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TLR2 Expression Is Regulated by MicroRNA miR-19 in Rheumatoid Fibroblast-like Synoviocytes

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Resident cells, such as fibroblast-like synoviocytes (FLS), play a crucial role in rheumatoid arthritis (RA). They are implicated in the inflammatory response and play a key role in osteoarticular destruction. Moreover, RA FLS spread RA to unaffected joints. Pathogen-associated molecular patterns and damage-associated molecular patterns have been found to activate RA FLS by interacting with pattern recognition receptors, such as TLR. RA FLS express a large number of TLR, and TLR2 was demonstrated to be involved in RA inflammation. Because microRNA have emerged as important controllers of TLR expression and signaling, the aim of this study was to evaluate their potential involvement in the control of TLR2 expression by RA FLS. We first showed that Tlr2 expression is strongly upregulated in RA FLS in response to TLR2 ligands. Using a microRNA microarray analysis, we identified one miRNA in activated RA FLS, miR-19b, which was downregulated and predicted to target Tlr2 mRNA. Downregulation of miR-19b and miR-19a, which belongs to the same cluster, was confirmed by real-time quantitative PCR. Transfection of RA FLS with miR-19a/b mimics decreased TLR2 protein expression. In parallel, we found that both IL-6 and matrix metalloproteinase 3 secretion was significantly downregulated in activated FLS transfected with either mimic. Moreover, using a luciferase assay, we showed that miR-19a/b directly target Tlr2 mRNA. Taken together, our data point toward an important role for miR-19a/b in the regulation of IL-6 and matrix metalloproteinase 3 release by controlling TLR2 expression, as well as provide evidence that miR-19a/b can act as negative regulators of inflammation in humans. The Journal of Immunology, 2012, 188: 454–461.

Rheumatoid arthritis (RA) is a systemic inflammatory disease that predominantly affects multiple peripheral joints. It is characterized by synovial inflammation, as well as bone and cartilage destruction. The development of RA is thought to result from interactions between genetic and environmental factors. Molecules of microbial origin have been found in the joints of patients (1, 2), where they trigger inflammation by activating various pattern recognition receptors. In addition, these pattern recognition receptors can be activated by noninfectious molecular patterns or damage-associated molecular pattern molecules, which are generated upon tissue injury. These include cellular components released from necrotic cells (HMGB1, microorganisms), extracellular molecules upregulated upon injury (Hsp90, ATP, S100 proteins), or extracellular matrix fragments, such as tenascin (3).

The TLR family is one of the best-characterized groups of innate immune receptors in terms of described ligands, signaling pathways, and functional relevance. TLRs are expressed both by immune cells and by resident cells of the joint, such as fibroblast-like synoviocytes (FLS), which play a crucial role in RA. FLS are implicated in the inflammatory response essentially by synthesizing cytokines, chemokines, prostanoids, NO, and proangiogenic factors (4–6). FLS also play a key role in osteoarticular destruction and take part in the differentiation and activation of osteoclasts by the RANK–RANK ligand pathway and through the release of PGE2 and IL-6 (7). Moreover, synovial fibroblasts spread RA to unaffected joints (8, 9). RA FLS express a large number of TLRs, such as TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, and TLR7. In basal conditions, RA FLS express Tlr3 and Tlr4, as well as Tlr2 and Tlr6 mRNAs (10).

With the assistance of the scavenger receptor CD36, TLR2 is implicated in cytokine and matrix metalloproteinase (MMP) release in response to ligands from Gram+ bacteria, such as glycolipids, lipopeptides, or GPI-anchored structures. Interestingly, TLR2 is the most significantly induced TLR in FLS treated with cyto- kines, such as TNF-α and IL-1β, or with TLR ligands, such as polyinosinic-polycytidylic acid, bacterial lipoprotein (BLP), and LPS (11). In addition, TLR2 levels are higher in macrophages isolated from RA synovium compared with macrophages isolated from control synovium (12). Further evidence for the involvement of TLR2 in RA was obtained in a mouse model of arthritis induced by streptococcal cell wall, in which animals deficient for Tlr2 were shown to develop reduced symptoms (13). Recent data...
suggested a potential role for TLR2 as a therapeutic target. Indeed, Rabeximod suppresses arthritis downstream of TLR2 stimulation by preventing the activation of inflammatory cells, most likely macrophages, in a time-dependent fashion (14). OPN301, a TLR2-specific mAb inhibits TLR2-mediated proinflammatory cytokine production (15). AP177, a molecule selected by the systematic evolution of ligands by exponential enrichment method, was demonstrated to antagonize TLR2 signaling by binding directly to TLR2 and to suppress IL-6 and IL-8 release by activated cells (16).

