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A Toll-like Receptor 1 Polymorphism Is Associated with Heightened T-helper 1 Inflammatory Responses and Antibiotic-Refractory Lyme Arthritis

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Abstract

Objective—Single-nucleotide polymorphisms (SNPs) have been identified in several genes encoding Toll-like receptors (TLRs) that alter immune function, inflammatory responses and disease susceptibility. The SNPs with best evidence for affecting immune function are TLR1 (1805GG), TLR2 (2258GA) and TLR5 (1174CT).

Methods—We studied the frequency and functional outcome of these polymorphisms in 248 patients with Lyme disease. Cytokine and chemokine levels were determined in serum of patients with erythema migrans (EM), joint fluid of patients with Lyme arthritis, and supernatants of *B. burgdorferi*-stimulated PBMC from Lyme arthritis patients, using multiplex assays.

Results—The frequency of TLR1-1805GG polymorphism was greater in patients with antibiotic-refractory arthritis compared with patients with EM or antibiotic-responsive arthritis. Early in the illness, EM patients with 1805GG, primarily those infected with *B. burgdorferi* RST1 strains, had higher serum levels of IFNγ, CXCL9 and CXCL10, and more severe infection than patients with 1805TG/TT. These inflammatory responses were amplified in patients with Lyme arthritis, and the highest responses were observed in antibiotic-refractory arthritis patients with 1805GG who had been infected with RST1 strains. When PBMC from Lyme arthritis patients were stimulated with a *B. burgdorferi* RST1 strain, the 1805GG group had significantly larger fold-increase in the levels of IFNγ, CCL2, CXCL9 and CXCL10, than the 1805TG/TT group. In contrast, the TLR2 and TLR5 polymorphisms did not vary among groups in frequency or function.

Conclusion—The TLR1-1805GG polymorphism in *B. burgdorferi* RST1-infected patients was associated with stronger T_H1-like inflammatory responses, which may set the stage for antibiotic-refractory arthritis.

INTRODUCTION

Lyme borreliosis, which is caused by the tick-borne spirochete *Borrelia burgdorferi* (1), usually begins with an expanding skin lesion, erythema migrans (EM). Months later, untreated patients in the U.S. often develop intermittent or persistent arthritis in a few large joints for a period of several years (2). Lyme arthritis can usually be treated successfully with a 4- to 8-week course of oral antibiotic therapy (3) or a 2- to 4-week course of intravenous (IV) antibiotics (3, 4), and the arthritis resolves. However, in a small percentage

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of cases, proliferative synovitis persists for months to several years after oral and IV antibiotic treatment (5), called antibiotic-refractory Lyme arthritis.

Antibiotic-refractory Lyme arthritis is associated with infection with certain *B. burgdorferi* genotypes, particularly 16s–23s rRNA intergenic spacer type 1 (RST1) strains (6). These strains stimulate macrophages to secrete significantly greater amounts of macrophage-associated cytokines and chemokines than RST2 or RST3 strains (7). However, persistent synovitis after antibiotic therapy does not seem to result from persistent infection; PCR and culture results for *B. burgdorferi* have been uniformly negative in synovial tissue obtained at synovectomy months after antibiotic treatment (8, 9). Rather, we have hypothesized that autoimmunity may play a role in antibiotic-refractory Lyme arthritis (10).

The innate immune response enables the host to differentiate self from pathogen, provides a rapid inflammatory response, and shapes the adaptive immune response (11). In recent years, single nucleotide polymorphisms (SNPs) have been identified in several genes encoding Toll-like receptors (TLRs) that lead to modified cellular immune responses, decreased cytokine production, and altered disease susceptibility (11, 12). The TLR SNPs with the best evidence for affecting immune function are TLR1 (1805GG) (13, 14), TLR2 (2258GA) (15, 16), and TLR5 (1174CT) (17–20).

The TLR1/TLR2 heterodimer recognizes triacylated lipids (Pam_3CSK_4) found on lipopeptides in a diverse array of pathogens, whereas TLR5 recognizes bacterial flagellar proteins. Of 17 SNPs identified in the TLR1 gene sequence, a thymine-to-guanine transversion at position 1805 was among the most common (13). It was found in 50% of European Caucasians, but in only 8% of Africans and none of Asians (13). The 1805TT to 1805GG transversion changes the amino acid at position 602 from an isoleucine (602II) to a serine (602SS). Compared with homozygous 1805TT or heterozygous TG, which are functionally similar, homozygous 1805GG leads to decreased numbers of TLR1 on the cell surface (14), impaired downstream signaling, and less cytokine production (13, 14). This less intense inflammatory response was associated with protection from tuberculoid leprosy (14, 21). This polymorphism would be expected to have a substantial impact on function and disease expression because of its location in the transmembrane domain of the receptor and its high frequency in Caucasian populations.

TLR2-2258GA and TLR5-1174CT are each found in about 10% of the Caucasian population (12, 15, 20). The TLR2 polymorphism, which results in an arginine-to-glutamine transversion at amino acid position 753 in the intracellular domain of the receptor, was associated with less cytokine production in vitro and reduced frequency of Lyme borreliosis in Europe (15). The TLR5 polymorphism, which resulted in a transversion of arginine to a stop codon at amino acid position 392 in the extracellular domain, abolished flagellin signaling in a transfected cell line (11, 20). This polymorphism was associated with down-regulated immune responses in Crohn's disease and lupus (17, 19), but did not seem to alter disease susceptibility in typhoid fever or chronic obstructive lung disease (18, 22).

In this study, we determined the frequency of TLR1-1805GG, TLR2-2258GA, and TLR5-1174CT in 248 patients from the northeastern U.S. with EM or antibiotic-responsive or antibiotic-refractory Lyme arthritis. *B. burgdorferi* has a number of outer-surface lipoproteins (Osps) with the Pam_3CSK_4 modification (23–[26]), which stimulate the TLR1/TLR2 heterodimer on APCs, and the organism has a number of flagellar proteins (24, 25), which presumably stimulate TLR5. Whereas the TLR2 and TLR5 polymorphisms were not significantly different in frequency or function among the Lyme disease groups, the TLR1-1805GG polymorphism in *B. burgdorferi* RST1-infected patients was associated with

stronger T_H1-like inflammatory responses, more symptomatic infection, and antibiotic-refractory arthritis.

