

MicroRNA-mediated Attenuation of Inflammation in NZB/W Lupus Mice

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Abstract

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production and deposition of nuclear self-antigen-containing immune complexes. Epigenetic factors, including altered microRNA (miRNA) expression, may contribute to aberrant immune cell function in SLE. miRNAs are small, noncoding RNAs that bind to the 3' untranslated region of target mRNAs resulting in post-transcriptional gene modulation. *IL-6*, an inflammatory cytokine overproduced by mesangial cells in SLE, contains a potential binding site for miR-let-7a. In order to examine if alterations in miR-let-7a expression can influence inflammatory mediator production in SLE, we isolated mesangial cell miRNAs from 8 and 32-week-old female New Zealand Black/White (NZB/W) mice. We found miR-let-7a expression was significantly increased in the mesangial cells of pre-diseased and actively diseased NZB/W mice compared to age-matched female New Zealand White (NZW) controls. Overexpression of miR-let-7a *in vitro* increased IL-6 production in LPS/IFN- γ -stimulated mesangial cells compared to the stimulated control. Due to the crucial role of miR-let-7a in cell division and inflammation, we investigated miR-let-7a-mediated proliferation and NF κ B activation in J774A.1 macrophages and MES 13 mesangial cells *in vitro*. Cell proliferation, retinoblastoma protein (Rb) phosphorylation, and NF κ B activation were increased in cells transfected with miR-let-7a and stimulated with LPS/IFN- γ . Expression and production of the cell cycle inhibitor E2F5 was decreased in stimulated cells overexpressing miR-let-7a. We found that the cell cycle promoter E2F2 and NF κ B target the miR-let-7a promoter. Next we sought to determine alterations in

specific disease-associated miRNAs in female NZB/W mice treated with hydroxychloroquine (HCQ) or prednisone (PRED) for 12 weeks beginning at 20 weeks-of-age. We found that treatment with HCQ or PRED induced unique changes to miRNA expression in multiple tissues. In order to identify specific miRNAs as disease-modifying agents and not merely disease correlates, further *in vitro* analyses confirmed HCQ or PRED-mediated inhibition of miRNAs is critical to alter the inflammatory response. Taken together, our results suggest that overexpression of miR-let-7a may contribute to hyperplasia and the proinflammatory response in SLE. Our studies indicate that lupus therapeutics may work, in part, by altering the expression of disease-associated miRNAs in immune cells and the urine.

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Attribution

Several colleagues contributed to the research and manuscripts presented in this dissertation by assisting with the experimental design, data collection/interpretation, and editing. A brief description of their contributions is included here.

Chapter 2: MicroRNA-let-7a expression is increased in the mesangial cells of NZB/W mice and increases IL-6 production *in vitro*

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Chapter 3: MicroRNA-let-7a promotes NFκB activation and E2F-mediated cell proliferation *in vitro*

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Chapter 4: Cellular and urinary microRNA alterations in NZB/W mice with hydroxychloroquine or prednisone treatment

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Chapter 1: Literature Review

MicroRNAs implicated in the immunopathogenesis of lupus nephritis

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1.2. Abstract

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the deposition of immune complexes due to widespread loss of immune tolerance to nuclear self-antigens. Deposition in the renal glomeruli results in the development of lupus nephritis (LN), the leading cause of morbidity and mortality in SLE. In addition to the well-recognized genetic susceptibility to SLE, disease pathogenesis is influenced by epigenetic regulators such as microRNAs (miRNAs). miRNAs are small, noncoding RNAs that bind to the 3' untranslated region of target mRNAs resulting in post-transcriptional gene modulation. miRNAs play an important and dynamic role in the activation of innate immune cells and are critical in regulating the adaptive immune response. Immune stimulation and the resulting cytokine milieu alter miRNA expression while miRNAs themselves modify cellular responses to stimulation. Here we examine dysregulated miRNAs implicated in LN pathogenesis from human SLE patients and murine lupus models. The effects of LN-associated miRNAs in the kidney, peripheral blood mononuclear cells, macrophages, mesangial cells, dendritic cells, and splenocytes are discussed. As the role of miRNAs in immunopathogenesis becomes delineated, it is likely that specific miRNAs may serve as targets for therapeutic intervention in the treatment of LN and other pathologies.

1.3 Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the loss of immune tolerance to nuclear self-antigens. The deposition of autoantibodies along the glomerular basement membrane results in immune complex (IC)–mediated glomerulonephritis (1). Mesangial cells, the primary immunoregulatory cells in the renal glomerulus, become activated due to the deposition of ICs. This recruits macrophages, B cells, T cells, and dendritic cells (DCs) to the kidney. Activated macrophages, mesangial cells, and DCs induce the maturation and activation of infiltrating T cells, which further activate macrophages and increase the B cell response (2). Lupus nephritis (LN) is the major cause of morbidity and mortality in patients with SLE, affecting up to 70% of SLE patients (3). Histological features include increased numbers of mesangial cells, overproduction of extracellular matrix, and inflammatory cell infiltration, which can lead to the development of sclerosis and fibrosis (4). Depending on the severity of disease, 10–30% of LN patients will progress to end-stage renal failure (5).

It has been shown that genetic predisposition coupled with known and unknown environmental factors contribute to the development of SLE (6, 7). Epigenetic defects have also been shown to play an important role in LN pathogenesis (8-10). Epigenetics, which includes microRNA (miRNA) regulation, refers to stable and heritable changes in gene expression that alter the phenotype but not the underlying DNA sequence itself. miRNAs are small, noncoding RNAs that endogenously regulate gene expression by partially binding to the 3' untranslated region (UTR) of target mRNAs (11-14). miRNAs contribute to diverse physiological and pathophysiological functions including cell developmental timing, cell cycle control, apoptosis, and carcinogenesis (15, 16). Hematopoiesis is fine-tuned by miRNAs at virtually every step (17). In the last decade, increasing evidence has shown that miRNAs are critical not only for the

regulation of immune cell development but also for modifying innate and adaptive immune responses (18).

A computational analysis performed on 72 lupus susceptibility genes in humans and mice revealed most lupus susceptibility genes contain numerous target sites for over 140 conserved miRNAs. Three miRNAs (miR-181, miR-186, and miR-590-3p) are predicted to target over 50% of all lupus susceptibility genes (19). Several studies have suggested that miRNAs play a role in the pathogenesis of LN by altering proinflammatory mediator production, innate immune cell responses, lymphocyte function, and Toll-like receptor (TLR) and NF κ B signaling pathways (20-24). For example, miRNAs can induce the expression of proinflammatory cytokines, dictating the magnitude and duration of the immune response (25, 26). miRNA dysregulation can result from genetic variation, hormonal influences, environmental triggers, or even the proinflammatory environment itself (27). Lipopolysaccharide (LPS) induces the expression of miRNAs and activates transcription factors that further regulate miRNA expression (28, 29). LPS has been shown to induce the expression of several miRNAs including miR-9, miR-132, miR-146, miR-155, and miR-let-7a (let-7a) (30-33). Dysregulated miRNA expression may represent an underlying trigger that induces multifactorial diseases such as SLE.

As pathogenic miRNAs are identified in LN pathogenesis, treatment strategies aimed at altering miRNA expression or signaling pathways may be employed to ameliorate disease pathogenesis in patients with SLE. Determining a patient's miRNA expression profile from the blood or urine may allow the use of targeted therapies to specifically modulate abnormal miRNA expression patterns in individuals suffering from lupus. It has been well-documented that lupus patients respond to immunosuppressive agents with varying degrees of efficacy (34). This has presented a major challenge in selecting the most effective treatment option. Determining how

particular therapeutics alter pathogenic miRNAs may ultimately provide a viable screening tool for specific, targeted therapy in SLE. In this review, we summarize the current data on miRNAs in the major immune cells as related to LN pathogenesis and examine the future directions in miRNA-based therapy for SLE.

1.4. miRNAs Broadly Implicated In Inflammatory Diseases

1.4.1. miR-21. miR-21 is induced upon inflammatory stimulation and is a key component of TLR, NFκB, and signal transducer and activator of transcription (STAT) signaling pathways (35-37). The 3' UTR of *programmed cell death 4 (PDCD4)* is a direct target of miR-21. PDCD4 is a proinflammatory protein that promotes NFκB activation and suppresses production of the anti-inflammatory cytokine IL-10. Overexpression of miR-21 in LPS-stimulated murine macrophages blocked NFκB activity, decreased PDCD4 production, and promoted the production of anti-inflammatory IL-10. PDCD4-deficient mice are protected from LPS-induced death, presumably by an IL-10-mediated reduction in NFκB activation (38).

miR-21 has been implicated in the immunopathogenesis of numerous inflammatory diseases (39). Using an *in vitro* model of diabetes, Kato et al. showed that miR-21 overexpression in glucose-stimulated mesangial cells prevented cell proliferation by downregulating *tumor suppressor phosphatase and tensin homolog (PTEN)*, whose 3' UTR contains a binding site for miR-21 (40, 41). miR-21 expression is induced by STAT3, a transcription factor activated by IL-6. miR-21 inhibition of PTEN leads to increased NFκB activation that is required to maintain the transformed state. miR-21, PTEN, NFκB, IL-6, and STAT3 are dynamic players in the positive feedback loop linking inflammation to cancer (35).

1.4.2. miR-146a. miR-146a may contribute to lineage determination in T cells as it is one of the only miRNAs that is differentially expressed in highly purified subsets of murine Th1 and Th2 cells (42). Lu et al. demonstrated that miR-146a knockout mice develop a fatal immune-mediated disease similar to Foxp3 knockout mice that are devoid of functional Treg cells (43). Although miR-146a knockout mice have increased Treg cells, their suppressive function is impaired. Treg cells without miR-146a (or Foxp3) acquire the ability to produce proinflammatory cytokines such as interferon- γ (IFN- γ). miR-146a deficiency in Treg cells caused an increase in STAT1 production, a key transcription factor required for Th1 effector cell differentiation. Because miR-146a regulates Treg cell suppressor function, the authors suggest that miR-146a maintains an optimal threshold of cytokine receptor-dependent activation of transcription factors that are necessary to suppress Th1 responses (43).

Since miR-146a has been reported to be an important negative regulator of acute responses during the activation of innate immunity, it has been suggested to play a regulatory role in the pathogenesis of SLE. miR-146a is induced by TLR activation (via LPS stimulation) and by proinflammatory mediators including tumor necrosis factor- α (TNF- α) and IFN- α (30). miR-146a negatively regulates type I IFN production and myeloid differentiation factor 88 (MyD88) pathway activation induced by TLR stimulation (44). Upon LPS-stimulated induction, miR-146a directly decreases TRAF6 and IRAK1 production, two signal transducers in the NF κ B activation pathway whose 3' UTRs contain multiple miR-146a target sequences (30, 45). Therefore, miR-146a reduces or terminates the inflammatory response through a negative-feedback loop by downregulating *TRAF6* and *IRAK1*.

Due to its importance in the control of inflammation, several studies have sought to determine if miR-146a-based therapy can improve disease outcome in lupus-prone animal

models or human patients. It was recently shown that treatment with the anti-inflammatory drug calcitriol alters the expression of miR-146a in SLE patients. Sera miR-146a expression, which is downregulated in patients with SLE, was significantly increased in patients after treatment with calcitriol for 6 months (46, 47). These findings suggest that the immunomodulatory effects exerted by calcitriol in patients with SLE may be due, in part, to alterations in miR-146a expression. In addition, sera levels of miR-146a may be used to monitor treatment response.

1.4.3. miR-155. Like miR-146a, miR-155 is vital to proper immune system functioning; it is highly expressed in Treg cells and is induced upon activation of T effector cells and myeloid cells (48, 49). miR-155 is induced in macrophages in response to both bacterial and viral antigens, functions in the hematopoietic compartment to promote the development of inflammatory T cells, and is required for DC production of Th17-promoting cytokines (28, 50). By developing miR-155-deficient mice, Rodriguez et al. found that miR-155 is required for the proper functioning of DCs, B cells, and T cells (51). The DCs of miR-155-deficient mice were unable to effectively activate T cells, indicating a defect in antigen presentation or costimulatory function. As they aged, the lungs of miR-155-deficient mice showed increased airway remodeling due to the increased numbers of lymphocytes in bronchoalveolar lavage fluids. The authors noted that these changes are similar to the lung fibrosis that often complicates systemic autoimmune processes with lung involvement (51). Zhou et al. examined the regulatory role of miR-155 in the regulation of plasmacytoid dendritic cell (pDC) activation and type I IFN production (52). They found miR-155 is upregulated upon TLR stimulation, providing another example of the link between stimulation, miRNA expression, and cellular activation. These

studies show that miR-155 is broadly implicated in LN pathogenesis and dysregulated miR-155 expression may play various roles in pathophysiology by altering immune cell function (52).

1.5. LN-associated miRNAs in Tissues

1.5.1. Renal tissue. miRNA expression profiles of renal tissue have gained much attention since Dai et al. provided a broad analysis of differentially expressed miRNAs in LN kidney biopsies (53). They identified 36 upregulated and 30 downregulated miRNAs in LN renal tissue compared to healthy controls. Their previous studies had identified 16 differentially expressed miRNAs in the peripheral blood mononuclear cells (PBMCs) of SLE patients, none of which constituted any of the 66 miRNAs identified in SLE kidney biopsies. These studies suggest that miRNA expression patterns are cell and organ specific (53, 54).

Lupus rodent models have revealed miRNAs implicated in LN pathogenesis (Table I). In the anti-Thy1.1 rodent model of glomerulonephritis, TGF- β and other cytokines and growth factors promote mesangial cell proliferation and activation, leading to mesangial proliferative glomerulonephritis (55). Denby et al. identified 2 miRNAs (miR-21 and miR-214) that are induced upon transforming growth factor- β (TGF- β) stimulation *in vitro* and characterized them further using the anti-Thy1.1 rat model (56). TGF- β -induced overexpression of miR-21 and miR-214 in tubular epithelial cells caused epithelial-mesenchymal transition (EMT)-like changes characterized by decreased *E-cadherin* expression and increased *α -smooth muscle actin* (*α -SMA*) and *collagen type I* expression. These changes are characteristic of proliferating cells and tissue remodeling (57). Blocking TGF- β downstream signaling in rat epithelial cells decreased the expression of miR-21 and miR-214 and prevented TGF- β -induced EMT by increasing *E-cadherin* expression and decreasing *α -SMA* and *collagen type I* expression. These

results suggest that TGF- β -induced miR-21 and miR-214 expression may contribute to extracellular matrix production and mesangial proliferative glomerulonephritis (56).

miRNAs that contribute to inflammation in chronic kidney disease (CKD) were recently examined in the B6.MRLc1 model, a congenic strain carrying a region of chromosome 1 derived from MRL/MpJ mice that develop IC-mediated glomerulonephritis (58). miR-146a expression was found to be significantly increased in B6.MRLc1 kidneys compared to healthy controls. B6.MRLc1 mice that expressed high levels of miR-146a expression showed severe glomerular and interstitial lesions including cell infiltration, tubular atrophy, and interstitial fibrosis. The lesions had increased macrophage and T cell infiltration as well as increased expression of cell-specific mRNAs associated with the development of renal lesions (*CD68* and *S100a4* for macrophages and fibroblasts, respectively) (59). miR-146a expression was also positively correlated with *IL-1 β* , *IL-10*, and *CXCL* expression. Because miR-146a is increased in the kidneys of B6.MRLc1 mice and continues to increase as they age, this model may be predisposed to increased miR-146a expression that initiates and perpetuates renal inflammation (59).

Research by Lu et al. confirmed that miR-146a is upregulated in glomerular tissue from LN patients and found that miR-146a is not overexpressed in LN tubulointerstitial tissue (60). miR-638 expression, on the other hand, was underexpressed in glomerular tissue but higher in tubulointerstitial tissue. Glomerular expression of miR-146a positively correlated with both estimated glomerular filtration rate (GFR) and histological activity index, determined from the sum of semi-quantitative scores of inflammation parameters (61). Increased tubulointerstitial expression of miR-638 was positively correlated with proteinuria and SLE Disease Activity Index (SLEDAI) score. While the correlation between changes in miRNA expression and

clinical disease severity suggests that these miRNAs may play a role in the pathogenesis of LN, it is currently unknown whether changes in miR-638 expression are pathogenic or an epiphenomenon (60).

1.5.2. PBMCs. While miR-146a was not initially reported to be decreased in SLE PBMCs, other miRNA expression screenings have revealed that miR-146a is significantly decreased in SLE patients and is inversely correlated with SLEDAI and IFN- α/β scores in SLE patients (46, 54, 62). Furthermore, *in vitro* studies by Tang et al. revealed that overexpression of miR-146a reduced type I IFN induction in PBMCs (62). They found that miR-146a negatively regulates both type I IFN production and TLR-stimulated downstream pathway activation by targeting the 3' UTR of *interferon regulatory factor-5 (IRF5)* and *STAT1*, key components in the type I IFN signaling cascade. The authors concluded that miR-146a deficiency is one of the causal factors in the abnormal activation of the type I IFN pathway in SLE (62).

A follow-up study identified a functional variant in the promoter of miR-146a that is associated with SLE disease risk; the promoter mutation resulted in decreased binding to the transcription factor ETS-1. Intriguingly, genome-wide association studies have identified an association between SLE risk and a functional variant of *ETS1*. The researchers observed additive effects of the risk alleles of miR-146a and *ETS1*, which suggests that individuals with 2 or more of these alleles are at a greater risk of developing SLE than those carrying only one allele (63).

Stagakis et al. identified 27 differentially expressed miRNAs in the PBMCs of SLE patients, 2 of which corresponded with the miRNAs identified by Dai et al. and 19 of which correlated with disease activity (23, 54). Of these disease-correlated miRNAs, eight were differentially expressed in T cells and 4 in B cells. Upregulation of miR-21 strongly correlated

with disease activity and activated T cells; inhibition of miR-21 reversed the activated T cell phenotype by increasing *PDCD4* expression (23). Another recent study found 7 abnormally expressed miRNAs (miR-145, miR-224, miR-150, miR-483-5p, miR-513-5p, miR-516a-5p, and miR-629) in SLE T cells compared to healthy controls. In a larger follow-up study, underexpression of miR-145 was confirmed and increased levels of *STAT1*, a target of miR-145, were observed in SLE T cells compared to healthy controls. Overexpression of miR-224 and decreased expression levels of its target, *apoptosis inhibitory protein 5 (API5)*, were also confirmed. T cells transfected with miR-224 *in vitro* were more susceptible to activation-induced apoptosis, indicating that SLE T cells overexpressing miR-224 may have an intrinsic defect that causes accelerated cell activation-induced apoptosis (64).

An additional study examining PBMC miRNAs found that decreased miR-125a expression in SLE patients contributed to increased KLF13 production by T cells. miR-125a has binding sites in the 3' UTR of *KLF13*, which belongs to the family of transcription factors that regulates the expression of the inflammatory chemokine *RANTES (CCL5)* in T cells. Increased *RANTES* expression is associated with persistent or recurrent organ inflammation due to its recruitment of T cells to inflammatory tissues. Increasing miR-125a levels in T cells from SLE patients *ex vivo* alleviated elevated *RANTES* expression. This study confirmed that underexpression of miR-125a contributes to the elevated expression of *RANTES* in SLE, increasing T cell recruitment to inflammatory tissues (65).

1.6. LN-associated miRNAs in Innate Immune Cells

The innate immune response provides the initial defense against infection by external pathogens and is predominantly mediated by macrophages, DCs, and neutrophils. The presence

of pathogens is commonly detected by macrophage and DC TLRs that bind conserved microbial products, triggering downstream signaling pathways to initiate inflammatory responses (66). Through TLR activation, ICs from lupus patients induce pDCs to secrete type I IFN (67). Activated DCs induce maturation and activation of infiltrating T cells, which further activates macrophages and increases the B cell response. The innate immune response, in particular DCs, promotes the activation of the adaptive immune system (68). Since miRNAs are critical for modifying innate and adaptive immune responses, dysregulated miRNA expression may represent an underlying cause to LN pathogenesis (Table I).

1.6.1. Macrophages/mesangial cells. miRNA expression is directly and indirectly altered after TLR activation and regulates macrophage signaling pathways that lead to the secretion of proinflammatory cytokines (28, 31, 69). Let-7a and miR-147 are directly induced upon LPS stimulation due to NF κ B binding sites in their promoter regions, which induces the expression of proinflammatory cytokines including TNF- α and IL-6 (29, 69). TNF- α , a critical cytokine involved in the response to LPS stimulation, increases miR-155 expression via JNK pathway activation, further increasing TNF- α production (28, 70, 71). Inhibition of JNK blocks the induction of miR-155, demonstrating that upregulated miR-155 expression in LPS-stimulated macrophages is indirectly due to JNK pathway activation (31, 69). These well-defined positive feedback loops demonstrate that stimulation-dependent miRNA expression induces cytokine production that further activates cells, which continues to alter miRNA expression.

Mesangial cells, the primary immunoregulatory cells resident to the renal glomerulus, possess phagocytic and contractile properties. Regulatory mechanisms of mesangial cells include a complex array of factors which control cell proliferation, survival, apoptosis, and GFR.

Mesangial cells from LN patients and lupus-prone mice have a heightened response to inflammatory stimulation (72, 73). Mesangial cells from NZB/W mice have been shown to produce significantly more chemokines in response to LPS stimulation than controls (74). Kato et al. demonstrated the involvement of miRNAs in mesangial cell activation (41). They determined that TGF- β activates Akt in glomerular mesangial cells by inducing miR-215a and miR-217, revealing a role for miRNAs in kidney disorders. We recently 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. Using *in vitro* techniques, we demonstrated that let-7a has a key role in regulating IL-6. Overexpression of let-7a increased IL-6 production in stimulated mesangial cells compared to nontransfected controls. 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. These data suggest increased let-7a expression may predispose lupus mice to increased inflammatory mediator production with immune stimulation (32).

1.6.2. Dendritic cells. Another significant immune cell that contributes to immunity in complex ways is the dendritic cell. DCs are widely considered to be critical for activating T cell responses, promoting B cell antibody production, and secreting cytokines in response to infections (75). In these ways they may direct autoimmunity and tolerance by serving as the primary antigen presenting cells (APCs) to initiate T cell autoimmunity, promoting B cell autoantibody production, and secreting proinflammatory cytokines. Altered function of DCs is known to play a major role in the development of autoimmunity (76). A recent study examining the functional characteristics of DCs in lupus patients found a significant increase in the

percentage of cytokine-producing DCs in addition to an increase in the amount of cytokine per cell in SLE patients compared with healthy subjects (77). pDCs are a specialized subset of DCs that are very active in IFN- α production, which promotes B cell differentiation into antibody-producing plasma cells (among many other functions). LN patients have been shown to have increased numbers of pDCs in the kidney as well as sustained IFN- α production (78).

The importance of TLR-induced miRNA expression in the regulation of pDC activation and type I IFN production has been examined. miR-155 and miR-155* (the complementary passenger strand in the miRNA duplex) were found to be the most strongly induced miRNAs in pDCs and were also differentially induced over time. The investigators found that miR-155* is induced before miR-155 and has biological activity. miR-155* induction after TLR stimulation increases IFN- α production by targeting *IRAKM*, which negatively regulates the TLR pathways by preventing the dissociation of IRAK1 and IRAK4 from MyD88 and the formation of IRAK1/TRAF6 complexes. The continual increase in miR-155 expression resulted in a reduction in IFN- α due to the targeting of *TAB2* by miR-155. *TAB2* regulates type I IFN production in pDCs upon TLR stimulation. Taken together, these results suggest there is cooperative involvement of both strands of the miRNA duplex in pDC activation (52).

1.7. LN-associated miRNAs in Adaptive Immune Cells

miRNAs were shown to be essential for altering the adaptive immune response in studies that conditionally depleted the enzyme Dicer from T or B cells. Dicer cleaves pre-miRNAs into double-stranded RNA products (duplexes) once they reach the cytoplasm (79). T cell Dicer depletion indicated miRNAs regulate diverse aspects of T cell biology, including basic cellular processes such as proliferation and survival as well as cell lineage decisions and cytokine

production during T helper cell differentiation (80). Dicer depletion in B cells resulted in the complete developmental block of B cells in the pro- to pre-B cell transition, affecting antibody diversity. These results indicate that miRNAs are critical for modifying adaptive immune responses and that irregular miRNA expression may represent an underlying cause to LN pathogenesis (Table I) (81).

1.7.1 Splenocytes. Although many miRNAs are expressed in T cell subsets, one study found 7 miRNAs (miR-16, miR-21, miR-142-3p, miR-142-5p, miR-150, miR-15b and let-7f) account for almost 60% of all T cell miRNAs. These miRNAs (except for miR-21) were downregulated in effector T cells compared to naïve cells. Memory T cell expression was similar to the expression seen in naïve T cells. miR-21 expression was higher in effector and memory T cells compared to naïve T cells, indicating that miRNAs are differentially expressed in hematopoietic lineages. These results suggest miRNAs may contribute dynamically to cell differentiation and the maintenance of cell identity (82).

It has recently been demonstrated that murine lupus models share a common disease-associated miRNA expression pattern despite strain differences in lupus susceptibility loci and clinical manifestation. In the MRL/*lpr* model, miR-146a was associated with disease development due to increased expression in splenocytes from 3–4-month-old mice compared to 1-month-old mice. miR-155 was found to be associated with disease development in both the MRL/*lpr* and the NZB/W models (83). An additional study investigated the relationship between IFN-accelerated disease, miRNAs, and B cell subsets in NZB/W mice due to the acceleration of disease by type I IFN in this model. Splenic and plasma miR-15a levels were elevated in diseased mice compared to pre-diseased mice. Increased autoantibody levels were

significantly correlated with increased miR-15a expression. The immunosuppressive B cell subset (B-10) was reduced following IFN treatment, yet had the highest miR-15a expression that increased with disease development. miR-15a expression in the pathogenic B cell subset (B-2) only increased upon disease onset. Although it is currently unknown whether changes in miR-15a expression are pathogenic or an epiphenomenon, these results suggest miR-15a is implicated in the development of SLE in NZB/W mice by directing the balance of splenic B cell subsets (84).

Pathogenic miRNAs have also been examined in the lymphocytes of B6.Sle123 mice. These mice spontaneously develop autoimmune disease characterized by autoantibodies, splenomegaly, and IC-mediated glomerulonephritis. They also have elevated ratios of CD4⁺ to CD8⁺ T cells. The expression of miR-21, which is upregulated in SLE T cells and has been shown to regulate apoptosis and cell proliferation pathways in part by targeting *PDCD4*, was found to be upregulated in B6.Sle123 splenocytes (23, 85). Short-term inhibition of miR-21 *in vivo* resulted in an approximate 20% decrease in *PDCD4* expression in naïve CD4⁺ T cells compared to T cells from control mice. Long-term inhibition of miR-21 *in vivo* significantly reduced splenomegaly in B6.Sle123 mice compared to the controls. In addition, the number of Fas receptor-expressing splenic B cells and the CD4⁺ to CD8⁺ T cell ratio were reduced, which suggests miR-21 inhibition skews the T cell ratio towards that of the nonautoimmune strain (85).

The overexpression of miR-148a has also been investigated in CD4⁺ T cells from patients with lupus as well as lupus-prone mice. Due to the importance of DNA methylation abnormalities in SLE pathogenesis, Pan et al. examined the roles of mir-21 and miR-148a in aberrant CD4⁺ T cell DNA hypomethylation (86). miR-21 and miR-148a downregulated *DNA methyltransferase 1 (DNMT1)* expression *in vitro* and *in vivo*, decreasing DNMT1 production in

T cells. Downregulation of *DNMT1* in CD4⁺ T cells contributes to lupus autoreactivity by inducing T cell DNA hypomethylation; this results in the overexpression of autoimmunity-associated genes including *lymphocyte function-associated antigen 1 (LFA-1 or CD11a)* and *CD70* (87). While a putative miR-148a binding site has been predicted in the 3' UTR of *DNMT1*, there are no predicted binding sites for miR-21. The researchers discovered miR-21 indirectly downregulated *DNMT1* expression by targeting its upstream regulator in the Ras-MAPK pathway, *RASGRP1*. Intriguingly, miR-148a directly downregulated *DNMT1* expression by targeting the protein coding region of its transcript. In addition, miR-21 and miR-148a induced the overexpression of methylation-sensitive, autoimmune-associated genes in CD4⁺ T cells including *CD70* and *LFA-1*. Furthermore, the investigators found that the effects were reversed when inhibitors of either miR-21 or miR-148a were transfected into CD4⁺ T cells isolated from SLE patients, implying hypomethylation in CD4⁺ T cells can potentially be alleviated by inhibiting these miRNAs (86).

