

The gene silencing of IRF5 and BLYSS effectively modulates the outcome of experimental lupus nephritis

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Systemic lupus erythematosus is a highly complex and heterogeneous autoimmune disease mostly mediated by B cells. It is characterized by circulating self-reactive antibodies that deposit and form immune complexes in kidney, leading to irreparable tissue damage and resulting in lupus nephritis. In a New Zealand Black X New Zealand White F1 mouse model, we tested two different small interfering RNA (siRNA) silencing treatments against interferon regulatory factor 5 (IRF5) and B cell-activating factor (BLYSS) expression and their combination in a second set of animals. The administration of these two siRNAs separately prevented the progression of proteinuria and albuminuria at similar levels to that in cyclophosphamide animals. These treatments effectively resulted in a reduction of serum anti-double-stranded DNA (dsDNA) antibodies and histopathological renal score compared with non-treated group. Treated groups showed macrophage, T cell, and B cell infiltrate reduction in renal tissue. Moreover, kidney gene expression analysis revealed that siRNA treatments modulated very few pathways in contrast to cyclophosphamide, despite showing similar therapeutic effects. Additionally, the combined therapy tested in a second set of animals, in which the disease appeared more virulent, exhibited better results than monotherapies in the disease progression, delaying the disease onset and ameliorating the disease outcome. Herein, we provide the potential therapeutic effect of both selective IRF5 and BLYSS silencing as an effective and potential treatment, particularly in early phases of the disease.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a complex, heterogeneous, and chronic autoimmune disorder. SLE is a B cell-mediated autoimmune disease in which autoantibodies, such as anti-double-stranded DNA (dsDNA) autoantibodies, form immune complexes that are deposited in different tissues, resulting in multiple inflammatory lesions and tis-

sue damage.^{1–3} One of the most serious related complications is lupus nephritis (LN), which can lead to severe proteinuria, chronic renal failure, and end-stage kidney disease. This complication remains the leading cause of short-term morbidity and mortality.⁴ The standard treatment for SLE is immunosuppression with corticosteroids and cyclophosphamide (CYP) and more recently with mycophenolate (Mofetil). However, these long-term treatments produce non-negligible adverse effects. Therefore, it is desirable to improve disease outcomes and quality of life by introducing new treatments with fewer side effects.^{1,2,5}

A feature of the immune system activation in SLE is the presence of increased circulating interferon (IFN)- α , which correlates with disease activity.⁶ Most patients with SLE show evidence of type I IFN increase, but frequently with dysregulated expression of genes in the IFN pathway. Furthermore, this IFN gene expression “signature” served as a marker for more severe diseases involving the kidneys.⁷ IFN production occurs when Toll-like receptors (TLRs) detect certain pathogen-associated molecular patterns (PAMPs); TLRs initiate signaling by inducing phosphorylation of the IFN regulatory factors (IRFs) type 3, 5, and 7 that, in turn, induce transcription of IFN-related genes.⁸ Once produced, type I IFNs exert an activating effect on dendritic cells (DCs), triggering higher expression of costimulatory molecules, major histocompatibility complex class I (MHC class I)/II proteins, chemokines, and chemokine receptors.⁹ IFN also

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Table 1. Sequence description and target localization of the siRNAs designed to screen for efficient mouse IRF5 mRNA silencing

Anti-mouse IRF5 siRNA	Mouse IRF5 mRNA-targeted region	Sequence (sense/antisense)
siRNA-1	143–162	5'-AGAAACUCUUCUUAUACCTT-3'/5'-GGUAUAUAGAAGAGUUUCUTT-3'
siRNA-2	1,114–1,135	5'-GUCAAGACGAAGCUCUUUATT-3'/5'-UAAAGAGCUUCGUCUUGACTT-3'
siRNA-3	1,246–1,267	5'-CGAGAGAAGAAGCUCUUAUACU-3'/5'-AGUAAUGAGCUUCUUCUCUCGGG-3'
siRNA-4	1,292–1,313	5'-GGUUGCUGCUGGAGAUGUUCU-3'/5'-AACAUCCAGCAGCAACCGG-3'
siRNA-5	774–795	5'-GAGAUCAAGUCCAGUACCGG-3'/5'-GGUACUGGAACUUGAUCUCTT-3'
siRNA-6	1,150–1,169	5'-UCAUAGAGCUCAUCCUGUUTT-3'/5'-AACAGGAUGAGCUCAUUGATT-3'
siRNA-7	376–395	5'-UCUACGAGGUCUGCUCCAATT-3'/5'-UUGGAGCAGACCUCGUAGATT-3'
siRNA-8	2,004–2,023	5'-UAAAUUCUACUUUCUGUCCTT-5'/5'-GGACAGAAAGUAGAAUUUATT-3'
siRNA-9	449–474	5'-GGAGGAGGAGGAAGAGGAAGAGCTC-3'/5'-AGCUCUCCUCUCCUCCUCCUCCUC-3'
siRNA-10	1,546–1,571	5'-GGACCUCUGGGACAGCCACGUAGA-3'/5'-UCUACGUGGCUGUCCAGGAGGUCAA-3'
siRNA-11	139–160	5'-UAUAGAAGAGUUUCUUUCC-3'/5'-GAAAAGAAACUCUUCUAUAUA-3'
siRNA-12	1,195–1,216	5'-AAAAGAAGAUUCUAAAAGGUG-3'/5'-CCUUUUGAGAUUCUUCUUUUGC-3'
siRNA-13	1,251–1,272	5'-GAGAAGAAGCUCUUAACUGUA-3'/5'-CAGUAAUGAGCUUCUUCUCTT-3'
siRNA-14	1,125–1,148	5'-AAGCUCUUUAGCCUAGAGCAGUU-3'/5'-CUGCUCUAGGCUAAAGAGCUUTT-3'

stimulates the activation of T and B cells, leading to antibody production.¹⁰

IRF5 genetic variants and disease-associated factors contribute to enhanced IRF5 expression in blood cells of SLE patients, with the risk of disease progression.^{11–14} IRF5 is necessary for the development of a lupus-like disease in both New Zealand Black X New Zealand White F1 (NZB/WF1) and MRL/lpr mice,^{15,16} and the permanent effect of IFN can be instrumental in perpetuation of the autoimmune process.^{16,17} Results from clinical studies in patients with SLE with anti-IFN- α monoclonal antibodies suggest that reduced expression of IFN-inducible genes could lead to a decrease in pro-inflammatory activity and clinical improvement.^{18–20}

B cell-activating factor (BLYSS) is a transmembrane glycoprotein in the tumor necrosis factor (TNF) ligand superfamily that is involved particularly in B cell maturation.^{21–23} Autoreactive B cell clones require higher levels of BLYSS for their survival. BLYSS overexpres-

sion causes SLE, and its inhibition can delay the disease in murine models. Therefore, BLYSS inhibition has recently become a promising therapeutic target for SLE.^{24–27} BLYSS expression is induced *in vivo* by type I IFN, but the exact molecular mechanisms and its relation to IRF5 remain unknown.²⁸ Comprehending how the expression of BLYSS is related to type I IFN system activation in SLE might provide important clues to understanding the mechanisms of the disease. BLYSS expression is directly downstream to the type I IFN signaling, and the members of the IRF family tightly regulate the expression of BLYSS. Thus, there is a critical interrelation between both networks. This leads to the hypothesis that *in vivo* blockade of both pathways concurrently could multiply their protective effects in LN.

RNA interference is a posttranscriptional genetic phenomenon inhibiting gene expression, and it is mediated by double-stranded RNA. Small interfering RNAs (siRNAs) act as leader sequences, joining nuclease activity protein complexes (RNA-induced silencing complex [RISC]), and the positioning in the mRNA target allows the specific target degradation. The main advantages of siRNAs are their high efficiency, potency, and specificity, thereby allowing their use at low concentrations with a highly specific gene expression inhibition. In addition, some chemical modifications result in siRNA molecules becoming resistant to degradation by nucleases and improving their distribution, half-life, and cellular uptake.^{29–31} Despite recent advances in delivering siRNA, the immunogenicity of siRNA and the delivery vehicle (Veh) remain challenging, especially when repeated dosing is needed to treat chronic or degenerative diseases. We used a murine-specific siRNA, chemically stabilized and cholesterol conjugated, in a model of autoimmune nephritis where we demonstrate that systemic blockade induced immune deactivation of the injured kidneys.¹

In the current study, the BLYSS and IRF5 blockade effects with our specifically designed siRNAs were analyzed in NZB/WF1 mice with established autoimmune nephritis. We hypothesized that blocking IRF5 expression would reduce the IFN-cascade-mediated signals and that the BLYSS blockade would reduce the secretion of antibodies and formation of immune complexes. Moreover, we developed the study with a multi-target strategy with these two functional and tested molecules, hypothesizing their additive effect.^{32,33} We analyzed the disease evolution and the modulation of renal histological lesions, mainly in the glomerular compartment, and tried to provide mechanistic insights focusing on renal gene signature. Furthermore, we assessed whether systemic siRNA administrations affected spleen cell populations.

