with* SoJIA. *Heterozygous -1082G/A alleles (p<0.001), and the GCC haplotype (p<0.001) on one allele protect from developing* SoJIA.

Conclusion

This suggests a central role of the immuno-regulatory cytokine IL-10 in the pathogenesis of SoJIA.

Key words

IL-10, systemic onset *JIA*, Still's disease, inflammation, polymorphism, SNP, cytokine

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Introduction

Systemic onset juvenile idiopathic arthritis (SoJIA), or "Still's disease", is a rare but severe cause of childhood disability (1-3), and accounts for approximately 5.8% of all JIA cases in Germany (Data from German JIA registry, 2007, personal conversation with M. Niewerth, Deutsches Rheuma-Forschungszentrum, Berlin, Germany). Other European countries and the USA report higher incidences of SoJIA up to 10% of JIA cases (1, 4, 5). Among the seven (sub-) groups of JIA, SoJIA is the most severe, and usually most difficult to treat. SoJIA is characterised by fevers, evanescent rash, generalised lymphadenopathy, hepato-/splenomegaly, and/or serositis in the absence of high titer autoantibodies (3). Previously, polymorphisms in other cytokine genes have been associated with SoJIA, including the interleukin 6 (*IL6*) (chromosome 7p21) and Macrophage migration inhibitory factor (*MIF*) (chromosome 22q11.2) genes (1, 7, 8).

Interleukin (IL-)10 plays an important role in controlling inflammation and instructing adaptive immune responses. Consequently, dysregulation in IL-10 expression is associated with susceptibility to infectious and autoimmune disease in humans and mice (9). IL-10 decreases the expression of cytokines, such as IL-1, IL-6, and tumour necrosis factor (TNF-) α, which are implicated in the development of autoinflammatory diseases, including SoJIA (1).

IL-10 expression can be stratified from low to high, based on SNP haplotype blocks upstream of *IL10* (9, 10, 11, 12) (Fig. 1). The majority of studies were focused on a series of three SNPs in the 5' proximal promoter. As the *IL10* promoter polymorphisms -1082G>A (rs1800896), -819C>T (rs1800871), and -592C>A (rs1800872) are in tight linkage disequilibrium, and result in three predominant (or "classical") haplotypes: ATA, ACC, and GCC. ATA and ACC are associated with low and medium IL-10 expression, whereas GCC is associated with high IL-10 expression (9, 13). Numerous studies demonstrated the correlation between *IL10* promoter SNPs and differential risk for a variety of infectious and autoimmune diseases, as well as cancer (9).

Several studies suggest that the *IL10* locus is under selective pressure from various pathogens during evolution (14, 15). Furthermore, the hypothesis that IL-10 production is genetically determined was suggested by the concordance of IL-10 levels in monozygotic twins, which implies that up to 70% of *IL-10* production is genetically determined (12). Nevertheless, IL-10 regulation in humans warrants further studies, since it is influenced by genetic, as well as other host factors, including epigenetic marks (9, 16).

In the present study, we investigated the possible link between the common *IL10* promoter haplotypes ATA, ACC and GCC with SoJIA. We identified an association of the ACC and GTC haplotypes with SoJIA. GCC alleles were more common in the control group.

Materials and methods

Patient and control samples

Seventy-four patient samples were collected by members of the Department of Paediatric and Adolescent Rheumatology, North-Western German Centre for Rheumatology, St. Josef-Stift Sendenhorst, and the Paediatric Rheumatology and Immunology Section, University Children's Hospital Dresden, University of Technology Dresden. Ethical approval for the study was obtained from the ethics commission of the University of Technology Dresden, and parents gave informed consent. An ethnically-matched healthy control population (Caucasian, of German origin) from first time blood donors (249 individuals) was used as controls. Prior to the main study, 120 healthy controls from Münster/Sendenhorst, North Rhine-Westphalia and 129 healthy controls from Dresden, Saxony were compared to screen for local differences in allele frequencies.

Genotyping

DNA from 74 SoJIA patients and 249 healthy controls was collected and purified, using EZ DNA II isolation kits (Zymo research). All samples were genotyped for three previously described SNPs: -1082G>A (rs1800896), -819C>T (rs1800871), and -592C>A (rs1800872).

