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Robust interferon signature and suppressed tissue repair gene expression in synovial tissue from patients with post-infectious, *Borrelia burgdorferi*-induced Lyme arthritis

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Abstract

In most patients with Lyme arthritis (LA), antibiotic therapy results in *Borrelia burgdorferi* pathogen elimination, tissue repair, and return to homeostasis. However, despite spirochetal killing, some patients develop proliferative synovitis, characterized by synovial hyperplasia, inflammation, vascular damage, and fibrosis that persists for months to several years after antibiotic treatment, called post-infectious LA. In this study, we characterized the transcriptomes of post-infectious LA patients' synovial tissue, the target tissue of the immune response. High-throughput RNA sequencing to a depth of ~30 million reads per sample was used to profile gene expression in synovial tissue from 14 patients with post-infectious LA, compared with 8 patients with other types of chronic inflammatory arthritis and 5 with minimally-inflammatory osteoarthritis (OA). Synovium from post-infectious LA and other inflammatory arthritides shared gene signatures associated with antigen presentation, innate immune responses, and cell-mediated immune activation, whereas these responses were diminished in OA synovium. Unique to post-infectious LA was a particularly robust interferon-gamma (IFN γ) signature. Moreover, this heightened IFN γ signature inversely correlated with expression of genes involved in repair of damaged tissue, including genes associated with stromal cell proliferation and differentiation, neovascularization, and extracellular matrix synthesis, which were markedly suppressed in post-infectious LA. Transcriptional observations were confirmed by cytokine profiling, histologic analyses, and clinical correlations. We propose that in patients with post-infectious LA, over-expression of IFN γ in synovium prevents appropriate repair of tissue damaged by *B. burgdorferi* infection, blocking return to tissue homeostasis long after completion of antibiotic therapy and resolution of active infection.

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CONFLICT OF INTEREST STATEMENT

None of the authors have any conflicts of interest to declare.

INTRODUCTION

Autoimmune diseases impact at least 7.6% of the U.S. population (Cooper, Bynum, & Somers, 2009), and the incidence of many immune disorders are increasing in developed countries (Bach, 2002). Infection may be an important “first hit” in development of autoimmunity (Mills, 2011; Root-Bernstein & Fairweather, 2014). However, establishing the etiology of an immune disorder according Koch’s postulates remains immensely challenging because of the many years that often separate the autoimmune trigger and clinical disease onset (Rosenblum, Remedios, & Abbas, 2015), and because of similarities between chronic infections, autoimmunity, and other inflammatory diseases (Baio et al., 2008; von Herrath, Fujinami, & Whitton, 2003).

Lyme disease (LD), caused by infection with the tick-borne spirochete *Borrelia burgdorferi*, serves as a unique model to study the causal relationships between immune responses to infection and subsequent development of immune-mediated disorders. LD is the most common vector-borne disease in the Northern Hemisphere, with an estimated 300,000 cases occurring annually in the U.S., primarily in the Northeast (Nelson et al., 2015). Lyme arthritis (LA) is the most common late-disease manifestation in the U.S. Although most patients respond well to antibiotic therapy and their arthritis resolves, proliferative synovitis may persist or worsen in some patients for months or years following bacterial clearance by 2–3 months of oral and IV antibiotic therapy, called post-infectious LA (Arvikar & Steere, 2015; Steere & Angelis, 2006). While spirochetal components may persist after antibiotic therapy, culture and PCR testing for *B. burgdorferi* have been uniformly negative in post-infectious LA synovial tissue (Li et al., 2011).

Post-infectious LA is an immune disorder characterized by vascular proliferation and damage, synovial hyperplasia, fibrosis, and accumulation of an inflammatory infiltrate within joint tissue and in surrounding synovial fluid, which persists or worsens without evidence of active infection (Johnston et al., 1985; Lawson & Steere, 1985; Steere, Duray, & Butcher, 1988). This is similar to the synovial lesion seen in the other chronic inflammatory arthritides such as rheumatoid arthritis (RA) (Smolen et al., 2018), except that the synovial lesion in LA often contains obliterative microvascular lesions (Johnston et al., 1985; Londono et al., 2014).

Several genetic and regulatory factors associated with host adaptive immune responses are implicated in the disease etiology of post-infectious LA. Certain human leukocyte antigen HLA-DRB1 alleles are the strongest genetic risk factor for post-infectious LA (Steere et al., 2006), as well as for RA (Plenge, 2009). In RA, the HLA associations are with “shared epitope” alleles (Viatte et al., 2015), whereas the associations in post-infectious LA are with a broader group of alleles (Steere et al., 2006). Both conditions are often accompanied by autoimmune T and B cell responses to proteins that are highly abundant in synovial tissue. In LA, the targets include endothelial cell growth factor (ECGF) (Drouin et al., 2013), matrix metalloproteinase-10 (MMP-10) (Crowley et al., 2016), apolipoprotein B-100 (APOB-100) (Crowley et al., 2015), annexin A2 (ANXA2) (Pianta et al., 2015). In RA, however, targets are primarily citrullinated proteins, such as vimentin, fibrinogen, type II

collagen, and enolase (Schellekens, de Jong, van den Hoogen, van de Putte, & van Venrooij, 1998).

In addition to adaptive immune factors, genetic and regulatory factors associated with innate immune responses also play a role. A polymorphism in toll-like receptor 1 (TLR1) has been identified as another genetic risk factor for excessive and sustained innate and Th1 adaptive inflammatory responses in post-infectious LA (Strle, Shin, Glickstein, & Steere, 2012). In addition, over-expression of miR-155, a pro-inflammatory microRNA downstream of TLR/NF- κ B signaling, is also associated with post-infectious LA (Lochhead et al., 2017). Because the trigger of post-infectious LA, *B. burgdorferi* infection, is known with certainty, LA is a natural human model to study the causal relationships between immune responses to infection, innate immune-mediated autoinflammation, and adaptive immune-mediated autoimmunity.

