# Interleukin (IL)-23 Receptor Is a Major Susceptibility Gene for Graves' Ophthalmopathy: The IL-23/T-helper 17 Axis Extends to Thyroid Autoimmunity

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**Context:** IL-23 and its receptor (IL-23R) guide T cells toward the T-helper 17 phenotype. IL-23R single nucleotide polymorphisms (SNPs) have been associated with several autoimmune diseases, including Crohn's disease and rheumatoid arthritis.

**Objective:** Our objective was to determine whether variants in the IL-23R gene are associated with Graves' disease (GD) and Graves' ophthalmopathy (GO).

**Design and Participants:** A total of 216 North American Caucasian GD patients and 368 healthy controls were genotyped for four SNPs spanning the IL-23R gene. SNPs rs11209026 and rs7530511 were genotyped using the TaqMan allelic discrimination assays (Applied Biosystems, Foster City, CA), and SNPs rs2201841 and rs10889677 were genotyped using a fluorescent-based restriction fragment length polymorphism method.

**Results:** The A allele of rs2201841 was present in 78.8% of GD patients with GO and 64.7% of controls [$P = 1.1 \times 10^{-4}$; odds ratio (OR) = 2.04]; the AA genotype was also significantly increased in GO patients compared with controls (62.5 and 41%, respectively; $P = 1.0 \times 10^{-4}$; OR = 2.4). The C allele of rs10889677 was present in 78.6% of GO patients and 64.5% of controls ($P = 1.3 \times 10^{-4}$; OR = 2.03), and the CC genotype was also significantly increased in GO patients *vs.* controls (62.1 and 41.0%, respectively; $P = 1.4 \times 10^{-4}$; OR = 2.36). The TT genotype of rs7530511 was significantly associated with GD, but not specifically with GO; it was present in 2.5% of GD patients and 0.3% of controls ($P = 0.02$; OR = 9.4). The rs11209026 SNP, which is the most strongly associated with Crohn's disease, was not associated with GD or GO in our data set.

**Conclusions:** Variants in the IL-23R gene are strongly associated with GO. These variants may predispose to GO by changing the expression and/or function of IL-23R, thereby promoting a proinflammatory signaling cascade. (***J Clin Endocrinol Metab*** **93: 1077–1081, 2008)**

Graves' disease (GD) represents one of the most common autoimmune diseases in the United States, with a population prevalence approaching 1% (reviewed in Ref. 1). Characterized by thyrotoxicosis, diffuse goiter, and the presence of stimulating TSH receptor antibodies, GD occurs, after a series of genetic and environmental events (2). An inherent genetic predisposition, in combination with external triggers, such as infection or iodine, result in the activation of thyroid-autoreactive CD4+ T cells, which infiltrate the thyroid gland. The thyroid infiltrating T cells activate B cells to secrete TSH receptor stimulating antibodies that induce thyrocyte proliferation and secretion of excess thyroid hormones, resulting in the classic hyperthyroid symptoms of GD. The past few years have borne witness to major advances in our understanding of the susceptibility to GD at the molecular genetic level. The major genes that are associated with GD include the major histocompatibility complex

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Abbreviations: CDC, Centers for Disease Control and Prevention; GD, Graves' disease; GO, Graves' ophthalmopathy; Jak, Janus kinase; MHC, major histocompatibility complex; OR, odds ratio; RA, rheumatoid arthritis; SNP, single nucleotide polymorphism; STAT, signal transducers and activators of transcription; Th, T helper.

(MHC) class II, human leukocyte antigen-DR genes (3), and more recently, the MHC class I, human leukocyte antigen-C genes (4), as well as five, additional, non-MHC genes (1).

Recently, the IL-23 receptor (IL-23R) gene has been a major susceptibility gene for several autoimmune diseases, including Crohn's disease (5), rheumatoid arthritis (RA) (6), and psoriasis (7). These data point to the *IL-23R* gene as a general autoimmunity gene in addition to CTLA-4 and PTPN22 (1). Therefore, we tested the *IL-23R* gene for association with GD and specifically with Graves' ophthalmopathy (GO) because cell-mediated immunity plays a central role in the pathogenesis of GO (8), similar to Crohn's disease and RA. Our analysis showed a significant association of the *IL-23R* gene variants with GD and an even stronger association with GO.

