

***IL10R1* loss-of-function alleles in rheumatoid arthritis and systemic lupus erythematosus**

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Abstract Objective

IL-10 is a pleiotropic cytokine involved in the regulation of innate and cell-mediated immunity and a key mediator within the disturbed SLE immune system. IL-10 binds to IL10R1, which is expressed on a variety of immune cells and activates the JAK-STAT pathway. Two (out of several known) genetic IL10R1 variants may alter IL-10 binding or signal transduction. Here we investigate the differential activity of these IL10R1 variants and their possible association with RA or SLE susceptibility.

Methods

IL10R1-wt, IL10R1-S138G, IL10R1-G330R, or IL10R1-S138G + G330R were cloned into pIRESpuro3 and transfected into HeLa cells. Single cell clones were tested for IL-10-induced SOCS3- and SLAM gene expression by real-time PCR. DNA from 182 RA patients, 222 SLE patients, and 250 healthy controls was genotyped by allele-specific PCR.

Results

A biphasic increase of SOCS3 mRNA was observed that peaked at 15 minutes and 4 hours after IL-10 stimulation. The presence of IL10R1 S138G and G330R showed a weaker induction of both SOCS3 and SLAM upon stimulation with IL-10. In RA a homozygous G330R genotype was more commonly present than in controls (15.4% vs. 7.6%; $p < 0.05$). In SLE the G330R allele frequency was also increased (36.3% vs. 30.0%; $p < 0.05$) without showing a gene-dose relationship at the genotype level.

Conclusions

Based on these results, both variants of the IL10R1 gene are loss-of-function alleles. IL10R1 G330R may possibly contribute to RA or SLE disease susceptibility in Caucasian populations.

Key words

Rheumatoid arthritis, systemic lupus erythematosus, single nucleotide polymorphism, interleukin-10 receptor, *IL10R1*.

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Abbreviations:

RA: rheumatoid arthritis;
 SLE: systemic lupus erythematosus;
 IL-10R: interleukin-10 receptor;
 SNP: single nucleotide polymorphism.

Introduction

IL-10 was originally identified as a murine B cell-derived stimulator of thymocytes and a T-helper 2 (Th2) lymphocyte-derived factor that suppressed cytokine production in Th1 cells (1). In humans, IL-10 is produced not only in Th2 cells, but also by a variety of immune and non-immune cells with strong anti-inflammatory effects on lymphocytes, macrophages, and dendritic and endothelial cells. In addition, IL-10 exhibits immuno-stimulatory activity, driving B cell proliferation and their differentiation into immunoglobulin-producing cells, and activating CD8⁺ T cells.

IL-10 levels in systemic lupus erythematosus (SLE) are significantly elevated and correlate with disease activity (2). As IL-10 is a strong stimulator of B cell maturation and immunoglobulin production, the increased IL-10 activity is considered to be a main feature of B cell hyperactivity in SLE (3). This hypothesis is supported by *in vivo* findings with neutralizing anti-IL-10 antibodies in a NZB/WF1 mouse model (4) and a murine anti-IL-10 antibody in a human pilot study (5).

The role of IL-10 signalling in rheumatoid arthritis (RA) has not yet been clearly evaluated. Pro-inflammatory cytokines such as TNF- α and IL-1 play a central role in the inflammatory process and it has been postulated that an imbalance towards a pro-inflammatory cytokine milieu in the joints may account for chronic inflammation and progression of disease. *In vitro* experiments have shown that IL-10 down-regulates TNF- α , IL-1, and interferon- γ in RA synovial membrane (6). In addition, IL-10 showed anti-inflammatory activity *in vivo* in collagen-induced arthritis, a well-characterized mouse model of RA (7). However, clinical trials in humans have been disappointing. Possible explanations include the short half-life of IL-10, inadequate synovial concentrations, an induction of interferon- γ (8), or immune complex formation (9). After the discovery of the polymorphic nature of the IL-10 promoter region, various investigators have explored the possible links between these promoter polymorphisms and susceptibility to

RA and SLE with, however, contrasting outcomes (10-12).

