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Bruton's Tyrosine Kinase Is Involved in miR-346-Related Regulation of IL-18 Release by Lipopolysaccharide-Activated Rheumatoid Fibroblast-Like Synoviocytes[1]

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MicroRNAs (miRNAs) have emerged as key players in the regulation of expression of target mRNAs expression. They have been associated with diverse biological processes, and recent studies have demonstrated that miRNAs play a role in inflammatory responses. We reported previously that LPS-activated fibroblast-like synoviocytes (FLS) isolated from rheumatoid arthritis (RA) patients express IL-18 mRNA but they do not release IL-18. Based on the observation that this inhibition was due to a rapid degradation of IL-18 mRNA, our group has conducted a study to identify miRNAs that could play a role in the "antiinflammatory" response of LPS-activated RA FLS. LPS challenge modulated the expression of 63 miRNAs as assessed by microarray analysis. Fifteen miRNAs were up-regulated, including miR-346, for which overexpression upon LPS treatment was validated by quantitative RT-PCR. We then transfected FLS with an antisense oligonucleotide targeting miR-346 and found that, in these conditions, IL-18 release could be measured upon LPS activation of FLS. Moreover, we also demonstrated that miR-346 indirectly regulated IL-18 release by indirectly inhibiting LPS-induced Bruton's tyrosine kinase expression in LPS-activated RA FLS. These findings suggest that miRNAs function as regulators that help to fine-tune the inflammatory response in RA. *The Journal of Immunology,* **2009, 182: 5088–5097.**

In rheumatoid arthritis (RA),[5] stimulation of innate immune cells, and especially organ-specific resident cells such as fibroblast-like synoviocytes (FLS), seems to be the primary fundamental event leading to the inflammatory response and to the development of organ-specific autoimmunity (1, 2).

Activation of FLS may be linked either to the cytokine environment, to cell-to-cell contacts, or to interactions between pathogen-associated molecular patterns (PAMPs) and pattern recognition receptors (PRRs) (3). FLS exert a proinflammatory activity, essentially by synthesizing cytokines, chemokines, prostanoids, and NO (4, 5). However, they also produce proangiogenic factors that help with the appearance of neovessels, thereby allowing the intrasynovial recruitment and migration of immune cells with the help of release of chemokines. FLS have a key role in osteoarticular destruction and take also part in the differentiation and activation of osteoclasts by the receptor activator of NF-κB (RANK)-RANK ligand pathway through the release of prostaglandin E_2 and IL-6 (6).

Conversely, FLS secrete little or no TNF-α and neither IL-1 nor IL-18, which are major cytokines implicated in the inflammatory response in RA. This "antiinflammatory" activity, which is specific to FLS, suggests the existence in these organ-specific resident cells of selective and complex mechanisms of regulation. We previously reported that LPS and protein I/II, a ligand of integrin $\alpha_5\beta_1$, induced IL-18 mRNA expression in both THP-1 monocytic cell line and in RA FLS, but in contrast to THP-1 cells, FLS did not release mature IL-18. Furthermore, our studies revealed that the lack of IL-18 release by RA FLS was due to a defect of translation of IL-18 mRNA into pro-IL-18 because of a rapid degradation of the IL-18 mRNA (7).

MicroRNAs (miRNAs) are an evolutionarily conserved class of endogenous small noncoding RNAs. Since their discovery, miRNAs have emerged as key players in the regulation of translation and degradation of target mRNAs (8–11). They provide an additional posttranscriptional mechanism by which protein expression can be regulated (12–14). The expression of ~10,000 genes, or 30% of the human genome, could potentially be regulated by miRNAs (15, 16). In mammals, miRNAs have been associated with diverse biological processes such as cell differentiation, cancer, regulation of insulin secretion, and viral infection (17, 18). Cells of the immune system must employ a multilayered control system to keep innate immunity and inflammation in check, and

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[5] Abbreviations used in this paper: RA, rheumatoid arthritis; Btk, Bruton's tyrosine kinase; FLS, fibroblast-like synoviocytes; LFM, leflunomide metabolite analog; miRNA, microRNA; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; UTR, untranslated region.

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recently a role for miRNAs has been proposed in the regulation of the inflammatory response in monocytes/macrophages (19). Taganov et al. (20), by using microarray studies, found an increased expression of three miRNAs (miR-132, miR-146, and miR-155) in response to LPS in THP-1 cells. They identified IL-1R-associated kinase 1 (IRAK1) and TNFR-associated factor 6 (TRAF6) as the key targets of miR-146 and proposed a model by which this miRNA regulates TLR signaling in response to various PAMPs. Tili et al. (21) later demonstrated that miR-155 plays an important role in innate immunity and is involved in the negative regulation of immune cells in response to LPS.

Based on these observations, our group has conducted a study to identify miRNAs that could play a role in the antiinflammatory response of LPS-activated RA FLS. To monitor the expression profile of miRNAs in RA FLS activated with LPS, we employed a microarray approach that detects 409 miRNAs of human origin. Fifteen miRNAs were overexpressed >2-fold in LPS-activated FLS, and among them we identified one miRNA, miR-346, that had partial sequence complementarity within the 3′ untranslated region (3′-UTR) region of the IL-18 mRNA. The up-regulation of miR-346 expression was validated by quantitative RT-PCR analysis. We then used antisense oligonucleotide molecules to block miR-346 activity; transfection of the inhibitor targeting miR-346 reestablished IL-18 release by LPS-activated FLS. Moreover, we also showed that transient transfection of miR-346 in THP-1 cells inhibited IL-18 secretion by these cells in response to LPS. Using a luciferase assay, we showed that miR-346 does not directly target IL-18 mRNA. In our search for another target that could explain the effect of miR-346 on IL-18, we also demonstrated that miR-346 inhibited indirectly Bruton's tyrosine kinase (Btk) expression at the transcriptional level in LPS-activated RA FLS and that indirect inhibition of Btk expression negatively regulated IL-18 release. These results suggest an important role of miR-346 in the control of IL-18 synthesis by LPS-activated FLS and indicate that its expression may be critical to prevent an excessive inflammatory response.

Materials and Methods

Reagents

Cell culture media (RPMI 1640, M199, and DMEM), FCS, L-glutamine, penicillin, streptomycin, amphotericin B, and TRIzol reagent were from Invitrogen. LPS from *Salmonella abortus equi* and type XI collagenase, leflunomide metabolite analog (LFM-A13), and anti-β-actin mouse IgG mAbs were obtained from Sigma-Aldrich. The LightCycler FastStart DNA Master SYBR Green I was from Roche Applied Science. The miScript system was obtained from Qiagen. Clear-MiR miRNA inhibitors were from Eurogentec. MiRIDIAN miR-346 mimic and miRIDIAN miRNA mimic negative control were supplied by Dharmacon/Perbio Science. Human dermal fibroblast Nucleofector kit and cell line Nucleofector kit V were from Amaxa. The enzyme immunoassay kits for human IL-18 detection was from MBL and for human IL-6 detection was from R&D Systems. Throughout this study, buffers were prepared with apyrogenic water obtained from Braun Medical. Anti-Btk mouse IgG mAbs were from BD Transduction Laboratories.

Cell culture

Human FLS were isolated from synovial tissues from four different RA patients at the time of knee joint arthroscopic synovectomy as described previously after informed consent was obtained from patients (22). The diagnosis was conformed to the revised criteria of the American College of Rheumatology (23). FLS cultures were done as previously described (24). Experiments were performed between the third and the ninth passage. During that time, cultures were constituted of a homogeneous population of fibroblastic cells, negative for CD16 as determined by FACS analysis. Cell number and cell viability were checked by the MTT test as described elsewhere (25). THP-1 cells (no. 88081201; European Collection of Cell Cultures) were cultured as described previously (26). HEK293 cells were purchased from the American Type Culture Collection and maintained in DMEM supplemented with 10% heat-inactivated FBS, 2 μM L-glutamine, 40 U/ml penicillin, and 50 μg/ml streptomycin.

Stimulation of cells for total RNA extraction

For miRNA studies, FLS and THP-1 cells (10^6 cells) were stimulated with 2 ml of medium alone or medium containing LPS (1 μg/ml). After a 3- or 6-h incubation period, total RNA was extracted using TRIzol according to the manufacturer's instructions. For IL-18 studies, cells were stimulated as previously described (6), and total RNA was extracted using TRIzol according to the manufacturer's instructions.

MiRNA microarray analysis

MiRNA microarray analyses were performed by Eurogentec using the MiRanalyser microarray, which contains 409 calibrated human miRNA oligonucleotides from the Sanger miRBase that wereHPLC purified and spotted in duplicate into two separate fields on 25 × 75-mm high-quality glass slides. The microarray data were submitted to the Minimum Information About a Microarray Experiment (MIAME) database (www.ebi.ac.uk/) with the accession no. E-MEXP-1970.

