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*J Immunol* 2003; 170:5578-5582; doi: 10.4049/jimmunol.170.11.5578

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Novel Variants of the IL-10 Receptor 1 Affect Inhibition of Monocyte TNF-α Production

Christoph Gasche,‡* Paul Grundtner,* Petra Zwirn,* Walter Reinisch,* Sarah H. Shaw,‡ Alexander Zdanov,‡ Usha Sarma,§ Lynn M. Williams,§ Brian M. Foxwell,§ and Alfred Gangl*

IL-10-deficient mice exhibit spontaneous enterocolitis and other symptoms akin to Crohn’s disease, indicating that IL-10 might regulate normal physiology in the gut. However, clinical trials with IL-10 in Crohn’s disease were disappointing, although some patients showed healing of intestinal mucosa. This study searched for genetic polymorphisms within the IL-10 pathway. We decided to screen for mutations of the IL-10R1 cDNA in healthy volunteers and Crohn’s disease patients and identified two novel variants: a serine 138-to-glycine (S138G) and a glycine 330-to-arginine (G330R) substitution. The allelic frequency in a European cohort was relatively high (16% for the S138G and 33% for the G330R), and S138G was in strong linkage disequilibrium with G330R. A similar allele frequency was found in a group of Crohn’s patients. In IL-10R1 G330R-expressing monocytes, the inhibitory effect of IL-10 on TNF-α production was diminished, indicating that this variant may be a loss-of-function allele. No such difference was observed between haplotypes 4 (G330R only) and 7 (S138G and G330R). In addition, these IL-10R1 variants had no influence on the IL-10R1 expression density. Structural analysis of the S138G variant revealed that the substitution of the human IL-10R1 chain plays a dominant role in mediating high affinity ligand binding and signal transduction, whereas the IL-10R2 subunit (previously described as cytokine receptor family 2–4) is thought to be required for signaling only (11, 12). Interaction of IL-10 with the IL-10R complex stabilizes dimerization of both IL-10R subunits, activates phosphorylation of the receptor-associated Janus tyrosine kinases, Janus kinase (JAK)4 and TYK2 (13, 14), and induces STAT3- and STAT1-mediated signal transduction (15–17). Deletion of genes of the IL-10 signaling pathway, such as the ablation of IL-10R2 or myeloid-specific deletion of STAT3, showed dysregulated inflammation similar to that seen in the IL-10 knockout intestine (11, 18). However, in humans, no inflammatory bowel disease-specific alteration of the IL-10 pathway has been identified (19).

Despite the evidence for an association between IL-10 deficiency and inflammatory bowel disease, clinical trials of IL-10 in Crohn’s have been disappointing (20–22). However, healing of intestinal Crohn’s disease lesions was observed in some patients. Moreover, no such mucosal healing or improvement in disease pathology was observed in placebo-treated controls (21). These findings led to the hypothesis that mucosal healing in inflammatory bowel disease upon IL-10 treatment could be dependent on genetic variations between subgroups of patients. Previous work has explored the possibility that the polymorphic nature of the IL-10 promoter region might be associated with intestinal inflammation in particular subgroups of patients (23–25). We chose to screen for mutations in the IL-10R1 and detected two novel variants that were shown to meet the criteria of coding single nucleotide polymorphisms (cSNPs).

Materials and Methods

Patients and samples

All patients before participation in the clinical trial gave informed, written consent, and additional consent was sought for genetic analysis, as approved by the institutional review board. Blood was drawn for RNA and DNA extraction from 10 control subjects, and seven Crohn’s disease patients, two of whom had a history of intestinal resection. Genotyping of

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0022-1767/03/$02.00

4 Abbreviations used in this paper: JAK, Janus kinase; SNP, single nucleotide polymorphism; cSNP, coding SNP, CI, confidence interval; MFI, median fluorescence intensity.
cSNPs was done in 310 DNA samples from European control subjects (mean age, 41 years (39–43); 55% female) and 100 European Crohn’s disease patients (mean age, 35 years (32–37); 53% female).

Screening of the IL-10R1 cDNA for sequence variants

Total RNA was extracted from PBMC using TRI-reagent (Molecular Research Center, Cincinnati, OH). One microgram of total RNA was used for the reverse-strand cDNA synthesis using the oligo(dT)15 primer and SuperScript II RNase H− reverse transcriptase (Life Technologies, Gaithersburg, MD). The entire IL-10R1 coding region was analyzed by PCR and cycle sequencing of four overlapping fragments. For analysis of the 5′-untranslated region, the 5′-rapid amplification of cDNA ends, abridged, anchored primer was used (Life Technologies). The following primer pairs were used: IL-10R1–1, 5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGG and 5′-GTCCAATGGCTCTACGCTTGGC; IL-10R1–3, 5′-TTCATTAGGACGCGTGTCCTCCTC-3′ and 5′-TGCTGTCCTAGGTAACCC-3′; and IL-10R1–4, 5′-CAGGTTACCTGGAGGACAG-3′ and 5′-TGCTCTTGGCCTAACTTCTG-3′.

