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Dysregulation of CD4+CD25hi+ T cells in the synovial fluid of patients with antibiotic-refractory Lyme arthritis

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Abstract

Objective—To explore the role of immune dysregulation in antibiotic-refractory Lyme arthritis, the phenotype, frequency and function of CD4+ T_H1 and T_H2 cells were compared in patients with antibiotic-responsive or antibiotic-refractory arthritis. In the latter condition, infection-induced autoimmunity is thought to have a pathogenic role.

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

Results—Critical differences between the 2 patient groups were found in the SF CD4+CD25hi+ populations, a subset of cells usually composed of FOXP3-positive T_H1 cells. In patients with antibiotic-refractory arthritis, this cell population often had fewer FOXP3-positive cells, and greater frequencies of FOXP3-negative (T_H1) compared to patients with antibiotic-responsive arthritis. Moreover, in the refractory group, CD4+CD25hi+ cells had significantly greater expression of GITR and OX-40, two co-receptors that augment T cell function. Suppression assays showed that CD4+CD25hi+ cells in patients with refractory arthritis did not effectively suppress proliferation of CD4+CD25– cells, or secretion of IFN- γ or TNF- α , whereas those from patients with responsive arthritis did. Finally, in the refractory group, higher ratios of CD25hi+FOXP3–/CD25hi+FOXP3+ cells correlated directly with longer post-treatment durations of arthritis.

Conclusion—Patients with antibiotic-refractory Lyme arthritis often had lower frequencies of T_H1, higher expression of activation co-receptors, and less effective inhibition of pro-inflammatory cytokines. This suggests that immune responses in these patients were excessively amplified leading to immune dysregulation and refractory arthritis.

There is increasing interest in the role of infection in triggering autoimmune diseases (1, 2). With infection, a pro-inflammatory response is induced to protect the host which includes the activation and expansion of innate and adaptive immune cells. However, this pro-inflammatory response must be properly down-regulated once the pathogen is controlled or eliminated to maintain tolerance and limit tissue pathology. In some individuals, these regulatory mechanisms do not work optimally, leading to pathogenic autoimmunity.

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AUTHORS 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.

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Therefore, identifying quantitative and qualitative differences in immune cells between patients who can properly down-regulate their immune response after infection from those who cannot is critical to our understanding of infection-induced autoimmunity.

Lyme arthritis, a late stage manifestation of infection with the tick-borne spirochete *Borrelia burgdorferi* (*Bb*) (3), provides a human model of infection that may lead to these two alternative outcomes (4). Most patients can be treated successfully with antibiotics, called antibiotic-responsive arthritis (5, 6). However, in a small percentage of patients, proliferative synovitis persists for months or years after 3 months of oral and IV antibiotics, called antibiotic-refractory arthritis (7). This outcome is postulated to result from persistent infection, retained spirochetal antigens, infection-induced autoimmunity, or a combination of these factors.

In animal models, a small number of attenuated spirochetes may survive despite 1 month of antibiotic therapy (8), but in patients with antibiotic-refractory arthritis, culture and PCR results for *Bb* in synovial tissue have been uniformly negative after 3 months of antibiotics (9). Additionally, in MyD88^{-/-} mice, which have a high pathogen load, spirochetal antigens are retained near cartilage surfaces after antibiotic therapy (10), but the relevance of this finding to human antibiotic-refractory arthritis is not yet clear. In the human disease, data supports the infection-induced autoimmunity model (7, 11, 12). For example, antibiotic-refractory arthritis is associated with specific HLA-DR alleles (particularly DRB1*0101 and 0401) (11), a risk factor commonly associated with autoimmune diseases. We postulate that these patients are unable to properly down-regulate their immune response with antibiotic therapy and apparent spirochetal killing leading to immune dysregulation and antibiotic-refractory arthritis.