Luciferase reporter constructs

To generate luciferase reporters plasmids, psiCHECK-2 (Promega) was modified by inserting the Gateway cassette C.1 (Invitrogen) at the 3′ end of the firefly luciferase gene (*f-luc*) into the *Xba*I site of psiCHECK-2. The 3′-UTR sequence of IL-18 was amplified from HEK293 cell genomic DNA and after addition of attB1 and attB2 sequences, the resulting PCR products were cloned into pDONR/Zeo and then recombined in the modified psiCHECK-2 vector using Gateway technology (Invitrogen). A similar construct was generated for the 3′-UTR sequence of Btk.

The primer sequences were (sense and antisense primers are indicated in respective order, and the anchor sequence used for the nested PCR is underlined): IL-18, 5′-AGAAAGCTGGGTCACATTATGAATTTTTTATTTGTTTA, 5′-AAAAAGCAGGCTCTATTAAAATTTCATGCCGGGC; attB1/2, 5′-ACAAGTTTGTACAAAAAAGCAGGCT, 5′-ACCACTTTGTACAAGAAAGCTGGGT; Btk, 5′-AAAAAGCAGGCTTGTCATGGATGAAGAATCCTG, 5′-AGAAAGCTGGGTCCTCCCCTCCCATCTTTATG.

Real-time quantitative RT-PCR

Total RNA isolated from FLS was reverse transcribed using the FirstStrand cDNA synthesis kit according to the manufacturer's instructions (Invitrogen). Real-time quantitative RT-PCR was performed in a total volume of 20 μl using the LightCycler FastStart DNA Master SYBR Green I and gene specific primers: *IL-18*, 5′-ATCAGGATCCTTTGGCAAGCTTGAATCTAAATTATC-3′ and 5′-ATAGGTCGACTTCGTTTTGAACAGTGAACATTATAG-3′; *GAPDH*, 5′-GGTGAAGGTCGGAGTCAACGGA-3′ and 5′-GAGGGATCTCGCTCGCTCCTGGAAGA-3′; *Btk*, 5′-GAAGCTGGTGCAGTTGTATG-3′ and 5′-TATACCCTCTCTGATGCCAG-3.

After an initial denaturing at 96°C for 10 min, the temperatures used were: 95°C for 10 s, 60°C for 15 s, and 72°C for 25 s for IL-18; 95°C for 15 s, 57°C for 10 s, and 72°C for 10 s for GAPDH; and 95°C for 10 s, 60°C for 30 s, and 72°C for 25 s for Btk using the LightCycler instrument (Roche Applied Science). Amplification products were detected as an increased fluorescent signal of SYBR Green during the amplification cycles. Results were obtained using SDS Software (PerkinElmer) and evaluated using Microsoft Excel. Melting curve analysis was performed to assess the specificity of PCR products.

Quantitative RT-PCR analyses for miRNAs were performed using the miScript system and the primers (Qiagen), and RNA concentrations were determined with a NanoDrop instrument (NanoDrop Technologies). One microgram of RNA per sample was used for the assays. Reverse transcriptase reactions and real-time quantitative PCR were performed according to the manufacturer's protocols. A U6 endogenous control was used for normalization. All reactions were run in triplicate in a LightCycler Instrument (Roche Applied Science). Relative expression was calculated using the comparative threshold cycle (*Ct*) method.

Northern blot analysis

Twenty micrograms of RNA per sample was resolved on a 15% urea-PAGE gel and transferred to a nylon membrane (Hybond NX; Amersham Biosciences). After UV cross-linking, the membrane was prehybridized in PerfectHyb Plus buffer (Sigma-Aldrich) at 42°C for 60 min and then hybridized with ^{32}P-labeled probes at 42°C overnight. After being washed, the membrane was exposed on a phosphorimager plate and the signal was quantified after scanning the plate on a Fuji FLA7000 phosphorimager. The membrane was stripped and rehybridized twice.

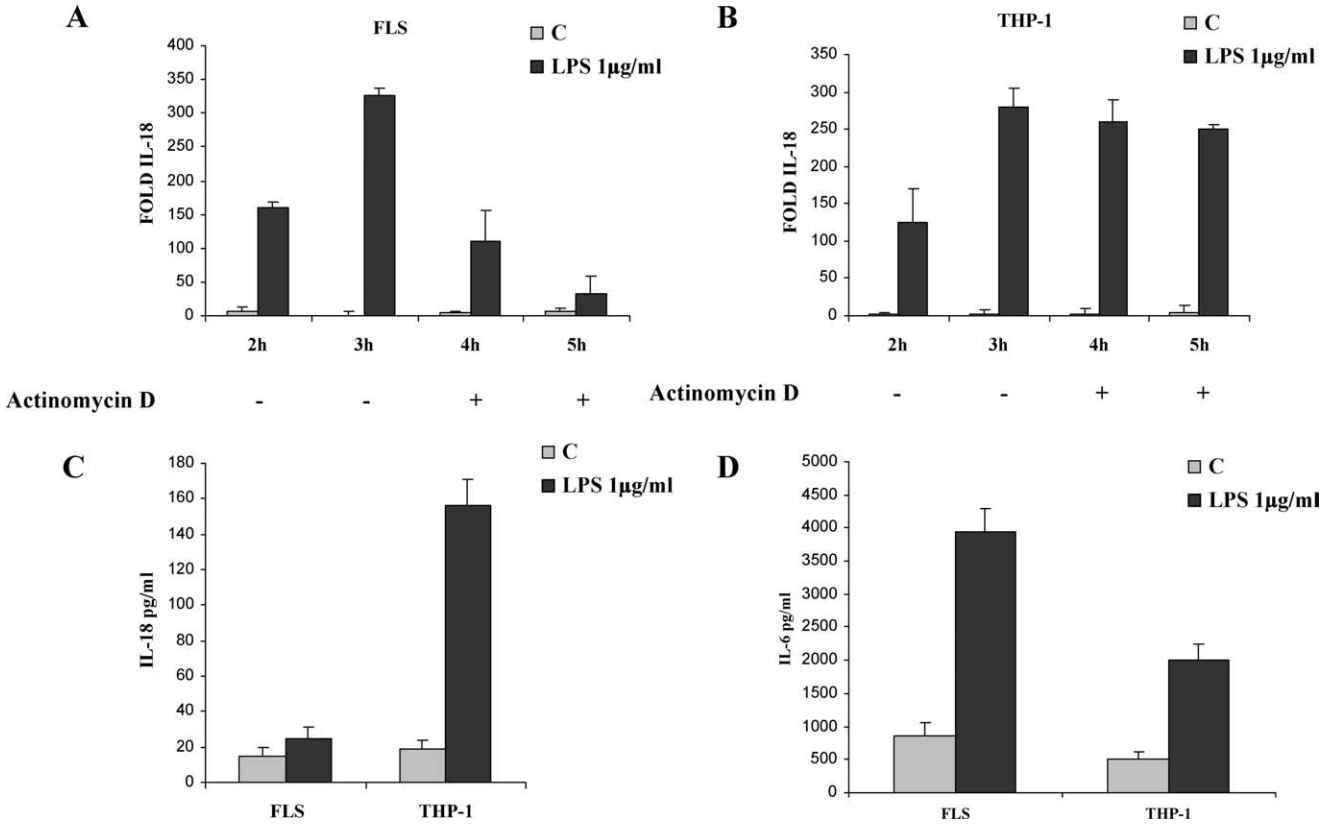

FIGURE 1. Effect of LPS on IL-18 mRNA expression and release by RA FLS and THP-1 cells. *A* and *B*, IL-18 mRNA levels were determined using quantitative RT-PCR in RA FLS (*A*) and THP-1 cells (*B*) activated with LPS (1 μg/ml) for 2 and 3 h, or incubated for another 1–2 h with actinomycin D. Results were normalized to GAPDH and expressed as the fold change compared with samples from cells incubated in medium (C). *C*, IL-18 release by activated RA FLS and THP-1 cells was determined by ELISA in culture supernatants, harvested 24 h after stimulation with LPS (1 μg/ml) or medium (C). *D*, IL-6 release was determined by ELISA in culture supernatants harvested 24 h after stimulation with LPS (1 μg/ml) or medium (C). Data are expressed as the mean of triplicate samples ± SD and are representative of three independent experiments.

Transfections and luciferase assay

The Clear-MiR anti-miR-346 used in our study was designed to inhibit efficiently the activity of miR-346. It consists of a sequence of 21 nucleotides complementary to the miRNA and was supplied by Eurogentec. The miR-346 mimic was supplied by Dharmacon.

Transient transfection of FLS with Clear-MiR miRNA inhibitor (200 nM) and reporter constructs (200 ng/ml) was performed using the human dermal fibroblast Nucleofector kit from Amaxa as previously described (27). Transient transfection of THP-1 cells with miR-346 mimic (200 nM) was performed using the cell line Nucleofector kit V from Amaxa. FLS and THP-1 cells were then plated in 24-well plates (2×10^5 cells/well and 10^6 cells/well, respectively). All assays were performed 24 h after transfection. Controls were conducted with the Clear-MiR negative control or with the mimic miRNA negative control. Transfection efficiency was evaluated with the pmaxGFP vector.

