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Lupus-prone New Zealand Black/New Zealand White F1 mice display endothelial dysfunction and abnormal phenotype and function of endothelial progenitor cells

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Summary

Patients with systemic lupus erythematosus (SLE) have an impairment in phenotype and function of endothelial progenitor cells (EPCs) which is mediated by interferon α (IFN- α). We assessed whether murine lupus models also exhibit vasculogenesis abnormalities and their potential association with endothelial dysfunction. Phenotype and function of EPCs and type I IFN gene signatures in EPC compartments were assessed in female New Zealand Black/New Zealand White F₁ (NZB/W), B6.MRL-Fas^{lpr}/J (B6/*lpr*) and control mice. Thoracic aorta endothelial and smooth muscle function were measured in response to acetylcholine or sodium nitropruside, respectively. NZB/W mice displayed reduced numbers, increased apoptosis and impaired function of EPCs. These abnormalities correlated with significant decreases in endothelium-dependent vasomotor responses and with increased type I IFN signature in EPC compartments. In contrast, B6/*lpr* mice showed improvement in endothelium-dependent and endothelium-independent responses, no abnormalities in EPC phenotype or function and downregulation of type I IFN signatures in EPC compartments. These results indicate that NZB/W mice represent a good model to study the mechanisms leading to endothelial dysfunction and abnormal vasculogenesis in lupus. These results further support the hypothesis that type I IFNs may play an important role in premature vascular damage and, potentially, atherosclerosis development in SLE.

Keywords

endothelial progenitor cells; endothelium; interferon-alpha; systemic lupus erythematosus

Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that affects primarily young women and is highly heterogeneous in its clinical presentation. Importantly, SLE is associated with a striking increase in the risk of premature cardiovascular (CV) complications due to accelerated atherosclerosis,⁽¹⁾ which significantly contributes to morbidity and mortality in this patient population.⁽²⁾ While traditional risk factors may play a role in this increased propensity, they do not seem to fully account for this complication.

Competing interests

The author(s) declare that they have no competing interests

Authors' contributions

ST, DD and JP performed all experiments and analyzed the data. ST drafted the manuscript. MJK conceived and designed the study and helped to draft the manuscript. All authors read and approved the final document.

(3) Indeed, the current proposal is that immune dysregulation characteristic of lupus plays the dominant role in the development of premature atherosclerosis. However, the exact etiology of this increased CV risk remains unclear.

A significant proportion of individuals with SLE have evidence of subclinical vascular disease which may precede the development of atherosclerosis. These subclinical abnormalities include the development of endothelial dysfunction (with preservation of vascular smooth muscle function), (4) arterial stiffness(5) and coronary perfusion abnormalities.(6) Endothelial dysfunction is a state wherein the vasodilatory, anticoagulant or anti-inflammatory properties of the endothelium are impaired and predisposes individuals to the development of atherosclerotic lesions.(7) As a potential mechanism explaining the development of endothelial dysfunction and accelerated atherosclerosis in SLE, our group and others have recently reported that patients with lupus develop a striking imbalance between vascular damage (apoptosis) and repair (vasculogenesis).(4,8,9) Indeed, SLE patients display decreased numbers and abnormal function of the cells involved in adult vasculogenesis: the endothelial progenitor cells (EPCs).(8) Importantly, similar abnormalities in EPCs have clearly been identified by numerous groups in various diseases characterized by an increased CV risk including diabetes mellitus, hypertension, tobacco use, aging and rheumatoid arthritis.(10–14) Furthermore, low EPC levels predict the occurrence of CV complications in the general population.(10) Therefore, the evidence that lupus EPCs are aberrant in their phenotype and function suggests that abnormal vascular repair may play a role in the development of premature vascular damage in this disease. Furthermore, we have recently reported that IFN- α , recently implicated as a major player in SLE disease pathogenesis and severity,(15) promotes abnormal EPC phenotype and function in human lupus.(8)