Another post-transcriptional modifier of *DNMT1*, miR-126, was found to be overexpressed in CD4⁺ T cells from SLE patients (88). Its degree of overexpression negatively correlated with DNMT1 protein levels. In addition, the expression of miR-142-3p and miR-142-5p was reduced to less than half in SLE CD4⁺ T cells compared to CD4⁺ T cells from healthy controls. miR-126, miR-142-3p, and miR-142-5p are predicted to target genes associated with SLE, which implicates their aberrant expression in CD4⁺ T cells in LN pathogenesis. Overexpression of miR-126 in primary CD4⁺ T cells from SLE patients contributed to T cell autoreactivity by targeting *DNMT1*, while inhibition in SLE patients resulted in T and B cell inactivation. Overexpression of miR-126 in primary CD4⁺ T cells from healthy donors resulted in the demethylation and upregulation of autoimmunity-associated genes including *CD11a* and

CD70, inducing T cell and B cell hyperactivity. These results demonstrate that overexpression of miR-126 can aberrantly induce splenocyte activity towards that of an autoimmune phenotype (88).

Decreased expression of miR-142-3p and miR-142-5p in SLE CD4⁺ T cells was confirmed in studies by Ding et al (89). *CD84* and *IL-10* are predicted targets of miR-142-3p, while *signaling lymphocytic activation molecule-associated protein (SAP)* is a potential target of miR-142-5p. When miR-142-3p was inhibited in CD4⁺ T cells from healthy donors, protein levels of CD84 and IL-10 increased. SAP protein production was decreased after inhibition of miR-142-5p. Inhibition in healthy donor CD4⁺ T cells caused T cell overactivation and B cell hyperstimulation. These results were reversed after transfection of the corresponding miRNA mimic. Overexpression in SLE CD4⁺ T cells decreased CD40L, inducible T cell costimulator (ICOS), IL-4, IL-10, and IL-21 protein levels, reduced T cell proliferation, and reduced IgG production compared to controls. These results indicate that reduced expression of miR-142-3p and miR-142-5p in CD4⁺ T cells of SLE patients contributes to T cell hyperactivity and B cell hyperstimulation (89).

1.8. Future Directions in LN Treatment

miRNAs are being recognized as potential therapeutic targets in the treatment of LN and other diseases as increasing numbers are identified as specific disease-modifying agents and not merely disease correlates. Recent studies have shown that exogenously increasing let-7a, a well-known tumor suppressor that is downregulated in many types of cancer, is effective in treating tumorigenesis by decreasing cell migration, invasion, and proliferation *in vitro* and *in vivo* (90-94). Intranasal let-7 administration reduced lung tumor formation in a murine model of lung

cancer (95). Tumorigenesis was suppressed in murine gastric cancer cells *in vivo* by overexpression of let-7a, which decreased cell proliferation by causing G₁ arrest (96).

Lupus therapeutics have recently been recognized for their ability to alter miRNA expression levels (97, 98). Once disease-associated miRNA expression is determined in patients with SLE, tailored therapies can be designed using immunosuppressant treatments that alter pathologic miRNAs. Examining miRNA expression profiles during the course of immunosuppressant therapy may more accurately assess treatment responsiveness. Since lupus susceptibility genes contain target sites for various miRNAs, future treatments may target multiple disease-associated miRNAs that synergistically contribute to LN pathogenesis. Additionally, pathogenic miRNA expression may be used to assess treatment feasibility. This will allow the use of targeted therapies to specifically modulate abnormal miRNA expression patterns in individuals suffering from lupus.

Circulating miRNAs have been used as diagnostic markers in the treatment and diagnosis of certain cancers (99, 100). Since the discovery of dysregulated miRNA expression in the serum and urine of SLE patients, the interest in using miRNAs as noninvasive biomarkers has increased (54, 97). One of the many advantages of using miRNAs as disease biomarkers is the availability of highly sensitive PCR detection methods and their low complexity compared with protein biomarkers (101). In addition, pathogenic miRNAs may be able to detect early SLE disease onset before clinical, pathological findings arise. Assessing miRNA expression in different tissues may alter our organ-specific and systemic understanding of SLE. For example, detecting alterations in urinary miRNA expression may offer valuable information regarding changes in the glomerular microenvironment, while pathogenic alterations in PBMCs may reveal the global state of the SLE patient.

1.8.1. Therapeutic modulation of miRNAs. Because of the vast and critical roles miRNAs perform in fundamental immune processes (and due to their dysregulated expression in many pathological conditions), they have become an increasingly attractive target for therapeutic modulation. While the endogenous delivery of miRNAs has had limited testing *in vivo*, the risk of altering unintentional targets remains high as a single miRNA can have multiple gene targets and these targets can have profound effects on a variety of miRNAs (102-104). The solution to this potential problem may lie in targeting miRNAs broadly associated with SLE such as miR-146a (42, 60, 62, 83, 105). Pan et al. therapeutically altered miR-146a levels using virus-like particles (VLPs) containing miR-146a, which were delivered via tail vein injection to lupus-prone BXSB mice (106). After administration of the miR146a-containing VLPs, high levels of miR-146a were detected in PBMCs, lung, spleen, and kidney tissues from BXSB and control mice. miR-146a therapy significantly reduced autoantibody, IFN- α , IL-1 β , IL-6, and total IgG production. Widespread restoration of miR-146a by VLPs was effective in ameliorating SLE progression in lupus-prone mice, providing a potential novel therapy for SLE treatment (106).

While the initial findings from studies that systemically increase miRNA levels are promising, a more effective treatment may utilize targeted delivery systems. A novel approach to manipulating mesangial miRNA expression alone could be employed by targeting the mesangial cell surface marker that is unique to the kidney glomeruli: Integrin $\alpha 8$ (107). Scindia et al. revealed that this molecule can be used to effectively target immunoliposomes to mesangial cells by tail vein injection (108). pDCs also possess a unique cellular marker: plasmacytoid dendritic cell antigen (PDCA) (109). In this way, pDCs may be specifically targeted instead of all splenocytes or PBMCs, considering a miRNA may not be differentially expressed in other

cell types found in the spleen or peripheral blood. For example, while miR-146a is decreased in PBMCs, it is upregulated in murine Th1 cells compared to naïve T cells and Th2 cells (62, 110).

1.8.2. Tailored therapy based on the patient's miRNA profile. Although glucocorticoids are the first-line treatment for a wide range of autoimmune diseases, up to 30% of patients with SLE are steroid-resistant, demonstrating persistent tissue inflammation despite treatment with high doses of steroids (111, 112). Disease-associated miRNAs may become unique biomarkers that help determine the course of the patient's immunosuppressant therapy. The use of miRNAs as selection markers for disease treatment is underway in the treatment of ovarian cancer.

Researchers found that let-7a expression was predictive of a patient's outcome after chemotherapy; let-7a expression differed substantially between the patients who did or did not respond to chemotherapy containing platinum and paclitaxel. The survival of patients with low let-7a expression was higher when they received platinum and paclitaxel in combination; patients with high let-7a expression did not have improved survival after adding paclitaxel to platinum-based therapy (113).

If disease-associated miRNAs are targeted, the treatment of SLE could be greatly improved. Steroid-resistant patients, amongst others, may benefit from tailored immunotherapy. Revealing miRNAs with therapeutic potential may provide insight in treating other inflammatory diseases as well. Polikepahad et al. showed that the inhibition of let-7 miRNAs in an experimental model of asthma *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 (114).

1.9. Conclusion

miRNAs are now recognized as key regulators of gene expression. A single miRNA, or even multiple miRNAs, may contribute to cell development and immunoregulation in diverse ways. Increasing evidence has shown that miRNAs are not only critical for the regulation of immune cell development but also for modifying innate and adaptive immune responses.

Evidence suggests that miRNAs are involved in LN pathogenesis by altering innate immune cell responsiveness, lymphocyte function, proinflammatory mediator production, and TLR and NF κ B signaling pathways.

Increasing evidence indicates that dysregulated miRNA expression in specific cell types contributes to LN immunopathogenesis. While it is becoming clear that miRNAs modulate components of inflammatory signaling cascades, it is not fully understood how miRNAs are regulated by different cell types in SLE. Overall, the possibility of altering miRNA expression in order to ameliorate disease remains promising. Studies that alter pathogenic miRNAs have shown that miRNA-based therapies have the potential for becoming therapeutic tools for the treatment of SLE and other diseases. As we learn more about the intricacies of miRNAs and epigenetics, targets for drug development will continue to emerge.

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Table I. miRNAs implicated in LN pathogenesis.

Cell or tissue type	miR ID(s)	Origin	Strain	Expression	Results	Mechanism(s)	Reference
Renal	21 & 214	R	WKY (anti-Thy1.1)	↑	Expression is induced by TGF- β in tubular epithelial cells <i>in vitro</i> and in renal tissue <i>in vivo</i>	Unknown	56
					Overexpression in tubular epithelial cells <i>in vitro</i> decreased <i>E-cadherin</i> expression and increased <i>collagen type I</i> and α - <i>SMA</i> expression	Unknown	56
	146a	M	B6.MRLc1	↑	Increased expression positively correlated with <i>IL-1β</i> , <i>IL-10</i> , and <i>CXCL</i> expression, severe glomerular and interstitial lesions, and T cell and macrophage infiltration	Unknown	59
		H	-	↑	Glomerular expression positively correlated with estimated GFR and histological activity index	Unknown	60
638	H	-	↑	Tubulointerstitial expression positively correlated with proteinuria and disease activity score	Unknown	60	

PBMCs	21	H	-	↑	Strongly correlated with disease activity and activated T cells	Unknown	23
					Inhibition <i>in vitro</i> reversed the activated T cell phenotype by increasing <i>PDCD4</i> expression	The 3' UTR of <i>PDCD4</i> is a target of miR-21	23
	125a	H	-	↓	Underexpression contributes to the elevated expression of <i>RANTES</i> (<i>CCL5</i>) in SLE, increasing T cell recruitment to inflammatory tissues	The 3' UTR of the <i>RANTES</i> upstream regulator <i>KFLI3</i> is a target of miR-125a, indirectly increasing <i>RANTES</i> expression	65
	145	H	-	↓	Decreased expression increased <i>STAT1</i> expression in SLE patients	The 3' UTR of <i>STAT1</i> is a target of miR-145	64
	146a	H	-	↓	Inversely correlated with disease activity and IFN- α/β scores	Unknown	62
					Overexpression reduced the induction and downstream effects of type I IFN	The 3' UTR of <i>IRF5</i> and <i>STAT1</i> are targets of miR-146a, reducing the induction of type I IFN	62
					Promoter variant associated with SLE disease risk	SLE-associated SNP (rs57095329) decreases miR-146a expression levels	63
					Positively correlated with GFR, CRP, and other renal function parameters; inversely correlated with proteinuria and SLEDAI	Unknown	46

PBMCs (continued)	155	H	-	↓	Positively correlated with GFR, CRP, and other renal function parameters	Unknown	46
	224	H	-	↑	Increased expression accelerated T cell activation-induced cell death by suppressing <i>API5</i> expression in SLE patients	The 3' UTR of <i>API5</i> is a target of miR-224	64
Mesangial cells	let-7a	M	NZB/W	↑	Increased expression throughout the lifetime of NZB/W lupus mice; overexpression increased <i>IL-6</i> expression and IL-6 production <i>in vitro</i>	The 3' UTR of <i>IL-6</i> is a target of let-7a; the exact mechanism of let-7a is unknown	32
Dendritic cells	155	H	-	↑	Induced by TLR stimulation after miR-155*; overexpression of miR-155 in normal pDCs significantly decreased <i>IFN-α</i> , <i>IFN-β</i> , and <i>TNF-α</i> expression	The 3' UTR of the type I IFN regulator <i>TAB2</i> is a target of miR-155, indirectly decreasing IFN-α and IFN-β	52
	155*	H	-	↑	Induced by TLR stimulation before miR-155; overexpression of miR-155* in normal pDCs significantly increased <i>IFN-α</i> , <i>IFN-β</i> , and <i>TNF-α</i> expression	The 3' UTR of the negative IFN regulator <i>IRAKM</i> is a target of miR-155*, indirectly increasing IFN-α and IFN-β	52
Splenocytes	15a	M	NZB/W	↑	Increased expression after disease was accelerated by IFN administration; differentially expressed in B cell subsets	Unknown	84

Splenicocytes (continued)	21	M	B6.Sle123	↑	Inhibition increased <i>PDCD4</i> expression in T cells and reversed splenomegaly, improving overall disease outcome	The 3' UTR of <i>PDCD4</i> is a target of miR-21	85
		M & H	MRL/ <i>lpr</i>	↑	Downregulated <i>DNMT1</i> expression in T cells	The 3' UTR of the <i>DNMT1</i> upstream regulator <i>RASGRP1</i> is a target of miR-21, indirectly downregulating <i>DNMT1</i>	86
	126	H	-	↑	Overexpression contributes to T cell autoreactivity by decreasing <i>DNMT1</i> expression	The 3' UTR of <i>DNMT1</i> is a target of miR-126	88
					Overexpression in healthy donors was sufficient for T cell autoreactivity and B cell hyperstimulation, while inhibition in SLE patients resulted in T and B cell inactivation	Unknown	88
	142-3p & 142-5p	H	-	↓	Underexpressed in SLE CD4 ⁺ T cells	Dysregulated DNA and histone methylation of the miR-142 promoter	89
					Underexpression in CD4 ⁺ T cells increased production of CD84, IL-10, and SAP	The 3' UTR of <i>CD84</i> and <i>IL-10</i> are targets of miR-142-3p; the 3' UTR of <i>SAP</i> is a target of miR-142-5p	89

Splenocytes (continued)	142-3p & 142-5p	H	-	↓	Inhibition in healthy donor CD4 ⁺ T cells caused T cell overactivation and B cell hyperstimulation, while overexpression in SLE CD4 ⁺ T cells had the opposite effect	Although CD84 and SAP stimulate T-B cell interactions, the exact mechanism of miR-142 is unknown	89
	146a	M	MRL/ <i>lpr</i>	↑	Increased expression associated with disease development	Unknown	83
	148a	M & H	MRL/ <i>lpr</i>	↑	Downregulated <i>DNMT1</i> expression in T cells	The protein coding region of the <i>DNMT1</i> transcript is a target of miR-148a	86
					Induced overexpression of autoimmune-associated, methylation-sensitive genes in CD4 ⁺ T cells including <i>CD70</i> and <i>LFA-1</i>	Inhibition of <i>DNMT1</i> results in DNA hypomethylation and the overexpression of methylation-sensitive genes	86
155	M	MRL/ <i>lpr</i> , NZB/W	↑	Increased expression associated with disease development	Unknown	83	

Abbreviations: H: humans; M: mice; R: rats

↑: increased expression; ↓: decreased expression

α-SMA: α-smooth muscle actin; API: apoptosis inhibitory protein; CRP: C-reactive protein; DNMT: DNA methyltransferase; GFR: glomerular filtration rate; IFN: interferon; IL: interleukin; IRAK: IL-1 receptor-associated kinase, IRF: interferon regulatory factor;

NZB/W: New Zealand Black/White; PBMCs: peripheral blood mononuclear cells; pDC: plasmacytoid dendritic cell; SNP: single-nucleotide polymorphism; PDCD: programmed cell death; SLE: systemic lupus erythematosus; SLEDAI: SLE Disease Associated Index; STAT: signal transducer and activator of transcription; TGF- β : transforming growth factor- β ; TTP: tristetraprolin

Chapter 2

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

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2.2. 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* mRNA 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 nontransfected 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.

2.3. 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, noncoding 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/uridylylate (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. In order 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.

2.4. Materials and Methods

2.4.1. Animals

Female NZB/W and NZW mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). 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).

2.4.2. 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 were 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 minutes (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, CA, USA) followed by incubation with goat anti-rabbit IgG magnetic microbeads (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, USA) and stored at -20°C until miRNA isolation.

2.4.3. 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, USA). 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, USA). 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 .

2.4.4. 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 μ L of 1ng/ μ L miRNA template. The negative control received 5 μ L of nuclease-free water. RT was performed in an iCycler (BioRad, Hercules, CA, USA) using the following parameter values: 16°C for 30 minutes, 42°C for 30 minutes, 85°C for 5 minutes, 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 minutes (1 cycle) and 95°C for 15 seconds/60°C for 60 seconds (40 cycles). Relative gene quantitation was determined using the comparative C_T ($\Delta\Delta C_T$) method (34). The ΔC_T was calculated using the endogenous control snoRNA202 and the $\Delta\Delta C_T$ was determined by calculating the fold change in let-7a expression between NZB/W and NZW mice. All samples were run in triplicate.

2.4.5. 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*.

2.4.6. Plasmids

The pMIR-REPORT empty vector (Ambion, Austin, TX, USA) 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, USA) 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.

2.4.7. Cell culture and transfection

A mouse mesangial cell line (MES 13) that is transgenic for SV40 was purchased from ATCC (Manassas, VA, USA). 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, USA). For serum-starving medium, FBS was absent from the complete growth medium. For immune stimulation, LPS (Sigma-Aldrich, St. Louis, MO, USA) and IFN- γ (Cedarlane Laboratories Limited, Burlington, NC, USA) 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 hours 24 hours 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, USA). The cells were serum-starved for 2 hours prior to transfection at which point they were transfected with the miRIDIAN let-7a mimic or hairpin inhibitor (Dharmacon RNAi Technologies, Lafayette, CO, USA). The final concentration of the miRNAs was 25nM unless otherwise noted. Nontransfected controls received complete growth medium only. The plates were incubated for 24 hours at 37°C at which time the media was removed and replaced with stimulating medium. Nonstimulated controls received complete growth medium. miRIDIAN miRNA mimic and hairpin inhibitor positive and negative controls were used (Dharmacon). These nontargeting 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 nontargeting siRNA control has no gene targets in mouse cells. Cells and supernatants were collected for analysis 24 hours post-stimulation. RNA and miRNAs were isolated as described above.

For DNA transfections, cells were serum-starved for two hours 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. Nontransfected controls received complete growth medium only. These experiments were performed separately with or without immune stimulation 24 hours post-transfection. Nonstimulated controls received

complete growth medium. The cell lysates were collected 48 hours post-transfection and stored at -80°C.

2.4.8. Cell viability

Cell viability was measured using a Vi-Cell (Beckman Coulter, Brea, CA, USA) 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.

2.4.9. 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, USA). 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, USA). The intact or mutant plasmid co-transfected with a nontargeting 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.

2.4.10. 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 μL of 1ng/ μL RNA template. The negative control received 10 μL of nuclease-free water. RT was performed in an iCycler using the following parameter values: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes, 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 ΔC_T was calculated using the endogenous controls snoRNA202 or GAPDH (for miRNAs or RNA samples, respectively), and then the $\Delta\Delta\text{C}_\text{T}$ was determined by calculating the fold change in expression between the transfected samples and the controls. All samples were run in triplicate.

2.4.11. 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, USA). The plate was read at 450 nm on a Spectramax 340PC microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). All samples were run in duplicate.

2.4.12. 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* $t_{1/2}$.

2.4.13. Statistical analysis

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

2.5. Results

2.5.1. 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 nonautoimmune 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.

2.5.2. Let-7a is increased in stimulated mesangial cells

In order 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. Nontargeting 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, nontargeting inhibitor control (NCi, Figure 1C), and nontargeting 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.

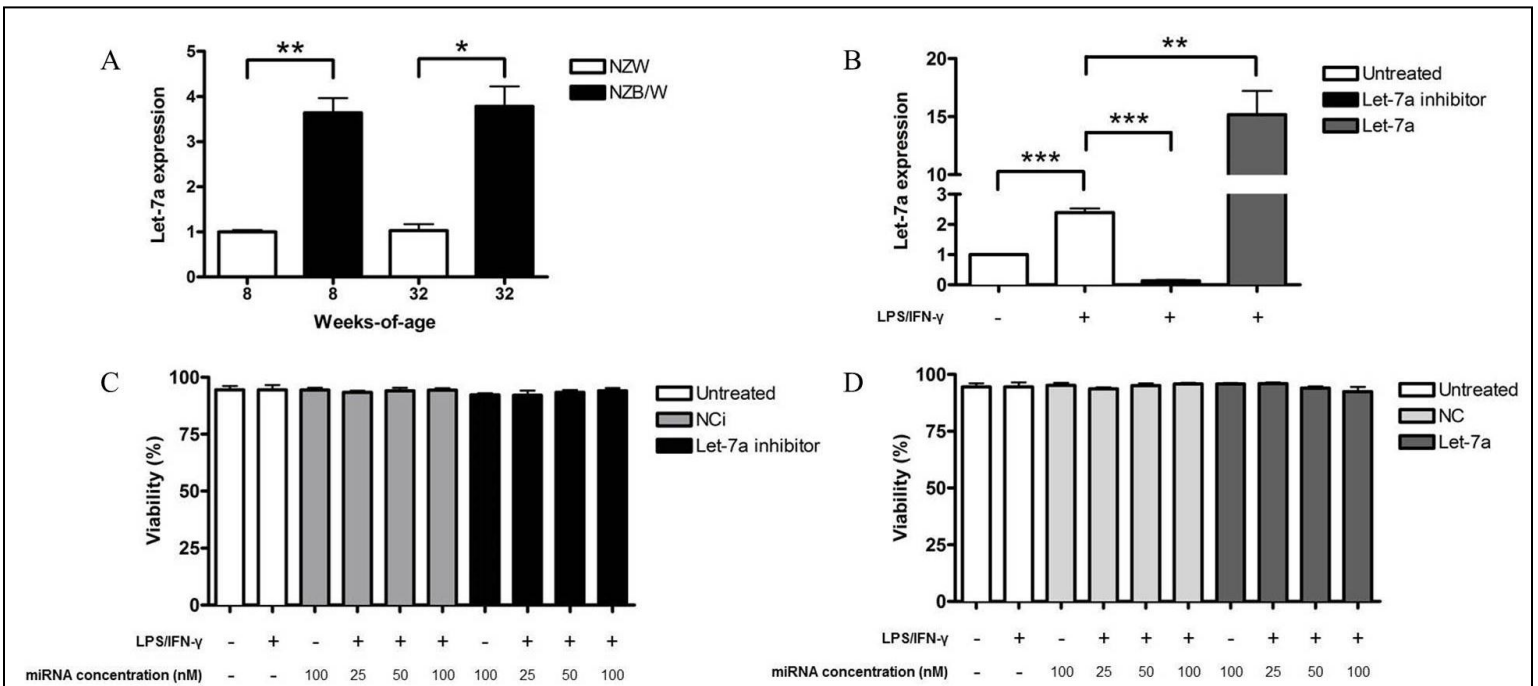


Figure 1. Let-7a expression is increased in the mesangial cells of 8 and 32-week-old NZB/W mice compared to age-matched, nonautoimmune NZW mice. (A) Real-time RT-PCR shows the increased expression of let-7a in the mesangial cells of 8-week-old NZB/W mice compared to age-matched NZW mice. Let-7a expression 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. Nontargeting 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 nontargeting 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 nontargeting mimic control (NC) and nontargeting 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. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

2.5.3. *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 (pMIR-IL6-Intact) or the mutant plasmid (pMIR-IL6-Mut) was co-transfected into cultured mesangial cells alone (untreated) or simultaneously with the let-7a mimic (let-7a), inhibitor (let-7a inhibitor), nontargeting mimic control (NC), or nontargeting 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 nonstimulated 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 nontargeting 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 nontargeting 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 nonstimulated 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.

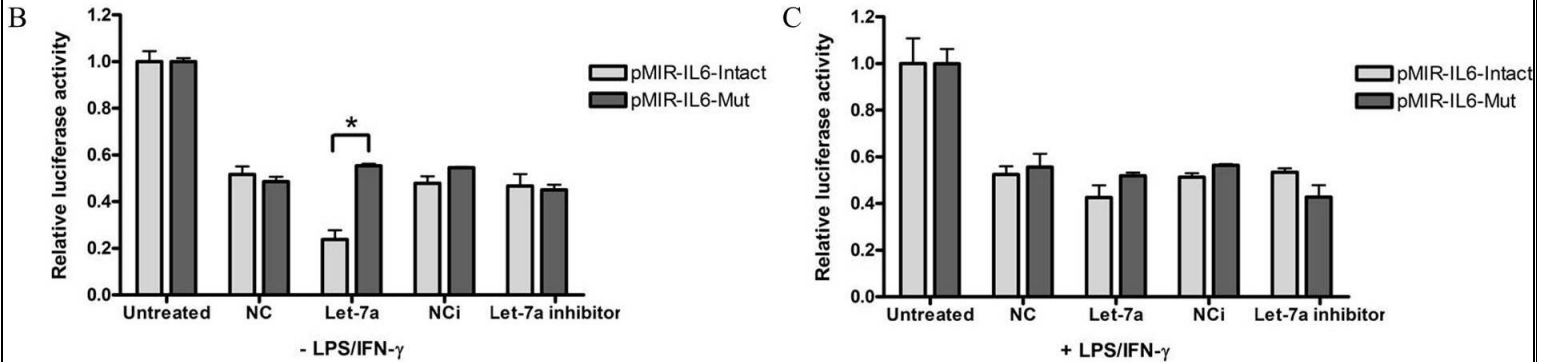
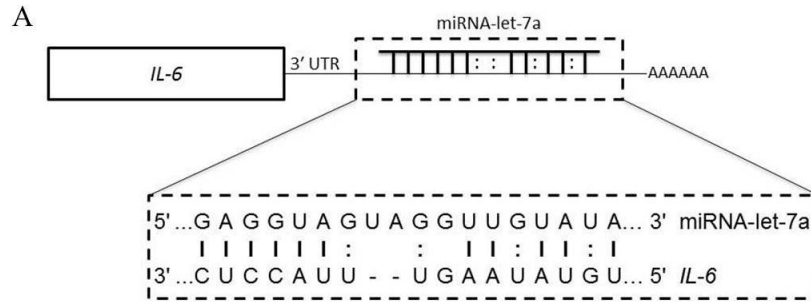


Figure 2. Let-7a partially binds to the 3' UTR of *IL-6*. (A) The 3' UTR of *IL-6* is a predicted target of let-7a. Vertical lines represent direct base pairing. Colons represent non-Watson-Crick base pairing (or wobble base pairing). (B) Luciferase activity is decreased in nonstimulated mesangial cells 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 nontargeting 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 nontargeting mimic control (NC) and nontargeting 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. * $p < 0.05$.

2.5.4. 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 nontargeting control (NC). IL-6 production was negligible in nonstimulated 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 (Figure 3A). When the stimulated cells were transfected with the let-7a inhibitor or nontargeting 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.

In order 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 nontargeting 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 nontargeting control (NC) for 24 hours at which time they were stimulated with LPS/IFN- γ for 6 hours. Fresh medium was added with the transcription inhibitor ActD (10 $\mu\text{g}/\text{mL}$) for 1, 2, or 3 hours to block transcription. The overall levels of *IL-6* were higher in the stimulated cells transfected with let-7a compared to the nontargeting control (Figure 3E). After 1 hour 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 hours post-ActD treatment compared to the 1 hour treatment. By 3 hours of ActD treatment, *IL-6* expression was similar to that of the nontargeting 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 hours 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 nontargeting 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.

