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MicroRNA-let-7a expression is increased in the mesangial cells of NZB/W mice and increases IL-6 production *in vitro*

Cristen B. Chafin¹, Nicole L. Regna¹, Rujuan Dai¹, David L. Caudell¹, and Christopher M. Reilly^{1,2}

¹Department of Biomedical Sciences & Pathobiology, Virginia-Maryland College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA

²Edward Via College of Osteopathic Medicine, Blacksburg, Virginia, USA

Abstract

Recent evidence supports a role for epigenetic alterations in the pathogenesis of systemic lupus erythematosus (SLE). MicroRNAs (miRNAs or miRs) are endogenous epigenetic regulators whose expression is altered in many diseases, including SLE. IL-6 is an inflammatory cytokine produced by mesangial cells during lupus nephritis (LN). *IL-6* contains a potential binding site for miRNA-let-7a (let-7a) in its 3' untranslated region (UTR). We found let-7a expression was significantly increased in the mesangial cells of pre-diseased and actively diseased New Zealand Black/White (NZB/W) mice compared to age-matched New Zealand White (NZW) mice. Overexpression of let-7a *in vitro* increased IL-6 production in stimulated mesangial cells compared to non-transfected controls. Inhibition of let-7a did not significantly affect immune-stimulated IL-6 production. When stimulated mesangial cells overexpressing let-7a were treated with the transcription inhibitor Actinomycin D (ActD), IL-6 was degraded faster, consistent with the direct targeting of the 3' UTR of *IL-6* by let-7a. Overexpression of let-7a increased the expression of tristetraprolin (TTP), an RNA-binding protein (RBP) that has 5 potential binding regions in the 3' UTR of *IL-6*. ActD inhibited the transcription of proteins including TTP that may contribute to the let-7a-mediated increase in immune-stimulated IL-6 production. These data show that NZB/W mice have higher let-7a expression than NZW mice and that increased let-7a expression *in vitro* increases IL-6 production in stimulated mesangial cells. Further studies examining the role of let-7a expression in inflammation are warranted.

Keywords

Immune stimulation; inflammatory mediators; mesangial cells; microRNAs; systemic lupus erythematosus

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Correspondence: Cristen B. Chafin, Center for Molecular Medicine and Infectious Diseases (CMMID), Virginia-Maryland College of Veterinary Medicine, 1410 Prices Fork Road, Blacksburg, VA 24061, USA. cchafin@vt.edu.

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Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by autoantibodies against nuclear antigens, including dsDNA [1]. Dysregulated apoptosis and inadequate clearance of cellular debris may contribute to autoimmune pathogenesis by causing prolonged exposure of the immune system to nuclear components [2]. Autoantibodies form complexes with nuclear antigens and sequester in target organs; this is particularly evident in the kidney glomeruli [3–5]. Pathogenic antibodies are preferentially deposited here, causing chronic inflammation leading to the development of glomerulonephritis. One of the hallmarks of proliferative lupus nephritis (LN) is mesangial cell proliferation [6,7]. Mesangial cells are the principle immunoregulatory cells in the glomerulus, possessing phagocytic and contractile capabilities [8]. In lupus, mesangial cells have been reported to be hyper-responsive to immune stimulation [9]. IL-6 plays a role in the pathogenesis of mesangial proliferative glomerulonephritis, activating mesangial cells to produce growth factors and cytokines that stimulate extracellular matrix deposition, a pathological characteristic that is upregulated in glomerular diseases [10,11].

In addition to the well-recognized genetic susceptibility to SLE, disease pathogenesis is also influenced by epigenetic factors including microRNAs (miRNAs or miRs) [12,13]. MiRNAs are short, non-coding RNA molecules that regulate gene expression by incomplete binding to the 3' untranslated region (UTR) of target mRNAs. Although the diverse mechanisms of miRNAs remain unclear, the biogenesis of these molecules has been well established [14,15]. One strand of the miRNA – miRNA duplex is preferentially assembled into the RNA-induced silencing complex (RISC), which subsequently acts on its target by translational repression or mRNA cleavage [16,17]. Conversely, miRNAs have also been shown to contribute to the upregulation of genes via stabilization of the target's mRNA or due to alterations in the cell cycle [18,19]. MiRNA-let-7a (let-7a) has gained attention due to its reported regulatory target, the 3' UTR of *IL-6* [20]. Although increased expression of let-7a has been reported in SLE and decreased expression has been reported in certain cancers, the precise outcomes are incompletely understood [21–23].

Short-lived inflammatory mediators such as cytokines have adenylate/uridylate (AU)-rich regions located in the 3' UTR of their mRNA transcripts [24]. Binding to these AU-rich elements (ARE) can activate several RNA decay pathways including decapping of the 5' cap structure, which exposes the body of the transcript to rapid exonucleolytic degradation [24,25]. ARE can be regulated by two classes of molecules: miRNAs and RNA-binding proteins (RBPs). RBPs specifically target AUUUA motifs in the mRNA's 3' UTR. Tristetraprolin (TTP), also known as zinc finger protein 36 (ZFP36), is an RBP that destabilizes mRNAs by binding to the ARE [26]. The outcomes of the direct and indirect interactions between miRNAs and RBPs require further characterization, particularly regarding whether these interactions alter the target gene's expression.

NZB/W mice are an established model used to study human lupus, derived from the first generation cross between New Zealand Black/BinJ (NZB) and New Zealand White/LacJ (NZW) mice. NZW mice do not develop disease, while NZB mice spontaneously develop hemolytic anemia, immune complex deposition of the IgG isotype, and excessive

lymphoreticular proliferation [27]. Female NZB/W mice show characteristics similar to human lupus including high titers of anti-nuclear antibodies, immune complex deposition, and proliferative glomerulonephritis [28,29]. Phenotypic disease typically begins to develop in the females of this strain around 20 weeks of age. Substantial pathologic changes become evident around 30 weeks of age. Mice 36 weeks of age and older show severe renal disease [30].

In this study, mesangial cell miRNAs were isolated from 8- and 32-week-old NZB/W mice to examine alterations in let-7a expression that may contribute to the production of IL-6 in the pre-diseased or diseased state. To examine further how epigenetics can influence inflammatory mediator production in SLE, the role of let-7a in inflammation was investigated using *in vitro* techniques.

Materials and methods

Animals

Female NZB/W and NZW mice were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were used in accordance with the Institutional Animal Care and Use Committee of Virginia Polytechnic Institute and State University (Virginia Tech) and housed in the AAALAC-accredited animal facility at the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM).

Isolation of mesangial cells

Eight- and 32-week-old NZB/W mice ($n = 5$) and age-matched NZW control mice ($n = 5$) were euthanized and the glomeruli from the mice in each group were pooled for mesangial cell isolation. This procedure was repeated three separate times for each experimental and control group. Briefly, the kidneys were removed and the cortical tissue was pooled and pressed through grading sieves (180, 150, and 75 μm mesh). The cells that were retained on the 75- μm filter was force-pressed through a 21-gauge needle and then pelleted by centrifugation. The cells were resuspended in a tube containing 750 U/mL Worthington type I collagenase solution and gently stirred in a water bath at 37 °C for 20 min [31]. The suspension was then pelleted by centrifugation and resuspended in MACS buffer (Miltenyi Biotec, Bergisch Gladbach, Germany) for magnetic cell separation.