PATIENTS AND METHODS

Study patients

TLR polymorphisms were determined using PBMC from 248 patients with EM or antibiotic-responsive or antibiotic-refractory Lyme arthritis. All patients met the criteria of the CDC for the diagnosis of Lyme disease (27). The Human Investigations Committees at Tufts Medical Center (1987–2002) and Massachusetts General Hospital (2002–2008) approved the studies, and all patients provided written informed consent.

From November 1987 through January 2008, 177 consecutive patients with Lyme arthritis (ages 12–79 years) who were treated with antibiotics according to the guidelines now recommended by the Infectious Diseases Society of America (28) were referred to us before, during, or after antibiotic treatment. Of the 177 patients, 101 had antibiotic-refractory arthritis and 76 had antibiotic-responsive arthritis. This distribution of refractory and responsive cases is reflective of our role as a referral center. These patients had pain and swelling of knees and positive IgG antibody responses to *B. burgdorferi*, determined by ELISA and western blotting (5). As a part of the study “Immunity in Lyme Arthritis”, patients’ serum samples, PBMC, and if available, joint fluid samples were obtained and frozen for subsequent determinations.

During the summers of 1998 through 2001, 115 patients with physician-identified EM were recruited at two field sites, one in Wakefield, RI (by N. Damle) and the other in East Lyme, CT (by V. J. Sikand), for a study of the diagnosis and pathogenesis of early Lyme disease. Of the 115 patients with EM, 93 had culture-confirmation of *B. burgdorferi* infection from skin biopsy specimens, and 71 of the culture-confirmed cases had PBMC available for TLR polymorphism typing.

Genotyping

PCR amplification-restriction fragment length polymorphism (RFLP) assays were used to determine the genotypes of TLR1 at position 1805 (13), TLR2 at position 2258 (15, 16), and TLR5 at position 1174 (20) (Figure 1). Frozen PBMC (5×10^6 cells/ml) were thawed in a 37°C water bath and washed with 50 ml of PBS. Total genomic DNA was isolated from washed cells using QIAamp DNA Mini Kit (Qiagen,Valencia, CA). Isolated genomic DNA (~50 ng) from each patient was amplified in a 50 μl reaction containing 200 μM of dNTP (Fisher Scientific, Springfield, NJ), 0.5 μM of forward and reverse PCR primers (IDT, Eugene, OR), and 2.5 units of HotStarTaq DNA polymerase (Qiagen). PCR reactions were performed with the following primers: for TLR1, the forward primer was CTTGATCTTCACAGCAATAAAATAAAGAGCATT and reverse primer was GGCCATGATACACTAGAACACACATCACT (13); for TLR2, the forward primer was GCCTACTGGGTGGAGAACCT and the reverse primer was GGCCACTCCAGGTAGGTCTT (15), and for TLR5, the forward primer was GGTAGCCTACATTGATTTGC, and the reverse primer was GAGAATCTGGAGATGAGGTACCCG (20). The PCR products were digested with PstI for TLR1, AciI for TLR2, and DdeI for TRL5 for 2h at 37°C. Undigested and digested PCR products for TLR1-1805GG, TLR2-2258GA, and TLR5-1174CT genotypes were electrophoresed on 1.5%, 2% and 3% agarose gels, respectively, and stained with ethidium bromide.

Determination of B. burgdorferi RST and OspC genotypes

In patients with EM, the *B. burgdorferi* 16s–23s rRNA intergenic spacer type (RST) genotype of the infecting strain was determined from skin biopsy isolates using nested PCR and RFLP, and the OspC type was determined using semi-nested PCR and sequencing techniques (29). The number of spirochetes in 1.5 mm biopsy samples was determined by QPCR (9). Since it is nearly impossible to culture *B. burgdorferi* from synovial fluid, the RST and OspC types were identified, when possible, from small amounts of spirochetal DNA sometimes present in joint fluid using semi-nested PCR, sequencing and RFLP analysis (6).

Cell culture of PBMC

For cell-culture experiments, an RST1 OspC type A isolate was grown to mid log phase in complete BSK medium (Sigma Aldrich, St. Louis MO) containing 6% rabbit serum. Spirochetes were washed 3 times in PBS and the numbers of organisms determined by optical density, as previously described (7, 30). PBMC were available from 41 of the 49 patients with Lyme arthritis in whom joint fluid samples were available. After thawing in 37°C water bath, cells were washed and counted using trypan blue exclusion. Viable cells were resuspended in RPMI medium supplemented with 10% human serum and 2mM L-glutamine, seeded at 2×10^5 cells per well onto round-bottom 96-well plates, and incubated with a *B. burgdorferi* RST1 isolate at a multiplicity of infection (MOI) of 25 organisms per cell for 5 days at 37°C and 5% CO_2. We have previously determined that these conditions were optimal for induction of the IFNγ-inducible chemokines by PBMC (data not shown).

Cytokine and chemokine determinations

PBMC from 41 patients with Lyme arthritis were cultured with *B. burgdorferi* RST1 strain (OspC type A) or Pam_3CSK_4 (InvivoGen, San Diego, CA) (300ng/ml) for 5 days in RPMI medium supplemented with 10% human serum and 2mM L-glutamine in absence of antibiotics. After incubation, protein levels of 6 chemokines (IL-8, CCL2, CCL3, CCL4, CXCL9 and CXCL10) and 6 cytokines (TNF, IL-1β, IL-6, IL-10, IFNγ and IFNα) were determined in cell culture supernatants (1:25 dilution) using bead-based multiplex assays (Millipore, Billerica MA) coupled with the Luminex-200 Analyzer (Luminex, Austin TX) and Upstate Beadview software (Millipore). In addition, the levels of 7 chemokines (IL-8, CCL2, CCL3, CCL4, CCL5, CXCL9, CXCL10 and CXCL13) and 5 cytokines (IL-1β, IL-6, IL-10, TNF, IFNγ) were measured in serum samples (undiluted) from 66 EM patients and in joint fluid samples (diluted 1:5) from 49 Lyme arthritis patients, using multiplex assays. Because the amounts of serum and joint fluid were limited, cytokine and chemokine levels in these samples were determined once.

Statistics

The frequencies of TLR1, 2 or 5 genotypes were compared in patients with EM, or antibiotic-refractory or antibiotic-responsive arthritis by chi-square analysis. Comparison of patients with symptoms or no symptoms was also performed using chi-square analysis. Cytokine and chemokine levels in serum from EM patients, in joint fluid from Lyme arthritis patients, or in supernatants from PBMC were compared by non-parametric Mann-Whitney rank sum test. SigmaStat version 3.0.1 software from SPSS was used for all statistical analyses. A P value of ≤ 0.05 was considered statistically significant.