It appears that the level of TLR2 needs to be tightly controlled to avoid a scenario where its deregulated expression will amplify the deleterious effect of inflammation on cell integrity. The modulation of gene expression can take place at several levels, among which regulation by microRNAs (miRNA) has gained increased interest in the recent years. miRNA are an evolutionarily conserved class of endogenous small noncoding RNAs. They are produced from long precursor molecules by the consecutive action of the RNase III enzymes Drosha and Dicer, before being loaded on an Argonaute protein within the RNA-induced–silencing complex. The mature miRNA acts a guide for RNA-induced–silencing complex to mediate destabilization and/or translational repression of target mRNAs (17, 18). The regulation of miRNA expression is itself controlled at various levels, such as transcription, processing, or stability (19), and can be influenced by various stress factors, including inflammation (20). In addition, emerging data have identified an important contribution of miRNA to the development and control of the inflammatory response that position these small noncoding RNAs at the heart of feedback and feed-forward loops controlling the inflammation process in both immune and nonimmune cells. Thus, inflammatory stimuli in pulmonary cells induce expression of miR-146a, which, in turn, promotes translational silencing of IL-8 and RANTES (21). Overexpression of miR-9 by activation of NF-kB leads to a feedback control of NF-kB–dependent acute inflammatory response (22). miR-147, which is induced by multiple TLR stimulation, attenuates TLR-induced inflammatory response in macrophages (23).

These data indicated that miRNA can exert negative effects in inflammatory pathways and this prompted us to look for miRNA directly targeting TLR2 and controlling cytokine release in response to TLR2 stimulation. In this article, we show that stimulation of RA FLS with BLP and LPS induces a decrease in expression of miR-19a and -19b and that this decrease is associated with upregulation of TLR2. Furthermore, we demonstrate that TLR2 is a direct target of miR-19a and -19b and that the down-regulation of miR-19b in BLP-activated RA FLS is associated with an increase in IL-6 and MMP3 release.

Materials and Methods

Reagents

Cell culture media (RPMI 1640, M199, and DMEM), FCS, t-glutamine, penicillin, streptomycin, amphotericin B, and TRIZol reagent were from Invitrogen (Cergy-Pontoise, France). LPS from Salmonella abortus equi was obtained from Sigma (Saint-Quentin-Fallavier, France). Synthetic bacterial lipopeptide Pam3CSK4 (BLP) was obtained from EMV Microcollections (Tübingen, Germany). The Lightcycler Faststart DNA Master SYBR Green and the miScript System, miRNA mimics, and AllStars negative control small interfering RNA (siRNA) were obtained from Qiagen (Courtabeuf, France). Human Dermal Fibroblast Nucleofector kit and negative control small interfering RNA (siRNA) were obtained from Amaxa (Cologne, Germany). The enzyme immunoassay kits for human IL-6 and MMP3 detection were from R&D (Lille, France).

For DNA and RNA purification, TRIzol reagent was from Millipore (Molsheim, France). Human Dermal Fibroblast Nucleofector kit was from Amaxa (Cologne, Germany). The Lightcycler Faststart DNA Master SYBR Green and the miScript System, miRNA mimics, and AllStars negative control small interfering RNA (siRNA) were obtained from Qiagen (Courtabeuf, France). Human Dermal Fibroblast Nucleofector kit was from Amaxa (Cologne, Germany). For DNA and RNA purification, TRIzol reagent was from Millipore (Molsheim, France). Human Dermal Fibroblast Nucleofector kit was from Amaxa (Cologne, Germany). The enzyme immunoassay kits for human IL-6 and MMP3 detection were from R&D (Lille, France).

