

## ORIGINAL ARTICLE

# The IL-10R1 S138G loss-of-function allele and ulcerative colitis

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Genetic predisposition is a risk factor for the development of inflammatory bowel diseases (IBDs). Disruption of the interleukin (IL)-10 pathway in mice causes intestinal inflammation similar to human IBD. Two common non-synonymous IL-10R1 variants, S138G and G330R, were cloned and expressed in HeLa and Ba/F3. A reduction in IL-10-induced STAT1 and STAT3 activation was seen for IL-10R1-S138G (but not IL-10R1-G330R) by phosphospecific western blotting in both cell types. When analyzing 52 world populations for the presence of IL-10R1 variants, a strong dissimilarity was found between major geographical regions. In addition, when 182 IBD-parent trios were genotyped for both variants, a reduced transmission of haplotype -7 (carrying the S138G variant allele) to offspring with ulcerative colitis (UC) was observed. This UC-protective effect of S138G was confirmed in a Hungarian cohort ( $n=185$ , allele frequency 11.6 versus 17.5%;  $P=0.017$ ) but not in an independent Belgian cohort ( $n=666$ , allele frequency 15.9 versus 15.5%;  $P=0.8$ ). In conclusion, the IL-10R1 S138G variant is a loss-of-function allele for IL-10-induced STAT1 and STAT3 activation but does not protect from UC susceptibility.

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**Keywords:** inflammatory bowel disease; ulcerative colitis; interleukin-10 receptor; single-nucleotide polymorphism; STAT

## Introduction

Genetic predisposition is a major risk factor for the development of inflammatory bowel diseases (IBDs (MIM 266600)). The genetic trait in IBD, both of Crohn's disease (CD) and ulcerative colitis (UC) is complex. Linkage studies led to the identification of nine independent susceptibility loci. *CARD15* (Nod2, IBD1 (MIM 605956)) was the first IBD gene to be identified.<sup>1–3</sup> Genotype–phenotype correlations revealed an association of *CARD15* polymorphisms with ileal CD.<sup>4</sup> *CARD15* does not increase the risk of developing colonic CD. Besides *CARD15*, many other genes have been linked to IBD<sup>5</sup> including an uncommon coding variant in the *IL-23R* that confers a protective effect.<sup>6</sup> The genetic background of IBD is diverse and accounts, at least in part, for the phenotypic appearance of the disease.

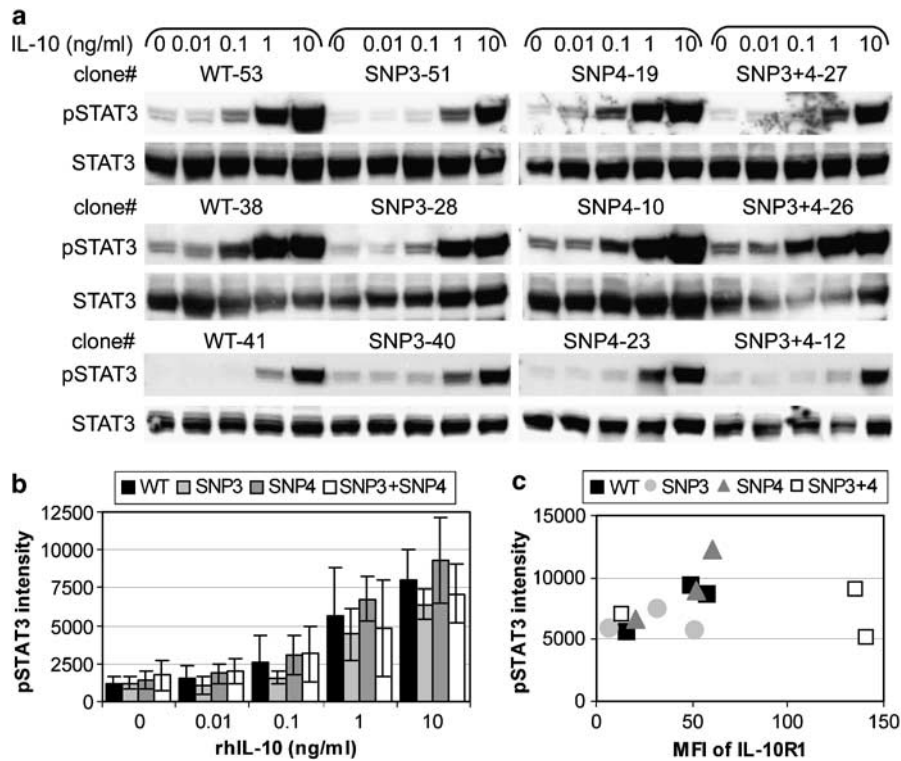
Interleukin-10 (IL-10 (MIM 146933)) is an anti-inflammatory immunomodulatory cytokine that regulates immune homeostasis specifically in the gut.<sup>7</sup> The main characteristic phenotype of the IL-10 knockout mouse is

spontaneous enterocolitis.<sup>8,9</sup> Silencing of genes of the IL-10 signaling pathway, such as the *IL-10 receptor 2* (*IL-10R2*) or target deletion of the IL-10 signal messenger *STAT3*, results in similar inflammatory responses in the intestine.<sup>10,11</sup> Together with the demonstration of IL-10 in the mucosa of patients with active CD,<sup>12</sup> these findings suggested that IL-10 controls the inflammatory response in the gut. So far, an IBD-specific alteration of the IL-10 signaling pathway has not been found in humans.<sup>13</sup>