RESULTS

Anti-mouse IRF5 and BLYSS siRNA selection

Fourteen and 12 siRNAs were designed to target different positions within the coding region of mouse IRF5 (Table 1) and BLYSS (Table 2) mRNA, respectively (see [Materials and methods](#) for procedures). Twenty-four hours after transfection into cells, IRF5 and BLYSS RNA expression was measured relative to 18s rRNA levels using quantitative real-time PCR and then normalized to the mRNA

Table 2. Sequence description and target localization of the siRNAs designed to screen for efficient mouse BLYSS mRNA silencing

Anti-mouse BLYSS siRNA	Mouse BLYSS mRNA-targeted region	Sequence (sense/antisense)
siRNA-1	466–487	5'-GCAGAGCUACCGAAGUUCAGC-3'/5'-UGAACUUCGGUAGCUCUGCAG-3'
siRNA-2	700–721	5'-CCUCAGAAACAUCAUCAAGA-3'/5'-UUGAAUGAUGUUUCUGAGGUU-3'
siRNA-3	176–197	5'-GCAGUUUCACAGCGAUGUCTT-3'/5'-GACAUCGCUUGAAACUGCTT-3'
siRNA-4	270–291	5'-GAAAGGAGAAGAUUGAAATT-3'/5'-UUUCAUAUCUUCUCCUUUCTT-3'
siRNA-5	562–583	5'-CACAAUCUCCAGCCGCGCCAC-3'/5'-GUGGCCGCGCUGGAGUUGUG-3'
siRNA-6	610–631	5'-UACCGAAGUUCAGCGACACCC-3'/5'-GGGUGUCGCUAACUUCGGUA-3'
siRNA-7	401–422	5'-UCACAGCGAUGUCCUUGUATT-3'/5'-UACAAGGACAUCGUGGATT-3'
siRNA-8	57–78	5'-UUCUGCAUCUUUUGCUACCTT-3'/5'-GGUAGCAAAAGAUGCAGAATT-3'
siRNA-9	266–287	5'-AUAUCUUCUCCUUUCUGGTT-5'/5'-CCGAGAAAGGAGAAGAUAUTT-3'
siRNA-10	720–741	5'-GACUGUCGAGCUGAUUGTT-3'/5'-CAAUCAGCUCGAGACAGUCTT-3'
siRNA-11	46–67	5'-UUUUGCUACCCUGAAGUAGGU-3'/5'-CUACUUCAGGUAGCAAAAGA-3'
siRNA-12	610–631	5'-UACAUCUUGUUCUGUUUCCUC-3'/5'-GGAAACAGACAAGAUGUAGA-3'

levels of the non-targeting control (Figures 1 and 2). Several of these siRNAs had significant knockdown efficacy. However, taking into account the replicates and the different doses that were tested in RAW 264.7 cell culture, siRNA-3 was finally chosen for IRF5 silencing (renamed as siIRF5); and in MCP11 cell culture, siRNA-4 was selected for BLYSS silencing (renamed as siBLYSS) for the subsequent *in vivo* study.

First set of experiments with siRNA monotherapy transfection

After siRNA selection, animals received either siIRF5 or siBLYSS in monotherapy and fully adhered to the established protocol. Different disease outcomes and cumulative survival were observed, depending on the treatments, as presented in Figures 3 and 4 and Table 3.

Effect of siRNA monotherapy transfection in LN model

Proteinuria levels increased progressively in Veh and scrambled non-silencing siRNA (SC) groups (Figure 3). By contrast, monotherapy-treated groups showed a significant diminution of proteinuria compared with Veh and SC groups at the end. Albuminuria levels followed a similar pattern as proteinuria. IRF5 and BLYSS groups had significantly lower proteinuria and albuminuria levels than the control groups.

According to histopathological evaluation, untreated animals developed typical and severe lesions of LN (Figure 4). siIRF5 treatment produced a global reduction in the evaluated lesions. Regarding si-

BLYSS treatment, the almost complete disappearance of glomerular deposits and a reduction in interstitial infiltrates, tubular atrophy, and interstitial fibrosis were prominent. BLYSS and intraperitoneal CYP groups displayed significantly less structural damage compared with the SC and Veh groups.

Levels of renal infiltrating CD3⁺ cells (T cell) were reduced in treated groups compared with the SC group, demonstrating their therapeutic effect. A reduction of CD45RA⁺ (B cell) infiltrate was only observed in siBLYSS treatment.

Anti-dsDNA antibody progression was also lower in treated groups. The significantly lowest increase was observed in the CYP group. The reduction of this circulating immunological marker of the disease paralleled the significant reduction of both renal immunoglobulin G (IgG) and complement 3 (C3) in all treated groups.

Analysis of peripheral blood, spleen, and kidney cell populations with flow cytometry

Peripheral blood analysis displayed different cell population changes, depending on the treatments. Animals treated with siIRF5 revealed a decrease in B cell population. Despite the reduction of CD4⁺ T cells, an increase in the CD3⁺ T cell population was observed, mainly due to the regulatory T cells (Tregs) expansion. The siIRF5 treatment also induced an increase of regenerating monocytes.

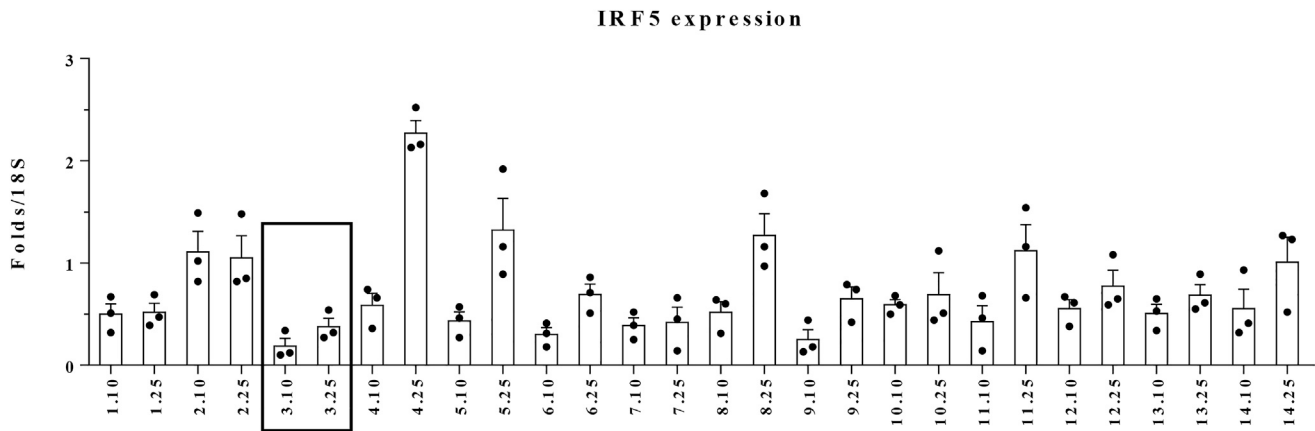


Figure 1. Knockdown efficiency of siRNA *in vitro* transfection (10, 15, and 50 nM) sequences against mouse IRF5

The synthesized siRNAs were assayed in RAW 264.7 cell line (macrophages). The amount of IRF5 mRNA was measured relative to 18s rRNA levels using qRT-PCR and then normalized to the mRNA levels of the non-targeting control, 24 h post-transfection. Results are given as mean \pm SEM from two independent experiments performed in duplicate.

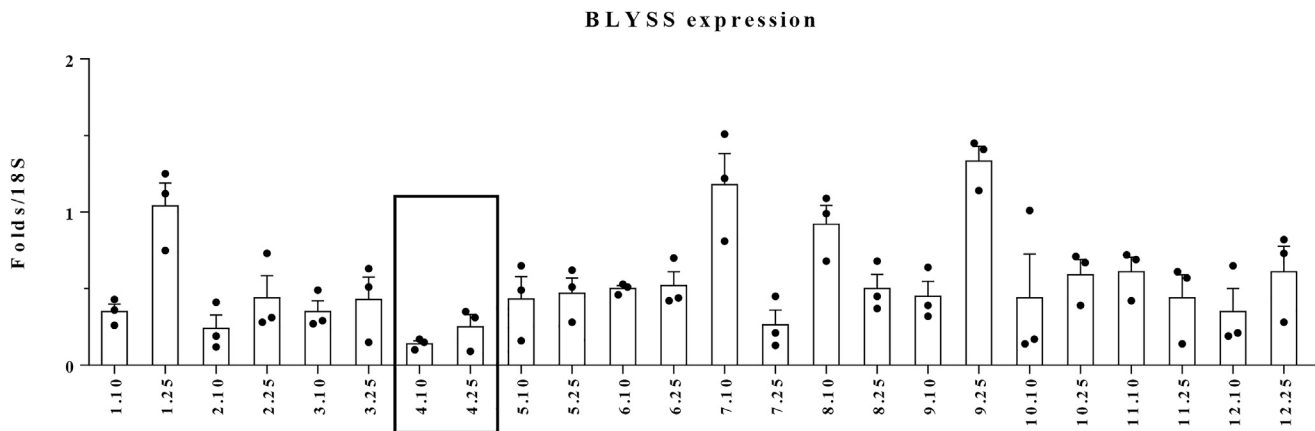


Figure 2. Knockdown efficiency of siRNA *in vitro* transfection (10, 15, and 50 nM) sequences against mouse BLYSS

The synthesized siRNAs were assayed in MCP11 cell line (B lymphocytes). The amount of BLYSS mRNA was measured relative to 18s rRNA levels using qRT-PCR and then normalized to the mRNA levels of the non-targeting control, 24 h post-transfection. Results are given as mean \pm SEM from two independent experiments performed in duplicate.

