Dysregulation of CD4+CD25^{high} T Cells in the Synovial Fluid of Patients With Antibiotic-Refractory Lyme Arthritis

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**Objective.** To examine the role of immune dysregulation in antibiotic-refractory Lyme arthritis by comparing the phenotype, frequency, and function of CD4+ T effector (Teff) cells and T regulatory (Treg) cells in patients with antibiotic-responsive arthritis and patients with antibiotic-refractory arthritis.

**Methods.** Matched peripheral blood and synovial fluid samples from 15 patients with antibiotic-responsive arthritis were compared with those from 16 patients with antibiotic-refractory arthritis, using flow cytometry, suppression assays, and cytokine assays.

**Results.** Critical differences between the 2 patient groups were observed in the synovial fluid CD4+CD25^{high} population, a cell subset usually composed of FoxP3-positive Treg cells. In patients with antibiotic-refractory arthritis, this cell population often had fewer FoxP3-positive cells and a greater frequency of FoxP3-negative (Teff) cells compared with patients with antibiotic-responsive arthritis. Moreover, the expression of glucocorticoid-induced tumor necrosis factor receptor and OX40 on CD4+CD25^{high} cells was significantly higher in the antibiotic-refractory group. Suppression assays showed that CD4+CD25^{high} cells in patients with antibiotic-refractory arthritis did not effectively suppress proliferation of CD4+CD25^{-} cells or secretion of interferon-\(\gamma\) and tumor necrosis factor \(\alpha\), whereas those cells in patients with antibiotic-responsive arthritis did suppress proliferation of CD4+CD25^{-} cells and secretion of interferon-\(\gamma\) and tumor necrosis factor \(\alpha\). Finally, in the antibiotic-refractory group, higher ratios of CD25^{high} FoxP3-negative cells to CD25^{high} FoxP3-positive cells correlated directly with a longer duration of arthritis after antibiotic treatment.

**Conclusion.** Patients with antibiotic-refractory Lyme arthritis often have lower frequencies of Treg cells, higher expression of activation coreceptors, and less effective inhibition of proinflammatory cytokines. This suggests that immune responses in these patients are excessively amplified, leading to immune dysregulation and antibiotic-refractory arthritis.

There is increasing interest in the role of infection in triggering autoimmune diseases (1,2). In the setting of infection, a proinflammatory response is induced to protect the host, which includes the activation and expansion of innate and adaptive immune cells. However, this proinflammatory response must be properly down-regulated once the pathogen is controlled or eliminated, in order to maintain tolerance and limit tissue pathology. In some individuals, these regulatory mechanisms do not function optimally, leading to pathogenic autoimmunity. Therefore, identifying quantitative and qualitative differences in immune cells between patients in whom the immune response is properly down-regulated after infection and those in whom the immune response is not properly down-regulated is critical to our understanding of infection-induced autoimmunity.
Lyme arthritis, which is a late-stage manifestation of infection with the tick-borne spirochete *Borrelia burgdorferi* (3), provides a human model of infection that may lead to these 2 alternative outcomes (4). In most patients, Lyme arthritis can usually be treated successfully with antibiotics; this is called antibiotic-responsive arthritis (5,6). However, in a small percentage of patients, proliferative synovitis may persist for ≥3 months after oral and intravenous antibiotic therapy; this is called antibiotic-refractory arthritis (7). This latter outcome is postulated to result from persistent infection, retention of spirochetal antigens, infection-induced autoimmunity, or a combination of these factors.

In animal models, a small number of attenuated spirochetes may survive despite antibiotic therapy for 1 month (8). Additionally, in a myeloid differentiation factor 88-deficient (MyD88<sup>−/−</sup>) mouse model, in which a high pathogen load develops, spirochetal antigens are retained near cartilage surfaces after antibiotic therapy (9). However, in patients with antibiotic-refractory arthritis, culture and polymerase chain reaction results for *B burgdorferi* in synovial tissue have been uniformly negative after ≥3 months of antibiotic treatment (10); disease-modifying antirheumatic drugs (DMARDs) used to treat patients with other autoimmune joint diseases have often been successful in resolving synovitis in these patients (7); specific HLA–DR alleles (particularly DRB1*0101 and DRB1*0401) are the greatest of the known genetic risk factors for a treatment-refractory outcome (11), a risk factor commonly associated with autoimmune diseases; and a subset of patients have autoreactivity to endothelial cell growth factor, the first autoantigen known to induce T cell and B cell responses in patients with Lyme disease (12). Therefore, we postulate that patients with antibiotic-refractory Lyme arthritis are unable to properly down-regulate their immune response despite apparent spirochetal killing due to excessive inflammation, immune dysregulation, and infection-induced autoimmunity.