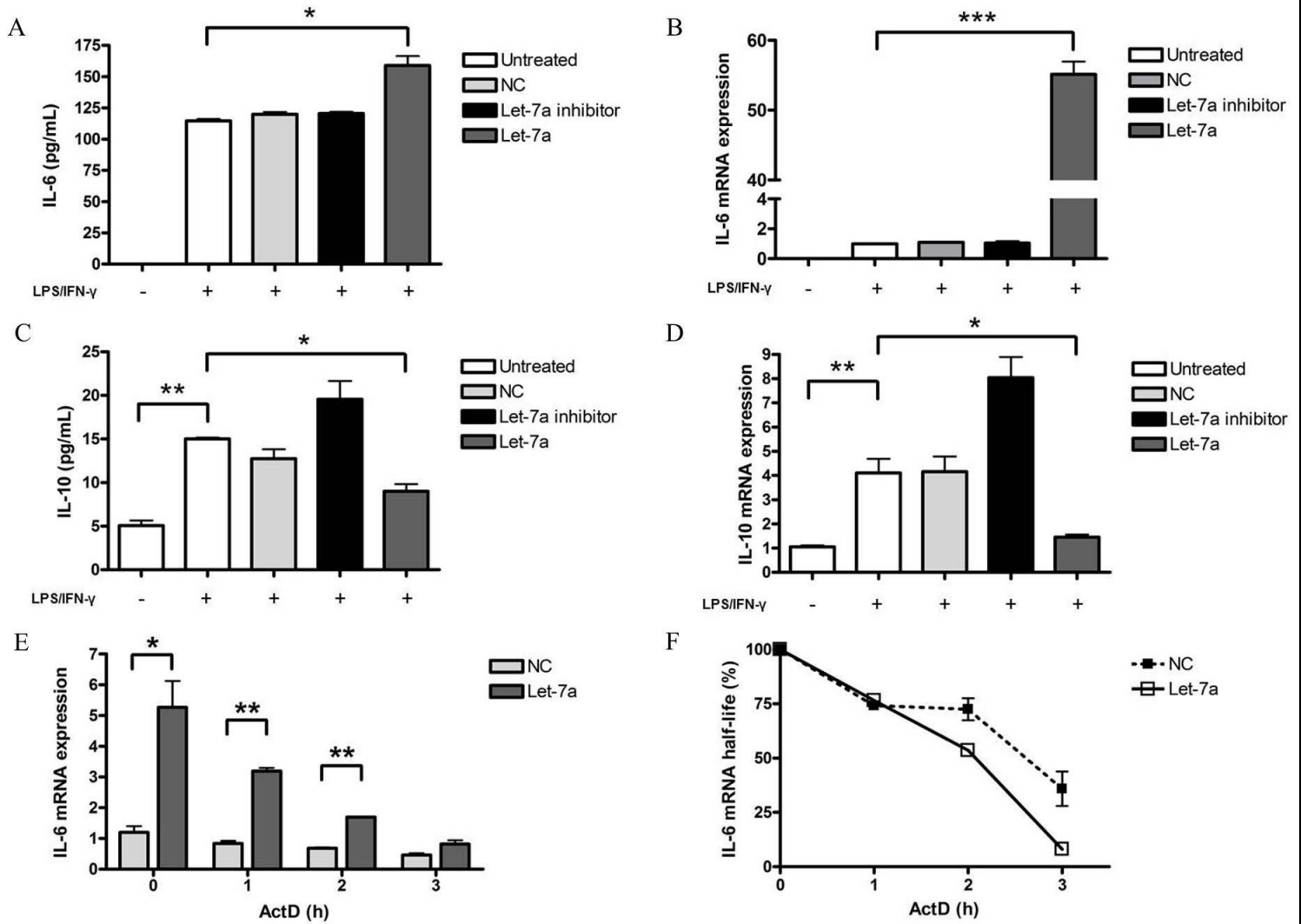


Figure 3. Exogenous delivery of let-7a increases IL-6 production in cultured mesangial cells. (A) Transfection of the let-7a mimic significantly increases IL-6 production relative to the stimulated control. Transfection of either the nontargeting control or the let-7a inhibitor has no effect on the production 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 nonstimulated control produces undetectable levels of *IL-6*. Transfection of any miRNA without stimulation produces undetectable levels of *IL-6* (data not shown). (C) Transfection of the nontargeting 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 hours, and treated with ActD for 1, 2, or 3 hours show a significant decrease in relative *IL-6* expression over time compared to the nontargeting control. (F) The $t_{1/2}$ of *IL-6* is not significantly longer in let-7a-transfected cells compared to the nontargeting control. The final concentration of the inhibiting or mimicking miRNAs was 25 nM. The nontargeting control (NC) is miR-67. The nontargeting 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. * $p < 0.05$. ** $p < 0.005$. *** $p < 0.0001$.

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

In order 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/uridylate (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). While 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, 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 hours of stimulation, *TTP* expression was significantly increased compared to the negative control (Figure 4E). After 12 hours of stimulation, *TTP* expression had increased over 8-fold. This demonstrates that let-7a increases *TTP* expression in stimulated mesangial cells.

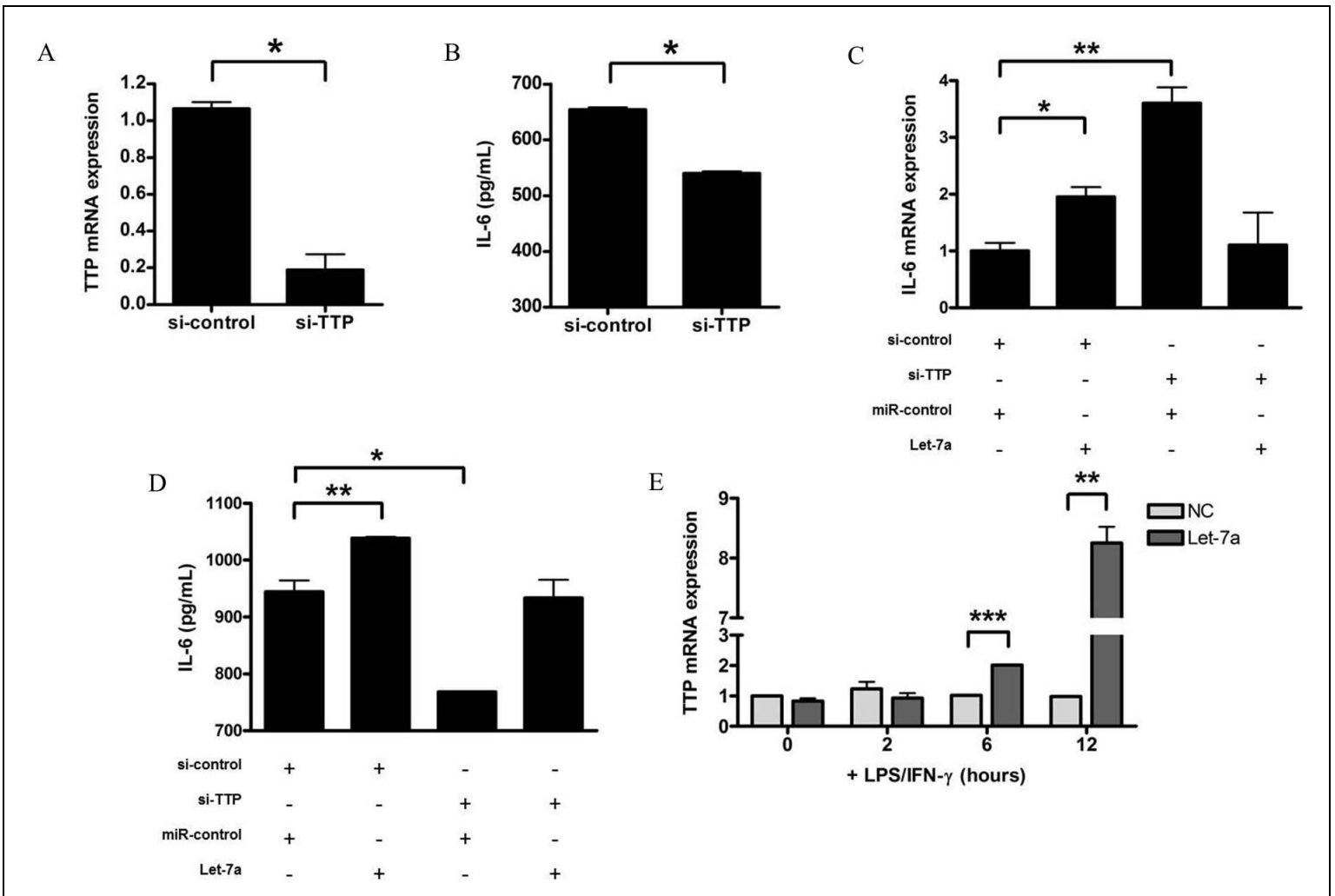


Figure 4. Let-7a upregulates IL-6 production through cooperation with TTP. (A) Real time RT-PCR shows *TTP* expression is significantly decreased after transfection of si-TTP compared to the nontargeting control. (B) *IL-6* expression is significantly increased after transfection of si-TTP compared to the nontargeting control. (C) *IL-6* expression is significantly increased when mesangial cells are transfected with either let-7a or si-TTP compared to the nontargeting 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 hours post-stimulation and continues to be upregulated after 12 hours of stimulation. The final concentration of the miRNAs or siRNAs was 25 nM. The nontargeting miRNA control (NC) is miR-67. The nontargeting 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. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

2.6. 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 responsive 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 nonautoimmune 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.

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 (61, 62). 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 (63). 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 (64). Serum levels of IL-6 are also elevated in this strain (65). While *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 (66, 67). TTP knockout mice develop a systemic inflammatory syndrome with severe arthritis and autoimmunity, as well as medullary and extramedullary myeloid hyperplasia (68). The development of autoimmunity is predominantly due to the resulting upregulation of TNF- α , an inducer of IL-6 production (69).

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 (70). Future experiments will examine the expression patterns of TTP in NZB/W mice as they develop disease compared to age-matched controls.

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.

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Chapter 3

MicroRNA-let-7a promotes NFκB activation and E2F-mediated cell proliferation *in vitro*

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3.2. Abstract

Epigenetic factors, including altered microRNA (miRNA) expression, may contribute to aberrant immune cell function in systemic lupus erythematosus (SLE). miRNA-let-7a (let-7a) has been shown to directly alter cell cycle progression and proinflammatory cytokine production. Due to the crucial role of let-7a in cell division and inflammation, we investigated let-7a-mediated proliferation and NF κ B translocation in J774A.1 macrophages and MES 13 mesangial cells *in vitro*. In immune-stimulated cells transfected with let-7a, cell proliferation was significantly increased over time. There was a significant increase in the number of immune-stimulated cells in S and G₂ phases. Immune-stimulated cells overexpressing let-7a had increased nuclear translocation of NF κ B. Bioinformatical analysis revealed that the E2F family, critical regulators of the G₁-S transition, has potential binding sites for let-7a in their mRNA transcripts. Let-7a overexpression significantly increased the expression of the cell cycle activator *E2F2* and increased retinoblastoma protein (Rb) phosphorylation in immune-stimulated cells. The expression of the cell cycle inhibitor *E2F5* was significantly decreased in let-7a-transfected cells that were immune-stimulated. Bioinformatical analysis revealed E2F2 and NF κ B are transcription factors predicted to regulate the let-7a promoter. We analyzed transcriptional regulation of let-7a by real-time RT-PCR using chromatin immunoprecipitation with E2F2 and NF κ B antibodies. There was an increase in E2F2 and NF κ B binding in DNA enriched for the let-7a promoter in immune-stimulated cells. Silencing E2F2 or NF κ B significantly decreased let-7a expression and IL-6 production in immune-stimulated cells. Taken together, our results suggest that overexpression of let-7a may contribute to hyperplasia and the proinflammatory response in SLE.

3.3. Introduction

Abnormal macrophage and mesangial cell function have been shown to contribute to SLE pathogenesis in lupus mouse models and patients with glomerulonephritis (1-4). Systemic lupus erythematosus (SLE) is a complex autoimmune disease characterized by high titers of autoantibodies against nuclear antigens (5). Due to endosomal trafficking, nuclear antigens trigger intracellular Toll-like receptors (TLRs), activating the canonical NF κ B pathway (6). The deposition of immune complexes in the renal glomeruli activates mesangial cells, the principle immunoregulatory cell in the glomerulus. Activated mesangial cells produce inflammatory and chemotactic mediators that target the kidney for monocyte, neutrophil, and lymphocyte infiltration (3, 7, 8). Activated mesangial cells and macrophages produce proinflammatory cytokines and chemokines that induce the maturation and activation of infiltrating T cells. T cells perpetuate the activation of innate immune cells and the B cell response. In lupus nephritis (LN), the sustained inflammatory response results in pathological features including cell proliferation, inflammatory cell infiltration, sclerosis, and fibrosis (9).

Cytokines and other stimuli induce the activation of the NF κ B family of transcription factors, which translocate into the nucleus to transcribe a variety of genes involved in inflammation, immune regulation, and apoptosis (10). The NF κ B p65 (RelA) subunit, along with c-Rel and RelB, contains a transcription activation domain allowing it to positively regulate gene expression (11). Activated NF κ B transcribes genes encoding proinflammatory cytokines like IL-6 that can continue to increase NF κ B activation via autocrine signaling (12, 13). Elevated levels of IL-6 have been observed in the sera and tissues of SLE patients (14). Renal IL-6 expression is positively correlated with increased mesangial proliferation, tubulointerstitial damage, and lymphocyte infiltration (15). In SLE, mesangial cells are hyperresponsive to

immune stimulation; when activated by proinflammatory mediators like IL-6, mesangial cells produce growth factors and cytokines that stimulate extracellular matrix deposition, a pathological characteristic that is increased in glomerular diseases (16-18).

Because cell proliferation is a common histological feature of LN, cell cycle regulators may contribute to SLE pathogenesis (19-21). The E2F family of transcription factors plays a critical role in the regulation of the G₁-S transition and can be subdivided into repressors and activators (22). Repressor E2Fs (such as E2F5) occupy E2F target promoters during quiescence and in early G₁ phase. As cells enter late G₁ phase, the repressor E2F complexes are replaced by activator E2Fs (such as E2F2) that have been released from retinoblastoma protein (Rb) due to its phosphorylation (23). E2F transcription factors regulate the expression of numerous genes needed for cell cycle entry and DNA synthesis (24). A genome-wide association study in peripheral blood mononuclear cells from SLE patients found that a significant number of SLE-associated genes contain E2F binding motifs in their promoters, which suggests transcription factors that regulate proliferation may play a role in SLE pathogenesis (25).

Epigenetics, including microRNA (miRNA) regulation, refers to stable and heritable changes in gene expression that are not dependent on DNA sequence (26). miRNAs are small, noncoding RNAs that function as endogenous regulators of gene expression by incompletely binding to the 3' untranslated region (UTR) of target mRNAs (27). miRNA expression is highly sensitive to inflammatory stimulation; lipopolysaccharide (LPS) induces the expression of miR-9, miR-132, miR-146, miR-147, miR-155, and miR-let-7a (let-7a) by activating transcription factors that regulate miRNA expression (28-33). miRNAs can contribute to the proinflammatory environment by inducing the expression of proinflammatory cytokines, perpetuating the magnitude and duration of the immune response (34, 35). Let-7a is implicated in SLE

pathogenesis due to its responsiveness to immune stimulation and its reported inflammatory targets; the expression of let-7a is increased after LPS stimulation and it is overexpressed in the kidneys of SLE patients and the mesangial cells of lupus mice (33, 36).

In addition to their vast role in immunology, miRNAs have been shown to contribute to alterations in the cell cycle (37, 38). Let-7a targets the mRNA transcripts of transcription factors including E2F2 and c-myc as well as the transcripts of cell cycle regulatory proteins such as cyclin D2 and Ras (39-42). Although it has recently been shown that miRNAs play a critical role in SLE pathogenesis, the precise mechanisms are largely unknown. In these studies, we used *in vitro* approaches to investigate let-7a-mediated alterations to cell proliferation through E2F activation and the proinflammatory response through NF κ B translocation.

3.4. Materials and Methods

3.4.1. Cell culture

A mouse macrophage cell line (J774A.1) and a mouse mesangial cell line (MES 13) were purchased from ATCC (Manassas, VA, USA). The cells were grown in 75-mm² culture flasks at 37°C in 5% CO₂. Macrophages were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin (Cellgro, Manassas, VA, USA). During serum starvation, cells were given DMEM containing 1% FBS and 1% penicillin-streptomycin (Cellgro). Mesangial cells were cultured 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). For serum-starving medium, FBS was absent from the complete growth medium. For immune stimulation, LPS (Sigma-Aldrich, St. Louis, MO, USA) and IFN- γ (Cedarlane Laboratories Limited, Burlington, NC, USA) were added to the complete medium at a final concentration of 1 μ g/mL and 100 ng/mL, respectively. For IL-6 stimulation, recombinant IL-6 (eBioscience, San Diego, CA, USA) was added to the complete medium at a final concentration of 100 ng/mL. Experiments were performed from passage 9 – 15. All experimental conditions were run in triplicate.

3.4.2. miRNA/siRNA transfection

Macrophages were transfected with Lipofectamine 2000 transfection reagent according to the manufacturer's protocol (Life Technologies, Grand Island, NY, USA). Mesangial cells were transfected with *TransIT*-siQUEST transfection reagent according to the manufacturer's protocol (Mirus, Madison, WI, USA). The let-7a mimicking RNA was used to endogenously alter miRNA levels (Dharmacon RNAi Technologies, Lafayette, CO, USA). The E2F2 siRNA (Santa

Cruz Biotechnologies, Santa Cruz, CA, USA), IL-6 siRNA (Life Technologies), or NFκB p65 siRNA (Life Technologies) were used to endogenously alter RNA levels. The nontargeting control miRNA (Life Technologies) or si-GENOME control #1 (Santa Cruz Biotechnologies) were used as controls. Cells and supernatants were collected for analysis 24 hours post-stimulation unless otherwise noted.

3.4.3. MTT assay

Cultured cells were plated in 96-well plates at 1×10^4 cells/well and transfected for 24 hours with either the nontargeting control or let-7a mimicking miRNA. Nonstimulated cells received fresh complete media daily. Immune-stimulated cells received complete media containing LPS/IFN- γ 1 – 5 days post-transfection. Viable cells were measured 1 – 5 days post-stimulation according to the manufacturer's protocol (Life Technologies). The plate was read at 540 nM on a microplate spectrophotometer. Each condition was performed in triplicate.

3.4.4. Cell cycle analysis

Cultured cells were plated in 6-well plates at 1×10^6 and serum-starved overnight to reset the population to G₀ phase. The cells were transfected with either the nontargeting control or let-7a mimicking RNA. The cells received either complete media or complete media containing LPS/IFN- γ 24 hours post-transfection. Cells were collected after 24 or 48 hours as previously described (43). Briefly, cells were collected and fixed overnight in 70% ethanol at -20°C. The cells were resuspended at a final cell density of 1×10^6 cells/mL in 1X PBS containing propidium iodide (PI, 40 μ g/mL, Enzo Life Sciences, Farmingdale, NY, USA) and RNase (100 μ g/mL, Amresco, Solon, OH, USA). The cell suspensions were incubated at 37°C for 30

minutes prior to flow cytometric analysis. Populations in G₀-G₁, S, and G₂-M phase were measured by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA) and the data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA). Each condition was performed in triplicate.

3.4.5. 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, USA). RNA and miRNAs were quantified on a spectrophotometer (Nanodrop, Thermo Scientific, Waltham, MA, USA) and were stored at -80°C.

3.4.6. Real-time RT-PCR

Let-7a expression was measured by real-time RT-PCR using TaqMan microRNA assays according to the manufacturer's protocol (Applied Biosystems). The TaqMan Small RNA Assays were used according to the manufacturer's protocol (Applied Biosystems). *E2F2*, *E2F5*, and *NFκB p65* expression were measured using TaqMan Gene Expression assays according to the manufacturer's protocol (Applied Biosystems). Data were analyzed using the comparative C_T ($\Delta\Delta C_T$) method as previously described (44). All samples were run in triplicate.

3.4.7. Bioinformatics analysis

A database search was performed as previously described to determine cell cycle-associated mRNAs whose 3' UTR contain binding sites for let-7a (33).

3.4.8. Computational analysis of the let-7a promoter

The DNA sequence of let-7a was obtained from the National Center for Biotechnology Information (NCBI, 93 nucleotides from 48538179 – 48538272 on chromosome 13, Accession NR_029725.1). The promoter sequence (1 kb upstream of the let-7a sequence) was determined using the BLAT genome browser gateway (NCBI Assembly MGSCv37). The DNA sequence was then analyzed with PROMO software to identify putative binding sites for different transcription factors (45, 46). E2F2 and NFκB binding sites upstream of the let-7a start site were identified.

3.4.9. Chromatin immunoprecipitation (ChIP)

The SimpleChIP Enzymatic Chromatin IP Kit was used for ChIP and DNA purification according to the manufacturer's protocol (Cell Signaling, Danvers, MA, USA). ChIP was performed using ChIP-validated E2F2 (Santa Cruz Biotechnologies) and NFκB p65 (Cell Signaling) antibodies. Normal rabbit IgG (Cell Signaling) served as the nontargeting control antibody. Each condition was performed in triplicate. The enrichment of let-7a promoter DNA sequences was analyzed by real-time RT-PCR. DNA was amplified using FAM – labeled primers complementary to the let-7a promoter. The PCR primers (E2F2 forward 5' GAGGCTTATAGCCCAGGTGTATCA 3', E2F2 reverse 5' CCTGTGTAATGAGGGAAAAGAAAA 3'; NFκB forward 5' GAAATGTTTGTGTGTGGTAGTCAG 3', and NFκB reverse 5' TCAGGGAATAAGGTTGCCTATCGC3') were designed to amplify 120–135 bp fragments from selected genomic regions. All samples were run in triplicate.

3.4.10. Western blot

Nuclear extracts were purified using the Nuclear Extract Kit according to the manufacturer's protocol (Active Motif, Carlsbad, CA, USA). Protein concentration was determined using the Bradford protein assay and equal amounts of protein were resolved by electrophoresis onto a Criterion TGX gel (Bio-Rad, Hercules, CA, USA). The gel was transferred to a 0.45 μm nitrocellulose membrane, blocked with 5% nonfat milk, and incubated overnight at 4°C with antibodies specific to pRb (#8516, Cell Signaling), Rb (#9313, Cell Signaling), E2F2 (sc-633, Santa Cruz Biotechnologies), E2F5 (sc-999, Santa Cruz Biotechnologies), pI κ B (#5A5, Cell Signaling), I κ B (#44D4, Cell Signaling), NF κ B (#S536, Cell Signaling), or β -actin (Ambion, Life Technologies, Grand Island, NY, USA). The blot was visualized using the Amersham ECL Plus Western Blotting Detection System (GE Healthcare, Piscataway, NJ, USA). For multiple visualizations, the blot was stripped by gently shaking for 30 minutes at 50°C in stripping buffer. The stripping buffer contained 20 mL of 10% sodium dodecyl sulfate (SDS, Sigma-Aldrich), 12.5 mL of 0.5 M Tris HCl (pH 6.8, Sigma-Aldrich), and 0.8 mL of 2-Mercaptoethanol (Sigma-Aldrich) mixed in 67.5 mL of distilled water. Western blots were quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA). β -actin served as the internal control.

3.4.11. NF κ B activation assay

Nuclear extracts were purified using the Nuclear Extract Kit according to the manufacturer's protocol (Active Motif). To detect NF κ B activation, the TransAM NF κ B p65 transcription factor assay kit was used according to the manufacturer's protocol using (Active

Motif). The plate was read at 450 nm on a Spectramax 340PC microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). All samples were run in duplicate.

3.4.12. ELISA

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

3.4.13. Statistical analysis

Statistical analysis was performed using Student's unpaired *t*-test (two-tailed). One-way analysis of variance (ANOVA) was used to analyze the MTT experiment. *P* values less than 0.05 were considered statistically significant.

3.5. Results

3.5.1. Let-7a increases cell proliferation in immune-stimulated cells by inducing S phase entry

In order to examine the physiological effects of let-7a overexpression on cell proliferation *in vitro*, macrophages (J774A.1) and mesangial cells (MES 13) were transfected and cell proliferation was measured over a 5 day period post-transfection. Cells were immune-stimulated 1 – 5 days post-transfection. Untransfected cells and cells transfected with a nontargeting control miRNA served as the controls. Transfection was confirmed by real time RT-PCR as previously described (Figure S1) (33). Without stimulation, there were no significant changes in macrophage or mesangial cell proliferation over time (data not shown). When J774A.1 macrophages were stimulated with LPS/IFN- γ , there was a significant increase in the number of let-7a-transfected cells over time compared to the control (Figure 1A). Similarly, there was a significant increase in the proliferation of stimulated let-7a-transfected MES 13 mesangial cells over time compared to the control (Figure 1B).

Cell cycle distribution was measured using flow cytometry post-transfection. In nonstimulated J774A.1 macrophages, let-7a did not cause any significant changes to cell cycle distribution compared to controls either 24 hours (data not shown) or 48 hours post-transfection (Figure 1C). In macrophages stimulated with LPS/IFN- γ for 24 hours, there were no differences in cell cycle distribution between untransfected macrophages or macrophages transfected with let-7a or the nontargeting control miRNA (data not shown). After 48 hours of stimulation, there was a significant decrease in the number of let-7a-transfected macrophages in G₁ phase and a significant increase in the number of let-7a-transfected cells in S and M phase compared to the control (Figure 1D). In nonstimulated MES 13 mesangial cells, cell cycle distribution was

unchanged 24 hours (Figure 1E) or 48 hours post-transfection (data not shown). After 24 hours of stimulation, there was a significant decrease in the number of stimulated let-7a transfected cells in G₁ phase compared to the controls, with a concomitant increase in the number of these cells in S and G₂ phases (Figure 1F). G₂ distribution remained significantly higher after 48 hours of stimulation (data not shown, $p < 0.05$). These data indicate that let-7a promotes stimulated cell proliferation by inducing cell cycle progression through S and G₂ phases.