BLYSS silencing also showed a B cell reduction alongside a CD3⁺ T cell increase; but in this case, the double-positive CD4⁺CD8⁺ T cells were the most expanded subpopulation. This treatment also increased the precursor DC (pDC) population.

Finally, animals treated with CYP revealed an increase of regulatory B cells (B-regs), regenerating monocytes, and pDC subpopulations. A reduction of CD4⁺ T cells was also observed; however, there was an increase in the total amount of CD3⁺ T cells.

Concerning spleen analysis, all treated groups showed an increase in B-reg and B1 cell populations.

By contrast, a reduction was seen regarding B2 cells, DC populations, and M1 macrophages. M2 macrophages were increased only in siBLYSS- and CYP-treated animals. With regard to CD3⁺ T cell population, only slight changes were noticed between groups.

Kidney tissue analysis showed a significant decrease in infiltrating B cells and macrophages in all treated groups. A reduction of CD3⁺ T cell and DC infiltrate was also revealed. IRF5-silencing group also exhibited an increase in B-reg recruitment.

The complete cytometry data are provided in the [Supplemental information](#).

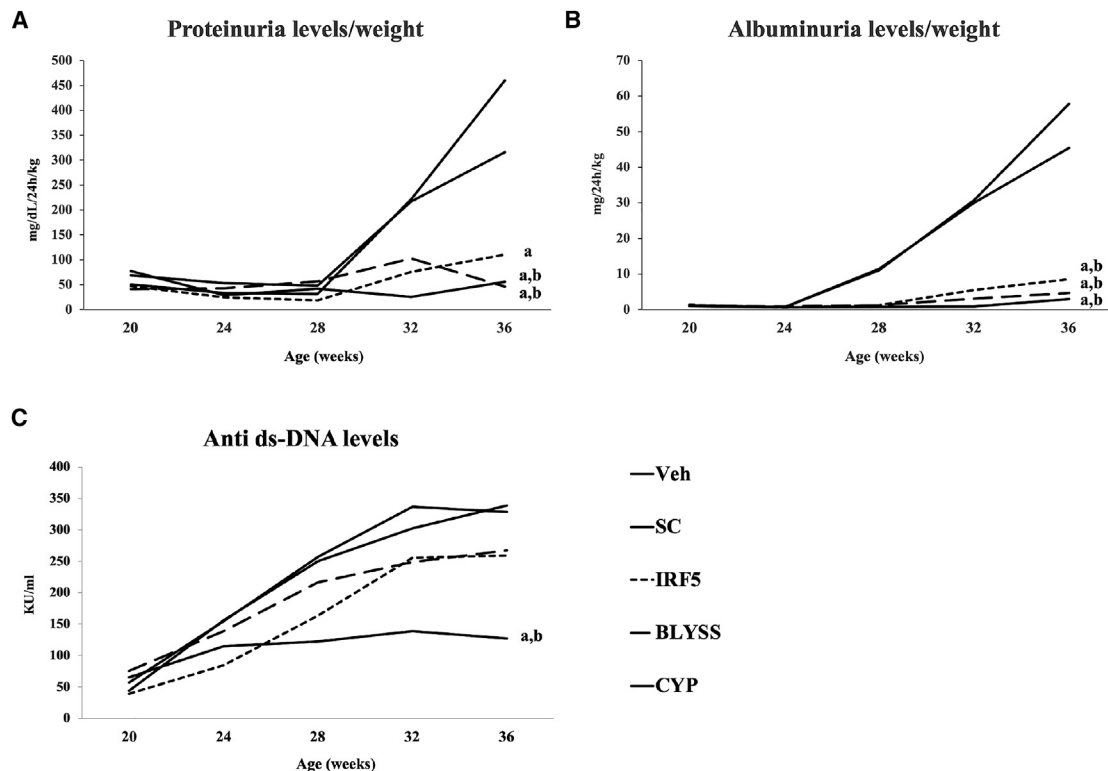


Figure 3. Functional renal parameters for the first set of experiments throughout the study for all treatments

(A and B) Functional renal parameters for the first set of experiments including (A) proteinuria and (B) albuminuria by weight. Values are expressed in mg/kg for 24 h. (C) Serum anti-dsDNA levels throughout the study for all treatments are expressed in kilo units/mL. Data are expressed as mean. a, $p < 0.05$ versus Veh; b, $p < 0.05$ versus SC.

Serum T helper 1 (Th1) and Th2 inflammatory cytokines

Levels of serum TNF, MCP1, interleukin-10 (IL-10), IL-6, and INF- γ were quantified in all surviving animals at the end of the study (Figure 5). Significant reductions of TNF levels in CYP and IRF5 groups and a moderate reduction in BLYSS group compared with the SC group were detected. MCP1 and IL-10 values were significantly decreased in all treated groups. IL-6 levels were also significantly reduced in IRF5 group and clearly, but not significantly, diminished in CYP and BLYSS groups. Finally, IRF5 was the group with the lowest INF- γ levels.

Gene expression analysis profile

An array of 384 genes involved in LN was configured. Following the development of the array, 23 genes were discarded for technical reasons and 361 genes were finally included. The results showed that 284 genes were significantly modulated by CYP: 32 by siBLYSS and only 18 by siIRF5. Curiously, only the *Tnfsf13* gene was significantly modified (downregulated in this case) by the two siRNA treatments.