Immune responses to *B. burgdorferi* infection, along with spirochetal interactions with host cells and tissue, result in inflammation and tissue damage, which must be repaired following pathogen clearance. This wound healing process involves many immune and stromal cell types, tumor growth factor-beta (TGF- β) and other tissue growth factors, and extracellular matrix (ECM) components (Martin, 1997). Tissue repair is a highly coordinated process occurring in stages, and is one of the most complex biological processes in humans (Gurtner, Werner, Barrandon, & Longaker, 2008). Within hours of damage, repair begins with inflammation to control the infection, progresses to formation of granulation tissue that occurs within days to weeks after injury, and concludes with tissue remodeling and strengthening of scar tissue that may take up to a year (Gurtner et al., 2008). When wound repair becomes dysregulated, chronic wounds (Singer & Clark, 1999) or tissue fibrosis (Ho, Lagares, Tager, & Kapoor, 2014) may develop that can lead to life-threatening conditions.

Using high-throughput RNA sequencing of synovial tissue, together with histologic and cytokine analyses, we found that the nature of inflammation in post-infectious LA was similar to that seen in other forms of chronic inflammatory arthritis, and distinct from that seen in patients with minimally inflammatory OA. Moreover, inflammation in post-infectious LA was dominated by a particularly robust interferon gamma (IFN γ) profile, but decreased expression of genes involved in repair of damaged tissue, including fibroblast and endothelial cell growth factors, TGF- β activation, and extracellular (ECM) components. In patients with post-infectious LA, we hypothesize that following resolution of *B. burgdorferi* infection, appropriate tissue repair is stalled by excessive IFN γ -mediated inflammation, preventing a return to tissue homeostasis. This results in vascular damage, autoimmune or autoinflammatory inflammatory processes, fibrosis, and synovial hyperplasia lasting for months to several years, even in the absence of active infection.

RESULTS

Characteristics of patient groups

During a 13-year period, from 2004 through 2016, synovial tissue was collected from 27 patients who underwent arthroscopic synovectomy or joint replacement surgery involving the knee, and in 1 LA patient, the ankle. The patients included 14 with post-infectious LA, 5

with RA, 2 with psoriatic arthritis (PsA), one with undifferentiated inflammatory monoarthritis (UIM), and 5 with osteoarthritis (OA). Patients were divided into three groups for this study: post-infectious LA, other forms of inflammatory arthritis (RA, PsA, and UIM), or minimally inflammatory OA. A summary of clinical and laboratory findings of each patient group is summarized in Table 1.

The 14 patients with post-infectious LA, a number of whom were adolescents, underwent synovectomies a median of 14.5 months after the start of antibiotic therapy, and their median total arthritis duration was 18 months. In contrast, patients with other forms of chronic inflammatory arthritis and OA had a median arthritis duration of 84 and 60 months, respectively; they were significantly older, and most underwent joint replacement procedures, or in one case, a synovectomy. At the time of surgery, synovial tissue from patients with LA was uniformly negative for *B. burgdorferi* infection by PCR and culture. Furthermore, of the 12 patients who received immunosuppressive DMARD therapy after antibiotic therapy, none showed reactivation of infection. Together, these results suggest that patients were no longer actively infected, and were in the post-infectious phase of the disease.

Characterization of the synovial lesion

Inflammation, fibrosis, and the degree of joint degradation were assessed in patients with post-infectious LA by histologic analyses (Fig 1) and magnetic resonance imaging (MRI) scanning. The primary locations of inflammatory infiltrates, as indicated by intense hematoxylin staining, were along the synovial lining and sublining (Fig 1, **inset i**), and surrounding the microvasculature, which often contained a mixture of obliterated vessels (Fig 1, **inset ii**) and inflamed intact vessels (Fig 1, **inset iii**). Synovial tissue also showed marked tissue fibrosis, indicated by intense eosin staining of extracellular matrix (ECM) proteins, and was most pronounced within perivascular regions, which also contained inflamed blood vessels (Fig 1, **insets i & ii**). Blinded scoring of stained sections showed that post-infectious LA patients tended to have higher scores for cell infiltrates than RA or OA patients, whereas their fibrosis scores were in between the higher scores in RA patients and the lower scores in OA patients. However, there was substantial variability, both between samples and within individual sections. MRI of the affected joints was available in 11 of the 14 post-infectious LA patients. Consistent with our previous description of radiologic findings in LA patients (Lawson & Steere, 1985), all patients had proliferative synovitis and moderate joint effusions, and 5 also had evidence of mild cartilage damage and/or bony erosion.

RNA expression profiles in LA synovial tissue are similar to other forms of chronic inflammatory arthritis

To identify dysregulated pathways associated with this disease pathology, high-throughput RNA sequencing (Hi-seq, Illumina) was performed using RNA isolated from synovial tissue biopsies from patients with post-infectious LA. Other forms of chronic inflammatory arthritis (RA, UIM, and PsA) and degenerative arthritis (OA) were used as comparison groups. A total of 1216 genes were differentially-expressed in synovial tissue in post-infectious LA compared with OA. In contrast, only 176 genes were differentially-expressed

in post-infectious LA compared with other forms of inflammatory arthritis (Tables S1–S3). Thus, the gene expression profile in the post-infectious LA synovial lesion was similar to that seen in the other inflammatory arthritides, but was dissimilar to that of the OA lesion.

To identify gene pathways dysregulated in post-infectious LA synovial tissue, differentially-expressed genes were analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis tool (Fig 2). In this analysis, pathways associated with immune activation were significantly upregulated in synovial tissue from patients with post-infectious LA, compared with OA, particularly those involved in antigen processing and presentation, T cell and natural killer (NK) cell activation, and pattern recognition receptor signaling. In contrast, pathways associated with tissue repair were down-regulated, such as Hippo signaling genes involved in development and cell proliferation (Pan, 2010), TGF- β signaling, and activation of the complement and coagulation cascades (Fig 2A). Although the general gene expression profiles were similar between LA and other forms of chronic inflammatory arthritis, several inflammatory response pathways were uniquely up-regulated in post-infectious LA, whereas metabolic pathways were suppressed (Fig 2B). These results underscore that the marked inflammation in post-infectious LA synovial tissue was quite similar to that of other immune disorders, such as graft vs. host disease or RA. However, post-infectious LA also had features that were distinct from OA or other forms of inflammatory arthritis, such as suppressed expression of genes involved in tissue repair, cell proliferation, and metabolism.