## Subjects and Methods

### Subjects

The project was approved by the institutional review board. A total of 216 North American Caucasian GD patients (176 females and 40 males) were studied. GD was diagnosed by: 1) documented clinical and biochemical primary hyperthyroidism requiring treatment, 2) a diffuse goiter, and 3) the presence of TSH receptor antibodies and/or a diffusely increased I-131 uptake in the thyroid gland. TSH receptor antibodies were measured by the Kronus RIA kit (Kronus, Boise, ID). Of the 216 GD patients, 104 (48.1%) had GO. The average age of onset of GD was 40.8 yr (range 3–78). Our controls consisted of 368 North American Caucasian individuals.

### Genotyping *IL-23R* single nucleotide polymorphisms (SNPs)

DNA was extracted from whole blood using the Puregene kit (Gentra Systems, Minneapolis, MN). Four SNPs spanning the *IL-23R* gene were used in our association studies, rs11209026 (R381Q), rs7530511 (P310L), rs2201841 (located in intron 7), and rs10889677 (located in the 3′ untranslated region 309 bases downstream the stop codon). SNPs rs11209026 and rs7530511 were genotyped using the TaqMan allelic discrimination assays (Applied Biosystems, Foster City, CA). The rs2201841 and rs10889677 SNPs were genotyped using a fluorescent-based restriction fragment length polymorphism method modified from Faragó *et al.* (6). For the restriction fragment length polymorphism analysis, we used the following primer pairs: forward primer *AGGGGATTGCTGGGCCATAT*; reverse primer *TGTGCCTGTATGTGTGACCA* for rs10889677 and forward primers *GGCCTATGATTATGCTTTTTCCTG* and reverse primer *GAACATAACCCTATTGACACCCTG* for rs2201841; and the following restriction enzymes: MnlI for rs10889677 and HpyF3I for rs2201841 (more information can be found in supplemental information A, which is published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

### Statistical analysis

Both allelic and genotypical frequencies were calculated and compared between patients and controls using the $\chi^2$ test and Fisher's exact test where appropriate. We used the Epi Info 3.4.2 software [Centers for Disease Control and Prevention (CDC), Atlanta, GA] for the statistical analyses. A $P$ value less than 0.05 was considered statistically significant.

### Power calculations

Power calculations were performed using the CDC simulation software (Epi Info, 3.3.2). We assumed the population frequency of the susceptibility alleles to be 64, 11, and 7% based on the population frequencies we observed in our controls for the four SNPs (Table 1). Our data set of 216 patients and 368 controls gave our study 80% power to

**TABLE 1.** Frequencies of alleles and genotypes of four *IL-23R* SNPs in GD patients and controls

| SNP | Allele/ genotype | GD (n = 216) | Controls (n = 368) | *P* value | OR (95% confidence interval) |
|---|---|---|---|---|---|
| rs2201841 | A | 315 (72.9%) | 476 (64.7%) | $3.6 \times 10^{-3}$ | 1.5 (1.12–1.92) |
| | G | 117 (27.1%) | 260 (35.3%) | | |
| | AA | 112 (51.9%) | 151 (41.0%) | 0.01[a] | 1.6 (1.09–2.20) |
| | GA | 91 (42.1%) | 174 (47.3%) | | |
| | GG | 13 (6.0%) | 43 (11.7%) | | |
| rs10889677 | C | 302 (72.9%) | 472 (64.5%) | $3.3 \times 10^{-3}$ | 1.5 (1.13–1.95) |
| | A | 112 (27.1%) | 260 (35.5%) | | |
| | CC | 108 (52.2%) | 150 (41.0%) | $9.7 \times 10^{-3}$[b] | 1.6 (1.1–2.25) |
| | CA | 86 (41.5%) | 172 (47.0%) | | |
| | AA | 13 (6.3%) | 44 (12.0%) | | |
| rs7530511 | C (Leu) | 341 (85.2%) | 653 (88.7%) | 0.09 | |
| | T (Pro) | 59 (14.8%) | 83 (11.3%) | | |
| | CC | 146 (73.0%) | 286 (77.7%) | | |
| | TC | 49 (24.5%) | 81 (22.0%) | | |
| | TT | 5 (2.5%) | 1 (0.3%) | 0.02[c] | 9.4 (1.07–214.4) |
| rs11209026 | G (Arg) | 375 (93.8%) | 687 (93.3%) | NS | |
| | A (Gln) | 25 (6.2%) | 49 (6.7%) | | |
| | GG | 177 (88.5%) | 320 (87.0%) | | |
| | AG | 21 (10.5%) | 47 (12.8%) | | |
| | AA | 2 (1.0%) | 1 (0.3%) | NS | |