The effect of IL-10 is mediated by its binding to the IL-10 receptor, which is a hetero-tetramer consisting of two IL-10 receptor 1 (IL10R1) chains and two IL10R2 chains (1). As in healthy subjects, *IL10R1* is expressed on the leukocytes of RA and SLE patients (13). The complex seems to mediate high affinity ligand binding and signal transduction through the activation of two receptor-associated kinases, JAK1 and Tyk2. Further downstream signaling involves STAT1, STAT3 and in some cells also STAT5 translocation to the nucleus and transcription activation of various response genes including SOCS3 (suppressor of cytokine synthesis 3) and SLAM (signaling lymphocytic activation molecule) (14, 15). We previously identified several variants of *IL10R1*, most importantly G330R and S138G (16). S138G is in strong linkage disequilibrium with G330R and structural analysis of the IL-10/IL10R1 complex indicated that S138G might influence the conformation of the *IL10R1* complex and thereby IL-10 binding. In this study we investigate the differential signaling activity of the *IL10R1* variants. In addition, we tested for the association of these *IL10R1* variants with RA and SLE susceptibility.

Materials and methods

Cloning of IL-10R1

As previously described, S138G and G330R result in four *IL-10R1* haplotypes (16, 17): wild-type nucleotides at both the S138G and G330R positions (haplotype-1), alleles carrying the S138G variant only (haplotype-3), alleles carrying the G330R variant only (haplotype-4), and alleles carrying both the S138G and G330R variants (haplotype-7). The coding sequence of *IL-10R1* haplotypes-1, -4 and -7 was obtained by the 5'-RACE method from two individuals. A FLAG-tag was introduced after the signal peptide by PCR mutagenesis. The haplotype-3 was generated from IL-10R1-WT by PCR mutagenesis. The FLAG-tagged receptor haplotypes and the cDNA of EGFP were cloned into the expression vector pIRESpuro3 (Clontech), resulting

Competing interests: none declared.

in the plasmids pIRESpuo3-IL10R1-WT, pIRESpuo3-IL10R1-S138G, pIRESpuo3-IL10R1-G330R and pIRESpuo3-IL10R1-S138G+G330R, respectively. The correct sequence was confirmed by cycle sequencing.

Transfection of HeLa cells

2×10^5 HeLa cells were transfected with 0.4 μ g of either pIRESpuo3-EGFP, pIRESpuo3 (mock), pIRESpuo3-IL10R1-WT, pIRESpuo3-IL10R1-S138G, pIRESpuo3-IL10R1-G330R, or pIRESpuo3-IL10R1-S138G+G330R using Effectene (Qia-gen) and selected with 1.5 μ g/ml puromycin (Sigma). Single cell clones were prepared by limiting dilution, identified by inverse microscopy, and analyzed for *IL-10R1* expression by flow cytometry. Three clones of each plasmid that showed homogeneous *IL-10R1* expression were further expanded and used for real-time PCR.

Real time PCR

HeLa clones were stimulated with serial dilutions (0, 0.1, 1, 10 ng/ml) of hIL-10 for 4 hours. Total RNA was extracted using TRIzol reagent (Invitrogen). cDNA synthesis was performed using the ThermoScript RT Kit (Invitrogen). TaqMan real-time PCR was performed from cDNA with SLAM, SOCS3 and GAPDH specific primers (VBC; 900 nM) and a fluorogenic probe (ABI; 200 nM) using an ABI Prism 7700 sequence detection system (Perkin Elmer). The sequences of primers and probes were the following: GAPDH-F-5'-CCTGAGCTGAACGGGAAGC-3'; GAPDH-R-5'-AGGTCCACCACT-GAGACGTTG-3'; GAPDH-probe: 6-FAM-CATGGCCTTCCGTGTC-CCCACT-TAMRA; SLAM-F-5'-CCATGTGGCTTACAGCTGGAG-3'; SLAM-R-5'-GGAGCTGTTGGCT-GGGTTC-3'; probe: 6-FAM-AAAAG-GCGGGCACCCACCA-TAMRA; SOCS3-F-5'-TTCTGATC-CGCGACAGCTC-3'; SOCS3-R-5'-TCCCAGACTGGGTCTTGACG-3'; probe: 6-FAM ACCAGCGCCACT-TCTTCACGCTCA-TAMRA. Amplification was performed in a total volume of 25 μ l for 45 cycles with denaturation at 95°C for 15 seconds and annealing/

