MAJOR ARTICLE

Elevated Levels of IL-23 in a Subset of Patients With Post–Lyme Disease Symptoms Following Erythema Migrans

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Background. The causes of post-Lyme disease symptoms are unclear. Herein, we investigated whether specific immune responses were correlated with such symptoms.

Methods. The levels of 23 cytokines and chemokines, representative of innate and adaptive immune responses, were assessed in sera from 86 antibiotic-treated European patients with erythema migrans, 45 with post-Lyme symptoms and 41 without symptoms, who were evaluated prior to treatment and 2, 6, and 12 months thereafter.

Results. At study entry, significant differences between groups were observed for the type 1 helper T cell (T_H1)–associated chemokines CXCL9 and CXCL10, which were associated with negative *Borrelia* cultures, and the type 17 helper T cell (T_H17)–associated cytokine interleukin 23 (IL-23), which was associated with positive cultures and the development of post-Lyme symptoms ($P \leq .02$). Moreover, of the 41 patients with detectable IL-23 levels, 25 (61%) developed post-Lyme symptoms, and all 7 with IL-23 levels ≥230 ng/mL had such symptoms. Furthermore, antibody responses to the ECGF autoantigen were more common in patients with post-Lyme symptoms ($P = .07$) and were correlated directly with IL-23 levels ($P = .02$). Despite the presence of post-Lyme symptoms, all posttreatment culture results were negative, antiborrelial antibody responses declined, and there were no objective signs of disseminated disease, suggesting that spirochetal eradication had occurred with treatment in all patients.

Conclusions. High T_H1-associated responses correlated with more effective immune-mediated spirochetal killing, whereas high T_H17-associated immune responses, often accompanied by autoantibodies, correlated with post-Lyme symptoms, providing a new paradigm for the study of postinfectious symptoms in a subset of patients with Lyme disease.

Keywords. Lyme disease; post–Lyme disease symptoms; inflammation; IL-23; T_H17.

Lyme disease in the United States is caused by *Borrelia burgdorferi* sensu stricto (*Bb*), whereas in Europe, the infection is due primarily to *Borrelia afzelii* and *Borrelia garinii* [1]. The most common sign of the infection is an initial expanding skin lesion, erythema migrans (EM), which is sometimes accompanied by flulike symptoms [2]. EM typically resolves with 10–21 days of oral antibiotics, and the majority of patients recover completely. However, about 10% of patients in Europe and the United States have persistent or new subjective symptoms, such as headache, fatigue, malaise, arthralgias, or myalgias, in the months after treatment, termed post–Lyme disease symptoms [3].

The term "post-Lyme symptoms" probably consists of >1 syndrome. At one end of the spectrum, one or a few subjective symptoms, such as malaise and fatigue or minor joint symptoms, may persist for several months after antibiotic treatment of EM. At the far end of the spectrum, patients may develop disabling joint and muscle pain, neurocognitive difficulties, and incapacitating fatigue that persist for years after Lyme disease [4–9]. This is sometimes called post–Lyme disease syndrome [4]. This area is further confused by

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the fact that "chronic Lyme disease" has become a diagnosis for disabling, medically unexplained symptoms, even when there is little or no evidence of past or present Lyme disease [4].

Pathogenetic mechanisms that account for post–Lyme disease symptoms remain unclear and are not likely to be the same in all patients. Four double-blind, placebo-controlled antibiotic trials have focused on the hypothesis that these symptoms may result from persistent infection [7–9]. In 2 trials, no significant differences were found between the antibiotic and placebo groups [7]. In the third trial, significant differences were noted only in fatigue for 1–6 months after therapy [8]. In the fourth study, significant differences were initially observed in fatigue and pain, but beneficial effects were not sustained [9]. Moreover, in all 4 trials, microbiologic measures of infection were negative. Finally, posttreatment culture results from EM skin lesions have been negative in almost all patients, including those with post-Lyme symptoms [3, 10].

Mechanisms other than active infection, including the possibility of immune system abnormalities, have also been considered. Heightened antineuronal antibody levels were reported in patients with disabling pain or neurocognitive or fatigue symptoms for years after Lyme disease [11]. In addition, in MyD88$^{-/-}$ mice, retained spirochetal antigens were proposed as a reason for joint symptoms after Lyme disease [12]. However, the causes for postinfectious phenomena after Lyme disease remain poorly understood.

Control of *Borrelia burgdorferi* sensu lato (*Bb*sl) infection requires both innate and adaptive immune responses [13]. EM skin lesions often contain high levels of interleukin (IL) 6, IL-10, and interferon (IFN)–γ, and the IFN-γ–inducible chemokines CXCL9 and CXCL10 [14], which orchestrate type 1 helper T cell (T_H1) responses [15]. In addition, spirochetes may stimulate the production of IL-23 [16, 17]. This cytokine promotes the proliferation and maintenance of type 17 helper T cells (T_H17), which are important for the control of extracellular pathogens, and have also been implicated in autoimmune phenomena [18–20]. Herein we assessed cytokine and chemokine profiles representative of innate and adaptive immune responses in serum samples from Slovenian patients with EM who were followed for 12 months to assess their posttreatment status.