The ability of cells to respond to IL-10 homodimers depends on the presence of two chains each of the homolog receptors IL-10R1 (MIM 146933)<sup>14</sup> and IL-10R2.<sup>15</sup> Both receptors belong to the cytokine receptor family type II that also includes interferon and IL-10 homolog receptors.<sup>16</sup> IL-10R1 plays a dominant role in mediating high-affinity ligand binding and signal transduction, whereas the IL-10R2 subunit is required for signaling only.<sup>10,15</sup> Binding of IL-10 to the IL-10R complex stabilizes dimerization of both IL-10R subunits, activates phosphorylation of the receptor-associated Janus tyrosine kinases, Jak1 (MIM 147795) and Tyk2 (MIM 176941),<sup>17,18</sup> and induces STAT1 (MIM 600555), STAT3 (MIM 102582) and in some cell types also STAT5 (MIM 601511 and 604260)-mediated signal transduction.<sup>19–21</sup>

Clinical trials of IL-10 in IBD have been disappointing.<sup>22–24</sup> However, IL-10 therapy was effective in a subset of CD patients and induced healing of ulcerated

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**Figure 1** IL-10-dependent activation of STAT3 in IL-10R1-expressing HeLa cell clones. HeLa were stably transfected with pIRESpuro3 constructs encoding IL-10R1 WT, IL-10R1 SNP3, IL-10R1 SNP4 and IL-10R1 SNP3 + SNP4, and single-cell clones were generated by limiting dilution. (a) Activation of STAT3 in IL-10R1-expressing HeLa cell clones was analyzed by western blotting. Cells were treated with serial dilutions of rhIL-10 for 30 min, protein concentration was measured, equal amounts of protein were loaded and homogenous transfer was controlled by reversible Ponceau-S staining. Membranes were stained for phospho-STAT3 (Y705) and STAT3 after stripping. Out of 45 single-cell clones that were tested for IL-10-induced activation of STAT3, three representative clones per IL-10R1 variant are shown. (b) The intensities of pSTAT3 bands were measured using the ImageJ software. Results from clones with the same IL-10R1 genotype were pooled and expressed as mean  $\pm$  standard deviation. On account of high clonal variations, only a trend toward reduced STAT3 activation levels in IL-10R1 SNP3- and IL-10R1 SNP3 + SNP4-expressing clones was seen. (c) IL-10R1 cell-surface expression density was analyzed by flow cytometry and the median fluorescence intensity was plotted against the intensity of the pSTAT3 band of the respective cell clones. Higher IL-10R1 expression levels did not correlate with higher pSTAT3 band intensities. IL-10, interleukin-10; SNP, single-nucleotide polymorphism.

intestinal mucosa in some cases. These observations led to the hypothesis that mucosal healing in IBD upon IL-10 treatment could be dependent on genetic variations between subgroups of patients. By screening for mutations in the IL-10 signaling pathway, we have identified earlier two common coding single-nucleotide polymorphisms (SNPs) on the *IL-10R1* that cause a serine 138 to glycine (SNP3) and a glycine 330 to arginine substitution (SNP4). These variants were not associated with CD but affected IL-10-dependent inhibition of tumor necrosis factor- $\alpha$  production in monocytes.<sup>25</sup> The aim of this study was to investigate the effect of these variants on IL-10 signal transduction, the distribution in various populations and their significance for UC susceptibility.