Ideally, lupus murine models would contribute to understanding the role of abnormal vascular repair and type I IFNs in the development of endothelial dysfunction and atherosclerosis in SLE. Unfortunately, the study of plaque formation in mice is complicated by the fact that this species is, in general, fairly resistant to the development of atherosclerotic lesions.(16) This general assessment also applies to murine lupus. Indeed, in contrast to human SLE, lupus-prone mice appear to be genetically-resistant to overt and florid diet-induced atherosclerosis, unless they are crossed to well-established murine models of atherosclerosis like the apolipoprotein E (ApoE) knock-out mouse or the low density lipoprotein (LDL) receptor knockout mouse.(17–20) In these settings, synergism in plaque development is observed, further indicating that lupus immune dysregulation may accelerate vascular damage in susceptible individuals. While florid atheroma formation has not been reported in murine lupus, a recent report indicates that the New Zealand Black/New Zealand White F₁ lupus model (NZB/W) may be more prone to develop endothelial dysfunction than control mice.(21) This finding suggests that this murine model is prone to developing vascular damage. However, whether abnormal EPC phenotype and function are also present in lupus-prone mice and are correlative to endothelial dysfunction has not been investigated.

The current study has evaluated the association of abnormal EPC phenotype and function with the development of endothelial dysfunction in murine lupus models. The studies were performed in two well-characterized lupus-prone mouse strains: NZB/W, a model where type I IFNs appear to play an important role in lupus development and severity,(22–27) and the B6.MRL-Fas lpr /J (B6/ lpr) model, where type I IFNs appear to be protective of disease severity and development.(28,29)

Materials and methods

Mice and tissue harvesting

Six- to eight- week old NZB/W, BALB/c, B6.MRL-Fas*lpr*/J (B6/*lpr*) and C57BL/6J (C57BL/6) females were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed in a specific pathogen-free barrier facility. Mice were killed at two time points: early disease before proteinuria development (labeled in the text as pre-nephritic or early disease stage), and active disease with proteinuria (labeled in the text as nephritic or active disease stage). Proteinuria was assessed using Uristix 4 (Siemens Healthcare Diagnostics Inc., Tarrytown, NY) following manufacturer's instructions. For NZB/W and control BALB/c, the early and late time points were 20 and 36 weeks, respectively; while for B6/*lpr* and control C57BL/6 this corresponded to 8 and 16 weeks of age, respectively. All animal protocols were reviewed and approved by the University of Michigan's Committee on Use and Care of Animals.

Assessment of vascular function

Post mortem, aortas were excised, cleaned and cut into 2-mm length rings. Endothelium was left intact and aortic rings were mounted in a myograph system (DMT-USA, Inc., Atlanta, GA). Vessels were bathed with warmed, aerated (95% O₂/5% CO₂) physiological salt solution (PSS). Aortic rings were set at 700 mg passive tension, equilibrated for 1 h and washed every 20 min. Prior to performing concentration response curves, the vessels were contracted with PSS containing 100 mmol/L potassium chloride (KPSS). Vessels were washed and contracted again with KPSS. After washing out excess potassium, vessels were contracted with phenylephrine (PE; 10⁻⁶ mol/l) and subsequently treated with acetylcholine (Ach; 10⁻⁷ mol/l) to test the integrity of the endothelium. Cumulative concentrations of PE (10⁻⁹ mol/l to 10⁻⁵ mol/l) were added to the bath to establish a concentration-response curve. The PE contraction was washed out with PSS, and the vessels were recontracted with PE at a concentration calculated to correspond to the EC80 and allowed to reach a stable plateau in the contraction. Ach (10⁻¹⁰ mol/l to 10⁻⁵ mol/l) was added cumulatively to the bath to examine endothelium-dependent relaxation. PE and Ach were washed out of the vascular preparation at the end of the concentration response, and the aortic rings were again recontracted with the PE EC80 and allowed to reach a stable plateau in the contraction. Endothelium-independent relaxation was assessed by the cumulative addition of sodium nitroprusside (SNP; 10⁻¹¹ mol/l to 10⁻⁶ mol/l) to the bath. Ach and SNP relaxation were expressed as a percentage of PE EC80 contraction.(30,31)

Quantification of EPCs

Spleens and long bones were harvested post mortem. Femurs and tibias were washed and epiphyses were excised and flushed with ice-cold MACS buffer (Miltenyi Biotech, Auburn, CA). Bone marrow cells and spleens were filtered through a 40 µm cell strainer (BD Bioscience, Bedford, MA) to obtain a single cell suspension. Bone marrow cells (30–60 × 10⁶) were depleted of lineage-positive cells using a mouse lineage depletion kit (Miltenyi), following the manufacturer's recommendations. Spleen cells were depleted of B and T cells using anti-CD3 (eBioscience San Diego, CA) and anti-CD19 (Biolegend San Diego, CA) monoclonal antibodies (mAbs), respectively, using a similar protocol that used for bone marrow depletion. Mononuclear cells were obtained from cardiac puncture blood by Histopaque 1083 density gradient (Sigma Aldrich, St. Louis, MO) and RBCs were lysed with 172 mM NH₄Cl₂ and 83.9 mM KHCO₃.

