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Cutting Edge: A Variant of the IL-23R Gene Associated with Inflammatory Bowel Disease Induces Loss of MicroRNA Regulation and Enhanced Protein Production

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IL-23R gene variants have been identified as risk factors for two major inflammatory bowel diseases (IBDs), Crohn’s disease and ulcerative colitis, but how they contribute to disease is poorly understood. In this study, we show that the rs10889677 variant in the 3'-untranslated region of the IL-23R gene displays enhanced levels of both mRNA and protein production of IL-23R. This can be attributed to a loss of binding capacity for the microRNAs (miRNAs) Let-7e and Let-7f by the variant allele. Indeed, inhibition and overexpression of these miRNAs influenced the expression of the wild type but not the variant allele. Our data clearly demonstrate a role for miRNA-mediated dysregulation of IL-23R signaling, correlated with a single nucleotide polymorphism in the IL-23R strongly associated with IBD susceptibility. This implies that this mutation, in combination with other genetic risk factors, can lead to disease through sustained IL-23R signaling, contributing to the chronicity of IBD. The Journal of Immunology, 2012, 188: 1573–1577.

Crohn’s disease (CD) and ulcerative colitis (UC) are severe inflammatory disorders of the gastrointestinal tract that are thought to result from dysregulated mucosal immune responses against ubiquitous commensal enteric bacteria in genetically susceptible individuals. From genome-wide association studies, IL-23R variants have been identified as risk factors for both CD and UC (1, 2).

IL-23R encodes one of the two chains comprising the functional IL-23R. The other chain, IL-12Rβ1, is also part of the functional IL-12R. The IL-23R is expressed on αβ and γδ T cells and on innate leukocytes (3).

The activity of IL-23, a heterodimeric cytokine comprising IL-12p40 and IL-23p19 (4), has been primarily linked to the Th17 cell subset (5, 6). Th17 cells are enriched for expression of IL-23R (7), and IL-23 plays an important role in the sustenance of Th17 cell responses in vivo (5). The activity of IL-23 is especially prominent in mucosal tissues, such as the intestine (8), highlighting IL-23R as a key player in the pathogenesis of inflammatory bowel disease (IBD) and the IL-23/Th17 pathway as an important regulator of intestinal homeostasis.

Neutralization of IL-23 has been shown to ameliorate and cure colitis in a number of mouse models of IBD (9, 10), and genetic ablation of Il23a clearly showed that IL-23 rather than IL-12 drives the Th1–IFN-γ inflammatory axis in the intestine (10). Furthermore, it has been demonstrated that IL-23 drives intestinal inflammation, in part, through inhibiting the development of induced Foxp3+ regulatory T cells in the intestine (11), but precisely how IL-23 controls this process is poorly understood. It has been suggested that IL-23 acts through direct effects on T cells, favoring the emergence of an IL-17A+IFN-γ+ population of T cells whereas inhibiting Foxp3 expression. However, a role for innate cells cannot be ruled out (12).

The association of single nucleotide polymorphisms (SNPs) in human IL-23R with two of the most prominent forms of IBD can be explained by effects on the function or expression of the receptor leading to dysregulation of intestinal inflammation. One of the SNPs (rs10889677) leads to a variant in the 3'-untranslated region (3' UTR) of the IL-23R mRNA.

Because the 3'UTR contains regulatory sequences that are sensitive to regulatory proteins and microRNAs (miRNAs), a variant in this region could affect the stability and translation of the mRNA, with direct effects on the amount of receptor protein expressed by a cell.

miRNAs are important regulators of gene expression, whereby the main function of miRNAs in mammalian systems is to decrease target mRNA levels (13). Each highly conserved mammalian miRNA probably targets several hundred distinct mRNAs (14), so it is probable that most miRNAs are controlled by miRNAs to some extent. miRNAs could therefore be as important as transcription factors in controlling the protein content of a cell. The expression of miRNAs is highly regulated, and they are therefore well placed to function as immunomodulators. Recently, evidence for a role for the Let-7f miRNA in the regulation of the IL-23R was found in human CD4 memory T cells (15).

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Abbreviations used in this article: CD, Crohn’s disease; IBD, inflammatory bowel disease; miRNA, microRNA; SNP, single nucleotide polymorphism; UC, ulcerative colitis; 3'UTR, 3'-untranslated region.
In this study, we analyzed the sequence differences between the 3' UTR of the rs10889677 variant and the wild type of the IL-23R gene, and showed a loss of binding capacity for the miRNAs Let-7e and Let-7f. This is associated with enhanced levels of mRNA and protein production of IL-23R by the variant allele, suggesting that this mutation can lead to altered and sustained signaling via the IL-23R, contributing to the chronicity of inflammation as seen in IBD and UC.

