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Lack of nitric oxide synthases increases lipoprotein immune complex deposition in the aorta and elevates plasma sphingolipid levels in lupus

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Abstract

Systemic lupus erythematosus (SLE) patients display impaired endothelial nitric oxide synthase (eNOS) function required for normal vasodilatation. SLE patients express increased compensatory activity of inducible nitric oxide synthase (iNOS) generating excess nitric oxide that may result in inflammation. We examined the effects of genetic deletion of *NOS2* and *NOS3*, encoding iNOS and eNOS respectively, on accelerated vascular disease in MRL/lpr lupus mouse model. *NOS2* and *NOS3* knockout (KO) MRL/lpr mice had higher plasma levels of triglycerides (23% and 35%, respectively), ceramide (45% and 21%, respectively), and sphingosine 1-phosphate (S1P) (21%) compared to counterpart MRL/lpr controls. Plasma levels of the anti-inflammatory cytokine interleukin 10 (IL-10) in *NOS2* and *NOS3* KO MRL/lpr mice were lower (53% and 80%, respectively) than counterpart controls. Nodule-like lesions in the adventitia were detected in aortas from both *NOS2* and *NOS3* KO MRL/lpr mice. Immunohistochemical evaluation of the lesions revealed activated endothelial cells and lipid-laden macrophages (foam cells), elevated sphingosine kinase 1 expression, and oxidized low-density lipoprotein immune complexes (oxLDL-IC). The findings suggest that advanced vascular disease in *NOS2* and *NOS3* KO MRL/lpr mice maybe mediated by increased plasma triglycerides, ceramide and S1P; decreased plasma IL-10; and accumulation of oxLDL-IC in the vessel wall. The results expose possible new targets to mitigate lupus-associated complications.

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Keywords

lupus; nitric oxide synthase; sphingosine 1-phosphate; sphingosine kinase; ceramide; oxidized LDL

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic multisystem autoimmune disease with a broad range of clinical manifestations. Distinctly, SLE is associated with accelerated atherosclerosis and increased risk of cardiovascular complication [1]. Noninvasive investigations have shown increases in intima-media thickness, carotid plaque build-up, and coronary artery calcifications in subjects with SLE [2]. Among SLE patients, there is a 5-10 fold increase in the risk of developing cardiovascular disease (CVD) compared to age-matched controls [3]. Manzi et al showed that 35-44 year-old women with SLE have an estimated 50-fold increased risk of myocardial infarction compared to age and sex-matched controls [4]. The underlying mechanisms responsible for the increase in morbidity and mortality due to SLE-related CVD are unclear.

Inflammation is believed to intensify the development of the atherosclerotic lesion and to contribute to acute rupture of plaques that occurs during acute myocardial ischemic events [5, 6]. Macrophage activation and intima-infiltration, and in turn atherogenesis, are often preceded by endothelial damage or dysfunction. Endothelial cell dysfunction represents the earliest indication of the development of CVD and is also a principal element of SLE [7]. Nitric Oxide (NO) secretion is required for normal endothelium-dependent vasodilation in SLE [8]. Endothelial dysfunction and specifically the impairment of NO secretion have been shown to result in increased vascular lesion formation, thus contributing to the severity of atherogenesis [9, 10]. While SLE patients display a defect in the function of endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS) is elevated in both endothelial cells and keratinocytes in association with disease activity [11]. It is known that *NOS2* expression is increased in response to inflammatory stimuli resulting in excess NO production which could promote inflammation and increase the risk of atherogenesis [12]. Likewise, elevated serum levels of NO and other reactive nitrogen and oxygen species (RNS/ROS) have been implicated in vascular lesion formation and endothelial functional defects [13, 14]. and are currently being pursued as possible new biomarkers for lupus [15].

NO plays an important role in maintaining cardiovascular homeostasis [16-18]. There is strong evidence that NO-activated pathways regulate many of the biological processes in which sphingolipids are involved [19, 20]. Sphingolipids are ubiquitous constituents of bio-membranes and their metabolic products sphingomyelin (SM), ceramide (Cer), sphingosine (Sph), and sphingosine 1-phosphate (S1P) has been implicated in the regulation of cell growth, differentiation, and apoptosis [21, 22]. A large number of agonists and stress signals, including elevated NO, induce the hydrolysis of SM resulting in the accumulation of Cer [19-21]. Moreover, it has been shown that secretory acid sphingomyelinase (S-ASMase) levels in the serum are up-regulated by tumor necrosis factor- α (TNF- α), interleukin-1 beta (IL-1 β) [23], lipopolysaccharides (LPS), and oxidative and nitrosative stress [24]. The intricacy of the role of sphingolipid signaling in the regulation of inflammatory responses and survival led us to posit that NO can influence sphingolipid metabolism that plays a role in the development and acceleration of the vascular disease. The roles of the NOS system *in vivo* have been investigated in pharmacological studies with iNOS inhibitors, and previous studies using the lupus mouse model MRL/lpr have shown that therapeutic inhibition of iNOS improved overall kidney function and increased survival rates [25], however in a non-iNOS-specific mechanism.

