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

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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 iRNAs.

Keywords

hydroxychloroquine; microRNAs; NZB/W mice; prednisone; systemic lupus erythematosus

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

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Conflict of Interest

The authors declare no financial or commercial conflict of interest.

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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 NFκB 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 NFκB 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.

2. Materials and Methods

2.1.1 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 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.

2.1.2 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.

2.2.1 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 .

2.2.2 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 $400 \times g$ 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 .

2.2.3 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 (61, 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.

2.2.4 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 $70 \mu\text{m}$ 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.

2.2.5 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 .

2.3.1 IgG ELISA—IgG_{2a} and total IgG protein levels in whole blood 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.

2.3.2 Anti-dsDNA ELISA—Anti-dsDNA levels in whole blood were measured by ELISA. High-binding plates were coated with 100 μ L of 5 μ g/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 μ L/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.

2.3.3 Cytokine ELISA—TNF- α , IL-6, and IL-10 protein levels in whole blood or 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.

2.3.4 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 by the comparative CT ($\Delta\Delta$ CT) method using snoRNA202 as the endogenous control (71). All samples were run in triplicate.

2.4.1 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. Experiments were performed from passage 9–15. All experimental conditions were run in triplicate.

2.4.2 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.

2.4.3. 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. Nonstimulated controls received complete growth medium. Cells and supernatants were collected for analysis 24 hours post-stimulation.

2.5 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.

3. Results

3.1 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 (50, 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 < 100 mg/dL throughout the study. Over the course of the study, vehicle control-treated NZB/W mice had significantly higher proteinuria scores than treated mice. Proteinuria scores for control mice were approximately 800 mg/dL at 26 weeks-of-age and 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 differences in IgG_{2a} production amongst the treatment or control groups (Figure 1B). There was a gradual increase in IgG_{2a} production as the mice aged. At 28, 32, and 36 weeks-of-age, there was a decrease in IgG_{2a} production when either the HCQ or PRED-treated group was compared to the control group. 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 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 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 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, 32, and 36 weeks-of-age, mice treated with HCQ had reduced anti-dsDNA antibody production compared to vehicle-treated mice. PRED-treated mice also had decreased anti-dsDNA antibody levels compared to controls at 28, 32, and 36 weeks-of-age. 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 although IL-6 production increased as the mice aged (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.

3.2 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 $2^{-\Delta\Delta CT}$ 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.

3.3 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.

3.4 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.

3.5 Immunosuppressants alter 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.

3.6 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.

4. 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 NF κ B-mediated mesangial cell activation and extracellular matrix production in glomerulonephritis (51, 87). The miR-21 promoter is regulated by NF κ B and STAT3, a transcription factor activated by IL-6, which suggests there may be a positive feedback loop connecting miR-21, NF κ B, 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 γ , 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.

5. 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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Highlights

- We measured miRNA alterations in NZB/W mice treated with HCQ or PRED.
- MiRNAs were measured in mesangial cells, splenocytes, PBMCs, and the urine.
- HCQ or PRED differentially altered miRNA expression in cells and tissues.
- MiRNA inhibition decreased HCQ or PRED's anti-inflammatory abilities *in vitro*.
- Individualized therapy may be utilized based on miRNA expression.

Figure 1A

Figure 1B

Figure 1C

Figure 1D

Figure 1E

Figure 1F

Figure 1G

Figure 1H

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 increased proteinuria with 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 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 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 less anti-dsDNA antibody production than mice treated with the vehicle alone. At 32 and 36 weeks-of-age, mice treated with HCQ or PRED had 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 where n = 5). Error bars represent the SEM. * $p < 0.05$. *** $p < 0.0001$.

Figure 2A

Figure 2B

Figure 2C

Figure 2D

Figure 2. HCQ or PRED treatment alters miRNA expression in PBMCs

A. At 28 and 32 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 28, 32, and 36 weeks-of-age in NZB/W mice treated with prednisone (PRED). B. The expression of miR-21 was significantly reduced at 28, 32, and 36 weeks-of-age in PBMCs from NZB/W mice treated with HCQ compared to controls. At 28 and 32 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. C. MiR-146a expression was significantly decreased at 28 weeks-of-age and returned to levels seen in the control at 32 and 36 weeks-of-age. MiR-146a expression was unaltered at any time point in NZB/W mice treated with PRED compared to control mice. D. At 28, 32, and 36 weeks-of-age, the expression of miR-155 was significantly decreased in NZB/W mice treated with HCQ or PRED. 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). Error bars represent the SEM. * $p < 0.05$. ** $p < 0.01$.

Figure 3A

Figure 3B

Figure 3C

Figure 3D

Figure 3E

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 significant 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. B. At 28, 32, and 36 weeks-of-age, there was a significant decrease in urinary miR-21 expression in NZB/W mice treated with HCQ or PRED compared to the vehicle control-treated mice. C. Urinary miR-146a expression was unchanged after treatment with HCQ or PRED. D. MiR-155 expression was significantly reduced in the urine of HCQ-treated NZB/W mice compared to controls at 28, 32, and 36 weeks-of-age. The expression of miR-155 was significantly reduced at 32 and 36 weeks-of-age in PRED-treated NZB/W mice compared to vehicle control-treated mice. 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). Error bars represent the SEM. $*p < 0.05$. $**p < 0.01$.

Figure 4A

Figure 4B

Figure 4C

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). Error bars represent the SEM. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.0005$.

Figure 5A

Figure 5B

Figure 5C

Figure 5D

Figure 5E

Figure 5F

Figure 5G

Figure 5H

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. * $p < 0.05$. ** $p < 0.01$.