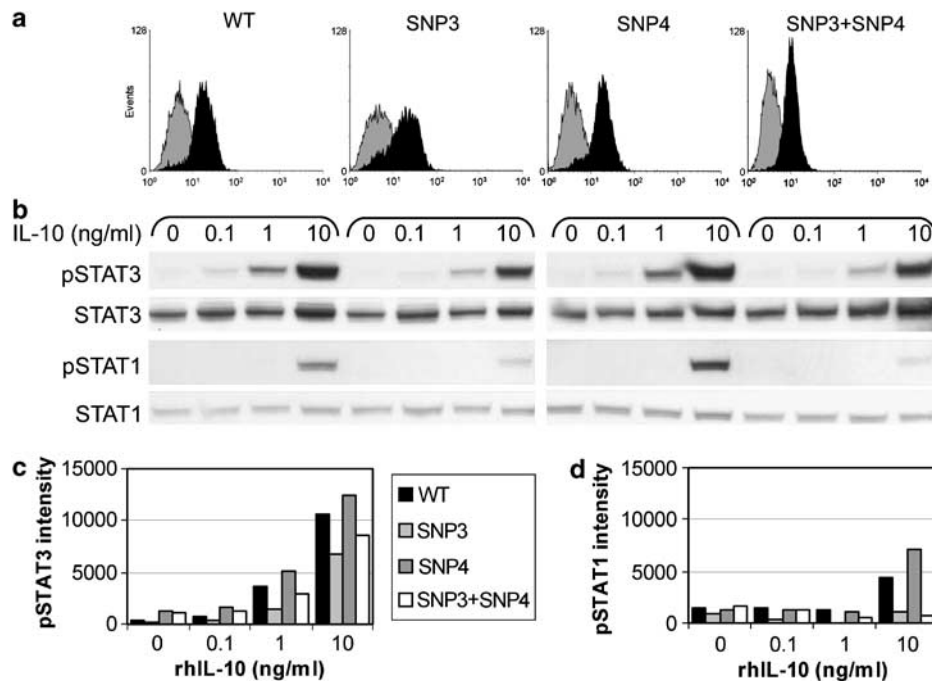
## Results

### SNP3 reduces activation of STAT3 and STAT1

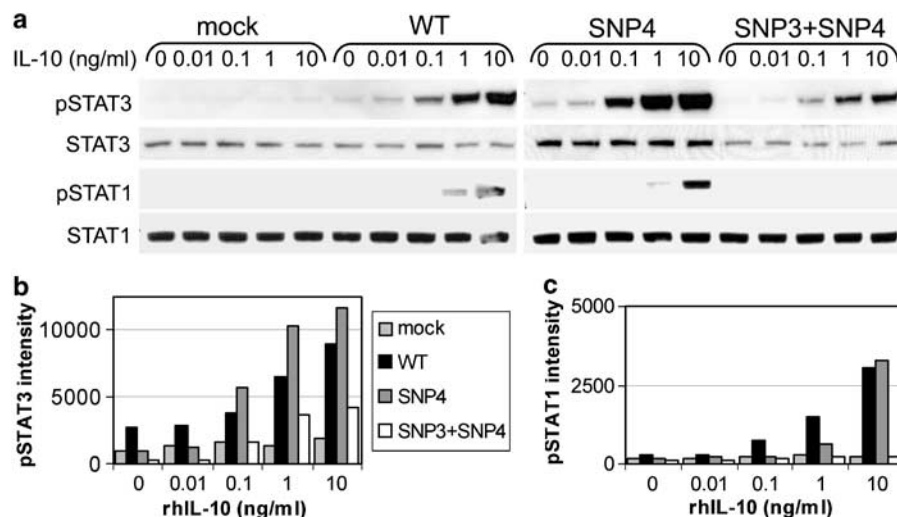
Interleukin-10 is critical in maintaining intestinal immune homeostasis. To test for changes in IL-10 signal transduction through common *IL-10R1* SNPs, we cloned four *IL-10R1* haplotypes into pIRESpuro3 and stably transfected HeLa cells. Forty-five single-cell clones that expressed IL-10R1 were analyzed for IL-10-dependent

STAT3 activation by phosphospecific western blot analysis. A dose-dependent increase of pSTAT3 was observed for all receptor variants with a high interclonal variation (Figure 1a). In *IL-10R1* SNP3 and *IL-10R1* SNP3 + SNP4 clones, a trend was seen toward a reduced STAT3 activation (Figure 1b). To test for differences in receptor density, IL-10R1 expression levels were compared by flow cytometry and related to the intensity of the pSTAT3 bands. No correlation was found between the median fluorescence intensity of IL-10R1 and the intensity of the pSTAT3 bands (Figure 1c;  $R^2 = 0.02$ ,  $P = 0.6515$ ).

To further exclude clonal variation of IL-10R1 expression, we investigated polyclonal HeLa cells. Cells expressing moderate densities of IL-10R1 WT, SNP3, SNP4 or SNP3 + SNP4 were sorted by flow cytometry and expanded for 2 days. Comparability of IL-10R1 expression levels was controlled at the time of IL-10 treatment (Figure 2a). Cells were stimulated with serial dilutions of rhIL-10 (0–10 ng ml<sup>-1</sup>), and the activation of the STAT3 and STAT1 was analyzed and quantified. STAT3 and STAT1 were activated in response to IL-10 in all cells in a dose-dependent fashion (Figure 2b). STAT3 activation was lower in SNP3- and SNP3 + SNP4-expressing cells (Figure 3c). This difference was even more pronounced for STAT1 activation, which was almost absent in



**Figure 2** IL-10-dependent activation of STAT3 and STAT1 in polyclonal IL-10R1-expressing HeLa cells. HeLa were stably transfected with pIRESpuro3 constructs encoding IL-10R1 WT, IL-10R1 SNP3, IL-10R1 SNP4 and IL-10R1 SNP3 + SNP4, and cells showing moderate expression densities were sorted by flow cytometry. Two days later, IL-10R1 expression was analyzed by flow cytometry. (a) Histogram overlays of unstained cells (gray) and cells stained with an anti-IL-10R1-PE antibody (black) are shown. Similar levels of IL-10R1 were expressed with all four constructs. (b) Activation of STAT3 and STAT1 in IL-10R1-expressing polyclonal HeLa cells was analyzed by western blotting. Cells were treated with serial dilutions of rhIL-10 for 30 min, protein concentration was measured, equal amounts of protein were loaded and homogenous transfer was controlled by reversible Ponceau-S staining. Membranes were stained for phospho-STAT3 (Y705), STAT3, phospho-STAT1 (Y701) and STAT1, respectively. STAT3 and STAT1 were activated in all IL-10R1-expressing HeLa cells. The intensities of pSTAT3 (c) and pSTAT1 (d) bands were measured using the ImageJ software. The pSTAT bands were not normalized to the STAT bands because the stripping procedure does not necessarily remove all bound antibodies, which could lead to an overestimation of the STAT bands in lanes where the pSTAT bands are more prominent. STAT3 activation was reduced and STAT1 activation was almost absent in IL-10R1 SNP3- and IL-10R1 SNP3 + SNP4-expressing cells, whereas both were slightly increased in IL-10R1 SNP4-expressing cells. IL-10, interleukin-10; SNP, single-nucleotide polymorphism.