Human and mouse mesangial cells have been shown to possess a surface marker that is unique to the kidney glomerulus: integrin $\alpha 8$ [32]. Its expression remains unchanged in mesangial cells whether the glomerulus is healthy or nephritic [33]. For these reasons, integrin $\alpha 8$ was selected as a target molecule for the identification and isolation of mesangial cells from the mixed cell population of the kidney.

For mesangial cell isolation, the mixed cell population was incubated with rabbit anti-mouse integrin $\alpha 8$ primary Ab (1:50 – Santa Cruz Biotechnologies, Santa Cruz, CA) followed by incubation with goat anti-rabbit IgG magnetic microbeads according to the manufacturer's protocol (Miltenyi Biotec). The cell suspension was applied to a magnetic column placed in the magnetic field of a MACS separator (Miltenyi Biotec) for the positive selection of

mesangial cells. The mesangial cells were resuspended in RNAlater (QIAGEN, Valencia, CA) and stored at -20°C until miRNA isolation.

Isolation of RNA and miRNAs

RNA and miRNAs were isolated using the *mirVana* miRNA isolation kit according to the manufacturer's protocol (Applied Biosystems, Carlsbad, CA). Briefly, the cells were lysed and mixed with acid-phenol: chloroform for organic extraction. The lysate was centrifuged to separate the organic phases. The upper aqueous phase was removed, mixed with 100% ethanol, and transferred onto a filter cartridge. The filtrate (collected by centrifugation) contained the miRNA fraction while the filter contained the RNA fraction depleted of small RNAs. RNA was eluted from the filter using 95°C elution solution. The filtrate containing the miRNAs was mixed with 100% ethanol, transferred to a second filter cartridge, and spun by centrifugation to collect the miRNAs on the filter. MiRNAs were eluted with 95°C elution solution. The eluates were quantified on a spectrophotometer (Nanodrop, Thermo Scientific, Waltham, MA). An aliquot was taken and diluted to $1\text{ ng}/\mu\text{L}$ for real-time RT-PCR. The eluted RNA and miRNAs were stored at -80°C .

Real-time RT-PCR: post-miRNA isolation

Let-7a expression was measured by real-time RT-PCR using TaqMan miRNA assays according to the manufacturer's protocol (Applied Biosystems). The RT master mix was combined with $5\text{ }\mu\text{L}$ of $1\text{ ng}/\mu\text{L}$ miRNA template. The negative control received $5\text{ }\mu\text{L}$ of nuclease-free water. RT was performed in an iCycler (BioRad, Hercules, CA) using the following parameter values: 16°C for 30 min, 42°C for 30 min, 85°C for 5 min, and 4°C until the thermal cycler was unloaded. The RT product was stored at -20°C until quantitative PCR was performed.

The TaqMan Small RNA Assays were used according to the manufacturer's protocol (Applied Biosystems). The following PCR parameters were used: 95°C for 10 min (1 cycle) and 95°C for 15 s/ 60°C for 60 s (40 cycles). Relative gene quantitation was determined using the comparative C_T ($-C_T$) method [34]. The C_T was calculated using the endogenous control snoRNA202 and the C_T was determined by calculating the fold change in let-7a expression between NZB/W and NZW mice. All samples were run in triplicate.

Bioinformatics analysis

A database search for all of the 3' UTR mRNA binding sites for let-7a was performed using the programs PicTar (www.pictar.org) and miRanda (www.microRNA.org). These databases showed that a predicted target site of let-7a is the 3' UTR of *IL-6*.

Plasmids

The pMIR-REPORT empty vector (Ambion, Austin, TX) was propagated using *Escherichia coli* (*E. coli*) and isolated using a QIAprep spin miniprep kit according to the manufacturer's protocol (QIAGEN). The pMIR-REPORT miRNA expression reporter vector system contained firefly luciferase under the control of a mammalian promoter with a miRNA target cloning region downstream of the luciferase translation sequence. The mouse *IL-6* 3' UTR

was amplified and cloned into the vector by Genewiz (South Plainfield, NJ) and designated pMIR-IL6-Intact. The 3' UTR region was determined using the National Center for Biotechnology Information (NCBI) nucleotide database (reference sequence NM_031168.1, 420 bases, position 668 to 1087). To generate the miRNA target site deletion mutant (pMIR-IL6-Mut), the 16 bases that constitute the let-7a target recognition sequence (position 975 to 990) were removed from the sequence, which was then amplified and cloned into the vector. The plasmids were transformed into competent *E. coli* cells and propagated and isolated as described above. All samples were run in triplicate.

Cell culture and transfection

A mouse mesangial cell line (MES 13) that is transgenic for SV40 was purchased from ATCC (Manassas, VA). The cells were grown in 75-mm² culture flasks at 37 °C in 5% CO₂ in a 3:1 mixture of DMEM and Ham's F12 medium with 14 mM HEPES, supplemented with 5% FBS and 1% penicillin-streptomycin solution (Cellgro, Manassas, VA). For serum-starving medium, FBS was absent from the complete growth medium. For immune stimulation, LPS (Sigma-Aldrich, St. Louis, MO) and IFN- γ (Cedarlane Laboratories Limited, Burlington, NC) were added to the complete medium at a final concentration of 1 μ g/mL and 100 ng/mL, respectively. Cells were treated with 10 μ g/mL Actinomycin D (ActD) for 1, 2, or 3 h post-stimulation (Sigma-Aldrich). Experiments were performed from passages 9–12. All experimental conditions were run in triplicate.

The cells were transfected with miRNAs using *TransIT*-siQUEST transfection reagent according to the manufacturer's protocol (Mirus, Madison, WI). The cells were serum-starved for 2 h prior to transfection at which point they were transfected with the miRIDIAN let-7a mimic or hairpin inhibitor (Dharmacon RNAi Technologies, Lafayette, CO). The final concentration of the miRNAs was 25 nM unless otherwise noted. Non-transfected controls received complete growth medium only. The plates were incubated for 24 h at 37 °C at which time the media was removed and replaced with stimulating medium. Non-stimulated controls received complete growth medium. MiRIDIAN miRNA mimic and hairpin inhibitor positive and negative controls were used (Dharmacon). These non-targeting controls are based on the miRNA-67 sequence found in *Caenorhabditis elegans* (*C. elegans*), which has minimal sequence identity with miRNAs in mice. TTP small interfering RNA (siRNA) and si-GENOME control #1 were used in the siRNA experiments (Santa Cruz Biotechnologies). The non-targeting siRNA control has no gene targets in mouse cells. Cells and supernatants were collected for analysis 24 h post-stimulation. RNA and miRNAs were isolated as described above.

For DNA transfections, cells were serum-starved for 2 h prior to transfection. The reporter plasmids were transfected into the cells using Effectene transfection reagent according to the manufacturer's protocol (QIAGEN). RNA transfections were performed simultaneously (as described above) in order to increase or decrease intracellular let-7a levels. Non-transfected controls received complete growth medium only. These experiments were performed separately with or without immune stimulation 24 h post-transfection. Non-stimulated controls received complete growth medium. The cell lysates were collected 48 h post-transfection and stored at –80 °C.