Activation of innate and adaptive immune responses in post-infections LA and other chronic inflammatory arthritides

A more detailed examination of differentially-expressed genes from immune response KEGG pathway analysis was conducted using Morpheus (Broad Institute) hierarchal clustering analysis (Fig 3). All patients with post-infectious LA and most patients with RA and other forms of chronic inflammatory arthritis, expressed very high levels of genes involved in antigen processing, cell-mediated cytotoxicity, and T cell receptor (TCR) signaling (Fig 3A), consistent with chronic stimulation of T cells and NK cells by antigen presenting cells (APCs) within the synovial lesion. Two patients with other forms of inflammatory arthritis (RA2 and PsA2) lacked this cell-mediated immune response gene signature. In these two patients, radiographs revealed end-stage degenerative changes in affected joints, the final result of these chronic inflammatory arthritides. Accordingly, their cell-mediated immune response pathway gene expression profiles were most similar to that seen in OA, the prototypic form of degenerative arthritis.

Genes encoding IgG heavy and light chains were expressed at very high levels in most LA patients, presumably due to localized production of IgG antibodies by plasma cells, which are highly abundant in synovial tissue (Londono et al., 2014). However, only a few genes involved in B cell activation were expressed in synovial tissue, suggesting that Lyme-associated B cell activation was occurring elsewhere, such as within secondary lymphoid tissue. In contrast, marked T and NK cell signatures indicated that these lymphocytes were highly activated (or reactivated) within synovial tissue itself.

In addition to adaptive immune responses, genes associated with innate immune responses were also highly expressed in patients with post-infectious LA (Fig 3B). Genes associated with pathogen recognition (e.g., TLRs and NOD2) and NF- κ B-responsive cytokines and chemokines were particularly elevated, despite lack of evidence of ongoing infection. As with the cell-mediated immune response, all patients with post-infectious LA, and most patients with other forms of chronic inflammatory arthritis had high expression of these innate immune response genes. Patients RA2 and PsA2, however, had innate response expression profiles most like that seen in OA. These results suggest that both adaptive/autoimmune and innate/autoinflammatory processes may play a role in post-infectious LA disease pathogenesis.

To confirm immune activation at the protein level, cytokines and chemokines associated with innate or Th1 and Th17 adaptive immune responses were measured in serum, synovial fluid (SF), and synovial tissue from 10 patients with post-infectious LA for whom matched samples were available. The levels of most cytokines, particularly those associated with Th1 responses (e.g., IFN γ , CXCL9, and CXCL10) and innate immune responses (e.g., IL-6, IL-8, and CCL2) were highly concentrated in synovial fluid compared with serum (Fig S1). Similarly, the levels of T cell chemokines CXCL9 and CXCL10, as well as a number of innate cytokines, were highly abundant in synovial tissue, consistent with findings in SF. These results are very similar to those from a recent report from our group using SF and serum from a much larger cohort of 141 LA patients (Strle et al., 2017). Thus, localized inflammatory responses in the joint, the site of the disease, are dominated by robust Th1 and innate immune activation, which is consistent with RNA-seq findings.

Suppression of tissue repair genes in synovium of post-infectious LA

Several KEGG pathways associated with tissue repair genes, including those involved in cell proliferation, differentiation, wound healing, and cell metabolism, were decreased in patients with post-infectious LA compared with the other 2 groups (Figure 2). Hierarchical clustering analysis showed that patients with OA had very high expression of genes associated with cell proliferation/differentiation and tissue remodeling, compared with patients with post-infectious LA. These included tissue growth factors such as TGF-family bone morphogenic proteins (BMPs), fibroblast growth factors (FGF), and insulin-like growth factor (IGF); cell signaling genes such as WNT proteins and adenylate cyclases (ADCY); and laminins. Collectively, these genes are associated with proliferation and terminal differentiation of stromal cells which play pivotal roles in angiogenesis and extracellular matrix (ECM) remodeling during tissue repair (Gurtner et al., 2008; Martin, 1997) (Fig 3C). In contrast, most patients with RA and other forms of inflammatory arthritis had a cell proliferation/differentiation profile like that seen in OA, with the exception of 2 patients (RA5 and UIM1), who were similar to post-infectious LA.

Expression levels of a number of genes associated with cell metabolism were also lower in post-infectious LA than in other groups. These included genes encoding insulin-like growth factor 1 (IGF1), leptin receptor (LEPR), lipid-binding enzymes, and proteins involved in fatty acid metabolism (Fig 3D). These genes are typically associated with adipogenesis, which is an important biological process during joint tissue repair (Mobasheri et al., 2017).

Lower expression of these genes in post-infectious LA indicates that there may be fewer mature adipocytes within the tissue, consistent with a reduction in synthesis of ECM (e.g., laminins). Taken together, these findings indicate that gene expression in post-infectious LA synovial tissue is dominated by cell-mediated immune activation, innate immune activation, and suppression of genes involved in tissue repair and cell metabolism.

Altered early response to wounding expression signature

Elevated immune response genes, accompanied by suppressed expression of tissue repair genes, indicated that healing of damaged tissue following infection may be impaired or blocked in patients with post-infectious LA. Therefore, comparative analysis of the 190 genes in the Gene Ontology Response to Wounding gene set (GO:0009611, Princeton University) was assessed in greater detail. Of the 190 genes in this gene set, 85 response to wounding genes (45%) were differentially-expressed in post-infectious LA compared with OA (Fig 4A). Approximately one-third of the 85 genes were suppressed (or under-expressed) in patients with post-infectious LA, including those involved in coagulation and clot formation, ECM synthesis, and response to vascular stress. These genes are typically associated with early stages of wound healing and formation of granulation tissue (Gurtner et al., 2008). In contrast, two-thirds of the 85 genes were over-expressed in post-infectious LA, compared with OA. These included genes involved in maintaining a pathogen-free wound healing environment, such as innate cytokines (e.g., IL-1 β , IL-8), pathogen sensing molecules (e.g., toll-like receptors), and anti-bacterial enzymes (e.g., superoxide dismutase, lysozyme). The altered response to wounding profile was less pronounced and more variable in tissue from patients with RA and other forms of inflammatory arthritis. Only 8 of 190 response to wounding genes were differentially-expressed between patients with post-infectious LA and other forms of chronic inflammatory arthritis, (Fig 4B), and 8 were differentially-expressed between patients with OA and the other inflammatory arthritides (Fig 4C).

