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MicroRNA expression shows inflammatory dysregulation and tumor-like proliferative responses in joints of patients with post-infectious Lyme arthritis

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Abstract

Objective—Lyme arthritis (LA) is caused by infection with *Borrelia burgdorferi* and usually resolves following spirochetal killing with antibiotics. However, in some patients, arthritis persists after antibiotic therapy. To provide insights into underlying pathogenic processes associated with post-infectious LA, we analyzed differences in microRNA expression between LA patients with active infection compared with those with post-infectious LA.

Methods—MicroRNA expression was assayed in synovial fluid (SF) from LA patients before and after oral and IV antibiotic therapy, and from synovial tissue from post-infectious LA patients obtained months after antibiotic therapy. SF and tissue from patients with other forms of arthritis such as rheumatoid arthritis and osteoarthritis were used for comparison groups.

Results—SF from LA patients during active infection had marked elevation in white blood cells, particularly polymorphonuclear leukocytes, accompanied by elevated miR-223 levels. In contrast, SF from post-antibiotic LA patients contained greater percentages of lymphocytes and mononuclear cells. Post-antibiotic LA SF also exhibited marked inflammatory (miR-146a, miR-155), wound repair (miR-142), and proliferative (miR-17~92) microRNA signatures, and higher levels of these microRNAs correlated with longer arthritis duration. miR-146a, miR-155, miR-142, miR-223, and miR-17~92 were also elevated in synovial tissue in late post-infectious LA, and let-7a was reduced, similar to RA.

Conclusions—During active infection, microRNA expression in SF reflected an immune response associated with bacterial killing, whereas in post-infectious LA, microRNA expression in SF and synovial tissue reflected chronic inflammation, synovial proliferation, and breakdown of wound repair processes, showing that the nature of the arthritis was altered after spirochetal killing.

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Bacterial infection normally elicits robust and effective immune responses. However, failure to resolve immune responses following pathogen clearance can result in tissue damage. These responses are tightly regulated by microRNAs (miRNAs), which are required to enhance or limit a large number of biological processes, including immune responses to infection (1).

miRNAs are small noncoding RNAs that bind the 3′ untranslated region of target mRNAs and inhibit translation, thereby acting as fine-tuners of gene expression (2). miRNAs provide robustness to gene regulation (3), and defects in miRNAs can contribute to a number of pathologies, including inflammatory and autoimmune diseases (1). For example, a recent miRNA study showed that rheumatoid arthritis (RA), a chronic inflammatory autoimmune disease, had the most significant enrichment of miRNA-target gene risk factors of any disease studied (4). Moreover, mouse studies have shown that miR-155 (5), miR-223 (6), and miR-146a (7) directly modulate experimental models of arthritis, consistent with the role of miRNAs and other epigenetic regulatory factors in human RA pathogenesis (8).

Using mouse models of Lyme disease, we have shown that miR-146a and miR-155 are upregulated during infection with the Lyme disease spirochete *Borrelia burgdorferi*, and act as negative (miR-146a) and positive (miR-155) regulators of immune activation (9, 10). These two miRNAs help to fine-tune the immune response, ensuring effective spirochetal killing while limiting tissue damage. Mice lacking either miR-146a or miR-155 have more severe Lyme arthritis (LA) or carditis, respectively, suggesting important roles for these miRNAs in balancing immune activation and tissue damage (9, 10).

Other miRNAs associated with immune regulation and cell proliferation also show altered expression in joints of infected mice that developed severe LA (9). These include miR-142, associated with immune modulation (11, 12) and tissue remodeling (13, 14); miR-17~92 cluster, associated with cell proliferation and oncogenesis (15–17); and let-7 family members, associated with tumor suppression (18). However, to date, no studies have examined the role of miRNAs in human Lyme disease.

Lyme arthritis (LA), the most common late manifestation of Lyme disease, is characterized by intermittent or persistent joint swelling and pain in one or a few large joints, especially the knee (19). In most patients, the arthritis resolves with spirochetal killing with oral or intravenous (IV) antibiotic therapy. However, in some patients, synovitis persists for months or years after antibiotic therapy. The synovial lesion in these patients shows synovial hypertrophy, vascular proliferation, and mononuclear cell infiltrates, which are also observed in other forms of chronic inflammatory arthritis, including RA (20). After antibiotic therapy, patients with antibiotic-refractory LA are treated with disease-modifying anti-rheumatic drugs (DMARDs), the standard treatment used for other inflammatory arthritides (21).

In this study, we assessed in LA patients expression of miRNAs that have been previously associated with arthritis pathogenesis (22). We report that during active infection, miRNA expression reflected an immune response associated with bacterial killing, and joint swelling resolved when that was accomplished. However, following antibiotic therapy and in the absence of active infection, a subset of patients transitioned to a marked proliferative

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synovitis, previously called antibiotic-refractory LA, but hereafter termed post-infectious LA. In these patients, miRNA expression profiles reflected inflammatory and proliferative dysregulation involved in post-infectious LA pathogenesis.