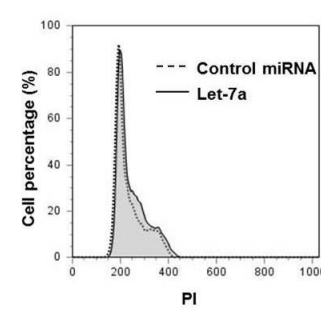
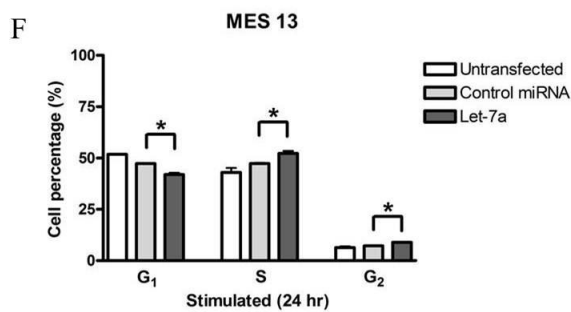
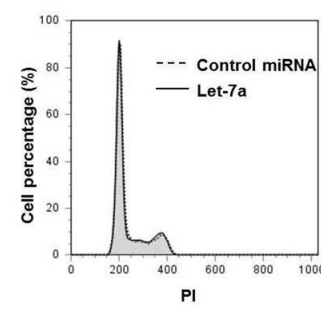
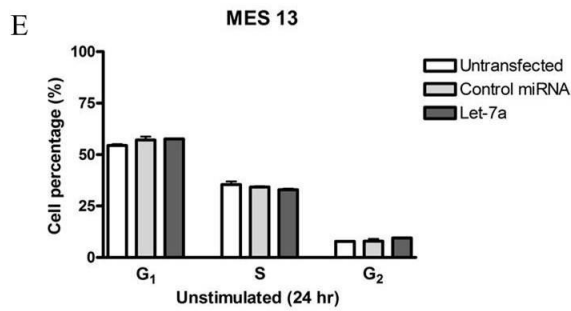
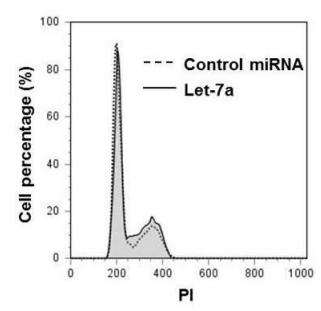
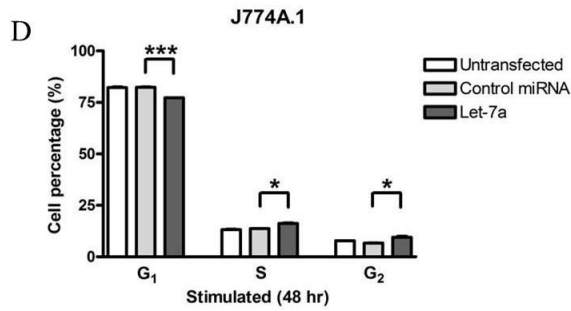
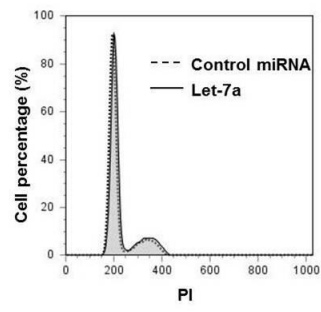
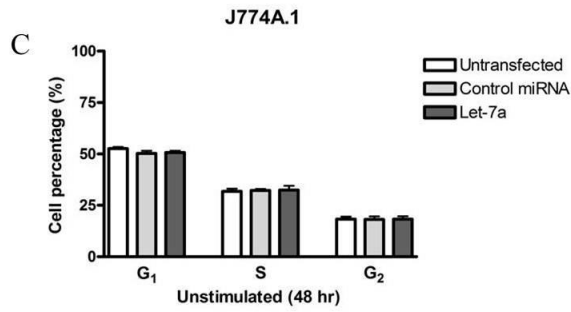
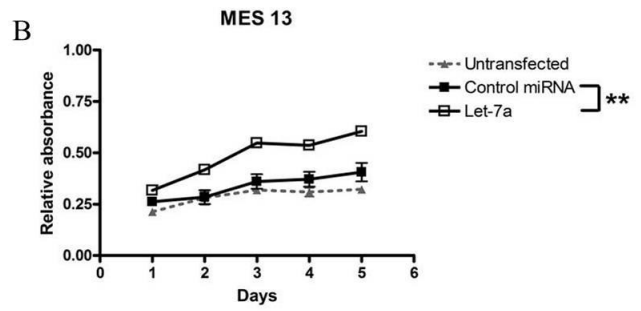
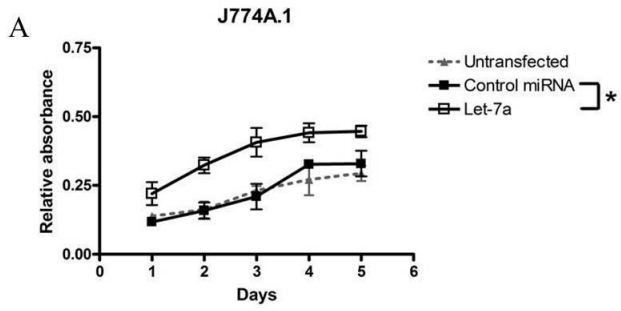


Figure 1. Let-7a induces immune-stimulated J774A.1 macrophages and MES 13 mesangial cells to proliferate by promoting entry into S phase. (A) In immune-stimulated J774A.1 macrophages, there is a significant increase in cell proliferation over time in let-7a-transfected macrophages compared to the transfected control. (B) Over time, there is a significant increase in the number of stimulated, let-7a-transfected MES 13 mesangial cells proliferating compared to the transfected control. (C) Cell cycle distribution is unchanged 48 hours post-transfection in nonstimulated J774A.1 macrophages. (D) In immune-stimulated J774A.1 macrophages, there is a significant decrease in the number of let-7a-transfected cells in G₁ phase and a significant increase in the number of let-7a-transfected cells in S and M phase. (E) Cell cycle distribution is unchanged 24 hours post-transfection in nonstimulated MES 13 mesangial cells. (F) In immune-stimulated MES 13 mesangial cells, there is a significant decrease in the number of let-7a-transfected cells in G₁ phase and a significant increase in the number of let-7a-transfected cells in S phase compared to the control. (A – F) represent 3 independent experiments run in triplicate. Flow cytometry image is representative of 3 independent experiments. PI: propidium iodide. The nontargeting miRNA is miR-67. The final concentration of the miRNAs was 25 nM. Error bars represent the SEM. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

3.5.2. Let-7a increases retinoblastoma protein (Rb) phosphorylation

Since we observed increased proliferation in cells overexpressing let-7a, we sought to determine the mechanism by which let-7a induces cell cycle entry. The E2F family of transcription factors plays a major role in the G₁ – S transition in the cell cycle.

Hyperphosphorylated Rb releases E2F activators to transcribe genes whose products are important in cell cycle progression. Due to differing states of phosphorylation, different sites of

phosphorylation, and different stoichiometry, total Rb can appear as one or two bands in Western blotting (47 – 49). In order to verify that let-7a induces E2F activation by increasing Rb phosphorylation (pRb), we measured pRb by Western blot in immune-stimulated cells post-miRNA transfection. Untransfected (U) cells and cells transfected with a nontargeting control miRNA (NC) served as the controls. In nonstimulated J774A.1 macrophages, there were no differences in pRb between untransfected macrophages or macrophages transfected with let-7a (L) or the nontargeting control miRNA (Figure 2A, Figure S2). In macrophages transfected with let-7a, there was an increase in pRb compared to the stimulated controls. pRb was undetectable in nonstimulated MES 13 mesangial cells (Figure 2B, Figure S3). There was a marked increase in pRb in immune-stimulated mesangial cells overexpressing let-7a.

While it has been established that IL-6 increases pRb, we recently showed that let-7a overexpression increases IL-6 production (33, 49). To confirm that let-7a-mediated phosphorylation of Rb is facilitated by IL-6, cells were treated with recombinant IL-6 and pRb was measured by Western blot. Cells were treated with IL-6 for 1 hour due to previous reports that have shown that phosphorylated Rb is predominant at this time (49). Compared to nonstimulated cells, J774A.1 macrophages had increased pRb after stimulation with IL-6 (Figure 2C, Figure S4). Similarly, there was an increase in pRb in MES 13 mesangial cells after IL-6 stimulation compared to the control (Figure 2D, Figure S5). To confirm that increased Rb phosphorylation is mediated by IL-6-mediated signaling, we next transfected J774A.1 macrophages and MES 13 mesangial cells with let-7a mimicking RNAs and IL-6 siRNA (si-IL-6). Cells were stimulated 24 hours post-transfection with LPS/IFN- γ . Untransfected cells and cells transfected with a nontargeting control miRNA served as the controls. Transfection efficiency was confirmed by RT-PCR (Figure 2E, F). Overall, there was an increase in pRb in

stimulated cells (Figure 2F, Figure S6). Compared to the stimulated control, there was a decrease in pRb when let-7a and si-IL-6 were cotransfected in J774A.1 macrophages. There was a slight decrease in pRb in macrophages cotransfected with the nontargeting control miRNA and si-IL-6. Similarly, there was a marked increase in pRb in MES 13 mesangial cells that were stimulated compared to nonstimulated cells (Figure 2H, Figure S7). There was a decrease in pRb in MES 13 mesangial cells cotransfected with let-7a and si-IL-6 compared to the stimulated controls. These results show that let-7a increases E2F activation by increasing pRb and mediates Rb phosphorylation via IL-6 in stimulated cells.

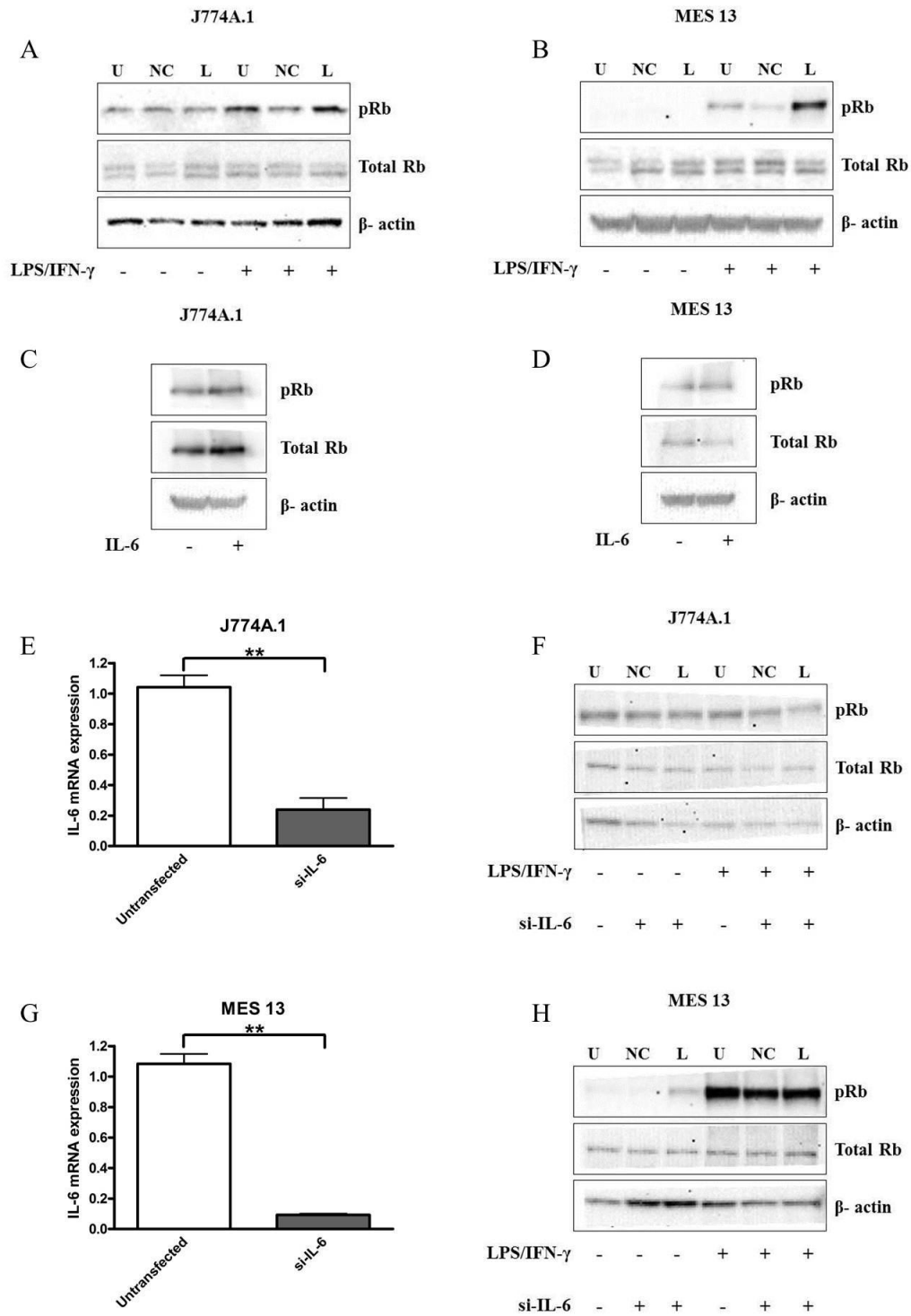


Figure 2. Let-7a regulates Rb phosphorylation. (A) Without immune stimulation, phosphorylated Rb (pRb) is unchanged in untransfected (U) or transfected J774A.1 macrophages. There is an increase in pRb in immune-stimulated macrophages transfected with

let-7a (L) compared to the transfected control (NC). (B) pRb is undetectable in untransfected (U) or transfected MES 13 mesangial cells that were not immune-stimulated. pRb is increased in stimulated, let-7a transfected (L) mesangial cells compared to the transfected control (NC). (C) pRb is increased in J774A.1 macrophages treated with recombinant IL-6 compared to nonstimulated cells. (D) There is an increase in pRb in MES 13 mesangial cells treated with IL-6 compared to the control. (E) Real-time RT-PCR shows *IL-6* expression is significantly decreased in immune-stimulated J774A.1 macrophages transfected with IL-6 siRNA (si-IL-6). (F) *IL-6* expression is significantly decreased in stimulated MES 13 mesangial cells transfected with si-IL-6. (G) There is a decrease in pRb when *IL-6* is knocked down in stimulated J774A.1 macrophages. (H) pRb is decreased when *IL-6* is knocked down in immune-stimulated MES 13 mesangial cells. (A – H) are representative of 3 independent experiments. β -actin served as the loading control. The nontargeting miRNA is miR-67. The final concentration of the miRNAs was 25 nM. Error bars represent the SEM. $*p < 0.05$. $**p < 0.01$. $***p < 0.0005$.

3.5.3. Let-7a alters the expression of the E2F family of transcription factors

Next we sought to determine if let-7a alters the expression of cell cycle regulators. We used independent computational programs as previously described to predict potential miRNA targets that may be involved in the G₁ – S transition (33). Let-7a was predicted to target the E2F transcription factor family, which has conserved let-7a binding sites in their 3' UTRs. We measured *E2F* expression in immune-stimulated cells post-transfection in order to determine the effects of overexpressed let-7a. J774A.1 macrophages and MES 13 mesangial cells were transfected with let-7a or the control miRNA and stimulated with LPS/IFN- γ 24 hours post-transfection. Real time RT-PCR was used to measure *E2F* expression. Western blot was used to

measure E2F production. Expression of the cell cycle activator *E2F2* was significantly increased in immune-stimulated J774A.1 macrophages transfected with let-7a compared to the control (Figure 3A). Western blot showed there was a decrease in E2F2 in nonstimulated macrophages transfected with let-7a (Figure 3B, Figure S8). However, when let-7a-transfected macrophages were immune-stimulated, E2F2 was unchanged compared to the stimulated controls. This is consistent with our previous work that showed the effect of let-7a on the target mRNA is altered upon immune stimulation (33). Expression of the cell cycle inhibitor *E2F5* was significantly decreased in let-7a-transfected macrophages that were immune-stimulated (Figure 3C). Western blot showed there was a decrease in E2F5 in nonstimulated or stimulated macrophages transfected with let-7a (Figure 3D, Figure S9). In MES 13 mesangial cells, *E2F2* expression was significantly increased in stimulated cells transfected with let-7a compared to the control (Figure 3E). *E2F2* was decreased in nonstimulated mesangial cells transfected with let-7a (Figure 3F, Figure S10). Like J774A.1 macrophages, *E2F2* was unchanged in let-7a-transfected mesangial cells that were stimulated compared to stimulated controls. *E2F5* expression was significantly decreased in immune-stimulated, let-7a-transfected mesangial cells (Figure 3G). Western blot showed there was a decrease in E2F5 in nonstimulated or stimulated cells (Figure 3H, Figure S11). Taken together, these results indicate that stimulated cells overexpressing let-7a have decreased *E2F5* expression and reduced E2F5 production. The increase in *E2F2* expression in stimulated cells overexpressing let-7a does not result in increased production of E2F2.

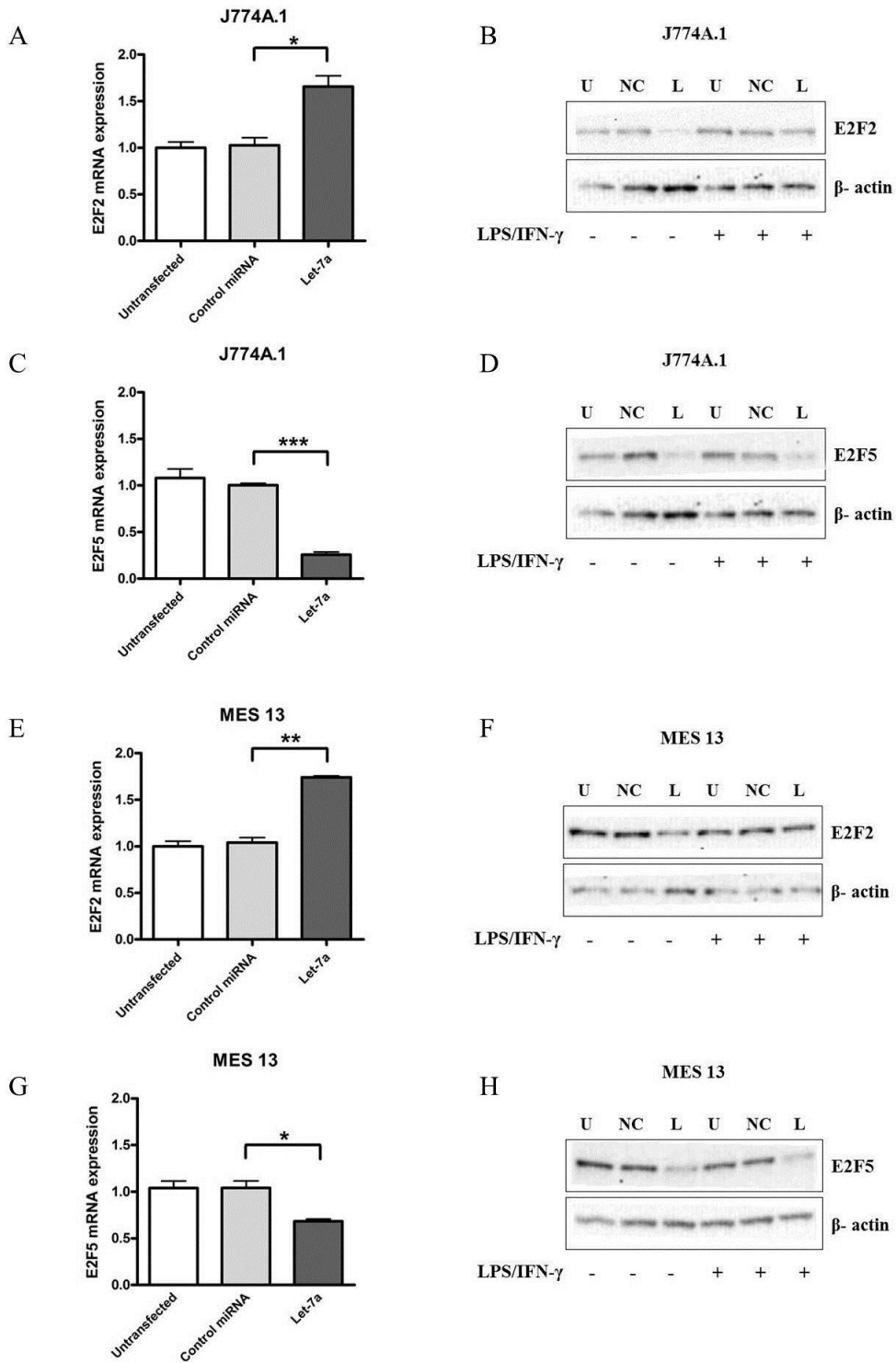


Figure 3. Let-7a targets the E2F family of transcription factors. (A) *E2F2* expression is significantly increased in immune-stimulated J774A.1 macrophages transfected with let-7a. (B) There is a decrease in *E2F2* in nonstimulated J774A.1 macrophages transfected with let-7a.

With LPS/IFN- γ stimulation, E2F2 is unchanged in untransfected or transfected cells. (C) Immune-stimulated J774A.1 macrophages overexpressing let-7a express significantly less *E2F5* compared to the control. (D) E2F5 is decreased in let-7-transfected J774A.1 macrophages with or without immune stimulation. (E) Stimulated MES 13 mesangial cells overexpressing let-7a express significantly more *E2F2* compared to the control. (F) E2F2 is decreased in nonstimulated MES 13 mesangial cells that were transfected with let-7a. E2F2 is unchanged in cells that were transfected with let-7a or the control miRNA and stimulated with LPS/IFN- γ . (G) *E2F5* expression is significantly increased in stimulated MES 13 mesangial cells transfected with let-7a. (H) E2F5 is decreased in nonstimulated or immune-stimulated MES 13 mesangial cells that were transfected with let-7a. (A, C, E, G) represent 3 independent experiments run in triplicate. (B, D, F, H) are representative of 3 independent experiments. β -actin served as the loading control. The nontargeting miRNA is miR-67. The final concentration of the miRNAs was 25 nM. Error bars represent the SEM. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.0005$.

3.5.4. The let-7a promoter is regulated by E2F2

We next examined potential transcription factors with binding sites in the let-7a promoter. We used computational analysis to identify putative binding sites for different transcription factors 1 kb upstream of the let-7a start sequence due to previous reports that 1 kb upstream is sufficient to induce promoter activity (50). E2F2 was selected for further analysis in let-7a-mediated cell proliferation after binding sites upstream of the let-7a start site were identified. We measured E2F2 bound to let-7a promoter-enriched DNA post-stimulation using real time RT-PCR and normal rabbit IgG as the control antibody. After ChIP, there were no differences in promoter binding in nonstimulated J774A.1 macrophages with either the control or

E2F2 antibody (Figure 4A). Compared to the nonstimulated control, there was an increase in E2F2 binding to DNA enriched for the let-7a promoter in macrophages stimulated with LPS/IFN- γ . Likewise, there were no differences in promoter binding in nonstimulated MES 13 mesangial cells with either the control or E2F2 antibody (Figure 4B). There was a significant increase in E2F2 binding to let-7a promoter-enriched DNA post-stimulation.

Because the let-7a promoter contains binding sites for E2F2, we next examined if knockdown of E2F2 altered let-7a expression in stimulated cells. Untransfected cells and cells transfected with a nontargeting siRNA served as the controls. Transfection of si-E2F2 significantly decreased *E2F2* expression in immune-stimulated J774A.1 macrophages, confirming transfection efficiency (Figure 4C). *E2F2* expression was also significantly decreased in stimulated MES 13 mesangial cells transfected with si-E2F2 (Figure 4D). Let-7a expression was significantly decreased when E2F2 was knocked down in immune-stimulated macrophages (Figure 4E) and mesangial cells (Figure 4F). In order to determine if IL-6 production is decreased by knocking down E2F2, we measured IL-6 production by ELISA. IL-6 was undetectable in nonstimulated cells (Figure 4G). With LPS/IFN- γ stimulation, IL-6 was significantly decreased in immune-stimulated J774A.1 macrophages after transfection with si-E2F2. IL-6 production was also significantly decreased when E2F2 was knocked down in stimulated MES 13 mesangial cells (Figure 4H). These results indicate that the let-7a promoter is regulated by E2F2 in stimulated cells.

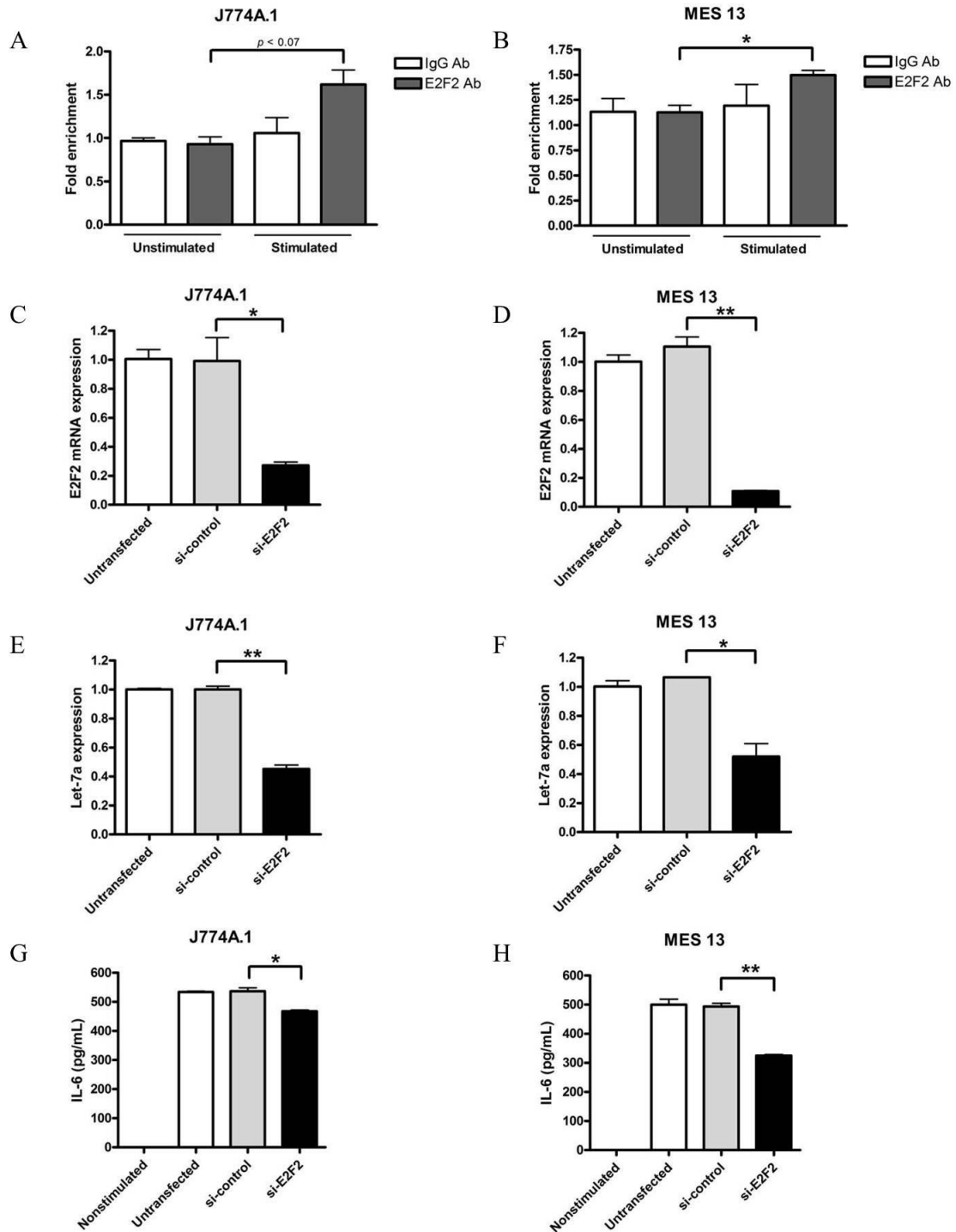


Figure 4. E2F2 targets the let-7a promoter and knockdown of E2F2 decreases let-7a expression and IL-6 production. (A) In nonstimulated J774A.1 macrophages, there are no differences in control or E2F2 antibody binding to DNA enriched for the let-7a promoter. In

immune-stimulated J774A.1 macrophages, there is an increase in E2F2 binding to let-7a promoter-enriched DNA compared to the nonstimulated control. (B) In nonstimulated MES 13 mesangial cells, there are no differences in let-7a promoter-enriched DNA between the control or E2F2 antibody. There is a significant increase in let-7a promoter-enriched DNA bound by E2F2 in stimulated MES 13 mesangial cells compared to the nonstimulated control. (C) Transfection of si-E2F2 significantly knocks down *E2F2* expression in immune-stimulated J774A.1 macrophages. (D) Transfection of si-E2F2 significantly knocks down *E2F2* expression in stimulated MES 13 mesangial cells. (E) Let-7a expression is significantly decreased in immune-stimulated J774A.1 macrophages after si-E2F2 transfection. (F) Let-7a expression is significantly decreased in stimulated MES 13 mesangial cells transfected with si-E2F2. (G) IL-6 production is significantly decreased in immune-stimulated J774A.1 macrophages after transfection with si-E2F2. (H) In stimulated MES 13 mesangial cells transfected with si-E2F2, IL-6 production is significantly decreased. (A – F) represent 3 independent experiments run in triplicate. (G – H) represent 3 independent experiments run in duplicate. The control antibody is normal rabbit IgG. The nontargeting siRNA control (si-control) has no gene targets in mouse cells. The final concentration of the siRNAs was 25 nM. Error bars represent the SEM. * $p < 0.05$. ** $p < 0.005$.

3.5.5. Let-7a increases NF κ B translocation in immune-stimulated cells

Since LPS/IFN- γ stimulation activates NF κ B via the canonical pathway, we measured let-7a-induced NF κ B p65 translocation by Western blot. Nonstimulated (NS) cells, untransfected (U) cells, and cells transfected with a nontargeting control miRNA (NC) served as the controls. We chose to measure the nuclear translocation of NF κ B 15, 30, and 60 minutes

post-stimulation due to previous reports that have indicated the phosphorylation of I κ B returns to baseline levels after 60 minutes of immune stimulation (51). There was a slight increase in NF κ B activation 30 minutes post-stimulation in let-7a-transfected (L) J774A.1 macrophages compared to the nontargeting control (Figure 5A, Figure S12). After 15 or 60 minutes of stimulation, there were no differences in NF κ B translocation in macrophages transfected with let-7a compared to the control miRNA. Compared to macrophages transfected with let-7a or the control miRNA, NF κ B activation was faster in stimulated macrophages that were not transfected. We next measured phosphorylated I κ B by Western blot. pI κ B was undetectable in macrophages stimulated with LPS/IFN- γ for 15 minutes (Figure 5B, Figure S13). There was a slight increase in pI κ B in let-7a-transfected cells stimulated for 30 minutes, which corresponds to increased NF κ B translocation at this time point. By 60 minutes of immune stimulation, there was a decrease in I κ B phosphorylation in macrophages transfected with let-7a compared to the control miRNA. Next we quantified let-7a-induced NF κ B p65 translocation by ELISA. NF κ B was undetectable in nonstimulated J774A.1 macrophages. As expected, NF κ B activation was significantly increased in macrophages compared to the control after 30 minutes of immune stimulation (Figure 5C). There were no significant differences in NF κ B activation 15 or 60 minutes post-stimulation compared to the transfected control.