The non-specificity of the NOS inhibitors and the compensatory interaction among the NOS isoforms (iNOS/eNOS) has obscured their role in the development of cardiovascular complications in SLE. To address this important issue, we have used the lupus mouse model MRL/lpr lacking the inducible NO (*NOS2* KO) and endothelial NO (*NOS3* KO). We investigated the effect of these knockouts on plasma levels of lipids, sphingolipids, and cytokines. We also examined histological changes of the vessel wall by performing immunohistochemistry on aortas obtained from these mice.

2. Materials and Methods

2.1. Mice

We adopted the mouse model MRL/MpJ-Fas^{lpr} (Jackson laboratories, Stock # 000485, 006825) (MRL/lpr) that is genetically predisposed to the development of SLE-like syndrome. Mice homozygous for the lymphoproliferation spontaneous mutation (Fas^{lpr}) show systemic autoimmunity, massive lymphadenopathy associated with proliferation of aberrant T cells, arthritis, and immune-complex glomerulonephrosis starting at about three months of age. To investigate the effect of iNOS on accelerated vascular disease in the SLE mouse model, *NOS2* KO mice were generated and were used in this study. Briefly, Female B6.129P2-*Nos2*^{tm1Lau}/J (Stock # 002609) mice from Jackson laboratories were backcrossed with lupus mouse model male MRL/MpJ-*Tnfrsf6*^{lpr}/J mice (changed by Jackson laboratories on 26-JAN-05 to MRL/MpJ-FAS^{lpr}/J). The F1 *NOS2* heterozygote generation was again backcrossed with MRL/MpJ-FAS^{lpr}/J and the resulting heterozygotes were backcrossed for a total of ten generations with the MRL/MpJ-FAS^{lpr}/J mice. Sixteen lupus susceptibility loci were followed by PCR using Speed Congenics and Touchdown PCR to ensure the specificity of the model.

To study the effect of eNOS on the development of vascular disease in the SLE mouse model, *NOS3* KO mice were generated using female B6.129P2-*Nos3*^{tm1Unc}/J (Stock # 002684) mice from Jackson laboratories backcrossed with cryo-recovered male MRL/MpJ-FAS^{lpr}/J (Stock # 000485) mice. The F1 *NOS3* heterozygote generation was again backcrossed with MRL/MpJ-FAS^{lpr}/J (Stock # 000485) mice. F1 *NOS3* heterozygotes from this backcross were backcrossed for a further ten generations with the cryo-recovered MRL/MpJ-FAS^{lpr}/J (Stock # 000485) to produce the *NOS3*/MRL/lpr mouse colony used in this study.

Sixteen lupus susceptibility loci were followed by PCR using Speed Congenics to ensure the specificity of the model. Genetic drift occurs in genetic strains, even within reputable animal vendors. Therefore, the resulting knockout homozygotes were compared to their counterpart *NOS2*^{+/+} MRL/lpr and *NOS3*^{+/+} MRL/lpr littermates to maintain the same MRL/lpr genotypic background. Therefore, *NOS2*^{+/+} MRL/lpr and *NOS3*^{+/+} MRL/lpr mice were used as counterpart controls for *NOS2* KO MRL/lpr and *NOS3* KO MRL/lpr mice, respectively. Thus, differences observed between groups would be isolated to the *NOS2* and *NOS3* mutations.

2.2. Ethical Aspects

No experiments performed on live animals (mice). Animals were euthanized by sedation with isoflurane followed by cervical dislocation or CO₂ inhalation. This method of euthanasia is approved by Ralph H. Johnson Veterans Affairs Institutional Animal Care and Use Committee, consistent with the recommendations of the Panel on Euthanasia of the American VMA. Mice were housed and cared for in The VA PHS Animal Welfare Assurance Number is A3428-01.

2.3. Determination of plasma lipid profile

Plasma samples were isolated from blood collected in EDTA-coated tubes. The plasma lipid profile analysis was performed using the Cholestec LDX® system (Cholestec Corporation, Hayward, CA). A volume of 35 µl plasma was used to determine high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) content, triglycerides (TRG), total cholesterol (TC), and also glucose levels in each mouse.