Cell viability

Cell viability was measured using a Vi-Cell (Beckman Coulter, Brea, CA) using the trypan blue dye exclusion method. The trypsin was neutralized with equal parts complete growth medium and the cell suspensions were loaded into Vi-Cell sample cups and analyzed. All samples were run in duplicate.

Luciferase assay

The activities of firefly (*Photinus pyralis*) and sea pansy (*Renilla reniformis*) luciferases were measured sequentially using the dual-luciferase reporter assay system according to the manufacturer's protocol (Promega Corporation, Madison, WI). After the normalization of protein, samples were loaded onto a high-quality opaque 96-well microplate in duplicate and measured on a Veritas microplate luminometer (Turner BioSystems, Inc., Sunnyvale, CA). The intact or mutant plasmid co-transfected with a non-targeting control miRNA (miR-67) served as the negative control. The relative luciferase activity was determined by first normalizing the expression relative to *Renilla* luciferase and then by setting the relative transcriptional activity of either plasmid (either pMIR-IL6-Intact or pMIR-IL6-Mut) to 1.

Real-time RT-PCR: post-transfection

Let-7a was measured as described above. *IL-6*, *IL-10*, and *TTP* expression were measured using TaqMan Gene Expression assays (Applied Biosystems). The RT master mix was mixed with 10 μ L of 1 ng/ μ L RNA template. The negative control received 10 μ L of nuclease-free water. RT was performed in an iCycler using the following parameter values: 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min, and 4 °C until the thermal cycler was unloaded. The RT product was stored at -20 °C until PCR was performed as described above. The C_T was calculated using the endogenous controls snoRNA202 or GAPDH (for miRNAs or RNA samples, respectively), and then the C_T was determined by calculating the fold change in expression between the transfected samples and the controls. All samples were run in triplicate.

ELISA

IL-6 and IL-10 protein levels in the cell supernatants were measured by ELISA according to the manufacturer's protocol (eBioscience, San Diego, CA). The plate was read at 450 nm on a Spectramax 340PC microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). All samples were run in duplicate.

Determination of mRNA half-life

The half-life ($t_{1/2}$) of *IL-6* was measured using the first-order kinetics equation $t_{1/2} = 0.693/k$, where k is the rate constant for mRNA decay determined by the slope of a semi-logarithmic plot of the concentration of mRNA over time (slope = k) [35]. The data from 3 independent experiments were taken to calculate *IL-6* $t_{1/2}$.

Statistical analysis

Statistical analysis was performed using Student's unpaired t -test (two-tailed). p values less than 0.05 were considered statistically significant.

Results

MiRNA-let-7a is increased in the mesangial cells of NZB/W mice

We isolated mesangial cells from young, pre-diseased (8-week-old) and actively diseased (32-week-old) NZB/W mice as well as age-matched, parental NZW mice. In preliminary studies, we performed microarray analyses on the isolated mesangial miRNAs. We chose to measure the relative expression of let-7a over time due to its reported involvement in inflammatory mediator production [20]. Prior reports have shown that let-7a is significantly upregulated in the kidneys of LN patients compared to healthy controls [21]. Let-7a is also significantly overexpressed in Treg cells of MRL/*lpr* mice compared to non-autoimmune mice [22]. Real-time PCR revealed that let-7a is significantly increased in pre-diseased and diseased NZB/W mice compared to age-matched controls (Figure 1A). Furthermore, there was no significant change in let-7a expression as the mice aged. These data suggest let-7a may be abnormally expressed in the mesangial cells of NZB/W lupus mice.

Let-7a is increased in stimulated mesangial cells

To examine further the expression of let-7a in inflammation, we stimulated cultured mesangial cells with LPS and IFN- γ . With LPS/IFN- γ stimulation, let-7a expression increased significantly (Figure 1B). Transfection of the let-7a inhibitor significantly reduced let-7a expression, indicating that transfection of the inhibitor was able to reduce the expression induced by LPS/IFN- γ stimulation. When the let-7a mimic was transfected, the relative intracellular concentration of let-7a was increased 15-fold. Non-targeting controls did not alter the relative expression of let-7a (data not shown). To verify that the transfection studies were not altering let-7a due to the induction of cell death, we performed a viability study using various concentrations of the let-7a mimic, let-7a inhibitor, non-targeting inhibitor control (NCi, Figure 1C), and non-targeting mimic control (NC, Figure 1D). We found that transfection of any miRNA at a final concentration up to 100 nM did not induce cell death. Due to its negligible effects on cell viability and its potent effect on intracellular concentrations, miRNAs were transfected to reach a final concentration of 25 nM unless otherwise noted.

IL-6 is a target of let-7a

To identify potential regulatory targets of let-7a, all known mouse mRNA 3' UTRs were scanned for putative binding regions of let-7a using miRanda-based computational ranking systems [36,37]. Two independent software programs predicted that several pro- and anti-inflammatory cytokines, including IL-6 and IL-10, may be regulated by let-7a. The 3' UTR of *IL-6* contains a complimentary binding site for let-7a (Figure 2A) and this miRNA has been shown to directly target *IL-6* [17].

We sought to determine if the 3' UTR of *IL-6* is indeed targeted by let-7a. The 3' UTR of *IL-6* was cloned into a firefly luciferase construct (designated pMIR-IL6-Intact). The 16 base pair binding site for let-7a was deleted in the mutant construct (designated pMIR-IL6-Mut). Either the intact or the mutant plasmid was co-transfected into cultured mesangial cells alone (untreated) or simultaneously with the let-7a mimic (let-7a), inhibitor (let-7a inhibitor), non-targeting mimic control (NC), or non-targeting inhibitor control (NCi). The

experiments were performed with or without immune stimulation in order to determine if there were any differences in let-7a binding due to inflammatory stimulation. Luminescence was measured by a dual-luciferase reporter assay system.

Overall, co-transfection with miRNAs caused a decrease in baseline luciferase activity. This may be due to decreased plasmid transfection efficiency when co-transfected with miRNAs. In non-stimulated cells co-transfected with the intact plasmid and let-7a mimic, luciferase activity significantly decreased (Figure 2B). Luciferase activity remained relatively unchanged when the cells were transfected with the intact plasmid and let-7a inhibitor or either non-targeting control. Additionally, relative transcriptional activity was unaltered when the deletion mutant (pMIR-IL6-Mut) was co-transfected with the let-7a mimic, inhibitor, or either non-targeting control.

Intriguingly, luminescence was unchanged relative to the control when the cells were immune-stimulated after co-transfection with the intact plasmid and let-7a mimic (Figure 2C). The decreased luminescence in the non-stimulated cells co-transfected with the intact plasmid and the let-7a mimic indicates the 3' UTR of *IL-6* is a direct target of let-7a. When let-7a is transfected with the intact plasmid in stimulated cells, unchanged luciferase activity indicates that let-7a does not cause degradation by binding to the 3' UTR of *IL-6*. This suggests that in immune-stimulated cells, let-7a does not bind to *IL-6* to induce degradation.