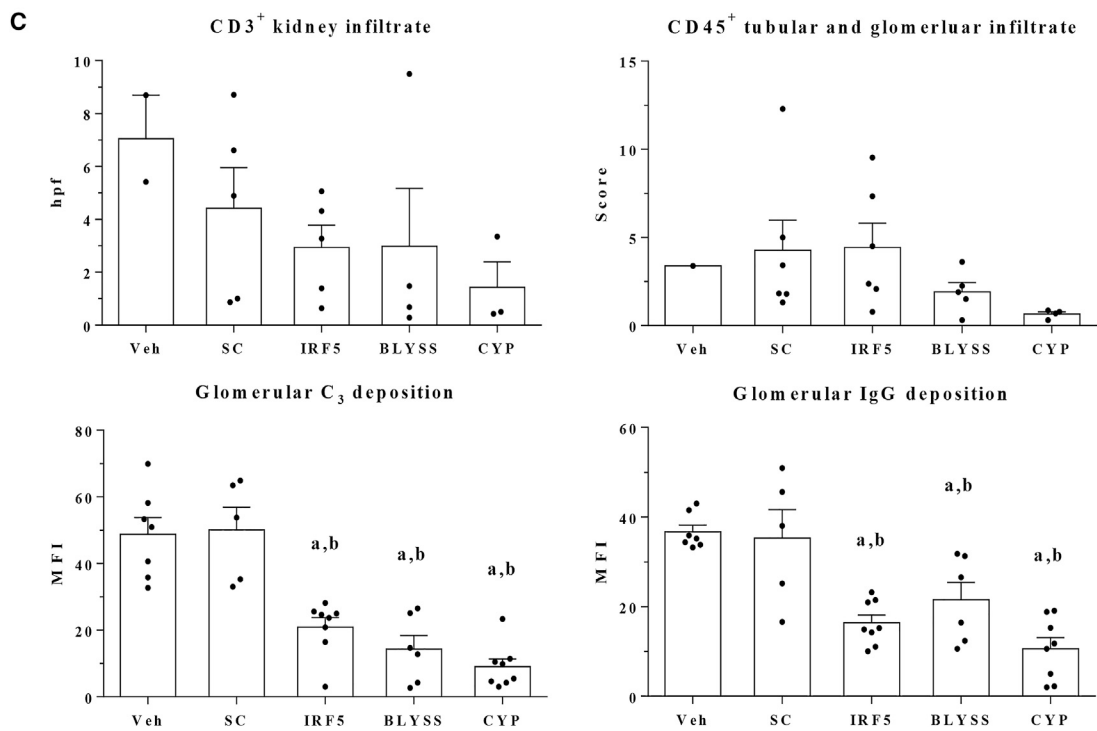
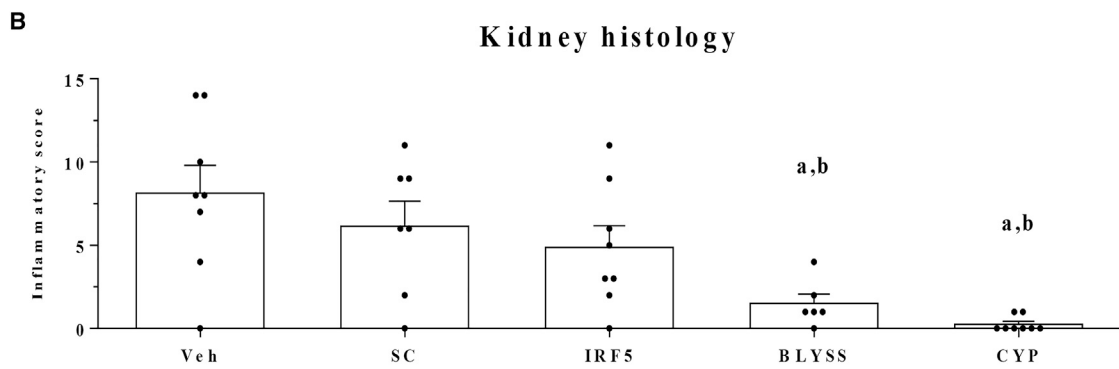
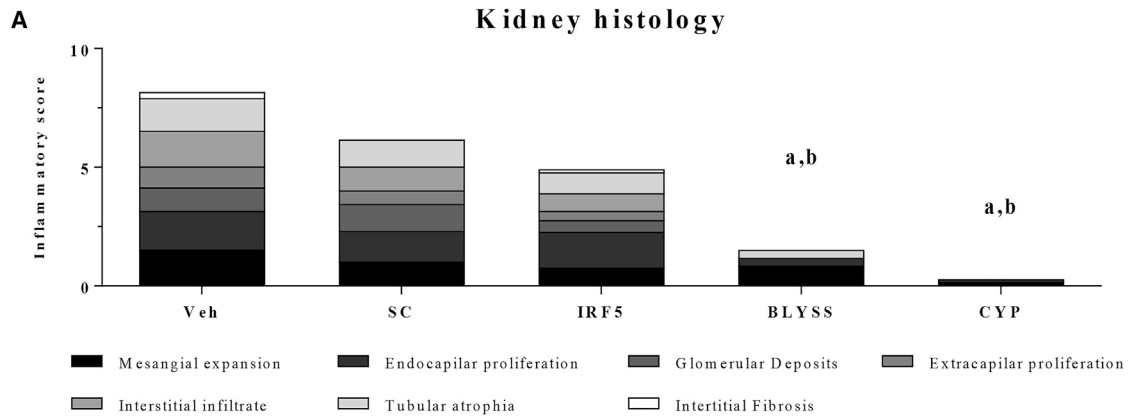
PathwAX results indicated that siIRF5 affected fourteen molecular pathways related to immune diseases such as diabetes and inflammation. It also affected six metabolism-related pathways, oxidation, and phosphorylation processes as the most significant. Of note is that it affected five pathways related to the processing of genetic information, activating genes related to the proteasome and inhibiting some genes

related to the synthesis, transport, and modification of RNA, and one single pathway related to cellular senescence process. siBLYSS modulated pathways related to metabolism and cellular adhesion and modulation pathways such as mitogen-activated protein (MAP) kinases, Janus kinase-signal transducer and activator of transcription (JAK-STAT), TNF, and cyclic MAP (cAMP). All the pathways related to genetic information processing are depleted with respect to modulated pathological pathways, which suggests that the groups have fewer connections in the real network than expected from the random model, indicating that they have crosstalk depletion. In this case, the association with SLE appears with a q value of $8,312e-9$, which includes 27 of the 32 significantly modulated genes analyzed in the array: *DID*, *Sod2*, and *Pdha1* are the genes with the most related pathways.

All of these results demonstrate that CYP treatment is strikingly active at the molecular level, but the designed siBLYSS and siIRF5 treatments cause a significant reduction in the disease evolution through the modulation of few targets. The list of all analyzed genes is provided in [Supplemental information](#).

Second set of experiments with siRNA monotherapy and combined transfection

A second set of experiments was undertaken to confirm the efficacy of the gene-silencing approach and to assess a potential synergistic or



(legend on next page)

Table 3. Survival differences between the two sets of experiments

	Initial n	Final n	Survival (%)
1st set			
Vehicle	8	8	100
SC	7	7	100
IRF5	8	8	100
BLYSS	8	6	75
CYP	8	8	100
2nd set			
Vehicle	8	6	75
SC	7	6	85.7
IRF5	8	7	87.5
BLYSS	8	8	100
DUAL	10	10	100
CYP	8	8	100

additive effect with the combined treatment. The cumulative survival in untreated groups was lower than that of the first set of experiments, which reveals greater disease virulence in this litter of animals (Table 3).

Validation of siRNA silencing *in vivo*

Expression of circulating IRF5 was significantly lower in the siIRF5 and intraperitoneal BLYSS-siRNA⁺IRF5-siRNA (DUAL) groups than in the untreated groups, as expected. Similar results occurred with circulating BLYSS. The treatments that significantly reduced these levels were in siBLYSS, CYP, and DUAL groups. Additionally, IFN- α was significantly reduced in siIRF5 and DUAL groups with respect to the SC group (Figure 6).

Effect of dual siRNA therapy in LN model

As seen in Figure 7, when compared with all treated groups, SC and Veh groups displayed a worse level of proteinuria. siRNA monotherapy provided a less aggressive worsening slope, while DUAL group offered clearly and significantly better proteinuria, quite similar to that of the CYP group, with steady low values throughout the study. Albuminuria levels were largely higher than the first SLE set, again indicating a greater virulence of the disease, but it followed the same profile as proteinuria. Additionally, levels of anti-dsDNA antibodies were higher in this second study. The progressive increase in Veh group was significantly higher than that in all treated groups.

Proteinuria excretion over 3 mg/24 h during the study was considered a negative disease outcome. In this regard, substantial differences be-

tween CYP and DUAL (only 12.5 and 20% proteinuria exceeding 3 mg/24 h, respectively) from the untreated groups (57.1% in SC and 87.5% in Veh) were observed (Table 4). Thus, DUAL therapy further provided better proteinuria outcome than monotherapy. Anti-dsDNA antibodies were also higher than in the first set. They progressively increased in Veh group and were significantly higher than in all treated groups.

According to the histopathological evaluation (Figures 8 and 9), a greater efficacy of siIRF5 was observed, without evidence of interstitial fibrosis and less cell proliferation than with the siBLYSS treatment. DUAL treatment showed lower histological score that was significantly different from Veh and similar to CYP.

CD3⁺, CD45RA⁺, and F4/80⁺ quantification in the DUAL group was lower than in siRNA monotherapy groups, although higher than in the CYP group. Interestingly, DUAL therapy reduced glomerular B cell to levels similar to CYP. Regarding F4/80⁺, siBLYSS reduced the infiltration better than siIRF5, with both better than Veh group. DUAL treatment showed significantly lower levels of T cells and macrophages than Veh.

DISCUSSION

These two studies with a well-recognized model of autoimmune nephritis have shown the reduction of the disease outcome and progression with both selective gene-silencing siRNA treatments. Moreover, the study with siRNA combination shows clearly better results in animals treated with DUAL therapy compared with siRNA monotherapy, substantiating an additive effect of IRF5 and BLYSS.

In the first set of experiments, mice treated with siIRF5 or siBLYSS displayed a better renal function than non-treated mice. These effects were accompanied by a significant reduction of circulating anti-dsDNA and renal histopathological lesions. Data also showed the same trend regarding C3 and IgG renal deposits as well as renal T cell infiltrate. These results validate the satisfactory LN protection using a highly targeted gene silencing, similar to the protection offered by CYP.

Interestingly, BLYSS silencing reduced the renal B cell infiltrate compared with untreated animals, while the IRF5 silencing did not. This phenomenon may be due to the lower hierarchy of the IFN signal in the B cell recruitment in this setting. IRF5 signal can be found mechanistically prior to BLYSS,^{34,35} but these results suggest that the BLYSS signal plays a more important role in the B cell recruitment than does the IRF5 signal. This fact also demonstrates that the BLYSS signal does not only depend on the previous IRF5 signal and the IFN

Figure 4. Renal histopathology parameters for the first set of experiments

(A) Semiquantitative inflammatory score values for hematoxylin and eosin stain. (B) Dot plot representation of total inflammatory score for each animal. (C) Cells per high-power field (HPF) values for renal immunofluorescence CD3⁺ infiltrate and semiquantitative score for CD45RA⁺ infiltrate staining. Mean fluorescence intensity values for glomerular C3 and IgG deposition were measured under a confocal microscope. Data are expressed as mean \pm SEM with individual dot plot representation. a, $p < 0.05$ versus Veh; b, $p < 0.05$ versus SC.