extension at 60°C for 1 minute. Samples were run in triplicate and their relative expression was determined by normalizing to the expression of the house-keeping gene GAPDH. Values obtained were compared to the level of expression in non-stimulated control samples. The relative expression was calculated by the delta Ct method.

Patients

For this study, 182 Caucasians from Austria diagnosed with RA according to the ACR revised criteria (18) were recruited from a tertiary hospital outpatient clinic. The cohort consisted of 146 female and 36 male patients and the mean age ($\pm$ SD) was 62 ($\pm$ 16) years. Recruited from the same clinic were 195 female and 27 male patients with SLE (mean age 48 $\pm$ 15 years) according to the 1997 updated criteria for the classification of SLE (19). The RA and SLE patients were compared to 250 healthy controls with the same ethnicity (100% Caucasians) recruited from the same regional area. Age and gender data for the control group are not available.

IL10R1 genotyping and detection of polymorphisms

After written informed consent for genetic analysis was obtained from each patient, blood was drawn for DNA extraction. Two allele-specific polymerase chain reactions (PCRs) were performed to detect *IL10R1* S138G and G330R, as described previously (16). The accuracy of both assays had been verified by comparison to cycle sequencing in 50 DNA samples. Haplotypes were analyzed from genotype data using an expectation-maximization algorithm as implemented by the program EH.

Statistical analysis

Allele and genotype frequencies were calculated by direct counting. The significance of the differences in allele frequencies was compared between RA patients and controls, and between SLE patients and controls using the standard χ^2 test (1df). Similarly, the significance of the differences in genotype frequencies was compared by the standard χ^2 test (2df). The χ^2 test (3df) was used to

test for significant differences between haplotypes. Hardy-Weinberg equilibrium tests were carried out using the χ^2 test to analyze the differences between the observed and expected genotype distributions. To compare the response to IL-10 between IL10R1-WT and any of the variant *IL10R1* receptors, a mixed-effects nested design model was used.

Results

IL-10R1 variants are loss-of-function alleles

To better understand the functional consequences of single amino acid substitutions of two common Caucasian *IL10R1* variants, we cloned four *IL10R1* haplotypes (IL10R1-wt, IL10R1-S138G, IL10R1-G330R, and IL10R1-S138G+G330R) into pIRESpuo3, stably transfected HeLa cells and analyzed the expression of IL-10 responsive genes in single cell clones.

First, HeLa clones that expressed IL10R1-WT were stimulated with IL-10 (10 ng/ml) and SOCS3 expression was analyzed in a time-dependent fashion. After an initial mRNA increase at 15 minutes, which is probably independent of transcriptional regulation, SOCS3 mRNA peaked at 4 hours and returned to background levels within 8 hours after IL-10 treatment (Fig. 1A). Next, HeLa cell clones that expressed IL10R1-WT, IL10R1-S138G, IL10R1-G330R or IL10R1-S138G+G330R were cultured with serial dilutions of IL-10 (0-10 ng/ml) and SOCS3 mRNA was compared at 4 hours (Fig. 1B). In clones expressing the IL10R1-WT, a dose-response curve was detected with a peak at 10ng/ml. HeLa clones that expressed any of the variant *IL10R1* receptors also displayed a dose response, but at significantly lower level than the WT receptor (S138G: $p=0.043$; G330R: $p=0.015$; S138G+G330R: $p=0.01$, by the mixed-effects nested design model; Fig. 1B).