Polymerase chain reaction (PCR) sequencing was used for genotyping. The *IL10* 5' SNP block was amplified, using the following primers: forward 5' GACAACACTACTAAGGCTTC 3', and reverse 5' GCTAACTTAG-GCAGTCACCT 3'. The cycling parameters consisted of an initial denaturation for 10 minutes at 94°C, followed by 35 cycles of 30sec denaturation at 94°C, 45sec annealing at 61°C and 45sec extension at 72°C, followed by a final extension step of 72°C for 5 minutes. PCR products were purified, using rAPid Alkaline Phosphatase cleaning (Roche-applied-science), and used as template for PCR sequencing.

The BigDyeTM terminator cycle sequencing reaction was purchased from Applied Biosystems to determine polymorphisms in the *IL10* gene promoter according to the protocol provided. Briefly, after mixing 2µL of the above PCR products (approximately 50ng DNA) with 0.5 µL primer (5ng), 2µL BigDye terminator ready reaction mix (containing Mg2+, pyrophosphatase, and DNA polymerase) and 2µl BigDye Teminator 10x Sequencing buffer and ddH_2O to a final reaction volume of 20µL, the following cycle conditions were applied: 25 cycles of 96°C for 30 sec, 15 sec at annealing temperature adapted to the primer pairs (54–62°C), and 60°C for 4 min (Trio Thermoblock, Biometra, Göttingen, Germany). The sequence was determined, using the ABI 310 automatic sequencer (Applied Biosystems). The promoter sequence of the *IL10* gene was determined in each patient and control sample. The DNA sequence of each patient was compared with the *IL10* promoter sequence of 249 healthy controls and the published sequence, using the ABI 310 sequence navigator software, version 1.0.1 (Applied Biosystems).

Association analysis

Rare haplotypes with frequencies <5% were excluded from statistical analysis. Statistical analysis was performed, using PASW Statistics 17.0 software (IBM). Statstical analysis was performed to compare single marker and haplotype frequencies in both groups, using Fisher's exact test. *P*-values <0.05 were considered to be significant.

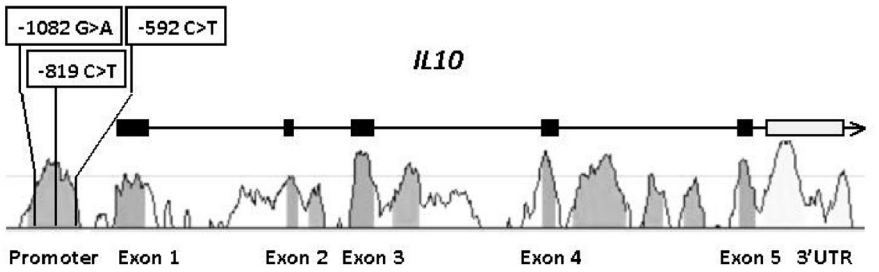
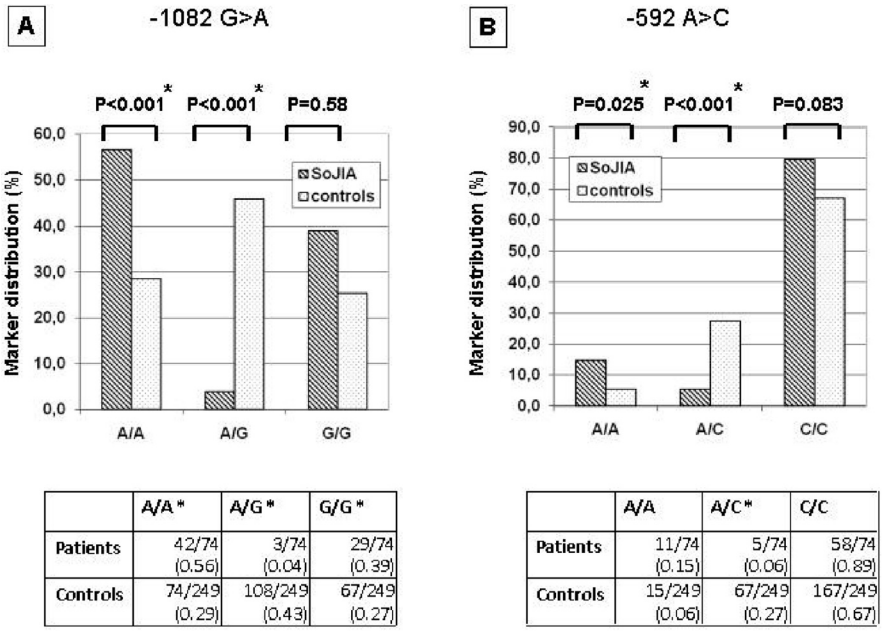


Fig. 1. Localisation of investigated promoter polymorphisms.
A vista alignment of the human and mouse *IL10* genes is given. Black boxes stand for exons, the grey box stands for the 3' untranslated region (3'*UTR*). The locations of the three invesitaged polymorphisms -1082 G>A, -819 C>T, -592 C>A are given.



