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Role of Interleukin-23 (IL-23) Receptor Signaling for IL-17 Responses in Human Lyme Disease

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Interleukin-23 (IL-23) is known to play a crucial role in the development and maintenance of T helper 17 cells. It has been previously demonstrated that IL-17 is involved in experimental Lyme arthritis, caused by Borrelia burgdorferi bacteria. However, the precise role of the IL-23 receptor (IL-23R) for the B. burgdorferi-induced IL-17 responses or human Lyme disease has not yet been elucidated. IL-23R single nucleotide polymorphism (SNP) rs11209026 was genotyped using the TaqMan assay. Functional studies were performed using peripheral blood mononuclear cells, and cytokines were measured using enzyme-linked immunosorbent assay (ELISA). Dose-dependent production of IL-23 and IL-17 by B. burgdorferi could be observed. Interestingly, when IL-23 bioactivity was inhibited by a specific antibody against IL-23p19, IL-17 production was significantly downregulated. In contrast, production of gamma interferon (IFN-γ) was not affected after the blockade of IL-23 activity. Moreover, individuals bearing a single nucleotide polymorphism in the IL-23R gene (Arg381Gln) produced significantly less IL-17 after B. burgdorferi stimulation compared with that of the individuals bearing the wild type. Despite lower IL-17 production, the IL-23R gene polymorphism did not influence the development of chronic Lyme disease in a cohort of patients with Lyme disease. This study demonstrates that IL-23R signaling is needed for B. burgdorferi-induced IL-17 production in vitro and that an IL-23R gene SNP leads to impaired IL-17 production. However, the IL-23R gene polymorphism is not crucial for the pathogenesis of chronic Lyme.

Lyme disease begins in most individuals with a localized skin infection (erythema migrans [EM]) caused by the pathogenic Borrelia burgdorferi spirochetes after transmission by an infected tick. When dissemination of B. burgdorferi occurs, the second stage of Lyme disease is established, which eventually leads to persistent Lyme disease. Several chronic inflammatory processes can be distinguished in Lyme patients, including inflammation of the central nervous system (neuroborreliosis), inflamed skin (acrodematitis chronica atrophicans [ACA]), or joint inflammation (Lyme arthritis) (30). The precise immunological mechanisms leading to the development of persistent Lyme disease are still unclear. While detection of live B. burgdorferi microorganisms in patients is difficult, chronic Lyme disease displays clinical similarities with autoimmune disorders such as rheumatoid arthritis (RA) and multiple sclerosis (MS), in which T cells are known to play important roles. Pathogenic Th17 cells (CD4+ T cells) play a prominent role in the pathogenesis of these diseases (8, 21, 23).

Of interest, proinflammatory cytokine interleukin-1β (IL-1β) is essential for the development of Th17 responses, and B. burgdorferi is a potent inducer of IL-1β (25). Recently, it was also demonstrated that caspase-1 is crucial for B. burgdorferi-induced IL-17 responses (26). In patients diagnosed with chronic Lyme disease, both mononuclear and T cells can be detected in inflamed tissues, which are able to produce IL-1β and IL-17 (29, 31). It was previously described that IL-17 is important for the development of experimental Lyme arthritis (7). Neutralization of endogenous IL-17 in gamma interferon (IFN-γ) knockout mice with Lyme arthritis resulted in a diminished cell influx, a reduction of swelling in the inflamed joints, and less production of proinflammatory cytokines. Since patients diagnosed with chronic Lyme disease often suffer from arthritis-like symptoms, Th17 cells and/or IL-17 might be involved. Not only could IL-17 be related to the development of Lyme arthritis, but other cytokines, such as IL-6, IL-1β, and IL-23, have also been linked to the inflammatory reaction caused by B. burgdorferi (10, 24, 32).