PATIENTS AND METHODS

Patients

The study "Immunity in Lyme arthritis" was approved by the Human Investigations Committee at Massachusetts General Hospital (MGH). All patients with Lyme disease met the Centers for Disease Control and Prevention criteria for *B. burgdorferi* infection (23); and those with RA, psoriatic arthritis, undifferentiated inflammatory monoarthritis, or osteoarthritis (OA) met validated criteria for those diseases (24). LA patients received antibiotic therapy according to an algorithm (25), as detailed in the guidelines of the Infectious Diseases Society of America (26).

Sample collection

Patient SF was centrifuged at 300g for 10 minutes, then at 3000g for 10 minutes, and stored at −80°C. Synovial tissue was collected from arthritis patients who underwent arthroscopic synovectomies. Tissue was placed immediately in RNA stabilization reagent and stored at −20°C.

RNA purification

RNA was recovered from 200 μl SF using a serum/plasmid miRNeasy kit, or from ~100 mg synovial tissue using a miRNeasy kit (Qiagen). Synovial tissue RNA quality was determined using a Bioanalyzer (Agilent).

Synovial fluid miRNA expression

miRNAs in synovial fluid were assayed using miScript Human Serum & Plasma 384HC miRNA PCR arrays (MIHS-3106ZG, Qiagen) on a Roche LC-480 light cycler. Expression was normalized to global geometric mean threshold cycle (Ct) of all expressed miRNAs (Ct cutoff=35).

Synovial tissue miRNA expression

Small RNA libraries were generated using the NEBNext multiplex small RNA library prep set for Illumina (New England Biolabs). Quality was determined using a Bioanalyzer (Agilent). Libraries were sequenced to a depth of ~2,500,000 50 base-pair reads (MiSeq Reagent Kit v2, Illumina). Library preparation, sequencing, and bioinformatics were performed by the MGH NextGen Sequencing and Bioinformatics Core Facilities.

Statistical and pathway analysis

Mann-Whitney exact test was used to determine differences in clinical data between groups. Welch's t-test was used to determine differences in miRNA expression between groups. Pearson's r coefficients were determined by correlation analysis. Differential expression analysis was used to determine differences in synovial tissue miRNA expression. miRNA/

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mRNA pathway analysis was performed using DIANA-mirPath v.2 (27). Statistical significance ($p<0.05$) was determined using PRISM v6 (Graph Pad).

RESULTS

Patients

During a 1.5-year period, from October 2014 through February 2016, 34 LA patients were enrolled in our study, 18 of whom had evaluable synovial fluid (SF) samples available. Additionally, from 2004 to 2015, 14 patients with LA underwent arthroscopic synovectomy from whom evaluable synovial tissue was available. Thus, for this study, 32 patients with LA were evaluated from whom it was possible to obtain extracellular miRNA from SF or miRNA from synovial tissue. These patients were representative of the spectrum of disease severity and treatment responses seen in this disease (28). Because inflammation in LA is localized to affected joints, we present data here only from patients in whom SF or synovial tissue was available.

Of the 18 patients from whom SF was collected, 5 were referred prior to antibiotic therapy when they had active *B. burgdorferi* infection (Group 1 pre-antibiotics, Table 1). The other 13 patients from whom SF was available were referred because of incomplete responses to oral doxycycline or IV ceftriaxone (Group 2, post-antibiotics). In these patients, SF was usually collected soon after oral or IV therapy when few, if any, spirochetes remained (29), and prior to starting DMARD therapy, usually methotrexate (MTX). In patients where multiple samples were available, the first sample collected was analyzed. miRNA from synovial tissue, a target tissue of this disease, was available from 14 patients who underwent arthroscopic synovectomies from 4–48 months (median 15.5 months) after oral and IV antibiotics (Group 3, synovectomy). In these patients, DMARDs were stopped several weeks prior to synovectomy.

Group 1

The 5 patients who were referred prior to antibiotic therapy (Group 1) had mild-to-severe knee swelling and pain for a median duration of 1 month prior to evaluation and the start of antibiotic treatment (Table 1). Typical of our previous cohorts, antibody titers to *B. burgdorferi* were high prior to therapy (30), and PCR results for *B. burgdorferi* DNA in SF were positive for 2 of the 4 patients (50%) tested (29). In addition, consistent with past experience (19, 28), the median white blood cell (WBC) count in SF was 25,760 cells/mm^3 with 90% polymorphonuclear leukocytes (PMNs). Three of the 5 patients resolved their arthritis during a one-month course of oral doxycycline, whereas 2 continued to have marked knee swelling. They were then treated with one month of IV ceftriaxone, and their arthritis resolved.

As determined by qRT-PCR, the 5 patients with active infection who were seen prior to antibiotic therapy had low levels of 5 of the 6 miRNAs measured in this study, including hematopoietic-specific miR-146a, miR-155, and miR-142 (14, 31), which are associated with myeloid cell effector function (Figure 1). Levels of miR-17 and miR-20a, part of the miR-17~92 oncomiR family involved in cell cycle regulation (15), were also low. The

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notable exception was high levels of miR-223, a hematopoietic-specific miRNA, which is abundantly expressed in PMNs (31). miR-223 is associated with down-regulation of acute inflammation (32) and tissue remodeling (33). This finding is consistent with high levels of PMNs present in SF from these patients.