	A/A *	A/G *	G/G *
Patients	42/74 (0.56)	3/74 (0.04)	29/74 (0.39)
Controls	74/249 (0.29)	108/249 (0.43)	67/249 (0.27)

	A/A	A/C *	C/C
Patients	11/74 (0.15)	5/74 (0.06)	58/74 (0.89)
Controls	15/249 (0.06)	67/249 (0.27)	167/249 (0.67)

Fig. 2. Single marker analysis of -1082 G>A(A) and -592 A>C(B).
Results of a single marker analysis of the *IL10* promoter polymorphisms -1082G>A (A) and -592A>C (B) are given. In SoJIA patients, homozygous -1082A/A, and -1082G/G alleles are significantly more frequent, compared to healthy controls. Healthy controls show significantly higher frequencies of heterozygous -1082A/G and -592A/C alleles, compared to SoJIA patients. Differences in the frequency of -592C/C between SoJIA patients and healthy controls were not significant. Significant differences between groups are marked with asterisks (*).

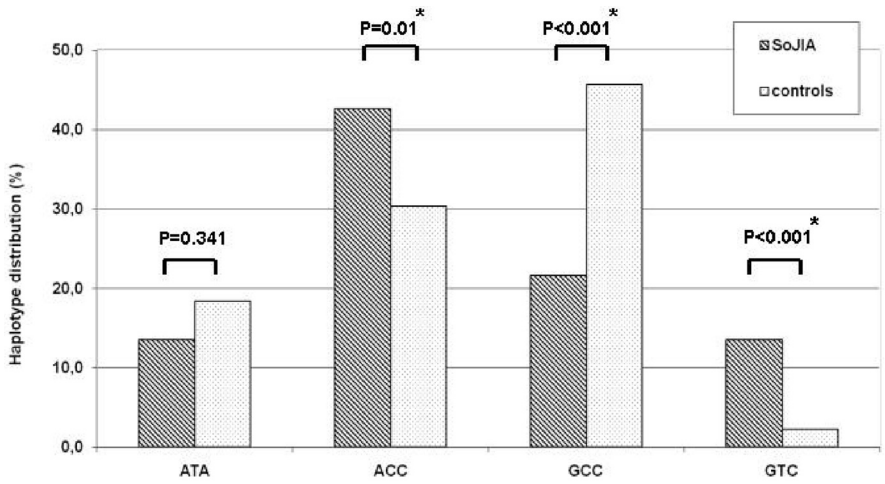
Results

Comparison of IL10 promoter polymorphisms in healthy controls from Munster and Dresden

One hundred and twenty healthy controls from Münster/Sendenhorst, North Rhine-Westphalia, and 129 healthy controls from Dresden, Saxony, were compared in order to screen for possible local differences in allele frequencies. No significant differences in allele distribution between the two groups were detected (Supplement 1). Thus, DNA samples from 129 Dresden and 120 Münster 1st time blood donors were combined to one group and used as control population for the main study (249 controls).

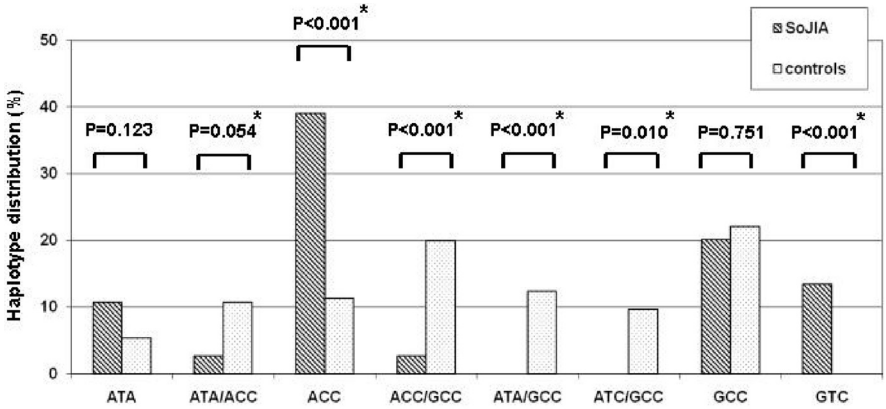
Comparison of IL10 promoter haplotypes in SoJIA patients and healthy controls