IL-23 is a heterodimeric member of the IL-12 family which shares the p40 subunit but contains a specific p19 subunit which can be recognized by the IL-23 receptor (27). Whereas IL-6 and IL-1β are necessary for induction of Th17 cells, IL-23 is responsible for the maintenance of this T helper cell population and production of IL-17 (1, 4, 18). In vitro studies revealed that only IL-1β and IL-23 are essential to generate Th17 cells (6). It has been demonstrated that IL-23 plays an important role in the induction of IL-17 after cells were stimulated in vitro with B. burgdorferi (19). It is also known that transgenic mice that overexpress IL-23 are spontaneously developing autoimmune disorders. Moreover, it was shown that IL-23 was involved in the development of murine Lyme arthri-
Bacterial cultures. The B. burgdorferi ATCC 35210 strain (B31) was cultured at 33°C in Barbour-Stoenner-Kelley (BSK)-H medium (Sigma-Aldrich) supplemented with 6% rabbit serum. Spirochetes were grown to late logarithmic phase and examined for motility by dark-field microscopy. Organisms were quantified by fluorescence microscopy after mixing 10^{-10} aliquots of culture material with 10 μl of an acridine orange solution. Bacteria were harvested by centrifugation of the culture at 7,000 × g for 15 min and washed twice with sterile phosphate-buffered saline (PBS; pH 7.4). Stock solutions were divided and stored at −20°C.

Isolation of human peripheral blood mononuclear cells and in vitro cytokine production. After obtaining informed consent, venous blood was drawn from the cubital vein of healthy volunteers and patients into EDTA tubes of 10 ml (Monovette). Peripheral blood mononuclear cells (PBMCs) were isolated according to standard protocols, with minor modifications. The PBMC fraction was obtained by density centrifugation of blood diluted 1:1 in PBS over Ficoll-Paque (Pharmacia Biotech). Cells were washed three times in PBS and resuspended in RPMI 1640 (Dutch modified) supplemented with 50 mg/liter gentamicin, 2 mM l-glutamine, and 1 mM pyruvate. Cells were counted in a Coulter Counter Z (Beckman Coulter) and adjusted to 5 × 10^{6} cells/ml. Mononuclear cells (5 × 10^{6}) in a 100-μl volume were added to round-bottom 96-well plates (Greiner) and incubated with either 100 μl of medium (negative control) or B. burgdorferi at a dose range between 1 × 10^{5} and 1 × 10^{7} spirochetes/ml. In some experiments, PBMCs and added stimuli were coincubated with antibody (anti-hIL-23p19 antibody; 5 μg/ml; R&D Systems) or control antibody (normal goat IgG; 5 μg/ml; R&D Systems). After 24 h or 7 days (in the presence of 10% human pool serum), supernatants were collected and stored at −20°C.

Study populations. (i) Healthy volunteers. Individuals used in this study were foresters from Geldersch Landschap and Kruonmoden in The Netherlands. In this cohort (n = 143 individuals), Lyme disease occurred as an occupational disease, and these persons were selected to participate due to their relatively high risk of infection with B. burgdorferi species. The individuals were between 23 and 73 years old, and the cohort consisted of 77% males and 23% females. Venous blood of these individuals was drawn after informed consent was obtained.

(ii) Lyme disease patients. From September 2008 until August 2010, individuals suspected of persistent Lyme disease were recruited from the outpatient clinic of the Department of Medicine, Radboud University Nijmegen Medical Centre. Patients were divided into three groups, based on diagnostic clinical criteria. “Proven” Lyme disease was diagnosed when patients displayed verified erythema migrans or ACA (B. burgdorferi PCR positive), arthritis (B. burgdorferi PCR or culture positive in synovial fluid), or meningitis (radiculitis, pleocytosis in liquor, or facialis paresis). At the time of inclusion, proven Lyme disease patients also displayed clinical signs, such as persistent tiredness, musculoskeletal complaints, paresthesia, memory and concentration disorders, or neuralgia. Probable Lyme disease was diagnosed for patients who were clinically comparable with the proven Lyme disease patients but did not display active disease during inclusion. Patients with Lyme disease history were known to suffer (>1 year ago) from clinical signs, such as EM, ACA, or arthritis, and had a positive Western blot or enzyme-linked immunosorbent assay (ELISA), but any of the described symptoms of disease were absent at the time of inclusion. “No Lyme” was diagnosed when EM, ACA, arthritis, or meningitis were absent and when B. burgdorferi Western blotting and ELISA were negative. These individuals often displayed persistent tiredness, musculoskeletal complaints, paresthesia, or memory and concentration problems.