Approximately 1 × 10⁶ lineage-depleted cells were incubated with mAbs against murine CD34 and murine VEGF-R2 (flk-1) (eBioscience) to determine the total number of EPCs, as described previously.(32) Similar experiments were performed with blood mononuclear

cells obtained from cardiac puncture. In additional experiments, bone marrow EPCs were further characterized by co-staining lineage-negative cells with mAbs to murine Sca-1 (eBioscience) and CD117 (Biolegend), as described previously.⁽¹⁾ EPC apoptosis was assessed by Annexin-V staining (BD Bioscience) following the manufacturer's recommendations. Fluorescence-activated cell sorter (FACS) was carried out using a FACSCalibur (BD Biosciences), followed by analysis with FlowJo (Treestar, Ashland, OR).

Assessment of EPC differentiation into mature endothelial cells

Bone marrow or spleen EPCs, isolated as described above, were plated onto fibronectin coated plates (BD Biosciences) at a density of 1×10^6 cells/cm² in EGM-2 Bulletkit media (Lonza Allendale, NJ) supplemented with 5% heat inactivated FBS. Media was changed after 72 h in culture, then every 48 h. When indicated, graded concentrations of recombinant murine IFN- α (PBL InterferonSource, Piscataway, NJ) were added to the freshly plated cells for the initial 72 h of culture. On day 7, cells were incubated with FITC-conjugated *Bandeiraea (Griffonia) Simplicifolia* Lectin I (Isolectin B4) (BS-1; Vector Laboratories Burlingame, CA) and 1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (diI)-acetylated LDL (ac-LDL; Biomedical Technologies, Stoughton, MA) for 4 h. Cells were analyzed by fluorescent microscopy using a Leica DMIRB fluorescent inverted microscope (Bannockburn, IL). Images were acquired with an objective magnification of $\times 10$, ($\times 100$ total magnification) using an Olympus DP30BW camera (Olympus Corporation, Tokyo, Japan) and the acquisition software Olympus-BSW (Olympus). Final processing was performed with Adobe Photoshop CS2 (San Jose, CA). Mature endothelial cells (ECs) were designated as those cells co-staining with BS-1 and ac-LDL, and were quantified in five to six random fields per well. Similar experiments were performed using anti-murine CD31 and anti-murine vascular endothelial growth factor receptor-2 (VEGFR2) (both from Biolegend) to confirm endothelial-cell specificity.

RNA isolation, real-time polymerase chain reaction and enzyme-linked immunosorbent assay for quantification of type I IFN-iinducible genes and proteins

RNA was isolated from bone marrow and spleen EPCs using Tripure (Roche Indianapolis, IN) following manufacturer's recommendations. cDNA was synthesized using MMLV RT (Invitrogen Carlsbad, CA) and 1 μ g of RNA using a MyCycler thermocycler (Bio-Rad, Hercules, CA). Six type I-IFN sensitive genes (ISGs) and one house-keeping gene (β -actin) were quantified by real-time polymerase chain reaction (PCR) using SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) as described previously.⁽²⁴⁾ Primer sequences were as follows:

Monocyte chemoattractant protein-1 (MCP-1)

AGGTCCTGTCATGCTTCTG (forward),
GGATCATCTTGCTGGTGAAT (reverse);

Myxovirus (influenza virus) resistance 1 (M \times 1)

GATCCGACTTCACTTCCAGATGG (forward),
CATCTCAGTGGTAGTCAACCC(reverse);

IFN regulatory factor 7 (IRF-7)

TGCTGTTTGGAGACTGGCTAT (forward),
TCCAAGCTCCCGGCTAAGT (reverse);

IFN-inducible protein 10 (IP-10)

ATCATCCCTGCGAGCCTAT (forward),
ATTCTTGCTTCGGCAGTTAC (reverse);

ISG15 ubiquitin-like modifier (ISG15)

CAGAAGCAGACTCCTTAATTC (forward),
AGACCTCATATATGTTGCTGTG (reverse);

IFN- γ

AGCGGCTGACTGAACTCAGATTGTA (forward),
GTCACAGTTTTTCAGCTGTATAGGG (reverse);

 β -Actin

TGGAATCCTGTGGCATCCTGAAAC (forward),
TAAAACGCAGCTCAGTAACAGTCCG (reverse).