Real-time quantitative PCR

Total RNA isolated from FLS was reverse transcribed using the First-Strand cDNA Synthesis Kit, according to the manufacturer’s instructions (Invitrogen). Real-time quantitative PCR (RT-qPCR) was performed in a total volume of 20 µl using SensiMix Plus SYBR kit (Quantace; Corbett Life Science) and gene-specific primers: Tlr2, 5'-GGCAGCAATCTCCTGTTG3' and 5'-AGAAAGCCTGAGTTCTGTTG3'; and Tlr4, 5'-TCGTTCTCTTTGCTGACGCTG3' and 5'-AGAAAGACTCCATTACACAC3'. PCR was performed using the Human Dermal Fibroblast Nucleofector kit and negative control small interfering RNA (siRNA) were obtained from Amaxa (Cologne, Germany). The enzyme immunoassay kits for human IL-6 and MMP3 detection were from R&D (Lille, France). For DNA and RNA purification, TRIzol reagent was from Millipore (Molsheim, France). Human Dermal Fibroblast Nucleofector kit was from Amaxa (Cologne, Germany). The enzyme immunoassay kits for human IL-6 and MMP3 detection were from R&D (Lille, France).

 Luciferase reporter constructs

To generate luciferase-based reporter plasmids, psiCHECK-2 (Promega) was modified by inserting the Gateway cassette C1 (Invitrogen) at the 3' end of the firefly luciferase gene (f-luc) into the Xba I site of psiCHECK-2. The 3' untranslated region (UTR) sequence of Tlr2 was amplified from HEK293 cell genomic DNA; after addition of attB1 and 2 sequences, the resulting PCR products were cloned into pDONR/Zero and then recombined in the modified psiCHECK-2 vector using Gateway technology (Invitrogen). The following primer sequences were used (sense and antisense primers are indicated in this respective order, and the anchor sequence used for the nested PCR is underlined): TLR2: 5'-AAAAAGCAGGTTCTCCCATATGTTAAGACCTTTTGGT3' and 5'-AGAAAAGCTGGTTGAGATTAAATGAGGATACAC3'; and TLR4: 5'-AAAAAGCAGGTTCTCCCATATGTTAAGACCTTTTGGT3' and 5'-AGAAAAGCTGGTTGAGATTAAATGAGGATACAC3'.

Cell culture

Human FLS were isolated from synovial tissues from four RA patients at the time of knee joint arthroscopic synovecctomy, as described previously, after informed consent was obtained (24). The diagnosis confirmed to the revised criteria of the American College of Rheumatology (25). FLS cultures were done, as previously described (26). Experiments were performed between passages three and nine. During that time, cultures consisted of a homogeneous population of fibroblastic cells, negative for CD16 as determined by FACS analysis. HEK293 cells were purchased from the American Type Culture Collection and maintained in DMEM supplemented with 10% heat-inactivated FBS, 2 mM t-glutamine, 40 U/ml penicillin, and 50 µg/ml streptomycin. Cell number and cell viability were checked by the MTT assay.

Stimulation of cells for total RNA extraction

FLS (104 cells) were stimulated with 2 µl medium alone or medium containing LPS (1 µg/ml) and BLP (1 µg/ml). After a 6- and 24-h incubation period, total RNA was extracted using TRIZol reagent, according to the manufacturer’s instruction.

Transfections and luciferase assay

Transient transfection of FLS with miR-19a and miR-19b mimics and Dicer siRNA (20 pM/sample) or with the AllStars negative-control siRNA was performed using the Human Dermal Fibroblast Nucleofector kit.
from Amaxa, as previously described (27). FLS were then plated in 24-well plates (2 \times 10^5 cells per well). All assays were performed at 48 h post-transfection. Transfection efficiency was evaluated with the PmaxGFP vector.

Transfection of HEK293 cells plated in 24-well plates (2 \times 10^5 cells per well) with reporter constructs and mir-19b/a mimics (200 nM) was performed using Lipofectamine 2000 (Invitrogen). After 48 h, cells were washed and lysed with proprietary lysis buffer (Promega), and luciferase (\( \ell \)-\( \ell \)) and Renilla luciferase (\( \ell \)-\( \ell \)) activities were determined using the dual-luciferase reporter assay system (Promega) and a luminometer. (Glomax; Promega). The relative reporter activity was obtained by normalization to \( \ell \)-\( \ell \) activity.