RESULTS

Frequencies of TLR genotypes in patients with Lyme disease

The 248 Caucasian patients with EM were evaluated at field sites and those with antibiotic-responsive or antibiotic-refractory Lyme arthritis were seen in our Lyme disease clinic over a 20-year period. All patients acquired the infection in the northeastern U.S. The frequencies of TLR1-1805GG, TLR2-2258GA, and TLR5-1174CT polymorphisms are shown in Figure 1. In a previous study (13), about half of a normal European Caucasian population had heterozygous TLR1-1805TG or the considerably less common homozygous TT, whereas the other half had the homozygous 1805GG polymorphism (Figure 1A). Similarly, in this study, the 1805GG polymorphism was found in 51% of the patients with EM. In contrast, the frequency of 1805GG was slightly less (47%) in those with antibiotic-responsive arthritis, but significantly greater (62%) in those with antibiotic-refractory arthritis. When the groups were compared, patients with antibiotic-refractory arthritis had a higher frequency of TRL1-1805GG than patients with EM (62% vs. 49%, odds ratio = 1.7, P=0.1) or antibiotic-responsive arthritis (62% vs. 47%, odds ratio = 1.9, P=0.05).

In a previous study (15), 88% of a Caucasian population had the TLR2-2258GG genotype, whereas 12% had the GA polymorphism at this position. In this study, 10% of the patients with EM had 2258GA, whereas 3% of the patients with antibiotic-responsive arthritis and 8% of those with antibiotic-refractory arthritis had this polymorphism, but these differences were not statistically significant (Figure 1B). Previously, about 90% of a Caucasian population had TLR5-1174CC, and about 10% had the 1174CT polymorphism (20). This distribution of the TLR5 genotypes was observed in each Lyme disease patient group (Figure 1C).

Cytokine and chemokine levels in serum of patients with EM

We next determined the functional consequences of these polymorphisms in patients with various manifestations of Lyme disease. Serum samples, obtained a median of 3 days after disease onset, were available from 66 of the 71 patients with EM in whom TLR polymorphisms were determined. Among the 66 EM patients, those with TLR1-1805GG had significantly higher levels of the IFN-γ inducible chemokines, CXCL9 and CXCL10, and tended to have higher levels of IL-6 (Figure 2A). Moreover, when patients were also stratified according to the *B. burgdorferi* genotype of the infecting strain, those with TLR1-1805GG who were infected with RST1 strains (also called OspC types A and B) (Figure 2B) had strikingly higher levels of CXCL9, CXCL10 and IL-6 than other patients. In addition, among patients infected with RST3 strains, the most diverse RST type (6, 29, 31), there was a trend toward higher levels of CXCL9 and CXCL10 among those with 1805GG, which was attributable to infection with an RST3 subtype OspC type I (data not shown) (7). In contrast, when chemokine and cytokine levels were stratified according to the TLR2-2258GA or TLR5-1174CT polymorphism, the values were similar in those with or without the polymorphism (data not shown).

Clinical correlations in patients with EM

In the northeastern U.S., EM is often accompanied by headache, neck stiffness, myalgias, arthralgias, fever, malaise and fatigue. When the presence or absence of these associated symptoms was correlated with chemokine and cytokine levels, the protein expression of IL-6, IFNγ, CXCL9 and CXCL10 was significantly higher in those with symptomatic illness (Figure 3A). Moreover, all 18 RST1-infected patients with the 1805GG polymorphism had symptoms compared with about 60–70% of patients in all other groups, including RST1-infected patients with 1805TG/TT and all RST2- or RST3-infected patients (Figure 3B). The difference between RST1-infected patients with 1805GG and each of the

groups with 1805TG/TT was statistically significant (in each instance, P<0.01). However, the numbers of organisms in EM lesions were similar regardless of the TLR1 polymorphism or *B. burgdorferi* genotype (Figure 3C). Moreover, the frequencies of disseminated infection, defined by a positive PCR result for *B. burgdorferi* DNA in blood or multiple EM lesions, did not differ significantly according to these host and spirochetal genotypes (data not shown). Thus, early in the illness, RST1-infected patients with TLR1-1805GG, which affects cells of the innate immune system, had greater adaptive T_H1-like inflammatory responses, and they were more likely to have symptomatic infection despite having similar numbers of spirochetes in EM skin lesions and similar frequency of disseminated infection.

Chemokine and cytokine levels in joint fluid of patients with Lyme arthritis

In a previous study (32), elevated levels of cytokines and chemokines in patients with Lyme arthritis were found only in joint fluid and not in serum samples. For this study, joint fluid samples were available in 49 of the 177 patients in whom TLR polymorphisms were determined. Of the 49 patients, 16 had antibiotic-responsive arthritis. Joint fluid samples in this group could only be obtained during the infectious period, prior to or soon after the start of antibiotics since joint effusions resolved soon thereafter. The remaining 33 patients had antibiotic-refractory arthritis and were referred to us because of lack of response to 1 or more courses of antibiotics. Thus, these samples were usually obtained in the post-antibiotic period, a median of 4 months (range, 0–23 months) after the start of antibiotics.

Despite differences in timing, the 33 patients with antibiotic-refractory arthritis, 22 (67%) of whom had TLR1-1805GG, had significantly higher levels of almost all of the chemokines and cytokines measured than the 16 patients with antibiotic-responsive arthritis, 8 of whom (50%) had 1805GG (Figure 4A). The refractory group had especially high levels of CXCL9, CXCL10, and IL-6. The levels of CXCL9 and CXCL10 were approximately 2-fold higher in refractory versus responsive patients, and the levels of these mediators were about 15-fold higher in patients with refractory arthritis than in those with EM (Fig. 2A).

Since differences in cytokine and chemokine levels in EM patients were found primarily in those with TLR1-1805GG who were infected with *B. burgdorferi* RST1 strains, we sought to stratify patients with Lyme arthritis according to both of these parameters. Because it has been nearly impossible to culture *B. burgdorferi* from joint fluid, we were only able to determine the *B. burgdorferi* genotype in 14 patients, 10 with antibiotic-refractory arthritis and 4 with antibiotic-responsive arthritis, in whom enough *B. burgdorferi* DNA was present in joint fluid to allow successful PCR amplification and RFLP analysis (6).