Group 2

The 13 patients who continued to have joint swelling after oral or IV antibiotic therapy exhibited marked variability in the severity and duration of their arthritis (Group 2), which is consistent with heterogeneity of treatment outcomes in human LA. These patients were referred because of incomplete responses to oral or IV antibiotic therapy 2.75 to 17 months after arthritis onset (Table 1). Of the 13 patient samples, 7 were collected after completion of oral antibiotic therapy but before IV antibiotic therapy, and 6 were collected after completion of both oral and IV antibiotic therapy, but before DMARD therapy. In contrast with patients in Group 1 who were seen prior to antibiotic therapy, those in Group 2 had significantly lower WBC counts in SF (p=0.008), fewer PMNs (p=0.004), and greater percentages of lymphocytes (p=0.008) and monocytes (p=0.028). Of the 13 patients, 2 resolved their arthritis after IV antibiotic therapy, whereas the other 11 did not. They were usually treated successfully with MTX, and none had reactivation of infection during or after immunosuppressive therapy.

In contrast with the low levels of most miRNAs found in Group 1, levels of miR 146a, 155, 142, 17, and 20a were higher in Group 2 than in Group 1 (Figure 1). This suggested that the nature of the arthritis had changed after spirochetal killing. The exception was miR 223, which was typically elevated in patients in both Groups 1 and 2. However, of the 5 patients in Group 1, the 2 who required IV antibiotic therapy for resolution of arthritis had higher levels of miR-142 and miR-223 (Figure 1, open circles) than the 3 patients who resolved their arthritis with oral therapy (Figure 1, closed circles). Conversely, of the 13 patients who were seen in the post-antibiotic period, the 2 whose arthritis resolved with IV therapy (Figure 1, open squares) had low-to-moderate values of miR-155 and miR-142 (Figure 1, closed squares).

For comparison, miRNAs were also assessed in SF from 4 patients with OA, a minimally inflammatory type of arthritis, and from 6 patients with RA, the prototypic form of chronic inflammatory arthritis. Levels of all 6 miRNAs in patients with RA were similar to those in LA patients in Group 2, whereas the low levels of most of these miRNAs in OA patients were similar to those in LA patients in Group 1 (Figure 1). miR-223 was high in all LA patients, and it was particularly high in RA patients, but very low in OA patients.

Correlation of arthritis duration and SF laboratory findings

When patients in Groups 1 and 2 were considered together, SF levels of miR-146a, miR-155, miR-142, miR-17, and miR-20a positively correlated with arthritis duration after start of oral antibiotic therapy (Figure 2A). Similar results were obtained with the estimated total duration of arthritis, but the data are not shown here because the date of arthritis onset was not always clear. Conversely, *B. burgdorferi* IgG antibody titers negatively correlated with miR-155 (r= −0.49, p=0.04), miR-146a (r= −0.49, p=0.04), and miR-142 levels (r=

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−0.54, p=0.02). Levels of miR-223, the only miR that was high in both Groups 1 and 2, did not correlate with arthritis duration.

When cell composition of SF from patient Groups 1 and 2 were considered together, changes in cellularity correlated with arthritis duration prior to sample collection (Figure 2B). Arthritis duration negatively correlated with WBC count (p=0.03) and the percent PMNs (p=0.002), and positively correlated with percent lymphocytes (p=0.04) and mononuclear cells (p=0.01). These results further suggested that the pathologic characteristics of the arthritis changed during transition from active infection to post-infectious LA, and that these miRNAs may have a role in arthritis pathogenesis.

Group 3

RNA from synovial tissue was available from 14 patients who underwent synovectomies for treatment of persistent synovitis (Group 3) a median of 15.5 months (range 4–48) after 2–3 months of antibiotic therapy (Table 1). Two patients (14%) required a second synovectomy; both patients declined DMARD therapy prior to their first synovectomy, whereas the other 12 patients did not. All 14 patients had negative culture and PCR results for *B. burgdorferi* and *B. burgdorferi* DNA (29). SF was not available for testing in these patients.

To assess miRNA expression in synovial tissue, the 14 LA samples were analyzed using miRNA-seq, allowing a global assessment of all miRNAs. miRNA-seq was not possible with the smaller amounts of RNA in cell-free SF. For comparison, synovial tissue was assessed from 8 patients with other forms of inflammatory arthritis (5 RA, 2 psoriatic arthritis, 1 undifferentiated inflammatory monoarthritis) and from 5 patients with minimally-inflammatory OA. A total of 73 miRNAs were differentially expressed in synovial tissue from post-infectious LA patients, compared with tissue from OA patients. A complete list of miRNAs differentially expressed in each patient group is provided (Supplemental Table S1).