2.4. Sphingolipid analysis by HPLC-MS/MS

Analysis of sphingolipids in plasma samples were performed by HPLC-MS/MS at the MUSC Lipidomics Shared Resource facility as described previously [26]. Sphingolipids measured were molecular species (C_n -) of Cer, and the sphingoid bases: Sph and dihydrosphingosine (dhSph), and their 1-phosphates (S1P and dhS1P). A volume of 100 µl plasma was used to determine the plasma sphingolipid profile in each mouse.

2.5. Determination of plasma cytokine levels

Plasma samples were processed in duplicate with a custom Bio-Rad Bio-Plex mouse cytokine reagent kit for interleukin-1 alpha (IL-1α), IL-1β, interleukin 6 (IL-6), interleukin 10 (IL-10), interferon gamma (IFNγ), and TNF-α according to manufacturer's instructions (Bio-Rad). Briefly, plasma samples were incubated with anti-cytokine-conjugated beads followed by incubation with biotinylated detection antibody. The reaction mixture was subsequently incubated with streptavidin-phycoerythrin and analyzed using a Luminex®-based multiplex analysis system from BioRad (BioPlex 200). Unknown cytokine concentrations were calculated by BioPlex Manager Software using standard curves derived from a recombinant cytokine standard. Data are expressed as fold change from control.

2.6. Gross examination and scoring of aortas

Mice were euthanized when they developed protein urea, a sign of developing complication in this model, and end stage of this disease. Aortas were then harvested and immediately fixed in 1% paraformaldehyde. After fixation surrounding fat was removed from the aorta using a dissecting microscope. Subsequently, the aortas were cut longitudinally and pinned to a wax-coated Petri dish. Tissue samples were stained using Sudan IV (50 µg/ml) for twenty minutes and de-stained 5 times for two minutes each time by using 70% ethanol. Tissue samples were scored blindly on scale of 1 to 4, the darker the stain the higher the score.

2.7. Immunohistochemistry

Aortas collected from mice were embedded in paraffin, sectioned, and mounted on glass slides. Hematoxylin and eosin staining (H & E) was performed to assess the general histology of blood vessels. Immunohistochemical analysis was performed to detect inflammatory cytokines and chemokines as described previously [27]. The anti platelet/endothelial cells adhesion molecules (PECAM-1) antibody (Santa Cruz Biotechnology) was used to determine the presence of activated platelet and endothelial cells in the vascular endothelium, and the antibody against F4/80 antigen (Abcam) was used to determine the presence of murine macrophages. Rabbit anti human oxidized low-density lipoprotein (oxLDL) IgG (affinity-purified, a gift from Dr. Virella, MUSC) was labeled with Alexa-633 (Invitrogen) according to manufacturer's instructions, and used to determine the presence of oxLDL. Alexa-488-labeled anti mouse IgG (Invitrogen) was used to determine the presence of immune complexes, and anti-nitro tyrosine (Upstate company, CA) to determine the presence of reactive nitrogen species. We also probed aortas for sphingosine kinase-1 (SK1), an enzyme that produces S1P, by using SK1 antibody (a gift from Dr. Kawamori, MUSC). For studies examining the presence of oxLDL-IC, all staining was performed using

VECTASTAIN® ABC Kit according to the manufacturer's protocol. Aortas from 8 mice from each group were studied.

2.8. Statistics

Data are expressed as mean $\pm$ SE, and significant differences between two groups were evaluated by Student's *t*-test, $P < 0.05$. We used a parametric test (Pearson), and a non-parametric test (Spearman) to test the significant differences between means in cytokine analysis.

3. Results

3.1. Increased levels of TRG in MRL/lpr mice with genetic deletion of *NOS2* or *NOS3*

Genetic deletion of *NOS2* or *NOS3* in MRL/lpr mice led to more rapid development of proteinuria and a shorter life expectancy than their counterpart controls (Supplemental table 1). Lipid profiles and glucose levels of plasma samples were obtained from *NOS2* KO and *NOS3* KO MRL/lpr mice and data revealed a significant increase in TRG in both *NOS2* and *NOS3* KO MRL/lpr mice compared to MRL/lpr controls (Fig 1A, B). *NOS2* but not *NOS3* KO MRL/lpr mice exhibited higher HDL-C levels compared to counterpart controls (Fig 1A, B).