METHODS

Selection of Patients

Initially, 937 patients ≥15 years of age with putative EM were evaluated for participation in clinical trials: an efficacy study of 15-day treatment with doxycycline vs cefuroxime axetil [3] and a trial of 10- vs 15-day treatment with doxycycline [10]. The studies were approved by the Medical Ethics Committee of the Ministry of Health of Slovenia and each patient provided written consent. All patients were evaluated at the Lyme Borreliosis Outpatient Clinic at the University Medical Center Ljubljana, Slovenia. After exclusions, 510 patients with a single EM lesion, as defined using modified Centers for Disease Control and Prevention criteria [3, 10], participated in the study.

Patients were assessed by the study physicians at baseline, and at 2, 6, and 12 months of follow-up about health-related difficulties, including fatigue, arthralgias, myalgias, headache, dizziness, malaise, irritability, nausea, or paresthesias. Symptoms that developed or worsened after the onset of EM that did not have another known medical explanation were regarded as post-Lyme symptoms. The severity of individual symptoms was graded by the subject on an 8-cm visual analogue scale (8 = most severe). Of the 510 patients, 159 (31%) had EM with associated symptoms, 62 (12%) had post-Lyme symptoms at 2 months, and 18 (4%) had symptoms 12 months after the start of antibiotics (Figure 1).

For this study, a total of 86 patients were selected from both treatment trials [3, 10], including all 45 of the 62 patients who reported at least 1 post-Lyme symptom after antibiotic therapy

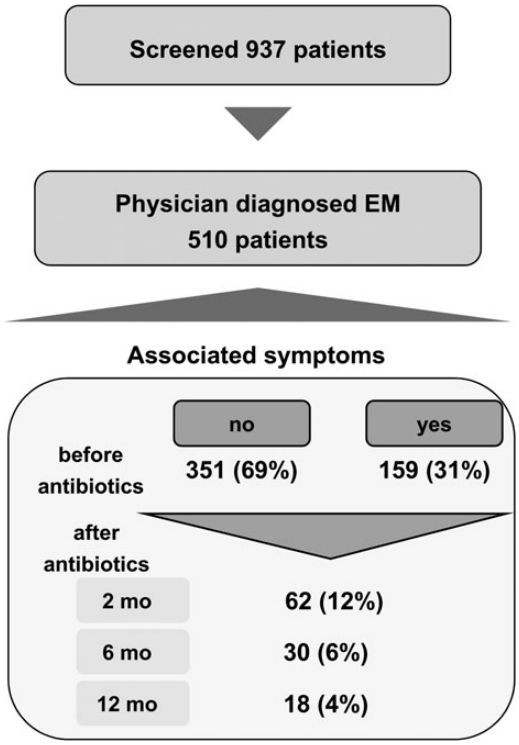

Figure 1. Selection of patients. Patients for this study were selected from 2 previous European studies [3, 10]. Of the 937 patients evaluated initially, 510 had erythema migrans and were treated with antibiotics and reevaluated over 12 months. Of the 510 patients, 62 had post-Lyme symptoms. For this study, sera were available from 45 of the 62 patients. For comparison, sera were randomly selected from 41 patients whose symptoms resolved with antibiotic therapy. Abbreviation: EM, erythema migrans.

in whom sufficient serum samples were still available from at least 2 follow-up visits (2, 6, or 12 months). For comparison, 41 patients were randomly selected from 296 patients who did not develop post-Lyme symptoms and from whom sera were still available at these time points. These 41 patients were representative of the larger patient cohort [3, 10]. In addition, serum samples were obtained from 22 healthy hospital personnel who did not have a history of Lyme disease, to provide the basal serum expression of the inflammatory mediators assessed in the study.

Sample Collection

Serum samples were obtained at each visit. An effort was made to collect an aliquot of serum for immune profiling analyses from each patient at each visit. After collection, serum aliquots were immediately stored at −80°C until use in this study.

Chemokine and Cytokine Determinations

Studies of the diagnosis and pathogenesis of early Lyme disease were approved by the Institutional Review Board at the Massachusetts General Hospital, Boston. The levels of 15 cytokines (IL-1β, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p40, IL-12p70, IL-17, IL-23, IL-21, IL-22, IL-27, IFN-α, and IFN-γ) and 11 chemokines (CCL2, CCL3, CCL4, CCL5, CXCL8, CXCL9, CXCL10, CXCL11, CXCL13, CCL19, and CCL21) were assessed in 1 complete experiment using Luminex (Millipore).

Serologic Analyses

To assess *Borrelia*-specific antibody responses, serum IgM/IgG antibodies to the *Borrelia* VlsE C6 peptide, which is largely conserved among the 3 pathogenic *Bb*sl species, were determined using the C6 (*B. burgdorferi*) enzyme-linked immunosorbent assay (Immunetics), according to the manufacturer's protocol. The anti-C6 antibody responses were determined for all patients and all time points in one complete experiment. Antibody responses were also determined to a recently identified human autoantigen in Lyme disease, endothelial cell growth factor (ECGF), as previously described [21]. To provide maximal specificity, a positive response was defined as >5 SD above the mean value in 10 normal control subjects.