Previously, our group showed that in patients with antibiotic-refractory arthritis, the percentage of CD4<sup>+</sup> FoxP3-positive Treg cells in synovial fluid (SF) correlated inversely with the duration of arthritis following antibiotic treatment (13), implying that lower numbers of Treg cells led to slower resolution of arthritis. Furthermore, suppression assays using cells from 2 patients with antibiotic-refractory arthritis showed that Treg cells from either peripheral blood (PB) or SF suppressed Teff cell proliferation equally well, but Teff cells from SF were more resistant to suppression compared with their PB counterparts. However, in that study, the expression of FoxP3 within various CD4<sup>+</sup> CD25<sup>+</sup> T cell subpopulations, the expression of activating or inhibitory T cell coreceptors, and the ability of Treg cells from these patients to suppress cytokine secretion were not determined.

In the current study, we compared the frequency, phenotype, and function of immune cells in the PB and SF of patients with antibiotic-responsive Lyme arthritis and patients with antibiotic-refractory Lyme arthritis. Critical differences between the 2 patient groups were observed in the CD4<sup>+</sup>CD25<sup>high</sup> T cell population in SF. This cell population in the antibiotic-refractory group often had lower frequencies of FoxP3-positive Treg cells, higher expression of activation coreceptors, and less effective inhibition of proinflammatory responses, leading to immune dysregulation and persistent synovitis.

**PATIENTS AND METHODS**

**Patients.** Synovial fluid mononuclear cells were available from 31 patients (15 with antibiotic-responsive Lyme arthritis and 16 with antibiotic-refractory Lyme arthritis) who were evaluated at our clinic between 2000 and 2010 (Table 1). Concomitant PB mononuclear cells were also available from 27 of the 31 patients. For comparison, PB samples were collected from 13 healthy control subjects.

The study, “Immunity in Lyme arthritis,” was approved by the Human Investigation Committees at Tufts Medical Center (2000–2002) and Massachusetts General Hospital (2002–2010). All patients met the Centers for Disease Control and Prevention criteria for the diagnosis of Lyme disease (14) and received antibiotic therapy according to the guidelines of the Infectious Diseases Society of America (15). After receiving antibiotic therapy, patients with antibiotic-refractory arthritis were treated with DMARDs or, sometimes, with synovectomies (7).

**Flow cytometric analysis of immune cell phenotype.** Peripheral blood cells or SF cells from all patients were stored in liquid nitrogen. For analysis, cells were resuspended in staining buffer (phosphate buffered saline with 2% fetal bovine serum). The cell Fc receptors were blocked by incubation with a human IgG antibody for 10 minutes at 4°C. Incubations with all subsequent antibodies were performed at room temperature and in the dark. After staining, cells were washed, fixed with 1% paraformaldehyde, and stored at 4°C until analyzed.

To enumerate the frequency of various immune cell types, cells were stained with anti-CD14, anti-CD19, anti-CD303, and anti–HLA–DR antibodies for 15 minutes, followed by anti-CD3, anti-CD4, and anti-CD11c antibodies for another 15 minutes. For Th1/Th2 cell polarization, cells were stimulated with phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin (1 μg/ml) in the presence of GolgiStop (BD Biosciences) for 5 hours at 37°C in 5% CO<sub>2</sub>. Afterward, cells were washed and incubated with anti-CD3 and anti-CD4 antibodies for 15 minutes, followed by fixation and permeabilization according to the manufacturer’s instructions (BD Biosciences).
IMMUNE DYSREGULATION AND ANTIBIOTIC-REFRACTORY LYME ARTHRITIS 1645