IL-6 and MMP3 release was measured in culture supernatants by a heterologous two-site sandwich ELISA, according to the manufacturer’s instructions.

Western blot
A total of 10^6 cells (FLS) was incubated for 24 h with BLP (1 \mu g/ml). Controls were performed with cells maintained in medium with 5% heat-inactivated FCS for 24 h. After stimulation, cells were centrifuged, and the pellets were resuspended for 20 min on ice in 300 ml ice-cold lysis buffer (1% Triton X-100, 20 mM Tris-HCl [pH 8], 130 mM NaCl, 10% glycerol, 1 mM sodium orthovanadate, 2 mM EDTA, 1 mM PMSF, and protease inhibitors). Lysates were centrifuged for 10 min at 14,000 \( \times \) g at 4 \( ^\circ \)C, and supernatants were subjected to SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membranes. Membranes were blocked using 5% BSA in TBS (20 mM Tris [pH 7.5], 150 mM NaCl) for 1 h at 25 \( ^\circ \)C. The blots were incubated with anti-TLR2 mouse IgG1 mAbs (IMG-319, clone 10305A5.138; Imgenex) for 2 h at 25 \( ^\circ \)C, followed by incubation with HRP-conjugated goat anti-mouse IgG mAbs (1 h at 25 \( ^\circ \)C) and detection by ECL (Amersham ECL Plus Western blotting Detection Reagents), according to the manufacturer’s instructions. To confirm the presence of equal amounts of proteins, bound Abs were removed from the membrane by incubation in 0.2 M glycine (pH 2.8) 0.5 M NaCl for 10 min at room temperature and reprobed with anti-GAPDH mouse mAbs (clone 6C5; Millipore).

Statistical analysis was performed using the Student t test. Values were compared between different groups in the experiment. The p values < 0.05 were considered statistically significant.

Results
LPS and BLP treatments increase expression of TLR2 in RA FLS
We first showed that the expression of Tlr2 mRNA is significantly enhanced in RA FLS treated with LPS and BLP. RA FLS were stimulated for 6 h with LPS from S. abortus equi (1 \mu g/ml) or with BLP (1 \mu g/ml), and RT-qPCR was performed on RNA isolated from both control and activated cells. We observed a 17–20-fold increase in Tlr2 transcripts in response to LPS and BLP treatment (Fig. 1A). In accordance with previous observations from other investigators (11), Tlr4 mRNA expression was only slightly affected by LPS or BLP treatment (Fig. 1B). To determine whether increased Tlr2 mRNA expression correlated with enhanced TLR2 protein expression, we performed Western blotting experiments. As shown in Fig. 1C, stimulation of RA FLS with either LPS or BLP led to an increased expression of TLR2. As expected from the moderate modification of Tlr4 mRNA expression after LPS and BLP activation, TLR4 protein expression was not modified (data not shown).

Role of miRNA in the regulation of TLR2 expression in RA FLS
To investigate the importance of miRNA in the control of TLR2 expression in RA FLS, we first used a global approach by evaluating the role of Dicer. Dicer is a cytoplasmic RNase III type endonuclease, required for the generation of most miRNA (28). RA FLS were transfected with an siRNA targeting Dicer at a concentration of 100 nM. A plasmid encoding the GFP protein was used to evaluate transfection efficiency. As assessed by RT-qPCR, Dicer mRNA expression level was efficiently downregulated upon transfection of Dicer-specific siRNA (Fig. 2A). Transfection of Dicer siRNA, but not of a nontargeting control siRNA, upregulated Tlr2 mRNA expression in RA FLS (Fig. 2B). Interestingly, Dicer knockdown did not impact Tlr4 mRNA (Fig. 2C).

miR-19b is downregulated in LPS- and BLP-activated RA FLS
Next, we searched for miRNA exhibiting misregulated expression in activated versus nonactivated RA FLS. For this, we analyzed the miRNA-expression profile of LPS-activated RA FLS using a DNA microarray containing 409 nucleotide probes complementary to miRNA of human origin, which was performed previously in our laboratory. The microarray data were submitted to the Minimum Information About Microarray Experiment database under accession number E-MEXP-1970 (http://www.ebi.ac.uk/microarray-as/aer/login) (27). After LPS challenge, this initial screening identified 17 downregulated miRNA, which are listed in Table I. An online search of the miRNA target database microCosm (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5) predicted several miRNA candidates involved in Tlr2 targeting. miR-19b (indicated in gray in Table I) was the only miRNA predicted to target Tlr2 and downregulated on the microarray analysis. Interestingly, miR-19b, together with miR-19a, which differs by only one nucleotide, was among the top 10 predictions of the algorithm.