3.2. Elevated plasma sphingolipids in MRL/lpr mice with genetic deletion of *NOS2* or *NOS3*

Plasma sphingolipid levels were analyzed using HPLC-MS/MS and, as shown in Figure 2, the sphingolipid profile changed significantly in *NOS2* KO and *NOS3* KO MRL/lpr mice relative to the corresponding MRL/lpr controls. Both *NOS2* KO and *NOS3* KO MRL/lpr mice had significantly higher levels of total Cer compared to their counterpart controls (Fig 2A, B). Significant increases were mainly in C₂₂-Cer and C₂₄-Cer levels. Intriguingly, C_{24:1}-Cer levels were about 2 fold higher in *NOS2* KO MRL/lpr mice compared to *NOS3* KO MRL/lpr mice and their counterpart controls (Fig 2A, B). Levels of the bioactive molecule S1P in MRL/lpr mice lacking *NOS2* or *NOS3* gene were significantly higher compared to their counterpart controls; however, levels of dhS1P were elevated in the *NOS2* but not the *NOS3* KO MRL/lpr mice (Fig 2C, D).

3.3. Decreased levels of the anti-inflammatory cytokine IL-10 in MRL/lpr mice with genetic deletion of *NOS2* or *NOS3*

To examine the effect of *NOS2* and *NOS3* deletion on the release of pro- and anti-inflammatory cytokines in the MRL/lpr lupus mouse model, plasma obtained from *NOS2* and *NOS3* KO MRL/lpr mice and counterpart controls were analyzed using a multiplex mouse cytokine detection assay. Interestingly, MRL/lpr mice with the genetic deletion of *NOS2* but not *NOS3* exhibited higher levels of IL-1 β and TNF- α compared to counterpart controls (Fig 3). Intriguingly, IL-10 levels were significantly lower in both *NOS2* and *NOS3* KO MRL/lpr mice compared to counterpart controls.

3.4. Increased lipid deposition in the aorta of MRL/lpr mice with genetic deletion of *NOS2* or *NOS3*

Gross examination of aortas using Sudan IV stain revealed that *NOS2* and *NOS3* KO MRL/lpr mice had significantly higher accumulation of lipid (red stain) in the blood vessel wall compared to their counterpart MRL/lpr controls as measured by average lipid deposition scores (Fig 4). The average score for both *NOS2* and *NOS3* counterpart control MRL/lpr mice was 1.2, with a range of 1-1.3 (representative score of 1 is shown as Fig 4A). The score for *NOS2* KO MRL/lpr mice was 2.5, with a range between 2-3.2, whereas for *NOS3* KO MRL/lpr mice the score was 1.8, with a range of 1-2.7.

3.5. Development of nodule-like lesions in the adventitia

Staining representative samples with H & E revealed that aortas obtained from both *NOS2* and *NOS3* KO MRL/lpr mice developed advanced nodule-like lesions in the adventitia (Fig 5). The lesions contained substantial lipid deposition within abundant foam cells (Fig 5), which were then confirmed to be lipid-laden macrophages. As shown in Figure 6, immunohistochemical staining of the aorta at the site of the lesion revealed the following characteristics:

3.5.1. Activated endothelial cells—PECAM-1 antibody was used to detect activated endothelial cells within dissected aortas of *NOS2* and *NOS3* KO MRL/lpr mice and their counterpart control. PECAM-positive cells were found at the endothelial side of the nodule-like lesion (Fig 6, *left panel*).

3.5.2. Macrophage infiltration—Macrophages were detected using the anti F4/80 antibody and were found mostly in nodule-like lesions in the adventitia of *NOS2* KO and *NOS3* KO MRL/lpr mouse aortas. The lesions of were heavily infiltrated with macrophages, which were loaded with lipids (Fig 6, *middle panel*).

3.5.3. Detection of SK1—Levels of the bioactive molecule S1P in MRL/lpr mice lacking *NOS2* or *NOS3* gene were significantly higher compared to their counterpart controls (Fig 2). Therefore, we examined the expression levels of SK1, the enzyme that generates S1P, in the mouse aortas. We have previously shown in an *in vitro* system that macrophages activated with oxLDL immune complexes (oxLDL-IC), but not oxLDL alone, display immediate translocation and release of SK1 extracellular [28]. This finding indicated that S1P may be generated extracellularly and may therefore promote cell survival and prolong cytokine release by activated macrophages. Intriguingly in this study we found that SK1 was present in abundance around the lipid-laden macrophages in the nodule-like lesion in the adventitia of *NOS2* KO and *NOS3* KO MRL/lpr mice (Fig 6, *right panel*).