These 73 differentially-expressed miRNAs were subjected to further analysis to determine which genes and pathways were predicted to be regulated by these miRNAs (Table 2) (27). Genes predicted to be regulated by 44 miRNAs over-expressed in LA synovial tissue were involved in cellular proliferation or regulation of inflammatory processes, such as T cell receptor signaling, B cell receptor signaling, and antibody-mediated phagocytosis. In contrast, the 29 miRNAs over-expressed in OA synovial tissue were predicted to be involved in tissue remodeling and cell proliferation, but not inflammation, consistent with the nature of OA. Thus, miRNAs in synovial tissue from post-infectious LA patients predominantly regulated 2 processes, inflammation and proliferation, whereas miRNAs in this tissue in OA regulated primarily proliferation.

As with all forms of chronic inflammatory arthritis, the synovial lesion in the 14 patients with post-infectious LA was characterized by massive, tumor-like proliferation of inflamed synovial tissue that can invade cartilage and bone (Figure 3). miRNA-seq analysis of inflamed synovial tissue showed high expression of many miRNAs that were also abundant in SF from post-antibiotic LA patients in Group 2. These included anti-inflammatory miR-146a and pro-inflammatory miR-155 (Figure 3A), associated with TLR/NF-κB inflammation (34), as well as hematopoietic-specific miRNAs, such as miR-142 and

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miR-223, associated with myeloid function, modulation of acute inflammation, and initiation of wound repair (Figure 3B) (11, 12, 14, 33). While expression levels of most miRNAs were similar between male and female patients, miR-146a was 2.9-fold higher in males and 1.4-fold higher in females in post-infectious LA synovial tissue compared with OA tissue.

As in SF, synovial tissue miRNA expression also contained a distinct oncogenic miRNA profile, and was similar between male and female patients. OncomiRs miR-17 and miR-20a were approximately 2.5- to 4-fold higher in post-infectious LA synovial tissue compared with OA tissue (Figure 3C); and let-7a and let-7c, tumor-suppressor miRNAs (18), were approximately 2-fold lower (Figure 3D). Thus, in these patients, the transition to the post-infectious phase was blocked by chronic inflammation, which stalled the wound repair process. Both inflammatory and proliferative miRNA responses were altered in these patients, indicative of development of an inflamed, tumor-like synovial lesion, analogous to a chronic synovial wound.

Correlation of miRNA signature in synovial tissue with disease duration

In contrast with findings in SF (Figure 2), expression of NF-κB-inducible miRNAs miR-146a and miR-155 did not significantly correlate with arthritis duration after start of antibiotic therapy (Figure 4). However, miR-223 and the oncomiR miR-17~92 cluster expression in tissue positively correlated with arthritis duration after the start of treatment, whereas let-7 family of tumor suppressor miRNAs negatively correlated with post-treatment arthritis duration (Figure 4). Each of these correlations was similar when analyzed with the estimated total arthritis duration (data not shown). Thus, in synovial tissue obtained late in the disease, miRNAs involved in proliferative responses correlated with arthritis duration, but chronically elevated inflammatory miRNAs did not.

These synovial tissue results showed that late in post-infectious LA, TLR/NF-κB-dependent miRNAs were constitutively expressed, and the tumor-associated miRNA signature became progressively more pronounced. This suggested that expression of these miRNAs reflected immune dysregulation in synovial tissue of patients with post-infectious LA, possibly perpetuating chronic inflammation and synovial proliferation in these patients.

DISCUSSION

In this study, patients who were seen prior to antibiotic therapy had a high number of infiltrating leukocytes in SF, primarily PMNs (Table I) and elevated levels of miR-223, which is highly expressed in PMNs (33). In contrast, they had very low levels of other hematopoietic-specific miRNAs, such as miR-146a, miR-155 and miR-142, similar to levels in SF in patients with OA (Figure 1). Low levels of these miRNAs, especially miR-146a and miR-155, suggested that inflammatory processes, such as TLR/NF-κB signaling, were tightly regulated in joints of these patients, despite the presence of spirochete-derived TLR ligands. Thus, during active infection, immune responses were focused on bacterial killing, primarily through controlled inflammation, antibody production, and PMN infiltration in SF of infected joints. After antibiotic therapy, most patients resolved their arthritis by down-

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regulating anti-bacterial responses, allowing for appropriate transition to wound repair and arthritis resolution.

We were unable to obtain SF or synovial tissue in the post-infectious phase from these LA patients, since their arthritis resolved during antibiotic therapy. However, we speculate that in such patients, the transition from infectious LA to the post-infectious phase occurs rapidly, progressing to beneficial wound repair responses after spirochetes have been killed. As a result, we would expect miRNA expression profiles after antibiotic therapy to look similar to those observed in OA patient synovial tissue, characterized by tissue repair with minimal inflammation.