Please note that when analyzing for alleles, the total number of alleles in each group equals the number of chromosomes (*i.e.* double the number of individuals). NS, Not significant.

[a] For the comparison of AA *vs.* GA + GG.

[b] For the comparison of CC *vs.* CA + AA.

[c] For the comparison of TT *vs.* CC + TC.

detect a difference between the patients and controls resulting in odds ratios (ORs) of more than 1.97, 2.3, and 2.6 (for 64, 11, and 7% allele frequencies, respectively), with an $\alpha$ of $1 \times 10^{-2}$.

## Results

### Association analysis of four *IL-23R* SNPs in GD

Table 1 shows the allele and genotype frequencies of the four *IL-23R* SNPs in GD patients and controls. SNPs rs2201841 and rs10889677 showed significant association with GD. The A allele of rs2201841 was present in 72.9% of patients and 64.7% of controls ($P = 3.6 \times 10^{-3}$; OR = 1.5), and the AA genotype was also significantly increased in patients *vs.* controls (51.9 and 41%, respectively; $P = 0.01$; OR = 1.6). The C allele of rs10889677 was present in 72.9% of patients and 64.5% of controls ($P = 3.3 \times 10^{-3}$; OR = 1.5), and the CC genotype was also significantly increased in patients *vs.* controls (52.2 and 41%, respectively; $P = 9.7 \times 10^{-3}$; OR = 1.6). In addition, the rare genotype TT of the rs7530511 SNP showed significant association with GD. The TT genotype was present in 2.5% of GD patients and 0.3% of controls ($P = 0.02$; OR = 9.4). The rs11209026 SNP, previously shown to be protective for Crohn's disease (*5*), was not found to be associated with GD. However, in the study that demonstrated the protective effect of rs11209026 in Crohn's disease, the rs2201841 and rs10889677 SNPs also showed significant associations with Crohn's disease (*5*).

### Subset analysis

Because cell-mediated immunity plays a central role in the pathogenesis of GO, similar to Crohn's disease and RA (*8*), we tested the four *IL-23R* SNPs for association with the subset of our GD patients that had ophthalmopathy. This analysis has shown that SNPs rs2201841 and rs10889677 are associated only with GD patients that have GO (Table 2). The frequency of the A allele of SNP rs2201841 was significantly higher in GO patients than controls ($P = 1.1 \times 10^{-4}$; OR = 2.04). Moreover, the frequency of the A allele was significantly higher in the GO patients than GD patients without GO ($P = 8.0 \times 10^{-3}$; OR = 1.8; Table 2). Similarly, the frequency of the C allele of SNP rs10889677 was significantly higher in GO patients than controls ($P = 1.3 \times 10^{-4}$; OR = 2.03) and GO patients when compared with GD patients without GO ($P = 5.6 \times 10^{-3}$; OR = 1.8). These data demonstrated that the association of these two SNPs is with GO. In contrast, the TT genotype of SNP rs7530511 was associated with GD both with and without GO, albeit the numbers are very small (Table 2).

### Confirmation study

To confirm our results, we tested the most strongly associated SNP, rs10889677, for association with GD and GO in an independent data set of 204 Caucasian GD patients from Eastern Europe and 223 matched controls. The C allele was present in 75.2% of patients and 67% of controls ($P = 8.0 \times 10^{-3}$; OR = 1.5). In the subset patients with GO, the C allele was present in 74.5% of patients compared with 67% of controls ($P = 0.055$; OR = 1.44), and the CC + CA genotypes were present in 97 of 102 (95.1%) of GO patients compared with 192 of 223 (86.1%) of controls ($P = 0.016$; OR = 3.1). Thus, the associations of rs10889677 with GD and GO were replicated in an independent data set.