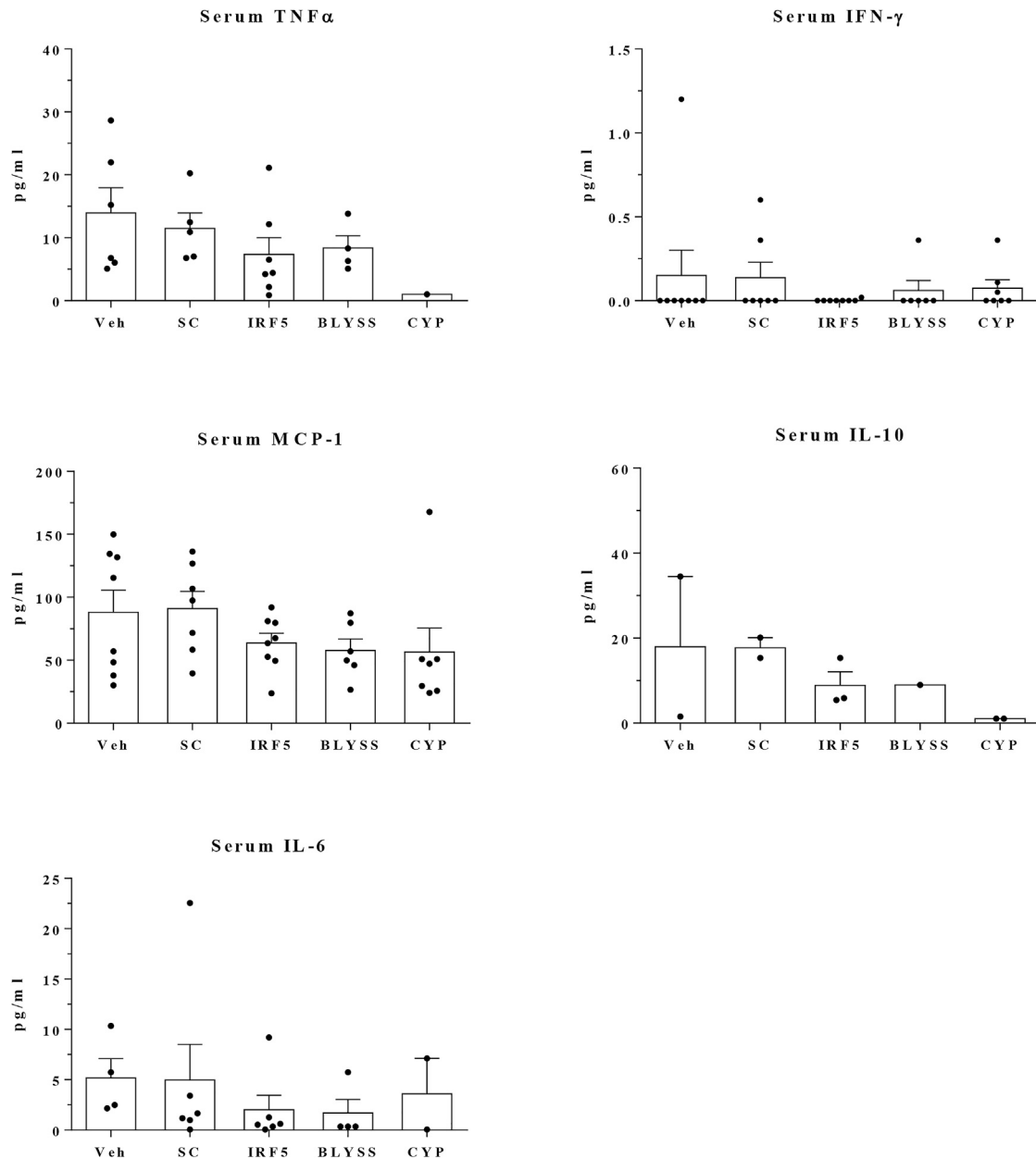


Figure 5. Serum levels of TNF- α , INF- γ , MCP1, IL-10, and IL-6 at the end of the first set of experiments for all treatments

Values are expressed in pg/mL. Data are expressed as mean \pm SEM with individual dot plot representation. a, $p < 0.05$ versus Veh; b, $p < 0.05$ versus SC.

release to exert its function. In fact, these results showed that BLYSS can act without the presence or in the presence of low levels of these cytokines. However, the modulatory effect conferred by IRF5 treatment represents an overall improvement, which gives credence to undertaking the DUAL treatment with both siRNAs.

Cell recruitment reduction in renal parenchyma was a relevant finding in this study, in accordance with the well-characterized excessive activation, signaling, and migration of both B/T cells in this disease.^{36,37}

Therefore, we looked more deeply into this cell dynamics by analyzing B cell subsets among other inflammation and autoimmunity subsets in renal tissue, spleen, and peripheral blood to assess the treatment differences. Renal cytometry confirmed the results in immunohistochemical B cell infiltrate. Cytometry also allowed us to discern different B cell subsets such as B1, B2, or B-regs. This approach elicited an expansion of B-regs under the IRF5 silencing. This shows that the overall increase in B cells observed in immunohistochemistry is due to the increase in B-regs in IRF5 group. Hence, treatment with siIRF5 involved an increase

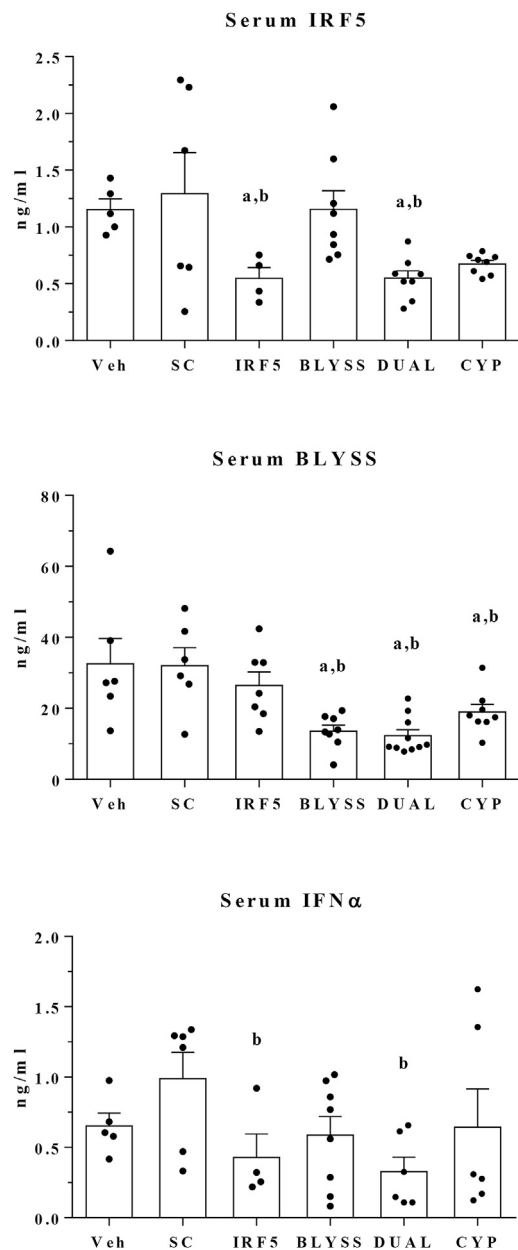


Figure 6. Endpoint serum circulating IRF5, BLYSS, and IFN- α levels for the second set of experiments

Values are expressed in ng/mL. Data are expressed as mean \pm SEM with individual dot plot representation. a, $p < 0.05$ versus Veh; b, $p < 0.05$ versus SC.

in B-regs that favored the palliation of the disease. This therapeutic effect given by siIRF5 treatment is like the effect observed with CYP, although it involves another pathway to alleviate the disease. In the case of siBLYSS silencing, no significant movements were observed in kidney-infiltrating B-regs. Regardless, an expected general reduction in kidney B cells was detected and attributable to the silencing of this B cell activation factor. An increase in B-regs and B1 cells in the spleen was also observed in both treated groups, as well as a reduc-

tion in B2 cells. These changes were associated with reduced release of anti-dsDNA antibodies, renal IgG, and C3 deposits, which is a particular occurrence in mouse and human LN.^{38–40} This phenomenon may be due to the immunomodulatory capacity of B1 cells as well as B-regs, which suppress IFN and TNF from T cells and release various regulatory cytokines.⁴¹ By contrast, B2 cells have been described as cells with a function contrary to those mentioned above.