Materials and Methods

Study objects

Blood and mouth swabs were collected from healthy volunteers who had given informed written consent, according to the Ethical Guidelines of the Departments of Molecular Cell Biology and Gastroenterology of the VU University Medical Center.

Genotyping of the SNP rs10889677 polymorphism

Genotyping for the MnlI polymorphism in the 3' UTR of the IL-23R was done by RFLP using primers that cover the MnlI restriction site.

Preparation of allotypic IL-23R 3' UTR constructs and reporter plasmids

The complete IL-23R 3' UTR between the TAG stop codon and the poly(A) tail corresponding to the nucleotides 1976 and 2812 (RefSeq NM_144701.2) of a CC homozygous individual was cloned in the TOPO vector (Invitrogen) and served as template for a recombinant PCR, in which the C nucleotide at the polymorphic site is specifically replaced by the A nucleotide. This way the constructs would differ only at the SNP site. The 3' UTRs were cloned in the HindIII and SpeI sites downstream of the luciferase reporter gene in the pMIR-Report vector (Ambion) and used as reporter plasmids. Luciferase determination was performed as described elsewhere (16).

Transfections

For transfection, HEK293T cells, K562 cells, or purified CD4 T cells were used as indicated. Cells were transfected using Lipofectamine2000 (Invitrogen). For specific inhibition of miRNA, 50 nM miRNA inhibitor (AM12304 hsa-let-7e or AM10902 hsa-let-7f; Ambion) or control (IP-004500-01-05; Applied Biosystems) was used. In the case of K562 cells, 50 nM miRNA mimics (PM12304 hsa-let-7e or PM10902 hsa-let-7f; Ambion) was used.

Electrophoresis and Western blotting

Cell extracts were denatured in Laemmli sampling buffer containing 1 M DTT (5 min at 100˚C) and separated by SDS-PAGE (12.5% polyacrylamide). Electrophoresis and Western blotting were performed as described elsewhere (16).

RT-PCR for quantification allele-specific IL-23R mRNA and Let-7e miRNA

Total RNA was extracted from the cells with TRIZol (Invitrogen). For Let-7e miRNA expression, the TaqMan MRNA Reverse Transcription Kit (Applied Biosystems) was used with the Let-7e–specific TaqMan MiRNA assay (assay ID: 002400; Applied Biosystems). Real-time PCR was performed on an ABI Prism 7900HT sequence detection system (PE Applied Biosystems), with GAPDH as endogenous reference.

For allele-specific miRNA quantification, cDNA was synthesized from total RNA using a Revert Aid First Strand cDNA synthesis kit (Fermentas Life Sciences). Quantification was done with an allele-specific genotyping assay (assay ID: C_11283764_10; Applied Biosystems) in TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems) on an ABI Prism 7900HT with the settings: absolute quantification and thermal profile 2 min at 50˚C, 10 min at 95˚C, followed by 40 cycles of 15 seconds at 92˚C and 1 min at 60˚C. Because the ratio of C to A is, by definition, 1 in heterozygous genomic DNA, each run included a serial dilution of heterozygous genomic DNA. This genomic DNA was taken as reference standard (lowest dilution arbitrarily set at 1:1,000). This prevents any possible bias induced by differences in efficiencies in the primer/probe sets.

Primary cell isolation and culture

LPS (026:B6; Sigma-Aldrich) stimulation was performed in whole blood at 100 pg/ml for 6 h at 37˚C. Erythrocytes were lysed, and cell pellets were taken up in TRIZol for RNA isolation. CD4+ T cells were isolated by Lymphoprep (Axis-Shield, Oslo, Norway) density gradient centrifugation and magnetic bead extraction with the CD4+ T Cell Isolation kit II (Miltenyi Biotec). Cells were activated with anti-CD3/CD28–coated beads (Dynabeads, Invitrogen) and cultured as indicated. Cells were lysed in Nonidet P-40 buffer (150 mM sodium chloride, 1.0% Nonidet P-40) for Western blot detection or in TRIZol for RNA isolation, respectively.

Statistics

Unless stated otherwise, the Mann–Whitney U test was used for statistical evaluation using GraphPad Prism version 4.00 for Windows (GraphPad, San Diego, CA).

Results and Discussion

The rs10889677 mutation of the IL-23R gene, associated with IBD, results in a polymorphism in the 3' UTR, whereby a C in the wild type allele is replaced by an A (1). Because the 3' UTR contains regulatory sequences that are sensitive to regulatory proteins and miRNA, such a replacement may affect the stability and translation of the mRNA with direct effects on the amount of receptor protein to be expressed by a cell.