Let-7a expression enhances immune-stimulated IL-6 production

To verify *IL-6* is a target of let-7a, we determined IL-6 protein production by ELISA after transfection with the let-7a inhibitor, mimic, or non-targeting control (NC). IL-6 production was negligible in non-stimulated cells (Figure 3A) or cells transfected with the let-7a inhibitor, mimic, or NC alone (data not shown). When the mesangial cells were stimulated with LPS/IFN- γ , IL-6 levels were elevated. When the stimulated cells were transfected with the let-7a inhibitor or non-targeting control, IL-6 protein production was not significantly affected compared to stimulation alone. When the stimulated cells were transfected with the let-7a mimic, IL-6 cytokine production was significantly increased compared to LPS/IFN- γ -only stimulation. *IL-6* expression was also significantly increased when the stimulated cells were transfected with the let-7a mimic (Figure 3B). Taken together, these results suggest let-7a is involved in the upregulation of IL-6 in an immune-stimulated environment.

To verify the functionality of the transfected miRNAs, IL-10, another reported regulatory target of let-7a, was measured post-transfection [38] (Figure S1). Compared to the stimulated control, IL-10 protein production was not significantly altered after stimulated cells were transfected with the non-targeting control (Figure 3C). When the stimulated cells were transfected with the let-7a inhibitor, IL-10 production was increased compared to LPS/IFN- γ -only stimulation. When the stimulated cells were transfected with the let-7a mimic, IL-10 cytokine production was significantly decreased compared to the stimulated control. As predicted, *IL-10* was increased after transfection with the let-7a inhibitor and significantly decreased after transfection with the let-7a mimic (Figure 3D). These data indicate that let-7a regulates IL-10 production and the transfected miRNAs are functional.

To examine if let-7a increases *IL-6* expression, cultured mesangial cells were transfected with the let-7a mimic or non-targeting control (NC) for 24 h at which time they were stimulated with LPS/IFN- γ for 6 h. Fresh medium was added with the transcription inhibitor ActD (10 μ g/mL) for 1, 2, or 3 h to block transcription. The overall levels of *IL-6* were higher in the stimulated cells transfected with let-7a compared to the non-targeting control (Figure 3E). After 1 h of ActD treatment, there was an approximate 40% reduction of *IL-6* in let-7a-transfected cells compared to the baseline levels. There was a 50% reduction of *IL-6* 2 h post-ActD treatment compared to the 1-h treatment. By 3 h of ActD treatment, *IL-6* expression was similar to that of the non-targeting control. Stimulated cells transfected with let-7a did not have a longer *IL-6* $t_{1/2}$ compared to the control (Figure 3F). After 3 h of ActD treatment, less than 10% of *IL-6* remained in the stimulated cells transfected with the let-7a mimic, while about 35% remained in cells treated with the non-targeting control. This suggests that in the presence of increased let-7a and the cessation of transcriptional activity, *IL-6* was degraded faster, consistent with the direct targeting of the 3' UTR of *IL-6* by let-7a.

Let-7a cooperates with TTP to upregulate IL-6 production

To explore the mechanism by which let-7a upregulates *IL-6* production, we examined the RNA-binding protein TTP in order to determine if let-7a and TTP jointly affect the production of *IL-6*. Like let-7a, TTP targets the 3' UTR of *IL-6* but its binding sites are in the adenylate/uridylylate (AU)-rich elements (ARE) [39]. When si-TTP was transfected into cultured mesangial cells, *TTP* expression was significantly reduced, indicating the inhibitor was able to knock down its expression (Figure 4A).

When TTP was silenced in stimulated mesangial cells, *IL-6* was significantly upregulated, demonstrating that *IL-6* is a target of TTP (Figure 4B). Although transfection of si-TTP and the control miRNA (miR-control) significantly increased *IL-6* expression (Figure 4C), *IL-6* protein production was significantly decreased (Figure 4D). As expected, *IL-6* expression and protein production increased in stimulated cells that were co-transfected with let-7a and the si-control. When let-7a and si-TTP were co-transfected, both *IL-6* expression and *IL-6* production returned to baseline levels (Figure 4C and D, respectively). This indicates that *IL-6* expression can be regulated by let-7a and TTP.

TTP was measured in stimulated mesangial cells that were transfected with let-7a. By 6 h of stimulation, *TTP* expression was significantly increased compared to the negative control (Figure 4E). After 12 h of stimulation, *TTP* expression had increased over 8-fold. This demonstrates that let-7a increases *TTP* expression in stimulated mesangial cells.

Discussion and conclusions

MiRNAs have gained appreciation as contributors to diverse physiological and pathophysiological functions including cell cycle control, cell development, and carcinogenesis; they may regulate up to 30% of all human mRNAs [40–42]. Altered miRNA expression profiles, found in many autoimmune diseases, have been identified in peripheral blood cells of SLE patients and to contribute to T cell autoreactivity in SLE [43–48]. Let-7a expression was recently shown to be upregulated in renal biopsies of LN patients as well as the Treg cells in lupus mice [21,22]. Although miRNAs are well-recognized for their

repressive action, they have also been shown to upregulate translation by various mechanisms [19,49,50]. The induction of inflammation using LPS and IFN- γ has potent mitogenic ability that may alter the effect miRNAs have on their targets. LPS has been shown to impact the expression of several miRNAs including miR-132, miR-146, miR-155, and miR-let-7a as indicated in our current studies [51]. It was recently shown that let-7a promoter activity is induced in response to LPS-stimulated NF- κ B activation [52].

The aim of these studies was to examine how epigenetics can influence inflammatory mediator production in SLE. We sought to determine if let-7a expression in mesangial cells is altered in NZB/W lupus mice compared to the non-lupus parental strain in either the pre-diseased or the diseased state. We isolated miRNAs from mesangial cells of 8- and 32-week-old NZB/W mice and compared the expression of let-7a to those of age-matched control mice. We found an increase in let-7a expression before clinical, pathologic disease was evident that remained elevated during active disease. Increased let-7a expression in the pre-diseased and diseased state may contribute to the increase in IL-6 production in young and old NZB/W mice. Using *in vitro* techniques, we demonstrated that let-7a has a key role in regulating *IL-6* expression. These data suggest an intrinsic defect in let-7a expression that may predispose lupus mice to increased inflammatory mediator production with immune stimulation. Increased let-7a expression in the mesangial cells of NZB/W mice can contribute to the increase in *IL-6* expression that is characteristic of SLE [53].

Our data suggest that ActD suspends the transcription of factors including NF- κ B that may be essential for let-7a to enhance IL-6 production. Let-7a induces more LPS/IFN- γ -stimulated NF- κ B production, resulting in increased IL-6. When transcriptional activity is blocked by ActD administration, let-7a quickly reduces *IL-6* expression. Our studies show that while let-7a does not stabilize *IL-6*, it acts to enhance immune-stimulated IL-6 production. Thus, it seems that let-7a upregulates *IL-6* expression due to unknown mechanisms, but targets *IL-6* for degradation when transcriptional activity is inhibited.