Dominant interferon response signature inversely correlates with tissue repair signature

In mice, dysregulated Type I (IFN α/β) or Type II IFN (IFN γ) production is associated with severe LA (Lochhead et al., 2012; Sonderegger et al., 2012; Whiteside et al., 2018). Therefore, Interferome analysis (Rusinova et al., 2013) was performed to identify the percentage of IFN-upregulated genes from those upregulated in LA synovial tissue compared with OA or other forms of chronic inflammatory arthritis (Fig 5A). When compared with OA synovial tissue, 37% of all genes up-regulated in post-infectious LA tissue were IFN-upregulated genes. In addition, compared with other forms of chronic inflammatory arthritis, about half of genes up-regulated in tissue from post-infectious LA were IFN-upregulated.

In murine LA, IFN-inducible gene expression correlates inversely with expression of genes associated with wound healing and tissue repair (Crandall et al., 2006). To compare gene expression patterns between individual patients, 4 gene signatures were generated and log-normalized for each patient sample. The first was an innate immune response signature of genes associated with pathogen recognition receptor (PRR) sensing pathways. The second was a cell-mediated immune response signature of genes associated with antigen processing

and presentation, NK cell activation, and T cell activation. The third was an interferon response signature of interferon-regulated genes. Finally, a tissue repair signature of genes associated with Hippo signaling, TGF- β signaling, coagulation, and wound healing was generated for each patient. Genes for these 4 signatures included those identified using the bioinformatics tools used in this study (KEGG pathway analysis, Response to Wounding Gene Ontology, or Interferome analysis), shown in Figures 2–5A (see Methods for a complete list of genes and method for normalization).

Correlation analysis was performed to assess the relationship between IFN responses and other gene signatures (Fig 5B). As expected, patients who had the highest expression of IFN-response genes in synovial tissue also expressed high levels of innate and cell-mediated immune response genes. At the protein level, expression of the IFN-response gene signature also correlated with the IFN-inducible T cell chemokine CXCL9 ($r=0.75$, $p=0.03$) in synovial tissue from post-infectious LA patients for whom both transcriptomics and cytokine data were available. In contrast, those with the highest expression of genes involved in tissue repair had relatively low expression of IFN-response genes. Thus, the IFN response signature positively correlated with other inflammatory response signatures, but inversely correlated with the tissue repair signature.

Comparisons of gene expression profiles with clinical, histologic, and microRNA findings

To identify clinical features most closely associated with the 4 gene signatures that were characteristic of post-infectious LA, innate immune response, cell-mediated immune response, interferon response, and tissue repair signatures were compared with clinical features of the disease (Fig 6). Longer duration of arthritis from the start of antibiotic therapy to synovectomy was associated with higher inflammatory gene signatures and lower tissue repair gene signatures. For example, the 3 patients with the longest arthritis duration (21 to 48 months) were among those with the highest inflammatory signatures and the lowest tissue repair signature. Other clinical features such as age, sex, or *B. burgdorferi* antibody responses did not correlate with these gene expression profiles. These findings emphasize that the marked inflammatory and suppressed tissue repair signatures may persist for several years after spirochetal killing with antibiotic therapy, and are likely the reasons for persistent synovitis.

Immunohistochemical staining of cell-lineage markers in tissue was conducted previously (Londono et al., 2014), and included synovial biopsies from 9 of the 14 patients in this study. Expression of tissue repair genes significantly correlated with staining of endothelial cell marker CD31 (PECAM), a marker of neovascularization of synovial tissue ($P=0.005$). In contrast, CD31 staining did not correlate with expression of innate immune response, cell-mediated immune response, or interferon-responsive genes, although there were modest, nonsignificant inverse trends. Correlations of gene expression profiles with markers for other cells, including lymphocytes, myeloid cells, or fibroblasts, did not achieve statistical significance. Thus, the suppressed tissue repair gene signature in post-infectious LA appears to strongly associate with impaired neovascularization.

MicroRNA (miRNA) expression profiling was also conducted previously using RNA collected from the same patients used in this study (Lochhead et al., 2017). In the previous

study, patients with post-infectious LA and those with other forms of chronic inflammatory arthritis had particularly high expression of pro-inflammatory miR-155 and low expression of cell-cycle regulator microRNA let-7a in synovial tissue, compared with OA tissue. Expression of innate immune response, cell-mediated immune response, and interferon response genes positively correlated with log-normalized expression of miR-155, while expression of tissue repair genes negatively correlated with miR-155 expression. In contrast, log-normalized let-7a expression negatively correlated with expression of inflammatory gene profiles, and positively correlated with expression of tissue repair genes. Thus, determinations of gene signatures here and previous measurements of cell lineage markers and microRNA expression profiles in synovium support the hypothesis that over-expression of inflammatory mediators and suppression of tissue repair mediators are central features of post-infectious LA.

DISCUSSION

Infection with *B. burgdorferi* elicits a robust immune response, which is necessary to control the infection. However, prolonged immune activation, as occurs in patients with post-infectious LA, may result in immune dysregulation and joint tissue damage. In these patients in whom arthritis persists despite 2–3 months of oral and/or IV antibiotic therapy, the nature of the arthritis is altered. Joint pathology in these patients is characterized by excessive inflammation, marked synovial hypertrophy, vascular damage, dysregulation of the CD4+ Teff/reg ratio, and increased T cell-dependent autoimmunity. Rather than additional antibiotic therapy, these patients usually respond well to treatment with immunosuppressive drug therapy, which is the standard of care for other forms of autoimmune arthritis, implying that this form of chronic inflammatory arthritis is no longer dependent on active infection for disease expression.

The current study provides the first detailed characterization of the synovial lesion transcriptome in post-infectious LA, based on synovial tissue collected from 14 patients over a 13-year period. Since most patients with LA resolve their arthritis with antibiotics or therapy with disease-modifying anti-rheumatic drugs (DMARDs), we were only able to collect synovium from patients who underwent synovectomies, which is a rare event. Importantly, synovium is the primary target tissue in this condition and in all forms of chronic inflammatory arthritis, including RA, ankylosing spondylitis, and psoriatic arthritis. However, unlike the other chronic inflammatory arthritides, the inflammatory trigger of LA, infection with *B. burgdorferi*, is known with certainty. Thus, study of the synovial lesion in LA is of paramount importance in providing mechanistic insights in post-infectious LA and may also provide insights about other chronic inflammatory joint diseases.