Previously, we showed that in patients with antibiotic-refractory arthritis, the percentage of CD4+FOXP3+ Treg cells in SF correlated inversely with the post-antibiotic duration of arthritis (13), implying that lower numbers of Treg led to slower arthritis resolution. Furthermore, suppression assays using cells from 2 patients with refractory arthritis showed that CD25-positive T cells (Treg) from PB and SF suppressed the proliferation of CD25-negative T cells (Teff) at a 1-to-1 ratio equally well, but CD25-negative T cells (Teff) from SF were more resistant to suppression than from PB. However, in this study, the expression of FOXP3 within various CD4+CD25 T cell subpopulations, the expression of activating or inhibitory T cell co-receptors, and the ability of these patients' Treg cells to suppress cytokine secretion were not determined.

In our current study, we compared the frequency, phenotype and function of immune cells in PB and SF from patients with antibiotic-responsive or antibiotic-refractory Lyme arthritis. Critical differences between the 2 patient groups were found in the CD4+CD25hi+ T cell population in SF. This cell population in the refractory group often had lower frequencies of Treg, higher expression of activation co-receptors, and less effective inhibition of pro-inflammatory responses, leading to immune dysregulation and persistent synovitis.

PATIENTS AND METHODS

Patients

SF mononuclear cells were available from 31 patients: 15 with antibiotic-responsive and 16 with antibiotic-refractory Lyme arthritis, who were evaluated in our clinic between 2000 and 2010 (Table 1). Concomitant PB mononuclear cells were also available from 27 of the 31 patients. For comparison, PB 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 Center 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 antibiotic therapy, patients with refractory arthritis were treated with DMARDs or sometimes with synovectomies (7).

Immune cell phenotype by flow cytometry

All patients' PB or SF cells were stored in liquid nitrogen. For analysis, cells were resuspended in staining buffer (PBS with 2% fetal bovine serum). Cells' 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 analysis.

To enumerate the frequency of various immune cell types, cells were stained with anti-CD14, CD19, CD303 and HLA-DR antibodies for 15 minutes, followed by anti-CD3, CD4 and CD11c antibodies for another 15 minutes. For Th1/Th2 polarization, cells were stimulated with phorbol 12-myristate 13-acetate (50ng/ml) and ionomycin (1ug/ml) in the presence of GolgiStop (BD Biosciences) for 5 hours at 37°C and 5% CO₂. Afterwards, cells were washed and incubated with anti-CD3 and CD4 antibodies for 15 minutes, followed by fixation and permeabilization as per the manufacturer's instructions (BD Biosciences) and stained with anti-IFN- γ and IL-4 antibodies for 30 minutes at 4°C. Memory phenotype and activation status of CD4⁺Teff cells was determined by staining with anti-CD45RO, CCR7, HLA-DR and OX-40 antibodies for 15 minutes, followed by anti-CD3, CD4, CD25 and GITR for another 15 minutes. Determination of Teff and Treg cells and the expression of CTLA-4, cells were first stained with anti-CD3, CD4 and CD25 antibodies for 15 minutes. Cells were then fixed and permeabilized as per the manufacturer's instruction for anti-FOXP3 (BD Biosciences). Afterwards, cells were stained with anti-FOXP3 and anti-CTLA-4 antibodies for 30 minutes. All flow cytometry analysis was performed with a LSRT Fortessa (BD Biosciences) and data were analyzed using FlowJo software.

Suppression assays experiments

SF CD4⁺CD25^{hi} T cells and CD4⁺CD25⁻ T cells were sorted by FACS Aria (Becton Dickinson, USA). The sorted populations had a purity >97% as determined by flow cytometry. CD4⁺CD25⁻ T cells (20,000 cells) were co-cultured alone or with different ratios of CD4⁺CD25^{hi} T cells in the presence of soluble anti-CD3 antibody (0.5ug/ml). Irradiated (5000 rad) PB cells from a healthy control subject were used as feeder cells. The cell cultures were incubated at 37°C and 5% CO₂ for 5 days then pulsed with ³H-thymidine (1μCi/well) for 18 hours. The suppressive capacity was determined by calculating the relative difference in proliferative response (mean of duplicated wells) between CD4⁺CD25⁻ T cells cultured alone or in the presence of CD4⁺CD25^{hi} T cells. Cell supernatants from the proliferation assays were assessed for IFN- γ , TNF- α , and IL-10 using bead-based Multiplex assays (Millipore), as previously described (16).