When chemokine and cytokine levels in the 10 patients with antibiotic-refractory arthritis (all of whom had 1805GG) were stratified according to *B. burgdorferi* genotype, the 5 patients who had been infected with RST1 strains had higher levels of most of the chemokines and cytokines measured, particularly of IFNγ, CCL2 and CXCL9, than those who had been infected with RST2 or RST3 strains (Figure 4B). The levels of CXCL9 in patients with 1805GG and antibiotic-refractory arthritis who had been infected with RST1 strain were the highest measured (median value, 263,000 pg/ml) in any group in this study. Moreover, the median duration from the start of antibiotics to resolution of arthritis among RST1-infected patients was 15 months (range 4–30 months) compared with 9 months (range 4–10 months) among those with RST2 or RST3 infection. However, because of small numbers, these differences in levels of inflammatory mediators or in duration of arthritis were not statistically significant. Because both the TLR2 and TLR5 polymorphisms were found in only a small percentage of patients, some of whom also had the TLR1 polymorphism, a meaningful comparison of cytokine and chemokine levels in these patients was not possible.

Stimulation of PBMC from patients with Lyme arthritis

Of the 49 patients in whom joint fluid was tested, 41 had PBMC available to assess the results of stimulation with a *B. burgdorferi* RST1 strain. In these experiments, in which cells were stimulated with similar numbers of organisms of the same strain, the chemokine and cytokine expression was similar in cells from patients with antibiotic-responsive or antibiotic-refractory arthritis, and therefore, the results were combined for presentation here. The PBMC from 12 patients with EM showed little, if any, recall response to *B. burgdorferi* antigens, and we did not test them further.

Among the 41 patients with Lyme arthritis, unstimulated PBMC secreted only basal amounts of each of the cytokines and chemokines tested regardless of the TLR1-1805GG genotype. When cells were stimulated with Pam3CSK4, the specific agonist for TLR1/TLR2, PBMC from those with 1805TG/TT had significantly greater fold-increase in the levels of innate immune response-associated mediators, IL-1β, IL-10, and CCL3, than patients with the 1805GG polymorphism (Figure 5A), and there was a trend in that direction for other chemokines and cytokines.

In contrast, when PBMC were stimulated with a *B. burgdorferi* RST1 isolate, most chemokines and cytokines, particularly the T_H1-like adaptive, immune response-associated mediators, IFNγ and the IFNγ-inducible chemokines CCL2, CXCL9 and CXCL10, were produced at a higher level in the 1805GG group ($P<0.001$) (Figure 5B). In contrast, cells from patients with either 1805GG or TG/TT produced only basal levels of IFNα. Thus, consistent with the results in joint fluid, *B. burgdorferi* RST1 stimulation of PBMC from patients with TLR1-1805GG strongly induced the secretion of IFNγ and the IFNγ-inducible chemokines, especially CXCL9, which seems to be important in setting the stage for antibiotic-refractory arthritis.

DISCUSSION

In this study, we assessed 248 patients, including 177 with Lyme arthritis, which represents the largest cohort available of patients with this manifestation of the illness. Patients with antibiotic-refractory arthritis had an increased frequency of a TLR1 polymorphism (1805GG) compared with patients with EM (OR = 1.7, P = 0.1) or antibiotic-responsive arthritis (OR = 1.9, P = 0.05). These P values are of borderline statistical significance, but the odds ratios are typical of what is found in rheumatoid arthritis or other autoimmune diseases in which multiple genetic factors (in this instance both spirochetal and host genetic factors) influence the outcome of the disease (33). More importantly, we found a functional difference in immune response and disease expression associated with TLR1-1805GG. Early in the illness, patients with EM who had 1805GG, primarily those infected with *B. burgdorferi* RST1 strains, had strikingly higher serum levels of IL-6, CXCL9 and CXCL10, and more symptoms than patients with 1805TG/TT. Although EM skin lesions from these patients were not available for analysis here, we have previously shown that these inflammatory mediators are also prominent in EM skin lesions (34, 35). Thus, early in the illness, RST1-infected patients with TLR1-1805GG, which affects cells of the innate immune system, had greater adaptive T_H1 inflammatory responses, and they were more likely to have symptomatic infection. In contrast, we did not find significant differences in frequency or function according to the TLR2-2274GA or TLR5-1174CT polymorphism.

The chemokine-cytokine profile in patients with EM who had TLR1-1805GG was amplified in joint fluid in patients with Lyme arthritis, particularly in those with 1805GG who had antibiotic-refractory arthritis. In these patients, the levels of CXCL9 and CXCL10 were at least 15-fold higher than in serum of patients with EM. Moreover, when PBMC from patients with Lyme arthritis were stimulated with the same *B. burgdorferi* RST1 strain, most

chemokines and cytokines, particularly IFNγ and the IFNγ-inducible chemokines, were produced at a higher level in the 1805GG group than in the TG/TT group, which demonstrated in cell culture the same cytokine-chemokine profile seen in patients. We previously demonstrated that synovial tissue from patients with antibiotic-refractory arthritis also had exceptionally high levels of CXCL9 and CXCL10 (32), which are chemoattractants for CD4+ and CD8+ T effector cells. These cells are the most numerous infiltrating cells in synovial lesions in antibiotic-refractory Lyme arthritis and in other forms of chronic inflammatory arthritis (36, 37).

This scenario is reminiscent of findings in *B. burgdorferi*-infected, TLR2-deficient C57BL/6 mice (38, 39). Compared with their wild-type counterparts, these mice developed more severe arthritis (the sole manifestation of the disease in mice), and their joint tissue had greater numbers of T cells, high levels of CXCL9 and CXCL10, and increased transcripts for a prototypical IFNγ-inducible gene, GTPase (*igtp*) (39). The authors concluded that TLR2 deficiency in *B. burgdorferi*-infected mice leads to increased numbers of T cells in joints, high levels of IFNγ, and overproduction of T cell attracting chemokines, CXCL9 and CXCL10, suggesting that the TLR2 pathway ordinarily modulates this IFNγ response. We did not see this effect in patients with the TLR2-2274GA polymorphism because it was present in only low frequency, it affected only one allele, and in contrast with the knockout mice, it did not abolish TLR2 function.

