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Modulation of renal disease in MRL/*lpr* mice by pharmacologic inhibition of inducible nitric oxide synthase

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Modulation of renal disease in MRL/*lpr* mice by pharmacologic inhibition of inducible nitric oxide synthase.

Background. MRL-MPJFas^{lpr} (MRL/*lpr*) mice spontaneously develop lupus-like disease characterized by immune complex glomerulonephritis and overproduction of nitric oxide (NO). Blocking NO production pharmacologically by a non-specific nitric oxide synthase (NOS) inhibitor ameliorated renal disease in MRL/*lpr* mice while genetically deficient inducible NOS (iNOS) mice developed proliferative glomerulonephritis similar to wild-type controls.

Methods. To clarify the role of iNOS in the pathogenesis of nephritis in MRL/*lpr* mice, we treated mice with two different NOS inhibitors. Either N^G-monomethyl-L-arginine (L-NMMA), a nonspecific NOS inhibitor, or L-N^6-(1-iminoethyl)lysine (L-NIL), an iNOS specific inhibitor, was administered in the drinking water from 10 through 22 weeks of age with disease progression monitored over time. Control mice received water alone.

Results. Both L-NMMA and L-NIL blocked NO production effectively in MRL/*lpr* mice. As expected, neither L-NNMA nor L-NIL had an effect on antibody production, immune complex deposition or complement activation. Although both NOS inhibitors decreased protein excretion, L-NMMA was more effective than L-NIL. Pathologic renal disease was significantly decreased at 19 weeks in both treatment groups. At 22 weeks the L-NIL treated mice, but not the L-NMMA mice, had significantly reduced renal disease scores compared to controls.

Conclusion. These results indicate that specific inhibition of iNOS blocks the development of pathologic renal disease in MRL/*lpr* mice.

MRL-MPJFas^{lpr} (MRL/*lpr*) mice spontaneously develop immune complex glomerulonephritis similar to that seen in human lupus [1, 2]. Interaction of immune complexes with resident glomerular mesangial cells triggers an inflammatory cascade characterized by mononuclear cell recruitment, mesangial cell proliferation and matrix protein accumulation [3]. Mediators released during renal inflammation include interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and nitric oxide (NO) [4, 5]. NO is produced in large quantities after exposure of infiltrating macrophages and resident mesangial cells to inflammatory mediators [6–8]. NO can react with O_2 radicals to form peroxynitrite with subsequent metabolism generating highly reactive and toxic hydroxyl radicals (HO•) [9, 10]. NO also may inhibit cell function by formation of iron-nitrosyl complexes in enzymes containing iron-groups or may nitrate tyrosine residues, thus altering the function of proteins such as catalase [11, 12].

Urine measurements of nitrate and nitrite (N/N, stable metabolites of NO) are a marker of endogenous NO production [13]. We have previously demonstrated that MRL/*lpr* mice have elevated levels of N/N in their urine compared to control strains [14]. This increase in urinary N/N begins at 10 to 12 weeks of age and predates the onset of proteinuria in these mice. Treatment with the non-specific NOS inhibitor (N^G-monomethyl-L-arginine, L-NMMA) blocks NO production [15], decreases proteinuria and yields lower pathologic renal scores in both NZB/NZW and MRL/*lpr* mice compared to controls [14, 16]. In contrast, MRL/*lpr* iNOS knockout mice develop similar renal disease to wild-type MRL/*lpr* mice [17]. To clarify these seemingly disparate results between pharmacologic inhibition of all NOS isotypes versus specific genetic inhibition of only iNOS production, we assessed the effect in MRL/*lpr* mice of specific pharmacologic inhibition of iNOS by using L-NIL, a specific inhibitor of iNOS, versus non-specific inhibition of all NOS isotypes using L-NMMA.

METHODS

Mice

Eight-week-old female MRL/*lpr* mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA), housed under specific pathogen-free conditions at the Ralph H. Johnson VAMC animal facility and provided

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autoclaved food and sterile water ad libitum. Mice were randomly tested and were serologically negative for common murine pathogens.

Treatment

At 10 weeks of age mice were placed on a defined N/N-free diet (Zeigler Brothers, Gardners, PA, USA) and given water containing L-NMMA (50 mmol/L) or L-N^6-(1-iminoethyl)lysine (L-NIL; 1 mg/mL). Controls received distilled water without additives. There was no difference in animal weights, food consumption or water consumption between the treatment groups. We have previously determined that the N/N-free diet alone has no effect on disease expression in MRL/*lpr* mice (Gilkeson, unpublished data).

Reagents

L-N^6-(1-iminoethyl)lysine (L-NIL) was a gift from Pharmacia Corporation, (St. Louis, MO, USA). L-NMMA was purchased from Cyclops (Pittsburgh, PA, USA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Nitrate/nitrite analysis

Nitrate/nitrite were measured in urine as previously described [13]. Briefly, urine samples were filtered using Centricon ultrafiltration tubes (Amicon, Beverly, MA, USA). Nitrate was converted to nitrite using nitrate reductase (Boehringer Mannheim, Indianapolis, IN, USA), and total N/N was determined by measuring nitrite via the Greiss reaction. Known amounts of N/N were used to generate a standard curve.

Urine protein excretion

Mice were placed in metabolic cages for 24-hour urine collections. To prevent bacterial growth, antibiotics (ampicillin, gentamicin, and chloramphenicol) were added to the collection tubes. Urinary protein excretion was determined by dipstick analysis (Roche, Indianapolis, IN, USA).