IL10 promoter haplotypes in 249 control individuals were compared to 74 SoJIA patients from the Department of Paediatric and Adolescent Rheumatology, North-Western German Centre for Rheumatology, St. Josef-Stift Sendenhorst, and the Paediatric Rheumatology and Immunology Section, University



Haplotype	Patients	Controls	P value
ATA	20/148 (0.13)	87/498 (0.18)	0.341
ACC	63/148 (0.42)	150/498 (0.31)	0.01 *
GCC	32/148 (0.21)	225/498 (0.45)	<0.001 *
GTC	20/148 (0.13)	10/498 (0.02)	<0.001 *

Fig. 3. *IL10* promoter haplotype analysis in SoJIA.
A single allele frequency, based on promoter haplotypes, is given. In *SoJIA* patients, ACC and GTC haplotypes are significantly more frequent, compared to normal controls. Healthy control individuals show a higher frequency of GCC alleles. Rare haplotypes with frequencies <5% are not displayed. Significant differences between groups are marked with asterisks (*).



Haplotype	Patients	Controls	P value
ATA	8/74 (0.108)	14/249 (0.05)	0.123
ATA/ACC	2/74 (0.02)	25/249 (0.10)	0.054
ACC	29/74 (0.39)	33/249 (0.13)	<0.001 *
ACC/GCC	2/74 (0.02)	51/249 (0.20)	<0.001 *
ATA/GCC	0/74 (0)	29/249 (0.12)	<0.001 *
ATC/GCC	0/74 (0)	19/249(0.10)	0.010
GCC	15/74(0.20)	56/249 (0.22)	0.751
GTC	10/74 (0.13)	0/249 (0)	<0.001 *

Fig. 4. *IL10* promoter haplotype analysis in SoJIA.
The paired allele frequency, based on promoter haplotypes, is given. In *SoJIA* patients homozygous alleles are more frequent, compared to normal controls. Homozygous ACC and GTC haplotypes are significantly more frequent, compared to normal controls. Healthy control individuals show a higher frequency of heterozygous alleles. The GCC haplotype on one allele, seems to protect from developing SoJIA, since ACC on one allele and GCC on the other is more frequent in healthy contols. Rare haplotypes with frequencies <5% are not displayed. Significant differences between groups are marked with asterisks (*).

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As a first step, single marker analyses of the -1082 A>G, and -592A>C polymorphisms were performed. A strong association of -1082A/A alleles (42/74 (0.56) *vs*. 74/249 (0.29), $p<0.001$) with SoJIA-was observed. Healthy controls showed a higher frequency of -1082A/G (3/74 (0.04) *vs*. 107/249 (0.46), $p<0.001$) (Fig. 2 A) alleles. At position -592, a higher frequency of -592 A/C alleles was detected in healthy controls, compared to SoJIA patients (67/249 (0.27) *vs*. 5/74 (0.06), $p<0.001$) (Fig. 2B).

Using a single allele based approach of the investigated haplotypes, that result from -1082G>A, -819C>T, and -592C>A polymorphisms, a strong association of ACC and GTC haplotypes with SoJIA was observed. Conversely, the 148 examined alleles of 74 SoJIA patients showed a higher frequency of ACC (63/148 (0.42) *vs*. 150/498 (0.31), $p=0.01$) and GTC (20/148 (0.13) *vs*. 10/498 (0.02), $p<0.001$) haplotypes, compared with controls. The high IL-10 expressing haplotype GCC showed a significantly higher frequency in the control group (32/148 (0.21) *vs*. 225/498 (0.45), $p<0.001$) (Fig. 3).