Statistical analysis

Quantitative data were analyzed by Mann-Whitney test for comparison between two groups and correlations between Treg frequencies and the duration of arthritis were sought by Pearson's correlation test. GraphPad Prism was used for all analyses. All P values were 2-tailed. P values of < 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 13 healthy control subjects. The cells from patients with antibiotic-responsive arthritis were obtained prior to or soon after the start of antibiotics when their joints were still infected with *Bb*. 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 illness.

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

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 SF of both patient groups was CD4⁺ IFN- γ -secreting T cells, and the frequencies of these cells were significantly greater in SF than 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 the *Bb* NapA protein (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 responsive and refractory groups (13). Therefore, in patients with responsive or refractory Lyme arthritis, SF were enriched with myeloid dendritic cells, a critical cell for shaping the adaptive immune response, and with CD4⁺IFN- γ -producing Th1 effector cells.

Memory phenotype of CD4⁺ T cells

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

Enumeration of CD4⁺ Teff and Treg cells

The presence of Treg is critical to immune tolerance (18) and Treg were initially identified based upon high CD25 expression (19–22). However, CD25 was subsequently found to be up-regulated also on recently activated CD4⁺ Teff cells. Therefore, dual staining for CD25^{hi} and FOXP3, a transcription factor that is essential for Treg development (23), is thought to be a more sensitive approach for Treg identification (24).

When CD4⁺ T cells were gated based upon CD25 expression, the PB of healthy control subjects contained mostly CD25-negative cells (median, 95%) (Figure 2A), whereas PB and SF from both patient groups had slight reductions in the frequencies of CD25-negative cells. In contrast, the frequencies of CD25^{hi}-positive cells were greater in the PB of both patient groups compared to healthy control subjects, and in the patient groups, the values were significantly greater in SF than PB.

When dual staining was performed for CD25 and FOXP3, critical differences were detected in the CD25hi+ population (Figure 2B). In the PB of healthy controls, the median frequency of CD25hi+FOXP3+ T cells was 91%; in the SF of patients with responsive arthritis it was only 72%, and in the refractory group it was even lower at 63%. Thus, the CD4+CD25hi+ T cell population in the SF of patients with antibiotic-refractory arthritis was associated with a lower frequency of FOXP3-positive cells (Treg) and higher numbers of FOXP3-negative cells (Teff) than that of patients with antibiotic-responsive arthritis.

Phenotypic characterization of CD4+ T cell subsets

Factors that can alter Treg and Teff functions include the expression of T cell co-receptors. For example, glucocorticoid-induced TNF receptor-related protein (GITR) and OX-40 both augment T cell function (25, 26), whereas cytotoxic T-lymphocyte antigen-4 (CTLA-4) inhibits T cell function (27).

In this study, the activation markers HLA-DR, GITR and OX-40 were expressed at higher levels in SF compared to PB, particularly in the CD25hi+ population; and patients with refractory arthritis had significantly higher levels of these markers on CD25hi+ cells in SF than patients with responsive arthritis (Figure 3A). In CD25hi+ cells, the median fluorescent intensity of HLA-DR was 8,450 in the refractory group compared with 4,962 in the responsive group ($P=0.03$); the median percentage of GITR+ cells was 42% compared with 25%, ($P<0.001$), and the median percentage of OX-40+ cells was 28% versus 20% ($P<0.01$). Additionally, 21% of the CD4+CD25hi+ T cell subsets in patients with refractory arthritis and 9% of this cell population in patients with responsive arthritis expressed both of these T cell co-receptors ($P=0.02$).

In contrast, the expression of the inhibitory co-receptor CTLA-4 was similar in SF in the responsive and refractory groups, and virtually all of the CD4+CD25hi+ cells expressed this co-receptor (Figure 3B). We also attempted to subtype the CD4+ T cell population based upon CD25 and FOXP3 expression, but intracellular staining for FOXP3 interfered with the binding of the anti-GITR and anti-OX-40 antibodies.

Functional studies of CD4+CD25- and CD4+CD25hi+ T cells

To assess the ability of CD25hi-positive cells to suppress CD25-negative cells, we purified these 2 T cell populations from the SF of patients with responsive or refractory arthritis. Because of the large numbers of cells required, it was only possible to conduct these experiments in 5 patients, but they represented the spectrum of disease durations associated with Lyme arthritis.