and stained with anti-interferon-γ (anti-IFNγ) and anti-interleukin-4 (anti-IL-4) antibodies for 30 minutes at 4°C. The memory phenotype and activation status of CD4+ T effector (Teff) cells were determined by staining with anti-CD45RO, anti-CCR7, anti–HLA–DR, and anti-OX40 antibodies for 15 minutes, followed by anti-CD3, anti-CD4, anti-CD25, and anti-glucocorticoid-induced tumor necrosis factor (TNF) receptor (anti-GITR) for another 15 minutes. For determination of Treg cells and Treg/Teff cells and the expression of CTLA-4, cells were first stained with anti-CD3, anti-CD4, and anti-CD25 antibodies for 15 minutes. Cells were then fixed and permeabilized according to the manufacturer’s instructions for anti-FoxP3 (BD Biosciences). Afterward, cells were stained with anti-FoxP3 and anti–CTLA-4 antibodies for 30 minutes. All flow cytometry analyses were performed with a LSRFortessa instrument (BD Biosciences), and data were analyzed using FlowJo software.

Suppression assay experiments. Synovial fluid CD4+CD25high T cells and CD4+CD25— T cells were sorted using a FACSAria (Becton Dickenson). The sorted populations had a purity of >97%, as determined by flow cytometry. CD4+CD25— T cells (20,000 cells) were cocultured alone or with different ratios of CD4+CD25high T cells in the presence of soluble anti-CD3 antibody (0.5 μg/ml). Irradiated (5,000 rad) PB cells from a healthy control subject were used as feeder cells. The cell cultures were incubated at 37°C in 5% CO2 for 5 days and then pulsed with 3H-thymidine (1 μCi/well) for 18 hours. The suppressive capacity was determined by calculating the relative difference in proliferative response (mean of duplicate wells) between CD4+CD25— T cells cultured alone or in the presence of CD4+CD25high T cells. Cell supernatants from the proliferation assays were assessed for IFNγ, TNFα, and IL-10 using bead-based Multiplex assays (Millipore), as previously described (16).

Statistical analysis. Quantitative data were analyzed by Mann-Whitney U test for comparison between 2 groups. Correlations between Treg cell frequencies and the duration of arthritis were determined by Pearson’s correlation test. GraphPad Prism software was used for all analyses. All P values were 2-tailed. P values less than or equal to 0.05 were considered significant.

RESULTS

Enumeration of immune cells in PB and SF. Using flow cytometry, we enumerated the percentages of various immune cell populations in PB and SF from 15 patients with antibiotic-responsive arthritis and 16 patients with antibiotic-refractory arthritis (Table 1), and for comparison, in PB from 10–13 healthy control subjects. The cells from patients with antibiotic-responsive arthritis were obtained prior to or soon after the start of antibiotic therapy, when their joints were still infected with B burgdorferi. In contrast, cells from patients with antibiotic-refractory arthritis were collected near or soon after the completion of antibiotic therapy but before treatment with DMARDs, during the putative autoimmune phase of the disease.

In the nonlymphocyte gate, the majority of cells in PB were monocytes in both the antibiotic-responsive and antibiotic-refractory groups, whereas the percentages of these cells were far lower in SF (Figure 1A). In contrast, the frequencies of myeloid dendritic cells were significantly greater in SF than in PB in both patient groups. Although the differences were much less dramatic, there also tended to be more plasmacytoid dendritic cells in SF than in PB in both groups.

Table 1. Clinical and demographic characteristics and treatment regimens in patients with Lyme arthritis

<table>
<thead>
<tr>
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<th>Antibiotic responsive (n = 15)</th>
<th>Antibiotic refractory (n = 16)</th>
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<tbody>
<tr>
<td>Age, years</td>
<td>36 (14–53)</td>
<td>29 (12–62)</td>
</tr>
<tr>
<td>No. of men/no. of women</td>
<td>8/7</td>
<td>11/5</td>
</tr>
<tr>
<td>Duration of arthritis, months</td>
<td>Prior to start of antibiotics</td>
<td>0.5 (0–4)</td>
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<td></td>
<td>From start of antibiotics to sample date</td>
<td>0.5 (0–3)</td>
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<td>From start of antibiotics to resolution</td>
<td>3 (1–3)</td>
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<td>12 (5–34)†</td>
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* Except where indicated otherwise, values are the median (range). Antibiotic-responsive arthritis was defined as resolution of arthritis within 3 months after ≥8 weeks of oral antibiotics or ≥4 weeks of intravenous antibiotics. Antibiotic-refractory arthritis was defined as persistent joint swelling for ≥3 months after the start of ≥8 weeks of oral antibiotics or ≥4 weeks of intravenous antibiotics, or usually both. † P < 0.0001 versus antibiotic responsive.