Measurement of anti-dsDNA Ab

Anti-dsDNA antibody (Ab) levels were measured by enzyme-linked immunosorbent assay (ELISA) as previously described [18]. Briefly, 96-well ELISA plates were coated with 5 μg/mL double-stranded calf thymus DNA (dsDNA) and incubated at 37°C overnight. The plates were then washed with phosphate-buffered saline (PBS) 0.05% Tween (PBS-T). Sera were added in serial dilutions, starting at 1/100 dilution to each well, and incubated for 45 minutes at room temperature (RT). After washing with PBS-T, horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (γ-chain specific; Sigma) was added and incubated for 45 minutes. After additional washing, a substrate solution containing 3,3′5,5′-tetramethylbenzidene (TMB; Sigma) was added in 0.1 mol/L citrate buffer (pH 4) and 0.015% H_2O_2. Absorption at OD_{380} was determined on a microtiter plate reader (Dynatech, McLean, VA, USA). Results are shown as the OD_{380} at a 1/100 dilution. Double stranded DNA was derived by S1 nuclease (Sigma) treatment of phenol extracted calf thymus DNA. Anti-glomerular basement membrane (GBM) antibodies were measured using a previously described ELISA-based assay [18].

Total Ig

Total IgG, IgG2a and IgG3 levels in the sera were determined by ELISA using a standard curve of known concentrations of total mouse IgG or specific isotypes. ELISA plates were coated with 1 μg/mL anti-mouse immunoglobulin (κ-chain specific; Southern Biotechnology, Birmingham, AL, USA) and incubated overnight at 4°C. Sera were added in serial dilutions starting at 1/1000 dilution. HRP-conjugated goat anti-mouse IgG (γ-chain specific; Sigma) or specific anti-isotype reagents were added, followed by TMB for color development. OD_{380} absorbance was measured as above.

Tail cuff measures

At 19 weeks of age, systolic blood pressures were measured by tail cuff. Briefly, mice were placed in restriction chambers on a warmed surface and allowed to acclimate to the surroundings. A rubber-sealed cuff was placed around the animal's tail and connected to a pressure transducer and recording computer (Visitech Systems, Apex, NC, USA). Ten separate pressure measurements were recorded and averaged for each mouse. A mercury manometer was used to calibrate the equipment before each set of recordings.

Pathology

At the time of sacrifice (19 and 22 weeks), the kidneys were removed. One kidney was fixed with buffered formalin, embedded in paraffin, then sectioned before staining with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) stain. The slides were read and interpreted in a blinded fashion grading the kidneys for glomerular inflammation, proliferation, crescent formation and necrosis. Interstitial changes and vasculitis were also noted. Scores from 0 to 3 were assigned for each of the features and then added together to yield a final renal score. For example, glomerular inflammation was graded as follows: 0 = normal; 1 = few inflammatory cells; 2 = moderate inflammation; and 3 = severe inflammation.

Immunofluorescence staining

The other kidney was snap frozen and cut into 4-μm thick sections. The deposition of IgG and C3 were analyzed in frozen sections by immunofluorescence after incubating the slides with FITC conjugated rabbit anti

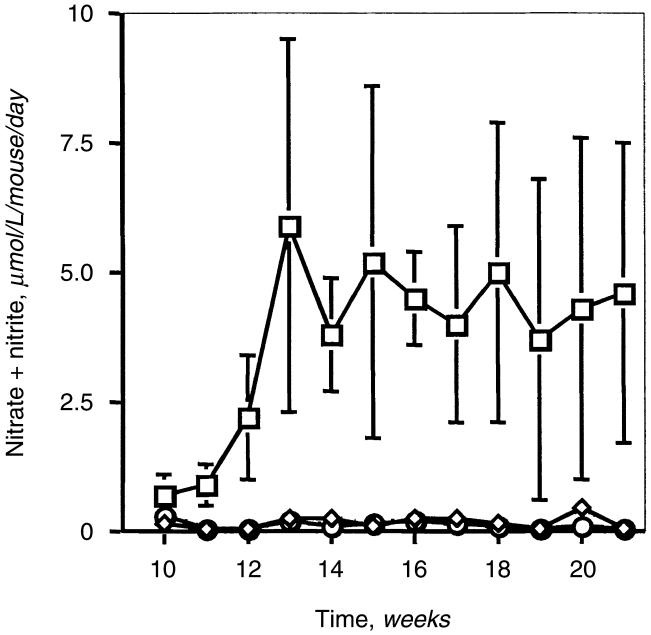

Fig. 1. Urinary nitrate and nitrite (N/N) excretion by MRL/*lpr* mice given L-NIL (1 mg/mL; ○) or L-NMMA (50 mmol/L; ◇) in distilled drinking water. Controls (□) received distilled water alone. Mice were placed in metabolic cages weekly for 24-hour urine collection from 10 to 21 weeks of age. Results shown are the mean ± SEM of 10 mice from 10 to 18 weeks (2 mice per cage) and 5 mice after 18 weeks (1 mouse per cage).

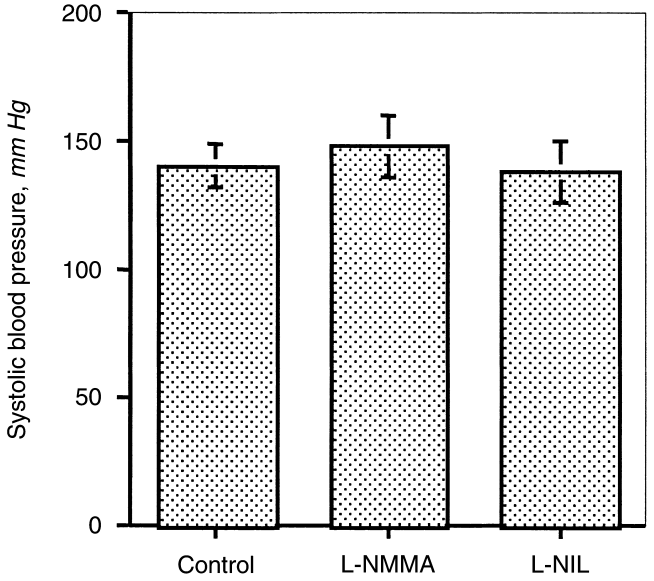

Fig. 2. Systolic blood pressure measurements in MRL/*lpr* mice given L-NIL (1 mg/mL) or L-NMMA (50 mmol/L) in distilled drinking water. Controls received distilled water alone. At 19 weeks mice were monitored for systolic blood pressures by tail cuff measurement. Results shown are the mean ± SEM of 10 mice.

mouse IgG (Sigma) and sheep anti mouse C3 (Sigma). IgG and C3 depositions were graded 0 to 3+ by a blinded observer.