Based on RNA sequencing of 45 RA synovial samples and clinical correlations, Orange et al., recently stratified RA patients into 3 distinct gene expression subtypes: 1) an inflammatory subtype with high levels of infiltrating leukocytes, 2) a low inflammatory subtype with a strong TGF β signature, and 3) a mixed subtype (Orange et al., 2018). Although small, our RA patient cohort captured this heterogeneity. Moreover, those in our RA group who had the low inflammatory/TGF β signature had marked joint damage late in the disease, and their gene expression profiles were like those in our OA cohort and in the

low inflammatory RA subgroup reported by Orange (Orange et al., 2018). OA synovium is known to exhibit chronic over-expression of TGF β and other tissue growth factors (van der Kraan, 2017), and has altered metabolism within the joint microenvironment (Mobasheri et al., 2017). Thus, whereas there is a range of gene expression profiles in RA, the gene expression profile in post-infectious LA is more uniform and is similar with that of the inflammatory RA subgroup. Although cartilage loss and bony erosion may occur in post-infectious LA (Lawson & Steere, 1985), it is generally a minor component of the disease.

The correlations between gene expression profiles in synovium and previous histologic (Londono et al., 2014) and microRNA (Lochhead et al., 2017) findings indicated that microRNA expression and expression of other gene signatures were co-regulated. For example, the inflammatory/Th1 signatures in post-infectious LA and the inflammatory RA subtype of RA are accompanied by elevated miR-155 expression. In contrast, the tissue repair/TGF β signature in the low inflammatory RA subtype and OA were accompanied by elevated expression of let-7a, which was also accompanied by marked CD31 staining in LA. It will be important to learn whether these microRNAs have value in predicting the development of post-infectious LA or in stratifying patients in RA subgroups, since different groups likely have distinct mechanisms of pathology and seem to respond differently to various types of DMARD therapy (Orange et al., 2018).

Although mice do not develop post-infectious LA, our findings in humans have similarities with gene expression in joints of arthritogenic strains of mice during *B. burgdorferi* infection and LA development. Arthritogenic C3H/HeN and C57BL/6 II10^{-/-} mice have robust up-regulation of IFN-response pathways in infected joints early in infection that negatively correlate with expression of tissue repair genes (Crandall et al., 2006). This phenotype is dependent on Type I IFN (IFN $\alpha\beta$) over-expression by myeloid cells in C3H mice (Lochhead et al., 2012; Ma et al., 2014; Miller, Ma, Crandall, Wang, & Weis, 2008; Paquette et al., 2017), and on Type II IFN (IFN γ) over-expression by T cells in B6 II10^{-/-} mice (Sonderegger et al., 2012; Whiteside et al., 2018). Conversely, wild-type C57BL/6 mice, which develop mildly inflammatory LA, have marked upregulation of tissue repair genes in infected joints, with only a modest upregulation of IFN-responsive genes (Crandall et al., 2006), similar to our OA cohort or the low inflammatory RA subgroup.

In humans, as in mice, these IFN or tissue repair processes are likely initiated relatively early during infection. A strong IFN expression profile is present in erythema migrans, the initial skin lesion of early LD (Marques et al., 2017), and an *in vitro* model of early infection using PBMCs stimulated with *B. burgdorferi* suggests that Type I IFN production by plasmacytoid dendritic cells and other APCs may initiate this early IFN response (Petzke, Brooks, Krupna, Mordue, & Schwartz, 2009). As spirochetes move from the site of infection in the skin to other affected tissues, tissue-specific IFN responses to *B. burgdorferi* infection are likely initiated by IFN γ -producing innate lymphocytes, such as NK and invariant NKT cells (Katchar, Drouin, & Steere, 2013). IFN γ production by invariant NKT cells is thought to play a protective role in limiting spirochete invasion of joint (Lee et al., 2010; Lee et al., 2014) and heart tissue (Hawley et al., 2012). However, excessively high IFN responses early in infection may also lead to adverse disease outcomes (Strle, Jones, Drouin, Li, & Steere, 2011). These findings indicate that the magnitude of Type I and/or Type II IFN responses

must be sufficient to mount an appropriate anti-bacterial immune response, but must also be tightly regulated to avoid excess inflammation-induced tissue pathologies (Lochhead et al., 2015).

In addition to its anti-bacterial activity, IFN γ likely plays an important role in suppressing TGF- β expression and promoting fibrinolytic responses during *B. burgdorferi* infection. A recent report by Santus and colleagues (Santus et al., 2017) showed that *Candida albicans* and *Staphylococcus aureus* skin infections are initially contained through NFAT/TGF- β -dependent collagen deposition, and are subsequently eliminated through IFN γ -dependent suppression of TGF- β and promotion of fibrinolysis. Analogous processes are likely occurring initially in *B. burgdorferi* infection, and are necessary for trapping and eliminating the pathogen from connective tissue (Duray & Steere, 1988; Johnston et al., 1985).

Connective tissue damage is likely initiated by endothelial cell and ECM damage that occurs during infection. *B. burgdorferi* directly interacts with vascular endothelial cells (Coburn, Magoun, Bodary, & Leong, 1998; Sellati, Burns, Ficazzola, & Furie, 1995; Szczepanski, Furie, Benach, Lane, & Fleit, 1990). This interaction elicits a pro-inflammatory endothelial cell response (Lochhead et al., 2012; Sellati, Abrescia, Radolf, & Furie, 1996; Wooten, Modur, McIntyre, & Weis, 1996) and promotes vascular leakage (Sellati et al., 1995). During the infection, spirochetes are trapped within obliterative microvascular lesions in synovial tissue (Johnston et al., 1985) and in other tissues (Cadavid, 2006), and infection results in significant damage to the microvasculature (Duray & Steere, 1988; Lalosevic, Lalosevic, Stojacic-Milosavljevic, & Stojacic, 2010; Lochhead et al., 2015). Vascular leakage is itself a pro-fibrotic process (Shea et al., 2017). Additionally, *B. burgdorferi* binds to soluble fibronectin (Niddam et al., 2017) and fibronectin within the joint (Lin et al., 2014; Lin et al., 2015), resulting in inflammation at sites of vessel injury and induction of clot formation, a process that is regulated by plasma fibronectin (Wang, Gallant, & Ni, 2016). Interestingly, obliterative microvascular lesions in LA are reminiscent of the vasculopathy in tertiary syphilis, which is caused by another pathogenic spirochete, *Treponema pallidum* (Derick & Hass, 1935), which expresses adhesion molecules that directly interact with host endothelial cells and ECM components (Parker et al., 2016). Thus, vascular leak-induced fibrosis may be particularly relevant to tissue damage caused by spirochetal infections.