Animal models of LA provide insights into these processes in human LA. When infected with *B. burgdorferi,* C57BL/6 mice develop only mild-to-moderately inflammatory LA (35). They rely on antibody responses to *B. burgdorferi* to control the infection, accompanied by robust wound repair responses. In contrast, *B. burgdorferi*-infected C3H/HeN mice develop severe arthritis, have a massive cellular immune response dominated by IFNαβ/STAT1 activation in joint tissue early in infection, and down-regulate wound repair responses (36). However, neither mouse strain has persistent arthritis after antibiotic therapy, as in human post-infectious LA.

Despite these differences, joints of C3H/HeN and C57BL/6 mice have similar numbers of spirochetes, indicating that the cellular response in C3H/HeN mice is maladaptive, worsening tissue damage without enhancing host defense (35). We suspect that in humans, genetic variables determine whether the response to *B. burgdorferi* infection elicits an appropriate wound repair response (B6-like) (37, 38) or a maladaptive inflammatory cellular response and arrest of wound repair processes (C3H-like) (39). For example, human patients who have a polymorphism in the TLR1 gene (1805GG), found primarily in the European Caucasian population, have higher levels of IFNγ/STAT1-dependent cytokines when infected with RST1 *B. burgdorferi* strains, and they have an increased frequency of post-infectious, antibiotic-refractory LA (40).

While all of the pre-antibiotic LA patients from Group 1 resolved their arthritis after completion of 1-to-3 months of antibiotic therapy, the LA patients from Groups 2 and 3 were tested in post-antibiotic phase when few, if any, spirochetes remained in affected joints. Compared with patients in Group 1, these patients had longer arthritis duration, fewer WBC and PMNs in SF, and greater frequency of lymphocytes and monocytes (Table I, Figure 2B). A number of miRNAs associated with NF-κB inflammation (miR-146a, miR-155), myeloid cell function and wound repair (miR-142, miR-223), and MYC-dependent cell cycle regulation (miR-17~92, let-7 family) were highly expressed the SF and synovial tissue in these patients (Figure 1, Figure 3). These results suggest that the inflammatory and proliferative processes occurring in the post-infectious synovial environment were indeed maladaptive, and were consistent with tumor-like characteristics of the synovial lesion, as has been described in RA (41).

The chronically high levels of miR-155 in SF and synovial tissue of patients with post-infectious LA provided valuable insights into the nature of their dysregulated inflammation.

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In mice, miR-155 is strongly up-regulated upon TLR/NF-κB activation, but is rapidly down-regulated in the presence of IL-10 in a STAT3-dependent manner (42). When infected with *B. burgdorferi,* $Il10^{-/-}$ mice lacking this IL-10/STAT3/miR-155 regulatory mechanism have very high miR-155 levels, as well as a strong IFNγ/STAT1 cytokine profile, enhancing cellular and humoral responses to infection (10). In contrast, infected C3H/HeN and C57BL/6 mice retain this IL-10/STAT3/miR-155 regulatory loop and have low miR-155 levels in joint tissue, despite differences between strains in LA severity (9).

Whereas $Il10^{-/-}$ mice down-regulated IFNγ/STAT1 responses in joints after spirochetes had been killed with antibiotics (43), some human LA patients have persistent inflammation months or years following completion of antibiotic therapy. We speculate than in post-infectious LA, elevated miR-155 indicates constitutive activation of pro-inflammatory pathways resulting from elevated cytokines such as IL-1β or TNFα, from retained pathogen-associated molecular patterns (44), or from host damage-associated molecular patterns. Mice that chronically over-express miR-155 spontaneously develop autoimmune T and B cell responses (45). Similarly, in human patients, chronic over-expression of miR-155 might also be a contributing factor in autoimmunity in human LA (46–50) and RA (22).

In this study, miRNA expression in post-infectious LA patients was most similar to expression in RA patients. Consistent with these results, elevated miR-155, miR-146a, and miR-223 expression has been shown previously in SF, synovial tissue, and synovial fibroblasts from patients with RA (22). Furthermore, mouse studies have demonstrated that miR-155 and miR-223 contribute to more severe experimental arthritis (5, 6), and miR-146a limits inflammation and tissue damage in experimental arthritis (7, 43). However, while the initial inflammatory triggers in RA are not clear, the initial trigger of LA is known with certainty, infection with *B. burgdorferi*. This makes it possible to study immune responses at their inception when the stage is being set for subsequent arthritis. Moreover, the comparison of patients with antibiotic-responsive LA or post-infectious LA provides a unique opportunity to implicate genetic and regulatory factors that lead to this disadvantageous outcome.

Our study population, which is based on referrals, was representative of the range of possible outcomes in LA. It is unusual that patients are referred prior to therapy with oral doxycycline, and none of the patients referred prior to therapy developed post-infectious LA, which occurs in fewer than 10% of patients with LA (19). Instead, patients are usually referred because of lack of response to oral or IV antibiotic therapy. Therefore, our patient cohort emphasizes the severe end of the spectrum of patients who do not respond well to antibiotic therapy. Moreover, SF cannot be obtained in all patients, and synovial tissue, the target tissue of the disease, is nearly always collected late in the post-infectious course in those who undergo arthroscopic synovectomies. Nevertheless, this study provides a novel assessment of miRNA expression in affected joints of human patients during the infectious and post-infectious phases of LA.