Statistical analysis

The unpaired Student *t* test or analysis of variance (ANOVA) followed by post hoc analysis was used to test for significant differences between groups. A *P* value of <0.05 was considered significant.

RESULTS

To determine if NO production was inhibited by treatment with the two NOS inhibitors, we measured N/N in the urine. In animals consuming a N/N-free diet, urinary excretion of N/N accurately reflects the endogenous production of NO [13]. At 10 weeks of age, all mice had low urinary N/N excretion. In the control mice, NO production increased beginning at week 12 and remained elevated for the duration of the study (Fig. 1). In contrast to the increase in urinary N/N excretion observed in the controls, mice that received either L-NMMA or L-NIL had minimal increases in N/N excretion over the same period of time.

L-NMMA treatment has known systemic effects including increasing blood pressure, in part, due to inhibition of endothelial NOS. We evaluated systolic blood pressures by tail cuff measurement to determine the effects of L-NMMA and L-NIL on blood pressure. At 19 weeks of age, tail blood pressure measures in the L-NMMA treated animals tended to be higher compared to the L-NIL and the control animals, although this increase was not statistically significant (Fig. 2).

To assess the role of NO inhibition on nephritis, we measured urine protein excretion by urine dipstick beginning at 10 weeks of age (Fig. 3). From 10 to 14 weeks of age, proteinuria remained low. After 14 weeks of age some mice in the control group began to develop proteinuria. The number of mice with proteinuria increased in the control group over time. Some of the animals in the L-NIL treatment group also began to have proteinuria after sixteen weeks of age. However, the L-NMMA treated animals did not develop proteinuria during the treatment. By dipstick analysis, measurements at week 20 showed that 80% of the control mice had urine protein levels of 100 mg/dL or greater. In the L-NIL treated mice, 40% showed urine protein of 100 mg/dL or greater.

The animals were assessed for differences in body, spleen and kidney weights at 19 and 22 weeks (5 per group), as reduced kidney size is another indicator of medical renal disease. Neither body weights nor spleen weights were significantly different in any of the treatment groups. However, the kidney and kidney-to-body weight ratios in the L-NIL and the L-NMMA treated mice at 19 weeks were significantly greater than the controls (Fig. 4). Furthermore, at 22 weeks of age the L-NIL

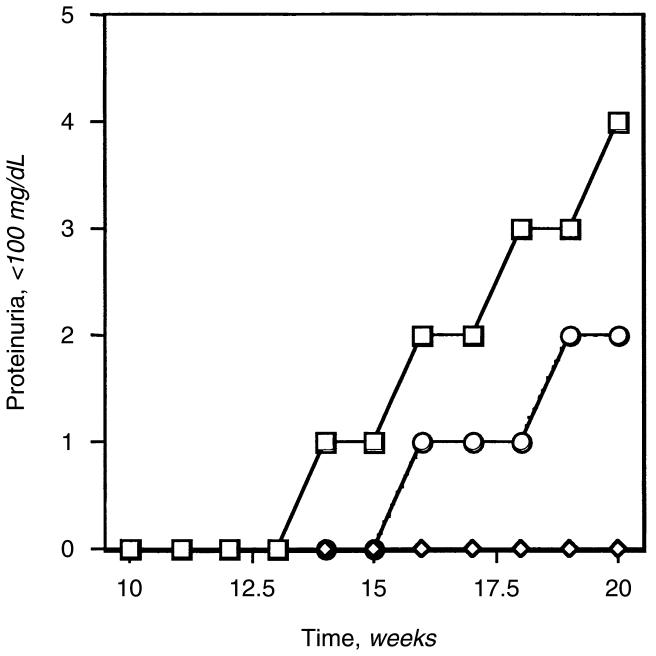

Fig. 3. Dipstick measurements of urine albumin from MRL/*lpr* mice given distilled water alone (control, □), L-NIL (1 mg/mL, ○), or L-NMMA (50 mmol/L, ◇) in the drinking water. Mice were placed in metabolic cages for 24-hour urine collection once a week from 10 to 20 weeks of age. From 10 to 18 weeks, 2 mice were placed in each metabolic cage (5 cages per group). After 18 weeks, 1 mouse was placed in each cage (5 mice per group). Each point represents the number of cages that were determined to have proteinuria with scores of greater than 100 mg/dL.

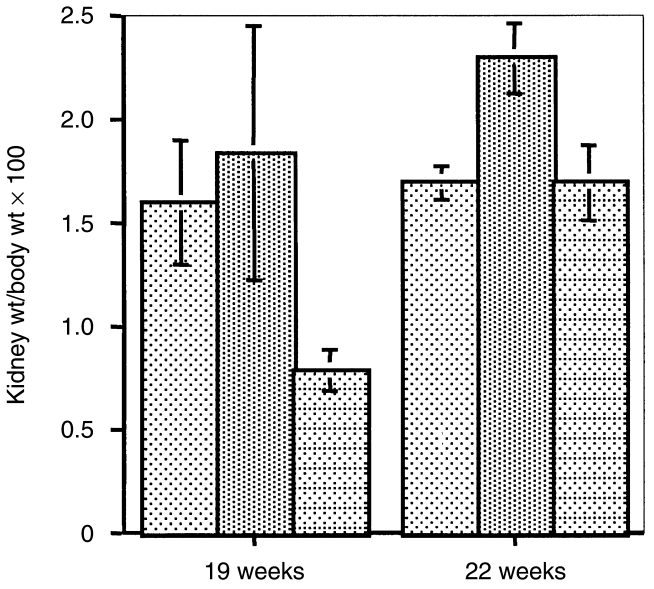

Fig. 4. Kidney weights relative to total body weights of MRL/*lpr* mice receiving drinking water alone (controls, ▨); L-NIL (1 mg/mL; ▩) or L-NMMA (50 mmol/L; ■) in distilled drinking water. The results are shown as kidney weights (×100) divided by total body weight at 19 and 22 weeks of age ($N = 5$; $^*P < 0.05$).

treated mice continued to have kidney-to-body weight ratios that were significantly greater than the controls.