When MES 13 mesangial cells were analyzed by Western blot, there was a slight increase in NF κ B p65 translocation in let-7a transfected cells that were immune-stimulated for 15 minutes (Figure 5D, Figure S14). There were no differences in NF κ B translocation at 30 or 60 minutes. We next measured phosphorylated I κ B by Western blot. In mesangial cells stimulated for 15 minutes, there was an increase in pI κ B in let-7a transfected cells, which is consistent with increased nuclear NF κ B translocation at that time (Figure 5E, Figure S15). pI κ B was unchanged

in cells stimulated for 30 or 60 minutes. When NFκB p65 activation was quantified by ELISA, there was a significant increase after 15 minutes of stimulation in mesangial cells transfected with let-7a (Figure 5F). There were slight yet insignificant increases in NFκB translocation at 30 and 60 minutes in let-7a-transfected cells. NFκB was undetectable in nonstimulated mesangial cells. Taken together, these data indicate that immune-stimulated cells overexpressing let-7a have increased NFκB activation compared to controls.

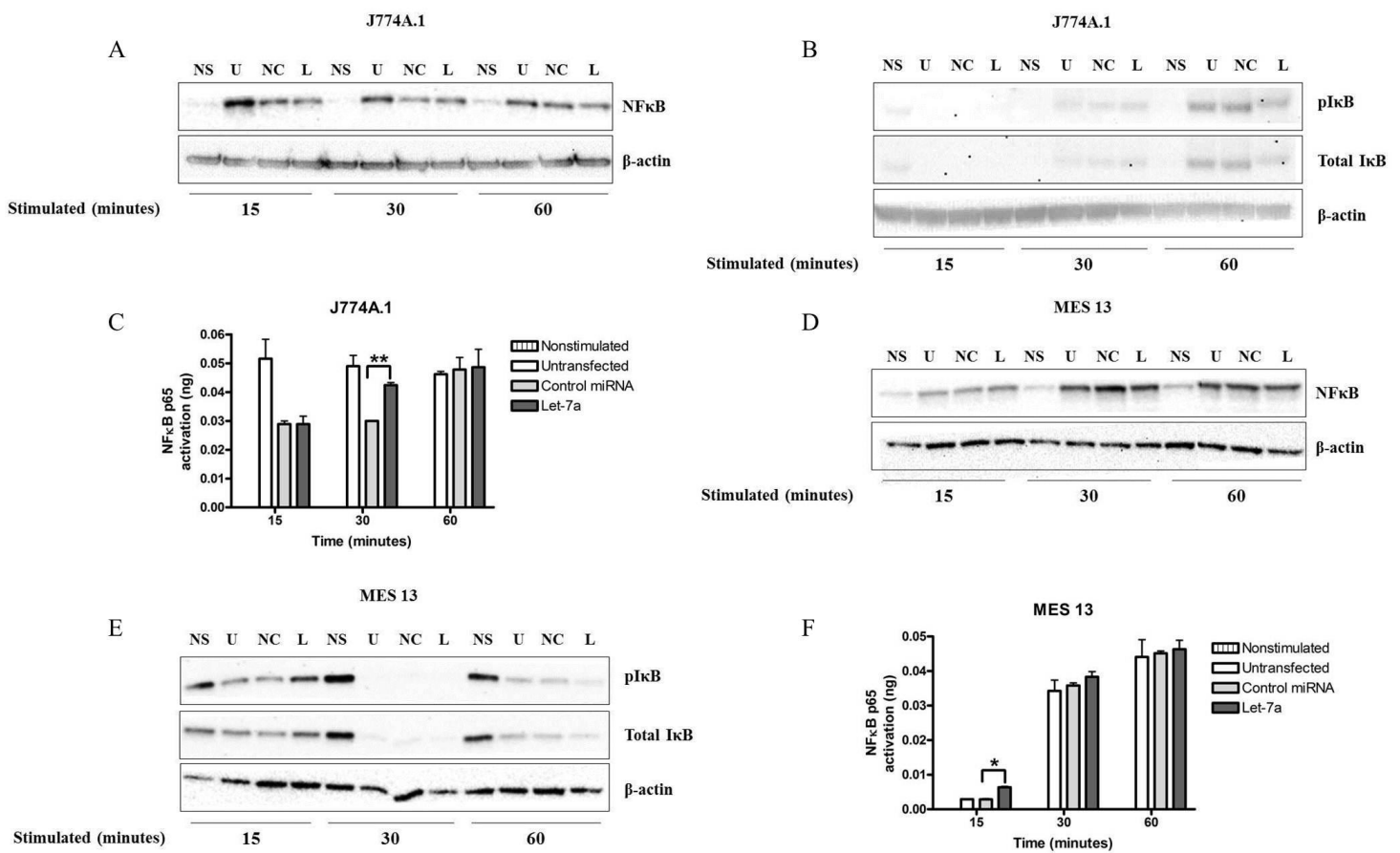


Figure 5. Let-7a increases NFκB translocation into the nucleus. (A) NFκB is almost undetectable in nonstimulated (NS) J774A.1 macrophages. After 30 minutes of immune stimulation, there is an increase in NFκB translocation in J774A.1 macrophages transfected with let-7a (L) compared to the transfected control (NC). Overall, cells that were stimulated but not

transfected (U) stimulated faster than cells that were transfected. (B) I κ B phosphorylation (pI κ B) is almost undetectable in nonstimulated (NS) J774A.1 macrophages. There are no differences in pI κ B in J774A.1 macrophages that were immune-stimulated for 15 minutes regardless of transfection status. After 30 minutes of immune stimulation, there is an increase in pI κ B in let-7a-transfected (L) J774A.1 macrophages compared to the untransfected (U) or transfected control (NC). There is a decrease in pI κ B in J774A.1 macrophages transfected with let-7a and immune-stimulated for 60 minutes. (C) NF κ B is undetectable in nonstimulated J774A.1 macrophages. NF κ B activation is significantly increased in J774A.1 macrophages 30 minutes post-stimulation compared to the transfected control. (D) NF κ B is almost undetectable in nonstimulated (NS) MES 13 mesangial cells. After 15 minutes of immune stimulation, NF κ B translocation is increased in let-7a-transfected MES 13 mesangial cells (L) compared to the transfected control (NC). (E) pI κ B is unchanged in nonstimulated (NS) MES 13 macrophages. There is a decrease in pI κ B in all samples 30 minutes post-stimulation compared to samples stimulated for 15 minutes. After 60 minutes of immune stimulation, pI κ B begins to return to levels seen at 15 minutes post-stimulation. (F) NF κ B is undetectable in nonstimulated MES 13 mesangial cells. NF κ B activation is significantly increased in MES 13 mesangial cells 15 minutes post-stimulation compared to the control. NF κ B activation is increased after 30 and 60 minutes of immune stimulation compared to controls. NF κ B is undetectable in nonstimulated cells. (A, B, D, E) are representative of 3 independent experiments. (C and F) represent 3 independent experiments run in duplicate. β -actin served as the loading control. The nontargeting miRNA is miR-67. The final concentration of the miRNAs was 25 nM. Error bars represent the SEM. * $p < 0.05$. ** $p < 0.01$.

3.5.6. The let-7a promoter is regulated by NFκB

It has recently been reported that the let-7a promoter contains NFκB binding sites that are highly responsive to the p65 subunit (50). Computational analysis verified putative binding sites for NFκB 1 kb upstream of the let-7a start sequence. We measured NFκB bound to let-7a promoter-enriched DNA after 15, 30, and 60 minutes of immune stimulation using real time RT-PCR and normal rabbit IgG as the control antibody. In macrophages immune-stimulated for 30 or 60 minutes, there was a significant increase in NFκB binding in DNA enriched for the let-7a promoter (Figure 6A). In mesangial cells, there was a significant increase in NFκB binding after 60 minutes of immune stimulation (Figure 6B). These data are consistent with previous reports that have shown increased NFκB binding to the let-7a promoter after 60 minutes of stimulation (13).

We next examined if knockdown of NFκB p65 alters let-7a expression in stimulated cells. Untransfected cells and cells transfected with a nontargeting siRNA served as the controls. Transfection of si-NFκB significantly decreased *NFκB p65* expression in immune-stimulated J774A.1 macrophages (Figure 6C) and MES 13 mesangial cells (Figure 6D), confirming transfection efficiency. Let-7a expression was significantly decreased when NFκB was knocked down in stimulated macrophages (Figure 6E). There was also a significant decrease in let-7a expression in stimulated mesangial cells (Figure 5F). These results confirm that the let-7a promoter is regulated by NFκB in immune-stimulated cells.

Several studies have shown that NFκB is an important mediator of IL-6 transcription (52, 53). In order to confirm that knocking down NFκB p65 decreases the production of IL-6, we measured IL-6 production by ELISA. IL-6 was undetectable in nonstimulated J774A.1 macrophages (Figure 6G). IL-6 was significantly decreased in immune-stimulated J774A.1

macrophages after transfection with si-NFκB p65. In nonstimulated MES 13 mesangial cells, IL-6 production was undetectable (Figure 6H). IL-6 production was also significantly decreased when NFκB p65 was knocked down in LPS/IFN-γ-stimulated MES 13 mesangial cells.

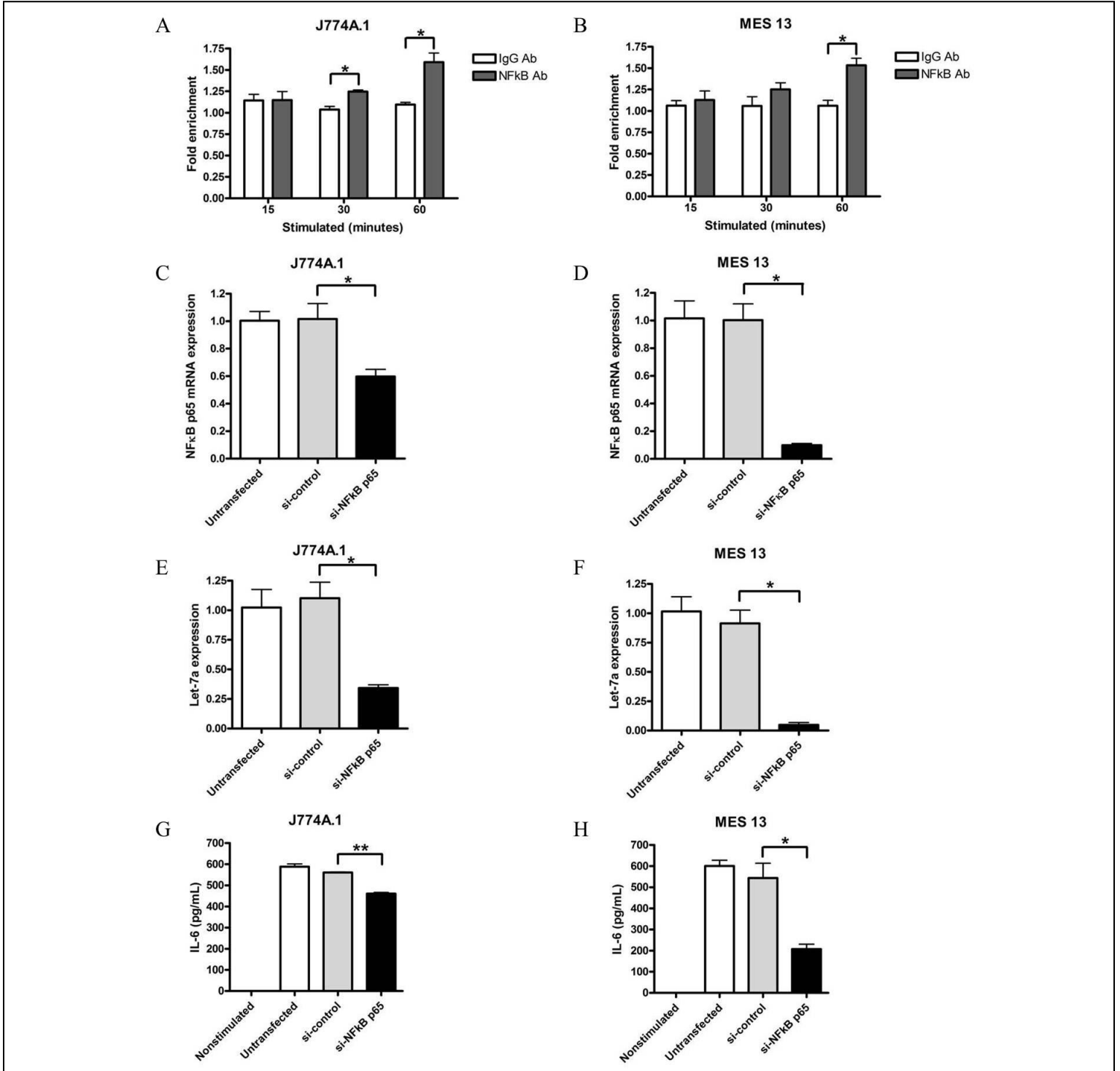


Figure 6. NFκB targets the let-7a promoter. (A) In J774A.1 macrophages immune-stimulated for 30 and 60 minutes, there is significantly more NFκB bound to DNA enriched for the let-7a promoter compared to the control antibody. (B) In MES 13 mesangial cells stimulated for 60 minutes, there is a significant increase in NFκB bound to let-7a promoter-enriched DNA compared to the control antibody. (C) Transfection of si-NFκB p65 significantly knocks down *NFκB p65* expression in immune-stimulated J774A.1 macrophages. (D) Transfection of si-NFκB p65 significantly knocks down *NFκB p65* expression in stimulated MES 13 mesangial cells. (E) Let-7a expression is significantly decreased in immune-stimulated J774A.1 macrophages after si-NFκB p65 transfection. (F) In stimulated MES 13 mesangial cells transfected with si-NFκB p65, let-7a expression is significantly decreased. (G) In stimulated J774A.1 macrophages transfected with si-NFκB p65, IL-6 production is significantly decreased. (H) IL-6 production is significantly decreased in stimulated MES 13 mesangial cells transfected with si-NFκB p65. (A – F) represent 3 independent experiments run in triplicate. (G – H) represent 3 independent experiments run in duplicate. The control antibody is normal rabbit IgG. The nontargeting siRNA control (si-control) has no gene targets in mouse cells. The final concentration of the siRNAs was 25 nM. Error bars represent the SEM. * $p < 0.05$. ** $p < 0.01$.

3.6. Discussion

One hallmark of SLE is cell proliferation due to inflammatory stimuli (54-56). Mesangial proliferation is one of the distinctive histological features of LN, which affects up to 70% of SLE patients (57, 58). miRNAs may contribute to LN pathogenesis by altering cell proliferation, proinflammatory mediator production, innate immune cell responses, and TLR and NFκB signaling pathways. In SLE, let-7a is overexpressed in renal tissue and mesangial cells and may contribute to elevated IL-6 production (33, 36). In these studies, we provide evidence that let-7a overexpression increases cell proliferation by increasing E2F activation through IL-6-mediated Rb phosphorylation. Cells overexpressing let-7a have increased expression of the cell cycle activator *E2F2* and decreased expression of the repressor *E2F5*. Let-7a post-transcriptionally modifies *E2F5* expression, promoting cell cycle entry. NFκB activation is increased in immune-stimulated cells overexpressing let-7a. In addition, our results indicate that the let-7a promoter is regulated by E2F2 and NFκB. In stimulated macrophages or mesangial cells, knocking down E2F2 or NFκB significantly decreased let-7a expression and IL-6 production.

Our results indicate that inflammation activates a positive feedback loop linking NFκB activation, let-7a overexpression, IL-6 production, and E2F-mediated cell proliferation. We propose the following autocrine signaling pathway that induces stimulated cells overexpressing let-7a to proliferate and increase cytokine production. When an immune complex binds to its receptor, the canonical NFκB pathway is initiated through IκB phosphorylation, enabling NFκB to translocate to the nucleus and transcribe genes including let-7a (amongst others) (50, 59). Let-7a is continually transcribed due to perpetual NFκB translocation in the proinflammatory environment of SLE. Increased let-7a expression perpetuates the inflammatory response by

increasing IL-6 production, which further increases the activation of NFκB (33). Increased IL-6 production also increases cell proliferation; IL-6 promotes the phosphorylation of Rb via activation of the JAK/STAT pathway (60). Phosphorylated Rb releases E2F to transcribe genes that are fundamental to cell cycle progression as well as let-7a (61). By increasing IL-6-mediated Rb phosphorylation, increased let-7a expression can contribute to increased cell proliferation.

While it has recently been shown that the expression of let-7a has major implications on cancer cell proliferation, the precise outcomes of overexpressed let-7a in SLE are still being elucidated (62-65). In these studies, we demonstrate that cells overexpressing let-7a have increased expression of the cell cycle activator *E2F2* and decreased expression of the repressor *E2F5*. Because the 3' UTR of E2F transcripts contain potential binding sites for let-7a, let-7a may be directly targeting E2F mRNA, leading to altered protein production (66). Conversely, let-7a may indirectly modify *E2F* expression by targeting upstream signaling cascades. Overall, the current studies indicate that let-7a overexpression in immune-stimulated cells increases E2F-mediated cell proliferation via IL-6-induced Rb phosphorylation. Increased mesangial cell proliferation leads to an increase in extracellular matrix deposition, which results in a reduced rate of glomerular filtration due to the loss of functioning nephrons (19). Because let-7a is overexpressed in various tissues and cell types in SLE, increased let-7a expression may contribute to increased cell proliferation and the resulting increase in extracellular matrix deposition that are characteristic of the disease (67).

Although dysregulated E2F expression has been demonstrated in tumorigenesis, the expression of E2F in SLE is largely unknown (68, 69). Genome-wide association studies have revealed that E2F may play a role in SLE pathogenesis by transcribing SLE-associated genes

(25). In the anti-Thy1.1 model of glomerulonephritis, the levels of cyclin A and cyclin-dependent kinase 2, products of E2F-mediated transcription, are significantly increased during mesangial proliferation (70). By administering antisense oligonucleotides (ODNs) containing the consensus E2F-binding sequence, studies have shown that inhibition of E2F is sufficient to reduce mesangial cell proliferation. E2F ODNs transfected into mesangial cells *in vitro* reduced serum-stimulated mesangial cell proliferation (71). *In vivo* administration of E2F ODNs after the induction of anti-Thy1.1 nephritis caused a reduction in glomerular cellularity compared to controls (72). Our results indicate that the let-7a promoter is regulated by E2F; knocking down E2F2 in stimulated macrophages and mesangial cells significantly decreased let-7a expression. Inhibition of E2F in mesangial cells may ameliorate glomerulonephritis, in part, by reducing the expression of let-7a.

Our studies show that NFκB activation is increased in immune-stimulated cells overexpressing let-7a. In addition, our results confirm that the let-7a promoter is regulated by NFκB.(50) In SLE, cells are continually stimulated by the proinflammatory microenvironment and binding of immune complexes, which may result in the overexpression of let-7a due to induction of the NFκB signaling pathway (73, 74). Let-7a increases NFκB translocation, leading to a positive feedback loop that induces more let-7a expression. Increased NFκB activation and increased let-7a expression also increase IL-6 production, which perpetuates this proinflammatory autocrine signaling pathway (33, 75). Studies have shown that blocking IL-6 downstream signaling suppresses the production of anti-dsDNA autoantibodies, reduces the proliferation of B cells, and partially downregulates T cells in SLE patients (16, 76). By blocking IL-6 binding, antibodies against IL-6 may reduce the proinflammatory cascade by reducing NFκB-induced overexpression of let-7a in mesangial cells.

The current studies identify a let-7a-mediated pathway that induces stimulated cells overexpressing let-7a to divide. By increasing IL-6 production, let-7a increases the phosphorylation of Rb, enabling E2F to transcribe genes involved in the G₁-S phase transition. We reveal a positive feedback mechanism in which immune stimulation induces NFκB activation, resulting in the upregulation of let-7a genes and a consequent increase in IL-6 production, which contributes to an increase in NFκB translocation into the nucleus. These results show that let-7a overexpression can contribute to the inflammatory response in diverse ways, including inflammatory mediator production and the induction of cell proliferation. Because the expression of let-7a is significantly increased with immune stimulation and is overexpressed in SLE, targeting let-7a may emerge as a new therapeutic intervention in the treatment of hyperplasia and inflammation in lupus.

3.7. References

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Chapter 4

Cellular and urinary microRNA alterations in NZB/W mice with hydroxychloroquine or prednisone treatment

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4.2. Abstract

Determining alterations to disease-associated miRNAs induced by specific therapeutics may allow the use of tailored therapy in lupus. We determined miRNA alterations in female NZB/W lupus mice treated with hydroxychloroquine (HCQ) or prednisone (PRED) for 12 weeks beginning at 24 weeks-of-age. B cell, PBMC, and urinary miR-let-7a expression were decreased with HCQ or PRED treatment. HCQ or PRED treatment reduced miR-21 expression in mesangial cells, T cells, pDCs, PBMCs, and the urine. miR-146a expression was reduced in mesangial cells with HCQ treatment and in pDCs with HCQ or PRED treatment. PRED treatment increased miR-155 expression in mesangial, B, and T cells and PBMCs yet decreased miR-155 expression in pDCs and the urine. *In vitro* studies confirmed that HCQ or PRED's anti-inflammatory actions are dependent on their ability to inhibit miRNA expression. Our studies indicate that lupus therapeutics may work, in part, by altering the expression of disease-associated miRNAs.

4.3. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production and deposition of nuclear self-antigen-containing immune complexes (1). Deposition along the glomerular basement membrane results in the development of immune complex – mediated glomerulonephritis due to the activation of complement and the local production of inflammatory mediators (2, 3). Mesangial cells, the primary resident immunoregulatory cells in the renal glomerulus, and dendritic cells (DCs) become activated and produce proinflammatory cytokines and chemokines, targeting the kidney for lymphocyte infiltration (4, 5). Plasmacytoid dendritic cells (pDCs), a specialized subset of dendritic cells, overproduce interferon- α (IFN- α), which promotes B cell differentiation into antibody-producing plasma cells (6). The innate immune response engages the adaptive immune response, perpetuating the inflammatory cascade in SLE. Due to their critical role in modifying innate and adaptive immune responses, dysregulated microRNA (miRNA or miR) expression may represent a trigger that induces SLE pathogenesis (7, 8). miRNAs are small, non-coding RNAs that partially bind to the 3' untranslated region (UTR) of target mRNAs, resulting in post-transcriptional gene modulation (9). miRNAs may contribute to SLE pathogenesis by altering proinflammatory mediator production, innate immune cell responses, lymphocyte function, and Toll-like receptor (TLR) and NF κ B signaling pathways (10-14).

Glucocorticoids (GC), a class of steroid hormones that includes prednisone (PRED), are a common treatment for rheumatic diseases due to their diverse anti-inflammatory and immunosuppressive effects (15). PRED mediates its anti-inflammatory properties by binding to near-ubiquitous glucocorticoid receptors (GR) that translocate into the nucleus and alter gene transcription (16). PRED inhibits nitric oxide synthesis, decreasing blood vessel dilation and

permeability (17). Treatment with PRED alters the balance of cytokines; the production of anti-inflammatory cytokines is amplified while the production of proinflammatory cytokines is reduced (18). GC are able to directly and indirectly suppress the activation of proinflammatory cytokine genes. GR can directly decrease proinflammatory cytokine production by blocking the binding of transcription factors such as AP-1 to proinflammatory promoters (19). GR are also able to inactivate MAP kinases including p38, an enzyme that is critical for NF κ B and AP-1 activation (20). Neutrophil migration is decreased with PRED treatment due to reduced chemokine production and adhesion molecule expression (21). PRED downregulates the expression of MHC class II receptors and T cell costimulatory molecules, decreasing antigen presentation and T cell activation, respectively (22). GC treatment induces G₁ cell cycle arrest and apoptosis in leukocytes (23). GR induce pro-apoptotic protein activation, including granzyme A, caspase-6, Bim, Bid and Bad, while repressing anti-apoptotic proteins such as Bcl-2, Mcl-1, and Bcl-xL (24, 25).

The antimalarial therapeutic hydroxychloroquine (HCQ) is associated with improved survival and reduced disease activity in SLE patients (26). Although the exact mechanisms of action are unknown, it has been established that HCQ increases intracytoplasmic and lysosomal pH, altering the immunological effects of leukocytes (15). Raising intracytoplasmic pH reduces cytokine production and antigen processing by decreasing the number of receptors on the cell's surface (27, 28). By increasing lysosomal pH, HCQ decreases phagocytosis, chemotaxis, and cell responsiveness to mitogenic stimuli (15, 29). Altering lysosomal acidification and disrupting endosomal maturation inhibits TLR activation; this results in downregulated IFN- α production and decreased antigen processing (26, 30). HCQ treatment has also been shown to have anti-proliferative effects. HCQ inhibits protein synthesis by intercalating with DNA and

RNA polymerases (31). HCQ can also bind to DNA itself, inhibiting immune complex formation *in vivo* (32).

Female NZB/W mice are an established model used to study human lupus, derived from the first generation (F1) cross between New Zealand Black/BinJ (NZB) and New Zealand White/LacJ (NZW) mice (33). NZB/W F1 mice show characteristics similar to human lupus including high titers of anti-nuclear antibodies, immune complex deposition, and proliferative glomerulonephritis (34, 35). Phenotypic disease typically begins to develop in the females of this strain around 20 weeks-of-age, progressing to severe renal disease by 36 weeks-of-age (36). It has been shown that diseased NZB/W mice treated with clinically efficacious doses of PRED have reduced blood urea nitrogen, serum creatinine, proteinuria, anti-dsDNA antibody production, total IgG production, IgG_{2a} production, and glomerular basement membrane thickness compared to untreated controls (37-39). In a mouse model of inflammation, HCQ treatment decreased TNF- α , IL-6, and IL-12 production, while *ex vivo* administration to murine macrophages reduced the expression of *TLR9* and the activation of NF κ B and AP-1 (40). HCQ therapy in SLE patients increased patient survival by reducing renal damage, the frequency of glomerulonephritis, and overall disease activity (26, 41). In patients with rheumatoid arthritis, treatment with HCQ reduced total IgG production and serum creatinine (42, 43).

It has been well-documented that lupus patients respond to immunosuppressive agents with varying degrees of efficacy, which has presented a major challenge in selecting the most effective treatment option (44, 45). Recent investigations have suggested that miRNA-based therapies that target aberrantly expressed miRNAs have the potential to become a promising new class of drugs (46, 47). While tailored miRNA-based therapy remains promising in the treatment of many diseases, SLE treatment may be improved in the near future by determining alterations

to disease-associated miRNAs induced by therapeutics currently in use. Existing SLE treatments may be utilized to target specific miRNAs that contribute to disease pathogenesis. In order to guide future clinical applications, further studies are needed to elucidate the alterations in miRNA expression that are induced by immunosuppressive therapy. We sought to determine the effects of lupus therapeutics on disease-associated miRNA expression in the urine and specific immunological cells in NZB/W female mice. We chose to measure a panel of miRNAs (miR-let-7a, miR-21, miR-146a, and miR-155) based on their reported implications in SLE pathogenesis (48-50). These miRNAs have been reported to be aberrantly expressed in various tissues and cell types in SLE patients and lupus mouse models (13, 51-61). By identifying cell-specific miRNA profiles and ascertaining how pharmacological agents modulate miRNAs to control inflammation, therapeutics that target cell-specific miRNAs may be utilized in the treatment of SLE.

4.4. Materials and Methods

4.4.1 Animals

Female NZB/W mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). 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). Mouse weight and proteinuria as determined by dipstick analysis (Uristix 4, Siemens Healthcare Diagnostics, Tarrytown, NY, USA) were measured every two weeks beginning at 16 weeks-of-age.

4.4.2 Immunosuppressant treatment *in vivo*

At 24 weeks-of-age, more than half of the mice had reached early disease (as determined by proteinuria scores from 30 – 100 mg/dL). Mice were randomly divided into 3 treatment groups (HCQ, PRED, or vehicle control). The HCQ and PRED-treated groups had 4 mice per cage (n = 8 per group) and the vehicle control-treated groups had 3 mice per cage (n = 6). Daily treatment began at 24 weeks-of-age with either HCQ (2.5 mg/kg p.o. daily; Sigma-Aldrich, St. Louis, MO, USA), PRED (1 mg/kg p.o. daily; Sigma-Aldrich), or the vehicle alone (water) (62-64). Mice were treated for 12 weeks (from 24 – 36 weeks-of-age) and euthanized at 36 weeks-of-age.