Real time PCR was carried out using an ABI PRISM 7900HT (Applied Biosystems). Transcripts were normalized using the β -actin gene. Expression of normalized genes was compared using a two-tailed Student's *t*-test. Data is shown as the relative fold change over control using $2^{-\Delta\Delta C_t}$ method as reported previously.(34)

To confirm findings at the protein level, an enzyme-linked immunosorbent assay (ELISA) was performed to quantify circulating MCP-1 (Biolegend) and IP-10 (RayBiotech Norcross GA) following manufacturer's recommendations.

Statistical Analysis

Unless otherwise specified, results represent mean \pm standard error of the mean (SEM) and all statistics were calculated using Student's *t*-test with GraphPad prism 5 (GraphPad Software, La Jolla, CA). For measurements of endothelial function, median effective concentration (EC₈₀) for agent-induced relaxation in aortic rings was calculated by non-linear regression analysis (GraphPad Prism, San Diego, CA). Comparisons in the dose response to EC₈₀ contractions were analyzed using a Student's *t*-test for each concentration of agonist.

Results**NZBW mice have impaired endothelium-dependent vasorelaxation**

Endothelium-dependent and endothelium-independent responses were similar between NZB/W and BALB/c mice at 20 weeks of age (not shown). However, similar to what has been reported in human SLE(4) and a previous study in these mice,(21) 36-week-old NZB/W mice display impaired endothelial-dependent vasorelaxation of pre-contracted thoracic aortas, as assessed by blunted responses to Ach when compared with age-matched BALB/c mice (Figure 1A). Endothelium-independent vasorelaxation in the NZB/W mice, assessed by response of pre-contracted thoracic aortas to SNP, was not impaired at the same time-point (Figure 1B).

In contrast, at both early and active disease time-points, B6/*lpr* mice displayed significant increases in endothelial-dependent and endothelial-independent vasorelaxation when compared to control C57BL/6 mice (Figure 1C and D and not shown). These improvements were seen even in those mice with well-established renal disease, as assessed by proteinuria

development. When examined during the active disease time-point, NZB/W and B6/*lpr* mice did not differ in their degree of proteinuria (625 ± 460.8 and 520 ± 372 mg/dl, respectively, $p =$ not significant). These results indicate that nephritic-stage NZB/W mice, but not B6/*lpr* mice with similar degrees of lupus disease activity develop significant endothelial dysfunction with preservation of vascular smooth muscle function.

NZB/NZW mice display reduced numbers and increased apoptosis of EPCs

At 20 weeks of age, EPCs were decreased in the bone marrow but increased in the spleen of NZB/W mice, when compared to BALB/c mice ($p < 0.01$). The depletion was enhanced by 36 weeks of age, when NZB/W EPCs were significantly decreased in both the bone marrow and spleen compartments ($p < 0.03$; Figure 2A). In contrast, B6/*lpr* EPC numbers did not significantly differ from control C57BL/6 mice when quantified in bone marrow and spleen at pre-nephritic and nephritic stages (Figure 2B). Circulating EPC numbers did not significantly differ between NZB/W or B6/*lpr* mice and control mice at either stage of the disease (not shown).

The decrease in bone-marrow EPCs in NZB/W mice at 20 and 36 weeks of age was associated with a significant increase in EPC apoptosis in this compartment, when compared with BALB/c mice ($p = 0.004$ and $p = 0.005$, respectively, Figure 2C). At 20 weeks of age, apoptotic EPCs also were increased in NZB/W spleen ($p = 0.05$), while no evidence of increased EPC death was detected in the blood of these mice. In contrast, B6/*lpr* mice showed no increases in EPC apoptosis during early or active disease in any of the compartments analyzed (Figure 2D). Similar results were observed when EPCs were quantified as lineage negative/Sca-1⁺/CD117⁺ cells (not shown).