In all 5 patients, CD25-negative cells from SF, when cultured alone, proliferated in response to anti-CD3 antibody, whereas CD25hi-positive cells were anergic (Figure 4A). In the 2 patients with antibiotic-responsive arthritis (patients 1 and 2), CD25hi-positive cells effectively inhibited the proliferation of CD25-negative cells (92-76%) at all cell ratios tested (Figure 4A), and suppressed the secretion of IFN- γ and TNF- α (Figure 4B). In addition, in patient 1, whose arthritis resolved within 1 month after the start of antibiotics, 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 his CD25hi+ cells in a dose dependent manner.

In comparison, patient 3, a patient with antibiotic-refractory arthritis whose synovitis resolved in 5 months while on DMARD therapy, her CD25hi-positive cells less effectively suppressed proliferation of her CD25-negative cells at the ratios tested (75-56%) (Figure 4A). Although IFN- γ was suppressed by her CD25hi+ cells at the 1-to-1 ratio, suppression was compromised at lower cell-to-cell ratios (Figure 4B) and TNF- α , though relatively low,

was not suppressed at all. In patients 4 and 5, patients who required synovectomies for resolution of antibiotic-refractory arthritis >1 year after antibiotic therapy followed by DMARD therapy, their CD25hi-positive cells suppressed the proliferation of their CD25-negative cells at the 1-to-1 ratio (61% and 86%, respectively). However, at lower ratios, suppression decreased and was completely lost at a ratio of 1-to-0.125. Moreover, large amounts of IFN- γ and TNF- α were produced, and production of these cytokines was not suppressed under any of the conditions tested. Furthermore, in patient 4, IL-10 levels were low, and its production was not inhibited by his CD4+CD25hi-positive 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 his initial sample, obtained 4 months after the start of antibiotic therapy, only 4% of his cells within the CD25hi+ cell population were FOXP3+. Nineteen months later, while receiving methotrexate and etanercept, 28% of his CD25hi+ cells were FOXP3+. Five months later, when DMARDs were stopped prior to a synovectomy of his knee, only 14% of his CD25hi+ T cells were FOXP3+. Thus, the majority of this patient's CD25hi+ T cells were likely Teff and not Treg, and this imbalance persisted throughout the course of his illness.

Correlation of the FOXP3-negative/FOXP3-positive T cell ratio in the CD25hi+ population and duration of arthritis

In the refractory group, the percentage of FOXP3-negative T cells in the CD25hi+ population in SF correlated directly with arthritis duration, as calculated from the sample date to the resolution of arthritis (hereafter called the post-antibiotic duration of arthritis) (Figure 5). Conversely, the percentage of FOXP3-positive T cells correlated inversely with this arthritis duration. When calculated as a ratio, the ratio of FOXP3-negative to FOXP3-positive cells in the CD25hi+ population correlated directly with post-antibiotic duration of arthritis, such that the higher the ratio, the longer the duration of arthritis ($P < 0.001$). One patient, in whom a synovectomy was performed 28 months after antibiotic treatment, was critically important in this clinical correlation. However, 4 other patients also underwent synovectomies 2 to 15 months after the completion of antibiotic therapy, and this date was used to define their arthritis resolution. These 4 patients had higher Teff-to-Treg ratios (circled data points) than most of the other patients. Thus, if their disease had not been treated surgically, it is likely that their durations of arthritis would have been longer, and the statistical correlation between the Teff-to-Treg ratios and duration of arthritis would have been even stronger. In contrast, no correlations were found between duration of arthritis and frequency of other immune cell populations in patients with refractory arthritis or between any immune cell populations in patients with 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 responsive arthritis properly down-regulated their immune response soon after spirochetal killing, whereas patients with antibiotic-refractory arthritis had persistent synovitis for months to years after 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 (*Bb*), but who had different clinical outcomes. We found that patients in the responsive and refractory groups had similar phenotypic changes in CD4+ T cell populations in SF, but the degree of

the changes was substantially greater in the refractory group. This result suggests there is a “tipping point” beyond which a protective immune response becomes pathogenic.