In the lymphocyte gate, the predominant cell type was CD4+ T cells (Figure 1B). When the polarization of CD4+ T cells was determined, the major cell type in the SF of both patient groups was CD4+ IFNγ-secreting T cells, and the frequencies of these cells were significantly higher in SF than in PB. In contrast, the percentages of CD4+ IL-4–secreting T cells were generally low. Additionally, although SF T cells from patients with Lyme arthritis were previously shown to express IL-17 in response to stimulation with B burgdorferi neutrophil-activating protein A (17), in our earlier study we showed that the percentages of IL-17–producing cells were usually low in PB or SF in both the antibiotic-responsive and antibiotic-refractory groups (13). Therefore, in patients with antibiotic-responsive or antibiotic-refractory Lyme arthritis, SF T cells were enriched with myeloid dendritic cells, which are critical for shaping the adaptive immune response, and with CD4+ IFNγ-producing Teff cells.

Memory phenotype of CD4+ T cells. In patients with antibiotic-responsive or antibiotic-refractory arthri-
virtually all CD4+ T cells in SF had a memory phenotype, which showed that SF contained almost entirely antigen-activated cells. In contrast, only approximately half of the CD4+ T cells in PB had a memory phenotype (Figure 1C). The percentages of naive T cells in PB were higher in patients than in healthy control subjects, presumably because memory cells had homed to inflamed joints.

Figure 1. Frequencies of various immune cell subsets in peripheral blood (PB) and synovial fluid (SF) samples from patients with antibiotic-responsive (Res) or antibiotic-refractory (Ref) Lyme arthritis and healthy control (HC) subjects. Matched PB and SF samples from 15 patients with antibiotic-responsive arthritis, 16 patients with antibiotic-refractory arthritis, and 10–13 healthy control subjects were analyzed by flow cytometry. The percentages of monocytes, myeloid dendritic cells, and plasmacytoid dendritic cells (A), various CD4+ T cell subsets (B), and naive and memory CD4+ T cells (C) are shown. The monocyte gate was based on forward and side scatter characteristics after the subtraction of activated T cells (CD3+) and B cells (CD19+). The lymphocyte gate was based on forward and side scatter characteristics. Each data point represents a single subject; horizontal lines show the median.

Figure 2. Frequencies of FoxP3+ T cells in the CD25high population. A, Frequencies of CD25−, CD25intermediate, and CD25high cells in the CD4+ T cell population in matched PB and SF samples from patients with antibiotic-responsive or antibiotic-refractory arthritis and in PB samples from healthy control subjects. B, Percentages of FoxP3-positive cells in the 3 CD25 T cell subsets. Each data point represents a single subject; horizontal lines show the median. See Figure 1 for definitions.
Enumeration of CD4+ Teff cells and Treg cells.

The presence of Treg cells is critical to immune tolerance (18), and Treg cells were initially identified based on high CD25 expression (19–22). However, CD25 was subsequently shown to be up-regulated also on recently activated CD4+ Teff cells. Therefore, dual staining for CD25high and FoxP3, a transcription factor that is essential for Treg cell development (23), is thought to be a more sensitive approach for identifying Treg cells (24).

When CD4+ T cells were gated based on CD25 expression, the PB of healthy control subjects contained mostly CD25− cells (median, 95%) (Figure 2A), whereas slight reductions in the frequencies of CD25− cells were observed in the PB and SF of both patient groups. In contrast, compared with healthy control subjects, the frequencies of CD25high cells were greater in the PB of both patient groups and even greater in SF, particularly in the antibiotic-refractory group.
When dual staining for CD25 and FoxP3 was performed, critical differences were detected in the CD25<sup>high</sup> population (Figure 2B). In the PB of healthy controls, the median frequency of CD25<sup>high</sup> FoxP3-positive T cells was 91%. In contrast, the frequency was only 72% in the SF of patients with antibiotic-responsive arthritis and was even lower (63%) in the SF of patients with antibiotic-refractory arthritis. Thus, the CD4<sup>+</sup> CD25<sup>high</sup> T cell population in the SF of patients with antibiotic-refractory arthritis was associated with a lower frequency of FoxP3-positive cells (Treg cells) and higher numbers of FoxP3-negative cells (Teff cells) compared with that in the SF of patients with antibiotic-responsive arthritis.