Statistical Analysis

Differences in cytokine and chemokine levels between groups were assessed using the Mann-Whitney rank-sum test. Samples with undetectable cytokine levels were not included in the analysis. Correlation between IL-23 levels and anti-ECGF or anti-C6 antibodies were assessed using Spearman correlation. Statistical analyses were conducted using the Sigma Stat version 3.0.1 software from SPSS. A P value of $\leq .05$ was considered statistically significant.

RESULTS

Clinical Characteristics of Patients at Study Entry

For this study, 86 patients with EM were selected, 45 with at least 1 post-Lyme symptom after antibiotic therapy, and 41 without posttreatment symptoms (Table 1). In addition to EM, approximately half of the patients had at least 1 associated symptom, such as headache, myalgias, arthralgias, malaise, or fatigue. Of the 86 patients, 47 (55%) had a positive EM skin biopsy culture for *Bb*sl (predominantly *B. afzelii*), and 55 (64%) had reactivity with the *Bb* VlsE C6 peptide. Altogether, 71 (83%) had laboratory documentation of *Bb*sl infection by culture or serology. When stratified according to subsequent development of post-Lyme symptoms, the 2 groups did not differ significantly in age, sex, duration of illness prior to enrollment, the number and intensity of EM-associated symptoms, or positive culture or serology result (Table 1). Thus, at the time of infection, patients who did or did not develop post-Lyme symptoms had similar clinical pictures.

Table 1. Clinical Characteristics of 86 Patients With Erythema Migrans at Study Entry

Characteristic	All Patients (N = 86)	No Post-Lyme Symptoms (n = 41)	Post-Lyme Symptoms (n = 45)
General characteristics			
Age[a]	53 (17–75)	52 (18–75)	56 (17–74)
Sex, No. female/male	55/31	24/17	31/14
Time from tick bite to EM, d[a]	16 (1–106)	16 (1–57)	15 (1–106)
Time from EM to study entry, d[a]	7 (1–62)	6 (1–62)	11 (1–60)
EM diameter, cm[a]	13 (3–86)	12 (5–50)	13 (3–86)
Symptoms at study entry			
No. of patients with symptoms	45 (51%)	20 (49%)	25 (56%)
No. of symptoms[a]	1 (0–6)	1 (0–5)	1 (0–6)
Intensity of symptoms[a,b]	4 (1–8)	4 (1–8)	5 (1–8)
Laboratory findings at study entry			
No. of patients with			
Positive *Borrelia* skin biopsy culture	47 (55%)	21 (51%)	26 (57%)
Positive VlsE C6 antibody response	55 (64%)	25 (61%)	30 (67%)
Positive culture or VlsE result	71 (83%)	31 (76%)	40 (89%)

Abbreviation: EM, erythema migrans.

[a] Data are expressed as median (range).

[b] Scale of 0–8, with 8 being the highest intensity.

Cytokine and Chemokine Levels at Study Entry

Of the 15 cytokines and 11 chemokines tested, the only significant differences between groups were in the levels of CXCL9, CXCL10, and IL-23. Therefore, only the results of these mediators are presented here. Of the 86 study patients, 85 had detectable serum levels of CXCL9 and 84 of CXCL10, which are chemoattractants involved in T_H1-like immune responses. In addition, 41 of the 86 patients had detectable levels of IL-23, a cytokine that is necessary for the proliferation and maintenance of T_H17 cells. The levels of other T_H17 mediators (IL-17, IL-21, IL-22, and IL-27) were below the limit of detection in most patients.

When cytokine and chemokine data in the 86 patients were stratified according to culture results at the initial visit, prior to the start of antibiotics, the 39 patients with negative *Borrelia* cultures tended to have higher levels of CXCL9 (1374 vs 847 pg/mL, $P = .1$), and they had significantly higher levels of CXCL10 (412 vs 229 pg/mL, $P = .02$) than the 47 culture-positive patients (Figure 2*A*). In contrast, the levels of IL-23 were significantly higher in the culture-positive group (217 vs 85 pg/mL, $P = .05$).

To correlate the inflammatory responses with disease severity, CXCL9, CXCL10, and IL-23 levels were stratified by the presence or absence of associated symptoms at study entry, prior to antibiotic therapy (Figure 2*B*). The levels of CXCL9 were significantly higher (1499 vs 668 pg/mL, $P < .0001$) in patients with symptoms compared to those without and a similar trend was observed for CXCL10 (370 vs 255 pg/mL, $P = .16$). In addition, IL-23 levels were significantly higher in those with symptoms (226 vs 128 pg/mL, $P = .003$). Thus, higher levels of