In post-infectious LA, vascular damage and fibrosis persist for months or years following spirochetal killing. Chronic up-regulation of anti-fibrotic IFN responses and suppressed pro-fibrotic tissue repair pathways indicate that a number of processes aimed at breaking down fibrotic tissue are dysregulated. Despite this robust anti-fibrotic response, post-infectious LA synovial tissue was often found to be quite fibrotic, indicating anti-fibrotic gene activation or suppression was not effective in these patients. Fibrosis is the result of dysregulated wound healing responses (Wynn & Ramalingam, 2012), suggesting that fibrosis in post-infectious LA is due to impaired tissue repair responses and development of a chronic synovial wound. However, these results also indicate that fibrosis in post-infectious LA is developing in a manner independent of activation of tissue growth factors, which are suppressed in post-infectious LA. The mechanisms of how this feedback loop is initiated and why it persists in the absence of a strong TGF- β response are not yet entirely clear.

Clues from both humans and animal models suggest that auto-inflammatory responses to damage-associated molecular patterns (DAMPs) may be contributing to chronic inflammation. In mice, accumulation of ECM and cellular debris, such as partially digested glycosaminoglycans, contribute to LA severity in C3H mice (Ma et al., 2014). Alternatively, pathogen-associated molecular patterns (PAMPs) may be acting as an inflammatory adjuvant, even when the bacteria have been killed. In mice, spirochetal antigens accumulate in joints following antibiotic therapy (Bockenstedt, Gonzalez, Haberman, & Belperron, 2012), and during infection, *B. burgdorferi* and host cells exchange lipids (Crowley et al., 2013), which may make these cells immune targets, even when spirochetes are no longer present (Crowley et al., 2016). The role of innate sensing of DAMPs and PAMPs in post-infectious LA is supported by genetic analysis, showing that a polymorphism in TLR1 is associated with significantly higher levels of IFN γ -inducible cytokines and with significantly greater likelihood of post-infectious LA (Strle et al., 2012).

It is also possible that autoimmune responses to Lyme-associated autoantigens contribute to chronic inflammation. For example, antibodies against ECGF (Drouin et al., 2013), which are associated with obliterative microvascular lesions (Londono et al., 2014), could be contributing to ongoing vascular inflammation, even after the infection has been cleared. Interestingly, autoantibodies to known Lyme-associated autoantigens ECGF, MMP-10, APOB-100, and ANXA2 may be detected at low levels in some patients with erythema migrans, generally without an accompanying T cell response (Crowley et al., 2015; Crowley et al., 2016; Drouin et al., 2013; Pianta et al., 2015). This initial response appears to be non-pathogenic and might even help to limit pro-inflammatory responses to DAMPs during tissue repair. However, under chronic inflammatory conditions, this benign or beneficial autoimmune response seems to become increasingly T cell-dependent and pathogenic. We have previously shown that in patients with post-infectious LA, a large percentage of the CD4⁺CD25^{hi} population, which ordinarily consist primarily of regulatory T cells, exhibit pro-inflammatory properties when restimulated, typical of effector T cells (Shen et al., 2010; Vudattu, Strle, Steere, & Drouin, 2013). Furthermore, in patients with post-infectious LA, but not in those with antibiotic-responsive LA, antibody responses to 3 Lyme-disease specific autoantigens (ECGF, MMP-10, and APOB-100) correlate directly with Th17 immune responses (Strle et al., 2017). Thus, unresolved innate immune responses arising from infection, autoinflammatory processes, or pathogenic tissue-specific autoimmunity, or a combination of the three, likely contribute to post-infectious LA pathogenesis. Although synovectomy was important in the resolution of arthritis in the patients reported here, post-infectious LA eventually resolves in all patients (Steere & Angelis, 2006). We postulate that without “danger signals” that are initially provided by live spirochetes, the innate and adaptive immune responses eventually regain homeostasis, and arthritis resolves.

The relationships between chronic vascular damage, tissue fibrosis, and autoimmunity seen in post-infectious LA may have broader implications for development of pathogenic autoimmunity in other conditions such as RA. An association between tissue damage signals and autoimmunity has been observed in RA (Nefla, Holzinger, Berenbaum, & Jacques, 2016), which may be initiated at sites of extra-articular inflammation and tissue damage from periodontal disease within the oral cavity (Arvikar et al., 2013; Potempa, Mydel, & Koziel, 2017), dysbiosis within the gut (Pianta et al., 2017; Van de Wiele, Van Praet,

Marzorati, Drennan, & Elewaut, 2016), or smoking-related lung fibrosis (Antoniou et al., 2013). However, in RA, autoimmunity usually develops into a life-long immune disorder, which does not happen in post-infectious LA, implying that in RA, antigenic triggers remain.

In a companion article published by Lochhead, et al in this issue, we used a combination of *ex vivo* and *in vitro* methods to identify cellular sources of IFN γ in synovial tissue. We also showed that fibroblast-like synoviocytes (FLS), the most common cell type in synovium, are an important target of this IFN γ response. Notably, FLS stimulated with IFN γ *in vitro* differentiate into immune effector cells, express MHC Class II HLA-DR molecules, and exhibit cytokine and chemokine responses that recapitulate conditions observed in synovial tissue presented in the current study. FLS are a key cell type during tissue repair and wound healing, and we propose that excessive IFN γ responses in the inflamed post-infectious synovial tissue alters FLS phenotype from tissue-remodeling myofibroblast-like synoviocytes into pro-inflammatory nonprofessional antigen presenting cells, thereby driving tissue-specific innate and adaptive immune responses seen in post-infectious LA.

In summary, LA is a disease wherein the transitions from infection to immune dysregulation and autoimmunity and corresponding wound repair responses can be observed longitudinally in individual patients with different outcomes, such as those who resolve their arthritis with antibiotics, and those who go on to develop post-infectious LA. Although spirochetes initially affect joints of both patient groups, one responds advantageously after antibiotics and the arthritis resolves, whereas the other does not. In the latter group, excessive IFN γ responses appear to block normal wound repair processes, resulting in proliferative synovitis that persists for months or years following antibiotic therapy. This model provides an opportunity to study immune disorders triggered by infection in ways not possible with other types of immune-mediated diseases where the inflammatory trigger is unknown. Thus, the lessons learned in LA, which revealed impaired wound repair as a key factor in the pathogenesis of the synovial lesion, may provide a new paradigm for studying disease pathogenesis in the other chronic inflammatory arthritides.