**Phenotypic characterization of CD4<sup>+</sup> T cell subsets.** Factors that can alter Treg cell and Teff cell functions include the expression of T cell coreceptors. For example, both GITR and OX40 augment Teff cell function (25,26), whereas CTLA-4 inhibits Teff cell function (27). In this study, the activation markers HLA–DR, GITR, and OX40 were expressed at higher levels in SF compared with PB, particularly in the CD25<sup>high</sup> population, and patients with antibiotic-refractory arthritis had significantly higher levels of these markers on CD25<sup>high</sup> cells in SF than patients with antibiotic-responsive arthritis (Figure 3A). In CD25<sup>high</sup> cells, the median fluorescence intensity of HLA–DR in SF was 8,450 in the antibiotic-refractory group compared with 4,962 in the antibiotic-responsive group (P = 0.03); the median percentages of GITR-positive cells were 42% and 25%, respectively (P < 0.001), and the median percentages of OX40-positive cells were 28% and 20%, respectively (P < 0.01). Additionally, 21% of CD4<sup>+</sup>CD25<sup>high</sup> T cells in patients with antibiotic-refractory arthritis and 9% of this cell population in patients with antibiotic-responsive arthritis expressed both of these T cell coreceptors (P = 0.02).

In contrast, expression of the inhibitory coreceptor CTLA-4 in SF was similar in the antibiotic-responsive and antibiotic-refractory groups, and virtually all of the CD4<sup>+</sup>CD25<sup>high</sup> cells expressed this coreceptor (Figure 3B). We also attempted to subtype the CD4<sup>+</sup> T cell population based on CD25 and FoxP3 expression, but intracellular staining for FoxP3 interfered with binding of the anti-GITR and anti-OX40 antibodies.

**Functional studies of CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>high</sup> T cells.** To assess the ability of CD25<sup>high</sup> cells to suppress CD25<sup>+</sup> cells, we purified these 2 T cell populations from the SF of patients with antibiotic-responsive arthritis and those with antibiotic-refractory arthritis. Because of the large numbers of cells required, it was possible to conduct these experiments in only 5 patients, but they represented the spectrum of disease durations associated with Lyme arthritis.

In all 5 patients, CD25<sup>+</sup> cells from SF, when cultured alone, proliferated in response to anti-CD3 antibody, whereas CD25<sup>high</sup> cells were anergic (Figure 4A). In the 2 patients with antibiotic-responsive arthritis (patients 1 and 2), CD25<sup>high</sup> cells effectively inhibited the proliferation of CD25<sup>+</sup> cells (from 92% to 76%) at all cell ratios tested (Figure 4A) and suppressed the secretion of IFNγ and TNFα (Figure 4B). In addition, in patient 1, whose arthritis resolved within 1 month after the start of antibiotic treatment, IL-10 levels remained high under all conditions tested, whereas in patient 2, whose arthritis resolved within 3 months, IL-10 levels were substantially lower and were suppressed even further by CD25<sup>high</sup> cells, in a dose-dependent manner.