Transfection of HEK293 cells plated in 24-well plates (2×10^5 cells/well) with reporter constructs and miR-346 mimic (200 nM) was performed using Lipofectamine 2000 (Invitrogen). After 48 h cells were washed and lysed with passive lysis buffer (Promega), and *f-luc* and *Renilla* luciferase (*r-luc*) 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 *r-luc* activity.

Cell numbers and cell viability were assessed using the MTT test. IL-18 release was measured in culture supernatants by a heterologous two-site sandwich ELISA according to the manufacturer's instructions.

Western blot

Cells (10^6; FLS and THP1) were incubated for various times (24 h) with LPS (1 μg/ml). Controls were performed with cells maintained in medium with 5% heat-inactivated FCS for 6 h. After stimulation, cells were centrifuged and the pellets were suspended for 20 min on ice in 300 μl of ice-cold lysis buffer (1% Triton X-100, 20 mM Tris-HCl (pH 8.0), 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°C, and supernatants were subjected to SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. Membranes were blocked using 1% BSA in TBS (20 mM Tris (pH 7.5), 150 mM NaCl) for 1 h at 25°C. The blots were incubated with anti-Btk mouse IgG mAbs (BD Transduction Laboratories) for 2 h at 25°C followed by incubation with HRP-conjugated goat anti-mouse IgG mAbs (1 h at 25°C) and detected by ECL (SuperSignal West Femto Maximum Sensitivity substrate; Pierce) 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 62.5 mM Tris (pH 6.7), 100 mM 2-ME, and 2% SDS for 30 min at 50°C and reprobed again with anti-β-actin (clone AC-74; Sigma-Aldrich) mouse mAbs.

Statistical analysis

Statistical analysis was performed using Student's *t* test. Values were compared between different groups in the experiment. A p value of <0.05 was considered statistically significant.

Results

LPS induced synthesis of IL-18 mRNA in RA FLS

In this study, we first verified the capacity of LPS from *S. abortus equi* to stimulate IL-18 mRNA expression in RA FLS. Quantitative RT-PCR was performed with RNA isolated from control and activated FLS. Stimulation with LPS at a concentration of 1 μg/ml resulted in an increasing amount of IL-18 transcript, which was detectable within 2 h and increased up to 3 h (the mean increases were 150-fold for 2 h and 300-fold for 3 h) (Fig. 1*A*). No constitutive expression of IL-18 mRNA was detectable in control cells. We verified also that this cytokine was not released in activated

cell supernatants: although LPS activation induced IL-6 secretion, activated FLS did not release any detectable amount of mature IL-18 in cell culture supernatants (Fig. 1, *C* and *D*). THP-1 cells were used as a control: upon LPS treatment, the level of IL-18 transcript was strongly up-regulated, which resulted in a high accumulation of IL-18 in the supernatant (Fig. 1, *B–D*). Moreover, using actinomycin D (cells were incubated with LPS for 3 h and then for another 1 and 2 h with actinomycin D at a concentration of 5 μg/ml), we observed a rapid decay of IL-18 mRNA (330-fold to 45-fold) in FLS cells but not in LPS-activated THP-1 cells (300-fold to 275-fold), which confirms the IL-18 mRNA instability in LPS-activated RA FLS (Fig. 1, *A* and *B*).

LPS induced synthesis of miRNAs targeting IL-18 mRNA in RA FLS

To examine the potential involvement of miRNAs in the regulation of IL-18 mRNA instability, we used two strategies. We first used a bioinformatic approach to identify potential miRNAs targeting IL-18 mRNA. An online search of the miRBase Target database (microrna.sanger.ac.uk/) demonstrated that 49 miRNAs have been predicted to potentially target the 3′-UTR region of the human IL-18 mRNA. In this approach, each miRNA is given a score, reflecting the local complementarity between miRNA seed sequences and the 3′-UTR region of the IL-18 mRNA. For this study, we focused on miRNA miR-197, as it showed the best score among all candidate miRNAs targeting IL-18 mRNA and it was predicted to form a thermodynamically stable complex with the considered mRNA (free energy = −25.35 kcal/mol).

We stimulated RA FLS with LPS (1 μg/ml) or medium and measured miR-197 levels by quantitative RT-PCR. We found that LPS did not induce miR-197 expression in RA FLS after 6 h of stimulation as compared with unstimulated control RA FLS (Fig. 2*A*).

In parallel, we analyzed the global expression profile of miRNAs using a microarray-based approach after exposure of RA FLS to LPS to identify LPS-induced miRNAs. FLS were stimulated with LPS (1 μg/ml) or medium for 6 h and the extracted RNAs were compared using a DNA microarray containing 409 oligonucleotide probes complementary to miRNAs of human origin. Fourteen miRNAs predicted to be able to target IL-18 mRNA were present on the array.

After LPS challenge, this initial screening identified 63 miRNAs, the expression levels of which were increased or attenuated in response to LPS. Data are presented on a scatter plot showing $\log_{10}$-transformed signal intensities for each probe on both channels for the Cy3-labeled media controls and Cy5-labeled samples stimulated with LPS (Fig. 2*B*).

Among the miRNAs showing an up-regulation following LPS treatment, 15 were up-regulated >2-fold. Within these 15 up-regulated miRNAs, 1 miRNA potentially targeting IL-18 mRNA showed a strong expression level in LPS-treated cells compared with control cells: miR-346 (>7-fold). The expression of miR-197, which was included in the array, was not modified after activation with LPS, confirming preceding results.

After LPS challenge, the array revealed also a strong up-regulation in the expression of miR-30d, miR-10a, and miR-125b. Interestingly, these miRNAs are predicted to target the 3′-UTR of IL-1β and TNF-α. The expression of 42 miRNAs was down-regulated, especially miR-633, miR-320, and let-7a (<0.5). The main results are presented in Table I.

To confirm the validity of miR-346 induction as assessed by the microarray analysis, quantitative RT-PCR was performed using primers that recognized their respective mature forms. Consistent with the microarray findings, miR-346 was strongly induced by

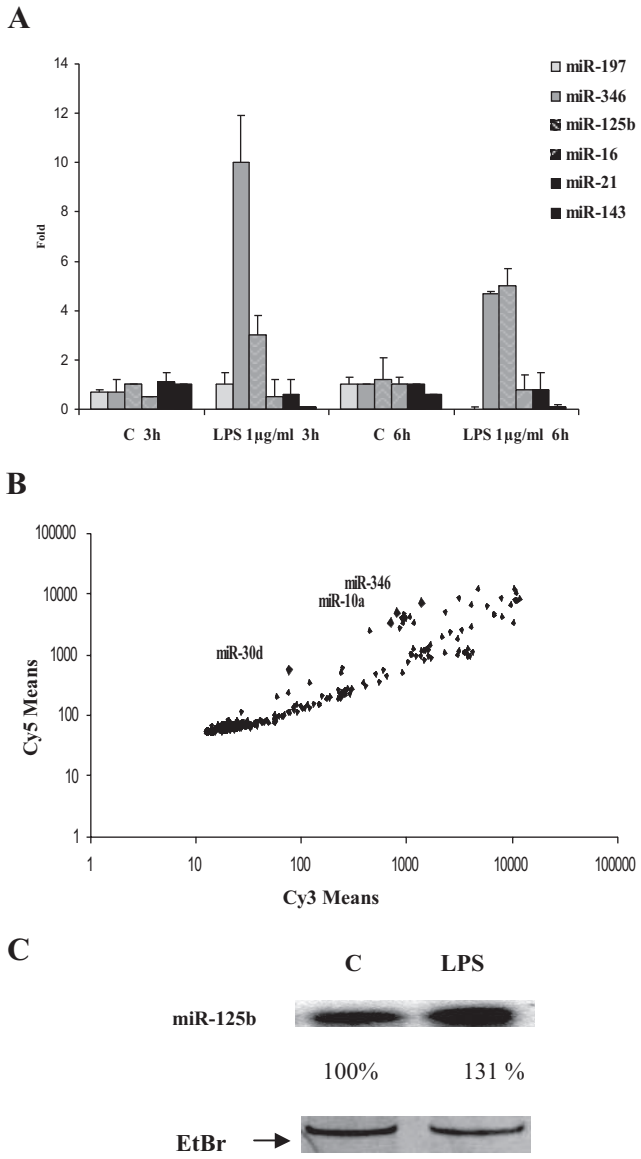

FIGURE 2. Microarray and quantitative RT-PCR analysis of miRNA expression in LPS-activated RA FLS. *A*, MiR-346, miR-125b, miR-197, miR-16, miR-21, and miR-143 levels were determined by quantitative RT-PCR in RA FLS stimulated with LPS (1 μg/ml) for 3 and 6 h. U6 small nuclear RNA was used as endogenous control for data normalization. The control (C) corresponded to untreated cells. Data are expressed as the mean of triplicate samples ± SD and are representative of three independent experiments. *B*, RA FLS were stimulated with medium or LPS (1 μg/ml) for 6 h. RNA was extracted and used in a microarray assay to determine the expression levels of 409 human miRNAs. Data are presented on a scatter plot showing $\log_{10}$-transformed signal intensities for each probe on both the channel for the Cy3-labeled control and the Cy5-labeled 6-h LPS-treated samples and represent the mean values. Each dot represents one miRNA probe. Main results are summarized in Table I. *C*, Northern blot analysis of miR-125b expression was performed under the same conditions with locked nucleic acid-modified oligodeoxynucleotides complementary to the indicated miRNAs. RNA was used as a loading control. The results are representative of three different experiments.