At 19 and 22 weeks of age, five mice from each group were sacrificed, kidneys removed and pathologic renal scores determined using a previously published scale for glomerular inflammation [18]. Figure 5 shows a representative kidney section from a control, L-NMMA and L-NIL treated mouse at 22 weeks of age. The control group showed significant pathologic renal findings including, diffuse glomerular hypercellularity, mesangial expansion, crescent formation, fibrinoid necrosis, glomerular hyalinization, and infiltration of inflammatory cells. Less renal disease was seen in the L-NMMA and the L-NIL treated mice as compared to controls. When the scores for disease activity were calculated, the mice in the L-NMMA and L-NIL groups had pathologic indices that were significantly less than the controls at 19 weeks (Table 1). At 22 weeks, the L-NIL treated mice continued to have significantly reduced pathologic renal scores as compared to controls; however, the renal pathology scores in the L-NMMA treated mice were not different from controls. Interstitial inflammation in the kidneys was similar in all three groups, as all *lpr* mice, regardless of strain background, have interstitial inflammation.

To determine the effects of NO inhibition on glomerular IC deposition, immunofluorescence analysis was preformed. Frozen sections were stained with fluorescein-conjugated and mouse IgG or C3 and scored by a blinded observer on a scale from 0 to 3. When the scores were averaged there was no qualitative or quantitative difference between the control, L-NIL, and L-NMMA treated mice at 19 or 22 weeks of age (Fig. 6).

To investigate the possible mechanisms for modulation of renal disease in L-NMMA and L-NIL treated mice, we measured levels of autoantibodies that are implicated in disease pathogenesis. Anti-dsDNA Abs and anti-GBM Abs are pathogenic in autoimmune nephritis that develops in MRL/*lpr* mice. We measured anti-ds DNA Abs in the serum by ELISA (Table 2). As the mice aged, anti-ds DNA Abs increased in all groups with no significant difference between the groups except at 22 weeks, where there was a decrease in anti-dsDNA antibodies in the L-NMMA treated mice. There was no significant difference in antibody titers to GBM in the three groups (Table 2). Furthermore, serum total IgG, IgG2a and IgG3 were similar in all three groups at all time points (data not shown).

At 22 weeks of age almost all MRL/*lpr* mice display external physical characteristics including excoriating dermatitis and necrosis of ear lobes. Figure 7 shows control mice with a typical skin rash. All the mice treated with L-NMMA displayed skin lesions that were similar to those in the control mice, whereas none of the mice treated with L-NIL had signs of skin involvement.

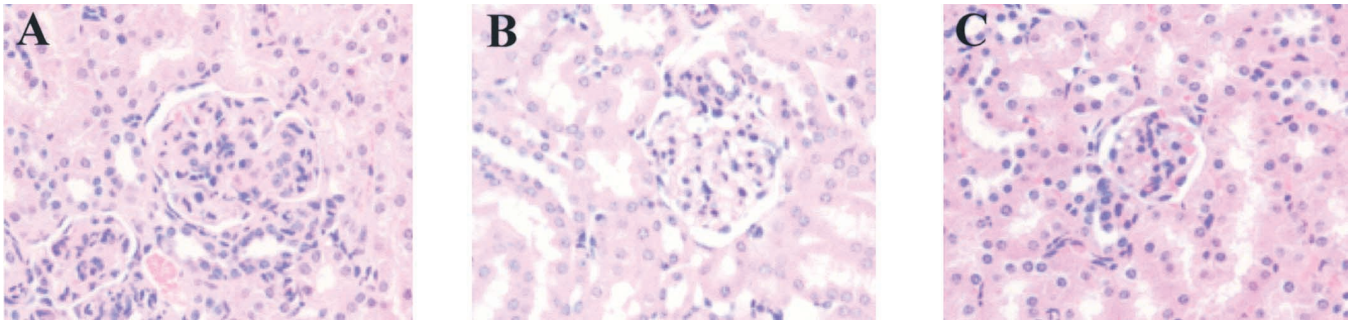

Fig. 5. Representative kidney section stained with H&E from a MRL/*lpr* mouse treated with L-NIL (1 mg/mL), or L-NMMA (50 mmol/L), or distilled water only at 22 weeks of age in (*A*) control, (*B*) L-NMMA and (*C*) L-NIL.

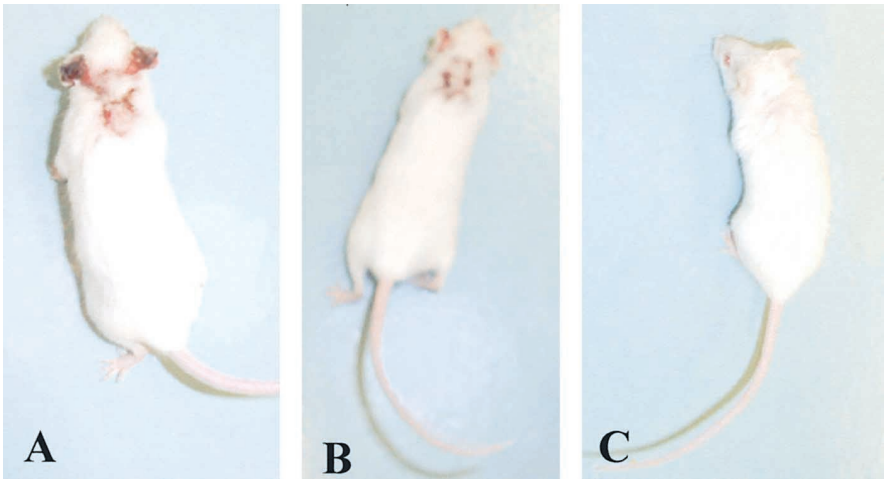

Fig. 7. Representative photographs of mice at 22 weeks of age. (*A*) Control MRL/*lpr* mice showing severe earlobe necrosis and skin dermatitis. (*B*) MRL/*lpr* mouse treated with L-NMMA (50 mmol/L) also showing earlobe necrosis and skin dermatitis. (*C*) MRL/*lpr* mouse treated with L-NIL (1 μg/mL) showing minimal signs of skin disease.