In the case of T cells, solid changes similar to immunohistochemistry were observed in the kidney in all treatments, where there was a reduction of these T cells compared with SC group. This phenomenon was observed in almost all other pro-inflammatory and immune-response-related cells, such as DCs and macrophages in the renal tissue and spleen. Macrophages were generally reduced in the kidney, but the M2 spleen subset, which is a subpopulation of macrophages with immunosuppressive activity, was significantly increased in CYP and BLYSS groups. Additionally, the inflammatory M1 subset in the spleen was reduced in all treated groups. The blood-circulating monocytes were also analyzed, and an increase of the Ly6C⁺-regenerating subset was observed in IRF5 and CYP groups. There were no essential differences in Treg population. Nevertheless, the largest differences were observed in the B cells and their subsets; as seen in other studies, this model should be more related to B cells and B-regs than Tregs.¹

Circulating levels of TNF, MCP1, IL-6, and IFN- γ were reduced in treated animals. Although few population changes were observed in peripheral blood, there appears to be a systemic effect with siRNA treatments. Analyzing these data, we can see the direct relation in IL-6 and even a reduction of IFN- γ with IRF5 silencing, thereby corroborating the silencing of this pathway.^{6,42,43} Regarding IL-10, the observed values showed a reduction in untreated groups. Higher circulating levels should be expected in treated groups. However, in the context of lupus, IL-10 is also related to the acceleration of renal disease, as high serum levels of IL-10 are found in patients with lupus.^{30,44} Thus, in this model, IL-10 expresses lupus disease activity, and the reduction of systemic inflammation leads to this cytokine reduction.

Information obtained from gene expression may evidence the different pathways implicated in lupus, and in particular, nephritis. Results showed that CYP, which is the gold standard treatment in this model, but with excessive short- and long-term systemic toxicities,⁴⁵ modifies many genes related to a wide range of pathways. On the contrary, both siRNAs modified fewer pathways, despite showing similar clinical beneficial results. Therefore, IRF5 and BLYSS silencing might be an effective and more specific treatment and by regulating fewer pathways, perhaps fewer side effects may appear.

A subsequent set of experiments was performed including the combination of both siRNAs. Despite being a widely used and verified model, differences in survival and disease outcome may be observed between litters. So, the reproducibility of a biological effect confirms its accuracy. Results in this second set of experiments showed a clearly

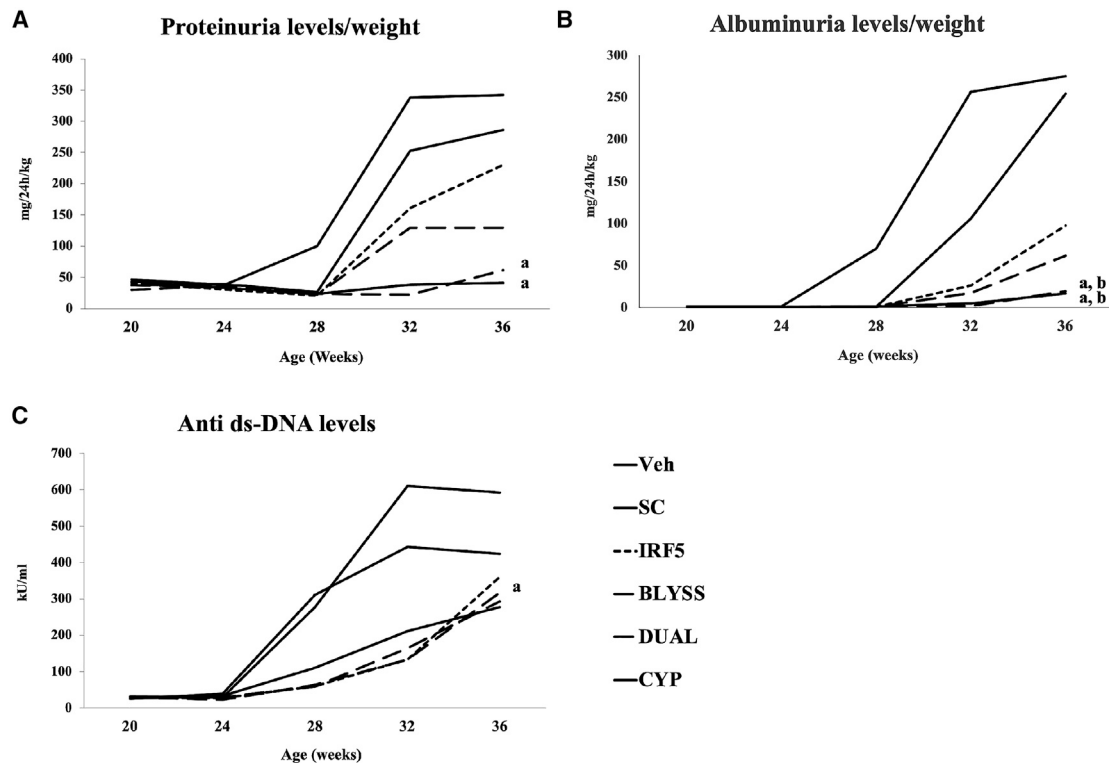


Figure 7. Functional renal parameters for the second set of experiments throughout the study for all treatments

(A and B) Functional renal parameters for the second set of experiments including (A) proteinuria and (B) albuminuria by weight. Values are expressed in mg/kg for 24 h. (C) Serum anti-dsDNA levels throughout the study for all treatments are expressed in kilo units/mL. Data are expressed as mean. a, $p < 0.05$ versus Veh; b, $p < 0.05$ versus SC.

more severe disease in untreated animals. In this less friendly scenario, both siIRF5 and siBLYSS monotherapy treatments reflected a lower disease reduction compared with the previous experiment, whereas CYP remains effective. Interestingly, the combination of both siRNAs offered a significant reduction in the disease at similar values to CYP, thus proving its additive potency.

Serum IRF5 and BLYSS levels revealed the efficacy in silencing those genes. Although BLYSS expression is not significantly conditioned on IRF5 expression, a connection between both targets must be considered due to the partial reduction in circulating BLYSS levels using siIRF5 and its observed protective effect. siIRF5-treated group also showed a significant reduction in IFN levels, thus proving its biological effect.

Conclusions

Our data strongly support the potential therapeutic effects of both selective IRF5 and BLYSS gene silencing as an effective new form of immune deactivation in the inflamed kidney of animals with spontaneous autoimmune nephritis akin to human LN, particularly when treatment is introduced during the early phases of the disease. It remains to be seen whether disease remission could be achieved through IRF5 and BLYSS gene interference initiated during the established phase of the disease, since patients with LN often debut with

established proteinuria and severe renal inflammation. Gene studies and their relationship may be the subject of future studies.

MATERIALS AND METHODS

siRNA design and screening

siRNA duplexes targeting the partial mouse IRF5 mRNA sequence (GenBank: NM_001252382; NCBI, Bethesda, MD, USA) and mouse BLYSS mRNA sequence (GenBank: NM_033622.2; NCBI, Bethesda, MD, USA) were designed. For the initial screening, fourteen siRNAs were synthesized to test the inhibition potential in IRF5 expression (Table 1) and twelve siRNAs were synthesized to test the inhibition potential in BLYSS expression (Table 2). A SC was used as control for off-target effects. To assess the mouse IRF5 mRNA-silencing efficiency of siRNA molecules, we used *in vitro* culture of a RAW 264.7 cell line (macrophages). To assess the mouse BLYSS mRNA-silencing efficiency, we used *in vitro* culture of an MPC-11 cell line (B lymphocytes).

In vitro transfection of siRNAs (10 and 25 nM) was performed using MIR6000 vector (TransIT-X2 Dynamic Delivery System, MirusBio, Madison, WI, USA) following the manufacturer's instructions. Briefly, after achieving an 80% cell confluence, siRNA complexes were previously prepared, with sufficient time for complexes to form, and transfected into cell culture. After 24 h of incubation, cells

Table 4. Number of animals in the second set of experiments with levels above 3 mg/dL concentration of proteinuria and the percentage they represent in each group

Group	Initial n	Proteinuria outcome n	Proteinuria outcome (%)
Vehicle	8	6	75
SC	7	4	57.1
IRF5	8	6	75
BLYSS	8	5	62.5
DUAL	10	2	20
CYP	8	1	12.5

were harvested to perform the knockdown assay of target gene expression.

siRNA properties

The siRNA BLYSS sequence used in this study consists of a 21-nucleotide sense and antisense strand. IRF5 and SC sequences used in this study consist of a 21-nucleotide sense strand and 23-nucleotide antisense strand, resulting in a two-nucleotide overhang at the 3' end of the antisense strand. The siRNA molecules were chemically stabilized with partial phosphorothioate backbone and 2'-O-methyl sugar modification on the sense and antisense strands. The oligonucleotide sequences were as follows: IRF5 sense strand, 5'-CGA GAG AAG CUC AUU ACU*Chol-3' and antisense strand, 5'-AGU AAU GAG CUU CUC UCZ*Z*G-3'; BLYSS sense strand, 5'-GAA AGG AGA UAU GAA AdTdT*-Chol-3' and antisense strand, 5'-UUU CAU AUC UUC UCC UUU X*W*dT-3'; and scrambled (SCR) sense strand, 5'-GUU GCA UUU CGU CUG GUA GGA*Chol-3' and antisense strand, 5'-UCC UAC CAG ACG AAA UGC AAX *Y*G-3', with Z as 2'-OMe-G, X as 2'-OMe-C, Y as 2'-OMe-U, dT as thymidine, the asterisk (*) as phosphorothioate, and Chol as cholesterol modification. Additionally, a conjugation of a cholesterol molecule to the 3' end of the sense strand of the siRNA molecule by means of a pyrrolidine linker was done. Scrambled siRNA was used as control. To generate siRNAs from RNA single strands, equimolar amounts of complementary sense and antisense strands were mixed and 100% annealed. Anti-IRF5, anti-BLYSS, and scrambled siRNAs were aliquoted to avoid freeze-thaw cycles and stored at -20°C .