LPS (the mean increases were 11-fold for 3 h and 5-fold for 6 h) (Fig. 2*A*); however, we were unable to confirm this result by Northern blot analysis (data not shown). MiR-125b was detected in activated cells either by Northern blot analysis or quantitative RT-PCR and the observed changes were confirmed by either means (Fig. 2, *A* and *C*). The validity of the results was also confirmed by

Table I. *MiRNA differentialy expressed between nonactivated RA FLS and LPS-activated RA FLS*[a]

Name	Intensity Ratio of Cy5/Cy3
hsa-miR-346	7.01
hsa-miR-10a	6.38
hsa-miR-10b	4.94
hsa-miR-30d	5.99
hsa-miR-30c-2, hsa-miR-30c-1	3.41
hsa-miR-487b	3.33
hsa-miR-596	3.07
hsa-miR-100	2.88
hsa-miR-30b	2.84
hsa-miR-125b	2.70
hsa-miR-508	2.67
hsa-miR-585	2.44
hsa-miR-563	2.36
hsa-miR-411	2.12
has-miR-155	1.7
hsa-let-7a	0.34
hsa-miR-320	0.32
hsa-miR-633	0.48

[a] Data are presented on intensity ratio of the Cy3-labeled media controls and Cy5-labeled samples stimulated with LPS.

quantitative RT-PCR using miR-16, miR-21, and miR-143, the expression of which was not modified by LPS in the array (Fig. 2*A*).

IL-18 synthesis is repressed posttranscriptionally by miR-346 in LPS-activated RA FLS

To study the functional consequence of miR-346 induction on IL-18-release by LPS-activated RA FLS, we performed miRNA inhibition experiments. We used an antisense oligonucleotide complementary to miR-346 to block its activity, and the Clear-MiR was used as a negative control. Oligonucleotide*s* were transfected in FLS at a concentration of 200 nM. A plasmid encoding GFP was cotransfected to evaluate transfection efficiency.

Transfection of miR-346 antisense molecules impaired endogenous miRNA expression by a factor of 2 as compared with miR-346 expression in activated cells transfected with an anti-miR control as assessed by quantitative RT-PCR analysis (Fig. 3*A*).

We also performed experiments demonstrating the fate of the IL-18 mRNA in RA FLS transfected with miR-346 antisense molecules or anti-miRNA control and incubated with LPS for 3 h and then for another 2 h with actinomycin D. As compared with the control, we observed a stabilization of IL-18 mRNA (Fig. 3*B*).

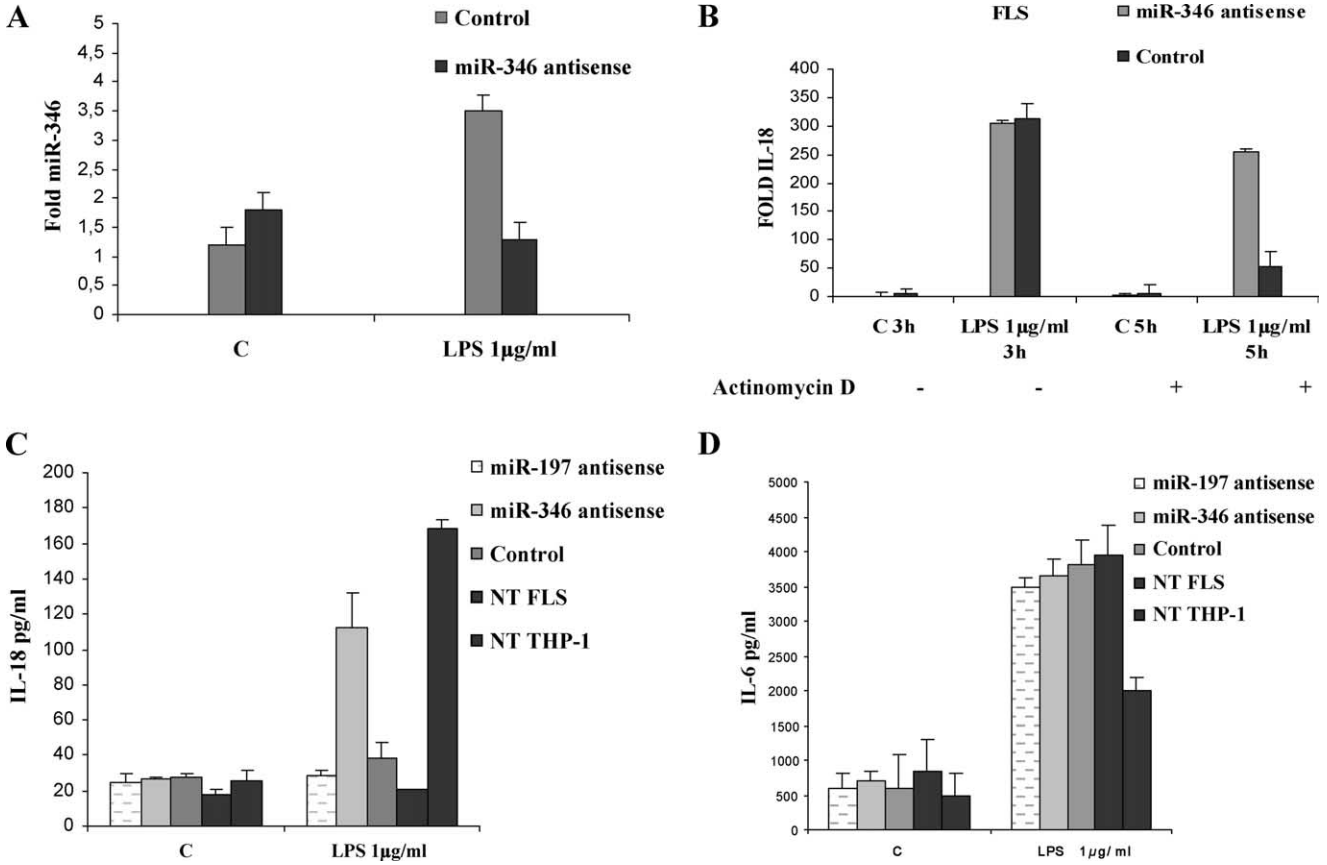

FIGURE 3. Effect of transfection of miRNA antisense molecules on IL-18 synthesis by RA FLS. *A*, RA FLS were transfected with miR-346 antisense molecules or with the Clear-MiR negative control (Control). LPS (1 μg/ml) activation of transfected RA FLS was performed 24 h posttransfection for 6 h. MiR-346 expression was determined by quantitative RT-PCR. U6 small nuclear RNA was used as endogenous control for data normalization. *B*, IL-18 mRNA levels were determined using quantitative RT-PCR in RA FLS transfected with miR-346 antisense molecules or with the Clear-MiR negative control (Control). LPS (1 μg/ml) activation of transfected cells was performed 24 h posttransfection for 3 h, and cells were then incubated for another 2 h with actinomycin D. Results were normalized to GAPDH and expressed as the fold change compared with samples from cells incubated in medium (C). *C* and *D*, RA FLS were transfected with miR-346, miR-197 antisense molecules, or with the Clear-MiR negative control (Control). LPS (1 μg/ml) activation of transfected RA FLS was performed 24 h posttransfection. Nontransfected RA FLS and THP-1 cells were used as negative and positive controls (NT). IL-18 and IL-6 release was determined by ELISA in culture supernatants harvested 24 h after stimulation with LPS (1 μg/ml) or medium (C). Data are expressed as the mean of triplicate samples $\pm$ SD and are representative of three independent experiments.

We next tested whether alteration of miR-346 cellular levels affected IL-18 protein release by LPS-stimulated FLS. We transfected cells with antisense molecules for 24 h and then measured IL-18 release by FLS stimulated with LPS (1 μg/ml) for 24 h at 37°C. Supernatants were tested for IL-18 secretion levels by ELISA measurements. As illustrated in Fig. 3*C*, treatment with LPS significantly induced IL-18 release by activated RA FLS transfected with antisense oligonucleotides targeting miR-346 (120 vs 25 pg/ml) as compared with activated FLS transfected with control oligonucleotides or with oligonucleotides targeting another miRNA (miR-197). A similar increase in IL-18 secretion was obtained in nontransfected LPS-activated THP-1 cells used as positive control. No IL-18 release was observed in nontransfected but LPS-activated RA FLS (Fig. 3*C*). The effect of transfection on cell viability was also determined by the MTT test, with no significant difference being observed for cells transfected with targeting and nontargeting antisense oligonucleotides (data not shown).