DISCUSSION

Given the conflicting results from our previous studies on the effect of pharmacologic inhibition versus genetic deficiency of iNOS in MRL/*lpr* mice, we performed these studies to define further the role that iNOS plays in renal disease in these mice. Two different agents that block NOS activity were used: L-NMMA, a non-specific NOS inhibitor, and L-NIL, a specific inhibitor of the iNOS isoform. Our results demonstrated that both L-NMMA and L-NIL significantly decreased pathologic renal scores when compared to controls. L-NIL was of greater efficacy in long-term control of proliferative renal disease than L-NMMA, but was less effective at blocking proteinuria. As expected, neither L-NIL nor L-NMMA decreased the serum levels of autoantibodies or affected the glomerular deposition of IgG/C3-containing immune complexes. These results indicate that specific pharmacologic inhibition of iNOS is beneficial in preventing lupus-like renal disease in MRL/*lpr* mice by inhibiting the inflammatory response induced by IC deposition.

Three distinct isoforms of NOS are known, including neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) enzymes [19–25]. The constitutive isoforms of NOS (nNOS and eNOS) are locally produced in the central and peripheral nervous system and in vascular endothelial cells and serve predominantly physiological functions. In contrast, iNOS is synthesized de novo in

Table 1. Renal pathology of kidneys from MLR/*lpr* mice given distilled water alone, L-NIL (1 mg/mL), or L-NMMA (50 mmol/L) in the drinking water

		Interstitial inflammation		
	Renal score	Focal	Diffuse	Vasculitis
19 weeks				
Controls	8.2 ± 2.7	1.8 ± 0.3	1.6 ± 0.5	3/5 mice
L-NMMA	2.7 ± 0.5[a]	2.6 ± 0.5	2.4 ± 0.4	4/5 mice
L-NIL	3.0 ± 1.5[a]	2.6 ± 0.5	1.5 ± 0.7	2/5 mice
22 weeks				
Controls	10.2 ± 1.7	2.0 ± 0.0	1.6 ± 0.5	3/5 mice
L-NMMA	9.5 ± 1.7	2.8 ± 0.3	2.2 ± 0.6	4/5 mice
L-NIL	5.2 ± 2.5[a]	2.0 ± 0.0	0.75 ± 0.3	2/5 mice

The kidney slides were interpreted in a blinded fashion and graded for glomerular inflammation, proliferation, crescent formation and necrosis. Data presented are the mean ± SEM of 5 mice in each group at 19 and 22 weeks of age ($P < 0.05$). The presence of interstitial inflammation is graded separately and vasculitis is noted.

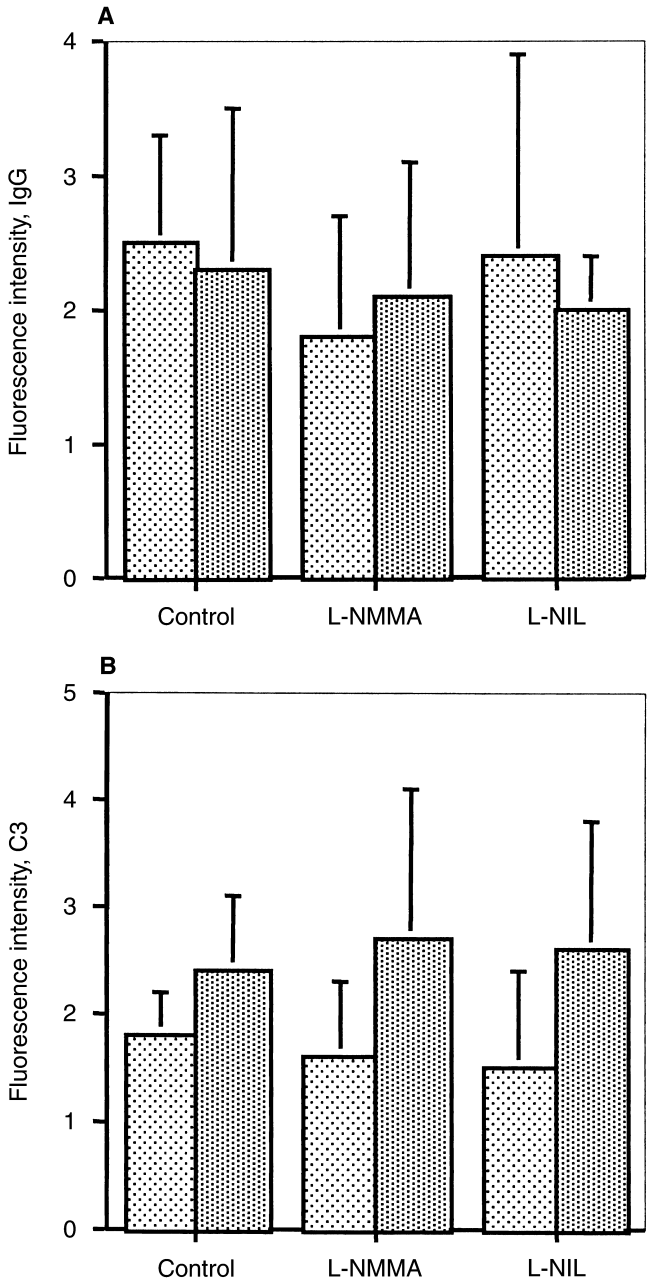

Fig. 6. Immunohistochemistry analysis of (*A*) IgG and (*B*) C3 deposition in the kidneys of MRL/*lpr* mice treated with L-NIL (1 mg/mL) or L-NMMA (50 mmol/L) in distilled drinking water. Controls received distilled water alone. Fluorescence was graded on a scale from 0 to 3 by a blinded observer and is shown as the ±SEM of 5 mice in each group at 19 (▩) and 22 (■) weeks of age.