While the exogenous delivery of let-7a significantly increased IL-6 production in immune-stimulated mesangial cells, the inhibition of let-7a did not reduce the expression of IL-6 compared to the stimulated controls. This suggests that although increased let-7a is sufficient to increase IL-6, inhibiting let-7a alone is not sufficient to decrease IL-6 production. If there is an inherent difficulty in reducing *IL-6* expression through one miRNA alone, a potential therapeutic approach to resolve elevated IL-6 levels in SLE may be to target mediators downstream of let-7a signaling. NF- κ B, a major proinflammatory transcription factor, is indirectly targeted by miRNAs at downstream sites in the signaling pathways it activates [54].

Here we provide evidence that let-7a and TTP cooperate in promoting IL-6 production in an immune-stimulated environment. Let-7a and TTP have different binding sites in the 3' UTR of *IL-6*; while let-7a has a single 16 base pair binding site, the 3' UTR of *IL-6* contains 5 scattered AUUUA pentamers, 3 of which have been shown to be essential for targeting TTP [39]. The differences in binding regions may contribute to the divergent effects they have on IL-6 production. The current studies show that silencing TTP increases *IL-6* expression yet

decreases protein production. Whether the knockdown of TTP in these experiments causes a decrease in mRNA stability or a reduction in translation efficiency is currently unknown.

While acknowledging the complexity of the underlying mechanism, other studies that have reported differences between mRNA levels and protein expression have suggested the cause may be discordant kinetics between mRNA induction and protein production [55,56]. The upregulation of IL-6 seen in these experiments may be due to increased *TTP* expression induced by let-7a, although TTP seems to have a minor role. It appears that let-7a increases IL-6 protein levels primarily by a TTP-independent mechanism. Nevertheless, our data indicate that stimulated mesangial cells respond to the knockdown of TTP with decreased IL-6 production. This suggests that IL-6 production is increased by TTP, an increase which may be due to TTP's ability to stabilize proteins. Sanduja et al. demonstrated overexpressing TTP in human cervical carcinoma cells significantly increased p53 levels due to TTP-mediated protein stabilization; this overexpression resulted in the induction of cellular senescence [57]. Given that TTP and let-7a are involved in cell cycle kinetics, modulating their expression may have potential implications on SLE mesangial cell hyperplasia [23,58,59].

Different contexts may explain why let-7 inhibits gene expression in some studies while it upregulates expression in others. The expression of the let-7 family of miRNAs is downregulated upon *Salmonella* infection, promoting IL-6 production in response to infection [38]. The constitutive overexpression of IL-6 increases let-7a expression in malignant epithelial cells, contributing to IL-6-mediated anti-apoptotic survival pathways [20]. Thus, factors such as infection and inflammation not only alter the expression of miRNAs but also modify the effects they have on downstream targets.

The divergent outcomes on IL-6 and IL-10 cytokine production in the current experiments may be due to the differences in the let-7a binding sites in the mRNA's 3' UTR. The 3' UTR of *IL-6* contains 11 direct base pairings with let-7a while the 3' UTR of *IL-10* contains 14 direct pairings. The location of the binding in the UTR differs between the cytokines as well. While let-7a binds near the 3' end of the IL-6 UTR (positions 319–323 of 420 nucleotides), it binds near the 5' end of the IL-10 UTR (positions 36–40 of 702 nucleotides). The location of binding on the miRNA itself is functionally important as well. Brennecke et al. determined there are 2 main groups of pairing sites which depend on whether the pairing occurs at the miRNA 5' or 3' end [60]. The group that pairs to the 5' end is further subdivided into those with good pairing to both 5' and 3' ends of the miRNA (canonical sites) and those with good 5' pairing but with little or no 3' pairing (seed sites). Intriguingly, let-7a belongs to the canonical group in regards to IL-10 but belongs to the seed site group in regards to IL-6. Brennecke et al. suggest that canonical sites are likely to be more effective than other site types because of their higher pairing energy.

Although *TTP* expression in the NZB/W mouse model is currently unknown, studies using cells derived from TTP knockout mice have confirmed TTP is not the sole regulator of inflammatory mediator mRNA stability but have implicated it in the development of autoimmunity [61,62]. TTP knockout mice develop a systemic inflammatory syndrome with severe arthritis and autoimmunity, as well as medullary and extramedullary myeloid

hyperplasia [63]. The development of autoimmunity is predominantly due to the resulting upregulation of TNF- α , an inducer of IL-6 production [64]. The dual role of TTP in post-transcriptional modification may explain why some studies show that the knockdown of TTP results in autoimmunity while our studies demonstrate greater *TTP* expression results in increased *IL-6* expression. It was recently discovered that TTP can change its mode of mRNA regulation from destabilizing to stabilizing depending on its phosphorylation state, revealing alternate post-transcriptional outcomes [65]. Future experiments will examine the expression patterns of *TTP* in NZB/W mice as they develop disease compared to age-matched controls.

The posttranscriptional regulation of IL-6 may play a key role in controlling inflammation through the activation of mesangial cells, B cells, and T cells. In human lupus patients, systemically elevated IL-6 levels are correlated with disease activity and anti-dsDNA levels [66,67]. IL-6 was shown to have a particularly close link with the renal manifestation of SLE due to its increased expression *in situ* along the glomeruli and tubules in lupus nephritis kidneys [68]. In the NZB/W mouse model, it has been demonstrated that B cells from these mice are hyper-responsive to IL-6 *in vitro*, producing anti-DNA antibodies once stimulated [69]. Serum levels of IL-6 are also elevated in this strain [70].

Because of its well-established role in the modulation of SLE, IL-6 is a potential therapeutic target. Administration of antibodies against the IL-6 receptor successfully suppressed the production of anti-dsDNA autoantibodies, reduced the proliferation of B cells, and downregulated anti-CD3-induced T-cell proliferation and mixed lymphocyte reactions in SLE patients [10,71]. MiRNAs may also have therapeutic potential in the treatment of SLE. Polikepahad et al. showed that the inhibition of let-7 miRNAs *in vivo* profoundly inhibited the production of allergic cytokines and the disease phenotype, indicating let-7a may be a potential therapeutic target in other diseases as well [72].

In summary, these results show that the expression of let-7a is significantly increased in pre-diseased and actively diseased NZB/W mice compared to controls and that *IL-6* expression is regulated by let-7a. Increased expression of let-7a may lead to enhanced expression of signaling molecules that induce or maintain *IL-6* expression in immune-stimulated mesangial cells. Furthermore, due to the upregulation of let-7a throughout the lifetime of NZB/W mice, we suggest that inhibition of let-7a in pre-diseased mice will result in improved kidney function as well as an improvement in disease outcome.