3.5.4. Detection of oxLDL, IgGs, and oxLDL-IC—Genetic deletion of *NOS2* or *NOS3* in MRL/lpr mice led to increased accumulation of oxLDL in the endothelium and in the tunica media, and abundance of auto-antibodies (IgGs) mainly in the nodule-like lesion in the adventitia mainly around the lipid-laden macrophages (Fig 7, *red and green, respectively*). The co-localization of Alexa-633-labeled anti oxLDL antibodies (Fig 7, *red*) with Alexa-488 anti mouse IgGs (Fig 7, *green*) indicates the formation of oxLDL-IC (Fig 7, *yellow*) in the lesion (Fig 7).

4. Discussion

In this study we used the MRL/lpr lupus mouse model with the deletion of either the *NOS2* or *NOS3* gene to investigate the mechanisms involved in developing accelerated vascular disease mediated by NO. By analyzing lipid deposition in the aorta of *NOS2* and *NOS3* KO MRL/lpr mice, we found that *NOS2* KO and *NOS3* KO MRL/lpr mice have higher lipid deposition in their aortas compared to their counterpart controls. The effects of atherogenic diet in both MRL/lpr and control mice has been previously reported [29]. It was shown that high fat diet induced hyperlipidemia, but levels of atherogenic apoB-containing lipoproteins (LDL) were much lower in the MRL/lpr mice, which suggested the formation of oxLDL in tissues [29]. The same study also reported enhanced lesion formation in the aortas of the high fat diet-fed mice. It has been also reported that ApoE deficient mice on cholesterol-enriched diets had higher levels of some plasma-borne ceramide species compared to the control diet [30]. The increased plasma-borne ceramide was correlated with a higher level of atherogenic oxLDL [30]. Therefore, changing the diet of *NOS2/NOS3* KO MRL/lpr mice

may be another means of changing plasma levels of ceramide species. Since the lupus MRL/lpr mice, with and without *NOS2* and *NOS3* deletion in this study were not on a high fat diet, inflammation and oxidative stress could have contributed to the acceleration of lipid deposition in the aorta in the *NOS2*, and *NOS3* KO mice.

Inflammatory diseases have been linked with atherogenic lipid profiles [31]. The lipid profile in SLE patients, described as the 'lupus pattern of dyslipoproteinemia', is characterized by elevated TRG, unchanged or slightly elevated LDL-C, and decreased HDL-C [32-35]. It has been also shown that HDL particle distribution and composition are abnormal and exhibit features of impaired atheroprotective properties in non-complicated SLE patients [36]. In our study, the MRL/lpr mouse has the typical mouse lipid profile with HDL being the major carrier of cholesterol and with very low levels of LDL cholesterol [37]. HDL-C levels were only increased in *NOS2* KO MRL/lpr mice compared to counterpart controls, while increased vascular lesions were observed in both *NOS2* and *NOS3* KO MRL/lpr mice. Therefore, it must be assumed that HDL in *NOS2* KO MRL/lpr mice is pro-inflammatory and is not atheroprotective. Importantly, it appears that the VLDL and LDL particles are not promptly cleared from the circulation. The presence of oxLDL-IC in the adventitia of blood vessels shows that the mice are under high oxidative stress creating oxLDL and oxLDL-IC.

The plasma profile of sphingolipid in lupus mice presented in this paper marks the first time such data has been reported. Although the mechanisms that drive changes in plasma levels of ceramide and S1P have yet to be determined, plasma sphingolipid profiles are of potentially diagnostic value [38, 39]. In lupus the disruption of reactive intermediate homeostasis may lead to a break in immune tolerance, increased tissue damage and altered enzyme function [40]. The role of oxidative stress on both increasing and decreasing sphingolipid metabolism has been reviewed by Won and Singh [41]. Therefore, a complex system involving the effect of oxidative stress on signaling intermediates that affect sphingolipid metabolism can be envisaged.

It has been shown that inflammatory cytokines such as TNF- α , and stress signals, including oxidative stress and elevated NO, induce the hydrolysis of SM resulting in the accumulation of Cer [42-45]. Additionally, the presence of NO inhibits ceramidase activity resulting in the accumulation of Cer [42-44]. Our data revealed that *NOS2* and *NOS3* KO MRL/lpr mice had significantly higher plasma levels of the bioactive molecule S1P as well as several Cer species compared to their counterpart controls. Genetic deletion of *NOS2* and *NOS3* and its consequent reduction in NO levels may allow for unrestricted ceramidase activity and thus accumulation of the substrate sphingosine for the enzymatic activity of SK1. We have previously reported that S1P is able to induce increases in released TNF- α , and prostaglandin E2 in macrophages *in vitro* [46]. Recently it has been also shown that S1P is involved in changes the vascular permeability and promotes cell recruitment by increasing leukocytes trafficking through a receptor mediated mechanisms [47]. We have performed further sphingolipid analysis on the lipoprotein fraction and lipid-free albumin-containing fraction of plasma samples obtained from the *NOS2* KO, *NOS3* KO and MRL/lpr control mice. The analysis revealed that dhS1P levels are elevated in the albumin containing-fraction of *NOS2* KO and *NOS3* KO mice compared to counterpart controls mice (data not shown). The mechanisms mediating the elevated levels of C_{24:1}-Cer and dhS1P in the MRL/lpr mice lacking iNOS but not eNOS remain to be investigated.