**Figure 3** IL-10-dependent activation of STAT3 and STAT1 in polyclonal EGFP- and IL-10R1-expressing Ba/F3 cells. Ba/F3 cells were stably transfected with pBI-EGFP encoding IL-10R1 WT, IL-10R1 SNP4, IL-10R1 SNP3 + SNP4 and empty pBI-EGFP (mock). Cells that co-expressed EGFP and IL-10R1 at moderate levels were sorted by flow cytometry. Two days later, EGFP expression was analyzed by flow cytometry and similar expression levels were observed for all constructs. (a) Activation of STAT3 and STAT1 in EGFP- and IL-10R1-expressing polyclonal Ba/F3 cells was analyzed by western blotting. Cells were treated with serial dilutions of rhIL-10 for 30 min, protein concentration was measured, equal amounts of protein were loaded and homogenous transfer was controlled by reversible Ponceau-S staining. Membranes were stained for phospho-STAT3 (Y705) or phospho-STAT1 (Y701) and STAT3 or STAT1 after stripping. The intensities of pSTAT3 (b) and pSTAT1 (c) bands were measured using the ImageJ software. Similar to HeLa cells (Figure 2), STAT3 activation was reduced and STAT1 activation was almost absent in IL-10R1 SNP3 + SNP4-expressing cells, whereas both were slightly increased in IL-10R1 SNP4-expressing cells. EGFP, enhanced green fluorescent protein; IL-10, interleukin-10; SNP, single-nucleotide polymorphism.

**Table 1** Allele frequencies of *IL-10R1* variants in 52 worldwide populations

Region	Population	Geographic origin	Sample (n)	SNP3	SNP4
Africa	Bantu South	South Africa	8	—	0.44
	San	Namibia	7	—	0.15
	Mandenka	Senegal	24	—	0.13
	Yoruba	Nigeria	25	—	0.22
	Biaka Pygmies	Central African Republic	36	0.01	0.13
	Mbuti Pygmies	Republic of Congo	15	—	0.27
	Bantu North-East	Kenya	12	—	0.13
North Africa	Mozabite	Algeria (Mzab)	30	0.05	0.27
Middle East	Bedouin	Israel (Negev)	49	0.07	0.26
	Druze	Israel (Carmel)	53	0.14	0.27
	Palestinian	Israel (Central)	52	0.08	0.37
Europe	Adygei	Russia Caucasus	16	0.16	0.25
	Russian	Russia	25	0.30	0.40
	Sardinian	Italy	28	0.05	0.13
	Tuscan	Italy	8	0.13	0.38
	North Italian	Italy (Bergamo)	14	0.11	0.32
	French Basque	France	24	0.06	0.06
	French	France	24	0.10	0.20
	Orcadian	Orkney Islands	16	0.16	0.19
Central/South Asia	Balochi	Pakistan	25	0.06	0.26
	Brahui	Pakistan	25	0.18	0.34
	Makrani	Pakistan	23	0.17	0.24
	Sindhi	Pakistan	25	0.18	0.28
	Pathan	Pakistan	22	0.14	0.34
	Burusho	Pakistan	24	0.46	0.46
	Hazara	Pakistan	28	0.16	0.23
	Kalash	Pakistan	23	0.06	0.13
East Asia	Uyгур	China	7	—	0.13
	Xibo	China	8	—	—
	Dai	China	8	—	—
	Lahu	China	10	—	—
	Naxi	China	10	—	—
	Tu	China	10	0.05	—
	Yizu	China	18	—	—
	Han	China	44	0.02	—
	Miaozu	China	9	—	—
	Tujia	China	10	—	—
	Mongola	China	9	—	—
	She	China	10	—	—
	Oroqen	China	8	—	—
	Daur	China	8	—	—
	Hezhen	China	10	—	0.1
	Japanese	Japan	29	—	—
	North-East Asia	Yakut	Siberia	24	0.02
South-East Asia	Cambodian	Cambodia	11	—	—
Oceania	Papuan	New Guinea	17	0.35	0.35
	Melanesian	Bougainville	22	—	—
America	Pima	Mexico	25	—	—
	Maya	Mexico	25	0.02	0.04
	Colombian	Colombia	13	—	—
	Karitiana	Brazil	24	—	—
	Surui	Brazil	21	—	—

Abbreviations: IL-10, interleukin-10; SNP, single-nucleotide polymorphism.

*IL-10R1* SNP3- and *IL-10R1* SNP3 + SNP4-expressing cells. These data demonstrate a decreased STAT activation through *IL-10R1* haplotypes -3 and -7, which are likely caused by the exchange of serine 138 to glycine of SNP3.