References

1. Perl A. Systems biology of lupus: mapping the impact of genomic and environmental factors on gene expression signatures, cellular signaling, metabolic pathways, hormonal and cytokine imbalance, and selecting targets for treatment. *Autoimmunity*. 2010; 43:32–47. [PubMed: 20001421]
2. Oates J, Gilkeson G. Mediators of injury in lupus nephritis. *Curr Opin Rheumatol*. 2002; 14:498–503. [PubMed: 12192244]
3. Weening J, D'Agati V, Schwartz M, Seshan S, Alpers C, Appel G, Balow J, et al. The classification of glomerulonephritis in systemic lupus erythematosus revisited. *J Amer Soc Nephrol*. 2004; 15:241–250. [PubMed: 14747370]

4. Rottman JB, Willis CR. Mouse models of systemic lupus erythematosus reveal a complex pathogenesis. *Veter Pathol.* 2010; 47:664–676.
5. Granholm NA, Cavallo T. Mechanism of localization of immune complexes in NZB/W mice with early nephritis. *Autoimmunity.* 1990; 8:17–24. [PubMed: 2129781]
6. Menè P. Physiology and pathophysiology of the mesangial cell. *Nefrologia.* 1996; 16:8–13.
7. Cove-Smith A, Hendry BM. The regulation of mesangial cell proliferation. *Nephron Exper Nephrol.* 2008; 108:e74–79. [PubMed: 18431073]
8. Seret G, Le Meur Y, Renaudineau Y, Youinou P. Mesangial cell-specific antibodies are central to the pathogenesis of lupus nephritis. *Clin Develop Immunol.* 2012; 8:Article ID 579670.
9. Peairs A, Dai R, Gan L, Shimp S, Rylander MN, Li L, Reilly CM. Epigallocatechin-3-gallate (EGCG) attenuates inflammation in MRL/lpr mouse mesangial cells. *Cell Mol Immunol.* 2010; 7:123–132. [PubMed: 20140007]
10. Tackey E, Lipsky PE, Illei GG. Rationale for interleukin-6 blockade in systemic lupus erythematosus. *Lupus.* 2004; 13:339–343. [PubMed: 15230289]
11. Eitner F, Westerhuis R, Burg M, Weinhold B, Grone HJ, Ostendorf T, Ruther U, et al. Role of interleukin-6 in mediating mesangial cell proliferation and matrix production *in vivo*. *Kidney Int.* 1997; 51:69–78. [PubMed: 8995719]
12. Saal S, Harvey SJ. MicroRNAs and the kidney: coming of age. *Curr Opin Nephrol Hypertens.* 2009; 18:317–323. [PubMed: 19424061]
13. Brooks WH, Le Dantec C, Pers JO, Youinou P, Renaudineau Y. Epigenetics and autoimmunity. *J Autoimmun.* 2010; 34:J207–219. [PubMed: 20053532]
14. Sato F, Tsuchiya S, Meltzer SJ, Shimizu K. MicroRNAs and epigenetics. *FEBS J.* 2011; 278:1598–1609. [PubMed: 21395977]
15. Asirvatham AJ, Magner WJ, Tomasi TB. miRNA regulation of cytokine genes. *Cytokine.* 2009; 45:58–69. [PubMed: 19121586]
16. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. *Nature Rev Genetics.* 2004; 5:522–531. [PubMed: 15211354]
17. Iliopoulos D, Hirsch HA, Struhl K. An epigenetic switch involving NF-kappaB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation. *Cell.* 2009; 139:693–706. [PubMed: 19878981]
18. Ma F, Liu X, Li D, Wang P, Li N, Lu L, Cao X. MicroRNA-466l upregulates IL-10 expression in TLR-triggered macrophages by antagonizing RNA-binding protein tristetraprolin-mediated IL-10 mRNA degradation. *J Immunol.* 2010; 184:6053–6059. [PubMed: 20410487]
19. Vasudevan S, Tong Y, Steitz JA. Switching from repression to activation: microRNAs can up-regulate translation. *Science.* 2007; 318:1931–1934. [PubMed: 18048652]
20. Meng FHR, Wehbe-Janek H, Smith H, Ueno Y, Patel T. The MicroRNA let-7a modulates interleukin-6-dependent STAT-3 survival signaling in malignant human cholangiocytes. *J Biol Chem.* 2007; 282:8256–8264. [PubMed: 17220301]
21. Dai Y, Sui W, Lan H, Yan Q, Huang H, Huang Y. Comprehensive analysis of microRNA expression patterns in renal biopsies of lupus nephritis patients. *Rheumatol Int.* 2008; 29:749–754. [PubMed: 18998140]
22. Divekar AA, Dubey S, Gangalum PR, Singh RR. Dicer insufficiency and microRNA-155 overexpression in lupus regulatory T cells: an apparent paradox in the setting of an inflammatory milieu. *J Immunol.* 2011; 186:924–930. [PubMed: 21149603]
23. Dong Q, Meng P, Wang T, Qin W, Wang F, Yuan J, Chen Z, et al. MicroRNA let-7a inhibits proliferation of human prostate cancer cells in vitro and in vivo by targeting E2F2 and CCND2. *PLoS ONE.* 2010; 5:e10147. [PubMed: 20418948]
24. Chen CY, Shyu AB. AU-rich elements: characterization and importance in mRNA degradation. *Trends Biochem Sci.* 1995; 20:465–470. [PubMed: 8578590]
25. Gao M, Wilusz CJ, Peltz SW, Wilusz J. A novel mRNA-decapping activity in HeLa cytoplasmic extracts is regulated by AU-rich elements. *EMBO J.* 2001; 20:1134–1143. [PubMed: 11230136]

26. Jing Q, Huang S, Guth S, Zarubin T, Motoyama A, Chen J, Di Padova F, et al. Involvement of microRNA in AU-rich element-mediated mRNA instability. *Cell*. 2005; 120:623–634. [PubMed: 15766526]
27. Ryan MJ, McLemore GR Jr, Hendrix ST. Insulin resistance and obesity in a mouse model of systemic lupus erythematosus. *Hypertension*. 2006; 48:988–993. [PubMed: 16982954]
28. Peutz-Kootstra CJ, de Heer E, Hoedemaeker PJ, Abrass CK, Bruijn JA. Lupus nephritis: lessons from experimental animal models. *J Labor Clin Med*. 2001; 137:244–260.
29. Burnett R, Ravel G, Descotes J. Clinical and histopathological progression of lesions in lupus-prone (NZB × NZW) F1 mice. *Exper Toxicol Pathol: Off J Gesellschaft fur Toxikologische Pathologie*. 2004; 56:37–44.
30. Theofilopoulos AN, Dixon FJ. Murine models of systemic lupus erythematosus. *Adv Immunol*. 1985; 37:269–390. [PubMed: 3890479]
31. Mene P, Stoppacciaro A. Isolation and propagation of glomerular mesangial cells. *Meth Mol Biol*. 2009; 466:1–15.
32. Scindia Y, Deshmukh U, Thimmalapura PR, Bagavant H. Anti-alpha8 integrin immunoliposomes in glomeruli of lupus-susceptible mice: a novel system for delivery of therapeutic agents to the renal glomerulus in systemic lupus erythematosus. *Arthritis Rheum*. 2008; 58:3884–3891. [PubMed: 19035491]
33. Hartner A, Schöcklmann H, Pröls F, Müller U, Sterzel RB. Alpha8 Integrin in glomerular mesangial cells and in experimental glomerulonephritis. *Kidney Inter*. 1999; 56:1468–1480.
34. Dai R, Phillips RA, Zhang Y, Khan D, Crasta O, Ahmed SA. Suppression of LPS-induced Interferon-gamma and nitric oxide in splenic lymphocytes by select estrogen-regulated microRNAs: a novel mechanism of immune modulation. *Blood*. 2008; 112:4591–4597. [PubMed: 18791161]
35. Collier J. Methods to determine mRNA half-life in *Saccharomyces cerevisiae*. *Meth Enzymol*. 2008; 448:267–284. [PubMed: 19111181]
36. Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, MacMenamin P, et al. Combinatorial microRNA target predictions. *Nat Genet*. 2005; 37:495–500. [PubMed: 15806104]
37. John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS. Human MicroRNA targets. *PLoS Biol*. 2004; 2:e363. [PubMed: 15502875]
38. Schulte LN, Eulalio A, Mollenkopf HJ, Reinhardt R, Vogel J. Analysis of the host microRNA response to *Salmonella* uncovers the control of major cytokines by the let-7 family. *EMBO J*. 2011; 30:1977–1989. [PubMed: 21468030]
39. Zhao W, Liu M, D'Silva NJ, Kirkwood KL. Tristetraprolin regulates interleukin-6 expression through p38 MAPK-dependent affinity changes with mRNA 3' untranslated region. *J Interfer Cytokine Res Off J Inter Soc Interferon Cytokine Res*. 2011; 31:629–637.
40. Mendell JT. MicroRNAs: Critical regulators of development, cellular physiology and malignancy. *Cell Cycle*. 2005; 4:1179–1184. [PubMed: 16096373]
41. Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer*. 2006; 6:857–866. [PubMed: 17060945]
42. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*. 2005; 120:15–20. [PubMed: 15652477]
43. Dai Y, Huang YS, Tang M, Lv TY, Hu CX, Tan YH, Xu ZM, Yin YB. Microarray analysis of microRNA expression in peripheral blood cells of systemic lupus erythematosus patients. *Lupus*. 2007; 16:939–946. [PubMed: 18042587]
44. Zhao S, Wang Y, Liang Y, Zhao M, Long H, Ding S, Yin H, Lu Q. MicroRNA-126 regulates DNA methylation in CD4(+) T cells and contributes to systemic lupus erythematosus by targeting DNA methyltransferase 1. *Arthritis Rheum*. 2010; 63:1376–1386. [PubMed: 21538319]
45. Hai-yan W, Yang L, Mei-hong C, Hui Z. Expression of MicroRNA-146a in peripheral blood mononuclear cells in patients with systemic lupus erythematosus. *Acta Academiae Medicinae Sinica*. 2011; 33:185–188.
46. Dalal SR, Kwon JH. The role of MicroRNA in inflammatory bowel disease. *Gastroenterol Hepatol*. 2010; 6:714–722.