Table 2. Serum anti-dsDNA and anti-GBM antibodies from MRL/*lpr* mice at 12, 16, 19 and 22 weeks of age after treatment with distilled water, L-NIL (1 mg/ml) or L-NMMA (50 mmol/L)

	Control	L-NMMA	L-NIL
Serum anti-dsDNA Ab levels			
12 weeks	0.31 ± 0.2	0.49 ± 0.4	0.44 ± 0.2
16 weeks	0.96 ± 0.4	0.89 ± 0.4	0.97 ± 0.3
19 weeks	1.2 ± 0.4	0.89 ± 0.4	1.5 ± 0.3
22 weeks	1.2 ± 0.5	0.55 ± 0.1[a]	1.0 ± 0.4
Serum anti-GBM Ab levels			
12 weeks	0.6 ± 0.2	0.70 ± 0.3	0.6 ± 0.2
16 weeks	1.2 ± 0.3	1.0 ± 0.4	1.1 ± 0.2
19 weeks	1.8 ± 0.3	1.2 ± 0.5	1.3 ± 0.3
22 weeks	1.4 ± 0.2	1.2 ± 0.3	1.3 ± 0.3

Results shown are the mean ± SEM of 10 mice from 10 to 19 weeks and 5 mice at 22 weeks ([a]$P < 0.05$). Abbreviations are in the text.

selected cell types after exposure to bacterial endotoxins or specific cytokines. The compounds used in these studies, L-NMMA and L-NIL, show different selectivity for the three isoforms of NOS. The IC_{50} values for L-NMMA are: iNOS 13.3 μmol/L, eNOS 5.2 μmol/L and nNOS 7.1 μmol/L, making L-NMMA a non-specific NOS inhibitor. The IC_{50} values for L-NIL are: iNOS 5.0 μmol/L, eNOS 135 μmol/L, and nNOS 54 μmol/L. Thus, L-NIL is 10 times more specific for iNOS than for nNOS and over 30 times more specific for iNOS than for eNOS. At the doses used in these studies, L-NIL was relatively specific for iNOS as evidenced by the lack of effect of L-NIL on blood pressure measures in the mice and the decreased levels of N/N in the urine of the animals.

The mechanisms responsible for the increased levels of NO in lupus mice are not completely known. Immune complex deposition in the kidney appears to predate N/N production and likely initiates the inflammatory cascade through complement and/or Fc receptor activation [26–29]. In the MRL/*lpr* mouse, macrophages infiltrate the glomerulus and secrete pro-inflammatory mediators such as IFN-γ and NO [6, 30, 31]. Mesangial cells also possess the ability to secrete pro-inflammatory mediators in response to inflammatory stimulation [32–35]. The relative contribution of macrophages versus mesangial cells to the production of inflammatory mediators and NO remains unclear.

Several studies have demonstrated beneficial effects of blocking NO production in glomerulonephritis [15, 36–38], whereas others have observed deleterious effects of NO inhibition [39, 40]. In a rat model of induced glomerulonephritis, anti-thymocyte serum (ATS) induces glomerulonephritis and acute mesangial cell proliferative glomerulonephritis. Studies by Narita et al showed that L-NMMA inhibited glomerulonephritis in rats given ATS and correlated these results with decreased mesangial cell lysis, TGF-β expression, and decreased extracellular matrix accumulation [41]. Similar to our data with both L-NMMA and L-NIL, these experiments indicate that pathologic renal disease can be treated distal to immune complex deposition. In contrast to our current studies showing a protective role with L-NIL treatment, studies by McCartney-Francis et al demonstrated that selective iNOS inhibition with L-NIL exacerbated erosive joint disease [42]. In our previous studies using mes-

angial cells, L-NIL addition did not lead to decreased iNOS protein expression [43], while in the studies by McCartney-Francis et al L-NIL decreased iNOS protein levels in arthritic joint tissue, indicating that other mechanisms aside from the known enzyme inhibitor effects of L-NIL may contribute to these disparate results.

Relatively specific iNOS inhibition was previously shown to have beneficial effects in another murine model of lupus. Yang et al reported that administration of aminoguanidine reduced NO and TGF-β production in NZB/NZW mice [44]. Glomerular sclerosis, mean glomerular cell number and urinary proteinuria also were decreased, showing beneficial effects of relative iNOS inhibition. Aminoguanidine, however, has other biologic effects independent of inhibiting iNOS activity that may have played a role in the beneficial effects seen in these experiments. L-NIL is significantly more specific for iNOS than aminoguanidine, allowing a clearer assessment of the effects of specific inhibition of iNOS on lupus-like glomerulonephritis.

A specific iNOS inhibitor would be preferred as a pharmacologic agent in humans due to the potential toxicity of non-specific NOS inhibitors [45, 46]. Although not clinically proven, elevation in blood pressure, possible central nervous system (CNS) dysfunction and impotence are predicted side effects of non-specific NOS inhibition [47]. Constitutively produced NO lowers glomerular pressure and thereby protects against pressure-induced renal injury. This effect on intra-glomerular pressure may account for the significant improvement in renal score at 22 weeks we observed in the L-NIL treated mice compared to the L-NMMA treated mice. In a recent review by Cattell et al [48], it was noted that NO could be either toxic or protective in immune glomerulonephritis depending on the amount of NO produced, which in turn depends on the NOS gene regulated, the production of other radicals, and the activity of arginase [49]. Local production of high concentrations of NO may induce injury while constitutive NO, critical for offsetting vasoconstriction in the injured glomerulus, is likely protective. For these reasons, targeting specifically the iNOS gene in lupus nephritis appears to be the preferred strategy.