Comparing the paired allele distribution in all homozygous and heterozygous individuals, a higher frequency of heterozygous alleles was found in controls (9/74 (0.12)), compared to SoJIA patients (156/249 (0.62)). A strong association of the homozygous ACC haplotype (29/74 (0.39) *vs*. 33/249 (0.13), $p<0.001$) with SoJIA was identified. In the healthy control group, a significantly higher frequency of heterozygous ACC/GCC (2/74 (0.02) *vs*. 51/249 (0.2), $p<0.001$), ATA/GCC (0/74 (0.00) *vs*. 29/249 (0.12), $p<0.001$) and ATC/GCC (0/74 (0.00) *vs*. 19/249 (0.08), $p=0.010$) alleles was seen. The frequency of homozygous GCC alleles was comparable in SoJIA patients and healthy controls (15/74 (0.20) *vs*. 56/249 (0.22), $p=0.751$) (Fig. 4).

Discussion

JIA is a group of heterogeneous diseases, with a wide range of symptoms and severity. Chronic inflammation and the elevation of (pro-) inflammatory

cytokines is a common feature to all groups of JIA, including SoJIA. Several studies have identified an imbalance of cytokines with any form of JIA (1, 17-20). The direct involvement of IL-1, IL-6, and TNF-α in systemic inflammation was shown by several groups, measuring serum and synovial fluid levels of SoJIA patients (4, 18, 21, 22, 23). Since IL-10 has a direct influence on IL-6 and TNF-α expression and has previously been shown to play a role in severe extended oligoarthritis (21), we investigated the association of *IL10* promoter polymorphisms with SoJIA.

In this study, we show an association of *IL10* promoter polymorphisms with SoJIA. The three *IL10* promoter variants -1082G>A (rs1800896), -819C>T (rs1800871), and -592C>A (rs1800872) form three predominant haplotypes (ATA, ACC and GCC). Next to these "classical" haplotypes, some rare haplotypes, including GTC, have been reported (24, 25). Here, we demonstrate an association of the low *IL10* expressing -1082G>A *IL-10* promoter SNP with *SoJIA*. This suggests an involvement of *IL10* in the dysregulation of (pro-)inflammatory cytokines, and $CD4^+$ T cells, which were suggested to play a central role in SoJIA (1, 4). This is in agreement with Fife *et al*.'s (1) finding, that SoJIA is associated with -1082A/A alleles, but not with -592A/A. In this study, the authors performed single marker analysis within the *IL10* promoter and additionally screened for SNPs in and around *IL19* and *IL20*. Fife and colleagues did not investigate *IL10* promoter haplotype blocks, which complicates a detailed comparison with our study. Other than Fife *et al*., we investigated sequence variants at positions -1082, -819, and -592, and were able to associate -1082A/A SNPs with possible ATA and ACC haplotypes. Interestingly, we found a strong association of SoJIA with the medium *IL10* expressing ACC haplotype, but not the low producing ATA haplotype. These findings are in agreement with results of Fife *et al*.'s study, since the authors found an association of SoJIA with -1082A/A, but not -592A/A *alleles*. GTC is a rare haplotype, with frequencies usually <3% in healthy controls (24, 25) (Fig. 3). The GTC haplotype has been reported to protect from developing nephropathy in type-2 diabetes (25). In this study, the GTC haplotype is highly enriched in the SoJIA population (13% of SoJIA patients have homozygous GCC haplotypes, Fig. 4). These findings suggest a role of the rare GTC haplotype in the development of SoJIA. In a further study in a Caucasian British JIA population, a relatively small cohort of SoJIA patients was included (26). In contrast to our findings, the authors did not see an association with the rare GTC haplotype. Possible explanations could be the small patient number of the studies (50 and 74 SoJIA patients), but also differences in the haplotype distribution of British and German SoJIA populations. Normal controls in the two studies did not show significant differences in the haplotype distribution, including the rare GTC haplotype (<3%). Thus, our findings could be an indication of an enrichment of the rare GTC haplotype in a German SoJIA cohort. Differences in haplotype distribution in different regions of the world have been reported (27) and could explain different disease associated haplotype frequencies in SoJIA patients in Great Britain and Germany. As, to our knowledge, no functional data is available for the GTC haplotype, further studies are necessary to investigate the association of this haplotype with high or low level IL-10 expression. Furthermore, GCC haplotypes are significantly more frequent in healthy controls. This suggests a dominant recessive and protective effect of GCC alleles, since heterozygous GCC haplotypes are significantly more common in the healthy control population. The "high expressing" GCC haplotype on one allele might lead to a reconstitution of an impaired IL-10 expression by the second allele. Donn *et al*. (26) reported comparable frequencies of heterozygous GCC haplotypes in their control population. Heterozygous GCC was more frequent in the SoJIA cohort, which further suggests regional differences in the haplotype distribution in SoJIA patients in Great Britain and Germany. Nonetheless, results need to be interpreted with caution, since patient numbers were relatively small in all studies, secondary to the rarity of the disease.