In comparison, patient 3, who had antibiotic-refractory arthritis with synovitis that resolved in 5 months while being treated with DMARDs, CD25<sup>high</sup> cells less effectively suppressed proliferation of CD25<sup>+</sup> cells (56–75%) at the ratios tested (Figure 4A). Although IFNγ in patient 3 was suppressed by CD25<sup>high</sup> cells at the 1:1 ratio, suppression was compromised at lower cell-to-cell ratios (Figure 4B), and, although the expression of TNFα was relatively low, it was not suppressed at all. In patients 4 and 5, both of whom required synovectomies for resolution of antibiotic-refractory arthritis 18 months and 28 months, respectively, after antibiotic therapy followed by DMARD therapy, CD25<sup>high</sup> cells suppressed the proliferation of their CD25<sup>+</sup> cells at the 1:1 ratio (61% and 86%, respectively). At lower ratios, however, suppression decreased and was completely lost at the 1:0.125 ratio. Moreover, large amounts of IFNγ and TNFα were produced, and production of these cytokines was not suppressed under any of the conditions tested. Furthermore, in patient 4, the levels of IL-10 were low, and production of IL-10 was not inhibited by CD4<sup>+</sup>CD25<sup>high</sup> cells, whereas in patient 5, IL-10 levels were relatively high, but production was suppressed in a dose-dependent manner.

Additionally, in patient 5, serial samples spanning a 2-year period were available for phenotypic analysis. In this patient’s initial sample, which was obtained 4 months after the start of antibiotic therapy, only 4% of cells within the CD25<sup>high</sup> cell population were FoxP3 positive. Nineteen months later, during treatment with methotrexate and etanercept, 28% of the patient’s CD25<sup>high</sup> cells were FoxP3 positive. Five months later,
when DMARDs were stopped prior to a knee synovectomy, only 14% of CD25hi T cells were FoxP3 positive. Thus, the majority of this patient’s CD25hi T cells were likely Teff cells and not Treg cells, and this imbalance persisted throughout the disease course.

**Correlation between the duration of arthritis and the ratio of FoxP3-negative to FoxP3-positive T cells in the CD25hi population.** In the antibiotic-refractory group, the percentage of FoxP3-negative T cells in the CD25hi population in SF correlated directly with ar-
Arthritis duration, as calculated from the sample date to the resolution of arthritis (hereafter called the post-antibiotic treatment duration of arthritis) (Figure 5). Conversely, the percentage of FoxP3-positive T cells correlated inversely with the post-antibiotic treatment duration of arthritis. When calculated as a ratio, the ratio of FoxP3-negative to FoxP3-positive cells in the CD25\textsuperscript{high} population correlated directly with the post-antibiotic treatment duration of arthritis, such that the higher the ratio, the longer the duration of arthritis ($P < 0.001$). One patient, who underwent synovectomy 28 months after completion of antibiotic treatment, was critically important in this clinical correlation. However, 4 other patients also underwent synovectomies 2–15 months after the completion of antibiotic therapy, and this date was used to define their arthritis resolution. These 4 patients had higher Teff cell–to–Treg cell ratios than most of the other patients. Thus, if these 4 patients had not been treated surgically, it is likely that the durations of arthritis would have been longer, and the statistical correlation between the Teff cell–to–Treg cell ratios and duration of arthritis would have been even stronger. In contrast, no correlations were observed between the duration of arthritis and the frequency of other immune cell populations in patients with antibiotic-refractory arthritis or between any immune cell populations in patients with antibiotic-responsive arthritis.

**DISCUSSION**

In this study, we gained insights into the different outcomes associated with Lyme arthritis by determining the phenotype and function of SF immune cells in patients with antibiotic-responsive or antibiotic-refractory arthritis. Patients with antibiotic-responsive arthritis properly down-regulated their immune response soon after spirochete killing, whereas patients with antibiotic-refractory arthritis had persistent synovitis for months to years after $\geq 3$ months of antibiotic treatment. Other models of human autoimmunity usually involve comparisons between patients and control groups. With Lyme arthritis, it is possible to compare immune cells from patients whose joints were or had been infected with the same microbe ($B$ burgdorferi) but who had different clinical outcomes. We observed that patients in the antibiotic-responsive and antibiotic-refractory groups had similar phenotypic changes in CD4$^+$ T cell populations in SF, but the degree of the changes was substantially greater in the antibiotic-refractory group. This result suggests that there is a “tipping point” beyond which a protective immune response becomes pathogenic.