These studies do provide clear evidence that inhibiting iNOS activity reduces proliferative glomerulonephritis in MRL/*lpr* mice. The current studies still leave us with an unexplained dichotomy of the effect of pharmacologic versus genetic inhibition of iNOS. There are a number of explanations for these contrasting results. The first is that there are significant differences between pharmacologic and genetic inhibition of a biologic system. For example, TNFαR1 knockout mice developed inflammatory arthritis similar to that of controls in the collagen induced model of rheumatoid arthritis [50]. However, anti-TNFα therapy (including soluble form of TNFαR1) represents the most successful class of therapeutic agents for rheumatoid arthritis to date [51, 52]. Thus, the results of knockout studies do not necessarily mean that the lack of effect of a knocked out gene on a disease process eliminates that gene as a good target for pharmacologic intervention.

An alternative explanation for the contrasting results of pharmacologic iNOS inhibition versus genetic ablation of iNOS is that both L-NMMA and L-NIL have additional effects on disease other than simply blocking NO production. Both agents are arginine analogs and block NO production by competing with arginine as a substrate for iNOS. Arginine is utilized by other biologic systems that may impact renal disease (for example, biologically active amines). It is possible, although we believe unlikely, that effects on these non-NOS pathways influence disease progression. Derivation of inhibitors of iNOS that are not arginine analogs would allow delineation of this possibility.

In summary, these studies demonstrate that iNOS derived NO plays a key role in the development of proliferative glomerulonephritis in MRL/*lpr* mice. Inhibition of all three isoforms of NOS leads to diminished proteinuria and lower renal pathology scores compared to untreated controls. Furthermore, treatment of MRL/*lpr* mice with a specific iNOS inhibitor results in long-term sustained significant improvement in renal scores and decreased skin disease as compared to controls. These studies suggest that specific iNOS inhibitors may have therapeutic value in the treatment of lupus nephritis.

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REFERENCES

1. Theofilopoulos AN, Kono DH: The genes of systemic autoimmunity. *Proc Assoc Am Phys* 111:228–240, 1999
2. Lindqvist AK, Alarcon-Riquelme ME: The genetics of systemic lupus erythematosus. *Scan J Immunol* 50:562–571, 1999
3. Couser WG, Nangaku M, Shankland SJ, Johnson RJ: Molecular mechanisms of experimental glomerulonephritis—An overview. *Nephrology* 3(Suppl): S633–S637, 1997
4. MacMicking J, Xie QW, Nathan C: Nitric oxide and macrophage function. *Ann Rev Immunol* 15:323–350, 1997
5. Yokoyama M, Hirata K, Kawashima S, Kawahara Y: Regulation of nitric oxide synthase gene expression by cytokines. *J Card Fail* 2(Suppl):S179–S185, 1996
6. Kashem A, Endoh M, Yano N, *et al*: Expression of inducible-NOS in human glomerulonephritis: The possible source is infiltrating monocytes/macrophages. *Kidney Int* 50:392–399, 1996
7. Mosley K, Waddington SN, Ebrahim H, *et al*: Inducible nitric