To further confirm these findings we analyzed the expression of SLAM in the same set of HeLa clones. SLAM has been identified as one of the strongest regulated genes downstream of IL-10. While the IL10R1-WT clones displayed an appropriate dose response to SLAM induction, no dose response was ob-

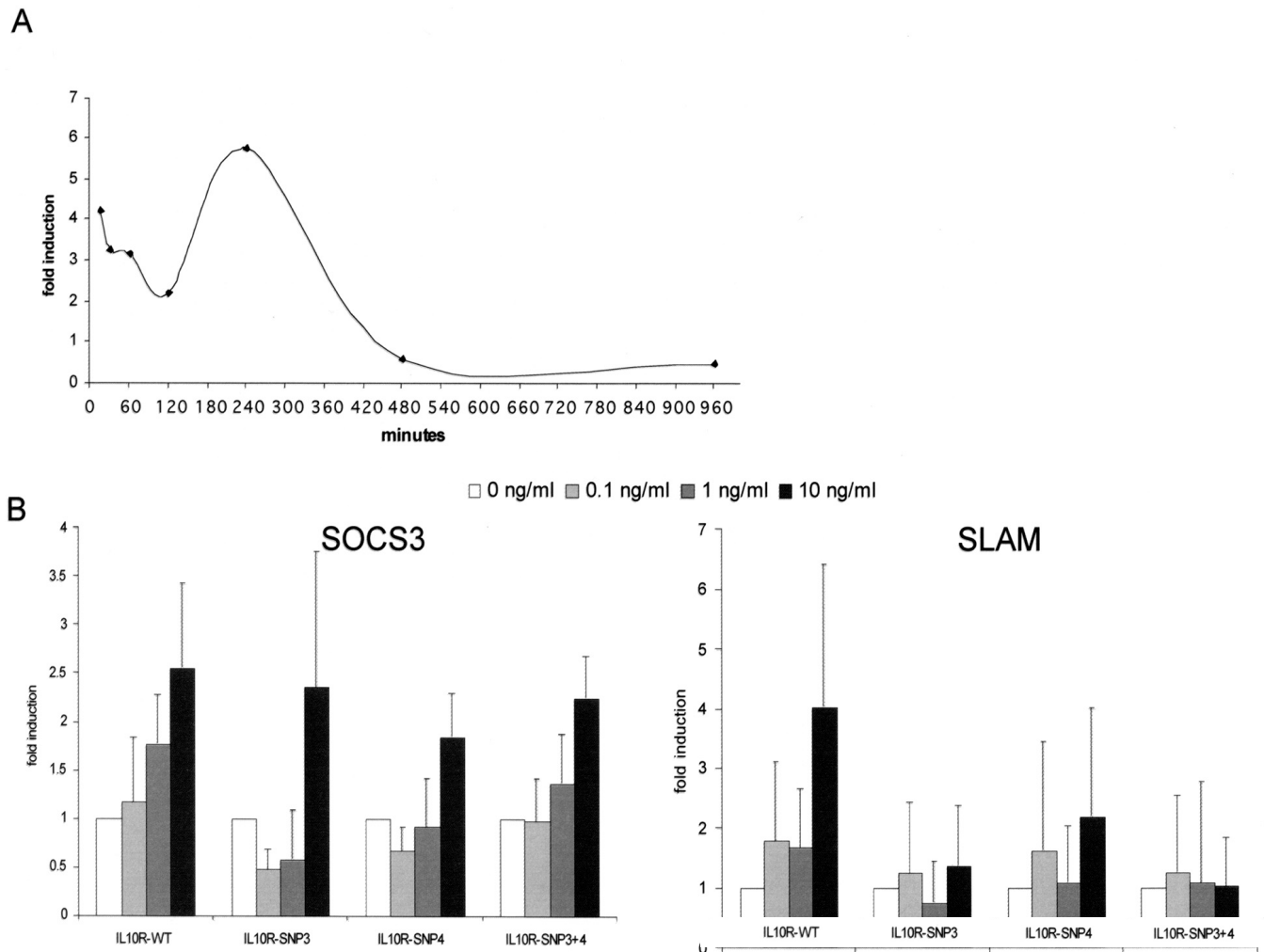


Fig. 1. *IL10R1* haplotypes alter IL-10-induced SOCS3 and SLAM mRNA expression.