The TLR1/TLR2 heterodimer recognizes lipopeptides both intracellularly and on the cell surface (40). Activation of these receptors in different compartments may result in distinct cytokine and chemokine profiles (40). However, it is not yet clear how *B. burgdorferi* infection in humans with the 1805GG polymorphism or in mice with TLR2 deficiency leads to heightened T_H1-like inflammatory responses. In culture or murine models, the TLR1/2 receptor complex has been shown to regulate cytokine production mediated by TLR1/TLR2 as well as other receptors. For example, in a dendritic cell culture system, TLR2 has been shown to regulate TLR4- and TLR7/8-mediated cytokine production by inhibiting the type I interferon amplification loop (41). Moreover, in *B. burgdorferi*-stimulated murine macrophages, a deficiency in a part of the TLR1/TLR2 complex resulted in persistent, elevated expression of inflammatory cytokines and chemokines due to decreased activation of p38 and reduced expression of the downstream suppressors of cytokine signaling (SOCS) proteins (42). Similarly, in our preliminary experiments, *B. burgdorferi*-stimulated PBMC from Lyme arthritis patients with 1805GG had significantly reduced p38 phosphorylation compared with cells from patients with TG/TT (data not shown). Therefore, a deficiency in TLR1 in human patients with 1805GG may cause decreased expression of p38 and SOCS, resulting in a loss of a balancing anti-inflammatory signal. Moreover, because *B. burgdorferi* stimulates multiple pathogen recognition receptors on innate immune cells (43), activation of other TLRs may cause a compensatory increase in a pro-inflammatory signal. In either case, this could result in higher levels of IFNγ and IFNγ-inducible chemokines seen here in patients with 1805GG.

We think that antibiotic-refractory arthritis is associated with TLR1-1805GG because this polymorphism is one of several factors that may lead to exceptionally high levels of IFNγ and IFNγ-inducible chemokines CXCL9 and CXCL10 in joint fluid and synovial tissue (32). We postulate that this heightened T_H1-like response leads to the recruitment of large numbers of CD4+ and CD8+ T effector cells, some of which may be autoreactive and not appropriately controlled by persistently low numbers of Treg (44). Since multiple factors may lead to exuberant Th1 responses, not all patients with antibiotic-refractory arthritis have the TLR1-1805GG polymorphism.

In conclusion, the northeastern United States is increasingly affected by *B. burgdorferi* infection (46, 47), and highly inflammatory RST1 (OspC types A or B) strains cause 30–50% of the infections in this region (29, 31). This *B. burgdorferi* genotype has a high transmission frequency among ticks (48), it may be increasing in frequency in nature, and it may be an important factor in the emergence of Lyme disease in epidemic form in the northeastern U.S. in the late 20th century (49, 50). We show here that a TLR1 polymorphism (1805GG), which is present in half of the Caucasian population, causes heightened T_H1 inflammatory responses primarily in *B. burgdorferi* RST1-infected patients. Thus, it is the combination of spirochetal and host genetic factors that leads to this host immune response in a subset of patients with Lyme disease. Rather than limiting infection-induced pathology, this heightened T_H1-like inflammatory immune response is associated with more symptomatic early infection, putative autoimmune phenomena, and antibiotic-refractory arthritis.

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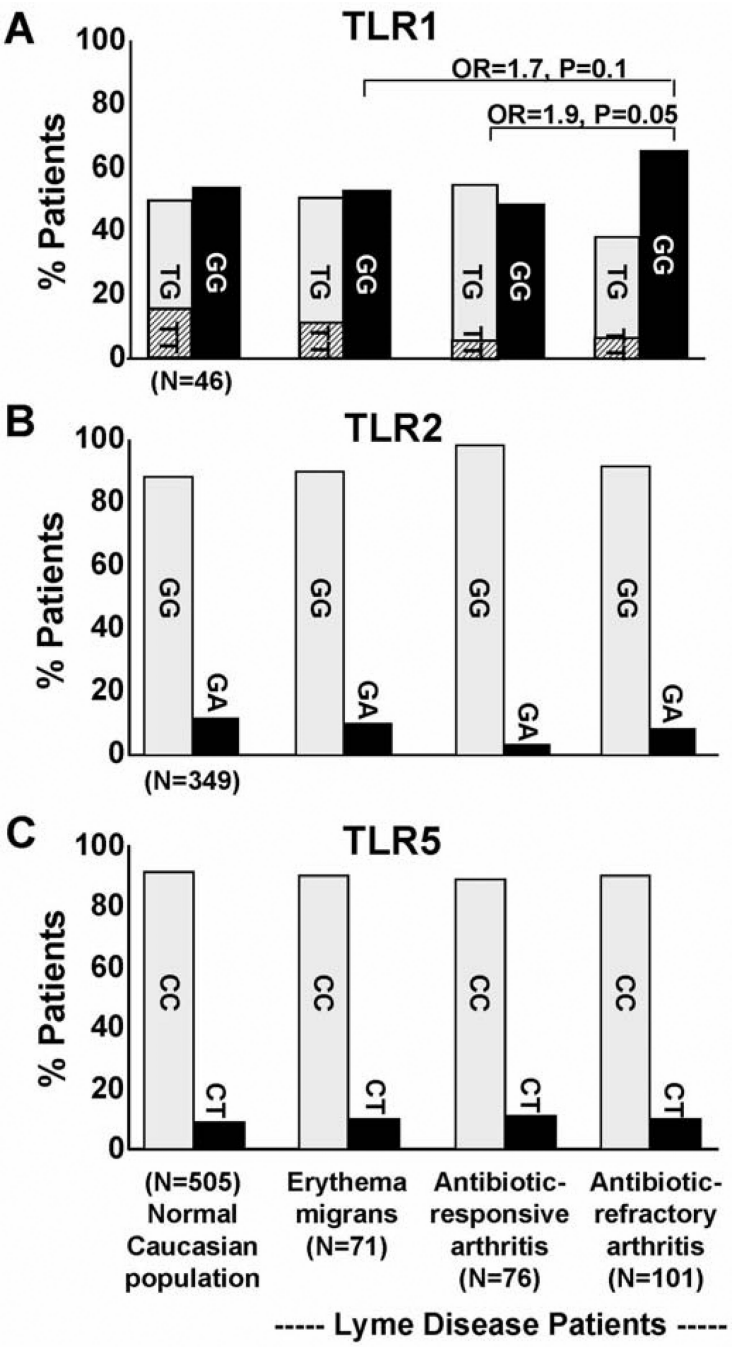

Figure 1.