To further confirm these results obtained in HeLa cells, the murine pro-B cell line Ba/F3 was transfected with the three prevalent *IL-10R1* haplotypes (haplotypes -1, -4 and -7). *IL-10R1* WT, *IL-10R1* SNP4 and *IL-10R1* SNP3 + SNP4

were cloned into pBI-EGFP, which then co-expressed enhanced green fluorescent protein (EGFP) and the *IL-10R1* haplotypes. This allowed us to sort EGFP-expressing cells to avoid a possible interference of anti-*IL-10R1* antibodies with receptor signaling. Similar to our results in HeLa, we found high clonal variation and a trend toward a reduced IL-10 responsiveness in the *IL-10R1* SNP3 + SNP4 clones. Similar to HeLa clones, the *IL-10R1* expression levels of Ba/F3 clones did not

**Table 2** Transmission disequilibrium testing of IL-10R1 variants in Belgian IBD patients

Allele	Transmitted	Non-transmitted	$\chi^2$	P-value
<b>IBD (n = 180)</b>				
SNP3				
1 <sup>a</sup>	56	29	8.58	<b>0.003</b>
2 <sup>b</sup>	29	56	8.58	<b>0.003</b>
SNP4				
1	83	71	0.94	0.33
2	71	83	0.94	0.33
Haplotypes				
1:1 (haplotype -1)	68	53	1.86	0.17
2:1 (haplotype -3)	1	3	1	0.32
1:2 (haplotype -4)	45	37	0.78	0.38
2:2 (haplotype -7)	22	43	6.78	<b>0.009</b>
<b>Crohn's disease (n = 133)</b>				
SNP3				
1	39	23	4.13	<b>0.042</b>
2	23	39	4.13	<b>0.042</b>
SNP4				
1	57	60	0.08	0.78
2	60	57	0.08	0.78
Haplotypes				
1:1 (haplotype -1)	44	45	0.01	0.92
2:1 (haplotype -3)	1	2	0.33	0.56
1:2 (haplotype -4)	37	24	2.77	0.10
2:2 (haplotype -7)	19	30	2.47	0.12
<b>Ulcerative colitis (n = 47)</b>				
SNP3				
1	17	6	5.26	<b>0.022</b>
2	6	17	5.26	<b>0.022</b>
SNP4				
1	26	11	6.08	<b>0.014</b>
2	11	26	6.08	<b>0.014</b>
Haplotypes				
1:1 (haplotype -1)	24	8	8	<b>0.005</b>
2:1 (haplotype -3)	0	1	1	0.32
1:2 (haplotype -4)	8	13	1.19	0.28
2:2 (haplotype -7)	3	13	6.25	<b>0.012</b>

Abbreviations: IBD, inflammatory bowel disease; IL-10, interleukin-10; SNP, single-nucleotide polymorphism.

Bold indicates significance level (P-value) below 0.05.

<sup>a</sup>1—wild-type allele.

<sup>b</sup>2—variant allele.

correlate with the intensities of the pSTAT3 activation ( $R^2 = 0.13$ ,  $P = 0.2088$ ). Polyclonal Ba/F3 cells that expressed EGFP and IL-10R1 were sorted for moderate EGFP expression. Cells were expanded for 2 days, treated with serial dilutions of rhIL-10 and analyzed for activation of STAT3 and STAT1. Both STAT3 and STAT1 were activated in response to IL-10 in a dose-dependent manner (Figure 3). IL-10R1 SNP3 + SNP4-expressing cells were less responsive, showing a significant reduction in STAT3 phosphorylation and no STAT1 phosphorylation at all. No uniform change was observed for IL-10R1 SNP4-expressing cells with slightly stronger STAT3 but weaker STAT1 activation (compared with wild type). These results corroborate our findings in HeLa and suggest that haplotypes carrying SNP3 (that is, haplotypes -3 and -7) display a loss-of-function for STAT1 and STAT3 activation, whereas IL-10R1 SNP4 has no or only minor effects on the intensity of IL-10-induced STAT activation.

### Worldwide distribution of IL-10R1 SNPs

To better understand the evolution of these IL-10R1 variants, DNA from 52 worldwide populations was analyzed. The amplification in 1033 of 1064 samples was informative for IL-10R1 SNP3 and in 1046 of 1064 for IL-10R1 SNP4, respectively. One hundred and forty-eight samples were positive for SNP3 (allele frequency, 0.08) and 308 for SNP4 (allele frequency, 0.171). In this collection, the distribution of IL-10R1 variants showed a strong dissimilarity between single populations and major geographical regions (Table 1). Although SNP4 was found in Sub-Saharan Africa, SNP3 evolved in Semite and Caucasian populations, which displayed the highest frequency for both the variants. Both SNPs were almost absent in native populations from East Asia and America.

### IL-10R1 S138G and UC susceptibility

Within the IL-10 pathway, IL-10R1 is a logical candidate gene for association with IBD. However, we were earlier unable to identify an association in a cohort of CD patients.<sup>25</sup> Here, we tested for an association of IL-10R1 with UC (and also tested CD in a different cohort). One hundred and eighty-two IBD triads (134 CD and 48 UC) were analyzed for IL-10R1 allele and haplotype transmissions (Table 2). Transmission disequilibrium testing revealed preferential transmission of the SNP3 wild-type allele to offspring ( $P = 0.003$ ). Alleles with haplotype -7 containing both variant alleles were significantly less likely to be transmitted to offspring ( $P = 0.006$ ). When subanalyses were performed, preferential transmission of both the wild-type alleles, SNP3 ( $P = 0.022$ ) and SNP4 ( $P = 0.014$ ), from parents to affected children was detected in UC. This was also reflected by preferred transmission of haplotype -1 ( $P = 0.005$ ), whereas haplotype -7 was less frequently transmitted to the affected children ( $P = 0.013$ ). These data indicate that the IL-10R1 wild-type allele may contribute to UC susceptibility. In CD, a borderline preferential transmission of the SNP3 wild-type allele was observed ( $P = 0.042$ ), but none was found for the SNP4 variant allele.