Genotyping of sequence variants

Two allele-specific PCRs were designed for detection of S138G (single nucleotide polymorphism (SNP3)) and G330R (SNP4) in genomic DNA. The primer sequences for IL-10R1–SNP3 are the following: P-138, 5′-TCAGCCCTCAAGTCTCATGTTAATCC-3′, Q-138, 5′-TGGTCCTATCATCACAAGGCTCTTG-3′, A-138, 5′-GGGGGGGGGGGCGGAGTATCGA-CATATGAAA-3′, and B-138, 5′-GGGCCGCCGGAGGATGCTAAGCTG-3′. Primer A-138 was specifically designed with R (A+G) to avoid interference with a G-to-A polymorphism at position 520 (SNP2; Table I) of the IL-10R1 cDNA (26). The primers for IL-10R1–SNP4 were the following: P-330, 5′-CCTTTACATCCATCTCAGGCGCTC-3′, Q-330, 5′-CTCGTTGAACCCGTGGTACAG-3′, A-330, 5′-GC GGGGGGGAAGAGGCTGGAACG-3′, and B-330, 5′-GGGGGC CGCCCGACCACGGGCTCCTTT-3′.

The PCR products of the SNP3 reaction are 464 bp (control PQ-330), 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). Forty cycles of amplification were performed with AmpliTaq Gold (Applied Biosystems, Foster City, CA). The accuracy of both assays was verified by comparison to cycle sequencing in 50 DNA samples. Because the S138G (SNP3) is located only 16 nt downstream of the already identified SNP2 (A520G; Table I) (26), allele-specific linkage analysis was performed between SNP3 and SNP2 in 90 control individuals who carried at least one copy of SNP3. Two allele-specific PCRs were performed using the primers P-138 and B-138 (SNP3 variant allele) or P-138 and B-138wet (SNP3 wild-type allele): 5′-GGGGGGGGGCGAAGTGACTGAAGATGCTT-3′ and 5′-GTCCAATGGCTCTACGCTTGGC; 5′-TTCATTAGGACGCGTGTCCTCCTC-3′ and 5′-TGCTGTCCTAGGTAACCC-3′; and 5′-CAGGTTACCTGGAGGACAG-3′ and 5′-TGCTCTTGGCCTAACTTCTG-3′.

Inhibition of monocyte TNF-α production

Monocytes from 61 volunteers were isolated by centrifugation elutriation and seeded at 2 × 105 cells/well in 96-well plates in triplicate. After 1-h preincubation, LPS (10 ng/ml; Sigma-Aldrich, Poole, Dorset, U.K.) and recombinant human IL-10 (100–0.01 ng/ml; R&D Systems, Abingdon, Oxon, U.K.) were added. Supernatants were harvested after 24-h incubation, and TNF-α expression was analyzed by ELISA (BD PharMingen, Cowley, Oxford, U.K.). The IL-10 concentrations at 50% TNF-α inhibition (IC50) and the maximal inhibition (IC100) were determined. IC50 values were calculated using Microcal Origin 5.0 (Microcal Software, Northampton, MA) and normalized for interbatch variation. DNA was extracted from the same donors and used for subsequent SNP genotyping.

IL-10R1 expression

PBMC of 70 volunteers were isolated from 8.5 ml of whole blood using Ficoll-Paque Plus (Amersham Pharmacia Biotech, Uppsala, Sweden). Cells were washed for 30 min at 4°C both with an anti-IL-10R1-PE mAb (clone 37607.11; R&D Systems), or an isotype control, and with anti-CD14-FITC mAb (MY4; Coulter, Miami, FL). Flow cytometry was performed on a FACScan (BD Biosciences, Mountain View, CA) using CellQuest acquisition software (BD Biosciences). Calculations were performed using WinMDI 2.8 (http://facs.scripps.edu). The IL-10R1 expression was determined as a percentage of IL-10R1-positive monocytes and as normalized median fluorescence intensity (MFI).