The transfection of antisense oligonucleotides did not impair FLS activation since nontransfected FLS as well as FLS transfected with targeting and nontargeting miRNA inhibitors produced IL-6 after 24 h of activation with LPS (Fig. 3*D*).

Taken together, these data demonstrate that miR-346 is implicated in the negative regulation of IL-18 synthesis in LPS-activated FLS.

MiR-346 repressed IL-18 synthesis in LPS-activated THP-1 cells

We next tested by transient transfection whether the presence of miR-346 affected IL-18 mRNA expression and IL-18 release in LPS-activated THP-1 cells. THP-1 cells did not express miR-346 when activated with LPS for 3 and 6 h as assessed by quantitative RT-PCR (Fig. 4*A*). We transfected THP-1 cells with miR-346 mimic for 24 h and incubated cells with LPS for 3 h and then for another 2 h with actinomycin D. As compared with the control, we observed that transfection of miR-346 mimic in THP-1 cells induces a degradation of IL-18 mRNA (Fig. 4*B*). We also measured IL-18 release by transfected cells stimulated with LPS (1 μg/ml) for 24 h at 37°C. As illustrated in Fig. 4*C*, cells transfected with miR-346 mimic showed a significant decrease of IL-18 release as compared with IL-18 secretion by activated cells transfected with the miRNA mimic negative control or nontransfected LPS-activated THP1 cells used as positive control. Collectively, these results confirm that miR-346 plays a role in the control of IL-18 release in a cell type-independent manner.

MiR-346 did not directly regulate the expression of IL-18

Computer analysis predicted that miR-346 might directly target the 3′-UTR of human IL-18 transcript (Fig. 5*A*), suggesting that this miRNA could play a direct role in IL-18 posttranscriptional regulation. To test the hypothesis of a direct regulation by miR-346, we cotransfected HEK293 cells with a luciferase reporter fused to IL-18 3′-UTR along with miR-346 mimic. FLS were also transfected with the same construct and then activated with LPS. Transfections in both kinds of cells did not result in a measurable change in luciferase activity (Fig. 5, *B* and *C*). These results indicate that miR-346 does not repress directly the expression of IL-18.

MiR-346 repressed Btk expression in LPS-activated RA FLS

Btk is a nonreceptor tyrosine kinase belonging to the Tec family of protein tyrosine kinases, and recent studies have shown that Btk is involved in the stabilization of various cytokine mRNAs such as TNF-α (28–30). FLS express Etk, which is implicated in IL-6 and IL-8 release by activated FLS, but they fail to express constitutively or after LPS activation either Btk mRNA or the mature protein. Surprisingly, we found by quantitative RT-PCR that transfection of FLS with the antisense oligonucleotides targeting miR-346 led to the induction of Btk mRNA expression after activation with LPS compared with activated cells transfected with the negative control (Fig. 6*A*). These results point to a putative effect at the transcriptional level. We also measured Btk expression by Western

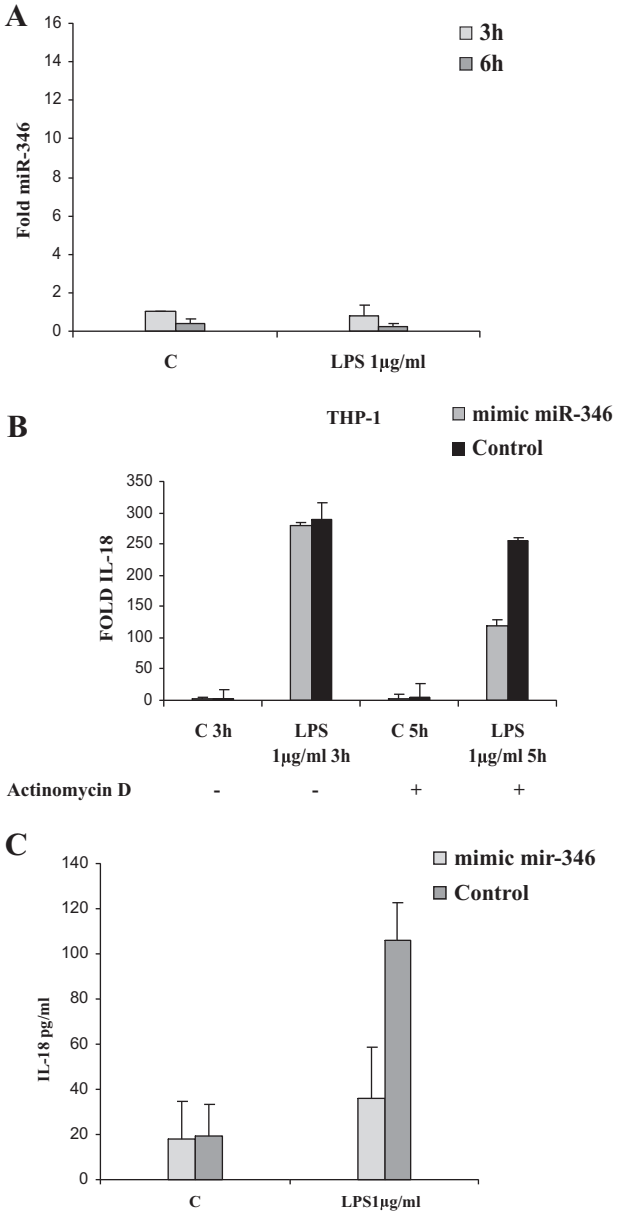

FIGURE 4. Effect of transfection of miR-346 mimic on IL-18 synthesis by THP-1 cells. *A*, MiR-346 expression was analyzed in THP-1 cells activated for 3 and 6 h with LPS (1 μg/ml) by quantitative RT-PCR. U6 small nuclear RNA was used as endogenous control for data normalization. *B*, IL-18 mRNA levels were determined using quantitative RT-PCR in THP-1 cells transfected with miR-346 mimic or with the miRNA mimic negative control (Control). Cells were activated with LPS (1 μg/ml) 24 h posttransfection for 3 h and incubated for another 2 h with actinomycin D. Results were normalized to GAPDH and expressed as the fold change compared with samples from cells incubated in medium (C). *C*, THP-1 cells were transfected with miR-346 mimic or with the miRNA mimic negative control (Control) and activated with LPS (1 μg/ml) 24 h posttransfection. IL-18 release was determined by ELISA in culture supernatants harvested 24 h after stimulation with LPS (1 μg/ml) or medium (C). Data are expressed as the mean of triplicate samples ± SD and are representative of three independent experiments.

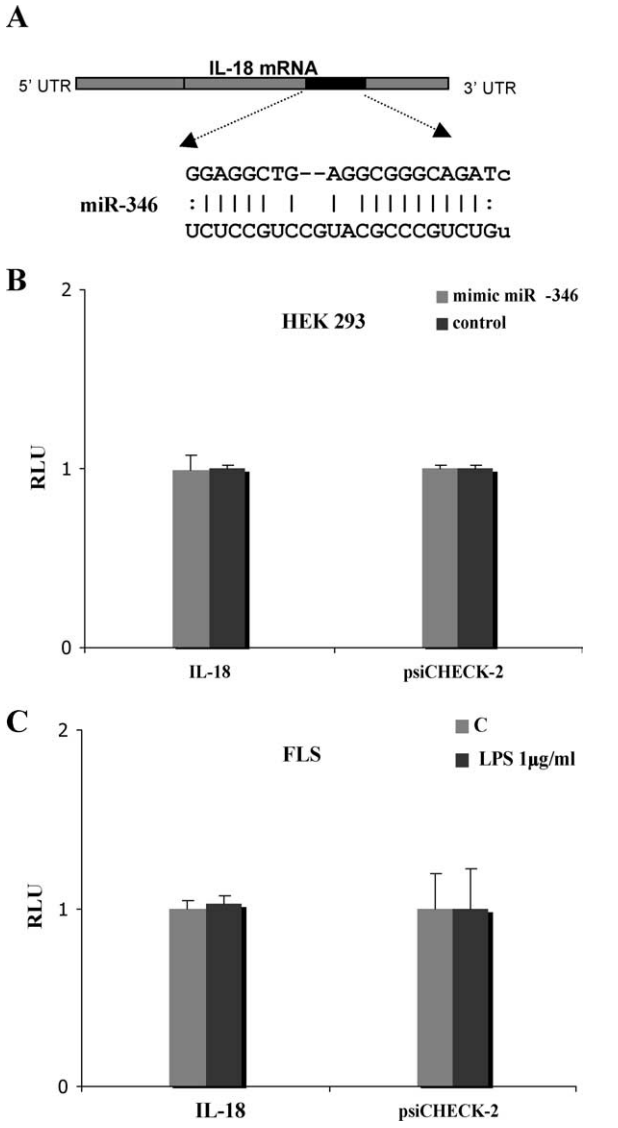

FIGURE 5. MiR-346 does not regulate the expression of IL-18 directly. *A*, Sequence alignment of miR-346 and its target sites in the 3′-UTR of IL-18 mRNA. *B* and *C*, Targeting of miR-346 to the 3′-UTR of IL-18 mRNA. A reporter construct with the potential binding site for miR-346 in the 3′-UTR of IL-18 was generated. HEK293 cells were transiently cotransfected with the reporter construct and either miR-346 mimic or miRNA mimic negative control (Control) (*B*); RA FLS were transfected with the reporter construct and activated with LPS (1 μg/ml) (*C*). Luciferase activities were measured for 48 h and normalized to the control psiCHECK-2 luciferase level. Bars represent the mean ± SD from three independent experiments.

blotting analysis of FLS stimulated with LPS for 24 h at 37°C. As shown in Fig. 6*B*, inhibition of miR-346 led also to a strong expression of the protein. Thus, these data collectively indicate that miR-346 inhibits Btk expression in LPS-activated RA FLS.