The critical differences between the 2 patient groups were observed in the CD4$^+$CD25\textsuperscript{high} cell population in SF. Under noninflammatory conditions, such as the PB of healthy individuals, this cell population consists primarily of FoxP3-positive Treg cells. However, CD25-positive Treg cells may lose FoxP3 expression and their repressor functions, particularly under highly inflammatory conditions (28). In the current study, the CD25\textsuperscript{high} population in SF in the antibiotic-refractory group tended to have higher percentages of FoxP3-negative (Teff) cells and lower percentages of FoxP3-positive (Treg) cells compared with that in the antibiotic-responsive group. Furthermore, higher Teff cell–to–Treg cell ratios correlated directly with a longer duration of arthritis post-antibiotic treatment in the antibiotic-refractory group.

Additionally, the antibiotic-refractory group had significantly greater expression of the activation markers.
GITR and OX40 on CD4+CD25^high T cells compared with the antibiotic-responsive group. Both GITR and OX40 are constitutively expressed in Treg cells, they are induced in Teff cells upon activation, and they are associated with increased Teff cell proliferation (25,26). Moreover, engagement of GITR leads to a loss of suppression (29–31), and in one study, this loss was shown to be attributable to the enhanced resistance of Teff cells to suppression (30). The role of OX40 on T cells is less clear, although it may also augment the resistance of Teff cells to suppression (32,33) and may inhibit the induction of Treg cells (33–35). Therefore, in patients with antibiotic-refractory arthritis, these 2 coreceptors likely contribute to pathogenicity by increasing proliferation of Teff cells in SF and enhancing their resistance to suppression by Treg cells. This concept is supported by our previous finding that Teff cells in the SF of patients with antibiotic-refractory arthritis were more resistant to suppression compared with their PB counterparts (13).

Functional studies using cells from the patients were consistent with the phenotypic differences in the CD4+CD25^high population between the 2 groups. In patient 1, who had antibiotic-responsive arthritis, SF CD25^high T cells inhibited the proliferation of CD25− T cells and suppressed the secretion of IFNγ and TNFα but not IL-10. Moreover, the FoxP3-negative–to–FoxP3-positive T cell ratio in this patient’s CD25^high population was 24:76, suggesting that he had 3-fold more Treg cells than Teff cells in this T cell subset. Therefore, in patients with antibiotic-responsive arthritis, the immune response in SF, combined with antibiotic therapy, leads to spirochetal killing, resolution of arthritis, and limited tissue pathology.

In contrast, in patients with antibiotic-refractory arthritis, CD25^high T cells were less able to inhibit the proliferation of CD25− T cells, and in 2 patients, whose disease was at the far end of the clinical spectrum, inhibition was totally lost at the lower ratios of CD25^high T cells. In patient 5, the FoxP3-negative–to–FoxP3-positive T cell ratio was 96:4, suggesting that most of this patient’s CD4+CD25^high cells were Teff cells. Even though the percentage of Treg cells was quite low, the fact that this patient’s CD25^high cells could suppress proliferation at a 1-to-1 ratio suggested that the Treg cells had enhanced regulatory function. However, in the joints of patient 5, Treg cells were likely overwhelmed by the excessive number of highly activated Teff cells. Moreover, these cells failed to inhibit IFNγ and TNFα secretion and often secreted only small amounts of IL-10. Earlier studies have shown that the levels of both TNFα and IFNγ were significantly higher in the SF of patients with antibiotic-refractory arthritis than in the SF of patients with antibiotic-responsive arthritis (36,37). Additionally, elevated levels of TNFα (38,39) and IFNγ (40) have been shown to inhibit T cell suppression. Therefore, although the immune responses of patients with antibiotic-refractory arthritis, combined with antibiotic therapy, also appear to result in spirochetal killing (9), the excessive proinflammatory environment in the joints of these patients leads to immune dysregulation, chronic synovitis, and substantial tissue pathology.

Multiple genetic factors presumably account for the high levels of proinflammatory cytokines or low levels of antiinflammatory cytokines in patients with antibiotic-refractory arthritis; these patients may have all or only some of these genes, accounting for the spectrum of disease. For example, patients with antibiotic-refractory arthritis who are homozygous for a specific TLR1 polymorphism, 1805GG, particularly when infected with highly inflammatory strains of B burgdorferi, have significantly higher levels of proinflammatory cytokines in SF, including TNFα and IFNγ, compared with the levels in patients with antibiotic-responsive arthritis (37). This highly inflammatory milieu likely leads to greater activation of T cells, including greater coreceptor expression. Going forward, it will be important to determine whether the SF of patients with antibiotic-refractory arthritis also has higher frequencies of CD14+CD16+ inflammatory monocytes, which are known to secrete higher levels of TNFα compared with classic CD14^highCD16− monocytes (41).