EPCs from NZB/W mice are impaired in their capacity to differentiate into mature ECs

The functional ability of murine EPCs was assessed by quantifying their capacity to differentiate into mature endothelial-like cells under *in vitro* proangiogenic stimulation. After plating equal numbers of cells, NZB/W bone marrow EPCs displayed a decreased capacity to differentiate into cells that express mature EC markers, both at 20 weeks and at 36 weeks (Figure 3), when compared to BALB/c mice. Similar to what we previously reported in human SLE,⁽⁸⁾ 7-day NZB/W cultures typically displayed scattered or clustered ECs but no EC monolayer formation, while BALB/c EPCs clearly differentiated into mature ECs with significant ac-LDL uptake and co-expression of BS-1. These findings were confirmed when other mature EC markers, CD31 and VEGFR-2 were examined (not shown). In contrast, B6/*lpr* bone marrow EPCs displayed no significant impairment in their capacity to differentiate into mature ECs, when compared with control C57BL/6 mice (not shown).

Type I IFN signatures are increased in NZB/W EPC compartments

To assess whether the increased apoptosis and decreased numbers of EPCs in NZB/W mice could be associated with their enhanced exposure or increased sensitivity to type I IFNs, the level of expression of six type I-IFN regulated genes was examined in the bone marrow and spleen EPCs of lupus-prone and control mice. As shown in Figure 4, 20- and 36-week old NZB/W mice display significant increases in expression of type I IFN inducible genes in both compartments, when compared with BALB/c mice. In contrast, B6/*lpr* mice display downregulation of various IFN-responsive genes in those same compartments, both at 8 and 16 weeks of age, when compared with control C57BL/6 mice (Figure 5). Increased systemic exposure to type I IFNs in NZB/W mice was confirmed at the protein level, as elevated expression of both circulating MCP-1 (Figure 6A, $p < 0.0081$) and IP-10 (Figure 6B, $p < 0.0113$) was detected in these mice, but not in B6/*lpr* mice, when compared with controls (Figure 6). These results indicate that NZB/W EPCs are exposed *in vivo* to increased levels

of type I IFNs and/or are more sensitive to the effects of these molecules when compared to control mice.

IFN- α induces cytotoxicity of murine EPCs

As type I IFNs are toxic to human EPCs and induce significant apoptosis of these cells,(8) we examined whether murine EPCs showed a similar sensitivity. Murine bone marrow (Figure 7) and spleen (not shown) EPCs from control BALB/c mice showed loss in their capacity to differentiate into mature ECs and increased death after *in vitro* exposure to recombinant IFN- α , recapitulating the phenotype observed in NZB/W EPCs (Figure 7).

Discussion

The results from this study suggest that the NZB/W murine model recapitulates the abnormal findings in EPC phenotype and function reported recently in human SLE.(8,9,35,36) Indeed, these mice have decreased numbers of EPCs in various compartments, increased bone marrow and spleen EPC apoptosis and decreased capacity of these cells to differentiate into mature ECs. Decreases in EPCs in NZB/W mice were already apparent in the bone marrow before overt clinical disease developed. The reasons for the initial increases in EPC numbers in the spleen of these mice before overt disease develops are unclear but may reflect aberrant homing of bone marrow EPCs or a differential response to ongoing vascular damage. Furthermore, even if EPCs were increased in the spleens of the mice at early disease time-points, a significantly higher number of these cells were apoptotic. The significant decreases in bone marrow and splenic EPCs that ensues as disease progresses could indicate exhaustion of the EPC pool in NZB/W mice in the context of accelerated apoptosis, similar to previously reported murine models of atherosclerosis.(37) The present study cannot assess whether the impairment in the differentiation of EPCs into a mature endothelium is solely secondary to accelerated EPC apoptosis or also results from functional abnormalities with regards to differentiation and this should be further explored in future studies.