To study whether this 3' UTR C-to-A conversion would affect the amount of RNA produced after stimulation, we used LPS-stimulated PBMCs from six individuals who had been typed as heterozygous for the rs10889677 SNP. Applying a SNP-specific typing assay, we could quantitatively compare the amount of IL-23R mRNA that had been tran-
The amount of IL-23R protein produced to tubulin as internal control for each homozygous individual.

As can be seen from Fig. 2, all persons with the rs10889677 3’UTR SNP on both chromosomes produced significantly more IL-23R protein than individuals homozygous for the wild type allele.

From the fact that the rs10889677 variant led to enhanced levels of both mRNA and protein, it seemed logical that this mutation had led to the loss of a regulatory miRNA site on the wild type allele. When the 3’UTR sequences of the wild type and variant were compared using the MicroCosm Database (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) (17), it became clear that the variant had led to the loss of a putative binding site on its 3’UTR for both the Let-7e and Let-7f miRNA (Fig. 3A). Because miRNA expression is spatially and temporally controlled, we first had to test whether miRNAs Let-7e and Let-7a were expressed in the two cell types. When T cells and monocytes isolated from peripheral blood were activated using CD3-CD28 beads or LPS, respectively, the expression of the two members of the Let-7 miRNA family was found in both cell types (Fig. 3B, 3C).

The Let-7e miRNA belongs to the Let-7 family, which is highly conserved across animal species (18) and plays important roles in the regulation of cell proliferation and differentiation. In humans, 13 different let-7 family members exist (19), and although a certain redundancy of their function has been suggested, it is clear that they represent different miRNAs with overlap in their target recognition sequence, but capable of differential interference with their target mRNAs (20).

To study the possible role of Let-7 miRNA in the regulation of IL-23R expression, we cloned the 3’UTR from both wild type and mutated allele, and inserted the clones in luciferase-containing vectors. These were transfected into the HEK293T cell line, expressing the miRNA Let-7e. Adding a specific inhibitor for miRNA Let-7e, we found that the expression of luciferase in the wild type allele, but not in the variant allele, was significantly upregulated (Fig. 4). These data confirmed the in silico analysis and show that the miRNA Let-7e can functionally interfere with the 3’UTR of the wild type IL-23R allele, and that this regulatory role is lost with the rs10889677 mutation. To extend these data to Let-7f and to effects on protein expression of IL-23R, we used two
approaches: K562 cells constitutively express IL-23R, are genetically homozygous for the C allele (A. Zwiers, unpublished observations), but lack both Let-7e and Let-7f miRNA (21). Transfecting these cells with Let-7e or Let-7f miRNA led to reduced expression of the IL-23R protein (Fig. 5). Conversely, we introduced inhibitors of both Let-7e and Let-7f miRNA into CD4 T cells from a donor homozygous for either the CC or AA allele. After stimulation of the cells with CD3-CD28 beads, this led to upregulation of IL-23R protein in the CC donor, but not in the AA donor (Fig. 6). Thus, by either introducing the two miRNAs or by specifically inhibiting their action, we could demonstrate that both Let-7e and Let-7f regulate the expression of the IL-23R at the protein level.

The Let-7 family was among the first miRNAs that were discovered (22), but only a few reports show a specific role for Let-7e and Let-7f. Interestingly, in macrophages, the expression of Let-7e is regulated by the protein kinase Akt1, under the influence of LPS, and targets the LPS receptor TLR4 (23). This way, Let-7e is involved in feedback inhibition of LPS signaling, comparable with the regulatory role of Let-7e that we have identified in IL-23R signaling.

The loss of miRNA regulation in the variant form of the IL-23R gene could have a direct effect on the amount of receptor expressed by activated cells, but could also have a profound effect on the duration of the expression because of lack of feedback regulation. This can lead to further stabilization of the Th17 cell population, for which IL-23 is crucial (6, 24), as well as for the downregulation of the differentiation of Foxp3-expressing regulatory T cells (25), leading to the more chronic inflammatory response as seen in CD and UC.

It cannot be completely excluded that differences in expression between the A and C allele in heterozygous, healthy donors may be caused by another SNP in the IL-23R gene that is in complete linkage disequilibrium with this polymorphism. This possibility, however, is very unlikely because as far as we know, no polymorphisms in known regulatory regions exist that are in 100% linkage with this SNP, as there is at least one haplotype block positioned between the haplotype block containing SNP rs10889677 and the haplotype block containing the 5’ transcriptional start site.

To our knowledge, this is the first time a link can be made between a mutation in the IL-23R gene that is strongly associated with IBD susceptibility and a clear effect on the functionality of the gene, in this case, through regulation via miRNA. These findings will be of importance to further understand the role of the IL-23R/Th17 axis in IBD and could help to develop better therapies.

**Disclosures**

The authors have no financial conflicts of interest.
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