oxide synthase induction in Thy 1 glomerulonephritis is complement and reactive oxygen species dependent. *Exp Nephrol* 7:26–34, 1999
8. Pfeilschifter J, Rob P, Mulsch A, *et al*: Interleukin 1 beta and tumour necrosis factor alpha induce a macrophage-type of nitric oxide synthase in rat renal mesangial cells. *Eur J Biochem* 203:251–255, 1992
9. Squadrito GL, Pryor WA: Oxidative chemistry of nitric oxide: the roles of superoxide, peroxynitrite, and carbon dioxide. *Free Radic Biol Med* 25:392–403, 1998
10. Kroncke KD, Fehsel K, Kolb-Bachofen V: Inducible nitric oxide synthase and its product nitric oxide, a small molecule with complex biological activities. *Biol Chem Hoppe Seyler* 376:327–343, 1995
11. Landino LM, Crews BC, Timmons MD, *et al*: Peroxynitrite, the coupling product of nitric oxide and superoxide, activates prostaglandin biosynthesis. *Proc Natl Acad Sci USA* 93:15069–15074, 1996
12. Stamler S, Gross SS, Moncada S: The role of nitric oxide and peroxynitrite in the pathogeneses of spontaneous murine autoimmune disease, in *The Biology of Nitric Oxide* (vol 5), London, Portland Press, 1996
13. Granger DL, Taintor RR, Boockvar KS, Hibbs JB Jr: Measurement of nitrate and nitrite in biological samples using nitrate reductase and Griess reaction. *Methods Enzymol* 268:142–151, 1996
14. Weinberg JB, Granger DL, Pisetsky DS, *et al*: The role of nitric oxide in the pathogenesis of spontaneous murine autoimmune disease: Increased nitric oxide production and nitric oxide synthase expression in MRL-*lpr/lpr* mice, and reduction of spontaneous glomerulonephritis and arthritis by orally administered N^G-monomethyl-L-arginine. *J Exp Med* 179:651–660, 1994
15. Cattell V, Cook T, Moncada S: Glomeruli synthesize nitrite in experimental nephrotoxic nephritis. *Kidney Int* 38:1056–1060, 1990
16. Oates JC, Ruiz P, Alexander A, *et al*: Effect of late modulation of nitric oxide production on murine lupus. *Clin Immunol Immunopathol* 83:86–92, 1997
17. Gilkeson GS, Mudgett JS, Seldin MF, *et al*: Clinical and serologic manifestations of autoimmune disease in MRL-*lpr/lpr* mice lacking nitric oxide synthase type 2. *J Exp Med* 186:365–373, 1997
18. Watanabe H, Garnier G, Circolo A, *et al*: Modulation of renal disease in MRL/*lpr* mice genetically deficient in the alternative complement pathway factor B. *J Immunol* 164:786–794, 2000
19. Forstermann U, Closs EI, Pollock JS, *et al*: Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions. *Hypertens* 23:1121–1131, 1994
20. Hattori R, Sase K, Eizawa H, *et al*: Structure and function of nitric oxide synthases. *Int J Cardiol* 47:S71–75, 1994
21. Geller DA, Billiar TR: Molecular biology of nitric oxide synthases. *Cancer Metastasis Rev* 17:7–23, 1998
22. Mashimo H, Goyal RK: Lessons from genetically engineered animal models. IV. Nitric oxide synthase gene knockout mice. *Am J Physiol* 277:G745–G750, 1999
23. Noris M, Remuzzi G: Physiology and pathophysiology of nitric oxide in chronic renal disease. *Proc Assoc Am Phys* 111:602–610, 1999
24. Andrew PJ, Mayer B: Enzymatic function of nitric oxide synthases. *Cardiovasc Res* 43:521–531, 1999
25. Bredt DS: Endogenous nitric oxide synthesis: Biological functions and pathophysiology. *Free Radic Res* 31:577–596, 1999
26. Veis JH, Yamashita W, Liu YJ, Ooi BS: The biology of mesangial cells in glomerulonephritis. *Proc Soc Exp Biol Med* 195:160–167, 1990
27. Sheerin NS, Sacks SH: The local production of complement in the pathogenesis of renal inflammation. *Nephrologie* 20:377–382, 1999
28. Grande JP: Mechanisms of progression of renal damage in lupus nephritis: Pathogenesis of renal scarring. *Lupus* 7:604–610, 1998
29. Waer M: The role of anti-DNA antibodies in lupus nephritis. *Clin Rheumatol* 9:111–114, 1990
30. Clark RB, Grunnet M, Lingenheld EG: Adoptively transferred EAE in mice bearing the *lpr* mutation. *Clin Immunol Immunopathol* 85:315–319, 1997
31. Cattell V, Lianos E, Largen P, Cook T: Glomerular NO synthase activity in mesangial cell immune injury. *Exp Nephrol* 1:36–40, 1993
32. Sakatsume M, Narita I, Yamazaki H, *et al*: Down-regulation of interferon-gamma signaling by gene transfer of Stat1 mutant in mesangial cells. *Kidney Int* 57:455–463, 2000
33. Salvemini D, Seibert K, Masferrer JL, *et al*: Endogenous nitric oxide enhances prostaglandin production in a model of renal inflammation. *J Clin Invest* 93:1940–1947, 1994
34. Beck KF, Eberhardt W, Frank S, *et al*: Inducible NO synthase: Role in cellular signalling. *J Exp Biol* 202:645–653, 1999
35. Fan X, Wuthrich RP: Upregulation of lymphoid and renal interferon-gamma mRNA in autoimmune MRL-Fas(*lpr*) mice with lupus nephritis. *Inflammation* 21:105–112, 1997
36. Muhl H, Pfeilschifter J: Tetrahydrobiopterin is a limiting factor of nitric oxide generation in interleukin 1 beta-stimulated rat glomerular mesangial cells. *Kidney Int* 46:1302–1306, 1994
37. Cattell V, Cook T: The nitric oxide pathway in glomerulonephritis. *Curr Opin Nephrol Hypertens* 4:359–364, 1995
38. Saura M, Lopez S, Rodriguez Puyol M, *et al*: Regulation of inducible nitric oxide synthase expression in rat mesangial cells and isolated glomeruli. *Kidney Int* 47:500–509, 1995
39. Waddington S, Cook HT, Reaveley D, *et al*: L-arginine depletion inhibits glomerular nitric oxide synthesis and exacerbates rat nephrotoxic nephritis. *Kidney Int* 49:1090–1096, 1996
40. Lianos EA, Guglielmi K, Sharma M: Regulatory interactions between inducible nitric oxide synthase and eicosanoids in glomerular immune injury. *Kidney Int* 53:645–653, 1998
41. Narita I, Border WA, Ketteler M, Noble NA: Nitric oxide mediates immunologic injury to kidney mesangium in experimental glomerulonephritis. *Lab Invest* 72:17–24, 1995
42. McCartney-Francis NL, Song X, Mizel DE, Wahl SM: Selective inhibition of inducible nitric oxide synthase exacerbates erosive joint disease. *J Immunol* 166:2734–2740, 2001
43. Reilly CM, Oates JC, Cook JA, *et al*: Inhibition of mesangial cell nitric oxide in MRL/*lpr* mice by prostaglandin J(2) and proliferator activation receptor-gamma agonists. *J Immunol* 164:1498–1504, 2000
44. Yang CW, Yu CC, Ko YC, Huang CC: Aminoguanidine reduces glomerular inducible nitric oxide synthase (iNOS) and transforming growth factor-beta 1 (TGF-beta1) mRNA expression and diminishes glomerulosclerosis in NZB/W F1 mice. *Clin Exp Immunol* 113:258–264, 1998
45. Spieker LE, Corti R, Binggeli C, *et al*: Baroreceptor dysfunction induced by nitric oxide synthase inhibition in humans. *J Am Coll Cardiol* 36:213–218, 2000
46. Qiu C, Baylis C: Endothelin and angiotensin mediate most glomerular responses to nitric oxide inhibition. *Kidney Int* 55:2390–2396, 1999
47. Sander M, Chavoshan B, Victor RG: A large blood pressure-raising effect of nitric oxide synthase inhibition in humans. *Hypertens* 33:937–942, 1999
48. Cattell V, Cook HT: Nitric oxide: Role in the physiology and pathology of the glomerulus. *Exp Nephrol* 1:265–280, 1993
49. Jansen A, Lewis S, Cattell V, Cook HT: Arginase is a major pathway of L-arginine metabolism in nephritic glomeruli. *Kidney Int* 42:1107–1112, 1992
50. Mori L, Iselin S, De Libero G, Lesslauer W: Attenuation of collagen-induced arthritis in 55-kDa TNF receptor type 1 (TNFR1)-IgG1-treated and TNFR1-deficient mice. *J Immunol* 157:3178–3182, 1996
51. Franklin CM: Clinical experience with soluble TNF p75 receptor in rheumatoid arthritis. *Semin Arthritis Rheum* 29:172–181, 1999
52. Harriman G, Harper LK, Schaible TF: Summary of clinical trials in rheumatoid arthritis using infliximab, an anti-TNFalpha treatment. *Ann Rheum Dis* 58:I61–64, 1999