**TABLE 2.** Frequencies of alleles and genotypes of four *IL-23R* SNPs in Graves' patients with and without ophthalmopathy

| SNP | Allele/ genotype | GO (n = 103) | GD no GO (n = 111) | Controls (n = 368) | *P* value (OR) (GO *vs.* Cont) | *P* value (OR) (GO *vs.* GD no GO) | *P* value (OR) (GD no GO *vs.* Cont) |
|---|---|---|---|---|---|---|---|
| rs2201841 | A | 164 (78.8%) | 151 (67.4%) | 476 (64.7%) | $1.1 \times 10^{-4}$ (2.04)[a] | $8.0 \times 10^{-3}$ (1.8)[a] | 0.45 |
| | G | 44 (21.2%) | 73 (32.6%) | 260 (35.3%) | | | |
| | AA | 65 (62.5%) | 47 (42.0%) | 151 (41.0%) | $1.0 \times 10^{-4}$ (2.4)[a] | $7.5 \times 10^{-3}$ (2.3)[a] | 0.86 |
| | GA | 34 (32.7%) | 57 (50.9%) | 174 (47.3%) | | | |
| | GG | 5 (4.8%) | 8 (7.1%) | 43 (11.7%) | | | |
| rs10889677 | C | 162 (78.6%) | 148 (66.7%) | 472 (64.5%) | $1.3 \times 10^{-4}$ (2.03)[a] | $5.6 \times 10^{-3}$ (1.8)[a] | 0.55 |
| | A | 44 (21.4%) | 74 (33.3%) | 260 (35.5%) | | | |
| | CC | 64 (62.1%) | 46 (41.4%) | 150 (41.0%) | $1.4 \times 10^{-4}$ (2.36)[a] | $2.5 \times 10^{-3}$ (2.3)[a] | 0.93 |
| | CA | 34 (33.0%) | 56 (50.5%) | 172 (47.0%) | | | |
| | AA | 5 (4.9%) | 9 (8.1%) | 44 (12.0%) | | | |
| rs7530511 | C (Leu) | 168 (83.2%) | 173 (87.4%) | 653 (88.7%) | | | |
| | T (Pro) | 34 (16.8%) | 25 (12.6%) | 83 (11.3%) | 0.03 (1.59)[a] | 0.24 | 0.60 |
| | CC | 70 (69.3%) | 76 (76.8%) | 286 (77.7%) | | | |
| | TC | 28 (27.7%) | 21 (21.2%) | 81 (22.0%) | | | |
| | TT | 3 (3.0%) | 2 (2.0%) | 1 (0.3%) | $9.0 \times 10^{-3}$ (11.23)[a] | 0.67 | 0.053[b] |
| rs11209026 | G (Arg) | 191 (94.6%) | 184 (92.9%) | 687 (93.3%) | 0.53 | 0.50 | 0.84 |
| | A (Gln) | 11 (5.4%) | 14 (7.1%) | 49 (6.7%) | | | |
| | GG | 90 (89.1%) | 87 (87.9%) | 320 (87.0%) | | | |
| | AG | 11 (10.9%) | 10 (10.1%) | 47 (12.8%) | | | |
| | AA | 0 (0.0%) | 2 (2.0%) | 1 (0.3%) | 0.60 | 0.15 | 0.053 |

Please note than when analyzing for alleles, the total number of alleles in each group equals the number of chromosomes (*i.e.* double the number of individuals). Cont, Control.

[a] Significant associations with IL-23R SNPs.

[b] Borderline association.