Finally, the similarities in miRNA expression noted here between post-infectious LA and other forms of chronic inflammatory arthritis, including RA, support the practice of treating post-infectious LA patients with DMARDs after appropriate antibiotic therapy (21).

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Currently, these patients are usually treated with 2–3 months of oral and IV antibiotic therapy followed by about 6–9 months of DMARD therapy, usually with MTX (21). With this regimen, we have not observed relapse of infection during the period of immunosuppressive DMARD therapy. However, this treatment algorithm requires a total of one year or more therapy, sometimes followed by months of physical therapy to regain normal function. miRNAs hold promise as potential biomarkers to identify LA patients who are developing maladaptive immune responses during the period of infection. In such patients, it will be important to learn whether simultaneous treatment with antibiotics and DMARDs, rather than sequential treatment with these medications, will reduce the period of therapy and improve outcome, creating a new paradigm in treatment of this form of chronic inflammatory arthritis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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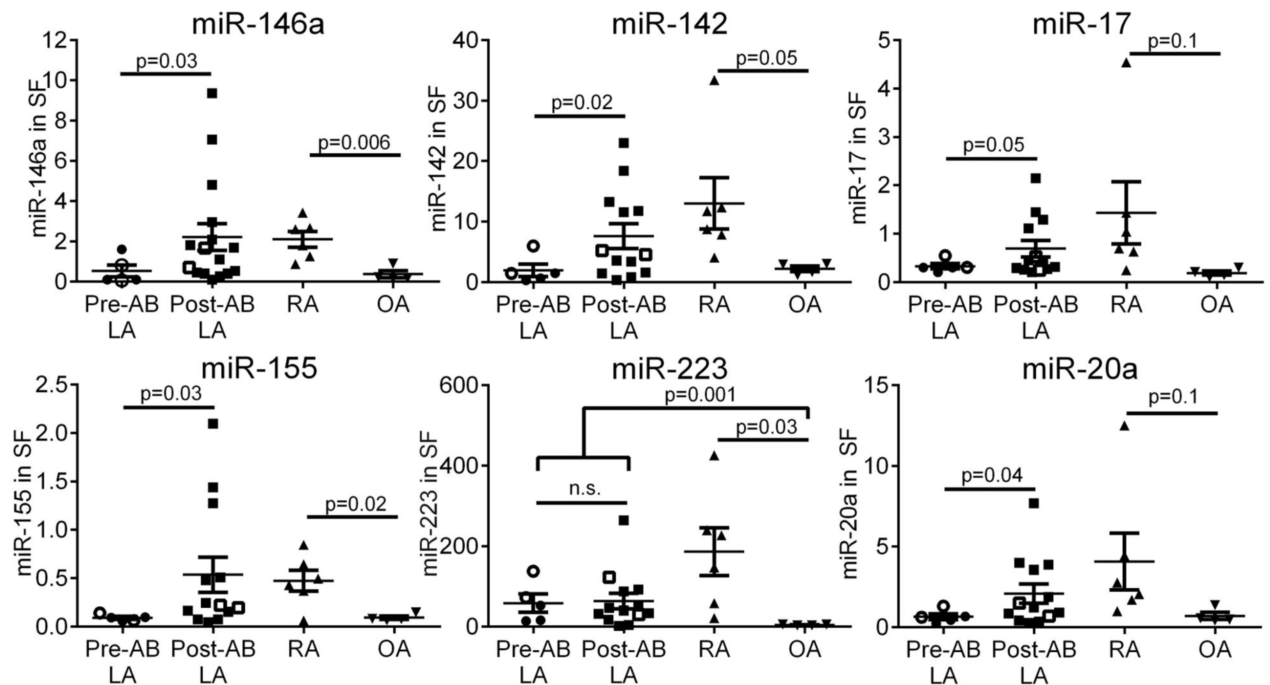

Figure 1.

Extracellular miRNA (ex-miR) levels in patient synovial fluid (SF). Ex-miRs from SF were quantified by qRT-PCR and normalized to global mean threshold cycle (Ct) of expressed ex-miRs (see methods). Open circles and squares indicate patients who required IV antibiotic therapy, but not DMARD therapy, for resolution of their arthritis. Statistically significant differences between groups were determined by Welch's t-test, with p values indicated. SF=synovial fluid; AB=antibiotics; LA=Lyme arthritis; RA=rheumatoid arthritis; OA=osteoarthritis; n.s.=not significant.