The critical differences between the 2 patient groups were in the CD4+CD25hi+ cell population in SF. Under non-inflammatory conditions, such as the PB of healthy individuals, this cell population consists primarily of FOXP3-positive Treg. However, CD25-positive Treg may lose FOXP3 expression and their repressor functions, particularly under highly inflammatory conditions (28). In our current study, the CD25hi+ population in SF in the antibiotic-refractory group tended to have higher percentages of FOXP3-negative cells (Teff) and lower percentages of FOXP3-positive cells (Treg) compared with that in the antibiotic-responsive group. Furthermore, higher ratios of Teff/Treg correlated directly with longer post-antibiotic duration of arthritis in the refractory group.

Additionally, the refractory group had significantly greater expression of the activation markers GITR and OX-40 on CD4+CD25hi+ T cells compared with the responsive group. GITR and OX-40 are both constitutively expressed on Treg, they are induced in Teff upon activation, and they are associated with increased T 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 due to the enhanced resistance of Teff to suppression (30). The role of OX-40 on T cells is less clear, though it may also augment the resistance of Teff to suppression (32, 33) and inhibit the induction of Treg (33–35). Therefore, in patients with refractory arthritis, these 2 co-receptors likely contribute to pathogenicity by increasing proliferation of Teff in SF, and enhancing their resistance to suppression by Treg. This belief is supported by our previous finding that Teff in the SF of patients with refractory arthritis were more resistant to suppression compared to their PB counterparts (13).

Functional studies with patients' cells were consistent with these phenotypic differences in the CD4+CD25hi+ population between these two patient groups. In patient 1 who had antibiotic-responsive arthritis, CD25hi-positive T cells from SF inhibited proliferation of his CD25-negative T cells, and suppressed IFN- γ and TNF- α secretion, but not IL-10 secretion. Moreover, the ratio of FOXP3-negative to FOXP3-positive T cells in his CD25hi+ population was 24-to-76, suggesting that he had 3 times more Treg than Teff 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, CD25hi-positive T cells were less able to inhibit the proliferation of CD25-negative T cells, and in 2 patients, who were at the far end of the clinical spectrum, inhibition was lost altogether at the lower ratios of CD25hi+ T cells. In patient 5, the ratio of his FOXP3-negative to FOXP3-positive T cells was 96-to-4, suggesting that most of his CD4+CD25hi-positive cells were Teff. Even though his percentage of Treg was quite low, the fact that his CD25hi+ cells could suppress proliferation at a 1-to-1 ratio suggested that his Treg had enhanced regulatory function. However, in his joint, his Treg cells were likely overwhelmed by the excessive number of highly activated Teff cells. Moreover, his cells failed to inhibit IFN- γ and TNF- α secretion, and often secreted only small amounts of IL-10. Earlier studies have shown that both TNF- α and IFN- γ levels were significantly greater in the SF of patients with refractory arthritis than in those with responsive arthritis (36, 37). Additionally, elevated levels of TNF- α (38, 39) and IFN- γ (40) have been shown to inhibit T cell suppression. Therefore, though the immune responses of patients with refractory arthritis, combined with antibiotic therapy, also appear to result in spirochetal killing (9), the excessive pro-inflammatory environment in these patients' joints leads to immune dysregulation, chronic synovitis, and substantial tissue pathology.

Multiple genetic factors presumably account for the high levels of pro-inflammatory cytokines or low levels of anti-inflammatory cytokines in patients with antibiotic-refractory arthritis, and these patients may have all or only some of these genes, accounting for the spectrum of disease. For example, patients with refractory arthritis who are homozygous for a specific TLR1 polymorphism 1805GG, particularly when infected with highly inflammatory strains of *Bb*, have significantly higher levels of pro-inflammatory cytokines in SF, including TNF- α and IFN- γ , compared with the levels in patients with responsive arthritis (37). This highly inflammatory milieu likely leads to greater activation of T cells, including greater co-receptor expression. Going forward, it will be important to determine whether patients with refractory arthritis also have greater frequencies of CD14+CD16+ inflammatory monocytes in their SF, which are known to secrete higher levels of TNF- α than CD14hi+CD16- classical monocytes (41).

