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 essay (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 (acrodermatitis 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-
tis (20). Induction of Lyme arthritis could not be observed when B. burgdorferi-exposed animals were treated with antibodies against the p19 subunit of IL-23. In addition, IL-17 levels in these animals were significantly decreased compared to those of control animals (27). Recently it has been demonstrated that a nonsynonymous single nucleotide polymorphism (SNP) in the IL-23 receptor (Arg381Gln; rs11209026) is associated with the protection from several autoimmune disorders, including Crohn’s disease, psoriasis, and rheumatoid arthritis (11, 16). This polymorphism is found in the cytoplasmic JAK-2 binding domain of the IL-23 receptor (IL-23R), and variations in this area are known to interfere within the binding of IL-23 to IL-23R (13). When variations occur, it is hypothesized that this will prevent activation of Th17 cells and thereby be protective against (chronic) disease. At this moment, IL-23 is used as a therapeutic target in the treatment of several autoimmune diseases, such as Crohn’s disease, psoriasis, and rheumatoid arthritis (12).

The precise role of IL-23 in Lyme disease patients has not been elucidated yet. We describe for the first time that IL-17 production by B. burgdorferi in humans is dependent on IL-23R signaling and genetic variation at the level of the IL-23R gene. In addition, we assessed whether the IL-23R Arg381Gln polymorphism contributes to the pathogenesis of chronic Lyme disease.

MATERIALS AND METHODS

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-μl aliquots of culture material with 10 μl of an acridine orange solution. Bacteria were harvested by centrifugation of the culture at 7,050 × 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 until use. B. burgdorferi was diluted in PBS to required concentrations, 1 × 10^5 to 1 × 10^6 spirochetes/ml. Viability after freezing was confirmed using dark-field microscopy.

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 (Monogel). 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 Compact; Sanquin Amsterdam; or R&D Systems, Minneapolis, MN), in accordance with the manufacturer’s protocol for whole blood, and dissolved in a final volume of 100 μl of medium (negative control) or 104 and 105 spirochetes/ml. After 7 days of incubation, a dose-dependent induction of IL-1α, IL-6, and IL-23 was observed in a dose-dependent manner. In addition, IL-1α, IL-6, IL-17, or IL-23 induce IL-17 production by PBMCs, we stimulated PBMCs for 7 days with IL-1α (Fig. 1B) as well as IL-23 (Fig. 1C) production could be determined IL-1α, IL-6, IL-17, or IL-23 induce IL-17 production by PBMCs, we stimulated PBMCs for 7 days with IL-1α (Fig. 1B) as well as IL-23 (Fig. 1C) production could be determined. Figure 1D shows that IL-1α, IL-6, IL-17, or IL-23 induce IL-17 production by PBMCs, we stimulated PBMCs for 7 days with IL-1α (Fig. 1B) as well as IL-23 (Fig. 1C) production could be determined.

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 × 10^3 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-$\gamma$, IL-1$\beta$, 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-$\gamma$ induction by B. burgdorferi, we examined whether the anti-IL-23 antibody modulates B. burgdorferi-induced IFN-$\gamma$ production (15). No differences in IFN-$\gamma$ 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-1$\beta$ and IL-6 production could be found when the anti-IL-23p19 antibody was added together with live B. burgdorferi/ml. Bars are means $\pm$ SEM of the means, and at least 6 healthy individuals were used. $**, P < 0.01$; $***$, $P < 0.001$, Mann-Whitney U test.

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-1$\beta$ 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 chronic Lyme disease has been suggested (7). Since we showed that the IL-23 pathway is crucial for *B. burgdorferi*-induced IL-17 production, we investigated whether individuals bearing the IL-23R Arg381Gln SNP have a different presentation of clinical signs of chronic Lyme disease. We analyzed a cohort of 228 individuals (foresters and Lyme patients) in total, in which several individuals were present that expressed clinical signs of chronic Lyme disease, including arthritis and skin disorders (ACA). We found little differences in the incidences of EM occurrence between individuals carrying the wild-type or heterozygous allele of the IL-23R gene (Table 1). Moreover, the percentages of anti-*B. burgdorferi* antibodies, both IgM and IgG, were similar in both genotypes of the IL-23 receptor. In addition, there was no decreased 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-23R</td>
</tr>
<tr>
<td>Total</td>
<td>188</td>
<td>40</td>
</tr>
<tr>
<td>Female/male</td>
<td>100/88</td>
<td>22/18</td>
</tr>
<tr>
<td>Age range (mean)</td>
<td>16–68 (48)</td>
<td>17–66 (50)</td>
</tr>
<tr>
<td>Lyme history</td>
<td>26/5</td>
<td>14/13</td>
</tr>
<tr>
<td>Possible Lyme disease</td>
<td>134/30</td>
<td>71/75</td>
</tr>
<tr>
<td>Proven Lyme disease</td>
<td>28/5</td>
<td>15/12</td>
</tr>
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Clinical signs or complaint

| EM                   | 87/21                | 46/53               |                       |
| Joint                | 108/17               | 57/43               |                       |
| Heart                | 29/7                 | 15/18               |                       |
| ACA                  | 12/2                 | 6/5                 |                       |
| ELISA IgG positive   | 77/17                | 41/43               |                       |
| ELISA IgM positive   | 26/8                 | 14/2                |                       |
| Blot IgG positive    | 84/21                | 45/53               |                       |
| Blot IgM positive    | 31/9                 | 16/23               |                       |

* Values are the numbers and percentages of patients unless otherwise noted.
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 vitro, 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 human patients 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 autoinflammatory diseases, such as gout and fever syndromes, and may represent a more attractive therapeutic target in chronic Lyme disease.

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