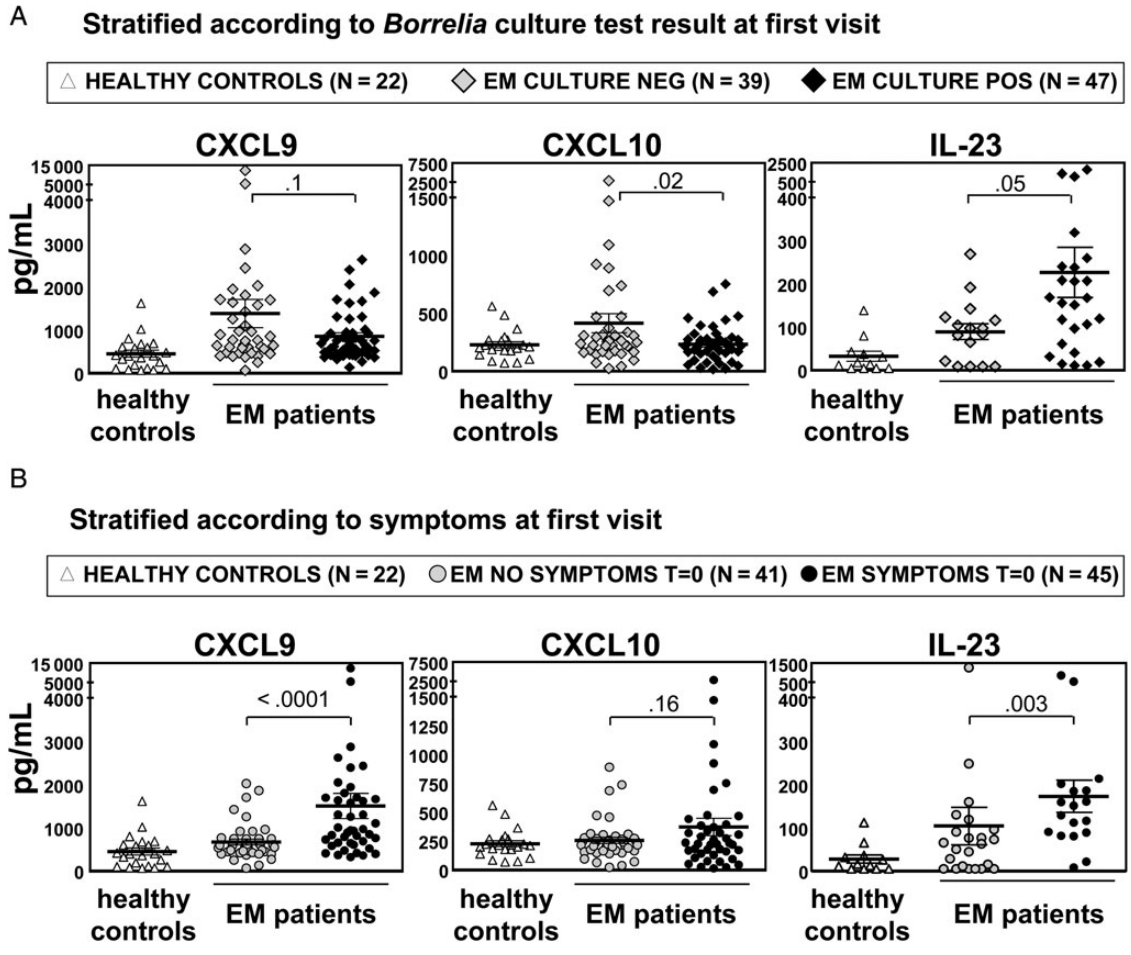

Figure 2. Cytokines and chemokines stratified according to *Borrelia* culture result or symptoms at first visit. Protein levels of the T_H1-associated chemokines CXCL9 and CXCL10, and of the T_H17-associated cytokine IL-23, were assessed in 86 patients with erythema migrans using bead-based multiplex assays. *A*, Patients were first stratified according to *Borrelia* culture test result at first visit, prior to antibiotic therapy. *B*, Patients were stratified according to associated symptoms at first visit. The bars represent the mean values and I-bars represent the standard error of the mean. *P* values for comparison of culture-positive vs culture-negative patients, and for comparison of patients with or without associated symptoms, are indicated in the graph. Abbreviations: EM, erythema migrans; IL-23, interleukin 23.

Table 2. Post-Lyme Symptoms During 2–12 Months After Treatment

Symptom	Post-Lyme Patients (n = 45)
No. of patients with symptoms[a]	
Arthralgia	23
Headache	18
Fatigue	18
Myalgia	11
Dizziness	5
Malaise	4
Irritability	2
Nausea	2
Paresthesias	2
No. of post-Lyme symptoms per patient,	
median (range)	2 (1–6)
No. of patients with symptoms at	
2 mo	32
6 mo	22
12 mo	12

[a] The number of patients who reported a given symptom at any of the follow-up time points. If a symptom was reported at >1 time point, it was only counted once here.

CXCL9 and CXCL10, which are involved in T_H1 immune responses, were associated with more symptomatic early infection and culture-negative results, whereas high levels of IL-23, a T_H17 mediator, were associated with more symptomatic infection and culture-positive results.

Cytokine and Chemokine Levels Stratified by Post–Lyme Disease Symptoms

Among the 86 patients, 45 had symptoms that persisted after the 2-week course of antibiotic therapy for EM, or they developed new symptoms in the weeks after treatment. The most common symptoms were arthralgia, headache, or fatigue, with a median number of 2 symptoms per patient (Table 2). When the 45 patients with post-Lyme symptoms were subdivided according to IL-23 levels, the group with detectable IL-23 responses tended to have more symptoms (median, 4 vs 2 symptoms, $P = .09$). The number of patients with post-Lyme symptoms decreased over time. By 12 months, only 12 patients still reported symptoms.