What antigens drive CD4+ Teff cells in Lyme arthritis? Initially, when *Bb* infection is active in joints, CD4+ T cells in SF respond briskly to *Bb* antigens (42, 43). Moreover, in BL/6 MyD88^{-/-} mice, which have high pathogen loads, *Bb* antigens are retained near cartilage surfaces after antibiotic therapy, and patellae homogenates from these mice induce macrophages to secrete TNF- α (10). However, patients with either responsive or refractory Lyme arthritis have low pathogen loads during the infection (9). Moreover, mouse models do not replicate the marked proliferative synovitis with inflammatory infiltrates seen in patients with antibiotic-refractory arthritis in the post-antibiotic period (4). Additionally, in these patients, T and B cell responses to *Bb* antigens decline after treatment (43, 44), while the levels of inflammatory cytokines in SF often increase (16, 36), suggesting that *Bb* antigens could not be the sole driver of post-infectious immune responses. Based upon the hypothesis that HLA-DR molecules in inflamed synovial tissue of patients with refractory arthritis present disease-related autoantigens, we recently identified the first autoantigen, endothelial cell growth factor (ECGF), that is a target of T and B cell responses in a subset of patients with antibiotic-refractory arthritis (12). However, 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 L.S. Walker known as “tuned suppression” (45). As applied here to Lyme arthritis, we propose that *Bb* infection initially induces proliferation and enhances the function of not only Teff, but also Treg. These changes occur due to the high pathogen load that stimulate TLRs, upregulate HLA-DR, and increase expression of TNF receptor superfamily members, which together alter T cell function. Initially, the 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 spirochetes are reduced or eradicated, Teff again become receptive to suppression (antibiotic-responsive arthritis), which Treg readily perform due to their increased numbers and enhanced function.

In some individuals though, it seems that this regulatory network breaks down due in part to 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 with enhanced resistance to Treg suppression, an imbalance in the Teff/Treg ratio, an excessive pro-inflammatory cytokine milieu, or a combination of these factors. Nevertheless, synovitis in most patients eventually resolves months to several years after antibiotic therapy, followed by 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, seems to allow the immune system to regain homeostasis, and the arthritis eventually resolves. Even in patients requiring synovectomies, the arthritis does not usually recur because innate immune signals associated with active infection are missing.

In summary, patients with antibiotic-responsive or antibiotic-refractory arthritis had similar phenotypic changes in the CD4+CD25hi+ cell population in SF, but the degree of the changes was substantially greater in the refractory group. These patients often had lower frequencies of Treg, higher expression of co-receptors that augment T cell function, and less effective inhibition of pro-inflammatory responses. This excessively amplified pro-inflammatory immune response leads to immune dysregulation and antibiotic-refractory Lyme arthritis.

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Figure 1.

Comparison of the frequencies of various immune cell subsets in patients with Lyme arthritis or healthy control subjects. Matched PB and SF samples from 15 antibiotic-responsive (Res) and 16 antibiotic-refractory (Ref) patients, and for comparison 11 to 13 healthy control (HC) subjects were analyzed by flow cytometry. **A**, Summary of the percentages of monocytes, myeloid dendritic cells and plasmacytoid dendritic cells; **B**, various CD4+ T cell subsets from each sample; **C**, and naïve and memory CD4+ T cells from each sample. The monocytic gate was based upon forward and side scatter characteristics after the subtraction of activated T cells (CD3+) and B cells (CD19+). The lymphocytic gate was based upon forward and side scatter characteristics. Symbols indicate individual subjects, horizontal bars represent the median of each group and significant differences are denoted. Statistical analyses were performed by using a Mann-Whitney test.

Figure 2.

In patients with refractory group, the percentage of FOXP3+ T cells in the CD25hi+ cell populations in SF was lower than in patients with antibiotic-responsive arthritis. **A**, Summary of the frequencies of CD25-, CD25int+ and CD25hi+ cells in the CD4+ T cell population in matched PB and SF patient samples, or PB of healthy control subjects. **B**, Summary of the percentages of FOXP3+ cells in the three CD25 T cell subsets. Symbols indicate individual subjects, horizontal bars represent the median of each group and significant differences are denoted. Statistical analyses were performed by using a Mann-Whitney test.