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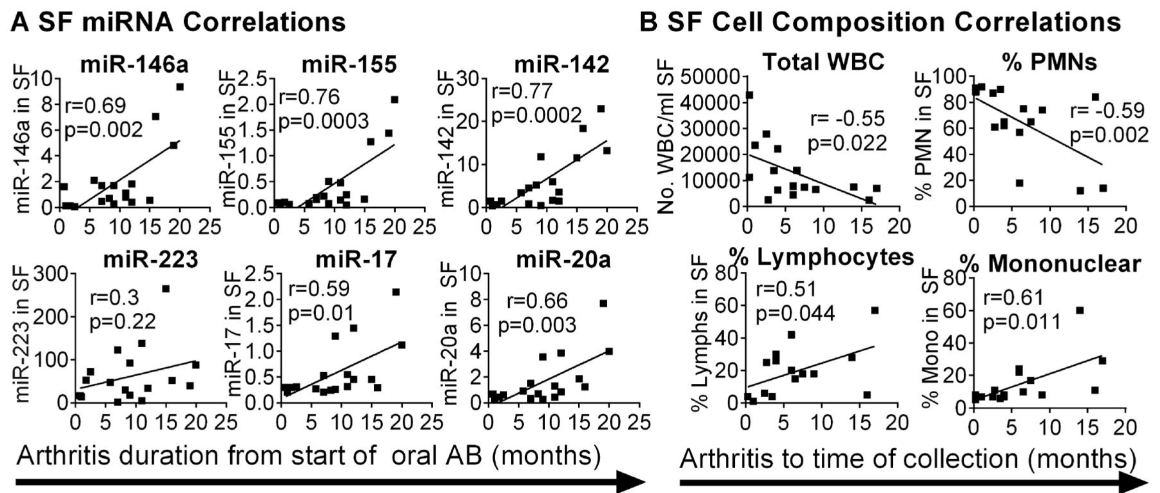

Figure 2.

Correlation between extracellular miRNA (ex-miR) levels, cell composition in synovial fluid (SF) and arthritis duration. Linear regression analysis was used to determine Pearson's r-values and p-values for clinical correlations between ex-miR expression in SF and arthritis duration after start of oral antibiotics (**A**), or between SF cell composition and duration of arthritis to time of SF collection (**B**). SF=synovial fluid; AB=antibiotics; WBC=white blood cells; PMNs=polymorphonuclear leukocytes.

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Figure 3.

Expression of miRNAs in synovial tissue. Arthroscopic images of synovitis in post-infectious LA, with inflamed villi (closed arrow) and invading synovial tissue (open arrow). (**A**) Expression of miR-146a and miR-155 (inflammatory response), (**B**) miR-142 and miR-223 (wound repair), (**C**) miR-17 and miR-20a (oncomiRs), and (**D**) let-7a and let-7c (tumor suppressors) in synovial tissue from arthritis patient groups (post-infectious LA, other inflammatory arthritis, osteoarthritis), normalized to miRNA copies per million reads. Statistically significant differences in expression between groups were determined by differential expression analysis ($p<0.05$). LA=Lyme arthritis; Inflamm. miRs=inflammatory miRNAs; oncomiRs=oncogenic miRNAs; Post-Inf LA=post-infectious LA; Infl Arth=other forms of inflammatory arthritis; OA=osteoarthritis.

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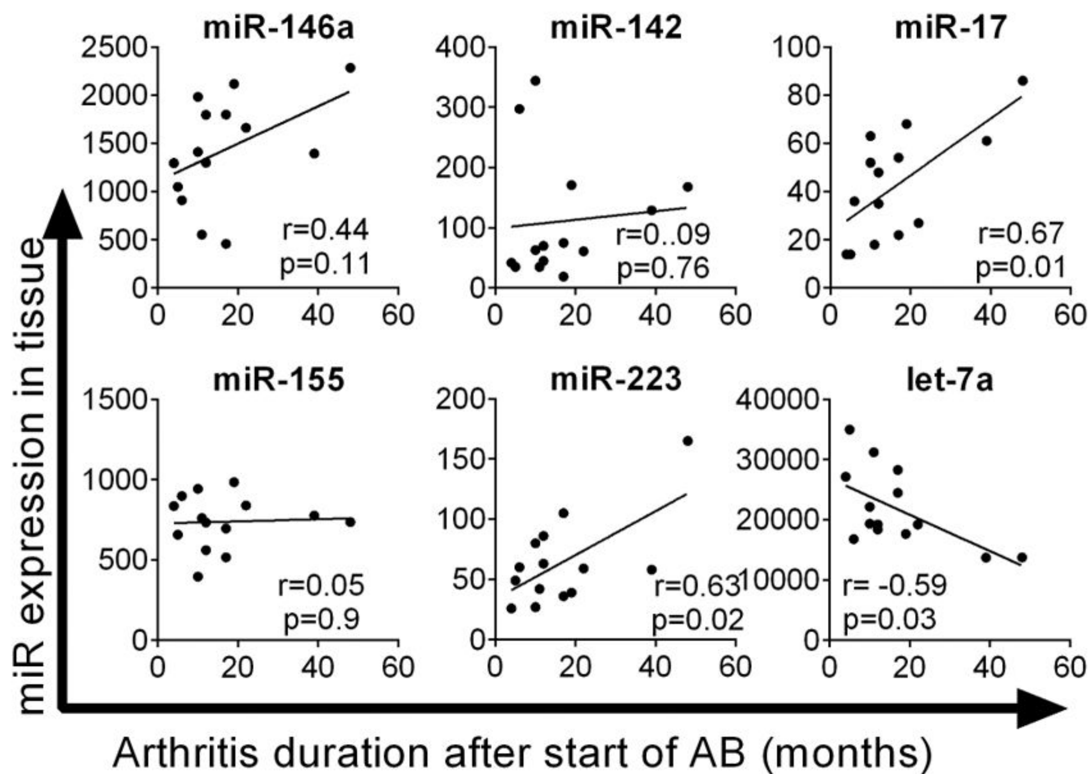

Figure 4.