We also found that in THP-1 cells transfected with miR-346 mimic, Btk expression was down-regulated (Fig. 6*B*) and that miR-346 did not repress Btk expression directly, as transfection of HEK293 cells with a luciferase reporter fused to Btk 3′-UTR along with miR-346 mimic did not result in a measurable change in luciferase activity (Fig. 6*C*).

Btk regulates IL-18 release in LPS-activated RA FLS

We therefore hypothesized that miR-346 inhibits Btk expression in RA FLS, which could result in the subsequent inhibition of IL-18.

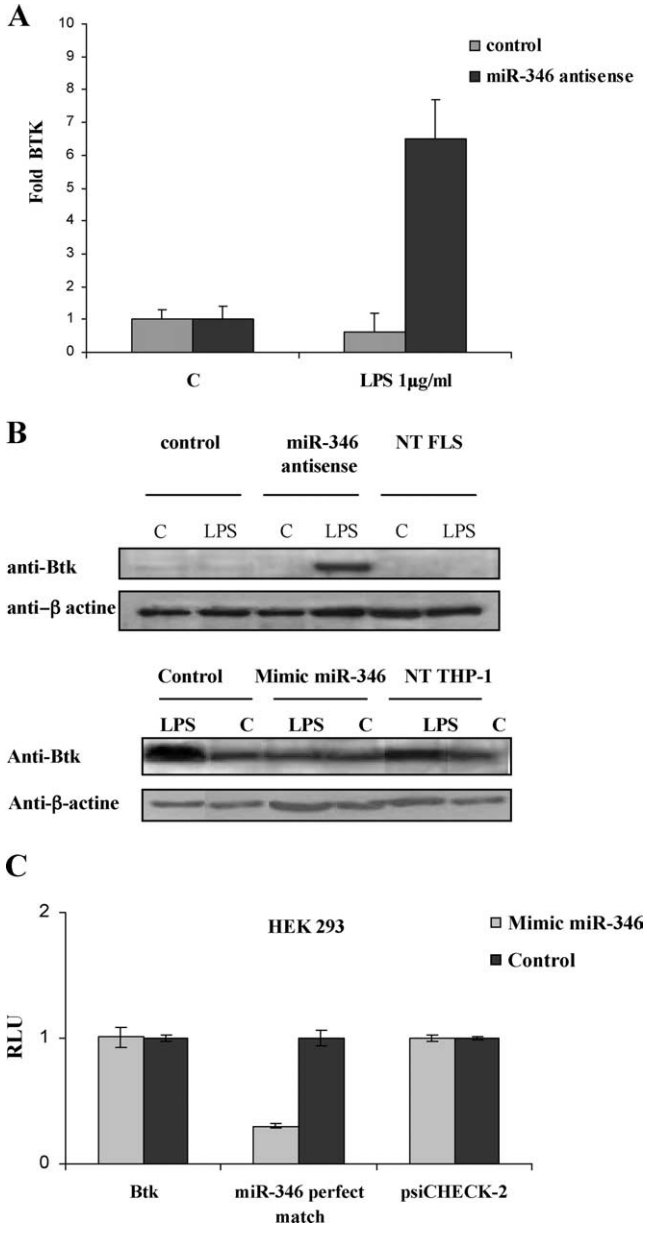

FIGURE 6. MiR-346 regulates the expression of Btk in activated RA FLS and THP-1 cells. *A*, Btk mRNA levels were determined using quantitative RT-PCR in RA FLS transfected with miR-346 antisense molecules or with the Clear-MiR negative control (Control). LPS (1 μg/ml) activation of transfected cells was performed 24 h posttransfection for 6 h. Results were normalized to GAPDH and expressed as the fold change compared with samples from cells incubated in medium (C). *B*, Btk expression was determined by Western blotting with anti-Btk mouse mAbs in FLS transfected with miR-346 antisense molecules or with the Clear-MiR negative control or in nontransfected FLS. Twenty-four hours posttransfection, FLS were either incubated in 5% FCS medium (C) or activated with LPS for 24 h. Similar experiments were performed with THP-1 cells transfected with the miR-346 mimic or the miRNA mimic negative control. For protein loading control, membranes were reprobed with anti-β-actin mouse mAbs. The results shown are representative of three separate experiments. *C*, MiR-346 does not regulate the expression of Btk directly. A reporter construct with the 3′-UTR of Btk or with the miR-346 perfect match were generated. HEK293 cells were transiently cotransfected with the reporter constructs and with either miR-346 mimic or miRNA mimic negative control (Control). Luciferase activities were measured for 48 h and normalized to the control psiCHECK-2 luciferase level. Bars represent the mean ± SD from three independent experiments.

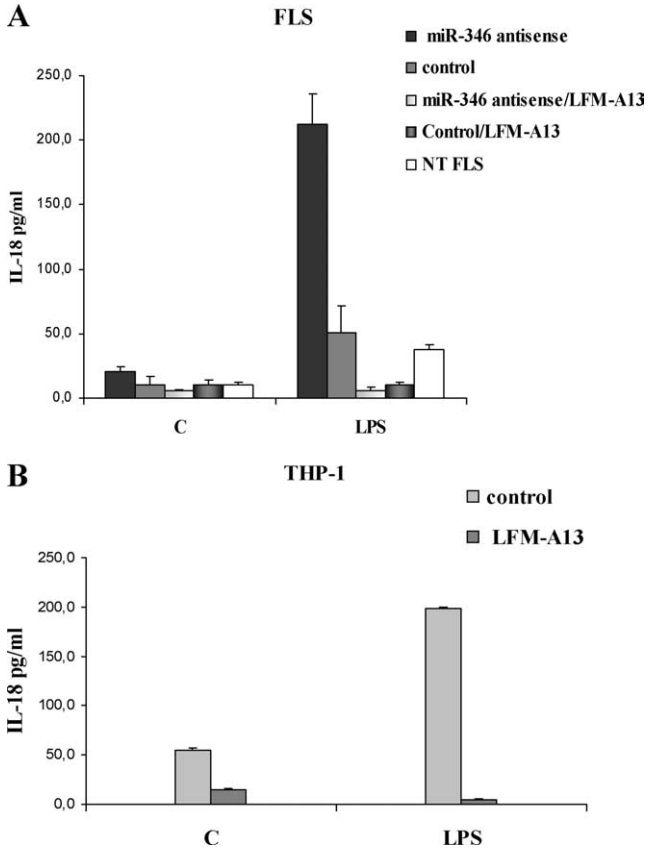

FIGURE 7. Btk regulates IL-18 expression. *A*, RA FLS were transfected with miR-346 antisense molecules or with the Clear-MiR negative control (Control) for 24 h. IL-18 release by FLS preincubated with LFM-A13 and then stimulated with LPS for 24 h was evaluated by ELISA. Nontransfected RA FLS were used as negative controls (NT). *B*, THP-1 cells was pretreated with LFM-A13 for 1 h before activation with LPS (1 μg/ml) for 24 h.

We transfected FLS with antisense molecules targeting miR-346 for 24 h and then examined IL-18 release by FLS pretreated with LFM-A13, a Btk inhibitor, before LPS stimulation. In this case, IL-18 production induced by LPS was impaired in cells treated with LFM-A13 when compared with cells not treated with the inhibitor (Fig. 7*A*). Similarly, in THP-1 cells activated by LPS in the presence of LFM-A13, IL-18 release was strongly inhibited (Fig. 7*B*). These results demonstrate that IL-18 release is Btk-dependent. Taken together, our data indicate an important role for miR-346 and Btk in the regulation of IL-18 release by RA FLS.

Discussion

The key findings in this study are that LPS induces the expression of miR-346 in PRR-activated synoviocytes from RA patients and that this miRNA negatively regulates the IL-18 response of FLS by inhibiting the transcription of the Btk gene. One important observation obtained from previous results was that PRRs-activated FLS, in contrast to other resident cells from the synovial membrane such as macrophages, have a more restricted cytokine profile: they up-regulate, for example, IL-18 mRNA, but release of this cytokine is impaired via translational suppression, and similar results were obtained with TNF-α and IL-1 (data not shown). IL-18 is normally produced from macrophage-like cells, but some epithelial cells such as gastric epithelial cells release this cytokine in response to *Helicobacter pilori* infection (31). These data suggest that IL-18 protein expression is tightly regulated depending of the origin of the cell. This study was designed to test the possibility that PAMPs might induce cellular miRNAs that target IL-18 transcripts in FLS and inhibit its release.