Frequency of selected TLR polymorphisms in patients with Lyme disease. Frequencies of polymorphisms in panel **A,** TLR1 (T1805G), panel **B,** TLR2 (G2258A), and panel **C,** TLR5 (C1174T) are shown in 248 patients with Lyme disease, 71 with EM, 76 with antibiotic-responsive, and 101 with antibiotic-refractory Lyme arthritis and in normal Caucasian populations. As reported in the literature, the 46 normal subjects for TLR1 were from Seattle (15); the 349 normal subjects for TLR2 were from Germany (17), and the 505 normal subjects for TLR5 were primarily from the Netherlands (22). Each polymorphism was determined by PCR amplification and restriction fragment length polymorphism (RFLP) techniques.

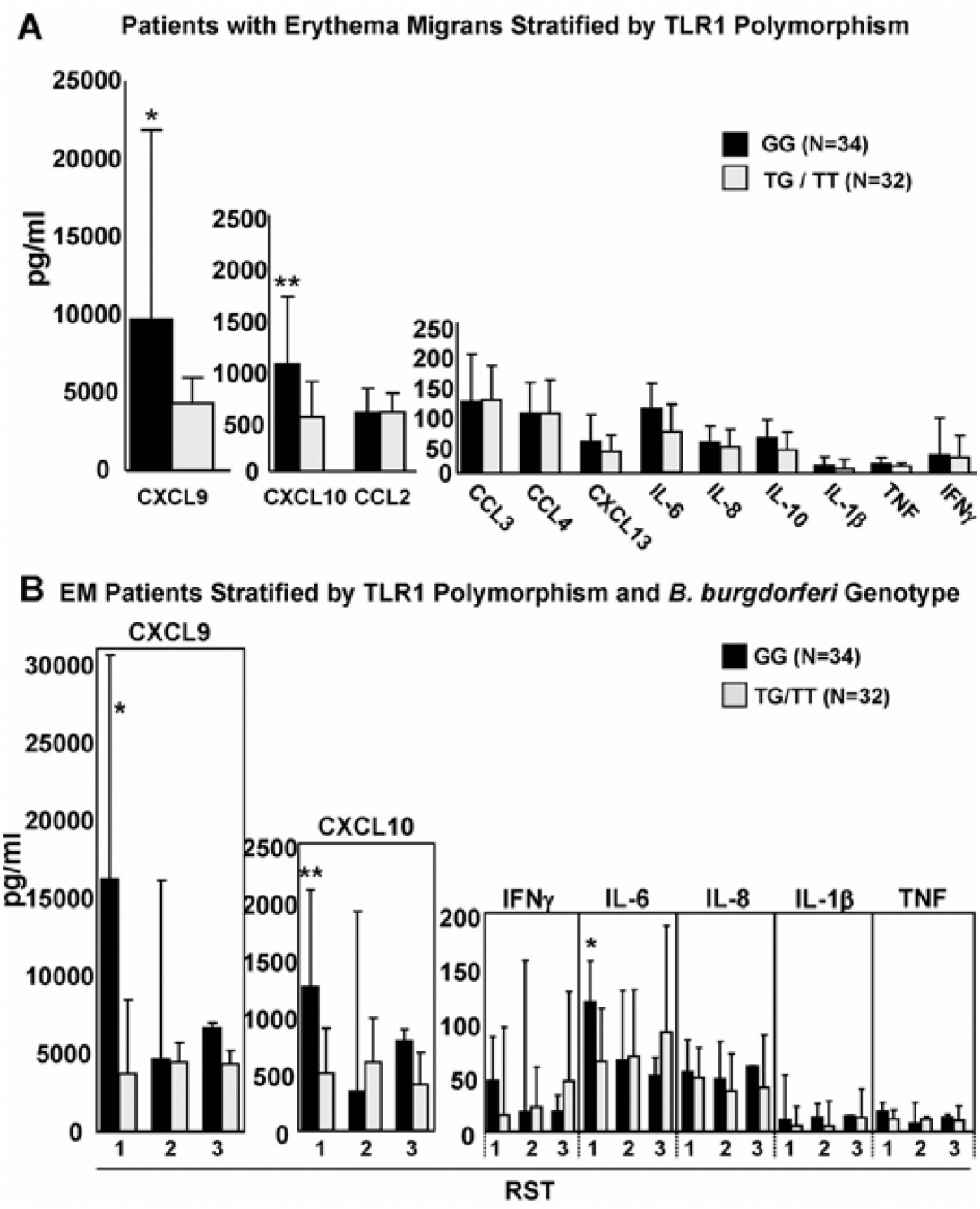

Figure 2.

Cytokine and chemokine levels in serum samples from patients with EM stratified by the TLR1-1805GG polymorphism. Protein levels of 7 chemokines and 5 cytokines were determined in sera from 66 patients with EM using bead-based multiplex assays. In panel **A,** the patients were first stratified according to TLR1-1805GG or 1805TG/TT genotypes. In panel **B,** the patients were further subdivided based on the *B. burgdorferi* RST genotype of the infecting strain. Of the 34 patients with 1805GG, 18 had RST1 infection, 14 RST2 infection, and 2 RST3 infection; of the 32 patients with 1805GT/TT, 12 had RST1 infection, 15 RST2 infection, and 5 RST3 infection. Bars show the median values and I-bars represent

the third quartile values. For comparison of 1805GG versus 1805TG/TT, *P ≤ 0.05, **P ≤ 0.01.