Lupus patients have increased levels of oxidized lipids (oxLDL and pro-inflammatory HDL) [35], up-regulation of adhesion molecules, and up-regulation of cytokines such as monocyte chemotactic protein (MCP-1), TNF- α , IFN γ , IL-1, and IL-12 [48-53]. These factors are believed to contribute to the accelerated development of atherosclerosis in SLE patients. We

found that *NOS2* KO MRL/lpr mice release higher levels of IL-1 β and TNF- α in the plasma than *NOS3* KO MRL/lpr mice. However, no obvious differences in size or structure of the lesion were recognized between *NOS2* and the *NOS3* KO MRL/lpr mice.

Elevated levels of the anti-inflammatory cytokine IL-10 in the serum of patients with SLE have been demonstrated in several studies [54, 55]. IL-10 has been also shown to be atheroprotective in mice [56], and to reduce *NOS2* expression [57] and increase *NOS3* expression [58] in different *in vivo* and *in vitro* models. Furthermore, IL-10 has been shown to decrease TNF α -induced reactive oxygen species [59] and to down-regulate endothelial adhesion molecules, thereby inhibiting the attachment of immune effectors known to drive atherogenesis [60]. Importantly, IL-10 KO MRL/lpr mice develop more severe lupus symptoms through enhanced production of IFN γ , and have shorter life expectancy [61]. However, anti-IL-10 therapy has been shown to down-modulate SLE by improving cutaneous lesions and joint symptoms, lowering the activity of immune and endothelial cells, and by decreasing the doses of administered prednisolone in these patients [62]. Our data revealed that genetic deletion of *NOS2* and *NOS3* resulted in a significant decrease in the plasma levels of IL-10 in the MRL/lpr mice compared to controls. Our findings are in agreement with a previous study that demonstrated that IL-10 could down-modulate murine lupus through inhibition of pathogenic Th1 cytokine responses [63]. However, it has also been reported that high serum levels of IL-10 in lupus patients correlated with increased disease activity and a corresponding increase in anti-dsDNA antibodies [64]. These conflicting data suggest a complex role for IL-10 in lupus pathology and requires further study to determine whether IL-10 may have any potential therapeutic benefit in lupus patients.

The previously published data on the effects of NO on IL-10 release is contradictory. Studies on activated human T cells *in vitro* have shown that NO-donors could inhibit the release of IL-10 [65]. Another study using isolated human monocytes showed that long-term incubation with an NO donor could inhibit IL-10 production in response to LPS challenge [66]. However, inhibition of iNOS by L-NG-monomethyl arginine in activated Jurkat cells inhibited IL-10 expression in a cAMP-dependent mechanism [67]. Thus, there appears to be no clear mechanism so far elucidated that can explain how *NOS2* and *NOS3* gene deletion can lead to lower plasma levels of IL-10.

In our study both *NOS2* KO and *NOS3* KO MRL/lpr mice developed nodule-like-lesions in the outer most layer of the arterial wall, the adventitia, and these lesions were infiltrated with macrophages. We found similar lesions in MRL/Lpr mice without the deletion of either *NOS2* or *NOS3* but with much less frequency and magnitude (data not shown). It has been shown that CD4 T cells are recruited to the adventitia, undergo local activation and subsequently orchestrate macrophage differentiation and function [68-70]. In giant cell arteritis, vascular infiltration occurs with influxes of large numbers of T cells and macrophages. Inflammatory cells accumulate in the arterial wall and induce a response-to-injury program that causes intimal hyperplasia leading to luminal occlusion [71, 72]. Therefore, we believe that accelerated vascular disease in lupus might be also mediated by the infiltration of the macrophages in the adventitia, which has yet to be examined in lupus patients.