We first performed an RT-qPCR analysis to confirm the downregulation of miR-19b observed with the microarray analysis. As

![FIGURE 1. Effect of BLP and LPS on TLR2 and TLR4 expression by RA FLS. Tlr2 mRNA (A) and Tlr4 mRNA (B) expression was determined by quantitative RT-PCR in RA FLS stimulated with BLP (1 \mu g/ml), LPS (1 \mu g/ml), and medium (C) for 6 h. Results were normalized to Gapdh and expressed as fold change compared with samples from cells incubated in medium (C). C, TLR2 and TLR4 expression in RA FLS, 24 h after stimulation with BLP, LPS, and medium (C), was determined by Western blotting with anti-TLR2 Abs. For protein loading control, membranes were reprobed with anti-GAPDH Abs. Data are expressed as the mean of triplicate samples ± SD and are representative of three independent experiments. **p < 0.01. ***p < 0.001.
shown in Fig. 3A, miR-19b was strongly downregulated in response to both LPS and BLP (Fig. 3A). Given the high sequence homology between miR-19a and miR-19b, it is difficult to estimate which of the two was detected by PCR. The primary transcript containing miR-19b derives from two genomic loci, which give rise to two miRNA clusters: mir-17–92 on chromosome 13 and mir-106a–363 on chromosome X (Fig. 3B). To confirm the specific downregulation of miR-19b, we measured the expression of its pre-miRNA, pre–miR-19b-1, and found that it was also significantly downregulated upon BLP or LPS treatment (Fig. 3C).

To assess whether the regulation of miR-19b was occurring at a transcriptional level, we used RT-qPCR to measure the expression level of pri-miRNA 17–92 in RA FLS and found that it was downregulated in response to BLP and LPS (Fig. 3D). This result also indicated that both miR-19a and -19b must be downregulated after LPS and BLP treatment. We failed to detect the cluster 106a–363 or the pre–miR-19b-2 (data not shown), which indicated that only the cluster on chromosome 13 is expressed in FLS cells.

In oral keratinocytes stimulated with heat-inactivated *Porphyromonas gingivalis*, miR-105 inversely regulates Tlr2 expression; this was demonstrated to occur through binding to the 3’-UTR of Tlr2 mRNA, thereby inhibiting its translation (29). miR-105 was not expressed in unstimulated RA FLS, as assessed by RT-qPCR, and activation with LPS or BLP did not modulate its expression (data not shown).

**miR-19a and miR-19b modulate TLR2 expression in BLP-activated RA FLS**

miRNA mediate posttranscriptional regulation via either mRNA destabilization or translation inhibition. To test whether miR-19b and miR-19a have an effect on the level of Tlr2 mRNA, we measured its expression by RT-qPCR in RA FLS transfected with miR-19a or -19b mimics. A plasmid encoding GFP was cotransfected to evaluate transfection efficiency. Forty-eight hours after transfection, cells were stimulated with BLP for 24 h. No significant difference in Tlr2 mRNA expression was found in cells transfected with the miR-19a or -19b mimics compared with cells transfected with the control mimics (Fig. 4A). The upregulation of Tlr2 mRNA after BLP treatment was not affected by the overexpression of miR-19a or -19b mimics compared with cells transfected with the control mimics (Fig. 4A). We also measured the stability of Tlr2 mRNA by incubating BLP-activated FLS treated with either control or miR-19b mimics (Fig. 4B).

We next tested whether expression of miR-19b and miR-19a affected TLR2 protein levels in RA FLS. We transfected control or miR-19b mimics in RA FLS, activated them with BLP or LPS, and measured TLR2 protein expression by Western blotting. As can be seen in Fig. 4C, we found that overexpression of miR-19b led to a global decrease in TLR2 protein in protein extracts from BLP- and LPS-activated cells (2- and 3-fold, respectively, as determined by densitometry analysis). These results indicated that miR-19a and miR-19b likely regulate the expression of TLR2 at the translational level.