4.4.3. Urinary miRNA isolation

Mice were placed in metabolic cages for 24-hour urine collection every four weeks beginning at 16 weeks-of-age. Mice were grouped in metabolic cages based on treatment

assignment. Urine miRNAs were purified using a urine microRNA purification kit according to the manufacturer's protocol (Norgen Biotek Corporation, Thorold, ON, CAN). miRNAs were quantified on a spectrophotometer (Nanodrop, Thermo Scientific, Waltham, MA, USA) and stored at -80°C.

4.4.4. PBMC miRNA isolation

Blood was collected from each mouse via saphenous vein puncture every four weeks beginning at 16 weeks-of-age. Blood was stored in RNAprotect animal blood tubes at -20°C according to the manufacturer's protocol (QIAGEN, Valencia, CA, USA). PBMCs were isolated using density-gradient centrifugation according to the manufacturer's protocol (GE Healthcare, Pittsburgh, PA, USA). Briefly, cells were diluted in buffer and carefully layered over Ficoll-Paque. Cells were spun by centrifugation at 400 x g for 30 minutes. The mononuclear cell layer was isolated from the interphase between the plasma and Ficoll-Paque layers. miRNAs were isolated using the RNeasy Protect animal blood kit according to the manufacturer's protocol (QIAGEN). miRNAs were quantified on a spectrophotometer (Nanodrop) and stored at -80°C.

4.4.5. Mesangial cell isolation

At the time of euthanization, mesangial cells were isolated from the kidneys as previously described (61). Briefly, minced cortical tissue was pressed through grading sieves, resuspended in a tube containing 750 U/mL Worthington type I collagenase solution (Alfa Aesar, Ward Hill, MA, USA) and gently stirred in a water bath at 37°C for 20 minutes (65). The cells were incubated with a mesangial-specific antibody, anti-mouse integrin $\alpha 8$ (1:50 – Santa Cruz Biotechnologies, CA, USA), and then incubated with goat anti-rabbit IgG magnetic microbeads

(Miltenyi Biotec, Auburn, CA, USA) (66). Mesangial cells were purified using MACS (Miltenyi Biotec) using positive selection. Mesangial cells were pooled amongst groups due to their low frequency in the kidney (67). Cells were resuspended in *RNAlater* (QIAGEN) and stored at -20°C until miRNA isolation.

4.4.6. Splenocyte isolation

B cells and T cells were isolated from the spleen using manual dissociation as previously described (68). Briefly, each spleen was gently dissociated using a circular motion across a sterile metallic screen (Sigma-Aldrich) in a petri dish containing ice-cold RPMI 1640 medium (Thermo Scientific). The single-cell suspension was passed through a metallic screen to remove the connective tissue. B cells were isolated using the Dynal Mouse B Cell Negative Isolation Kit according to the manufacturer's protocol (Invitrogen, Life Technologies, Grand Island, NY, USA). T cells were isolated using the Pan T Cell Isolation Kit II according to the manufacturer's protocol (Miltenyi Biotec). Cells were resuspended in *RNAlater* and stored at -20°C until miRNA isolation.

pDCs were isolated from a portion of each spleen using anti-murine plasmacytoid dendritic cell antigen-1 (mPDCA-1) microbeads according to the manufacturer's protocol (Miltenyi Biotec). Briefly, a portion of the spleen was placed in a petri dish containing 2 mg/mL Collagenase D solution (Roche Applied Science, Indianapolis, IN, USA). The spleen was injected with Collagenase D solution, minced, and incubated at 37°C for 30 minutes. Cells were further disaggregated by being pressed through a 70 µm strainer. The cells were purified by MACS using a pDC-specific antibody, mPDCA-1, which is specifically expressed on pDCs (69).

pDCs were pooled amongst groups due to their low frequency in the spleen (70). Cells were stored in *RNAlater* (QIAGEN) until RNA isolation.

4.4.7. Isolation of cellular miRNAs

miRNAs were isolated using the *mirVana* miRNA isolation kit according to the manufacturer's protocol (Applied Biosystems, Life Technologies). miRNAs were quantified on a spectrophotometer (Nanodrop) and stored at -80°C.

4.4.8. IgG ELISA

IgG_{2a} and total IgG protein levels in whole blood were measured by ELISA according to the manufacturer's protocol (eBioscience). The plate was read at 450 nm on a Spectramax 340PC microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). All samples were run in duplicate.

4.4.9. Anti-dsDNA ELISA

Anti-dsDNA levels in whole blood were measured by ELISA. High-binding plates were coated with 100 µL of 5 µg/mL calf thymus DNA (Sigma-Aldrich) in saline-sodium citrate (SSC) buffer and incubated overnight at 37°C. Plates were washed 3 times with 1X PBS (Thermo Scientific) containing 0.05% Tween-20 (Sigma-Aldrich) and then blocked for 1 hour with 1X PBS containing 0.05% Tween-20 and 1% BSA (Sigma-Aldrich). Blood samples were added to the plate at a 1:100 dilution, followed by a two-fold serial dilution. The plates were incubated for 45 minutes at 37°C. The plates were washed as described above and incubated with 200 µL/well of HRP-conjugated goat anti-mouse IgG gamma chain specific Ab (1:4000,

Southern Biotech, Birmingham, AL, USA). Plates were washed as described above. TMB substrate (Pierce, Thermo Scientific, Rockford, IL, USA) was added to the wells and the plate was read at 380 nm on a Spectramax 340PC microplate spectrophotometer (Molecular Devices). A final dilution of 1:400 was reported.

4.4.10 Cytokine ELISA

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

4.4.11. Real-time RT-PCR

miRNA expression was measured by real-time RT-PCR using TaqMan microRNA assays according to the manufacturer's protocol (Applied Biosystems) as previously described (61). miRNAs were diluted to reach 10 ng per reaction. TaqMan Small RNA Assays were used according to the manufacturer's protocol (Applied Biosystems). Relative gene quantitation was determined using the comparative C_T ($\Delta\Delta C_T$) method using snoRNA202 as the endogenous control (71). All samples were run in triplicate.

4.4.12 Cell culture

A mouse macrophage cell line (J774A.1) and a mouse mesangial cell line (MES 13) were purchased from ATCC (Manassas, VA, USA). The cells were grown in 75-mm² culture flasks at

37°C in 5% CO₂. Macrophages were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin (Cellgro, Manassas, VA, USA). During serum starvation, cells were given DMEM containing 1% FBS and 1% penicillin-streptomycin (Cellgro). Mesangial cells were cultured 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). For serum-starving medium, FBS was absent from the complete growth medium. For immune stimulation, LPS (Sigma-Aldrich, St. Louis, MO, USA) and IFN- γ (Cedarlane Laboratories Limited, Burlington, NC, USA) were added to the complete medium at a final concentration of 1 μ g/mL and 100 ng/mL, respectively. Experiments were performed from passage 9 – 15. All experimental conditions were run in triplicate.

4.4.13 miRNA inhibitor transfection

Macrophages were transfected with Lipofectamine 2000 transfection reagent according to the manufacturer's protocol (Life Technologies, Grand Island, NY, USA). Mesangial cells were transfected with *TransIT-siQUEST* transfection reagent according to the manufacturer's protocol (Mirus, Madison, WI, USA). The cells were serum-starved for 2 hours prior to transfection at which point they were transfected with the miRIDIAN hairpin inhibitor or nontargeting control at a final concentration of 25 nM (Thermo Fisher Scientific Biosciences Inc., Chicago, IL, USA). The non-targeting control is based on the miRNA-67 sequence found in *Caenorhabditis elegans* (*C. elegans*), which has minimal sequence identity with miRNAs in mice (Thermo Fisher Scientific Biosciences Inc.). Non-transfected controls received complete growth medium only. The plates were incubated for 24 hours at 37°C.

4.4.14. Immunosuppressant treatment *in vitro*

The cells were serum-starved for 2 hours and then treated with HCQ (100 uM final), PRED (1 mM final), or the vehicle alone (water) for 2 hours due to previous work that has indicated this is sufficient to reduce inflammatory mediator production (unpublished data) (72). The media was removed and replaced with immune-stimulating medium. Non-stimulated controls received complete growth medium. Cells and supernatants were collected for analysis 24 hours post-stimulation.

4.4.15. Statistical analysis

Linear regression was used to test for significant differences in proteinuria, IgG production, cytokine production *in vivo*, and anti-dsDNA production. Student's unpaired *t*-test (two-tailed) was used to test for significant differences in miRNA expression and cytokine production *in vitro*. The Mann–Whitney two-tailed U test for nonparametric measures was used to test for significance in the anti-dsDNA titration curve. *P* values less than 0.05 were considered statistically significant.

4.5. Results

4.5.1 HCQ or PRED treatment decreases disease progression in NZB/W mice

miRNAs have gained appreciation as contributors to both normal cell development and SLE disease pathogenesis (50, 58, 73, 74). Altered miRNA expression in the blood and kidneys of lupus patients suggests miRNAs may be used as diagnostic biomarkers for LN (75, 76). These studies were performed in order to assess urinary and immune cell miRNA alterations mediated by SLE therapeutics. We chose to use hydroxychloroquine (HCQ) and prednisone (PRED) because they are effective therapeutics with different mechanisms of action that have been approved for the treatment of SLE (77-81). The pharmacokinetics for each therapeutic agent have been well-documented, allowing us to use efficacious dosages to treat disease (82).

Female NZB/W F1 mice were weighed and proteinuria was measured by dipstick analysis every two weeks beginning at 16 weeks-of-age. At 16 weeks-of-age, all mice had proteinuria from 0 – 30 mg/dL (Figure 1A). More than half of the mice reached early disease (proteinuria from 30 – 100 mg/dL as determined by dipstick analysis) at 24 weeks-of-age. At 24 weeks-of-age, mice were randomly divided into treatment groups and daily treatment began with either HCQ (2.5 mg/kg p.o. daily), PRED (1 mg/kg p.o. daily), or the vehicle control (62-64). Mice were treated for a total of 12 weeks. Mice treated with either HCQ or PRED maintained proteinuria scores \leq 100 mg/dL throughout the study. Vehicle control-treated NZB/W mice had proteinuria scores of approximately 800 mg/dL at 26 weeks-of-age that increased to over 1500 mg/dL by 36 weeks-of-age. One control mouse died at 31 weeks-of-age.

We next measured IgG_{2a} and total IgG antibody production in mice over time. At 16, 20, and 24 weeks-of-age, there were no significant differences in IgG_{2a} production amongst the treatment or control groups (Figure 1B). There was a gradual increase of IgG_{2a} production as the

mice aged. At 28 weeks-of-age (after 4 weeks of treatment), there was a significant decrease in IgG_{2a} production when either the HCQ or PRED-treated group was compared to the control group. By 32 and 36 weeks-of-age, there was a significant decrease in IgG_{2a} production in mice treated with HCQ or PRED compared to the mice that received the vehicle control alone. In addition, treatment with either therapeutic returned IgG_{2a} production to levels seen in pre-diseased mice. Vehicle-treated control mice had increased IgG_{2a} production as they aged. There were no significant differences in total IgG production between the treatment groups and controls at 16, 20, or 24 weeks-of-age (Figure 1C). At 28, 32, and 36 weeks-of-age, there was a significant decrease in total IgG production when either the HCQ or PRED-treated group was compared to the controls. While the levels of total IgG in HCQ or PRED-treated mice returned to levels seen in pre-diseased mice, total IgG production continued to increase in the control mice as they aged.

Next, we measured anti-dsDNA antibody levels from the whole blood as the mice aged (Figure 1D). There were no significant differences in anti-dsDNA levels between the groups before treatment began. As mice aged from 16 – 24 weeks, anti-dsDNA antibody production increased over time. There were increased anti-dsDNA antibody levels in vehicle-treated mice through 36 weeks-of-age. At 28 weeks-of-age, mice treated with HCQ had significantly reduced anti-dsDNA antibody production compared to vehicle-treated mice. PRED-treated mice had decreased anti-dsDNA antibody levels compared to controls. At 32 and 36 weeks-of-age, there was a significant decrease in anti-dsDNA antibody production in mice treated with HCQ or PRED compared to the control mice. Serum titrations at 36 weeks revealed that HCQ or PRED-treated mice had anti-dsDNA titers 2 – 4 times that of vehicle-treated mice (Figure 1E). There

were no significant differences between the control and HCQ-treated mice ($p < 0.09$) or control mice and PRED-treated mice ($p < 0.51$).

We next measured pro- and anti-inflammatory cytokine production in the mice over time. While there was an increase in TNF- α production from 16 – 24 weeks-of-age, there were no differences between the groups before treatment began (Figure 1F). At 28, 32, and 36 weeks-of-age, HCQ and PRED-treated mice had decreased TNF- α production compared to vehicle-treated controls. Similarly, from 16 – 24 weeks-of-age, there were no differences in IL-6 production between the groups (Figure 1G). From 28 – 36 weeks-of-age, IL-6 production continued to increase in vehicle-treated mice. Treatment with HCQ or PRED returned IL-6 production to levels seen in pre-diseased mice. Control mice maintained IL-10 levels ≤ 132 pg/mL throughout the study (Figure 1H). Mice that were treated with HCQ or PRED had increased IL-10 production at 28, 32, and 36 weeks-of-age. Taken together, these results indicate that the doses of HCQ and PRED used were able to ameliorate disease in NZB/W mice.

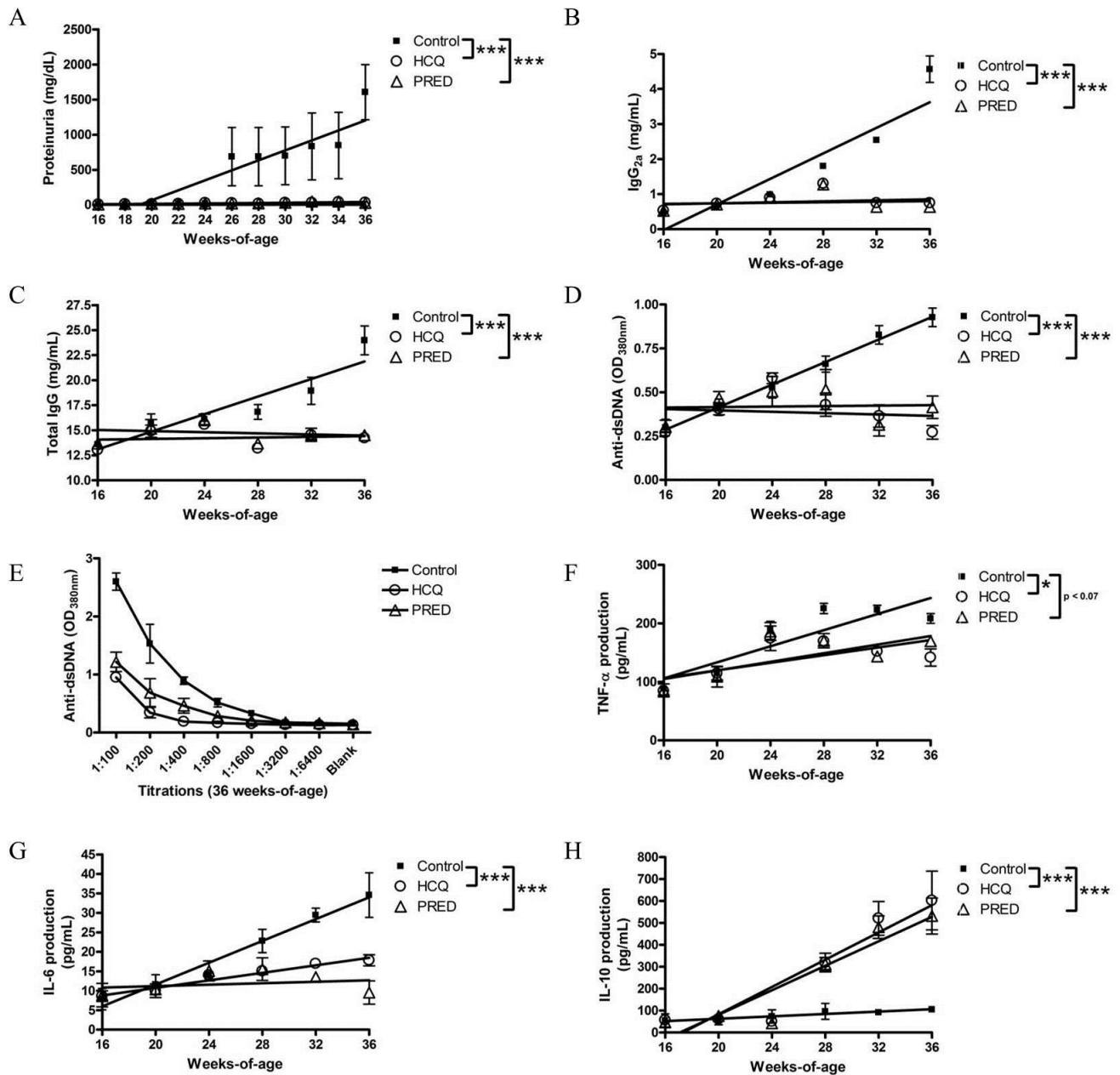


Figure 1. HCQ or PRED treatment decreases disease progression in NZB/W mice. A. At 16, 20, and 24 weeks-of-age, NZB/W mice had proteinuria from 0 – 30 mg/dL as measured by dipstick analysis. From 28 – 36 weeks-of-age, hydroxychloroquine (HCQ) or prednisone (PRED)-treated mice maintained proteinuria from 0 – 100 mg/dL. Control mice had proteinuria measurements of approximately 700 mg/dL at 28 weeks-of-age that increased to approximately

1600 mg/dL at 36 weeks-of-age. B. ELISA showed that all NZB/W mice had similar IgG_{2a} production from 16 – 24 weeks-of-age. HCQ or PRED-treated mice had reduced significantly reduced IgG_{2a} production compared to controls from 28 – 36 weeks-of-age. C. ELISA showed that all NZB/W mice had similar total IgG production from 16 – 24 weeks-of-age. From 28 – 36 weeks-of-age, mice treated with either HCQ or PRED had significantly decreased total IgG production compared to controls. D. ELISA showed that all NZB/W mice had similar anti-dsDNA production from 16 – 24 weeks-of-age. At 28 weeks-of-age, HCQ-treated mice had significantly less anti-dsDNA antibody production than mice treated with the vehicle alone. At 32 and 36 week-of-age, mice treated with HCQ or PRED had significantly less anti-dsDNA antibody production compared to controls. E. Titration of whole blood from NZB/W mice for anti-dsDNA antibody production at 36 weeks-of-age. F. ELISA showed that NZB/W mice had similar TNF- α production from 16 – 24 weeks-of-age. Mice treated with either HCQ or PRED had decreased TNF- α production from 28 – 36 weeks-of-age. G. There were no differences in IL-6 production from 16 – 24 weeks-of-age. From 28 – 36 weeks-of-age, mice that received HCQ or PRED had less IL-6 production compared to vehicle-treated controls. H. Control mice maintained IL-10 levels ≤ 132 pg/mL throughout the course of the study. HCQ or PRED-treated mice had increased IL-10 production compared to controls. The lines represent the regression lines for each data set (A – D, F – G). N = 8 for HCQ or PRED-treated mice. N = 6 for vehicle control-treated mice (except at the 36 weeks-of-age time point were n = 5). * $p < 0.05$. ** $p < 0.01$. *** $p < 0.005$.

4.5.2 HCQ or PRED treatment alters miRNA expression in PBMCs

Mouse whole blood was collected every 4 weeks beginning at 16 weeks-of-age. PBMCs were isolated using density-gradient centrifugation and miRNA expression was measured using real time RT-PCR. We chose to measure a panel of miRNAs (miR-let-7a, miR-21, miR-146a, and miR-155) based on their reported implications in SLE pathogenesis using real time RT-PCR (13, 51-59, 61, 83). Relative expression was determined by the $\Delta\Delta CT$ method using the endogenous miRNA control snoRNA202 (71). Before treatment at 16, 20, and 24 weeks-of-age, there were no significant differences in PBMC miRNA expression when either HCQ or PRED-treated mice were compared to the control mice (Figure 2A – D). At 32 and 36 weeks-of-age, there was a significant decrease in urinary miR-let-7a expression in HCQ-treated mice compared to the control mice (Figure 2A). There was a significant decrease in PBMC miR-let-7a expression at 36 weeks-of-age in mice treated with PRED compared to the mice that received the vehicle alone. Over time, miR-let-7a expression increased in mice that receive the vehicle control alone. Compared to the control group, miR-21 expression was significantly decreased in the PBMCs of HCQ-treated mice at 32 weeks-of-age (Figure 2B). Treatment with PRED significantly decreased miR-21 expression at 28, 32, and 36 weeks-of-age. There was an increase in PBMC miR-21 expression in vehicle-control treated mice as they aged, which is consistent with reports of increased miR-21 expression in the PBMCs of SLE patients (13). HCQ treatment significantly did not significantly alter miR-146a expression (Figure 2C). PRED treatment increased miR-146a expression in the PBMC of NZB/W mice at 32 and 36 weeks-of-age. While HCQ treatment did not alter PBMC miR-155 expression, treatment with PRED increased PBMC miR-155 expression compared to controls at 32 and 36 weeks-of-age (Figure 2D). PBMC miR-146a and miR-155 expression decreased as control mice aged, which

coincides with previous reports that have correlated decreased miR-146a or miR-155 in the sera with SLE disease activity (59). Taken together, these results indicate that HCQ and PRED alter the expression of SLE-associated miRNAs in PBMCs. Immunosuppressant therapy can significantly alter PBMC miRNA expression in as little as four weeks of treatment.

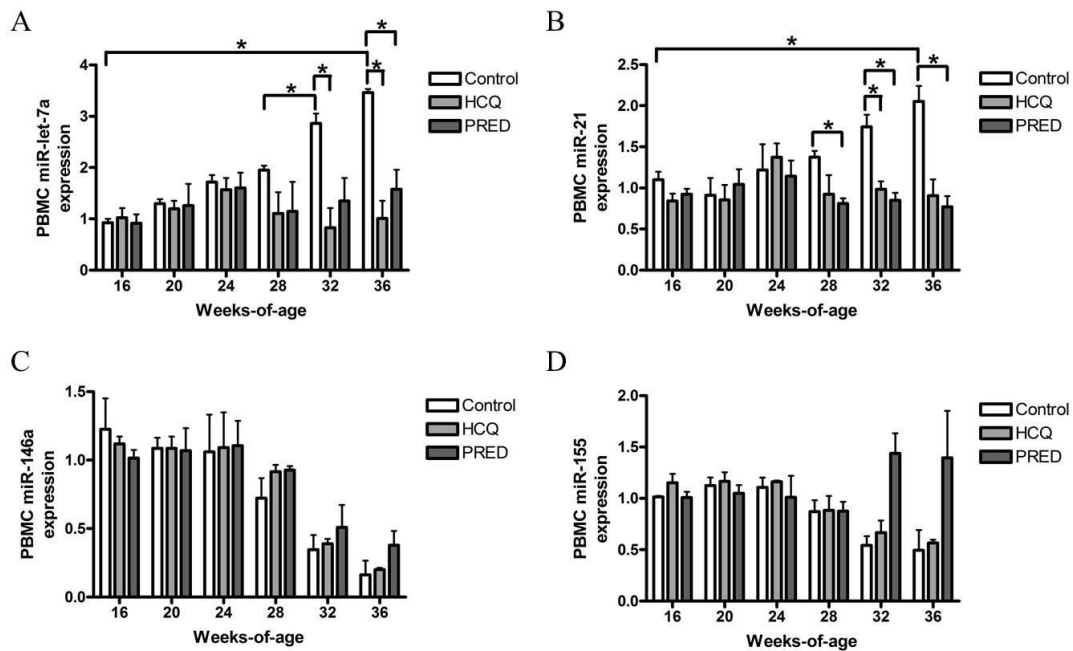


Figure 2. HCQ or PRED treatment alters miRNA expression in PBMCs. A. At 32 and 36 weeks-of-age, miR-let-7a expression was significantly decreased in PBMC from NZB/W mice treated with hydroxychloroquine (HCQ) compared to mice treated with the vehicle control alone. miR-let-7a expression was significantly decreased at 36 weeks-of-age in NZB/W mice treated with prednisone (PRED). Over all, miR-let-7a expression increased in control mice as they aged. B. The expression of miR-21 was significantly reduced at 32 weeks-of-age in PBMCs from NZB/W mice treated with HCQ compared to controls. At 28, 32, and 36 weeks-of-age, miR-21 expression was significantly decreased in NZB/W mice treated with PRED compared to mice treated with the vehicle control alone. The expression of miR-21 increased in control

NZB/W mice over time. C. There was an increase in miR-146a expression at 32 and 36 weeks-of-age in NZB/W mice treated with PRED compared to control mice. Overall, PBMC miR-146a expression decreased as the mice aged. D. At 32 and 36 weeks-of-age, the expression of miR-155 was increased in NZB/W mice treated with PRED. There was a decrease in miR-155 expression in control mice over time. N = 8 for HCQ or PRED-treated mice. N = 6 for vehicle control-treated mice (except at the 36 weeks-of-age time point were n = 5). * $p < 0.05$. ** $p < 0.01$.

4.5.3. HCQ and PRED treatment alters urinary miRNA expression

We next measured urinary miRNA expression of the treated and control groups over the course of the 20 week study using real time RT-PCR. Before treatment at 16, 20, and 24 weeks-of-age, there were no significant differences in urinary miRNA expression when either HCQ or PRED-treated mice were compared to the control mice (Figure 3A – D). At 28 weeks-of-age, there was a significant decrease in urinary miR-let-7a expression in HCQ-treated mice compared to the control mice (Figure 3A). miR-let-7a expression was lower in HCQ or PRED-treated mice at 28, 32, and 36 weeks-of-age compared to mice that received the vehicle alone. There was an increase in miR-let-7a expression in vehicle control-treated mice as they aged. Compared to the control group, miR-21 expression was significantly decreased in the urine of PRED-treated mice at 32 and 36 weeks-of-age (Figure 3B). The expression of miR-21 was lower in HCQ or PRED-treated mice at 28, 32, and 36 weeks-of-age compared to mice that received the vehicle alone. As control mice aged, there was an increase in urinary miR-21 expression. Although miR-146a expression increased over time, neither HCQ nor PRED treatment significantly altered miR-146a expression in the urine of NZB/W mice (Figure 3C). Compared to controls, treatment with

PRED significantly decreased urinary miR-155 expression at 36 weeks-of-age (Figure 3D). The expression of miR-155 increased in control mice over time. Increased urinary miR-146a and miR-155 expression have been reported in SLE patients and lupus mice compared to healthy controls (59, 60). These results indicate that HCQ and PRED decrease urinary levels of miR-let-7a, miR-21, and miR-155. Like PBMC miRNA expression, immunosuppressant therapy can alter urinary miRNA expression in as little as four weeks of treatment.

4.5.4. HCQ or PRED treatment alters mesangial miRNA expression

When the mice were euthanized at 36 weeks-of-age, mesangial cell miRNAs were isolated from the pooled kidneys from each group. Neither HCQ nor PRED treatment significantly altered mesangial miR-let-7a expression (Figure 3E). Mice treated with HCQ had significantly decreased miR-21 expression compared to the control mice. There was also a decrease in miR-21 expression in PRED-treated mice compared to the control. While PRED did not significantly alter the expression of miR-146a in mesangial cells, there was a decrease in miR-146a expression in mice treated with HCQ. miR-155 expression was significantly increased in the mesangial cells of HCQ-treated mice. There were no significant differences in mesangial miR-155 expression in mice treated with HCQ compared to the control. These results indicate that HCQ and PRED alter the expression of miR-21, miR-146a, and miR-155 differently in mesangial cells. While HCQ treatment reduces miR-21 and miR-146a expression, treatment with PRED reduces miR-21 expression and increases miR-155 expression.