Structural analysis of the IL-10/IL-10R1 complex

Atomic coordinates of the IL-10/IL-10R1 complex have been used to generate a tentative structural model of the IL-10R1 S138G variant. At the beginning, coordinates form a computer model of the complex (27), and at the end, coordinates of the crystal structure (28) were used. Program O (29) was used for analysis, and the program RIBBONS (30) was used to generate Fig. 3.

Statistical analysis

Tests for conformity to Hardy-Weinberg equilibrium were done using a standard x2 (1 df) 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 Crohn’s disease patient and control groups using 2 × 2 tables and standard x2 tests (1 df). Similarly, the significance of the differences in genotype frequencies was compared between the Crohn’s disease patient and control groups using 2 × 3 tables and standard x2 tests (2 df). Haplotype frequencies were estimated from genotype data using an expectation-maximization algorithm as implemented by the program EH (31). Haplotype frequencies were compared between Crohn’s disease patient and control groups using a 2 × 4 table and standard x2 test (3 df). Linkage disequilibrium between SNP marker alleles were derived from the 2 × 2 table using the measure D’ (32). Results from monocyte inhibition and IL-10R1 expression studies were compared using the Kruskal-Wallis and the Mann-Whitney test.

Results

Novel IL-10R1 variants

Based on our hypothesis that an intestinal response to IL-10 therapy in Crohn’s disease patients might be genetically defined, we screened for mutations in IL-10R1. IL-10R1 cDNA (GenBank accession no. U00672) was sequenced from 10 unrelated control subjects and from seven Crohn’s disease individuals. In five of seven Crohn’s disease patients and 3 of 10 control subjects, a G-to-A transition at position 1112 was detected causing a substitution of glycine (G)330 (mature protein numbering) to arginine (R) (G330R or SNP4). All but one of these patients were heterozygous for this mutation. In the patient with the homozygous mutation, one allele carried an additional A536G mutation, which causes a substitution of serine (S138) to glycine (S138G or SNP3). We also detected a silent substitution at G586A in a single allele (SNP5). Seven alleles were found with the previously described silent variation at G520A (SNP2) and none at G241A (26). Six additional SNPs had been submitted to the National Center for Biotechnology Information SNP database (GenBank accession no. NM_001558), two of which also cause an amino acid substitution (SNP6 and SNP8; Table I).

Table I. SNPs in the IL-10R1 cDNA

<table>
<thead>
<tr>
<th>Nucleotide Position</th>
<th>Amino Acid Position</th>
<th>PubMed Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNP1 G241A</td>
<td>A60A</td>
<td>rs2229112</td>
</tr>
<tr>
<td>SNP2* G520A</td>
<td>A153A</td>
<td>rs3135931         (rs2256111)</td>
</tr>
<tr>
<td>SNP3* A536G</td>
<td>S138G</td>
<td>rs3135932</td>
</tr>
<tr>
<td>SNP5* G586A</td>
<td>P175R</td>
<td>rs228054</td>
</tr>
<tr>
<td>SNP6 A731G</td>
<td>I203V</td>
<td>rs228055</td>
</tr>
<tr>
<td>SNP7 C1033T</td>
<td>T324T</td>
<td>rs229115</td>
</tr>
<tr>
<td>SNP4* G1112A</td>
<td>G330R</td>
<td>rs229113</td>
</tr>
<tr>
<td>SNP8 C1320T</td>
<td>S420L</td>
<td>rs229114</td>
</tr>
<tr>
<td>SNP9 C1929T</td>
<td>3′ UTR</td>
<td>rs752174</td>
</tr>
<tr>
<td>SNP10 A331G</td>
<td>3′ UTR</td>
<td>rs11263</td>
</tr>
<tr>
<td>SNP11 A3524G</td>
<td>3′ UTR</td>
<td>rs9610</td>
</tr>
</tbody>
</table>

* Described in this article.

UTR, Untranslated region.
Population genotyping

Two multiplex PCR assays were developed for genotyping of SNP3 and SNP4 in larger populations (Fig. 1). For the variant G330R (SNP4), there were 204 of 620 variant alleles (32.9%), and for variant S138G (SNP3), there were 100 of 620 variant alleles (16.1%; Table II). For both variant sites, genotype proportions did not significantly deviate from Hardy-Weinberg equilibrium. At the SNP4 locus, we found 10.6% (10.9% expected) homozygous variant, 44.6% (44.2% expected) heterozygous, and 44.8% (44.9% expected) homozygous wild-type individuals ($\chi^2 = 0.03; p = 0.86$). At the SNP3 locus, there were 3.2% (2.5% expected) homozygous, 25.8% (26.9% expected) heterozygous and 71.0% (70.5% expected) homozygous wild-type individuals ($\chi^2 = 0.70; p = 0.40$). SNP3 was in strong linkage disequilibrium with SNP4 ($D^\prime = 0.94; p < 0.001$). Four hundred and twelve (66.5%) of all alleles were wild-type at both SNP3 and SNP4 (haplotype 1), 108 (17.4%) alleles carried SNP4 only (haplotype 4), 4 (0.6%) alleles carried SNP3 only (haplotype 3), and 96 (15.5%) carried both SNP3 and SNP4 variants (haplotype 7). We also examined the IL-10R1 SNP status in 100 Crohn’s disease patients. In this population, genotype, allele, and haplotype frequencies of SNP3 and SNP4 were similarly to those of controls (Table II).