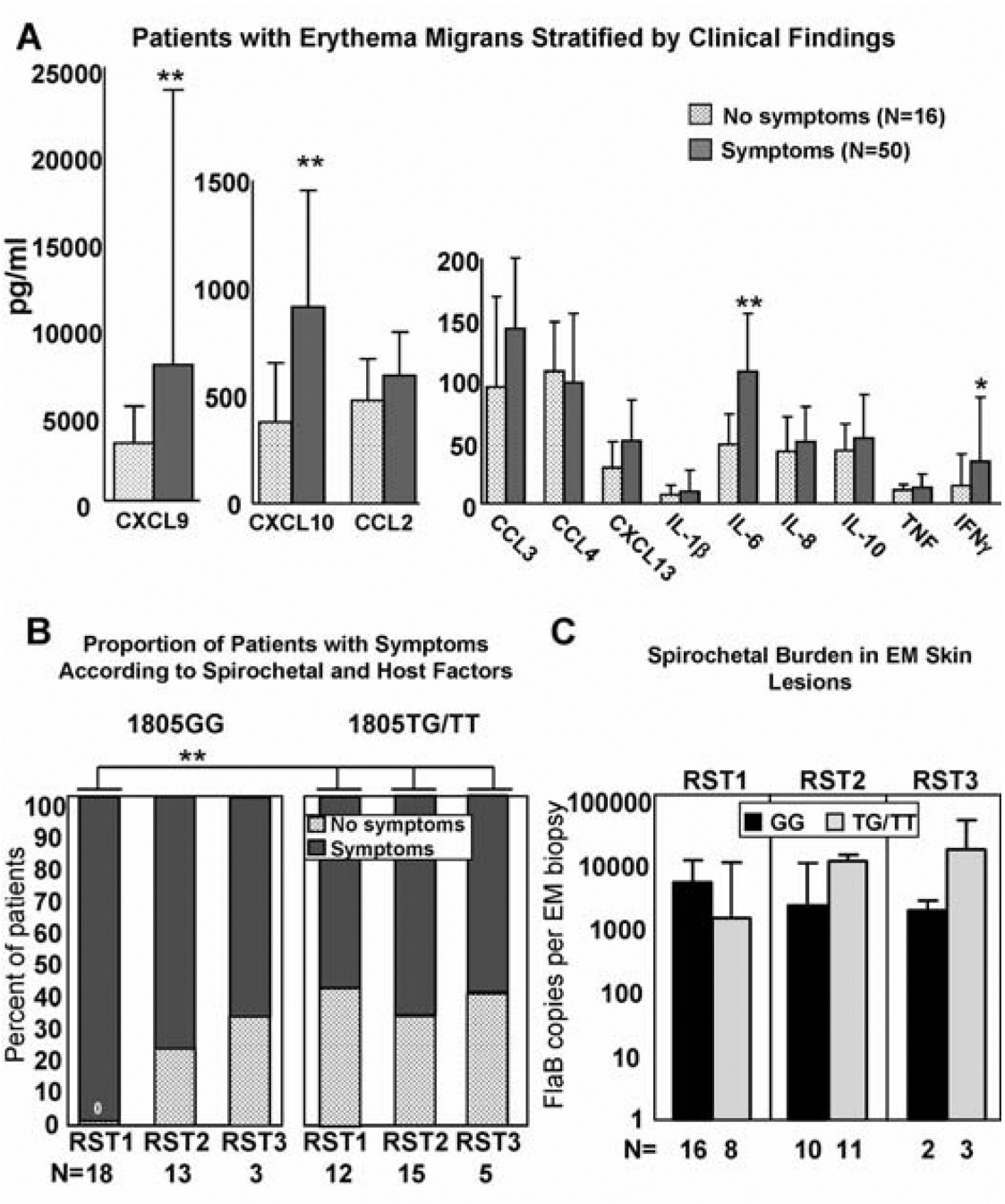

Figure 3.

Clinical correlations in patients with EM. In panel **A,** protein levels of 7 chemokines and 5 cytokines were compared in 66 patients according the presence or absence of symptoms. In panel **B,** the proportion of 66 patients with or without associated symptoms was correlated with the 1805GG polymorphism and RST genotype of the infecting strain. The differences between RST1-infected patients with 1805GG compared with RST1-, 2-, or 3-infected patients with 1805 TG/TT were statistically significant, whereas the differences among RST1-, 2-, or 3-infected patients with 1805GG were not. In panel **C,** the pathogen burden in EM skin lesions from 50 patients, quantified by QPCR detection of the *B. burgdorferi flaB*

gene, was stratified by the 1805GG polymorphism and RST genotype. In panels **A** and **C,** bars show median values and I-bars represent the third interquartile range. For comparison of 1805GG versus 1805TG/TT, *P ≤ 0.05, **P ≤ 0.01.