### Replication in two independent IBD cohorts

To confirm this association between IL-10R1 variants and UC, a Hungarian cohort of 185 UC and 451 CD patients and a Belgian cohort of 666 UC patients were genotyped and compared with two sets of matched controls (Table 3). In the Hungarian cohort, both SNP3 (11.6%) and SNP4 (23.8%) were less frequent in UC than in the controls (17.5 and 33.3%,  $P = 0.017$  and  $P = 0.005$ , respectively). No differences were seen in individuals with CD, which was in agreement with our earlier study.<sup>25</sup> In the large Belgian cohort, however, no difference was observed between UC and controls, thereby rejecting the hypothesis that IL-10R1-S138G may protect from UC.

## Discussion

Several experimental studies implicate that the IL-10 signaling pathway protects from intestinal inflammation. Foremost, the IL-10<sup>-/-</sup> mice were one of the first knockout models with an IBD-like phenotype. In the absence of IL-10, mice develop a Th1-mediated inflammation of the mucosal surfaces that is most severe in the

**Table 3** Allele, genotype and haplotype frequencies of *IL-10R1* SNP3 and *IL-10R1* SNP4 in Hungarian and Belgian populations

Allele or genotype	Hungary				Belgium			
	Controls (n = 315)	CD (n = 451)	P-value <sup>a</sup>	UC (n = 185)	P-value <sup>a</sup>	Controls (n = 393)	UC (n = 666)	P-value <sup>a</sup>
<i>SNP3 genotype</i>								
1 <sup>b</sup> -1 (n/%)	213/67.7	314/69.6		143/77.3		280/71.2	466/70.3	
1-2 <sup>c</sup> (n/%)	94/29.8	128/28.4		41/22.2		104/26.5	183/27.6	
2-2 (n/%)	8/2.5	9/2.0	0.86	1/0.5	<b>0.036</b>	9/2.3	14/2.1	0.91
<i>SNP3 allele</i>								
1 (n/%)	520/82.5	756/83.8		327/88.4		664/84.5	1115/84.1	
2 (n/%)	110/17.5	146/16.2	0.56	43/11.6	<b>0.017</b>	122/15.5	211/15.9	0.81
<i>SNP4 genotype</i>								
1-1 (n/%)	138/43.8	209/46.3		108/58.4		175/44.5	303/45.6	
1-2 (n/%)	144/45.7	196/43.5		66/35.7		187/47.6	298/44.9	
2-2 (n/%)	33/10.5	46/10.2	0.49	11/5.9	<b>0.005</b>	31/7.9	63/9.5	0.56
<i>SNP4 allele</i>								
1 (n/%)	420/66.7	614/68.1		282/76.2		537/68.3	904/68.1	
2 (n/%)	210/33.3	288/31.9	0.60	88/23.8	<b>0.002</b>	249/31.7	424/31.9	0.91
<i>Haplotypes</i>								
1:1 (haplotype -1) (n/%)	417/66.2	608/67.4		281/75.9		530/68.0	894/67.4	
2:1 (haplotype -3) (n/%)	3/0.5	6/0.7		1/0.3		3/0.4	9/0.7	
1:2 (haplotype -4) (n/%)	103/16.3	148/16.4		46/12.4		130/16.7	221/16.7	
2:2 (haplotype -7) (n/%)	107/17.0	140/15.5	0.8	42/11.4	<b>0.013</b>	116/14.9	202/15.2	0.87

Abbreviations: CD, Crohn's disease; IL-10, interleukin-10; SNP, single-nucleotide polymorphism; UC, ulcerative colitis. Bold indicates significance level (P-value) below 0.05.

<sup>a</sup>Compared with controls.

<sup>b</sup>1—wild-type allele.

<sup>c</sup>2—variant allele.

colon and also involves the small intestine.<sup>8</sup> In addition, disruption of *CRFB4*, the gene that encodes CRF2-4 (now called IL-10R2, the second chain of the IL-10 receptor complex), also causes colitis.<sup>10</sup> Cell type-specific disruption of the IL-10 signal messenger *STAT3* in neutrophils and macrophages, and overexpression of the non-IL-10 signal transducer *STAT4* showed similar inflammatory responses in the intestine.<sup>11,26</sup> Thus, abnormalities of IL-10 itself and/or IL-10-mediated signaling lead to enterocolitis in mice. We chose a candidate gene approach to test whether variants in the IL-10 pathway are associated with human IBD. By screening for coding mutations in the *IL-10* and the *IL-10R1* genes, we have identified earlier two SNPs on the *IL-10R1*<sup>25</sup> that did not associate with CD. In this study, we identified a reduced transmission of the S138G (SNP3) allele from parents to offspring with UC. These data were confirmed in a medium-size Hungarian population but not in a large Belgian cohort. We conclude that the two common *IL-10R1* variants do not protect from the development of UC. It is likely that the significant results are caused by a type-1 error due to the small sample size. However, it is important to note that we cannot rule out that the *IL-10R1* locus is associated with disease, as this would require testing of more SNPs across the gene and its vicinity.