All patients were tested for B. burgdorferi infection by IgG and IgM ELISAs and Western blotting. Genotyping was performed on DNA isolated from 228 patients between 16 and 80 years old. The study population was 46% male and 54% female. After informed consent was obtained, blood was drawn as described before. All experiments were conducted according to the principles expressed in the Declaration of Helsinki.

Isolation of genomic DNA and single nucleotide polymorphism analysis. DNA was isolated using the Gentra Puregene blood kit (Qiagen), according to the manufacturer’s protocol for whole blood, and dissolved in a final volume of 100 μl. PCR amplification of IL-23R gene fragments bearing the polymorphism Arg381Gln was performed using a predesigned TaqMan SNP genotyping assay (Applied Biosystems) in 25-μl reaction mixtures containing 2 μl of genomic DNA as well as primers, two specific probes (with either the VIC or FAM [6-carboxyfluorescein] label), and universal PCR 2× Master mix (Applied Biosystems). Cycling conditions were 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 95°C for 15 s and 1 min at 60°C. Fluorescence intensities were corrected using a postread/pread method for 1 min at 60°C before and after the amplification. The software automatically plotted genotypes based on a two-parameter plot with an overall success rate of >95%. Intermediate samples were excluded from the analysis.

Cytokine measurements. Concentrations of human IL-1β, IL-6, IL-17, or IFN-γ were determined using either specific or commercial ELISA kits (PeliKine Compact; Sanquin Amsterdam; or R&D Systems, Minneapolis, MN), in accordance with the manufacturers’ instructions. Detection limits were 40 pg/ml, except for IFN-γ ELISA (12 pg/ml). IL-23 was measured using Ready-SET-Go! ELISA from eBioscience, with a sensitivity of 15 pg/ml.

Statistical analysis. Data are expressed as means ± standard errors of the means (SEM) unless mentioned otherwise. Differences between experimental groups were tested using the Mann-Whitney U test performed on GraphPad Prism 4.0 software (GraphPad). P values of ≤0.05 were considered significant and are marked with an asterisk.

RESULTS

Exposure of PBMCs to Borrelia burgdorferi induces IL-17 production. To determine the capacity of live B. burgdorferi to induce proinflammatory cytokine production by freshly isolated peripheral blood mononuclear cells (PBMCs), cells were stimulated with spirochetes in a dose range of 1 × 10^{2} to 1 × 10^{7} spirochetes/ml. After 7 days of incubation, a dose-dependent production of IL-17 was detected (Fig. 1A). Since it is known that for the development of Th17 cells and IL-17 production IL-1β, IL-6, and IL-23 are needed, we analyzed the production of these cytokines after B. burgdorferi exposure. IL-1β (Fig. 1B) as well as IL-23 (Fig. 1C) production could be observed in a dose-dependent manner. In addition, B. burgdorferi is a potent inducer of IL-6 production (Fig. 1B). To confirm that both IL-1β and IL-23 are important for IL-17 production by PBMCs, we stimulated PBMCs for 7 days with IL-1β and IL-23. Figure 1D shows that IL-1β and IL-23 induce IL-17 production. In addition to IL-17, IL-22 was also detected in large amounts (Fig. 1D). When the medium was used as a
background control, neither IL-17 nor IL-22 could be measured in the supernatants (data not shown).