SNP3 is located 16 nt downstream of the previously identified SNP2 (A520G; Table I). In Asians, the allele frequency for SNP2 was reported to be between 55 and 60% (33). In our control population (European Caucasian), the SNP2 allele frequency was 46%. All (100%) of the 100 SNP3 variant alleles and 30% of the SNP3 wild-type alleles carried SNP2.

Structural analysis of SNP3

Modeling of the IL-10/IL-10R1 complex revealed that S138 is located on the C-terminal domain D2 at /H9251. Modeling of the IL-10/IL-10R1 complex revealed that S138 is located on the C-terminal domain D2 at /H9251. This is the only hydrogen bond on this side of the outer surface of the interdomain junction. The disruption of this hydrogen bond upon exchange of S138 to G could produce certain conformational rearrangements in this area.

Effect of IL-10R1 variants on monocyte inhibition

To further elucidate the effect of polymorphisms in the IL-10R1 on receptor function, we compared the ability of IL-10 to inhibit LPS-induced TNF-α production in human monocytes derived from individuals with two IL-10R1 wild-type alleles (wt/wt; n = 29) with that with one wild-type and one IL-10R1 SNP3 and/or SNP4 allele

![FIGURE 1. SNP3 (S138G) and SNP4 (G330R) genotyping by allele-specific PCR. Multiplex PCR (SNP3, A; SNP4, B) was designed to generate a nonspecific product along with allele-specific amplicons, a wild-type and a variant fragment. Lane 1, 100-bp DNA marker (Life Technologies); lane 2, homozygous carrier; lane 3, wild-type individual; and lane 4, heterozygous carrier. Numbers on the right indicate the size of the amplicons.](image)

![FIGURE 2. Structure of the IL-10/IL-10R1 complex and interaction sites of S138. In the IL-10/IL-10R1 complex, S138 is located on the C-terminal domain D2 at a helix A before loop 5, which interacts with IL-10. The hydroxyl oxygen on S138 forms a hydrogen bond to the peptide link N133-D134, and the side chain of D134 interacts with the side chain of H71 of the N-terminal domain D1. The IL-10 domain is shown in red; the receptor molecule is yellow; S138, N133, and H71 are shown as stick-and-ball models (atom color code: C, green; N, blue; and O, red); and hydrogen bonds are shown as dashed lines. Receptor loops interacting with the IL-10 are marked in accordance with Josephson et al. (28).](image)
sensitive to IL-10-mediated inhibition of TNF-α production in vitro.

Haplotyp 4 carries SNP4, and haplotype 7 carries SNP4 and SNP3. To test for a functional difference between haplotypes 4 and 7, we performed subgroup analysis within the heterozygous group (wt/mut). The median normalized IC50 value from heterozygous individuals with haplotype 4 (0.94 (95% confidence interval (CI), 0.55–1.27); n = 14) was higher than that from wild-type individuals (0.55 (95% CI, 0.53–1.0); n = 29), though not different than that from individuals with haplotype 7 (0.34 (95% CI, 0.45–1.75); n = 13; p = 0.76). Likewise, the maximal TNF-α inhibition from heterozygous individuals with haplotype 4 (91% (95% CI, 83.2–96.6%)) was lower than that from wild-type individuals (95% (95% CI, 92.8–97.3%); p = 0.017) but not different than that from individuals with haplotype 7 (95% (95% CI, 86.7–98.3%), p = 0.35).