At study entry, prior to antibiotic therapy, the 45 patients who subsequently developed post-Lyme symptoms had similar levels of CXCL9 and CXCL10 compared with the 41 patients without posttreatment symptoms (Figure 3*A*), and the levels remained similar at the follow-up time points (Figure 3*B*). In contrast, IL-23 levels were significantly higher in the post-Lyme group at study entry, and remained significantly higher at each posttreatment visit ($P \leq .04$). Moreover, of the 41 patients with detectable IL-23 levels, 25 (61%) had post-Lyme symptoms, and all 7 patients with IL-23 levels ≥230 ng/mL had such symptoms.

Culture and Serology Results Stratified by Post–Lyme Disease Symptoms

Because patients with high IL-23 values were more often culture-positive (Figure 2), we used culture and serologic analyses to evaluate whether patients had persistent infection. All 47 patients who were culture-positive for *Bb*sl at study entry had undergone rebiopsy close to the original biopsy site 2 months after the start of antibiotics [3, 10]. All repeat cultures in patients with or without post-Lyme symptoms were negative (Figure 4*A*). In addition, the anti-VlsE C6 peptide antibody responses were similar in patients who developed post-Lyme symptoms and in those who did not (Figure 4*B*), and these responses declined similarly in both groups over subsequent months (Figure 4*C*). Moreover, there were no objective signs of persistent infection or disseminated disease in either group. Thus, although high T_H1-mediated immune responses seemed to be more effective in spirochetal killing than T_H17-mediated responses, antibiotic therapy at the time of EM appeared to result in spirochetal eradication in all patients, including those with post-Lyme symptoms.

Antibody Responses to ECGF Stratified by Post–Lyme Disease Symptoms

An alternate hypothesis is that dysregulated T_H17 responses may predispose patients to autoimmune phenomena. At study entry, 10 of the 45 patients (22%) with post-Lyme symptoms had antibody responses to ECGF, a recently identified autoantigen in Lyme disease [21], compared with 3 of the 41 patients (7%) who did not have post-Lyme symptoms ($P = .07$; Figure 5*A*), a difference of possible significance. Moreover, in patients with post-Lyme symptoms, IL-23 levels correlated directly with the magnitude of anti-ECGF antibody response ($P = .02$; Figure 5*B*). In contrast, there was no correlation between IL-23 and anti-ECGF antibodies in patients whose symptoms resolved with antibiotics. Furthermore, antibody responses to a *Borrelia*-specific VlsE C6 peptide did not correlate with IL-23 levels in either patient group (Figure 5*C*). Thus, anti-ECGF antibody responses were more common in patients with post-Lyme symptoms, and they correlated directly with higher IL-23 levels, suggesting that post-Lyme symptoms could be associated with persistent T_H17 responses in the absence of live spirochetes.

DISCUSSION

*Bb*sl infection induces a complex immune response that drives the clinical signs and symptoms of disease. Our findings here suggest that T_H1 and T_H17 responses are differentially activated in patients with EM. A T_H1 response, characterized by elevated