The worldwide distribution of *IL-10R1* alleles revealed relatively high frequencies of SNP3 in populations from the Europe, Middle East, Central/South West Asia and in Papuan from New Guinea, which provide protection from UC in such populations. The SNP3 gradient between Sub-Saharan Africa and the Middle East and the absence in East Asian and native American popula-

tions indicate that the evolution of this SNP occurred somewhere in this region in a period after hunter-gatherers had settled in Asia (that is, 'out of Africa 2').<sup>27</sup>

Functional studies in *IL-10R1* SNP3-expressing HeLa cells displayed reduced activation of STAT1 and also STAT3. By using a distinct plasmid in Ba/F3 cells, we were able to sort EGFP expression, thereby avoiding a possible interference of *IL-10R1* antibody (that was used for cell sorting). As our structural model has predicted,<sup>25</sup> the exchange of serine 138 to glycine in the *IL-10R1* results in the elimination of a hydrogen bond that is important for the proper orientation of the extracellular receptor domain. Therefore, *IL-10R1* SNP3 may influence the conformation of the IL-10/*IL-10R1* complex leading to decreased binding affinity and downstream signaling of IL-10. In all our assays, *IL-10R1* SNP3 displayed reduced IL-10 signaling activity. We may conclude that SNP3 is a loss-of-function variant that affects the *IL-10R1* haplotype -7. Upon activation by its ligand, the *IL-10R1* S138G allele causes diminished STAT3 and almost absent STAT1 phosphorylation, which may lead to changes in the regulation of IL-10-dependent genes. If IL-10 therapy returns (for example, as genetically modified lactobacilli<sup>28</sup>), the *IL-10R1* SNPs are prime candidates for pharmacogenetic studies.

## Materials and methods

### Cloning of *IL-10R1*

As described earlier, SNP3 and SNP4 result in four *IL-10R1* haplotypes:<sup>25</sup> wild-type nucleotides at both

SNP3 and SNP4 positions (haplotype -1), alleles carrying the SNP3 variant only (haplotype -3), alleles carrying the SNP4 variant only (haplotype -4) and alleles carrying both SNP3 and SNP4 variants (haplotype -7). The coding sequence of *IL-10R1* haplotypes -1, -4 and -7 was obtained by the 5'-rapid amplification of cDNA ends 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, Mountain View, CA, USA) resulting in the plasmids pIRESpuro3-EGFP, pIRESpuro3-IL-10R1-WT, pIRESpuro3-IL-10R1-SNP3, pIRESpuro3-IL-10R1-SNP4 and pIRESpuro3-IL-10R1-SNP3 + 4, respectively. In addition, the cDNAs of the FLAG-tagged *IL-10R1* haplotypes were cloned into pBI-EGFP (Clontech) resulting in the plasmids pBI-EGFP-IL-10R1-WT, pBI-EGFP-IL-10R1-SNP4 and pBI-EGFP-IL-10R1-SNP3 + 4. The correct sequence of the various vectors was confirmed by cycle sequencing.

#### Transfection of HeLa and Ba/F3 cells

HeLa cells (kindly provided by Dr J Hutchinson, Institute of Molecular Pathology, Vienna, Austria) were maintained in RPMI medium containing 10% fetal calf serum. Ba/F3 cells were obtained from the German collection of microorganisms and cell cultures (DSMZ GmbH, Braunschweig, Germany) and maintained in RPMI containing 10% fetal calf serum supplemented and  $10 \text{ ng ml}^{-1}$  murine IL-3. HeLa cells ( $2 \times 10^5$ ) were transfected with  $0.4 \mu\text{g}$  of pIRESpuro3-EGFP, pIRESpuro3 (mock), pIRESpuro3-IL-10R1-WT, pIRESpuro3-IL-10R1-SNP3, pIRESpuro3-IL-10R1-SNP4 or pIRESpuro3-IL-10R1-SNP3 + 4 using the effectene transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's recommendations and selected with  $1.5 \mu\text{g ml}^{-1}$  puromycin (Sigma, Munich, Germany). Single-cell clones were prepared by limiting dilution, identified by inverse microscopy and analyzed for IL-10R1 expression by flow cytometry. Clones that showed homogeneous IL-10R1 expression were further expanded. Similarly, Ba/F3 cells were electroporated with the pTet-Off plasmid encoding the tetracycline-dependent transactivator and selected with  $400 \mu\text{g ml}^{-1}$  geneticin (Invitrogen, Carlsbad, CA, USA). Single-cell clones were prepared by limiting dilution. One clone with suitable tetracycline-dependent transactivator expression was additionally transfected with pBI-EGFP, pBI-EGFP-IL-10R1-WT, pBI-EGFP-IL-10R1-SNP4 or pBI-EGFP-IL-10R1-SNP3 + 4 together with the pTK-hyg plasmid for selection in mammalian cells. Clones were screened for EGFP and IL-10R1 expression by flow cytometry, and those showing both EGFP and IL-10R1 expression were further expanded.