**miR-19a and miR-19b repress IL-6 and MMP3 production in BLP-activated RA FLS**

To examine the consequence of Tlr2 regulation by miR-19a and -19b, we next tested whether their overexpression could affect II-6 mRNA expression and IL-6 release in BLP-activated RA FLS. IL-6 is a cytokine with widespread activities, is one of the central

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**Table I. Downregulated miRNA following LPS activation of RA FLS**

<table>
<thead>
<tr>
<th>Name</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>has-mir-494</td>
<td>0.679</td>
</tr>
<tr>
<td>has-mir-335</td>
<td>0.556</td>
</tr>
<tr>
<td>has-mir-633</td>
<td>0.467</td>
</tr>
<tr>
<td>has-mir-29b</td>
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<tr>
<td>has-let-7a</td>
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<tr>
<td>has-mir-22</td>
<td>0.684</td>
</tr>
<tr>
<td>has-mir-21</td>
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<tr>
<td>has-mir-299</td>
<td>0.719</td>
</tr>
<tr>
<td>has-mir-24</td>
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</tr>
<tr>
<td>has-mir-19b</td>
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</tr>
<tr>
<td>has-mir-203</td>
<td>0.609</td>
</tr>
<tr>
<td>has-mir-222</td>
<td>0.690</td>
</tr>
<tr>
<td>has-mir-656</td>
<td>0.517</td>
</tr>
<tr>
<td>has-let-7e</td>
<td>0.760</td>
</tr>
<tr>
<td>has-mir-620</td>
<td>0.549</td>
</tr>
<tr>
<td>has-mir-320</td>
<td>0.325</td>
</tr>
</tbody>
</table>

Mean expression was obtained from microarray data collected previously (38).
mediators of inflammation, plays a major role in RA, and is an important therapeutic target (30). We measured IL-6 mRNA levels in RA FLS transfected with either miR-19a or miR-19b mimics for 48 h and then activated with BLP for 6 h. Compared with the control, we observed that transfection of miR-19a and miR-19b mimics impaired the induction of IL-6 mRNA expression in response to BLP (Fig. 5A). A similar experiment was performed with LPS instead of BLP; however, in this case, cells transfected with the miR-19a and -19b mimics showed no alteration in IL-6 mRNA expression in response to LPS (Fig. 5B). In parallel, we measured the secretion of IL-6 by ELISA and found that it was significantly downregulated in BLP-activated FLS transfected with miR-19a and -19b mimics (Fig. 5C) but was not modified in LPS-activated FLS (Fig. 5D). We also measured the secretion of MMP3, which is one of the major MMPs involved in invasion and cartilage destruction in RA. We found that, as for IL-6, MMP3 secretion was downregulated in BLP-activated FLS transfected with miR-19a and -19b mimics (Fig. 5E) but was not modified in LPS-activated FLS (Fig. 5F). Taken together, these data demonstrated that the Tlr2 regulation by miR-19a and -19b has an effect on IL-6 and MMP3 release by BLP-activated RA FLS.

miR-19a and miR-19b directly regulate TLR2 expression

To verify whether the predicted binding site for miR-19a or -19b within Thr2 mRNA was functional (Fig. 6A), we generated luciferase reporter constructs (psiCHECK-2) that contain the firefly luciferase gene fused to the entire 3'-UTR of Thr2 and the Renilla luciferase for normalization. We also generated luciferase reporter constructs in which we inserted a mutant version of Thr2 3'-UTR to disrupt the predicted binding site for miR-19a or -19b. Before testing these constructs in HEK293 cells, we measured the expression levels of miR-19a and -19b in these cells. As can be seen in Fig. 6B, HEK293 cells express much higher levels of both miRNA than RA FLS. Therefore, we cotransfected the psiCHECK-2 constructs with control or miR-19a and -19b antisense 2'-O-methylated oligoribonucleotides. In the presence of specific antisense oligonucleotides, we observed an upregulation of the Thr2 3'-UTR–controlled luciferase sensor but not of the luciferase sensor fused to the mutated Thr2 3'-UTR (Fig. 6C). Altogether, these data suggested that Thr2 mRNA is a direct target for posttranscriptional regulation by miR-19a and miR-19b.