*B. burgdorferi*-induced IL-17 production is dependent on IL-23. To investigate whether IL-17 production by *B. burgdorferi* (Fig. 1A) is indeed dependent on IL-23, we neutralized endogenous IL-23 using an antibody against IL-23p19. While exposure of PBMCs to $1 \times 10^5$ live *B. burgdorferi* spirochetes/ml induced high IL-17 production in the presence of an isotype control antibody, almost no IL-17 production could be detected when IL-23 was neutralized by the anti-IL-23p19 antibody (Fig. 2A). To examine whether a blockade of IL-23 influences the production of cytokines other than IL-17, we analyzed IFN-γ, IL-1β, and IL-6. Both IL-23 and IL-12 belong to the IL-12-family of cytokines and share a p40 subunit. Since it is known that IL-12 is involved mainly in IFN-γ induction by *B. burgdorferi*, we examined whether the anti-IL-23 antibody modulates *B. burgdorferi*-induced IFN-γ production (15). No differences in IFN-γ levels could be observed after *B. burgdorferi* exposure to PBMCs for 7 days in combination with the anti-IL-23p19 antibody (Fig. 2B). In addition, no differences in IL-1β and IL-6 production could be found when the anti-IL-23p19 antibody was added together with live *B. burgdorferi* to PBMCs (Fig. 2C and D).

PBMCs isolated from individuals bearing the IL-23R Arg381Gln polymorphism produce less IL-17 after exposure to *B. burgdorferi*. To further dissect the role of IL-23 in the production of IL-17, PBMCs isolated from individuals carrying different alleles of the Arg381Gln SNP of the IL-23R subunit of the IL-23R gene were stimulated for 24 h or 7 days with *B. burgdorferi* (Fig. 3). Healthy individuals heterozygous for the IL-23R Arg381Gln polymorphism displayed a significantly lower production of IL-17 than individuals homozygous for the wild-type allele (Fig. 3A). Interestingly, no differences could be observed in IL-1β or IL-6 production after 24 h of PBMC stimulation between individuals bearing wild-type or heterozygous alleles (Fig. 3B and C, respectively). As expected, no
differences in IL-23 production after *B. burgdorferi* stimulation by PBMCs from both wild-type and heterozygous individuals could be detected (Fig. 3D).

**IL-23R Arg381Gln polymorphism does not influence clinical signs of chronic Lyme disease.** IL-17 is linked to the pathogenesis of chronic inflammatory diseases, and a fundamental role for IL-17 in *B. burgdorferi*-induced Th17 or IL-17 production was not yet described. We identify that IL-23 is crucial for the IL-17 production by PBMCs after exposure to *B. burgdorferi* spirochetes. Blockade of IL-23R using neutralizing IL-23p19 antibodies resulted in a highly significant suppression of IL-17 production after *B. burgdorferi* stimulation. Of interest, we demonstrated that individuals carrying a nonsynonymous SNP of IL-23R (Arg381Gln) showed a significantly reduced IL-17 production after cell stimulation with *B. burgdorferi*. Despite the downmodulation of the IL-17 response by the IL-23R Arg381Gln polymorphism, there was no difference in clinical or serological markers of acute Lyme disease or expression of signs of chronic Lyme disease in individuals bearing the IL-23R Arg381Gln SNP.

**DISCUSSION**

We assessed the role of the IL-23/IL-23R pathway for the induction of IL-17 by *B. burgdorferi* spirochetes in humans. IL-23 plays an important role in the induction and maintenance of Th17 cells, but its role for *B. burgdorferi*-induced Th17 or IL-17 production was not yet described. We identify that IL-23 is crucial for the IL-17 production by PBMCs after exposure to *B. burgdorferi* spirochetes. Blockade of IL-23R using neutralizing IL-23p19 antibodies resulted in a highly significant suppression of IL-17 production after *B. burgdorferi* stimulation. Of interest, we demonstrated that individuals carrying a nonsynonymous SNP of IL-23R (Arg381Gln) showed a significantly reduced IL-17 production after cell stimulation with *B. burgdorferi*. Despite the downmodulation of the IL-17 response by the IL-23R Arg381Gln polymorphism, there was no difference in clinical or serological markers of acute Lyme disease or
the occurrence of chronic symptoms of Lyme between B. burgdorferi-exposed individuals carrying wild-type or heterozygous alleles. These results indicate that carrying the IL-23R SNP is probably not essential for the development of chronic Lyme disease.