RESEARCH METHODS

Ethics Statement

The study “Immunity in Lyme arthritis” was approved by the Human Investigations Committee at Massachusetts General Hospital (MGH), according to principles for medical research involving human subjects expressed in the World Medical Association Declaration of Helsinki. Written informed consent was obtained from all participants 18 years of age or older, or from a parent or guardian of participants under the age of 18 on their behalf.

Patients

All patients with Lyme disease met the Centers for Disease Control and Prevention criteria for *B. burgdorferi* infection (Centers for Disease & Prevention, 1995); and those with RA, psoriatic arthritis, undifferentiated inflammatory monoarthritis, or osteoarthritis (OA) met validated criteria for those diseases (Aletaha et al., 2010). LA patients received treatment

according to an algorithm (Steere & Angelis, 2006), as detailed in the guidelines of the Infectious Diseases Society of America (Wormser et al., 2006).

Determination of inflammation and fibrosis

Synovial lesion biopsies were collected and frozen at the time of surgery. Biopsies were sectioned and stained using hematoxylin and eosin, and imaged using an AxioScan.Z1 scanner (Zeiss). Images from post-infectious LA, RA, and OA patients were assigned a random code and blindly scored by ACS for infiltrate and fibrosis severity, with 4 being the most severe, and 1 being mild.

RNA purification

Tissue biopsies were collected at the time of synovectomy and placed immediately in RNA-later and stored at -20°C . RNA was recovered from ~100 mg synovial tissue using a miRNeasy kit (Qiagen). Ribosomal RNA was depleted using RiboZero reagent (New England Biolabs). Synovial tissue RNA quality was determined using a Bioanalyzer (Agilent), and low-quality RNA samples were excluded from this study.

High-throughput RNA sequencing

RNA libraries were constructed using the NEBNext Ultra RNA Prep Kit for Illumina (New England Biolabs). Libraries were sequenced to a depth of 25–35 million paired-end, 50 base-pair reads (Hi-Seq PE50 Reagent Kit, Illumina). Transcriptome coverage was estimated at ~40%. Library preparation, sequencing, and bioinformatics were performed by the MGH NextGen Sequencing and Bioinformatics Core Facilities.

Bioinformatics analysis of gene expression

Copies per million reads (CPMR) was used to normalize expression between patients. The following web-based bioinformatics tools were used for gene expression analysis: Database for Annotation, Visualization, and Integrated Discovery v.6.8 (Huang da, Sherman, & Lempicki, 2009), a functional annotation tool; Morpheus (Broad Institute, software.broadinstitute.org/morpheus/), a matrix visualization and analysis platform; Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (GenomeNet, www.genome.jp/kegg/pathway) (Kanehisa & Goto, 2000), a tool to identify enriched biological pathways; Gene Ontology (GO, www.geneontology.org/page/go-enrichment-analysis) (Ashburner et al., 2000), a gene enrichment analysis tool; INTERFEROME v2.0 (interferome.its.monash.edu.au/interferome) (Rusinova et al., 2013), a tool to compare gene expression profiles with a searchable database of interferon-regulated genes.

Determination of gene pathway signatures

Gene pathway signatures for each sample were determined by calculating the median value of log-transformed expression of differentially-expressed genes identified by bioinformatics analysis used in this study, normalized to geometric mean gene expression. Genes not expressed in all synovial tissue samples were excluded from analysis.

Cell-mediated immune response gene signature included genes differentially-expressed between post-infectious LA and OA from the following KEGG pathways: antigen

processing and presentation, natural killer cell-mediated cytotoxicity, and T cell receptor signaling (B2M, CARD11, CD244, CD247, CD248, CD28, CD3D, CD3E, CD40LG, CD48, CD74, CD8A, CIITA, FASLG, FCGR3B, GRAP2, GZMB, HCST, HLA-A, HLA-B, HLA-C, HLA-DOA, HLA-DPA1, HLA-DPB1, HLA-DQA1, HLA-DQA2, HLA-DQB1, HLA-DRA, HLA-DRB1, HLA-DRB5, HLA-F, ICAM1, ITGAL, ITK, KLRD1, LCK, LCP2, NFKBIA, PDCD1, PRF1, PRKCB, PRKCQ, RAC2, RASGRP1, SH2D1A, TAP1, TAP2, TNFSF10, ZAP70).

Innate sensing gene signature included genes differentially-expressed between post-infectious LA and OA from the following KEGG pathways: NOD-like receptor signaling, Toll-like receptor signaling, and cytosolic DNA sensing (BIRC3, CCL13, CCL2, CCL4, CCL5, CCL8, CD80, CXCL1, CXCL10, CXCL2, CXCL9, IL1B, IL8, MEFV, NFKBIA, NOD2, STAT1, TLR2, TLR8, TNFAIP3).

Interferon signature included a subset of genes upregulated in post-infectious LA, compared with OA, from genes within the Interferome dataset (ADAR, B2M, CIITA, CXCL10, CXCL9, GBP1, GBP2, GBP3, GBP4, GBP5, IFI27, IFIH1, IRF1, IRF2, IRF9, ISG20, OAS2, OASL, STAT1, TRIM21, TRIM26).

Wound repair gene signature included genes upregulated in OA, compared with post-infectious LA, from the Hippo signaling, TGF- β signaling, complement and coagulation cascade, and basal cell carcinoma KEGG pathways; those upregulated in OA vs. post-infectious LA from the response to wounding GO gene set; and genes upregulated in RA/UM/PSA vs. post-infectious LA from AMPK signaling, adipocytokine signaling, regulation of lipolysis in adipocytes, and PPAR signaling KEGG pathways (ADCY2, ADCY5, ADORA3, AOX1, AR, BMP4, BMP5, BMP7, C7, CD36, CDO1, CFD, FABP4, FBLN5, FGF13, FGF18, FST, FZD8, GDF5, GDF7, HHIP, IGF1, IGFBP4, IGSF10, IL17D, LAMA2, LAMC3, LEPR, LIPE, LOX, LPL, LYVE1, NBL1, NRG1, NTRK1, OLR1, PCSK1, PLIN1, PROS1, SCD, SCG2, SCUBE1, SERPINE1, SLC11A1, SORBS1, SPP1, TREML1, VCAN, WNT11, WNT5B, WNT9A).