IL-10R1 expression on monocytes

To exclude the possibility that the difference in monocyte inhibition is due to an altered IL-10R1 expression, we analyzed the IL-10R1 expression on monocytes. The percentage of IL-10R1-positive monocytes and the MFI were compared between wt/wt (n = 29), wt/mut (n = 34), and mut/mut (n = 6) individuals. In the respective groups, 12.4% (95% CI, 3.7–24.4), 12.2% (95% CI, 6.1–23.1), and 27.1% (95% CI, 1.8–50.0) of monocytes expressed IL-10R1 (p = 0.32). The MFI were 1.7 (95% CI, 1.4–3.0) in wt/wt, 2.1 (95% CI, 1.6–3.0) in wt/mut, and 3.1 (95% CI, 1.3–4.3) in mut/mut individuals (p = 0.38). No difference was seen between the haplotype 4 and 7 subgroups of the heterozygous individuals.

Discussion

This investigation has revealed three previously unrecognized polymorphisms of the IL-10R1 (SNP3, SNP4, and SNP5), two of which result in an amino acid substitution. One of the cSNPs, SNP4, was found in approximately one-third of investigated alleles and is located in a section of the cytoplasmic domain of the IL-10R1 (aa 282–389), which, when deleted in mice, leads to increased JAK/STAT activation and consequently to increased downstream events such as cell proliferation, modulation of TNF-α production, and HLA-DR expression (34). SNP3 is in strong linkage disequilibrium with SNP4. In a European Caucasian cohort, approximately one-half of SNP4 alleles also carried SNP3. Allelic genotyping studies did not show a different SNP3 and SNP4 allele frequency in a matched Crohn’s disease population, indicating that IL-10R1 variants do not confer susceptibility to Crohn’s disease. However, Crohn’s disease is not a homogenous disease phenotype, and different disease locations and behaviors have been recognized (35). Indeed, mutations in nucleotide-binding oligomerization domain 2/caspase recruitment domain 15 are susceptibility factors only for ileal but not for colonic Crohn’s disease (36). For IL-10R1 variants, it will be necessary to perform subgroup analyses in larger series before rejecting the hypothesis that IL-10R1 variants may confer genetic susceptibility to Crohn’s disease. Preliminary data from our laboratory point to a protective effect of the IL-10R1 variants in ulcerative colitis (37).

The results from monocyte inhibition experiments reveal a functional change by these cSNPs. Monocytes from individuals homozygous for the IL-10R1 variants showed significantly higher IC50 for IL-10-induced inhibition of TNF-α production and a 20% lower maximal inhibition. The effect was dose dependent, because heterozygous carriers also displayed lower IL-10 responsiveness when compared with that of wild-type individuals. To further dissect a functional role of SNP3, heterozygous individuals were grouped into haplotype 4 and haplotype 7 carriers, but no difference was detected. This indicates that the SNP4 polymorphism may act as a loss-of-function allele, with and without the presence of SNP3.

Receptor staining for flow cytometry revealed that the IL-10R1 haplotypes 1, 4, and 7 are expressed at a similar percentage and density on monocytes. However, the huge interindividual variation could have obstructed possible effects of these haplotypes. Indeed, we observed a little tendency toward a higher median receptor expression in the variant haplotypes. At the moment, we cannot rule out that these variants, specifically SNP4, have a stabilizing effect on receptor expression or increased receptor recycling. However, it is very unlikely that the reduction in IL-10-mediated inhibition of TNF-α production, which was observed for the variant haplotypes, is caused by a reduced receptor expression. These findings support the hypothesis that SNP4 acts as a true loss-of-function allele.

Analyses of the structure of the IL-10/IL-10R1 complex point to a possible influence of the S138G variant on the conformation of the IL-10/IL-10R1 complex. S138 does not make any direct contacts with the ligand; however, its position at the C terminus of the α helix A, covering the interdomain hydrophobic core of the receptor before loop 5, which is involved in the interaction with IL-10, implies that it may be important for the proper orientation of the receptor domains D1 and D2 upon binding with IL-10. The exchange of S to G with elimination of the hydrogen bond between S138 and the peptide link N133-D134 may initiate a change in the mutual orientation of the receptor domains or in the conformation of loops 3 and 5, both of which could affect the downstream signal of the IL-10R complex through impaired IL-10 binding.

For the class II cytokine receptor family, the downstream events within the JAK/STAT signaling pathway are well described, but not sufficient to explain the anti-inflammatory actions of IL-10 (17, 38). The complex regulation of TNF-α inhibition by IL-10 involves both transcriptional and posttranscriptional mechanisms (39) and possibly also induction of heme oxygenase-1, a stress-inducible protein (40). However, the receptor-adjacent events of such mechanisms are obscure. It is currently too early to speculate on how the IL-10R1 variants could affect the regulation of TNF-α expression. It is also unclear whether they alter STAT3 activation. We anticipate further answers from cells that are transfected with various IL-10R1 alleles.
References