Table 1. Clinical characteristics of patients with a suspicion of chronic Lyme disease, divided on the basis of the IL-23R SNP

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. of patients with:</th>
<th>% of patients with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type IL-23R</td>
<td>Heterozygous IL-23</td>
</tr>
<tr>
<td>Total</td>
<td>188</td>
<td>40</td>
</tr>
<tr>
<td>Female/male</td>
<td>100/88 (48)</td>
<td>22/18 (50)</td>
</tr>
<tr>
<td>Age range (mean)</td>
<td>16–80 (48)</td>
<td>17–66 (50)</td>
</tr>
<tr>
<td>Lyme history</td>
<td>26</td>
<td>5</td>
</tr>
<tr>
<td>Possible Lyme disease</td>
<td>134</td>
<td>30</td>
</tr>
<tr>
<td>Proven Lyme disease</td>
<td>28</td>
<td>5</td>
</tr>
<tr>
<td>Clinical signs or complaint</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EM</td>
<td>87</td>
<td>21</td>
</tr>
<tr>
<td>Joint</td>
<td>108</td>
<td>17</td>
</tr>
<tr>
<td>Heart</td>
<td>29</td>
<td>7</td>
</tr>
<tr>
<td>ACA</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>ELISA IgG positive</td>
<td>77</td>
<td>17</td>
</tr>
<tr>
<td>ELISA IgM positive</td>
<td>26</td>
<td>8</td>
</tr>
<tr>
<td>Blot IgG positive</td>
<td>84</td>
<td>21</td>
</tr>
<tr>
<td>Blot IgM positive</td>
<td>31</td>
<td>9</td>
</tr>
</tbody>
</table>

*Values are the numbers and percentages of patients unless otherwise noted.

IL-23 is known to play an important role for the induction and maintenance of Th17 cells and the production of IL-17 (1, 4, 18). It has been demonstrated that B. burgdorferi-induced IL-17 production in mice is dependent on IL-23 (19). Cells from mice lacking bioactive IL-23 (p19 deficiency) were unable to produce IL-17. Here, we showed that human PBMCs incubated with a neutralizing anti-IL-23p19 antibody produced significantly smaller amounts of IL-17. IL-23 is a member of the IL-12 family, and IL-12 itself is a cytokine which is known to drive IFN-γ production (15). Blockade of endogenous IL-12 ameliorates murine Lyme arthritis (2), and T helper 1 cell responses (e.g., IFN-γ) promote the development of Lyme arthritis. However, these studies used anti-IL-12p40 antibodies that neutralize both IL-12 and IL-23, and it therefore cannot be excluded that IL-23 blockade, rather than that of IL-12, led to decreased severity of Lyme arthritis in these mice through reduction of IL-17. We found that a specific IL-23p19 blockade or an Arg381Gln polymorphism in IL-23R does not modulate IFN-γ production by B. burgdorferi in primary cells.

B. burgdorferi spirochetes are potent inducers of IL-17 production by human PBMCs. This is in line with previous reports that demonstrated enhanced IL-17 production in both murine and human T cells, after stimulation with outer surface proteins isolated from B. burgdorferi spirochetes (17). Here, we demonstrated that human PBMCs exposed to live B. burgdorferi spirochetes produce robust IL-17 levels, which is in line with previous reports showing that microbial components, especially those originating from B. burgdorferi species, are po-
tent inducers of Th17 and/or IL-17 (9, 17, 22). Interestingly, it was shown that IL-17 contributes to the development of murine Lyme arthritis and that blocking of the IL-17 pathway leads to amelioration of this chronic disease (7). These data clearly indicate that Th17 and IL-17 are valid target candidates for therapeutic approaches in Lyme disease. This is in line with recent reports that anti-IL-17 treatment of RA patients results in suppression of disease activity (14).

As shown before, human PBMCs exposed to *B. burgdorferi* produce proinflammatory cytokines, such as IL-1β and IL-6, in a dose-dependent manner. These proinflammatory cytokines are known to be necessary for an optimal Th17/IL-17 response. Next to that, it was also demonstrated that human mononuclear cells (PBMCs) release IL-23 after stimulation with live *B. burgdorferi* spirochetes (3). However, this group did not show a dose-dependent induction of IL-23 production by *B. burgdorferi* species. So far, it was demonstrated only that murine bone marrow-derived dendritic cells (BMDCs) were able to produce IL-23 after exposure to *B. burgdorferi* species (19). In addition, human neutrophils were able to induce IL-23 after stimulation with neutrophil-activating protein A (NapA) isolated from *B. burgdorferi* (9). Interestingly, when neutrophils were exposed to *B. burgdorferi*-derived outer surface protein A (OspA), which is commonly used as a *B. burgdorferi* antigen, IL-23 production was not observed (9). In the present study, we used viable *B. burgdorferi* in order to stimulate the IL-23 production by PBMCs.