An initial microarray screen identified 63 miRNAs, the expression of which was increased or attenuated in FLS in response to LPS. This approach revealed a promising result insofar as one miRNA predicted to target IL-18 transcript was considerably up-regulated: miR-346. This was suggested by an online search of the miRBase Target database maintained by the Sanger Institute, which predicts potential target sequences of miRNAs. The change in expression of miR-346 was then validated by quantitative RT-PCR but not by Northern blot. In fact, some mature miRNAs are produced at low levels and are not detected by Northern blot, but they are still functional, suggesting that high levels of mature miRNAs are not necessary for their efficient inhibition (32). A small variation in one component that is upstream of a transduction cascade can have strong effects in the end.

When comparing our microarray data with previous studies from others, we found similarities as well as differences. Recently, Taganov et al. analyzed miRNA expression in a monocytic cell line treated with LPS (20). They found that miR-146a, miR-155, and miR-132 were endotoxin-responsive genes. Similar results were obtained by Stanczyk et al. (33) in RA FLS activated with either TNF-α or TLR ligands such as LPS, bacterial lipopeptide, and poly(I:C). They also observed an up-regulation of miR-146a and miR-155. Similarly, we also found that miR-155 is up-regulated in response to LPS, but more faintly than miR-346. These results confirm the important role of miR-155 in innate immune response (34). However, the arrays of Stanczyk et al. (33) never revealed the up-regulation of miR-346, which was one of the most overexpressed miRNAs in RA FLS activated with LPS. A simple possible explanation for this discrepancy could be that their screen did not include the oligonucleotide probe for miR-346: only 200 sequences complementary to mammalian miRNAs were tested in the Taganov et al. (20) studies. It is also possible that miR-346 was not detected in cells treated for 8 h with LPS. In fact, quantitative RT-PCR analysis indicated that miR-346 reached its highest level by 3 h and was still present but at reduced levels by 6 h. To assess the reproducibility of the data, the array was performed twice with FLS isolated from different patients, and we found in every instance that miR-346 was overexpressed. Of note, we could not detect miR-146a/b in LPS-activated RA FLS, but it was demonstrated that this miRNA family, which is implicated in TLR signaling and in endotoxin tolerance, has a rather complicated mode of regulation of expression (35). Weber et al. (36) also reported the up-regulation of miR-346, as well as miR-197, in follicular thyroid carcinoma compared with follicular adenoma. They linked the up-regulation of these two miRNAs to cellular proliferation. We did not observe a significant effect of miR-346 on cell viability as assessed by MTT assay.

IL-18 is present in significantly elevated levels in the synovium of RA patients and is mainly produced by macrophages and dendritic cells. Its expression was demonstrated to correlate with the inflammatory phase of the disease. Studies with RA synovial cell culture have shown increased production of NO as well as TNF-α, IFN-γ, and IL-6 after stimulation with IL-18. This suggests that IL-18 is a pre-TNF-α cytokine (37–39). The expression of the IL-18 gene is regulated at different levels, such as control of promoter activity, degradation of mRNA, and posttranslational regulation through processing of the inactive precursor pro-IL-18 to an active form via activation of caspase 1 (40). Two different promoters are controlling the expression of IL-18, one constitutively active and one inducible by LPS. Additionally, IL-18 mRNA does not contain an AU-rich element within the 3′-UTR region, which

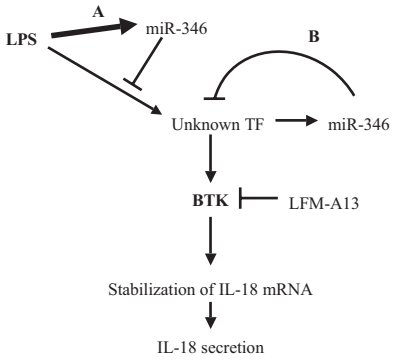

FIGURE 8. Model of action for miR-346 and Btk in IL-18 secretion regulation. See text for details. TF indicates transcription factor.

is a motif known to promote rapid mRNA degradation, and this implies that IL-18 mRNA may have a longer half-life than ARE-containing transcripts (41, 42). However, we demonstrated that IL-18 mRNA is very instable in LPS-activated RA FLS and that this instability negatively regulates IL-18 release (6).

It was thus important to investigate whether the expression levels of miR-346 could modulate IL-18 expression. RA FLS were transfected with the antisense miR-346 inhibitor. For the first time, we demonstrated that inhibition of endogenously expressed miR-346 by an anti-miR-346 oligoribonucleotide markedly increased IL-18 expression. Further evidence of miR-346 inhibitory effect on IL-18 release was obtained by transient transfection of miR-346 mimic in LPS-activated THP-1 cells. Taken together, these data clearly suggest that one of the functions of miR-346 is to turn off IL-18 expression in response to LPS.

We also demonstrated, using luciferase reporter constructs containing the 3′-UTR of the human IL-18 gene, that miR-346 does not directly regulate the expression of IL-18. A general principle regarding miRNAs is that each miRNA can potentially regulate a wide spectrum of protein-coding genes. This suggests that miRNA can be involved in the regulation of multiple independent physiological processes. Thus, it was tempting to speculate that miR-346 may use different mechanisms to down-regulate at a posttranscriptional level the expression of IL-18. Several reports have established that Btk is implicated in TLR signaling and regulates TNF-α synthesis by stabilizing its mRNA, without having any effect on IL-6 or IL-12 release (28). FLS do not express either Btk mRNA or the mature protein but Etk, another Tec kinase, which was demonstrated to be implicated in IL-6 and IL-8 release in response to integrin $\alpha_5\beta_1$ and TLR4 stimulation (27). Results obtained here demonstrated that transient transfection of miR-346 antisense induced a strong expression of Btk in FLS and reestablished IL-18 release. Moreover, inhibition of induced Btk by LFM-A13 led to the impairment of IL-18 secretion by RA FLS. These data demonstrated the implication of miR-346 in Btk expression that results in IL-18 release.

Interestingly, as in THP-1 cells transfection of miR-346 mimic diminished protein expression, we investigated whether miR-346 was capable of a direct inhibition of Btk by using a luciferase reporter assay. A direct inhibition was not observed. Thus, as previously demonstrated with FLS, miR-346 inhibits IL-18 secretion by an indirect mechanism involving the regulation of Btk. The interaction between miR-346 and Btk must involve other yet-undefined factors acting probably at the transcriptional level.

We propose a model in which LPS would induce expression of both Btk and miR-346 through two distinct transcription factors (Fig. 8*A*) or through a common transcription factor (Fig. 8*B*). The induced Btk would then act on stabilizing IL-18 mRNA, which would ultimately result in its secretion. Blocking Btk either chemically (with the inhibitor LFM-A13) or by overexpressing miR-346 (in THP-1 cells) would therefore result in the inhibition of IL-18 secretion. The action of miR-346 could be upstream of the transcription factor inducing Btk expression (Fig. 8*A*) or, alternatively, miR-346 could regulate its own transcription factor in a feedback loop manner (Fig. 8*B*). The latter model would explain why miR-346 is only transiently detected after LPS challenge of FLS cells and seems to gradually disappear.

In conclusion, our findings provide evidence that miRNAs, which are induced by bacterial ligands, can act as potential negative regulators of inflammation and may be novel targets for immunomodulating inflammatory responses in humans. These data also demonstrated that cytokine secretion by resident cells of target organs of autoimmunity can be negatively regulated at the post-transcriptional level by miRNAs. Understanding these complex controls has important implications for the development of future therapeutic applications.

Disclosures

The authors have no financial conflicts of interest.