Genetic deletion of *NOS2* or *NOS3* in MRL/lpr mice led to increased accumulation of oxLDL in the endothelium and in the tunica media, and abundance of auto-antibodies (IgGs) mainly in the lesion (adventitia) around the lipid-laden macrophages. The co-localization of Alexa-633-labeled anti oxLDL antibodies with Alexa-488 anti-mouse IgGs indicates the formation of oxLDL-IC in the lesion. We have previously reported that oxLDL-IC promote

cell survival and induce inflammatory responses in human U937 monocytic cells [28, 73] possibly mediated by inhibition of NO production [74].

It has been recently suggested that expansion of macrophages necessary for pathogen control or wound repair can occur without recruitment of potentially tissue-destructive inflammatory cells. It has been found that local macrophage proliferation (F4/80 negative) rather than recruitment from the blood (F4/80 positive) is fundamental in T helper-related inflammation [75]. We have shown in Figure 6 that F4/80-positive macrophages were found mostly in the center of the lesion. Furthermore, SK1 was present in abundance around the F4/80-negative macrophages in the periphery of the lesion. We hypothesize that oxLDL-IC-induced S1P can lead to local macrophage proliferation. It has been previously shown that the more advanced the atherosclerotic lesion, the greater the cell infiltration in the adventitia [76], predominantly infiltration of B lymphocytes [76, 77]. The increased SK1 levels in the aortic lesions may be generating S1P promoting B-cell infiltration and production of antibodies that will form oxLDL-IC. We therefore hypothesize that the increased cell damage in the nodule-like lesion could expose more auto-antigens that are being recognized by auto-antibodies generated in the lesion. Thus, our data suggest that accelerated vascular disease in *NOS2* and *NOS3* KO MRL/lpr lupus mice could be mediated by oxLDL and oxLDL-IC accumulation. It is probable that the elevated levels of oxLDL in MRL/lpr models paired with the KO of NOS encoding genes would be sufficient to induce the advanced vascular disease.

As expected, RNS, as determined by anti 3-nitro-L-tyrosine (3NT) staining, were barely detected in the blood vessels of *NOS2* KO and *NOS3* KO MRL/lpr mice, while counterpart controls displayed relatively high RNS levels (data not shown). It has been shown that NO scavenges ROS through formation of peroxynitrite that nitrates cellular proteins, forming 3NT [78-80]. This suggests that reduction in NO levels might lead to increased ROS concentration that may generate higher levels of oxLDL.

Recent studies have suggested that NO and sphingolipids interact in pathways that can act as positive feedback mechanisms [19, 20]. It has been shown for example that NO donors can up-regulate SK1 mRNA, protein, and activity in human endothelial cells [81]. This linkage of signaling events involve various sphingolipids, especially Cer and S1P [24]. Further studies on this relationship are sought to fully understand the interactions between NO and sphingolipids. It has been shown that the mutual regulation of sphingomyelinases and eNOS acts to control crucial patho-physiological processes such as inflammation [82]. This interaction may be able to explain the relatively higher levels of SK1 seen in *NOS2* and *NOS3* KO mice compared to their counterpart controls. Moreover, the low NO levels may have an inverse effect on the production and secretion of SK1 in infiltrating macrophages at the nodule-like lesions in lupus mice. It has been shown that inhibition of S1P signaling using FTY720 (fingolimod) suppresses the development of autoimmunity in MRL/lpr mice [83]. Another study showed that FTY720-treatment of lupus mice (BSXB mice) may improve their lifespan by inhibiting inflammatory processes involved with antibody deposition in the glomerulus [84]. It is therefore possible that inhibition of S1P signaling may also inhibit atherosclerotic plaque development caused by deposition of oxLDL-IC.

5. Conclusions

Our results provide evidence that advanced vascular disease in MRL/lpr mice with a genetic deletion of *NOS2* or *NOS3* is mediated at least in part by increased levels of TRG and total cholesterol, increased levels of Cer species and S1P, and decreased levels of the anti-inflammatory cytokine IL-10. Moreover, absence of either iNOS or eNOS might lead to the development of nodule-like lesions in the adventitia of aorta. Thus, restoring the eNOS

function may be a fruitful approach in the treatment of SLE patients. Further studies characterizing the sphingolipid pathway and related regulating enzymes in SLE patients are required, as these investigations might determine a target enzyme that will allow us to increase the survival rate, decrease the flare ups of the disease, and consequently stop its deleterious complications.

Supplementary Material

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Highlights

- ▶ Lupus mice lacking *NOS2* or *NOS3* developed lesions in the aortic adventitia.
- ▶ The lesions displayed SK1, macrophage infiltration and oxLDL-IC accumulation.
- ▶ *NOS2* or *NOS3* deletion in lupus mice decreased levels of anti-inflammatory IL-10.
- ▶ Plasma levels of ceramide species and pro-inflammatory S1P were increased.
- ▶ Restoration of *NOS3* function may be a future approach in the treatment of lupus.