47. Pauley KM, Cha S, Chan EK. MicroRNA in autoimmunity and autoimmune diseases. *J Autoimmun.* 2009; 32:189–194. [PubMed: 19303254]
48. Tufekci KU, Oner MG, Genc S, Genc K. MicroRNAs and Multiple Sclerosis. *Autoimmune Dis.* 2011; 27:Article ID 807426.
49. Rusk N. When microRNAs activate translation. *Nat Meth.* 2008; 5:122–123.
50. Vasudevan S, Tong Y, Steitz JA. Cell-cycle control of microRNA-mediated translation regulation. *Cell Cycle.* 2008; 7:1545–1549. [PubMed: 18469529]
51. Zhou R, Hu G, Liu J, Gong AY, Drescher KM, Chen XM. NF-kappaB p65-dependent transactivation of miRNA genes following *Cryptosporidium parvum* infection stimulates epithelial cell immune responses. *PLoS Pathogens.* 2009; 5:e1000681. [PubMed: 19997496]
52. Wang DJ, Legesse-Miller A, Johnson EL, Collier HA. Regulation of the let-7a-3 promoter by NF-kappaB. *PLoS ONE.* 2012; 7:e31240. [PubMed: 22348059]
53. Mihara M, Nishimoto N, Ohsugi Y. The therapy of autoimmune diseases by anti-interleukin-6 receptor antibody. *Expert Opin Biol Ther.* 2005; 5:683–690. [PubMed: 15934843]
54. Asirvatham AJ, Gregorie CJ, Hu Z, Magner WJ, Tomasi TB. MicroRNA targets in immune genes and the Dicer/Argonaute and ARE machinery components. *Mol Immunol.* 2008; 45:1995–2006. [PubMed: 18061676]
55. Chege D, Chai Y, Huibner S, McKinnon L, Wachihi C, Kimani M, Jaoko W, et al. Evaluation of a quantitative real-time PCR assay to measure HIV-specific mucosal CD8+ T cell responses in the cervix. *PLoS ONE.* 2010; 5:e13077. [PubMed: 20949096]
56. Collison LW, Pillai MR, Chaturvedi V, Vignali DA. Regulatory T cell suppression is potentiated by target T cells in a cell contact, IL-35- and IL-10-dependent manner. *J Immunol.* 2009; 182:6121–6128. [PubMed: 19414764]
57. Sanduja S, Kaza V, Dixon DA. The mRNA decay factor tristetraprolin (TTP) induces senescence in human papillomavirus-transformed cervical cancer cells by targeting E6-AP ubiquitin ligase. *Aging.* 2009; 1:803–817. [PubMed: 20157568]
58. Al-Haj L, Blackshear PJ, Khabar KS. Regulation of p21/CIP1/WAF-1 mediated cell-cycle arrest by RNase L and tristetraprolin, and involvement of AU-rich elements. *Nucl Acids Res.* 2012; 40:7739–7752. [PubMed: 22718976]
59. Zhou JY, Ma WL, Liang S, Zeng Y, Shi R, Yu HL, Xiao WW, Zheng WL. Analysis of microRNA expression profiles during the cell cycle in synchronized HeLa cells. *BMB Rept.* 2009; 42:593–598.
60. Brennecke J, Stark A, Russell RB, Cohen SM. Principles of microRNA-target recognition. *PLoS Biology.* 2005; 3:e85. [PubMed: 15723116]
61. Jalonen U, Paukkeri EL, Moilanen E. Compounds that increase or mimic cyclic adenosine monophosphate enhance tristetraprolin degradation in lipopolysaccharide-treated murine j774 macrophages. *J Pharmacol Exp Ther.* 2008; 326:514–522. [PubMed: 18469159]
62. Carrick DM, Lai WS, Blackshear PJ. The tandem CCCH zinc finger protein tristetraprolin and its relevance to cytokine mRNA turnover and arthritis. *Arthritis Res Ther.* 2004; 6:248–264. [PubMed: 15535838]
63. Taylor GA, Carballo E, Lee DM, Lai WS, Thompson MJ, Patel DD, Schenkman DI, et al. A pathogenetic role for TNF alpha in the syndrome of cachexia, arthritis, and autoimmunity resulting from tristetraprolin (TTP) deficiency. *Immunity.* 1996; 4:445–454. [PubMed: 8630730]
64. Theofilopoulos AN, Lawson BR. Tumour necrosis factor and other cytokines in murine lupus. *Ann Rheum Dis.* 1999; 58:I49–55. [PubMed: 10577973]
65. Fahling M, Persson AB, Klinger B, Benko E, Steege A, Kasim M, Patzak A, et al. Multi-level regulation of HIF-1 signaling by TTP. *Mol Biol Cell.* 2012; 23:4129–4141. [PubMed: 22918951]
66. Kirou KA, Lee C, Crow MK. Measurement of cytokines in autoimmune disease. *Meth Mol Med.* 2004; 102:129–154.
67. al-Janadi M, al-Balla S, al-Dalaan A, Raziuddin S. Cytokine profile in systemic lupus erythematosus, rheumatoid arthritis, and other rheumatic diseases. *J Clin Immunol.* 1993; 13:58–67. [PubMed: 8445045]
68. Herrera-Esparza R, Barbosa-Cisneros O, Villalobos-Hurtado R, Avalos-Diaz E. Renal expression of IL-6 and TNFalpha genes in lupus nephritis. *Lupus.* 1998; 7:154–158. [PubMed: 9607638]