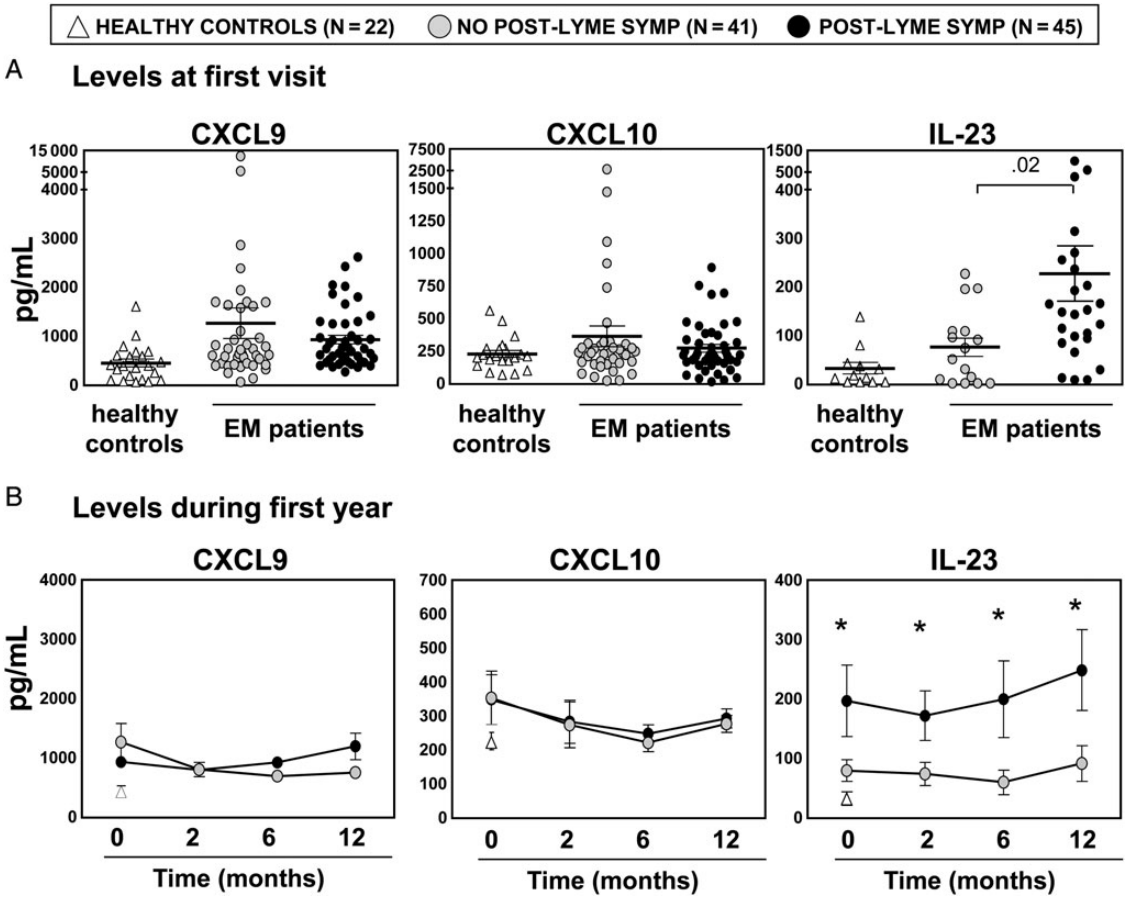

Figure 3. Cytokines and chemokines stratified according to post-Lyme symptoms. Serum protein levels of the T_H1-associated chemokines CXCL9 and CXCL10, and of the T_H17-associated cytokine IL-23, were stratified according to patients who developed post-Lyme symptoms during 2–12 months after the start of antibiotic therapy, and those whose symptoms resolved with antibiotics. *A*, Levels of CXCL9, CXCL10, and IL-23 during the first visit, prior to antibiotic therapy. *B*, Levels of these mediators during the first year. The bars in *A* and the dots in *B* represent the mean values, and the I-bars represent the standard error of the mean. For comparison of patients with post-Lyme symptoms and those with no post-Lyme symptoms, $^*P \leq .05$, or as indicated in the graph. Abbreviations: EM, erythema migrans; IL-23, interleukin 23; Symp, symptoms.

levels of CXCL9 and CXCL10, the chemoattractants for $CD4^+$ and $CD8^+$ T cells, appears to be the predominant response in clearing the infection. At the first visit, prior to antibiotics, CXCL9 and CXCL10 levels were detectable in serum of virtually all patients (>97%), and high levels of these chemokines were associated with negative *Bb*sl culture results in EM skin biopsies, presumably due to lower numbers or lack of viable spirochetes [22, 23]. The consequence of these elevated T_H1 mediators was more symptomatic early infection.

In comparison, T_H17 responses, characterized by IL-23 levels, were found in only a subset of patients (43%). These patients were more often culture-positive and symptomatic, suggesting that T_H17 responses were not as effective in spirochetal killing, but were capable of causing disease-associated pathology. Moreover, in the subset of patients with post-Lyme symptoms, IL-23 levels were elevated at enrollment and remained elevated during the 12-month follow-up period. The levels of other T_H17-associated mediators, including IL-17, which primarily regulates the differentiation of T_H17 cells, were undetectable in most patient samples, implying that T_H17-cell expansion rather than de novo differentiation plays a role in these responses.

The main function of IL-23 is to drive the proliferation and survival of T_H17 cells, which are important in host defense against extracellular pathogens [18–20]. However, aberrant IL-23/T_H17 responses have been implicated in several autoimmune conditions, including rheumatoid arthritis, inflammatory bowel disease, lupus, and type 1 diabetes [18–20]. The understanding of T_H17/IL-23-mediated immunity in Lyme disease is limited. In animal models, mice and hamsters vaccinated with killed *Bb* and then infected with live spirochetes developed severe destructive arthritis [24], which was ameliorated by

A **Number of patients with positive *Borrelia* skin biopsy culture**

	ALL PATIENTS (N = 86)	NO POST-LYME SYMPTOMS (N = 41)	POST-LYME SYMPTOMS (N = 45)
FIRST VISIT	47 (55%)	21 (51%)	26 (57%)
2 MONTHS	0	0	0