What antigens drive CD4+ Teff cells in Lyme arthritis? Initially, when B burgdorferi infection is active in patients’ joints, CD4+ T cells in SF respond briskly to B burgdorferi antigens (42,43). In MyD88−/− C57BL/6 mice, which have high pathogen loads, B burgdorferi antigens are retained near cartilage surfaces after antibiotic therapy, and patellae homogenates from these mice induce macrophages to secrete TNFα (10). However, patients with either antibiotic-responsive or antibiotic-refractory Lyme arthritis have low pathogen loads during the infection (9), and mouse models do not replicate the marked proliferative synovitis with inflammatory infiltrates seen in patients with antibiotic-refractory arthritis in the post–antibiotic treatment period (4). Additionally, in these patients, T cell and B cell responses to B burgdorferi antigens decline after treatment (43,44), whereas the levels of inflammatory cytokines in SF often increase (16,36), suggesting that B burgdorferi antigens could not be the sole driver of
postinfectious immune responses. Based on the hypothesis that HLA–DR molecules in the inflamed synovial tissue of patients with antibiotic-refractory arthritis present disease-related autoantigens, we recently identified endothelial cell growth factor as the first antigen that is a target of T cell and B cell responses in a subset of patients with antibiotic-refractory arthritis (12). We are continuing our search for additional disease-related antigens, because it is likely that responses to multiple antigens are necessary for an antibiotic-refractory outcome.

Our data support a model of immune regulation recently put forth by Walker, known as “tuned suppression” (45). When this model is applied to Lyme arthritis, we propose that B. burgdorferi infection initially induces proliferation and enhances the function of not only Teff cells but also Treg cells. These changes occur due to the high pathogen load that stimulates Toll-like receptors, up-regulates HLA–DR, and increases expression of TNF receptor superfamily members, which together alter T cell function. Initially, highly activated effector CD4+ T cells have enhanced resistance to suppression, thereby allowing the host, in combination with antibiotic therapy, to eliminate the infection. However, once the number of spirochetes is reduced or once spirochetes are eradicated, Teff cells again become receptive to suppression (antibiotic-responsive arthritis), which Treg cells readily perform due to their increased numbers and enhanced function.

In some individuals, however, it seems that this regulatory network breaks down due to part in the development of immunity to self antigens, causing the “antigen load” to remain high (antibiotic-refractory arthritis). In these patients, factors that contribute to immune dysregulation may include Teff cells with enhanced resistance to Treg cell suppression, an imbalance in the Teff cell:Treg cell ratio, an excessive proinflammatory cytokine milieu, or a combination of these factors. Nevertheless, synovitis in most patients eventually resolves months to several years after antibiotic therapy followed by treatment with DMARDs such as methotrexate, which are thought to inhibit T cell activation (46). This inhibition, in the absence of live spirochetes to act as an adjuvant, appears to allow the immune system to regain homeostasis, and the arthritis eventually resolves. Even in patients requiring synovectomies, the arthritis usually does not recur, because innate immune signals associated with active infection are missing.

In summary, patients with antibiotic-responsive arthritis and those with antibiotic-refractory arthritis had similar phenotypic changes in the CD4+CD25high T cell population in SF, but the degree of the changes was substantially greater in the antibiotic-refractory group. Patients with antibiotic-refractory arthritis often had lower frequencies of Treg cells, higher expression of coreceptors that augment Teff cell function, and less effective inhibition of proinflammatory responses. This excessively amplified proinflammatory immune response leads to immune dysregulation and antibiotic-refractory Lyme arthritis.

ACKNOWLEDGMENTS

We thank Dr. Lisa Glickstein for helpful discussions, Michael Waring and Adam Chicoine from the Ragon Institute for their help with fluorescence-activated cell sorting, and Colleen Squires for help with preparation of the manuscript.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Drouin had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Vudattu, Steere, Drouin.

Acquisition of data. Vudattu, Strle.

Analysis and interpretation of data. Vudattu, Steere, Drouin.

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