**Discussion**

We and other investigators reported that in RA FLS, TLR2 signaling was increased in response to LPS and BLP and that this could be involved in the inflammatory response in RA (31). Because the most obvious point to control this pathway is at the level of receptor expression, this study was aimed at identifying whether miRNAs might be involved in the control of TLR2 expression. Our results established that stimulation of RA FLS with BLP and LPS decreases miR-19a and -19b expression, which correlates with upregulation of Thr2. Furthermore, our results in-
indicated that suppression or induction of miR-19a and -19b caused reciprocal alterations in IL-6 release induced by BLP-mediated TLR2 activation.

Many studies showed that TLR stimulation can modulate miRNA expression. Usually, miRNA are upregulated in response to TLR2 and TLR4 activation, and this is the case for miR-155,

**FIGURE 5.** miR-19a/b repress IL-6 and MMP3 production in BLP-activated RA FLS. IL-6 mRNA expression was determined by RT-qPCR in RA FLS transfected with miR-19a and -19b mimics or with an AllStars negative control siRNA (control) and then activated (for 6 and 24 h) with BLP (1 μg/ml) (A) or LPS (1 μg/ml) (B) for 48 h posttransfection. Control cells were incubated with medium (C). Results were normalized to Gapdh and expressed as fold change compared with samples from cells incubated in medium (C). C and D. IL-6 release was determined by ELISA in culture supernatants after stimulation of RA FLS in the same conditions as in A and B. E and F, MMP3 release was determined by ELISA in culture supernatants after stimulation of RA FLS in the same conditions as in A and B. Data are expressed as the mean of triplicate samples ± SD and are representative of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 6.** miR-19a and -19b directly regulate TLR2 expression. A. Sequence alignment of miR-19a and -19b and their target sites in the 3’-UTR of Tlr2 mRNA. B. miR-19a and -19b relative expression levels were determined by RT-qPCR in RA FLS and HEK293 cells. U6snRNA was used as endogenous control for data normalization. C. miR-19a and -19b directly target the 3’-UTR of Tlr2 mRNA. Luciferase reporter constructs with wild-type or mutated sequence (for miR-19a and -19b binding sites) were generated. HEK293 cells were transiently cotransfected with reporter constructs and either miR-19a or -19b antisense molecules. Luciferase activities were measured 48 h after transfection and normalized to luciferase expressed by the control psiCHECK-2 vector devoid of 3’-UTR sequences. Data are expressed as the mean of triplicate samples ± SD and are representative of three independent experiments. *p < 0.05.
miR-146, miR-147, and miR-9 (32). Moreover, we previously demonstrated that miR-346 is upregulated in RA FLS in response to LPS and that this upregulation participated in the control of IL-18 and Tnf-α mRNA stability and cytokine release (27, 33). Conversely, we found in this study that activation of TLR2 and TLR4 by their respective ligands led to a downregulation of miRNA, including miR-19a and miR-19b, by a currently unidentified mechanism. This regulation has a proinflammatory effect because it allows an increase in TLR2 expression. Such a decrease was also observed for other miRNA, such as let-7i, miR-125b, and miR-98, in response to TLR4 stimulation (32).

An important observation in this study is that the effect of miR-19a and -19b on TLR2 expression occurs at a translational level in RA FLS. Although transfection of miR-19a and -19b mimics had no significant impact on Tlr2 mRNA levels, it caused a decrease at the protein level, as shown by Western blotting. In addition, inhibition of miR-19a and -19b with antisense oligonucleotides significantly increased the activity of a luciferase reporter containing the 3′-UTR of Tlr2. A mutation in the binding sequence completely abrogated the regulation of the luciferase reporter by miR-19a and -19b. These results indicated that, in BLP-activated RA FLS, miR-19a and miR-19b play a role in the control of TLR2 expression by regulating mRNA translation.

There is only limited evidence that TLR are directly regulated by miRNA (32). Nevertheless, it was shown that Tlr4 mRNA is regulated by members of the let-7 miRNA family. Let-7i was by miRNA (32). Nevertheless, it was shown that Tlr4 mRNA expression by regulating mRNA translation.