(A) Induction of *SOCS3* mRNA in response to IL-10. A HeLa single cell clone that expresses IL-10R1-WT was stimulated with IL-10 (10 ng/ml). Cells were collected after 15, 30, 60, 120, 240, 480, and 960 minutes, mRNA was extracted and reverse transcribed, and *SOCS3* expression was analyzed by real time PCR. The relative expression (fold induction) was determined by the delta delta CT method and normalization to both the housekeeping gene GAPDH and to its non-stimulated baseline expression. After an initial increase at 15 min, *SOCS3* mRNA dropped at 120 minutes post-stimulation and peaked at 240 minutes. This time point was chosen for the successive comparative analysis.

(B) *IL10R1* variant haplotypes lowered the expression of *SOCS3* and *SLAM* in response to IL-10. Eleven HeLa cell clones, two expressing IL10R1-WT, three expressing IL10R1-S138G (IL10R1-SNP3), three expressing IL10R1-G330R (IL10R1-SNP4) and three expressing IL10R1-S138G+330R (IL10R1-SNP3+4) were cultured with serial concentrations of IL-10. Cells were collected after 4 hours (according to the maximum response in the time-course experiment) and analyzed for *SOCS3* (left) and *SLAM* (right) mRNA expression. Samples were run in triplicate and the relative expression (fold induction) was determined by normalization to both the housekeeping gene GAPDH and to expression in non-stimulated controls (delta delta CT method). A dose response was observed for both *SOCS3* and *SLAM* mRNA induction upon increasing the IL-10 concentrations (0–10 ng/ml). This dose response of *SOCS3* and *SLAM* mRNA induction was reduced, however, in cells expressing the variant receptors when compared to the wild type receptor.

P-values were calculated for *SOCS3*- and *SLAM* mRNA by the mixed-effects nested design model (*SOCS3*: S138G: *p*=0.043; G330R: *p*=0.015; S138G+G330R: *p*=0.01; and *SLAM*: S138G: *p*=0.018; G330R: *p*=0.014; S138G+G330R: *p*=0.015, respectively).

served with any of the variant IL10R1-expressing clones, the type of response also being significantly different from the IL10R1-WT (S138G: *p*=0.018; G330R: *p*=0.014; S138G+G330R: *p*=0.015, by the mixed-effects nested design model; Fig. 1B). These findings indicate that both S138G and G330R are loss-of-function mutations for IL10 signaling specifically at lower (more physiologic) IL-10 concentrations.

Weak association of *IL10R1*-G330R with autoimmune disease

As IL-10 signaling has been implicated in the pathogenesis of both RA and SLE, we examined *IL10R1* S138G and *IL10R1* G330R in small (single center) cohorts with RA and SLE, and compared the findings with matched controls. For both variant sites, genotype proportions did not deviate from Hardy-Weinberg equilibrium. In addition,

allele frequencies did not show major deviations from the controls (Table I). In RA the G330R allele was slightly more common (35.2% vs. 30.0%). Genotype analyses showed a gene-dose relationship, with twice as many homozygous G330R carriers (15.4% vs. 7.6%). In SLE, the *IL10R1* G330R allele was also higher (36.3%), although without displaying a gene-dose relationship at the genotype level. In

Table I. Comparison of IL10R1 genotype distribution, allele frequency and haplotype in healthy controls, rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE).