The function of both IL-17 and IL-23 in the development of human chronic Lyme disease is not demonstrated yet. Although it has been suggested that both IL-17 and IL-23 may be important mediators in chronic Lyme disease, this was not confirmed in functional studies (5). In rheumatoid arthritis (RA), it was already demonstrated that the IL-17/IL-23 axis plays an important role and that individuals with the single nucleotide polymorphism in IL-23R are protected against the development of RA (16). However, the functional mechanisms that mediate these findings were not investigated until now. This is the first study that describes the functional consequences of the IL-23R Arg381Gln SNP. It was already proposed that this polymorphism might interfere with JAK-STAT binding and therefore inhibit the activation of Th17 cells (13).

In our study, IL-17 production by PBMCs from individuals with the IL-23R SNP is indeed significantly reduced after stimulation with live *B. burgdorferi* spirochetes.

Despite the significant consequences of this SNP for the release of IL-17 in *vivo*, individuals bearing the IL-23R Arg381Gln polymorphism were not protected from developing clinical signs of acute or chronic Lyme disease. The infection rates of individuals carrying the wild type or the IL-23R SNP were similar, indicating that the risks of symptomatic *B. burgdorferi* infection in the situation of high exposure were equal between individuals bearing the various IL-23R genotypes. In addition, the percentages of individuals with anti-*B. burgdorferi* antibodies (IgG or IgM) were roughly the same (Table 1). Moreover, in a cohort of adults examined for a suspicion of chronic Lyme disease, individuals heterozygous for the IL-23R polymorphism showed symptoms of chronic Lyme disease, such as ACA or cardiac problems, similar to those in individuals homozygous for the wild-type allele. Interestingly, the incidence of joint problems was lower in the group heterozygous for the polymorphism (43% versus 54%) than in the group with the wild-type allele. However, this difference was not found to be significant. It was shown before that the TLR2 Arg753Gln polymorphism may protect for the development of late-stage Lyme disease due to a reduced signaling via TLR2/TLR1 (28). Several reports showed that both *B. burgdorferi* and Pam3Cys (TLR2 ligand originating from *B. burgdorferi*) are potent inducers of IL-23. Therefore, lacking functional TLR2 could have led to reduced IL-23 production followed by lower Th17 responses upon exposure to *B. burgdorferi*. This hypothesis is not supported by the lack of influence of the clinical presentation by the IL-23R polymorphisms (Table 1).

Since the IL-17 blockade was shown to be effective in suppressing murine Lyme disease in IFN-γ-deficient mice, it was tempting to speculate about the therapeutic value of anti-IL-17 antibodies in human Lyme disease (7). Anti-IL-17 antibodies are in development for treatment of RA patients, and the results are promising (14). Here, we demonstrated that a polymorphism resulting in a nonfunctional IL-23R leads to reduced IL-17 production and did not influence the susceptibility or severity of Lyme disease in humans. While this may be an argument against therapeutic usage of anti-IL-17 antibodies in chronic Lyme disease, one important aspect may be represented by the degree of IL-17 blockade attained by therapeutic antibodies. This may be much higher than that of the IL-23R polymorphism.

In conclusion, the contribution of the IL-23/IL-17 axis to the development of the chronic stages of Lyme disease is limited, and other pathological pathways need to be explored in the future. Apart from IL-17, *B. burgdorferi* spirochetes are potent inducers of IL-1β, which is a classic proinflammatory cytokine (5). The latter cytokine is linked to the development of several autoimmune diseases, such as gout and fever syndromes, and may represent a more attractive therapeutic target in chronic Lyme disease.

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