Figure 3.

Activation markers are more highly expressed in the CD4+CD25hi+ T cell population in the SF of patients with antibiotic-refractory versus antibiotic-responsive arthritis. **A**, Summary of the mean fluorescent intensity (MFI) of HLA-DR expression and the percent positive for the activation markers GITR and OX-40 in CD4+CD25 T cell subsets in matched PB and SF samples from Lyme arthritis patients, and PB of healthy control subjects. **B**, Summary of the percent positive for the inhibitory marker CTLA-4 in the various CD4+CD25 T cell subsets in patients and healthy control subjects. Symbols indicate individual subjects, horizontal bars represent the median of each group and significant differences are denoted. Statistical analyses were performed by using a Mann-Whitney test.

Antibiotic-Responsive Lyme Arthritis

Antibiotic-Refractory Lyme Arthritis

Patient (Start of antibiotics to arthritis resolution in months)

Figure 4.

CD4+CD25hi+ T cells from the SF of patients with antibiotic-refractory arthritis less effectively inhibit proliferation, and secretion of IFN- γ and TNF- α than cells of patients with antibiotic-responsive arthritis. The cells from the 2 patients with antibiotic-responsive arthritis (patients 1 and 2) were obtained prior to or soon after the start of antibiotics, whereas cells from the 3 patients with antibiotic-refractory arthritis (patients 3, 4 and 5) were collected near or soon after the completion of antibiotic therapy, but before treatment with DMARDs. **A**, The duration of arthritis from the start of antibiotics to the resolution of arthritis is indicated above the graphs for each patient. CD4+CD25- and CD4+CD25hi+ T cells isolated from patients' SF by FACS were cultured alone or together at various CD25- to CD25hi+ T cell ratios. After stimulation with plate bound anti-CD3 antibody, proliferation (3 H-thymidine incorporation) was measured (counts per minute (CPM)). All samples were tested in duplicate. **B**, Cell culture supernatants were collected from all sample wells and analyzed for IFN- γ , TNF- α or IL-10 using a multiplex cytokine assay. The ratio of FOXP3-negative to FOXP3-positive T cells in the CD4+CD25hi+ population, in SF are indicated for patients 1, 3 and 5. For the other two patients these data were not available (N.A.).

Figure 5.

In patients with antibiotic-refractory arthritis, the frequency of SF FOXP3-negative T cells correlated directly, whereas the frequency of FOXP3-positive cells correlated inversely with the duration of arthritis. Frequencies of FOXP3-negative (left graph) or FOXP3-positive (center graph) T cells in the CD4+CD25hi+ population were plotted against the duration of arthritis as calculated from the sample date to the resolution of arthritis. The ratio of FOXP3-negative to FOXP3-positive T cells (right graph) in the CD4+CD25hi+ T cell population also correlated with the duration of arthritis. The circled data points indicate the 5 patients who underwent synovectomies. All cell frequencies were obtained from Figure 3. Statistical analyses were performed by using Pearson's correlation.

Table 1

Clinical and demographic characteristics and treatment regimens of patients with Lyme arthritis.

	Lyme Arthritis	
	Antibiotic-Responsive*	Antibiotic-Refractory[§]
Number of patients	N = 15	N = 16
Median age in years (range)	36 (14–53)	29 (12–62)
Number of men/women	8/7	11/5
Year at onset of arthritis (range)	2007 (2000 – 2010)	2005 (2001 – 2010)
Median duration of arthritis		
Prior to start of antibiotics (months)	0.5 (0–4)	1 (0–9)
From start of antibiotics to sample date (months)	0.5 (0–3)	5 (1–18) [¶]
From start of antibiotics to resolution (months)	3 (1–3)	12 (5–34) [¶]

* Defined as resolution of arthritis within 3 months after 8 weeks of oral antibiotics or 4 weeks of IV antibiotics.

[§] Defined as persistent joint swelling for 3 months after 8 weeks of oral antibiotics or 4 weeks of IV antibiotics, or usually both.

[¶] For the comparison of responsive versus refractory Lyme arthritis, P<0.0001.