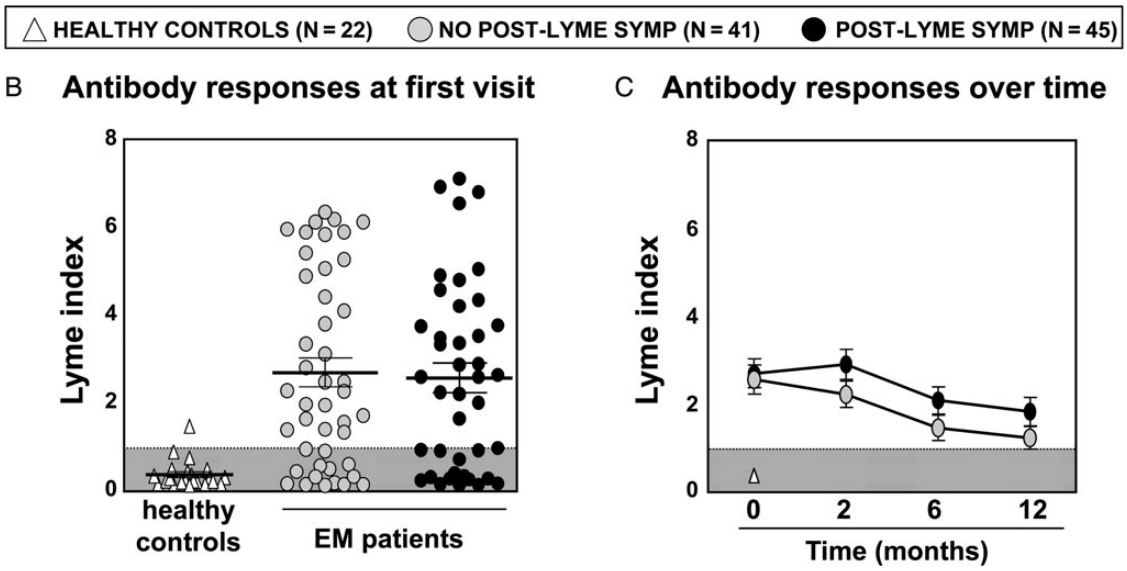

Figure 4. *Borrelia burgdorferi* sensu lato skin biopsy culture results and antibody responses to *Borrelia* VlsE C6 peptide in patients with post-Lyme symptoms. *Borrelia burgdorferi* sensu lato culture results and antibody responses to VlsE C6 peptide were stratified according to presence or absence of post-Lyme symptoms. *A*, Number of patients with a positive *B. burgdorferi* sensu lato culture result at first visit, and at rebiopsy at the 2-month visit. *B*, Antibody responses to *Borrelia* VlsE C6 antigen at first visit, and *C*, these antibody responses during 12 months after treatment. The shaded region represents the range of values that were considered negative. Data are expressed as a Lyme index, according to the manufacturer's instructions. The bars in *B* and the dots in *C* represent the mean values, and the I-bars represent the standard error of the mean. Abbreviations: EM, erythema migrans; Symp, symptoms.

neutralizing antibodies against IL-17 [25] or IL-23 [26], implying that T_H17 responses were associated with more severe and prolonged disease.

In human patients, $CD4^+$ T cell subsets, including T_H17 cells, have been studied in patients with antibiotic-responsive or antibiotic-refractory Lyme arthritis. In both groups, IFN-γ–positive T_H1 cells were the predominant population in joint fluid, but in some patients with antibiotic-refractory arthritis, up to 25% of synovial fluid T cells were T_H17 cells [27]. Furthermore, in human cell culture models, the *Borrelia* protein NapA induced the secretion of several T_H17-associated mediators, including IL-17 and IL-23 [16, 28]. Our findings here demonstrate that T_H1 is the predominant response in this infection, but a subset of patients develops T_H17 responses, which may be associated with more severe and prolonged symptoms.

Additionally, in this study, untoward T_H17 responses were sometimes associated with autoimmune phenomena. Autoantibodies to ECGF, the first reported autoantigen in Lyme disease that is a target of T-cell and B-cell responses, often develop early in the illness in patients with EM [21]. However, as observed with murine models in which persistent autoantigen stimulation leads to more active T-regulatory cells and resolution of disease [29, 30], the autoimmune responses in our patients appear to subside, and postinfectious symptoms usually resolve.

In this study of European EM patients, most of whom were infected with *B. afzelii*, the most common post-Lyme symptoms were arthralgia, headache, and fatigue. These symptoms were not incapacitating, and typically resolved within months after antibiotic therapy. It will be important to determine whether some patients with post-Lyme symptoms in the United States,