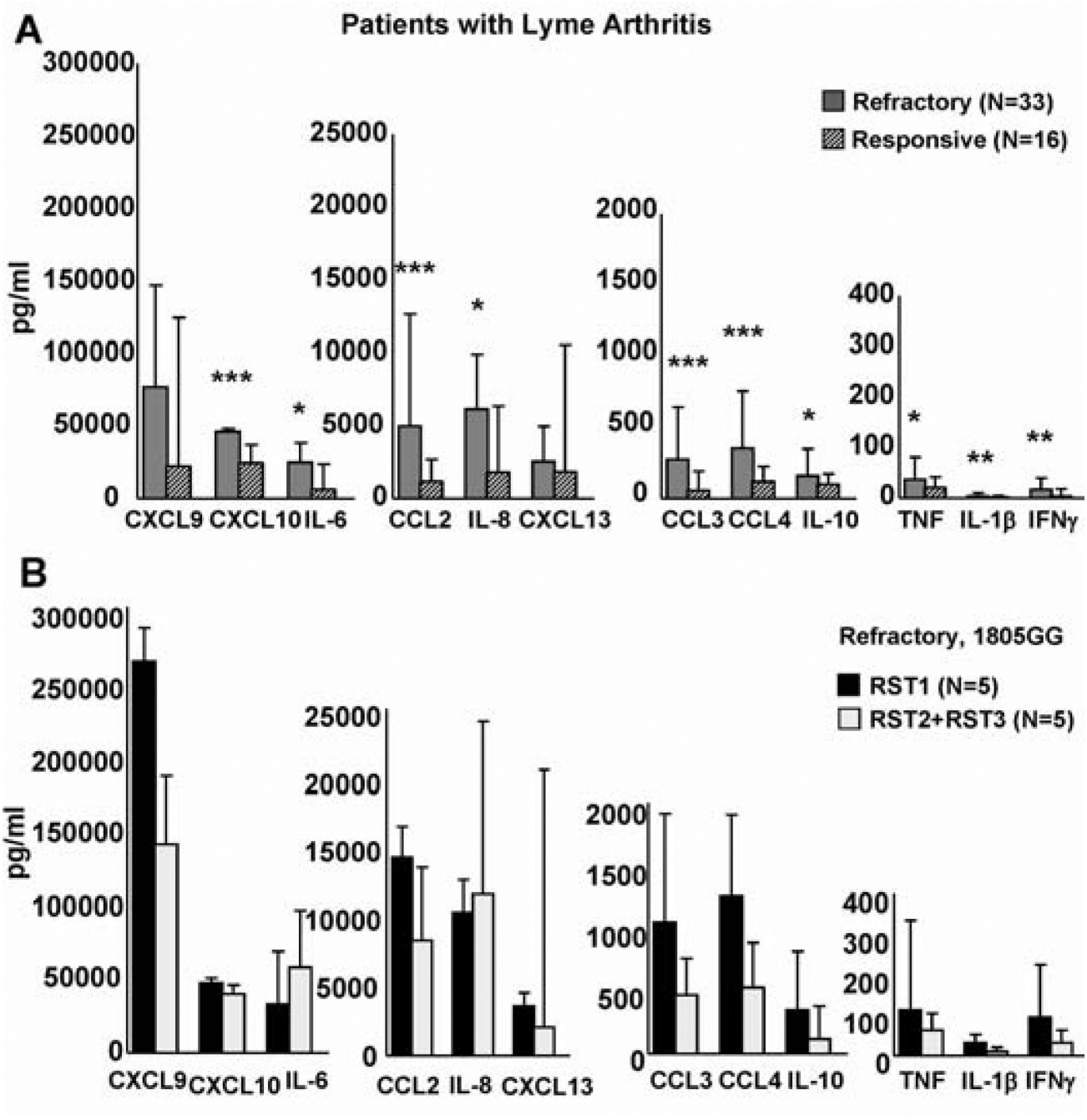

Figure 4.

Cytokine and chemokine levels in joint fluid of patients with Lyme arthritis. Protein levels of 7 chemokines and 5 cytokines were measured in joint fluid samples from 49 patients with Lyme arthritis using bead-based multiplex assays. In panel **A,** the patients were stratified in antibiotic-responsive or antibiotic-refractory groups. In panel **B,** the 10 patients with antibiotic-refractory arthritis, all of whom had 1805GG, were stratified according to the infecting strain. Because of small numbers, the RST2 and RST3 groups were combined for comparison with the RST1 group. Bars show median values and I-bars represent third interquartile ranges. For each comparison, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

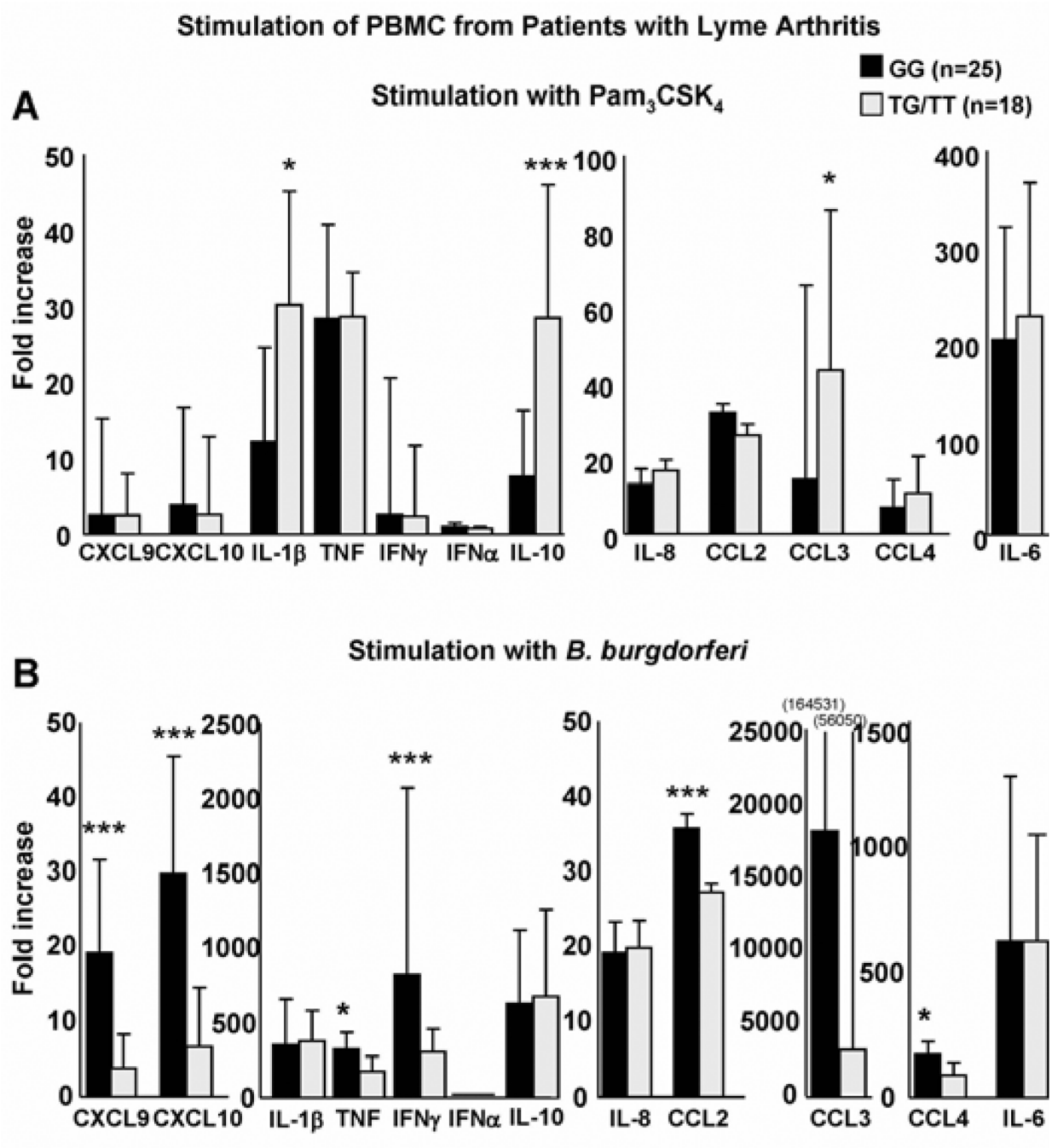

Figure 5.

Stimulation of PBMC from patients with Lyme arthritis. Protein levels of 6 chemokines and 6 cytokines in culture supernatants were assessed in PBMC from 43 patients with Lyme arthritis; 25 were 1805GG and 18 were TG/TT, using bead-based multiplex assays. Cells were stimulated with Pam3CSK4 (300 ng/ml) (panel **A**), or with an RST1 (OspC type A) isolate of *B. burgdorferi* at an MOI of 25 spirochetes per cell (panel **B**), in each instance for 5 days. Data are calculated as fold increase in levels of cytokines and chemokines (stimulated values divided by unstimulated values). Bars show median values and I-bars

represent third interquartile ranges. For comparison of 1805GG versus 1805TG/TT, *P ≤ 0.05, ***P ≤ 0.001.