## Discussion

IL-23, a recently discovered heterodimeric cytokine, enjoys a similar functionality to IL-12 in promoting cellular immunity, enhancing lymphocyte proliferation, inducing interferon-γ production, and promoting T-helper (Th) 1 cell differentiation by dendritic cells. However, unlike IL-12, IL-23 acts primarily on CD4+ T-cells that have already been exposed to antigen, to sustain long-term cellular immunity by promoting survival and effector cytokine production of Th1 memory cells (9). The receptor for IL-23 is expressed in a distinct subpopulation of Th1-derived T cells, which produce IL-17, thus earning them designation Th17 cells. IL-17 is a potent proinflammatory cytokine, acting on stromal endothelial cells and monocytes, inducing other proinflammatory mediators such as IL-8, chemokines, TNF, and granulocyte monocyte colony-stimulating factor. These cytokines result in a rapid recruitment of neutrophils, which normally serve to control an acute infection. However, dysregulation in the production of IL-17 in tissues can lead to chronic inflammatory responses and tissue damage. Therefore, it is not surprising that IL-17 has been found in affected tissues in several human autoimmune conditions, including multiple sclerosis, RA, and psoriasis (reviewed in Ref. 10).

IL-23R is a type I transmembrane protein, sharing homology with other cytokine receptors. The extracellular region contains a signal sequence, an N-terminal immunoglobulin-like domain, and two cytokine receptor domains, containing a WQPWS motif that is similar to the signature WSXWS motif of other cytokine receptors. Although the 252 amino acid intracellular region of IL-23R has no inherent catalytic activity, there are, however, seven tyrosine residues that can be phosphorylated; three are potential Src homology 2 domain-binding sites, and two are signal transducers and activators of transcription (STAT) binding sites (9, 11). Binding of IL-23 to its receptor leads to the activation of Janus kinases (Jak2 and Tyk2), which phosphorylate IL-23R at discrete locations and, thus, form docking sites for the STATs. Subsequently, the Jaks phosphorylate the STATs, allowing them to dimerize and translocate to the nucleus where they activate the transcription of key proinflammatory genes such as IL-17 and interferon-γ (9).

Although IL-23 signals through several molecules, one that is of particular interest is STAT4. STAT4 has been a key cytokine driving autoimmune responses, as demonstrated in STAT4 deficient mice that showed resistance to disease in several models of autoimmunity (12–16). Thus, it is possible that up-regulation of IL-23R, by certain SNP alleles in the gene, could increase STAT4 signaling, thereby conferring risk for autoimmunity (10, 17). Indeed, recently several SNPs in the *IL-23R* gene have been shown to be associated with autoimmune and inflammatory conditions, including Crohn's disease (5), RA (6), and psoriasis (7). One group from Spain did not find an association of the *IL-23R* gene with RA, perhaps due to population differences (18).

SNP rs11209026 is specific for Crohn's disease, whereas rs10889677 and rs2201841 have been shown to confer risk for both Crohn's disease and RA. Intriguingly, these latter two SNPs are the same SNPs we found to be associated with GO. However, rs7530511 was associated with GD, but not specifically with GO. In contrast, the rs11209026 SNP did not show an association with GD or GO in our cohort (Tables 1 and 2). However, this SNP has not been proven to be the causative SNP in Crohn's disease. Thus, different variants in the *IL-23R* gene may predispose to different autoimmune conditions.

While the biological impact of these SNPs on the expression and functionality of IL-23R is currently unknown, it is apparent that the SNPs represent an important genetic link in the complexity of GD and GO. One may postulate several mechanisms by which SNPs in the *IL-23R* gene can change the function of the receptor itself, leading to GO:

1. SNPs in *IL-23R* (possibly rs10889677, which is located in the 3′ untranslated region) can cause overexpression of the receptor (*e.g.* by increasing mRNA stability), driving differentiation of Th1 helper T cells toward a Th17 subpopulation, resulting in an increased release of IL-17 from these cells. This would subsequently lead to the release of other cytokines (*e.g.* TNF), causing chronic inflammation of the involved organ, such as the eyes in GO.
2. SNPs in the *IL-23R* gene (possibly rs7530511, which is located very close to one of the WQPWS motifs) can cause a change in the binding affinity of IL-23 to its receptor, altering IL-23R activation.

In summary, our study demonstrated a significant association of variants in the *IL-23R* gene with GO. This is the first time that a unique susceptibility gene has been identified for GO. This finding supports the proposed model for GO in which Th1 responses predominate early in the disease (8) since IL-23R is involved in driving the CD4+ T cells of a Th1 subtype into IL-17 secreting Th17 cells. In view of the cardinal importance of IL-23 in the differentiation of Th17 cells, the IL23/Th17 axis may play an important and, as of yet, under-investigated role in GO.

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