	Controls (%) (n=250)	RA (%) (n=182)	<i>p</i> *	Odds ratio (C.I. 95%)	SLE (%) (n=222)	<i>p</i> *	Odds ratio (C.I. 95%)
G330R genotype							
MT	19 (7.6)	28 (15.4)	0.04		23 (10.4)	0.09	
HT	112 (44.8)	72 (39.6)			115 (51.8)		
WT	119 (47.6)	82 (45.1)			84 (37.8)		
G330R allele							
MT	150 (30.0)	128 (35.2)	0.11	1.27 (0.95-1.69)	161 (36.3)	0.04	0.75 (0.57-0.99)
WT	350 (70.0)	236 (64.8)			283 (63.7)		
S138G genotype							
MT	7 (2.8)	6 (3.3)	0.95		7 (3.2)	0.27	
HT	69 (27.6)	49 (26.9)			76 (34.2)		
WT	174 (69.6)	127 (69.8)			139 (62.6)		
S138G allele							
MT	83 (16.6)	61 (16.8)	0.95	1.01 (0.70-1.45)	90 (20.3)	0.15	0.78 (0.56-1.09)
WT	417 (83.4)	303 (83.2)			354 (79.7)		
Haplotypes							
Haplotype 7	80 (16.0)	57 (15.7)	0.13		85 (19.1)	0.23	
Haplotype 4	70 (14.0)	71 (19.5)			75 (16.9)		
Haplotype 3	3 (0.6)	4 (1.1)			4 (0.9)		
Haplotype 1	347 (69.4)	232 (63.7)			280 (63.1)		

*Frequencies were compared using χ^2 analysis.

MT: homozygous; HT: heterozygous; WT: wildtype; C.I.: confidence interval.

Haplotype 1: WT/WT; haplotype 3: S138G/WT; haplotype 4: WT/G330R; haplotype 7: S138G/G330R.

fact, most patients were heterozygous G330R carriers. S138G alleles were quite common in SLE (20.3%) without reaching statistical significance.

Discussion

IL-10 signaling has been described to be of pathogenetic importance in both RA and SLE. Here we studied the functional consequence of single amino acid substitutions of two common Caucasian *IL10R1* variants and their possible association with disease susceptibility in RA and SLE. Both *IL10R1* gene variants reduced the induction of SOCS3 and SLAM. As SOCS3 is involved in the IL-10-dependent inhibition of LPS-induced macrophage activation, this finding is in line with our previous observation in G330R-homozygous monocytes, which were less sensitive to IL-10-mediated suppression of LPS-induced TNF- α production than G330R-heterozygous or *IL10R1* wild type cells (16). We assume that *IL10R1* G330R might contribute to the insufficient control of inflammation and possibly to RA susceptibility (as demonstrated by a higher [borderline significance] frequency of homozygosity at the G330R site). In

this regard *IL10R1* G330R seems to be a recessive allele, an observation that is similar to our previous findings in hepatic fibrosis (20).

In contrast to RA, our alternative hypothesis predicted a higher presence of the wild type *IL10R1* allele in SLE. Unexpectedly, however, the *IL10R1* G330R allele was more frequently found in SLE than in controls. The G330R increase was mainly due to the accumulation of heterozygous G330R carriers. As G330R is a recessive allele, the increase in the heterozygous population might reflect the effect of an unrecognized dominant allele that is in linkage disequilibrium with G330R. S138G may only partially account for this effect, since it was increased but did not reach statistical significance. This observation points to the presence of other variants at this gene locus, which confer moderate disease susceptibility and are associated with the G330R allele. Since the monocytes of heterozygous G330R individuals do not show biologically meaningful differences in response to IL-10 (16), we would not expect impaired IL-10 signaling in SLE. The increased presence of G330R may reflect

an 'innocent bystander effect' without direct pathogenetic meaning. So far one other group has analyzed the genetic contribution of *IL10R1* SNPs in SLE. This group in Japan investigated two synonymous SNPs [G241A (SNP1) and G520A (SNP2)], which did not lead to amino acid substitution and may therefore not influence receptor function (21). They did not find any association between the SNP1 or SNP2 and the development of SLE.

In summary, our data support the hypothesis that coding variants in *IL10R1* reduce IL-10 signal transduction and that *IL10R1* G330R may contribute to RA and SLE susceptibility in Caucasian populations. As a next step, transmission disequilibrium testing in RA trios could confirm the relevance of our finding. Greater knowledge of the effects of IL-10 in RA and SLE may also help to improve our understanding of the pathogenetic role of this cytokine in both phenotypes.

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