References

1. Firestein, G. S. 2003. Evolving concept of rheumatoid arthritis. *Nature* 423: 356–361.
2. Muller-Ladner, U., C. Ospelt, S. Gay, O. Distler, and T. Pap. 2007. Cells of the synovium in rheumatoid arthritis: synovial fibroblasts. *Arthritis Res. Ther.* 9: 223–230.
3. Al-Okla, S., C. Chatenay-Rivauday, J. P. Klein, and D. Wachsmann. 1999. Involvement of $\alpha_5\beta_1$ integrins in interleukin 8 production induced by oral viridans streptococcal protein I/IIf in cultured endothelial cells. *Cell. Microbiol.* 1: 1157–1168.
4. Filer, A., K. Raza, M. Salmon, and C. D. Buckley. 2008. The role of chemokines in leucocyte-stromal interactions in rheumatoid arthritis. *Front. Biosci.* 13: 2674–2685.
5. Pierer, M., J. Rethage, R. Seibl, R. Lauener, F. Brentano, U. Wagner, H. Hantzschel, B. A. Michel, R. E. Gay, S. Gay, and D. Kyburz. 2004. Chemokine secretion of rheumatoid arthritis synovial fibroblasts stimulated by Toll-like receptor 2 ligands. *J. Immunol.* 172: 1256–1265.
6. Müller-Ladner, U., J. Kriegsmann, B. N. Franklin, S. Matsumoto, T. Geiler, R. E. Gay, and S. Gay. 1996. Synovial fibroblasts of patients with rheumatoid arthritis attach to and invade normal human cartilage when engrafted into SCID mice. *Am. J. Pathol.* 149: 1607–1615.
7. Zeisel, M. B., L. N. Neff, J, Randle, J. P. Klein, J. Sibilia, and D. Wachsmann. 2005. Impaired release of IL-18 from fibroblast-like synoviocytes activated with protein I/II, a pathogen-associated molecular pattern from oral streptococci, results from defective translation of IL-18 mRNA in pro-IL-18. *Cell. Microbiol.* 174: 7393–7397.
8. Bartel, D. P. 2004. MicroRNAs: genomics, biogenesis, mechanism and function. *Cell* 116: 281–297.
9. Griffith-Jones, S., R. J. Grocock, S. van Dongen, A. Bateman, and A. J. Enright. 2006. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res.* 34: D140–D144.
10. Chen, K., and N. Rajewsky. 2007. The evolution of gene regulation by transcription factors and microRNAs. *Nat. Rev. Genet.* 8: 93–103.
11. Garneau, N. L., J. Wilus, and C. J. Wilusz. 2007. The highways and byways of mRNA decay. *Nat. Rev. Mol. Cell Biol.* 8: 113–126.
12. Filipowicz, W., S. N. Bhattacharyya, and N. Sonenberg. 2008. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat. Rev. Genet.* 9: 102–114.
13. Eun, J. L., B. Myungwon, G. Yuriy, D. J. Brackett, G. J. Nuovo, and D. Thomas. 2008. Systematic evaluation of microRNA processing patterns in tissues, cell lines, and tumors. *RNA* 14: 3–42.
14. Pillai, R. S., S. N. Bhattacharyya, and W. Filipowicz. 2007. Repression of protein synthesis by miRNA: how many mechanisms? *Trends Cell. Biol.* 17: 118–126.
15. Rajewsky, N. 2006. MicroRNA target predictions in animals. *Nat. Genet.* 38: 8–13.
16. Lewis, B. P., C. Burge, and D. P. Bartel. 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120: 15–20.
17. Farazi, T. A., S. A. Juranek, and T. Tuschl. 2008. The growing catalog of small RNAs and their association with distinct Argonaute/Piwi family members. *Development* 135: 1201–1214.
18. Liang, Y., D. Ridzon, L. Wong, and C. Chen. 2007. Characterization of microRNA expression profiles in normal human tissues. *BMC Genomics* 8: 166.
19. Sonkoly, E., M. Sthahle, and A. Pivarcsi. 2008. MicroRNAs and immunity: novel players in the regulation of normal immune function and inflammation. *Semin. Cancer Biol.* 18: 131–140.

20. Taganov, K. D., M. P. Boldin, K. J. Chang, and D. Baltimore. 2006. NF-κB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc. Natl. Acad. Sci. USA* 103: 12481–12486.
21. Tili, E., J. J. Michaille, A. Cimino, S. Costinean, C. D. Dumitru, B. Adair, M. Fabbri, H. Alder, C. G. Liu, G. A. Calin, and C. M. Croce. 2007. Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-α stimulation and their possible roles in regulating the response to endotoxin shock. *J. Immunol.* 179: 5082–5089.
22. Dechanet, J., J. L. Taupin, P. Chomarat, M. C. Rissoan, J. F. Moreau, J. Banchereau, and P. Miossec. 1994. Interleukin-4 but not interleukin-10 inhibits the production of leukemia inhibitory factor by rheumatoid synovium and synoviocytes. *Eur. J. Immunol.* 24: 3222–3228.
23. Arnett, F. C., S. M. Edworthy, D. A. Bloch, D. J. Macshane, J. F. Fries, N. S. Cooper, L. A. Healey, S. R. Kaplan, M. H. Liang, H. S. Luthra, et al. 1988. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum.* 31: 315–324.
24. Neff. L., M. Zeisel, J. Sibilia, M. Scholler-Guinard, J. P. Klein, and D. Wachsmann. 2001. NF-κB and the MAP kinases/AP-1 pathways are both involved in interleukin-6 and interleukin-8 expression in fibroblast-like synoviocytes stimulated by protein I/II, a modulin from oral streptococci. *Cell. Microbiol.* 3: 703–712.
25. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65: 55–63.
26. Chatenay-Rivauday, C., I. Yamodo, M. Sciotti, J. A. Ogier, and J. P. Klein. 1998. The A and the extended V N-terminal domains of streptococcal protein I/IIf mediate the production of TNF-α in the monocyte cell line THP-1. *Mol. Microbiol.* 29: 39–48.
27. Semaan, N., G. Alsaleh, J. E. Gottenberg, D. Wachsmann, and J. Sibilia. 2008. Etk/BMX, a Btk family tyrosine kinase, and Mal contribute to the cross-talk between MyD88 and FAK pathways. *J. Immunol.* 180: 3485–3491.
28. Horwood, N. J., T. H. Page, J. P. Mcdaid, C. D. Palmer, J. Campbell, T. Mahon, F. M. Brennan, D. Webster, and B. M. Foxwell. 2006. Bruton's tyrosine kinase is required for TLR2 and TLR4-induced TNF, but not IL-6 production. *J. Immunol.* 176: 3635–3641.
29. Doyle, S. L., C. A. Jefferies, C. Feighery, and L. A. J. O'Neill. 2007. Signaling by Toll-like receptors 8 and 9 requires Bruton's tyrosine kinase. *J. Biol. Chem.* 282: 36953–36960.
30. Lee, K. G., S. Xu, E. T. Wong, V. Tergaonkar, and K. P. Lam. 2007. Bruton's tyrosine kinase separately regulates NFκB p65RelA activation and cytokine interleukin (IL)-10/IL-12 production in TLR9-stimulated B cells. *J. Biol. Chem.* 283: 11189–11198.
31. Yamauchi, K., I. J. Choi, H. Lu, H. Ogiwara, D. Y. Graham, and Y. Yamaoka. 2008. Regulation of IL-18 in *Helicobacter pylori* infection. *J. Immunol.* 180: 1207–1216.
32. Obernosterer, G., P. J. F. Leuschner, M. Alenius, and J. Martinez. 2006. Post-transcriptional regulation of microRNA expression. *RNA* 12: 1161–1167.
33. Stanczyk, J., D. M. L. Pedrioli, F. Brentano, O. Sanchez-Pernaute, C. Kolling, R. E. Gay, M. Detmar, S. Gay, and D. Kyburz. 2008. Altered expression of microRNA in synovial fibroblasts and synovial tissue in rheumatoid arthritis. *Arthritis Rheum.* 58: 1001–1009.
34. O'Connell, M. R., K. D. Taganov, M. P. Boldin, G. Cheng, and D. Baltimore. 2007. MicroRNA-155 is induced during the macrophage inflammatory response. *Proc. Natl. Acad. Sci. USA* 104: 1604–1609.
35. Taganov, K. D., M. P. Boldin, and D. Baltimore. 2007. MicroRNAs and immunity: tiny players in a big field. *Immunity* 26: 133–137.
36. Weber, F., R. E. Teresi, C. E. Broelsch, A. Frilling, and C. Eng. 2006. A limited set of human microRNA is deregulated in follicular thyroid carcinoma. *J. Clin. Endocrinol. Metab.* 91: 3584–3591.
37. Gracie, J. A., R. J. Forsey, W. L. Chan, A. Gilmour, B. P. Leung, M. R. Greer, K. Kennedy, R. Carter, X. Q. Wei, D. Xu, et al. 1999. A proinflammatory role for IL-18 in rheumatoid arhthritis. *J. Clin. Invest.* 104: 1393–1401.
38. Lotito, A. P. N., C. A. A. Silva, and S. B. V. Mello. 2007. Interleukin-18 in chronic joint diseases. *Autoimmun. Rev.* 6: 253–256.
39. Cho, M. L., Y. O. Jung, Y. M. Moon, S. Y. Min, C. H. Yoon, S. H. Lee, S. H. Park, C. S. Cho, D. M. Jue, and H. Y. Kim. 2005. Interleukin-18 induces the production of vascular endothelial growth factor (VEGF) in rheumatoid arthritis synovial fibroblast via AP-1-dependent pathways. *Immunol. Lett.* 103: 159–166.
40. Mariathasan, S., and D. M. Monack. 2007. Inflammasome adaptors and sensors: intracellular regulators of infection and inflammation. *Nat. Rev. Immunol.* 7: 31–40.
41. Bufler, P., F. Gamboni-Robertson, T. Azam, S. H. Kim, and C. A. Dinarello. 2004. Interleukin-1 homologues IL-1F7b and IL-18 contain functional mRNA instability elements within the coding region responsive to lipopolysaccharide. *J. Biochem.* 381: 503–510.
42. Jing, Q., S. Huang, S. Guth, T. Zarubin, A. Motoyama, J. Chen, F. Di Padova, S. C. Lin, H. Gram, and J. Han. 2005. Involvement of microRNA in AU-rich element-mediated mRNA instability. *Cell* 120: 623–634.