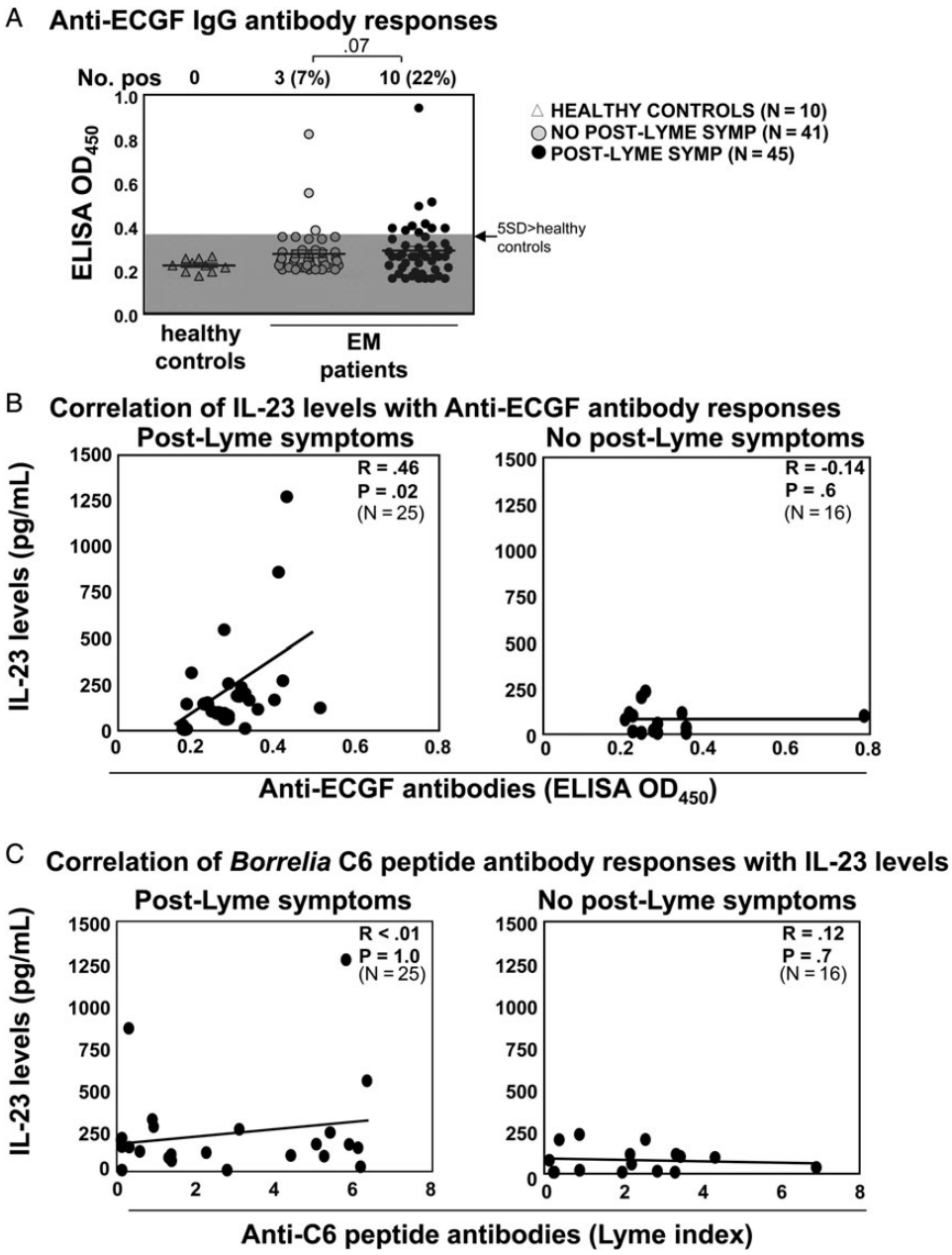

Figure 5. Antibody responses to a human autoantigen, endothelial cell growth factor (ECGF), in patients with erythema migrans at first visit stratified according to post-Lyme symptoms. *A*, Frequency of a positive antibody response to ECGF as defined by antibody levels >5 standard deviation above the mean of values in healthy control subject. Shaded region represents the values that were considered negative. *B*, Magnitude of the anti-ECGF antibody response was correlated with IL-23 levels in patients with post-Lyme symptoms (left panel) or no post-Lyme symptoms (right panel). *C*, Antibody responses against *Borrelia* VlsE C6 peptide were correlated with IL-23 levels in patients with post-Lyme symptoms (left panel), or those without (right panel). The bars in panel *A* represent the mean values and the I-bars represent the standard error of the mean. *P* values and correlation coefficients are indicated in the graphs. Abbreviations: ECGF, endothelial cell growth factor; ELISA, enzyme-linked immunosorbent assay; EM, erythema migrans; IgG, Immunoglobulin G; IL-23, interleukin 23; OD_{450}, optical density; Symp, symptoms.

including those with more debilitating symptoms, also have untoward T_H17 responses associated with such symptoms.

Study limitations include possible bias in patient selection due to testing only a subset of the study population. However, we included serum samples from all available patients with post-Lyme symptoms (n = 45) and we randomly selected samples from 41 of the 296 patients without post-Lyme symptoms; this group was representative of the larger cohort [3, 10]. We also did not develop comparison groups of patients who had similar symptoms following other manifestations of Lyme

disease or other illnesses. However, the goal of the current study was to test a uniquely well-characterized population of EM patients in whom cultures and long-term follow-up were conducted systematically in those who did or did not develop post-Lyme symptoms. This study design made possible the most cogent comparison group for patients with post-Lyme symptoms. Finally, coinfection with other tick-borne agents is a potential confounding variable in EM patients. In Slovenia, the other diseases transmitted by *Ixodes ricinus* ticks include tick-borne encephalitis, human granulocytic anaplasmosis, and, very rarely, tularemia. All patients in our study had a single EM lesion sometimes accompanied by nonspecific symptoms. They did not have the clinical characteristics of tick-borne encephalitis or human granulocytic anaplasmosis; they were rarely febrile, and they did not have thrombocytopenia or leukopenia. Thus, we do not think that the patients in this study had coinfection.

In summary, a subset of patients with EM may have immune dysregulation reflected by the persistently elevated levels of IL-23, resulting in post-Lyme disease symptoms. These observations offer a new paradigm for the study of patients with postinfectious symptoms following Lyme disease.

Notes

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Author contributions. K. S., F. S., and A. C. S. designed the study. K. S. conducted the experiments and the data analyses for this study. F. S. and D. S. conducted the initial clinical trials [3, 9], and provided the patient samples and clinical information. E. E. D. helped with the design and interpretation of ECGF experiments. K. S., A. C. S., F. S., and E. E. D. helped with writing of the manuscript. All authors reviewed and approved the manuscript.

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Potential conflicts of interest. All authors: No reported conflicts.

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