Mice, study design, and follow-up

First set of experiments with siRNA transfection individually to assess the effectiveness of siRNA in LN model and the potential mechanisms implicated

Five-month-old NZB/WF1 mice (The Jackson Laboratory, Charles River, Barcelona, Spain) were randomly assigned into five groups. In this genetically modified mouse model, the renal disease began early in the animal life, and there was a burst of clinical symptoms later within 20–28 weeks. Next, we decided to begin treatments at week 24 as follows: CYP, intraperitoneal cyclophosphamide (n = 8); IRF5, intraperitoneal IRF5-siRNA (n = 8); BLYSS, intraperitoneal BLYSS-siRNA (n = 8); SC (n = 7) intraperitoneal scrambled siRNA; and Veh, intraperitoneal PBS as non-treated group (n = 8).

Second set of experiments to retest effectiveness of siRNA individually in LN model and to assess the superiority of combined siRNA transfection

Five-month-old NZB/WF1 were randomly assigned into six groups. At six months of age, treatments were initiated as follows: CYP, intraperitoneal CYP (n = 8); IRF5, intraperitoneal IRF5-siRNA (n = 8); BLYSS, intraperitoneal BLYSS-siRNA (n = 8); DUAL, intraperitoneal BLYSS-siRNA+IRF5-siRNA (n = 10); SC, intraperitoneal scrambled siRNA (n = 7); and Veh, intraperitoneal PBS as non-treated group (n = 8).

All siRNA molecules were administered 50 μg twice weekly following our experience with other siRNAs as described previously.^{1,46} A volume of 200 μL of PBS was administered twice weekly. CYP was administered in 50 mg/kg doses every 10 days. Mice were treated for 12 weeks. Body weight was determined weekly from the beginning to the end of follow-up. Mice were placed in metabolic cages to collect 24 h urine specimens before the onset of treatment and then monthly thereafter. Blood was obtained from the tail vein at monthly intervals. Kidneys were processed for histological and biochemical studies at the end of the study or at death.

The experiments were carried out in accordance with current European Union (EU) legislation on animal experimentation and were approved by CEEA (Animal Experimentation Ethics Committee), the Institutional Ethics University of Barcelona (UB) Committee for Animal Research, and the Animal Experimentation Commission of the Generalitat de Catalunya (Catalonian Government). Mice were housed in a room at constant temperature with a 12-h dark/12-h light cycle, had free access to tap water, and were fed a standard laboratory diet.

Renal function: Urinary total proteins, albumin, and creatinine

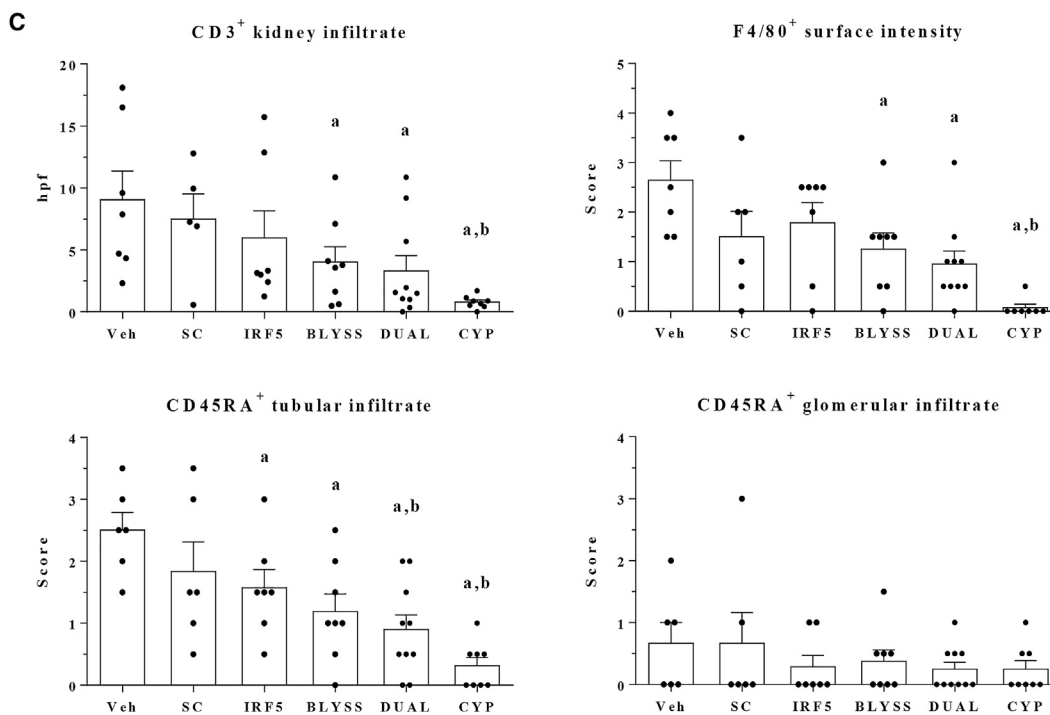
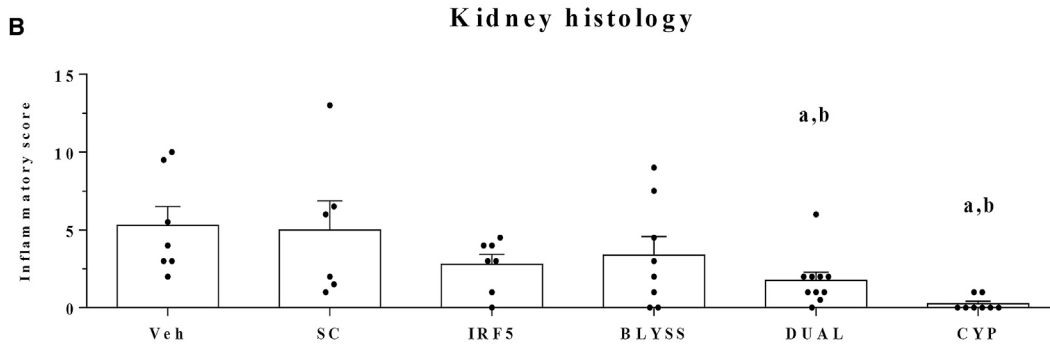
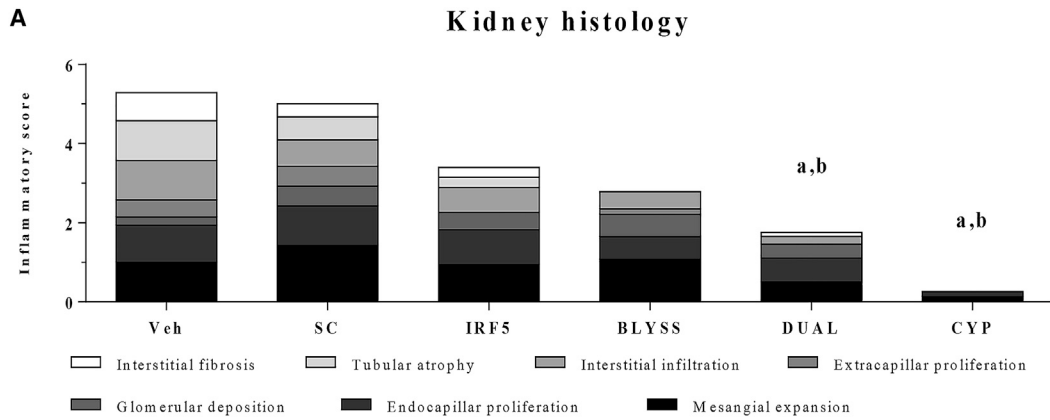
Twenty-four hour urinary protein was determined by pyrogallol red reaction, and 24 h urinary creatinine measurement was performed following Jaffe's reaction (Autoanalyzer AU400, Olympus, Hamburg, Germany) in the Veterinary Clinical Biochemistry Laboratory of Universitat Autònoma de Barcelona. Twenty-four hour urinary albumin was determined using a commercially available ELISA kit (Active Motif, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The intensity of the fluorescent signal is directly proportional to the albumin concentration in the sample.

Serum ELISA for cytokine and immunoglobulin analysis

Serum IL-12, TNF- α , IFN- γ , and MCP1 cytokines were measured by using a FACSCanto II cytometer with a cytometric bead array kit (CBA mouse inflammation kit) in accordance with manufacturer's instructions. Data were acquired and analyzed using BD FCAP software and CBA software (BD Biosciences, San Jose, CA, USA).