69. Mihara M, Takagi N, Takeda Y, Ohsugi Y. IL-6 receptor blockage inhibits the onset of autoimmune kidney disease in NZB/W F1 mice. *Clin Exp Immunol.* 1998; 112:397–402. [PubMed: 9649207]
70. Finck BK, Chan B, Wofsy D. Interleukin 6 promotes murine lupus in NZB/NZW F1 mice. *J Clin Invest.* 1994; 94:585–591. [PubMed: 8040314]
71. Liang B, Gardner DB, Griswold DE, Bugelski PJ, Song XY. Anti-interleukin-6 monoclonal antibody inhibits autoimmune responses in a murine model of systemic lupus erythematosus. *Immunology.* 2006; 119:296–305. [PubMed: 17067309]
72. Polikepahad S, Knight JM, Naghavi AO, Oplt T, Creighton CJ, Shaw C, Benham AL, et al. Proinflammatory role for let-7 microRNAs in experimental asthma. *J Biol Chem.* 2010; 285:30139–30149. [PubMed: 20630862]

Figure 1.

Let-7a expression is increased in the mesangial cells of 8- and 32-week-old NZB/W mice compared to age-matched, non-lupus NZW mice. (A) Real-time RT-PCR shows the increased expression of let-7a in the mesangial cells of 8-week-old NZB/W mice compared to age-matched NZW mice. Let-7a expression is significantly increased in the mesangial cells of 32-week-old NZB/W mice compared to age-matched NZW mice. Let-7a expression does not change in NZB/W or NZW mice as they age. (B) Real-time RT-PCR shows let-7a expression is increased in cells after stimulation with LPS and IFN- γ . Let-7a is significantly increased after transfection of the let-7a mimic and is significantly decreased after transfection of the let-7a inhibitor. Non-targeting controls did not alter the relative expression of let-7a (data not shown). (C–D) Cell viability remains unchanged after stimulation alone or stimulation post-transfection of increasing amounts of the let-7a inhibitor (C), mimic (D), or non-targeting controls. (A) represents 3 independent isolations where the glomeruli were pooled ($n = 5$ mice) for mesangial cell isolation. (B–D) represent 3 independent experiments run in triplicate. The non-targeting mimic control (NC) and non-targeting inhibitor control (NCi) are miR-67 and the inhibitor of miR-67, respectively. The final concentration of the miRNAs was 25 nM unless otherwise noted. Error bars represent the SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2.

Let-7a partially binds to the 3' UTR of *IL-6*. (A) The 3' UTR of *IL-6* is a predicted target of let-7a. Vertical lines represent direct base pairing. Colons represent non-Watson-Crick base pairing (or wobble base pairing). (B) Luciferase activity is decreased in non-stimulated mesangial cells after co-transfection with the intact *IL-6* 3' UTR (pMIR-IL6-Intact) and let-7a mimic. Luciferase activity is unchanged when the intact plasmid is co-transfected with the let-7a inhibitor or when the deletion mutant plasmid (pMIR-IL6-Mut) is co-transfected with the let-7a mimic or inhibitor. The non-targeting controls (NC or NCi) have no effect on the relative luciferase activity of pMIR-IL6-Intact or pMIR-IL6-Mut. (C) After immune stimulation, relative luciferase activity is unchanged relative to the control upon transfection

with pMIR-IL6-Intact and the let-7a mimic. The final concentration of the inhibiting or mimicking miRNAs was 25 nM. The non-targeting mimic control (NC) and non-targeting inhibitor control (NCi) are miR-67 and the inhibitor of miR-67, respectively. (B–C) represent 3 independent experiments run in duplicate. Error bars represent the SEM. * $p < 0.05$.

Figure 3.

Exogenous delivery of let-7a increases IL-6 production in cultured mesangial cells. (A) Transfection of the let-7a mimic significantly increases IL-6 production relative to the stimulated control. Transfection of either the non-targeting control or the let-7a inhibitor has no effect on the production of IL-6. (B) Real-time RT-PCR shows the levels of *IL-6* are significantly increased after transfection of the let-7a mimic. The inhibition of let-7a produces levels of *IL-6* relative to the expression seen in the stimulated controls. The non-stimulated control produces undetectable levels of *IL-6* (data not shown). (C) Transfection of the non-targeting control has no effect on the production of IL-10. IL-10 protein production increases after transfection of the let-7a inhibitor. Transfection of the let-7a mimic significantly decreases IL-10 production relative to the stimulated control. (D) Real-time RT-PCR shows the levels of *IL-10* are increased after transfection of the let-7a inhibitor. Transfection of the let-7a mimic significantly decreases IL-10 expression compared to the stimulated control. Transfection of any miRNA without stimulation produces levels of *IL-10* comparable to the control (data not shown). (E) Mesangial cells transfected with the let-7a mimic, stimulated with LPS/IFN- γ for 6 h, and treated with ActD for 1, 2, or 3 h show a significant decrease in relative *IL-6* expression over time compared to the non-targeting control. (F) The $t_{1/2}$ of *IL-6* is not significantly longer in let-7a-transfected cells compared to the non-targeting control. The final concentration of the inhibiting or mimicking miRNAs was 25 nM. The non-targeting control (NC) is miR-67. The non-targeting inhibitor control (NCi) does not have a significant effect on any experimental condition (data not shown). (A–F) represent 3 independent experiments run in duplicate (A, C) or triplicate (B, D–F). Error bars represent the SEM. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$.

Figure 4.

Let-7a upregulates IL-6 production through cooperation with TTP. (A) Real time RT-PCR shows *TTP* expression is significantly decreased after transfection of si-TTP compared to the non-targeting control. (B) *IL-6* expression is significantly increased after transfection of si-TTP compared to the non-targeting control. (C) *IL-6* expression is significantly increased when mesangial cells are transfected with either let-7a or si-TTP compared to the non-targeting control. When the cells are co-transfected with let-7a and si-TTP, relative *IL-6* expression returns to the expression seen in the stimulated control. (D) IL-6 production is significantly increased when mesangial cells are transfected with let-7a. IL-6 production is significantly decreased after transfection of si-TTP. When the cells are co-transfected with let-7a and si-TTP, IL-6 production returns to the levels seen in the stimulated control. (E) *TTP* expression is significantly increased in let-7a-transfected mesangial cells 6 h post-stimulation and continues to be upregulated after 12 h of stimulation. The final concentration of the miRNAs or siRNAs was 25 nM. The non-targeting miRNA control (NC) is miR-67. The non-targeting siRNA control (si-control) has no gene targets in mouse cells. (A–E) represent 3 independent experiments run in triplicate (A–C, E) or duplicate (D). Error bars represent the SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.