Fig 1. Increased levels of triglycerides in MRL/lpr mice with genetic deletion of *NOS2* or *NOS3* Cholestec LDX® system was used to analyze the lipid profile and glucose levels of plasma samples obtained from *NOS2* KO (-/-) (**A**) and *NOS3* KO (-/-) (**B**) MRL/lpr mice and their counterpart MRL/lpr control (+/+) mice. Data expressed as mean $\pm$ SE (n=10 mice for each group); *denotes significantly different from counterpart control group ($P < 0.05$). TC, total cholesterol; TRG, triglycerides; GLU, glucose.

Fig 2. Elevated plasma sphingolipids in MRL/lpr mice with genetic deletion of *NOS2* or *NOS3* HPLC-MS/MS was used to analyze sphingolipid profile of plasma samples obtained from *NOS2* KO (-/-) (**A**) and *NOS3* KO (-/-) (**B**) MRL/lpr mice and their counterpart MRL/lpr control (+/+) mice. Sphingolipids measured were molecular species (C_n-) of ceramide (Cer), and the sphingoid bases: sphingosine (Sph) and dihydrosphingosine (dhSph), and their 1-phosphates (S1P and dhS1P). Data expressed as mean ± SE (n=10 mice for each group); *denotes significantly different from counterpart control group ($P < 0.05$).

Fig 3. Decreased levels of the anti-inflammatory cytokine IL-10 in MRL/lpr mice with genetic deletion of *NOS2* or *NOS3*

Bio-Plex mouse cytokine kit (Bio-Rad) were used to analyze cytokine panels of plasma samples obtained from *NOS2* KO (-/-) and *NOS3* KO (-/-) MRL/lpr mice and their counterpart MRL/lpr control mice. Data expressed as fold of change from control of duplicate values (n=8 mice for each group);*denotes significantly different from counterpart control group ($P < 0.05$).

Fig 4. Increased lipid deposition in the aorta of MRL/lpr mice with genetic deletion of *NOS2* or *NOS3*

Aortas obtained from *NOS2* and *NOS3* KO (-/-) MRL/lpr mice and their counterpart MRL/lpr control (+/+) mice were fixed in 1% paraformaldehyde, stained using Sudan IV (A), and scored on a scale 1.0-4.0 based on the intensity of lipid deposition (B); aortas from control mice with no staining detected in their aortas scored 0.0. Arrows indicate regions of lipid deposition (red stain). Data expressed as mean ± SE (n=10 mice for each group); *denotes significantly different from counterpart control group ($P<0.05$).

Fig 5. Development of nodule-like lesions in the adventitia

Hematoxylin and eosin staining was used for general histological examination of aortas obtained from representative *NOS2* KO (-/-) (**upper panels**) and *NOS3* KO (-/-) (**lower panels**) MRL/lpr mice and their counterpart MRL/lpr control (+/+) mice. *Black arrow heads* denote the outer layer of adventitia; *e*, endothelium; *m*, tunica media; *L*, lesion area.

Fig 6. Lesions characterized by activated endothelial cells, macrophage infiltration, and abundance of extracellular sphingosine kinase 1

Immunohistochemistry was performed on aortas from *NOS2* KO and *NOS3* KO MRL/lpr mice; aorta sections were probed using anti PECAM-1 antibodies to detect activated endothelial cells, the anti mouse macrophage F4/80 antibody, and a polyclonal anti sphingosine kinase 1 (SK1) antibody. *Brown color* denotes positive reaction for the particular probe. White arrow denotes similar area magnified in middle and right panels. Black arrow heads denote the sites of activated endothelial cells.

Fig 7. Increased oxLDL, IgGs, and oxLDL immune complexes in aortas of *NOS2* and *NOS3* KO MRL/lpr mice compared to MRL/lpr controls

Immunohistochemistry were performed on aortas obtained from *NOS2* KO (-/-) (A) and *NOS3* KO (-/-) (B) MRL/lpr mice and their counterpart MRL/lpr control (+/+) mice using Alexa-633-labeled affinity-purified polyclonal anti-oxLDL IgG antibody (red) and Alexa-488 goat anti mouse IgG antibody (Invitrogen) (green). Tissue sections were imaged using a Zeiss LSM confocal fluorescence microscope. Arrows denote sites of oxLDL immune complexes accumulation around macrophages inside nodule-like lesion (yellow); *e*, endothelium; *m*, tunica media.