Analysis of phenotypic peripheral blood, spleen, and renal cell populations with flow cytometry

Spleen and blood samples were processed in order to analyze the different composition of immune cells. Peripheral blood was collected



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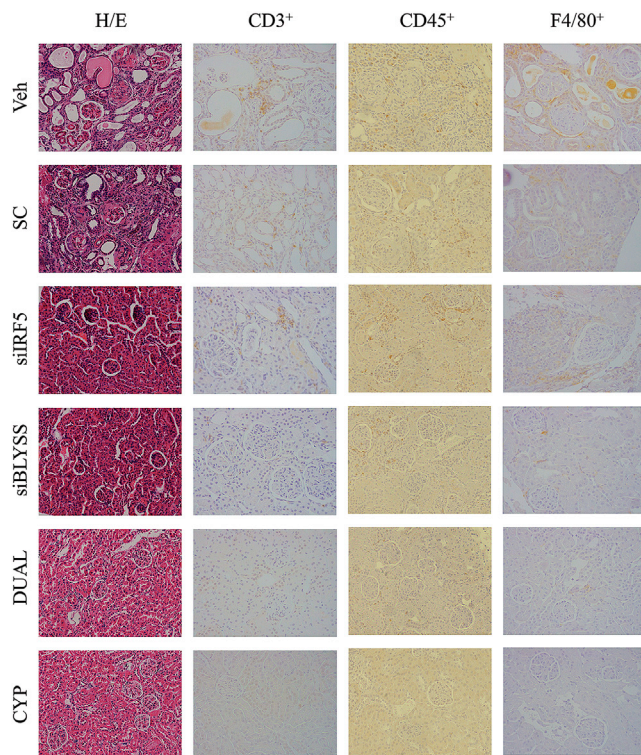


Figure 9. Effect of different treatments in the mouse kidneys from the second set of experiments

Representative histological images of hematoxylin and eosin staining and CD3⁺, CD45RA⁺, and F4/80⁺ infiltrate using immunohistochemistry.

in heparin for flow cytometry protocol. Spleens were collected in PBS for the splenocytes isolation by FicollH (GE Healthcare, Uppsala, Sweden) density gradient and cryopreservation at -80°C . For quantifying the percentage of different populations, cells were thawed, washed, and recovered by standard methods.

A volume of 100 μL of peripheral blood or 2×10^5 splenocytes were added in every cytometry tube for flow cytometry protocol. Cytometry tubes were incubated in the dark (25 min at room temperature) with antibodies. Study of populations was performed by using a BD FACSCanto II cytometer and analyzed by BD FACSDiva software (BD Biosciences, San Jose, CA, USA).

To characterize the different cell populations, anti-CD45R (clone RA36B2), anti-CD23 (clone B3B4), anti-IgD (clone 11-26c.2a), anti-CD19 (clone 1D3), anti-CD5 (clone 53-7.3), anti-CD1d (clone 1B1), anti-CD3 (clone 145-2c11), anti-CD4 (clone RM4-5), anti-CD8a (clone 53-6.7), anti-PD-1 (clone 29F.1A12), anti-CD3e (clone

145-2c11), anti-CD25 (clone PC61), anti-Fox P3 (clone FJK-16 s), anti-IL-17a (clone TC11-18H10), anti-ROR γ (clone Q31-378), anti-CD11c (clone HL3), anti-PDL-1 (clone MIH5), anti-PDL-2 (clone TY25), anti-F4/80 (clone 6F12), anti-CD11b (clone M1/70), anti-CD86 (clone GL1), anti-CD206 (clone MR6F3), anti-CD115 (clone AFS98), and anti-Ly6C (clone 1G7.G10) were used. All antibodies were provided by BD Biosciences (San Jose, CA, USA) and MiltenyBiotec (Bergisch Gladbach, Germany), conveniently titrated, mixed together, and formulated for optimal staining performance.

Renal lupus histopathology

For histological analysis, 1- to 2-mm-thick coronal slices of kidney were fixed in 4% formaldehyde and embedded in paraffin. For light microscopy, 3- to 4- μm -thick tissue sections were stained with hematoxylin and eosin and periodic acid-Schiff. To determine the extent of renal damage, two pathologists blinded of information analyzed all kidney biopsies. Characteristic glomerular active lesions of LN were evaluated: mesangial expansion, endocapillary proliferation, glomerular deposits, extracapillary proliferation, and interstitial infiltrates as well as tubulo-interstitial chronic lesions, tubular atrophy, and interstitial fibrosis. Lesions were graded semiquantitatively using a scoring system from 0 to 3 (0 = no changes, 1 = mild, 2 = moderate, 3 = severe). Finally, a total histological score was derived from the sum of all the described items.

Paraffin tissue sections were stained for CD3 (Abcam, Cambridge, UK) CD45RA (Abcam, Cambridge, UK), and F4/80 (LabClinics, Barcelona, Spain). Sections were de-paraffined and hydrated. The sections were blocked and immunoperoxidase labeled using a Vectastain ABC kit and the avidin biotin blocking kit (Vector Laboratories, Burlingame, CA, USA) in accordance with the manufacturer's protocol. Peroxidase-conjugated antibody staining was followed by diaminobenzidine substrate development (Sigma-Aldrich, Madrid, Spain). To quantify CD45RA (tubule and glomeruli) and F4/80 expression, a semiquantitative intensity score from 0 to 3 was used. For quantifying CD3 expression, at least 15 high-power fields were counted, and the mean value was expressed.

Renal immunofluorescence studies

Slices of kidneys were fixed in 4% paraformaldehyde, embedded in Tissue Tec optimal cutting temperature compound (Sakura, Alphen aan den Rijn, the Netherlands), and stored at -80°C . Fluorescent staining of 5 μm cryostat sections was used for confocal microscopy to quantify glomerular IgG and C3 deposition. Sections were directly stained with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma-Aldrich, Madrid, Spain), and FITC-conjugated C3 (Nordic Immunology, Tilburg, the Netherlands). For analysis of C3 and IgG deposition, at least 10 glomeruli were visualized and photographed with an immunofluorescence confocal microscope

Figure 8. Renal histopathology parameters for the first set of experiments

(A) Semiquantitative inflammatory score values for hematoxylin and eosin stain. (B) Dot plot representation of total inflammatory score for each animal. (C) Cells per HPF values for renal immunofluorescence CD3⁺ infiltrate and semiquantitative score for F4/80⁺ infiltrate and CD45RA⁺ tubular and glomerular infiltrate staining. Data are expressed as mean \pm SEM with individual dot plot representation. a, $p < 0.05$ versus Veh; b, $p < 0.05$ versus SC.

(TCS-SL spectral microscope, Leica Microsystems, Mannheim, Germany). Fluorescence was quantified and normalized with Simulator confocal software (Leica Microsystems) and is expressed as mean fluorescence intensity.

RNA extraction, RT, and gene expression analysis: Quantitative real-time PCR

For the screening of IRF5 and BLYSS siRNAs in cell culture, mRNA expression of target genes was quantified by TaqMan real-time PCR (ABI Prism 7700 Sequence Detection System, Applied Biosystems, Madrid, Spain) using the comparative cycle threshold method (Applied Biosystems, Madrid, Spain).

For molecular studies, the kidney was immediately snap-frozen in liquid nitrogen and stored at -80°C . RNA was extracted from kidney with PureLink RNA Mini Kit (Invitrogen, Barcelona, Spain) in accordance with the manufacturer's instructions. RNA purity was analyzed on a spectrophotometer (ND-1000V3.3, NanoDrop Wilmington, DE, USA). All samples had an A260/280 ratio >1.8 . RNA was stored at -80°C . A total of 500 ng of RNA was used to do the reverse transcription using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK) following the manufacturer's instructions.

Kidney tissue expression of immune-inflammatory mediators was quantified by TaqMan Low Density Array microfluidic cards (Applied Biosystems, Warrington, UK) using the comparative cycle threshold method. Controls, which were composed of distilled water, were negative for target and reference genes.

Pathway analysis of single gene sets was set up using online <https://pathwax.sbc.su.se/> web server,⁴⁷ which applies the BinoX algorithm to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and FunCoup networks.

Statistical analysis

One-way analysis of variance (ANOVA) with post hoc tests was performed to compare proteinuria, albuminuria, and anti-dsDNA antibodies throughout the follow-up and gene expression and circulating cytokines at sacrifice. To compare histological data, the non-parametric Kruskal-Wallis test was used. A p value <0.05 was considered significant. Data are expressed as mean \pm SEM.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.omtn.2021.03.019>.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.G. and J.T.; siRNA design, E.R. and J.M.A.; siRNA synthesis, A.A. and R.E.; animal studies, J.G., E.R., N.B., and L.d.R.; pathological analysis, M.G. and R.T.; gene array analysis, J.G., E.R., and P.F.; other laboratory techniques, J.G., E.R., N.B., P.F., N.L., and J.M.C.; data analysis, J.G., E.R., J.M.G., and J.T.; writing – original draft, J.G., L.d.R., and P.F.; writing – review & editing, J.G., P.F., J.M.G., and J.T.; funding acquisition, J.M.G. and J.T.; supervision, J.M.G. and J.T.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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