Quantification of cytokines and chemokines

The levels of 10 cytokines and chemokines associated with innate and adaptive immune responses were assessed in matched serum, synovial fluid, and synovial tissue samples from 10 patients with post-infectious LA. Cytokine/chemokine determinations were performed in sera (diluted 3-fold), synovial fluid (diluted 5-fold), and in 10 μ g of synovial tissue with PBS as the diluent. The levels of each mediator were assessed in one complete experiment using bead-based multiplex assays (EMD) and a Luminex 200 instrument, following the manufacturer's protocol.

Immunohistochemical staining and scoring of biopsies

Sections of synovial biopsies were previously analyzed by immunohistochemistry (Londono et al., 2014) to probe for markers of T cells (CD3), B cells (CD20cy), macrophages (CD68), plasma cells (VS38c), myeloid dendritic cells (CD38), follicular dendritic cells (Ber-MAC-DRC), endothelial cells (CD31), and fibroblasts (vimentin), and included 9 of the 14 patients

used in this study. Staining intensity was ranked in a blinded fashion from lowest (1) to highest (9), and were correlated with gene expression profiles using PRISM v.6 (Graph Pad).

Statistical analysis

Differential expression analysis (custom bioinformatics R script) was used to determine differences in synovial tissue mRNA expression, adjusting for false discovery error using Benjamini-Hochburg correction (adjusted p-value <0.05). Clustering of genes was performed using one minus Pearson correlation analysis. Mann-Whitney exact test was used to determine differences in clinical data between groups. Correlations between patient gene profile signatures, microRNA expression, and antibody staining were determined using Pearson's r coefficient analysis. Friedman's paired test with Dunn's multiple comparison correction was used for paired measurements from the same patient. Statistical significance (p<0.05) was determined using PRISM v6 (Graph Pad) or statistical analysis algorithms from web-based bioinformatics platforms used in this study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1. Histologic assessment of the synovial lesion.

Representative H&E-stained section of a biopsy from a patient with post-infectious LA is shown, highlighting the inflammatory infiltrate along the synovial lining and sublining (i), areas of fibrosis surrounding ablated microvascular lesions (ii), and intact inflamed microvasculature (iii). Comparative analysis of infiltrate and fibrosis severity was conducted for 14 patients with post-infectious LA, 3 patients with RA, and 3 patients with OA, with 4 being severe and 1 being mild. Statistically significant differences between groups were determined by Mann-Whitney t-test, with p-values indicated in the figure.

Fig 2. Pathways enriched in synovial tissue from patients with post-infectious LA, other inflammatory arthritides, or OA.

KEGG pathways enriched in synovial tissue from (A) post-infectious LA compared with osteoarthritis (OA), or (B) other forms of inflammatory arthritis including rheumatoid arthritis (RA), undifferentiated inflammatory monoarthritis (UIM) and psoriatic arthritis (PSA) are shown for 14 patients with post-infectious LA, 5 patients with RA, 1 patient with UIM, 2 patients with PSA (RA, UIM, and PSA grouped together), or 5 patients with OA. Statistically significant differences between groups were determined by t-test, adjusting for false discovery error using Benjamini-Hochburg correction (adjusted p-value<0.01).

Fig 3. Gene expression comparisons between patients with post-infectious LA, other forms of inflammatory arthritis, and OA.

Hierarchical clustering analysis is shown for genes differentially-expressed between post-infectious LA and OA, associated with (A) cell-mediated immune response KEGG pathways (antigen processing and presentation, NK cell-mediated cytotoxicity, and TCR signaling pathways), (B) innate immune response (NOD-like receptor signaling and Toll-like receptor signaling pathways), (C) cell proliferation and differentiation (HIPPO signaling and TGF signaling pathways), or (D) metabolic pathways (PPAR signaling, lipolysis, adipocytokine signaling, AMPK signaling pathways). Log-transformed maximally-expressed genes are indicated in red (row max), and minimally-expressed genes are indicated in blue (row min). Clustering of genes was performed using one minus Pearson correlation analysis.

A Interferon-regulated genes in post-infectious LA **B Interferon response signature compared with other gene signatures**

Fig 5. Robust interferon profile in patients with post-infectious LA is associated with suppressed tissue repair signature.

(A) Interferon-upregulated and other upregulated genes in post-infectious LA were compared with OA or other forms of inflammatory arthritis. (B) Log-normalized expression of genes from the interferon response signature were compared with expression of genes from the innate immune response signature, the cell-mediated immune response signature, or the tissue repair signature for each sample using correlation analysis. Statistically significant correlations were determined by t-test with Pearson's r coefficient analysis (p -values are indicated in figure).

Fig 6. Comparisons between pathway gene signatures and other markers of disease.

Pathway gene signatures for each patient were compared with arthritis duration after the start of antibiotic therapy (all post-infectious LA patients), neovascularization by ranked staining of endothelial cell marker CD31 (9 post-infectious LA patients), and log-normalized expression of miR-155 (all patients), or log-normalized expression of let-7a (all patients) by correlation analysis. Correlations were determined by t-test using Pearson's analysis (Pearson's r and p values are indicated in figure).

Table 1.

Summary of characteristics and clinical findings of patient groups.

Patient group (no. patients)	Post-inf. LA (n=14)	RA/PsA/UIM (n=8)	OA (n=5)
Age, median (w/range)	20 (11–58)	49.5 (39–73)*	56 (50–80)*
Male/Female no. of pts.	7/7	4/4	3/2
Oral AB (months)	2 (1–4.5)	NA	NA
IV AB (months)	1 (1–2)	NA	NA
Received DMARDs	12/14	7/8	NA
Arthritis duration (months)	18 (4–48)	84 (12–252)*	60 (24–132)*
--prior to start of AB	0.5 (0–20)	NA	NA
--after start of AB	14.5 (4–48)	NA	NA
ESR (normal <13 mm/hr)	9 (1–43)	19 (10–40)	NA
CRP (normal <7 mg/dL)	4.7 (0.6–17.4)	7.6 (6.4–19.1)	NA
<i>Bb</i> IgG titer	19,200 (800–25,600)	NA	NA
No. WB bands	9 (5–10)	NA	NA
No. tissue biopsies <i>Bb</i> PCR positive	0/14	NA	NA
-- <i>Bb</i> culture positive	0/14	NA	NA

Statistically significant differences between groups determined by Mann-Whitney t test (* $p < 0.05$). Numbers represent median (range) unless otherwise stated.