#### Flow cytometry

Cells were stained with a phycoerythrin-conjugated anti-IL-10R1 antibody (PharMingen, San Diego, CA, USA). Unspecific binding to cells was blocked with 20% fetal calf serum for 10 min at room temperature. IL-10R1 and EGFP expressions were analyzed on a FACScan (Becton Dickinson, San Jose, CA, USA). Fluorescence-activated cell sorting of stably transfected cells was performed on an FACSAria (Becton Dickinson).

#### Western blot analysis

Protein extracts were prepared using extraction buffer containing protease and phosphatase inhibitors (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2 mM EDTA, 0.2% NP-40, 10% glycerol, 50 mM  $\beta$ -glycerophosphate, 50 mM NaF, 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM  $\beta$ -mercaptoethanol and  $5 \mu\text{g ml}^{-1}$  each of pepstatin, leupeptin and aprotinin; all obtained from Sigma). Protein concentration was measured on a spectrophotometer (Hitachi, Pleasanton, CA, USA) using the protein assay from (Bio-Rad, Hercules, CA, USA). Equal amounts of proteins were separated on pre-cast denaturing 4–12% gradient polyacrylamide gels (Invitrogen) and transferred to a polyvinylidene fluoride membrane (Bio-Rad) by electroblotting. Homogeneous loading and transfer of proteins were controlled by reversible Ponceau-S staining. Membranes were blocked with 5% non-fat dried milk and incubated overnight with the primary antibody. Horseradish peroxidase-conjugated secondary antibodies (horseradish peroxidase-conjugated anti-rabbit from Amersham Biosciences, Piscataway, NJ, USA and horseradish peroxidase-conjugated anti-goat from Invitrogen) were applied before developing with ECL (Amersham Biosciences). After detection of phosphoproteins, membranes were stripped using stripping buffer (2% SDS, 100 mM  $\beta$ -mercaptoethanol, 50 mM Tris (pH 6.8)) for 10 min at  $55^\circ\text{C}$ . The detection of non-phosphorylated proteins was performed as described for the phosphoproteins. The following primary antibodies were used: Phospho-STAT3 (Tyr 705), Phospho-STAT1 (Tyr 701), STAT1 (all from Cell Signaling Technology, Danvers, MA, USA) and STAT3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Band intensity was quantified using the ImageJ program (<http://rsb.info.nih.gov/ij/>).

#### DNA samples

All patients gave informed written consent and the study was as approved by the local ethics committee. Genomic DNA was genotyped from three populations as described below. First, samples from 188 Belgian IBD patients (49 UC, 135 CD and 4 indeterminate colitis) and 887 controls were analyzed for the presence of *IL-10R1* SNP3 and *IL-10R1* SNP4. From 182 (48 UC and 134 CD) out of these patients, matched DNA samples from both parents were available and also genotyped. The CD patients had been characterized according to the Vienna Classification.<sup>29</sup> Second, an independent Hungarian population of 636 IBD patients (185 UC and 451 CD) and 315 controls was genotyped and third, a large Belgian cohort of 666 UC patients and 393 (different) controls.

In addition, 1064 DNA samples from 52 world populations that had been characterized earlier by typing of 377 autosomal microsatellites<sup>30</sup> were kindly provided by the Foundation Jean Dausset (CEPH)<sup>31</sup> and screened for the presence of *IL-10R1* SNPs. Inconclusive samples were tested at least twice.

#### IL-10R1 genotyping

Two allele-specific PCRs were used for the detection of SNP3 (S138G or S159G when including the 21-aa signal peptide; rs3135932) and SNP4 (G330R or G351R including the 21-aa signal peptide; rs2229113) in genomic DNA as described earlier.<sup>25</sup> The PCR products of the SNP3 reaction are 464 bp (control PQ-138), 337 bp

(wild-type AQ-138) and 183 bp (variant PB-138). The PCR products of the SNP4 reaction are 515 bp (control PQ-330), 318 bp (wild-type AQ-330) and 248 bp (variant PB-330).

#### Statistical analysis

Tests for conformity to Hardy–Weinberg equilibrium were performed using a standard  $\chi^2$  test (1 degree of freedom (d.f.)) to test differences between observed and expected genotype distributions for both SNPs based on control population allele frequencies. The significance of the differences in allele frequencies was compared between the patient and control groups using  $2 \times 2$  tables and standard  $\chi^2$  tests (1 d.f.). Similarly, significance of the differences in genotype frequencies was compared between the patient and control groups using  $2 \times 3$  tables and standard  $\chi^2$  tests (2 d.f.). Haplotypes were estimated from genotype data using an expectation–maximization algorithm as implemented by the program EH.<sup>32</sup> Haplotype frequencies were compared between patient and control groups using  $2 \times 4$  tables and standard  $\chi^2$  test (3 d.f.). Correction for multiple testing was also not performed as both SNPs are in strong linkage disequilibrium. Disease association of *IL-10R1* SNPs was analyzed by transmission disequilibrium testing using the Genehunter v2.1.<sup>33</sup> Student's *t*-test was used for comparing STAT activation in different clones. Statistical significance was assumed when  $P < 0.05$ .

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