The pathophysiological effect of this finding is still not entirely clear, but the relevance of our findings is suggested by the role of IL-10 in immune regulation. Multiple autoimmune and infectious diseases were shown to be associated with dysregulation of IL-10 expression. Shin *et al*. reported that certain *IL10* alleles were predictive for HIV progression rates (28) and showed that the *SNPs* in this haplotype block display unique DNA-protein-binding patterns. The authors reported reduced protein binding to homozygous -592AA alleles, compared to heterozygous -592A/C alleles, to SP-1 binding sequences. SP-1 is a transcription factor that strongly promotes IL-10 expression by macrophages and monocytes and binds to GC rich regions within the proximal promoter of the *IL-10* gene (29). In the present study, healthy controls showed a significantly higher frequency of -592A/C alleles (23/100 (0.23)), compared to SoJIA patients (5/74 (0.06)). This might lead to

Supplement 1. Comparison of allele frequencies in Münster/Sendenhorst and Dresden control individuals (1st time blood donors). No significant differences between both groups were detected, using Fisher's exact test. Haplotype

	Münster/Sendenhorst	Dresden	*p*-value
ACC	74/240 (0.30)	76/258 (0.29)	0.49
ACA	0/240 (0.00)	2/257 (0.007)	0.77
ATA	42/240 (0.18)	45/258 (0.17)	1.00
ATC	2/240 (0.008)	16/258 (0.06)	0.06
GCC	118/240 (0.49)	197/258 (0.41)	1.00
GCA	0/240 (0.00)	1/258 (0.004)	0.09
GTA	2/240 (0.008)	3/258 (0.01)	1.00
GTC	2/240 (0.008)	8/258 (0.03)	0.11

alternate IL-10 expression and strongly suggests that allelic variants may influence protein recruitment to regulatory regions and gene expression.

It is important to note, that individual *IL10* promoter SNPs could be markers of larger haplotypes that may extend several hundred kilobases over the entire *IL10* gene cluster (11, 12, 30) rather than directly influencing binding of regulatory proteins. Indeed, a recent study was unable to find a link between *IL10* promoter SNPs and IBD, but did find a strong association between a SNP 3' to the *IL10* gene and ulcerative colitis (31). An association of the *IL10* -1082G>A polymorphism and *IL20* -468C>T polymorphisms with SoJIA has been shown (1), but warrants further study and more extensive analysis of the *IL10* gene cluster. Jones and Flavell (28) identified distal enhancer elements in the mouse *Il10* cluster, and we previously reported the presence of activation dependent intergenic transcripts between the *IL10* and *Il19* genes in murine macrophages (9). These findings suggest the presence of tissue specific regulatory elements in the *IL10* cluster. Polymorphisms in and around these regions could alter cytokine expression and influence the course of (auto-) inflammatory diseases, including SoJIA.

SNPs with putative function within the entire *IL10* cluster may be in linkage disequilibrium or a part of extended haplotypes. This highlights the necessity to characterise the molecular and genomic requirements for appropriate cell type-specific IL-10 expression in health and disease.

Apart from genetic control mechanisms and promoter polymorphisms, which were addressed in this study, other mechanisms, such as chromatine structure and DNA methylation were shown to play a role in the regulation of different cytokine genes, including *IL10* (9). These mechanisms could influence the different clinical courses and outcomes of patients with identical or similar promoter haplotypes (9).

Conclusion

In the present study, we demonstrate a strong association of homozygous -1082A/A (rs1800896) *IL10* alleles, and the ACC and GTC haplotypes with SoJIA. Heterozygous -1082G/A (rs1800896) *IL10* promoter alleles and GCC haplotypes seem to protect from developing SoJIA. This suggests a central role of the immuno-regulatory cytokine IL-10 in the pathogenesis of SoJIA. To our knowledge, this is the first study that shows an enrichment of the rare GTC haplotype in patients with autoimmune disease. Further studies are needed to investigate the functional relevance of the GTC haplotype on IL-10 expression.

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