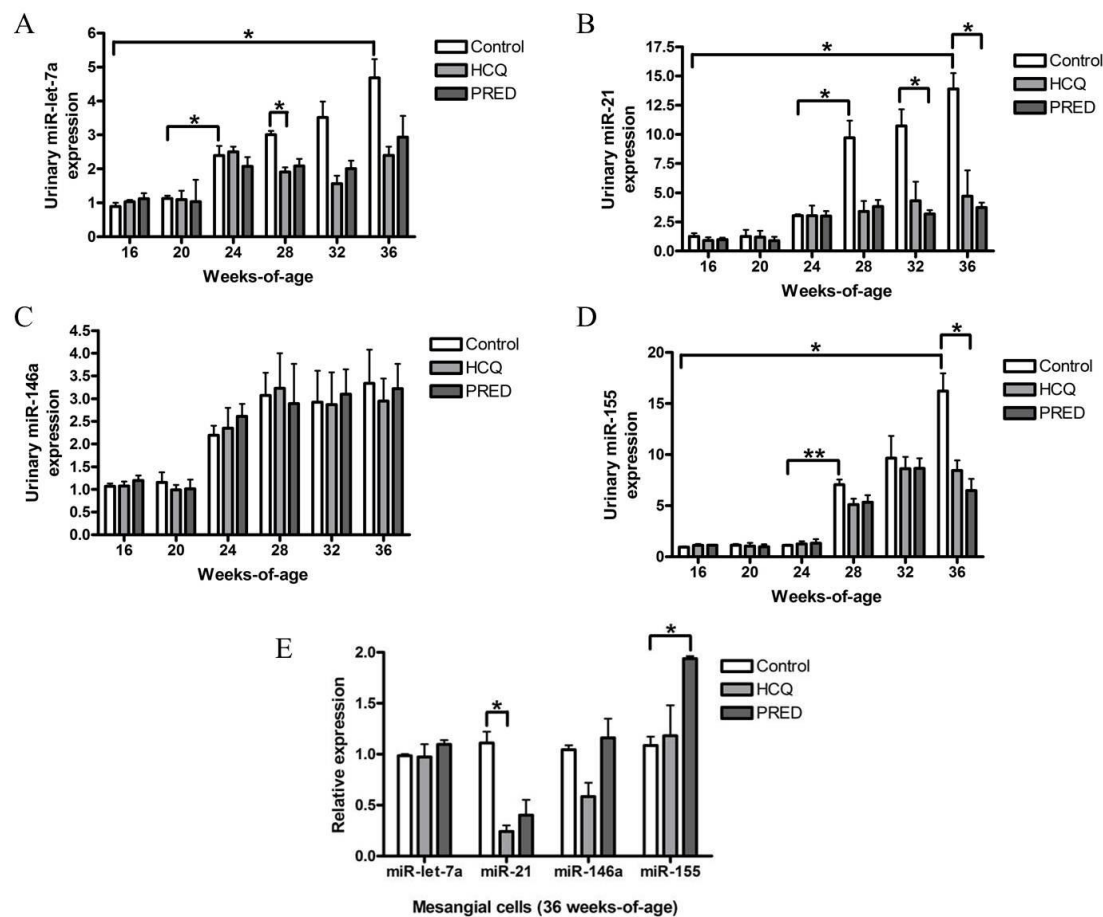


Figure 3. HCQ or PRED alters urinary and mesangial cell miRNA expression. A. Urinary miR-let-7a expression was significantly reduced at 28 weeks-of-age after treatment with hydroxychloroquine (HCQ). There was a decrease in miR-let-7a expression in the urine of prednisone (PRED)-treated NZB/W mice compared to controls at 28, 32, and 36 weeks-of-age. Urinary miR-let-7a expression increased in control NZB/W mice over time. B. At 28, 32, and 36 weeks-of-age, there was a decrease in urinary miR-21 expression in NZB/W mice treated with HCQ or PRED compared to the vehicle control-treated mice. miR-21 expression increased in control mice as they aged. C. Although urinary miR-146a expression increased over time, neither HCQ nor PRED treatment altered its expression. D. miR-155 expression was reduced in the urine of HCQ-treated NZB/W mice compared to controls at 36 weeks-of-age. The

expression of miR-155 was significantly reduced at 36 weeks-of-age in PRED-treated NZB/W mice compared to vehicle control-treated mice. Urinary miR-155 expression increased in control mice over time. E. Mesangial miR-let-7a expression was unaffected by HCQ or PRED treatment in NZB/W mice. miR-21 expression was significantly decreased following HCQ treatment compared to the expression in control mice. PRED treatment reduced the expression of miR-21. Mesangial miR-146a expression was reduced after HCQ treatment in NZB/W mice compared to controls. miR-146a expression was unchanged following treatment with PRED. Treatment with HCQ did not affect miR-155 expression in mesangial cells. miR-155 expression was significantly increased in NZB/W mice after PRED treatment. For HCQ or PRED-treated mice, n = 2 cages of 4 mice each. For vehicle control-treated mice, n = 2 cages of 3 mice each from 16 – 32 weeks-of-age. At 36 weeks-of-age, n = 2 cages (one containing 2 vehicle control-treated mice and the other containing 3 vehicle control-treated mice). * $p < 0.05$. ** $p < 0.01$.

4.5.5. HCQ or PRED treatment alters miRNA expression in splenocytes

B cells, T cells, and pDCs were isolated from the spleen when the mice were euthanized at 36 weeks-of-age. B cell and T cell miRNAs were isolated from a portion of each mouse spleen. A portion of the spleen was pooled from each group and pDC miRNAs were isolated. Treatment with HCQ or PRED significantly decreased miR-let-7a expression in splenic B cells compared to the control (Figure 4A). Neither treatment altered miR-21 or miR-146a expression in mice. While treatment with HCQ had no effect on miR-155 expression, treatment with PRED significantly increased miR-155 expression in B cells. In T cells, neither treatment altered miR-let-7a expression compared to controls (Figure 4B). HCQ and PRED-treated mice had significantly decreased splenic T cell miR-21 expression compared to control mice. Neither

treatment altered miR-146a expression in mice. PRED treatment significantly increased miR-155 expression in T cells. In pDCs, HCQ or PRED treatment had no effect on miR-let-7a expression (Figure 4C). Treatment with HCQ or PRED significantly reduced miR-21, miR-146a, and miR-155 expression in pDCs. These results indicate that HCQ and PRED differentially alter the expression of splenic miRNAs implicated in SLE pathogenesis. HCQ and PRED treatment decreased miR-let-7a expression in B cells, yet PRED also increased miR-155 expression. Similarly, while treatment with HCQ or PRED decreased miR-21 expression in T cells, PRED also increased T cell miR-155 expression. Overall, pDCs were the most susceptible to expression changes in the miRNAs examined. HCQ or PRED treatment significantly decreased miR-21, miR-146a, and miR-155 expression in pDCs.

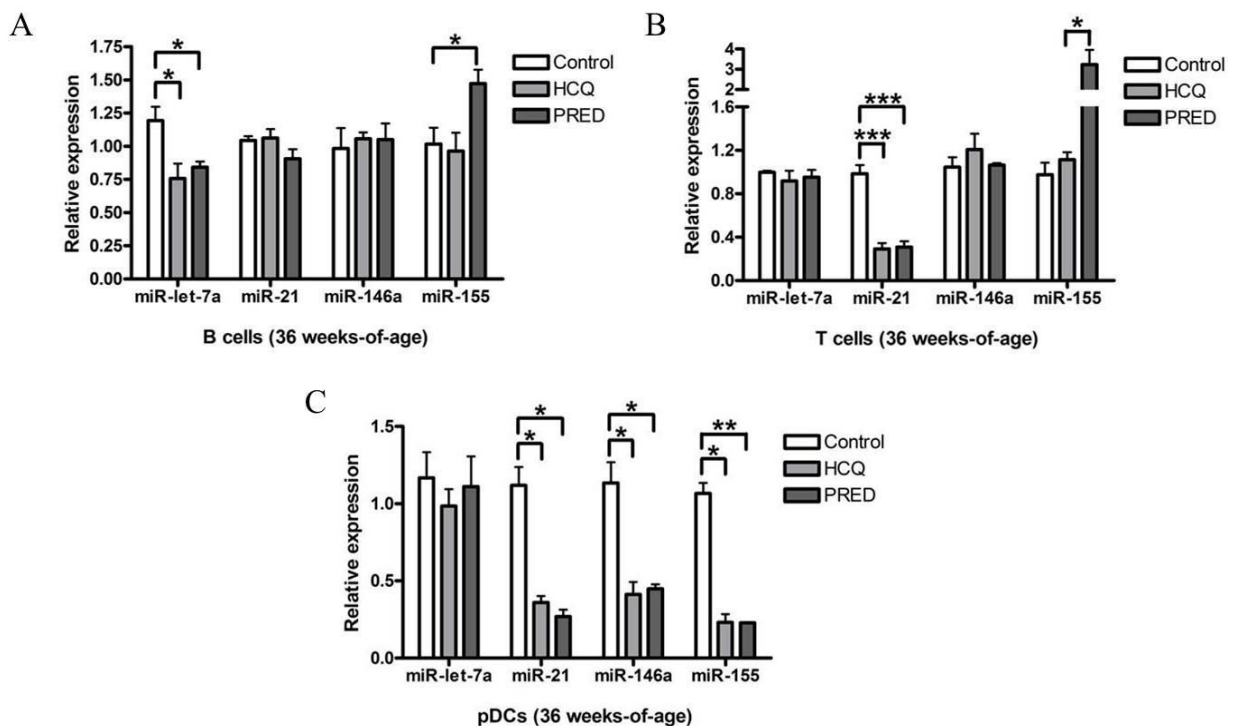


Figure 4. HCQ or PRED treatment alters miRNA expression in splenocytes. A. miR-let-7a expression was significantly reduced in B cells after NZB/W mice were treated with

hydroxychloroquine (HCQ) or prednisone (PRED). Compared to controls, miR-21 and miR-146a expression were unchanged in NZB/W mice after HCQ or PRED treatment. HCQ did not affect the expression of miR-155. PRED significantly increased miR-155 expression in the B cells of NZB/W mice compared to controls. B. miR-let-7a expression was unchanged in the T cells of HCQ or PRED-treated NZB/W mice compared to vehicle control-treated mice. miR-21 expression was significantly decreased after HCQ or PRED treatment. miR-146a expression was unaltered by HCQ or PRED treatment in T cells. HCQ treatment did not alter T cell miR-155 expression in NZB/W mice compared to control mice. miR-155 expression was significantly increased after treatment with PRED. C. miR-let-7a expression was unchanged in pDCs after treatment with HCQ or PRED compared to controls. The expression of miR-21, miR-146a, and miR-155 were significantly decreased in pDCs from NZB/W mice treated with HCQ or PRED. For B and T cells, n = 8 for HCQ or PRED-treated mice and n = 5 for vehicle control-treated mice. For pDCs, n = 2 cages of 4 mice each for HCQ or PRED-treated mice. For vehicle control-treated mice, n = 2 cages (one containing 2 mice and the other containing 3 mice). * $p < 0.05$. ** $p < 0.01$. *** $p < 0.005$.

4.5.6 HCQ or PRED-mediated inhibition of miRNAs is critical to alter the inflammatory response *in vitro*

We next examined if specific miRNAs contribute to the anti-inflammatory mechanisms mediated by HCQ or PRED treatment. Because our results largely indicate that HCQ or PRED treatment decreases disease-associated miRNA expression, we transfected J774A.1 mouse macrophages and MES 13 mouse mesangial cells with miRNA inhibitors in order to determine if knocking down miRNAs alters HCQ or PRED's ability to suppress the inflammatory response.

Transfection efficiency was confirmed by real time RT-PCR (Figure 5A – D). Cells were immune-stimulated 24 hours post-transfection with LPS/IFN- γ . Cytokine production was measured in the cell supernatants by ELISA 24 hours post-stimulation. IL-6 was measured to determine changes to proinflammatory cytokine production, while IL-10 was measured to determine anti-inflammatory cytokine alterations.

IL-6 was undetectable in nonstimulated J774A.1 macrophages (Figure 5E). In immune-stimulated macrophages transfected with the miR-let-7a inhibitor, there was a decrease in IL-6 production. This is consistent with previous studies that have indicated miR-let-7a overexpression significantly increases IL-6 production (61). Treatment with HCQ or PRED significantly decreased IL-6 production in macrophages. In HCQ-treated macrophages with knocked down miR-146a expression, there was a significant decrease in IL-6 production. Similarly, there was a decrease in IL-6 production in macrophages transfected with the miR-146a inhibitor and treated with PRED. In macrophages transfected with the miR-let-7a inhibitor, there was a significant increase in IL-6 production. These results suggest that HCQ or PRED therapy combined with miR-146a inhibition may be utilized to greatly reduce IL-6 production in SLE. In addition, reduced IL-6 production by J774A.1 macrophages induced by PRED treatment is dependent on its ability to decrease miR-let-7a expression.

In J774A.1 macrophages, IL-10 production was unchanged amongst groups with or without stimulation (Figure 5F). There was a significant increase in IL-10 production with immune stimulation. Treatment with HCQ or PRED reduced IL-10 production in all of the treatment groups. Macrophages transfected with the miR-155 inhibitor and treated with HCQ had decreased IL-10 production. PRED-treated macrophages with knocked down miR-155 expression had significantly reduced IL-10 production. Macrophages transfected with the miR-

21 inhibitor and treated with PRED had increased IL-10 production. This suggests that miR-21 inhibition by PRED may not be necessary for PRED to increase IL-10 production. Alternatively, our results may indicate that miR-21 is essential for PRED to mediate decreased IL-10 production in macrophages as GCs have been shown to decrease monocyte IL-10 production (84). These results also suggest that HCQ or PRED-induced IL-10 production is greatly diminished by knocking down miR-155 in macrophages.

IL-6 production was undetectable in nonstimulated mesangial cells (Figure 5G). HCQ or PRED treatment significantly reduced IL-6 production in immune-stimulated mesangial cells. There was an increase in IL-6 production in HCQ-treated mesangial cells transfected with the miR-21 inhibitor. miR-146a inhibition in HCQ-treated mesangial cells resulted in a significant increase in IL-6 production. There was a significant increase in IL-6 production in mesangial cells transfected with the miR-21 inhibitor and treated with PRED. Taken together, these results suggest that the mechanism by which HCQ or PRED reduce IL-6 production in mesangial cells *in vitro* may be due to their ability to decrease miRNA expression.

IL-10 production was unchanged between the treatment groups in nonstimulated MES 13 mesangial cells (Figure 5H). There was a significant increase in IL-10 production in immune-stimulated mesangial cells. HCQ or PRED treatment significantly reduced IL-10 production. With HCQ treatment, there was a slight increase in IL-10 production in mesangial cells transfected with the miR-21, miR-146a, or miR-155 inhibitor. In PRED-treated mesangial cells, there was a slight increase in IL-10 production when miR-21 or miR-155 expression was knocked down. These results indicate that miRNA inhibition by HCQ or PRED is not necessary for mesangial cells to increase IL-10 production.

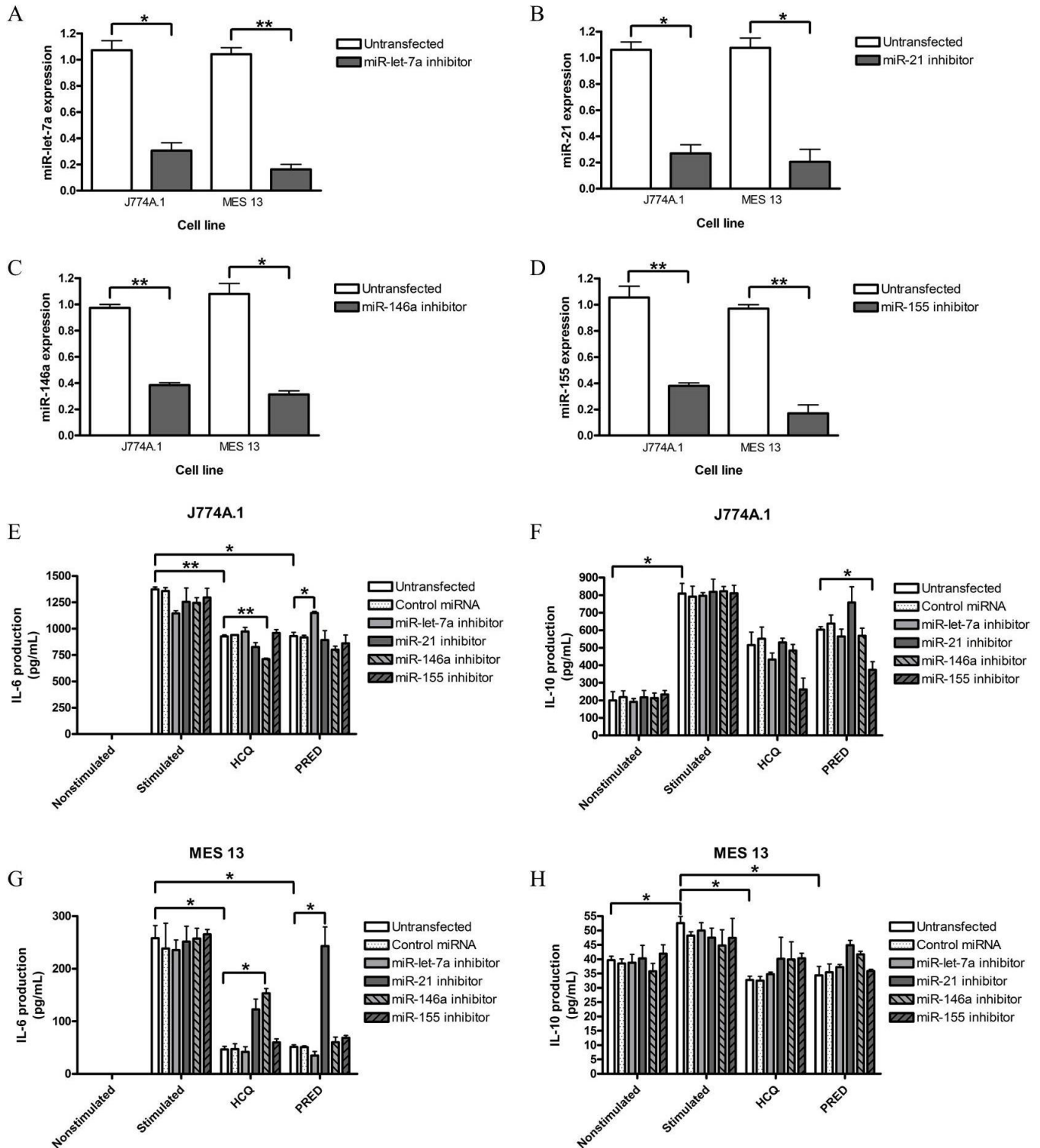


Figure 5. HCQ or PRED-mediated inhibition of miRNAs is critical to alter the inflammatory response *in vitro*. A. Transfection of the miR-let-7a inhibitor significantly reduced miR-let-7a expression in J774A.1 macrophages and MES 13 mesangial cells. B. miR-

21 expression was significantly reduced in J774A.1 macrophage and MES 13 mesangial cells transfected with the miR-21 inhibitor. C. Transfection of the miR-146a inhibitor significantly reduced miR-146a expression in J774A.1 macrophages and MES 13 mesangial cells. D. miR-155 expression was significantly reduced after transfection of the miR-155 inhibitor in J774A.1 macrophages and MES 13 mesangial cells. E. IL-6 was undetectable in J774A.1 macrophages without immune stimulation. Treatment with HCQ or PRED significantly decreased IL-6 production compared to the stimulated control. Compared to the untransfected control, miR-146a inhibition and HCQ treatment significantly reduced IL-6 production in macrophages. IL-6 production was significantly increased in macrophages transfected with the miR-let-7a inhibitor and treated with PRED. F. IL-10 production was unchanged amongst treatment groups with or without immune stimulation in J774A.1 macrophages. miR-155 inhibition with HCQ treatment reduced IL-10 production compared to the untransfected control. IL-10 production was significantly decreased in macrophages transfected with the miR-155 inhibitor and treated with PRED. G. IL-6 was undetectable in MES 13 mesangial cells without immune stimulation. Treatment with HCQ or PRED significantly reduced IL-6 production compared to the stimulated control. Mesangial cells transfected with the miR-21 inhibitor and treated with HCQ had increased IL-6 production. HCQ-treated mesangial cells transfected with the miR-146a inhibitor had significantly increased IL-6 production compared to the untransfected control. miR-21 inhibition significantly increased IL-6 production with PRED treatment. H. IL-10 production was unchanged in MES 13 mesangial cells with or without immune stimulation. Compared to the stimulated control, HCQ or PRED treatment significantly reduced IL-10 production. IL-10 production was slightly increased in stimulated mesangial cells transfected with the miR-21, miR-146a, or miR-155 inhibitor and treated with HCQ. There was a slight increase in IL-10

production in miR-21 or miR-146a-transfected mesangial cells treated with PRED. (A – D) represent 3 independent experiments run in triplicate. (E – H) represent 3 independent experiments run in duplicate. The nontargeting miRNA was miR-67. The final concentration of the miRNAs was 25 nM. Cells were treated with a final concentration of 100 uM HCQ or 1 mM PRED. Error bars represent the SEM. * $p < 0.05$. ** $p < 0.01$.

4.6. Discussion

Epigenetic regulation by miRNAs has been shown to contribute to both normal and abnormal protein expression (74, 85, 86). As pathogenic miRNAs are identified in LN pathogenesis, treatment strategies aimed at altering miRNA expression may be employed to ameliorate disease in patients with SLE. These studies were performed in order to assess HCQ and PRED- mediated alterations to the expression of urinary and immune cell miRNAs implicated in SLE pathogenesis. In order to identify specific miRNAs as disease-modifying agents and not merely disease correlates, we performed further *in vitro* studies to confirm the direct contributions of disease-associated miRNAs to HCQ and PRED's anti-inflammatory mechanisms of action.

Our *in vivo* studies show that treatment with HCQ significantly reduces miR-21 expression in mesangial cells. miR-21 has been shown to regulate innate immune cell activity by altering apoptosis and cell proliferation pathways (52). Overexpressed miR-21 may contribute to NFκB-mediated mesangial cell activation and extracellular matrix production in glomerulonephritis (51, 87). The miR-21 promoter is regulated by NFκB and STAT3, a transcription factor activated by IL-6, which suggests there may be a positive feedback loop connecting miR-21, NFκB, and IL-6 (88, 89). Our *in vitro* results demonstrate that inhibiting miR-21 in mesangial cells may be necessary for HCQ to decrease IL-6 production. It has been established that HCQ therapy preferentially alters the production of macrophage-mediated cytokines over Th cell-mediated cytokines, targeting the production of cytokines including IL-6 to a greater extent than IL-2 and IL-4 (72, 90). This suggests that the preferential decrease of IL-6 production by HCQ may be mediated by its ability to decrease miR-21 expression. By

decreasing miR-21 expression, HCQ may contribute to decreased mesangial cell proliferation and reduced inflammatory cell infiltration through decreased production of IL-6.

Our results indicate that HCQ or PRED treatment decreases miR-21 expression in PBMCs, T cells, and the urine. Overexpressed miR-21 has been reported in the PBMCs and T cells of SLE patients and is strongly correlated with SLE disease activity (13, 53). Inhibition of miR-21 in SLE T cells *in vitro* reversed the activated T cell phenotype by increasing IL-10 production (13). It has been shown that GC treatment differentially regulates IL-10 production based on the cell type, increasing production by B and T cells while decreasing production by monocytes (91). The GC-mediated increase in IL-10 production by B and T cells may have contributed to the overall increase in IL-10 production in the blood from PRED-treated NZB/W mice in the current studies (91, 92). Our results show that PRED treatment in J774A.1 macrophages decreases IL-10 production, which is consistent with decreased IL-10 production by monocytes treated with GCs (84). Our results also demonstrate that PRED-treated macrophages transfected with the miR-21 inhibitor have increased IL-10 production. This may indicate that miR-21 inhibition by PRED is necessary for PRED to decrease IL-10 production in macrophages. Taken together, these results provide a link between miR-21 and the regulation of innate and adaptive immunity by PRED.

Because miR-let-7a has been shown to regulate the 3' UTR of cytokine transcripts including *IL-6* and *IL-10*, PRED therapy may alter the proinflammatory environment in SLE by decreasing miR-let-7a expression (61, 93, 94). We found that miR-let-7a expression is reduced in PBMCs, B cells, and the urine after PRED treatment. We also found that miR-let-7a inhibition in J774A.1 macrophages significantly increased IL-6 production with PRED

treatment, which further implicates miR-let-7a in IL-6 production in SLE. Reduced IL-6 production with PRED treatment may be mediated by its ability to reduce miR-let-7a expression.

Our current studies show that the expression of miR-146a remains largely unchanged after treatment with HCQ or PRED in NZB/W mice. Treatment in pDCs and mesangial cells were the only exceptions. miR-146a expression was significantly reduced in pDCs following treatment with HCQ or PRED while HCQ treatment reduced miR-146a expression in mesangial cells. Our *in vitro* results indicate that HCQ's ability to reduce IL-6 production by mesangial cells is mediated by its ability to decrease miR-146a expression. In MES 13 mesangial cells, miR-146a inhibition with HCQ treatment caused a significant increase in IL-6 production, which is consistent with recent reports that have shown miR-146a inhibition *in vitro* increases IL-6 production via p38 MAPK phosphorylation (95). These results indicate that although miR-146a is largely unaltered with HCQ or PRED treatment, miR-146a inhibition by HCQ is necessary in order to reduce IL-6 production in mesangial cells.

SLE patients with dysregulated miR-146a expression may benefit from immunosuppressant therapy that directly alters miR-146a expression. miR-146a is largely implicated in LN pathogenesis due to its regulation of both type I IFN production and TLR-stimulated downstream pathway activation (96, 97). It has recently been shown that serum miR-146a expression, which is downregulated in patients with SLE, is increased upon treatment with the anti-inflammatory drug calcitriol (59). These findings suggest that alterations to miR-146a expression may contribute to the immunoregulatory effects of calcitriol in patients with SLE. Like calcitriol, other therapeutics may mediate their anti-inflammatory properties through the alteration of miR-146a expression. Methotrexate (MTX) is an immunosuppressant that inhibits enzymes involved in purine metabolism, which results in the inhibition of T cell activation and

suppression of intercellular adhesion molecule expression (15). MTX is an effective treatment for SLE patients who are antimalarial-resistant, which indicates disparate mechanisms of action between MTX and HCQ (98). Because HCQ treatment did not alter miR-146a expression in the current studies, this suggests that MTX may be effective in altering miR-146a in SLE.

Our results demonstrate that changes in urinary miR-let-7a and miR-21 expression appear to correlate with PBMC miRNA expression changes over the course of treatment; treatment with either HCQ or PRED significantly decreased the expression of miR-let-7a and miR-21 over time. Because the expression of urinary and PBMCs miRNAs do not correlate in healthy tissue, these results suggest that altered expression of urinary and PBMC miR-let-7a and miR-21 may be used as biomarkers in the treatment of SLE (99). Pathogenic miRNAs in the urine or circulation may be used to detect early SLE disease onset before clinical, pathological findings arise. In addition, assessing miRNA expression in either the urine or PBMCs may offer valuable information regarding changes in the overall state of the SLE patient, providing insight to the effectiveness of a therapeutic regimen.

The interest in using miRNAs as non-invasive biomarkers has increased due to the discovery of dysregulated miRNA expression in the serum and urine of SLE patients (60, 75). Disease-causing miRNAs may become unique biomarkers that help determine the course of the patient's immunosuppressant therapy. The use of miRNAs as selection markers for disease treatment is underway in the treatment of ovarian cancer and nasopharyngeal carcinoma (100, 101). In ovarian cancer patients, the survival of patients with low miR-let-7a expression was higher when they received platinum and paclitaxel in combination; patients with high miR-let-7a expression did not have improved survival after adding paclitaxel to platinum-based therapy, indicating that miR-let-7a expression was predictive of a patient's outcome after chemotherapy

(100). It was recently shown that a particular miRNA signature was associated with nasopharyngeal carcinoma survival. Serum miR-22, miR-572, miR-638 and miR-1234 expression levels combined with tumor-node-metastasis had better prognostic value than tumor-node-metastasis alone (101). By identifying individual miRNA profiles in different cells and understanding how pharmacological agents modulate miRNAs and control inflammation, therapeutics that target specific miRNAs in specific cells may be utilized.

Due to the advancement of the first miRNA-targeting drug to clinical trials, therapeutic alteration of miRNAs may be a viable treatment option for patients with SLE and other diseases in the near future (46). miR-let-7a has been successfully targeted *in vivo* in order to treat inflammatory diseases including prostate cancer and asthma (102, 103). Administration of miR-21 antisense oligonucleotides delayed gastric cancer growth *in vitro* (104). Although most studies examine therapeutic modulation of miRNAs in cancer, there is evidence that clinically altering miRNAs in SLE is a viable treatment option. Global restoration of miR-146a by viral-like particles has been shown to be an effective treatment in lupus-prone BXSB mice, significantly reducing autoantibody, IFN- α , IL-1 β , IL-6, and total IgG production (47). Overall, miRNA-based therapies are an emerging class of drugs with great potential in the treatment of SLE and other inflammatory diseases.