Modulation of TLR2 expression in human gingival epithelial cells by miR-105 was reported recently (29). The investigators showed that regulation occurs via binding to the 3′-UTR of Tlr2 mRNA, leading to translational inhibition. They also found that miR-19b was differentially expressed but to a lesser extent than miR-105. In contrast to this study, we could not detect expression of miR-105 in nonactivated or activated RA FLS. Tlr2 3′-UTR has overlapping binding sites for miR-19 and miR-105 (miR-19: nt 335–358 and miR-105: nt 331–355), indicating that this binding site is accessible in vivo and plays a critical role in the post-transcriptional regulation of Tlr2 mRNA. Why is miR-105 not expressed in FLS? miR-105 is located on Xq28 in the intronic region of Gabra3A gene. Lee et al. (36) reported that miR-105 belongs to a group of miRNA that are detectable in their mature form only in certain cell types and tissues, whereas their precursors are expressed in all or most cell/tissues. This could explain the differential expression of miR-105 between epithelial cells and FLS.

miR-19 belongs to the cluster mir-17–92, which is located within the nonprotein-coding gene C13orf25 at 13q31.3. Essential roles for this cluster in the development of heart, lungs, and B cells were recently established. Mouse embryos lacking this cluster are characterized by a deficiency of pre-B cells but not earlier B cell progenitors, whereas other hematopoietic cells are largely unaffected (37). In humans, the cluster mir-17–92 is amplified in several types of lymphoma, solid tumors, and neuroblastoma and plays a key role in the control of cell cycle and cell death (38). In particular, miR-17 and miR-20a target p21 and the proapoptotic factor Bim (39), and recent data from Mestdagh et al. (40) indicated that this cluster is a potent inhibitor of TGF-β signaling by acting on TGFBR2, SMAD2, and SMAD4. In this study, we found that the expression of the mir-17-92 cluster was downregulated in BLP-activated RA FLS and that the expression of one of its paralogs, the mir-106a–363 cluster, is undetectable. Unlike the mir-17–92 cluster, this cluster is most often undetectable or expressed at trace levels in various cell types, which indicates that it might have more cell-specific functions. This also suggests that the mir-106a–363 cluster plays no role in regulating the expression of TLR2 in RA FLS. It is also worth noting that the mir-17–92 cluster contains multiple miRNA of the mir-19 family; according to in silico predictions, these miRNA should have a redundant activity. Indeed, both miR-19a and miR-19b acted on TLR2 protein expression, as observed in our experiments. This raises the possibility that the expression of Tlr2 mRNA may be subjected to a regulation by multiple miRNA of a same cluster, which might increase the efficiency of this regulation.

The role of the mir-17–92 cluster in inflammation is largely unknown. miR-19a, which is overexpressed in esophageal squamous cell carcinoma, was recently shown to directly target Tnf-α mRNA (41). Results obtained in our study demonstrated that miR-19a and miR-19b negatively regulate the synthesis of IL-6, because their overexpression leads to a strong reduction in IL-6 synthesis in BLP-activated RA FLS. These data indicated that these two miRNAs are anti-inflammatory and that their downregulation in response to TLR2 stimulation is crucial for the development of an inflammatory response. This effect is exclusively dependent of TLR2 regulation, because the transfection of miR-19a and -19b in FLS stimulated with LPS (which triggers TLR4 signaling) had no effect on IL-6 synthesis. IL-6 is a major cytokine implicated in RA and a current therapeutic target (30). This cytokine was recently shown to be directly targeted by two miRNA: let-7 and miR-365 (42–44). IL-6 can also be indirectly regulated, as demonstrated by Stanczyk et al. (45), who linked the expression levels of IL-6 to that of miR-203. Moreover, we also showed that miR-19a and miR-19b regulate the expression of MMP3 and that their downregulation will promote cartilage invas and destruction. Our results demonstrated a new way of controlling the synthesis of IL-6 and MMP3 by modulating the expression of TLR2.

Taken together, our data point toward an important role for miR-19a and miR-19b in the regulation of IL-6 and MMP3 release by controlling TLR2 expression. Our findings provide clear evidence that miR-19a and miR-19b, which are induced by bacterial ligands, can act as negative regulators of inflammation in humans.

Disclosures

The authors have no financial conflicts of interest.

References