Correlations between miRNA expression in synovial tissue and arthritis duration. Linear regression analysis was used to determine Pearson's r-values and p-values for clinical correlations between miRNA expression in tissue and arthritis duration after the start of oral antibiotics. miR=miRNA; AB=antibiotics.

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Table 1

Patient study participant characteristics*

	Group 1 Pre-antibiotics (n=5)	Group 2 Post-antibiotics (n=13)	Group 3 Synovectomy (n=14)
Sex (F/M)	1/4	4/9	7/7
Age (range)	49 (34–71)	45 (17–76)	19.5 (11–58)
B. burgdorferi infection			
Bb IgG titer, median (range)	6400 (1600–25600)	25600 (800–25600)	19200 (6400–25600)
No. PCR+/No. tested	2/4	0/7	0/14
Arthritis duration, months, median (range)			
Arthritis duration prior to start of AB	1 (0.25–2.5)	2 (0.5–13)	0.75 (0.25–20)
Arthritis duration to sample collection	1 (0.25–2.5)	**6.5 (2.75–17)**	**15.5 (4–48)**
Total arthritis duration	3.5 (1–13)	**12.5 (8–26)**	**15.5 (4–48)**
Arthritis resolution, No. of patients (%)			
Resolved after oral AB	3/5 (60)	0/13 (0)	0/14 (0)
Resolved after IV AB	2/2 (100)	2/13 (15)	0/14 (0)
Resolved after DMARDs (MTX)	NA	9†/11‡ (82)	0/12§ (0)
Resolved after synovectomy	NA	1/1‡ (100)	12/14§ (86)
Synovial fluid characteristics, median (range)			
Effusion (ml)	10 (5–66)	42 (10–95)	NA
Total WBC count	25760 (11282–42793)	**7247 (2480–22180)**	NA
% PMNs	90 (87–92)	**63.5 (12–90)**	NA
% Lymphocytes	4 (1–6)	**22.5 (4–57)**	NA
% Mononuclear	7 (5–8)	**11 (6–60)**	NA

*Statistically significant differences between groups ($p<0.05$) were determined by Mann-Whitney U-test and are indicated in bold.

†DMARD given was usually methotrexate (MTX), one patient declined DMARD therapy, and her arthritis resolved 6 months following completion of IV antibiotic therapy.

‡One patient failed to resolve arthritis after 6 months of DMARD therapy and underwent synovectomy.

§Two patients who declined DMARD therapy prior to synovectomy required a second synovectomy.

AB=antibiotics; IV=intravenous; DMARDs=disease-modifying anti-rheumatic drugs; MTX=methotrexate; WBC=white blood cell; PMN=polymorphonuclear leukocyte.

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Table 2

Pathway analysis of miRNA expression in post-infectious LA vs. OA synovial tissue*

	p-value	#genes/#miRs
Regulated in LA		
Pathways in cancer	1.2e-53	187/34
Wnt signaling	5.0e-26	91/34
TGFB signaling	2.9e-18	57/30
TCR signaling	2.8e-16	59/31
Cell cycle regulation	5.9e-14	68/31
BCR signaling	6.6e-12	42/28
P53 regulation	6.9e-11	37/29
FcGR-med. phagocytosis	1.1e-10	35/30
Regulated in OA		
Focal adhesion	1.3e-47	102/18
PI3K-Akt signaling	2.4e-33	149/19
ErbB signaling	4.1e-26	51/16
Pathways in cancer	3.2e-20	145/18
Insulin signaling	2.9e-19	64/16
Ubiquitin-mediated proteolysis	4.7e-18	65/17
HIF-1 signaling	1.5e-17	53/17
mTOR signaling	7.5e-16	33/15

*Genes predicted to be regulated by miRNAs over-expressed in synovial from post-infectious LA or OA patients were analyzed by DIANA-mirPath pathway analysis (see Methods). The top 8 pathways, ranked by p-value, are listed, along with number of genes (#genes) in those pathways predicted to be targeted by miRNAs (#miRs).

LA=Lyme arthritis; OA=osteoarthritis; Wnt=wingless-related integration site; TGFB=tumor growth factor beta; TCR=T cell receptor; BCR=B cell receptor; P53=tumor protein 53; FcGR=Fc fragment of IgG receptor; PI3K= phosphatidylinositol-4,5-bisphosphate 3-kinase; Akt=protein kinase B; ErbB=epidermal growth factor receptor tyrosine kinase family; HIF-1=hypoxia-inducible factor 1; mTOR=mechanistic target of rapamycin.