4.7. Conclusion

Overall, our studies indicate that lupus therapeutics may work, in part, by altering the expression of disease-associated miRNAs in various tissue and cell types. Determining how particular therapeutics alter pathogenic miRNAs may ultimately provide a viable screening tool for specific, targeted therapy in SLE. miRNAs associated with disease may become unique biomarkers that help determine the course of the patient's immunosuppressant therapy. In addition, examining miRNA expression profiles during the course of immunosuppressant therapy may more accurately assess a patient's responsiveness to treatment.

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Future Directions

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5.2. Proposed experiments

5.2.1. *In vitro* experiments

We have recently shown that cells overexpressing miRNA-let-7a (let-7a) have increased expression of *IL-6* due to increased NF κ B activation (1). Future studies should determine the mechanism by which let-7a increases NF κ B nuclear translocation. It has recently been shown that let-7a targets the 3' untranslated region (UTR) of κ B-Ras2, a member of the Ras family of proteins (2). κ B-Ras2 inhibits NF κ B signaling by binding directly to I κ B α , inhibiting I κ B kinase (IKK)-dependent phosphorylation and subsequent degradation (3). Therefore, let-7a can promote nuclear translocation of NF κ B by binding to the 3' UTR of κ B-Ras2, facilitating IKK phosphorylation of I κ B α . Future experiments could confirm this proposed mechanism of let-7a-mediated NF κ B activation.

Our current studies have shown that cells overexpressing let-7a have increased *E2F2* expression (1). Because the precise mechanisms are unknown, future studies should determine the direct or indirect pathways through which let-7a increases the expression of *E2F2*. Let-7a may bind directly to the 3' UTR of *E2F2*, stabilizing the construct for translation (4). Alternatively, let-7a may target upstream mediators of E2F transcription. Retinoblastoma protein (Rb), p53, and p21 are implicated in the transcription of E2F family members (5). We have shown that cells overexpressing let-7a have increased Rb phosphorylation. Future investigations could examine the role of hyperphosphorylated Rb in E2F transcription. Like E2F, p53 plays a critical role in the cell cycle and is implicated in systemic lupus erythematosus (SLE) (6). Actively diseased SLE patients have higher levels of p53 in their peripheral blood mononuclear cells (PBMCs) compared to healthy controls (7). The effects of increased let-7a on p53-mediated transcription of E2F could be examined. The let-7a promoter contains potential

binding sites for p53 and the 3' UTR of p53 contains potential binding sites for let-7a, suggesting a potential positive feedback loop that may exacerbate increased let-7a, *E2F*, and *p53* expression in SLE (8-10).

We have shown that cells overexpressing let-7a proliferate more over time (1). Future experiments could investigate the mechanism by which let-7a increases cell proliferation. Let-7a has been shown to contribute to the constitutively increased phosphorylation of signal transducer and activator of transcription 3 (STAT3), a transcription factor activated by IL-6 (11). Phosphorylation and nuclear entry of STAT3 induces the transcription of genes involved in differentiation, cell activation, proliferation, and survival (12). Let-7a increases STAT3 phosphorylation by directly targeting and inhibiting neurofibromatosis 2 (NF2), a known regulator of STAT3 activation (13). By directly targeting the 3' UTR of *NF2*, let-7a may promote the expression of proliferation-associated genes induced by STAT3. Therefore, future experiments could examine the role of let-7a-mediated inhibition of NF2. These studies would provide evidence linking let-7a overexpression with increased IL-6 production, STAT3 activation, and cellular proliferation.

5.2.2. *In vivo* experiments

We have recently shown that hydroxychloroquine (HCQ) or prednisone (PRED) treatment alters the expression of specific miRNAs in New Zealand Black/White (NZB/W) mice (14). Because other immunosuppressants may work, in part, by altering disease-associated miRNAs, future studies should examine the effects of other lupus therapeutics on NZB/W miRNA expression. The immunosuppressant drug mycophenolate mofetil (MMF) has been successfully used to treat SLE by reversibly inhibiting inosine 5'-monophosphate dehydrogenase

(IMPDH), an enzyme involved in the *de novo* synthesis of guanine nucleotides (15). In NZB/W mice, MMF inhibited autoantibody formation, blocked the glycosylation of proteins involved in leukocyte adhesion, and showed a selective anti-proliferative effect on B and T cells (16). Like MMF, methotrexate (MTX) inhibits enzymes involved in purine metabolism, which results in the inhibition of T cell activation and suppression of intercellular adhesion molecule expression (17). Lupus mice treated with MTX had decreased proteinuria and increased life span compared to controls (18). MMF or MTX treatment may act to decrease cellular activation, in part, by decreasing miR-21 expression. Overexpressed miR-21 has been reported in the PBMCs and T cells of SLE patients and is strongly correlated with SLE disease activity (19, 20). Upregulation of miR-21 in T cells from SLE patients increased T cell activation (19). Studies have shown that silencing miR-21 *in vitro* reverses the activated T cell phenotype by reducing the expression of cell surface molecules that are vital to T cell activation (21). Therefore, the mechanism by which MMF or MTX decrease T cell proliferation and activation may be mediated by decreased miR-21 expression.

Our current studies have shown that the expression of miR-146a remains largely unchanged after treatment with HCQ or PRED in NZB/W mice (14). miR-146a is implicated in LN pathogenesis due to its regulation of both type I interferon (IFN) production and Toll-like receptor (TLR)-stimulated downstream pathway activation (22, 23). If future studies determine that therapeutics such as MMF or MTX increase miR-146a expression, this could be a major development in elucidating their anti-inflammatory mechanisms of action. These therapeutics could be utilized in the treatment of SLE patients with dysregulated miR-146a expression. MTX is especially promising due to reports that have shown it is an effective treatment for SLE patients who are antimalarial-resistant, which indicates disparate mechanisms of action between

MTX and HCQ (24). Previous studies have shown that overexpression of miR-146a in SLE PBMCs reduced type I IFN induction by targeting the 3' UTR of *interferon regulatory factor-5* (*IRF5*) and *STAT1* (25). Since miR-146a negatively regulates type I IFN production and myeloid differentiation factor 88 (MyD88) pathway activation, increased miR-146a expression induced by MMF or MTX may inhibit the production and downstream effects of IFN- α in SLE.

Future studies could also verify specific cellular and urinary miRNA alterations in strains that develop lupus differently than the NZB/W model, including the MRL/*lpr* or B6.MRLc1 models. MRL/*lpr* mice lack the *lpr* gene, which results in a defective Fas molecule and reduced Fas-mediated apoptosis of activated B and T cells (17). As a result, MRL/*lpr* mice develop massive lymphoproliferation, high titers of anti-dsDNA antibodies, and lethal glomerulonephritis (26). The B6.MRLc1 model is congenic strain carrying a region of chromosome 1 derived from MRL/MpJ mice that develop immune complex – mediated glomerulonephritis (27). Confirming altered miRNA expression in other models of SLE may reveal a common miRNA expression profile induced by a particular immunosuppressant therapy across the lupus models.

Future *in vivo* studies could use urinary or PBMC miRNA profiles of NZB/W mice as unique biomarkers that determine the course of their immunosuppressant therapy. By determining the expression of disease-associated miRNAs from the urine or blood of NZB/W mice as they age, mice can be treated with a therapeutic that is known to target the expression of a specific miRNA. For example, urinary miR-155 expression has been shown to be significantly increased in SLE patients compared to healthy controls (28). Our previous studies have shown that PRED treatment significantly reduces miR-155 expression in the urine of NZB/W mice (14). To determine the baseline expression of miR-155 in healthy mice, urinary miR-155 expression would be measured from NZW mice, the parental strain that does not develop disease (29).

NZB/W mice that have increased urinary expression of miR-155 would be assigned to either the PRED group or the vehicle control group. In addition, NZB/W mice that have urinary expression of miR-155 similar to the NZW controls would be assigned to either the PRED group or the vehicle control group. The group allocation would be based on each mouse's baseline miRNA expression from 16 – 24 weeks-of-age.

At the end of the studies, mice would be assessed for differences in total body weight, spleen weight, and spleen-to-body weight ratios. Histological evaluation will focus primarily on the kidney for changes in the glomerulus (size and cellularity), tubules (epithelial changes and protein casts), as well as the interstitium (inflammation). Alterations in kidney deposition would be examined using fluorescent antibodies for complement component 3 (C3) and IgG. This will allow direct correlation with changes in kidney architecture and function. NZB/W mice with increased urinary miR-155 expression that were treated with PRED would be expected to have decreased proteinuria, spleen-to-body weight ratios, C3 and IgG deposition, and reduced kidney inflammation and cellularity. Based on our previous work, the expression of urinary miR-155 would be expected to decrease with PRED treatment (14). Either vehicle-treated group (NZB/W mice that did or did not have increased urinary miR-155 expression) would be expected to be severely diseased. If mice with normal urinary miR-155 expression are less diseased than controls with PRED treatment, this may indicate that altered miR-155 expression induced by PRED treatment is an epiphenomenon. Urinary miR-155 may not be an appropriate biomarker for PRED therapy selection. On the other hand, if these mice have increased kidney inflammation at the end of the study based on histological scoring, this would indicate that individuals with increased urinary miR-155 expression may benefit more from PRED therapy than those with normal urinary miR-155 expression. These studies would show that urinary or

PBMC miRNA profiles can be used as unique biomarkers to determine the choice of immunosuppressant therapy. In addition, these studies would determine if the expression of specific miRNAs is predictive of treatment outcome.

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Supplemental Figures

A.2. Supplemental figures

A.2.1. Chapter 2

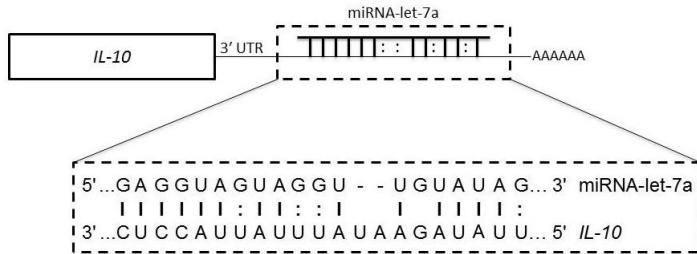


Figure S1. The 3' UTR of *IL-10* is a predicted target of let-7a. Vertical lines represent direct base pairing. Colons represent non-Watson-Crick base pairing (or wobble base pairing).

A.2.2. Chapter 3

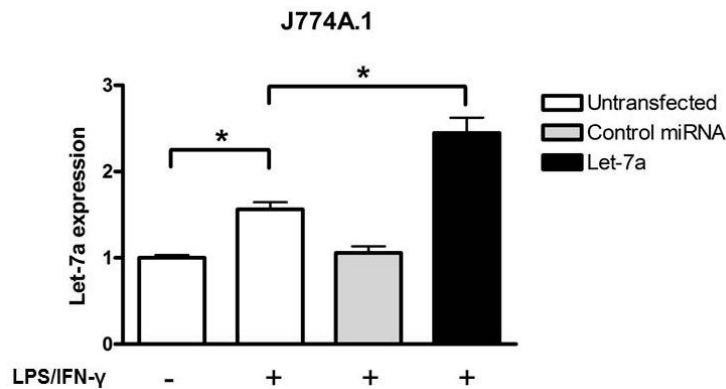


Figure S1. Real-time RT-PCR shows let-7a expression is increased in J774A.1 macrophages after stimulation with LPS and IFN- γ . The nontargeting miRNA control did not alter the relative expression of let-7a. Let-7a is significantly increased after transfection of the let-7a mimic. The final concentration of the miRNAs was 25 nM. The nontargeting miRNA is miR-67. Error bars represent the SEM. * $p < 0.05$.

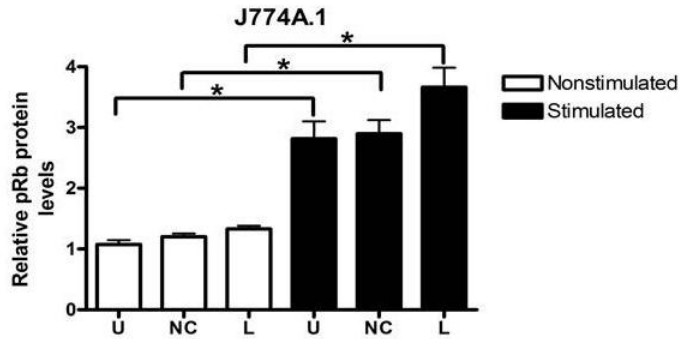


Figure S2. Western blot quantitative densitometry shows there is a significant increase in pRb in immune-stimulated cells compared to nonstimulated cells. There is an increase in pRb in let-7a-transfected J774A.1 macrophages that were immune-stimulated compared to the stimulated controls. Densitometry data was normalized to the intensity of β -actin bands. The final concentration of the miRNAs was 25 nM. The nontargeting miRNA is miR-67. Error bars represent the SEM. $*p < 0.05$.

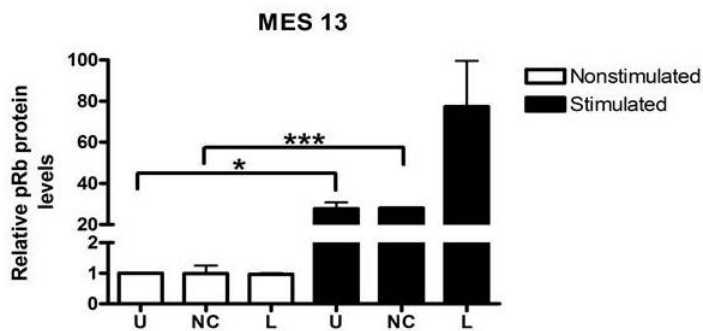


Figure S3. Western blot quantitative densitometry shows there is an increase in pRb in immune-stimulated cells compared to nonstimulated cells. There is an increase in pRb in let-7a-transfected MES 13 mesangial cells that were immune-stimulated compared to the stimulated controls. Densitometry data was normalized to the intensity of β -actin bands. The final concentration of the miRNAs was 25 nM. The nontargeting miRNA is miR-67. Error bars represent the SEM. $*p < 0.05$.

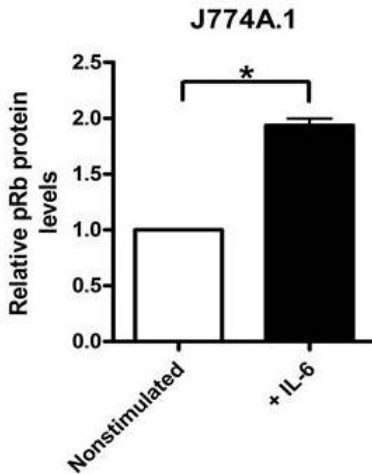


Figure S4. Western blot quantitative densitometry shows there is a significant increase in pRb in J774A.1 macrophages stimulated with IL-6 compared to nonstimulated cells. Densitometry data was normalized to the intensity of β -actin bands. The final concentration of the miRNAs was 25 nM. The nontargeting miRNA is miR-67. Error bars represent the SEM. $*p < 0.05$.

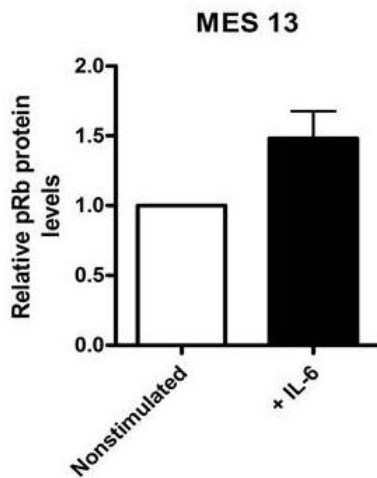


Figure S5. Western blot quantitative densitometry shows there is an increase in pRb in IL-6-stimulated MES 13 mesangial cells compared to nonstimulated cells. Densitometry data was normalized to the intensity of β -actin bands. The final concentration of the miRNAs was 25 nM. The nontargeting miRNA is miR-67. Error bars represent the SEM. $*p < 0.05$.

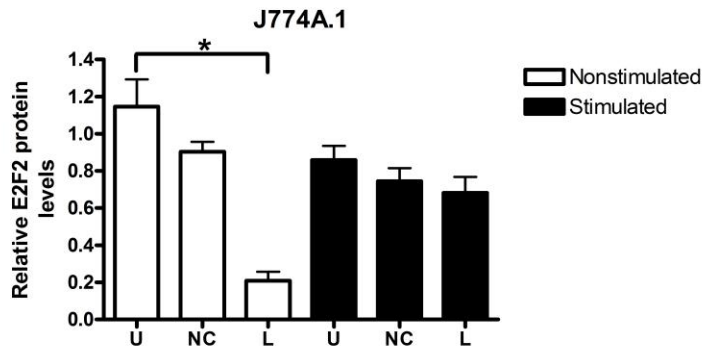


Figure S6. Western blot quantitative densitometry shows there is a significant decrease in E2F2 in nonstimulated J774A.1 macrophages overexpressing let-7a. E2F2 is unchanged in stimulated cells. Densitometry data was normalized to the intensity of β -actin bands. The final concentration of the miRNAs was 25 nM. The nontargeting miRNA is miR-67. Error bars represent the SEM. *** $p < 0.0005$.

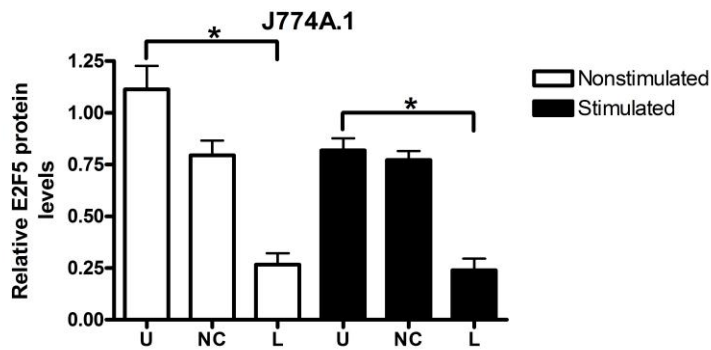


Figure S7. Western blot quantitative densitometry shows there is a significant decrease in E2F5 in nonstimulated or stimulated J774A.1 macrophages overexpressing let-7a. Densitometry data was normalized to the intensity of β -actin bands. The final concentration of the miRNAs was 25 nM. The nontargeting miRNA is miR-67. Error bars represent the SEM. * $p < 0.05$.

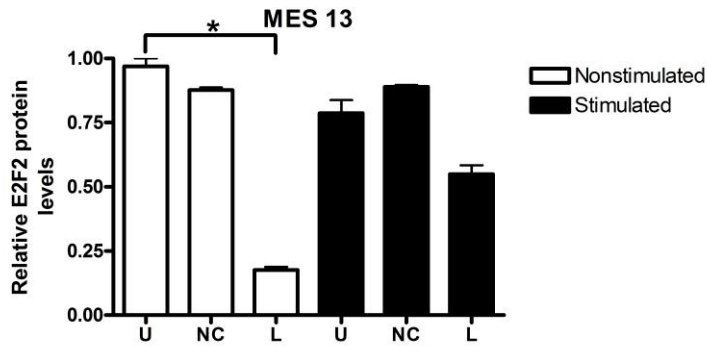


Figure S8. Western blot quantitative densitometry shows there is a significant decrease in E2F2 in nonstimulated MES 13 mesangial cells overexpressing let-7a. E2F2 is unchanged in stimulated cells. Densitometry data was normalized to the intensity of β -actin bands. The final concentration of the miRNAs was 25 nM. The nontargeting miRNA is miR-67. Error bars represent the SEM. $*p < 0.05$.

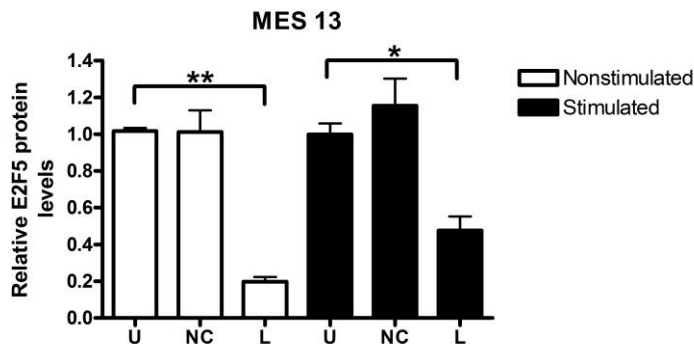


Figure S9. Western blot quantitative densitometry shows there is a significant decrease in E2F5 in nonstimulated or stimulated MES 13 mesangial cells overexpressing let-7a. Densitometry data was normalized to the intensity of β -actin bands. The final concentration of the miRNAs was 25 nM. The nontargeting miRNA is miR-67. Error bars represent the SEM. $*p < 0.05$.

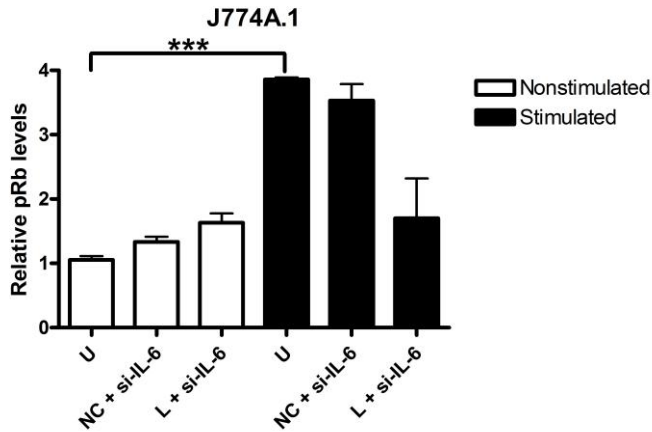


Figure S10. Western blot quantitative densitometry shows there is an increase in pRb in immune-stimulated cells compared to nonstimulated cells. There is a decrease in pRb in let-7a-transfected J774A.1 macrophages that were immune-stimulated compared to the stimulated controls. Densitometry data was normalized to the intensity of β -actin bands. The final concentration of the miRNAs was 25 nM. The nontargeting miRNA is miR-67. Error bars represent the SEM. $*p < 0.05$.

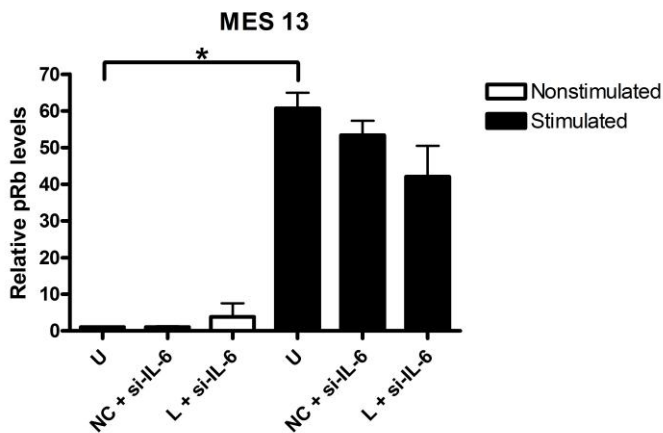


Figure S11. Western blot quantitative densitometry shows there is an increase in pRb in immune-stimulated cells compared to nonstimulated cells. There is a decrease in pRb in let-7a-transfected MES 13 mesangial cells that were immune-stimulated compared to the stimulated

controls. Densitometry data was normalized to the intensity of β -actin bands. The final concentration of the miRNAs was 25 nM. The nontargeting miRNA is miR-67. Error bars represent the SEM. * $p < 0.05$. ** $p < 0.01$.

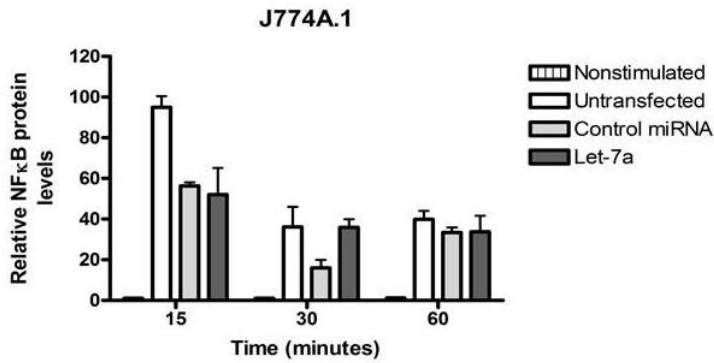


Figure S12. Western blot quantitative densitometry shows there is an increase in NF κ B in let-7a-transfected J774A.1 macrophages that were immune-stimulated for 30 minutes compared to cells transfected with the control miRNA. Densitometry data was normalized to the intensity of β -actin bands. The final concentration of the miRNAs was 25 nM. The nontargeting miRNA is miR-67. Error bars represent the SEM.

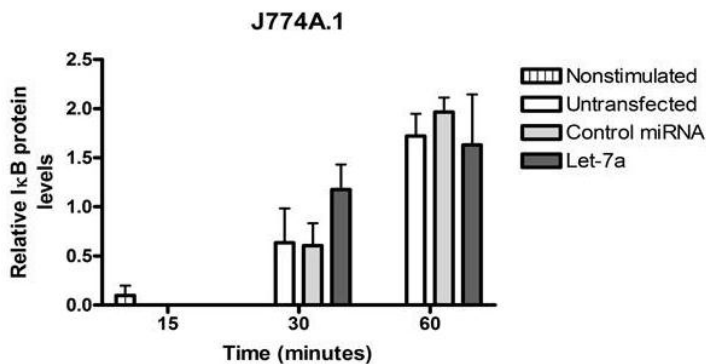


Figure S13. Western blot quantitative densitometry shows there is an increase in pI κ B in let-7a-transfected J774A.1 macrophages that were immune-stimulated for 30 minutes compared to

controls. Densitometry data was normalized to the intensity of β -actin bands. The final concentration of the miRNAs was 25 nM. The nontargeting miRNA is miR-67. Error bars represent the SEM. $*p < 0.05$.

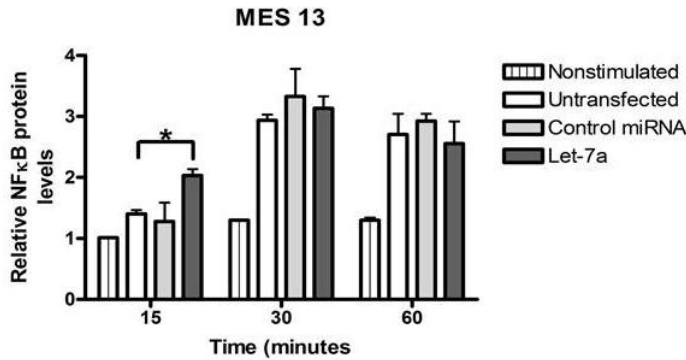


Figure S14. Western blot quantitative densitometry shows there is a significant increase in NF κ B in let-7a-transfected MES 13 mesangial cells that were immune-stimulated for 15 minutes compared to the untransfected control. Densitometry data was normalized to the intensity of β -actin bands. The final concentration of the miRNAs was 25 nM. The nontargeting miRNA is miR-67. Error bars represent the SEM. $*p < 0.05$.

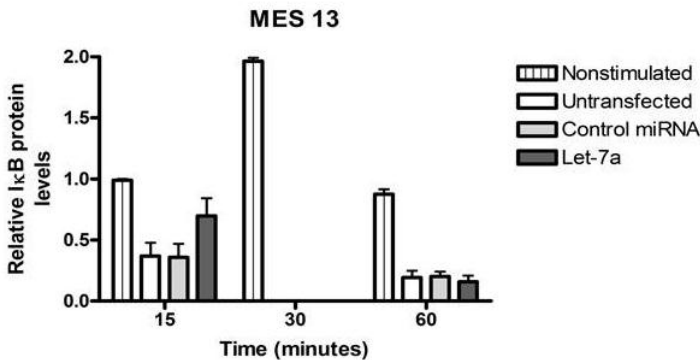


Figure S15. Western blot quantitative densitometry shows there is an increase in I κ B in let-7a-transfected MES 13 mesangial cells that were immune-stimulated for 15 minutes compared to

controls. Densitometry data was normalized to the intensity of β -actin bands. The final concentration of the miRNAs was 25 nM. The nontargeting miRNA is miR-67. Error bars represent the SEM. $*p < 0.05$.