The results from this study also support previous findings that EPCs play a key role in vascular health,(10,11,38,39) as the development of endothelial dysfunction in the NZB/W mice correlates with decreased EPC numbers and enhanced EPC death. While we did not evaluate atherosclerosis as an outcome in these mice, given the relative resistance of most murine lupus strains to the development of this complication, NZB/W mice did exhibit impaired endothelium-dependent vasorelaxation with preserved endothelium-independent vascular smooth muscle relaxation, similar to what has been reported observed in human SLE.(4) This was observed without exposing the mice to a Western diet which could have further enhanced these abnormalities.

Endothelial dysfunction is considered to be one of the earliest events in the development of atheroma and a promoter of disease progression and trigger for CV events.(7,40,41) Abnormal vasomotor responses of the endothelium may be attributable to possible alterations in the underlying physical properties of the vessel wall and/or the vascular tone in the setting of chronic inflammation, as proposed to occur in various human systemic autoimmune diseases.(5) While renal dysfunction has been associated with the development of endothelial dysfunction and abnormal EPC phenotype and function(42–45) it is unlikely that lupus nephritis significantly contributes to these abnormalities in the NZB/W mice, as B6/*lpr* mice with similar degrees of renal dysfunction and established SLE features did not display these vascular abnormalities. Further, while the B6/*lpr* phenotype can enhance the development of atherosclerosis when compounded by ApoE deficiency,(18) our results indicate that these mice are not impaired in their vasoregulatory function and display normal markers of vasculogenesis, unlike what has been described in human SLE.(4,8) The lack of

endothelial and EPC dysfunction in B6/*lpr* mice in the active disease stage also indicates that the abnormalities observed in NZB/W mice are not primarily explained by lupus disease activity. This is consistent with the human model where, even lupus patients with no evidence of disease activity, display significant abnormalities in EPC phenotype and function as well as striking endothelial dysfunction.(4,8)

The mechanisms that lead to abnormal EPC phenotype and function in the NZB/W mice but not in the B6/*lpr* mice remain to be defined and may be multifactorial, including an enhanced resistance to apoptosis in the latter. However, B6/*lpr* mice are also known to have increased spontaneous apoptosis of peripheral blood mononuclear cells through nitric oxide(46) and to generate increased levels of nucleosomes.(47) This suggests that other apoptotic pathways are likely preserved and functional in these mice, as supported by previous studies.(48) Genetic differences, autoantibodies, immune complexes and other immunological abnormalities could certainly play differential roles in vascular damage in both models and need to be further examined. The differences in endothelium-dependent vasorelaxation observed between the NZB/W and B5/*lpr* mice may also be related to differences in other factors, including inducible and endothelial nitric oxide synthase expression and potential variabilities in autoantibody profiles. While these possibilities were not assessed directly in this study, future studies should test the effects of non-selective and iNOS-selective inhibitors on endothelial function in lupus-prone mice.(49,50)

Type I IFNs have been proposed to play an important role in disease development and progression in NZB/W mice and derived congenic strains,(23) whereas the B6/*lpr* mice do not appear to depend on type I IFNs for their disease progression and may actually be protected by these cytokines.(28) Our group has shown previously that, upon exposure to recombinant IFN- α , human bone marrow and circulating EPCs undergo striking increases in apoptosis and a decreased capacity to differentiate into mature ECs upon proangiogenic stimulation.(8) Indeed, the abnormalities observed in human control EPCs upon exposure to IFN- α recapitulate the phenotype observed in human SLE EPCs.(8) Furthermore, neutralization of type I IFNs *in vitro* results in restoration of the normal capacity of human lupus EPCs to differentiate into mature ECs.(8) While the precise mechanisms leading to aberrant EPC phenotype and function and endothelial dysfunction in the NZB/W mice remain to be determined and may be multifactorial, an attractive hypothesis is that, similar to human lupus, type I IFNs are main players in the induction of the observed EPC dysfunction and vasomotor abnormalities. Indeed, the presence of endothelial dysfunction was associated with an increased type I IFN signature in the NZB/W EPC compartments, while decreases in these signatures were observed in B6/*lpr* mice. This is consistent with a recent report where splenic mononuclear cells from NZB/W, but not B6/*lpr*, lupus-prone mice at the pre-autoimmune stage were found to have increased expression of many known type I IFN-regulated genes.(26) Further, control bone marrow murine EPCs lose the ability to differentiate into a mature endothelium when exposed to IFN- α , and acquire a similar phenotype to what we have now observed in NZB/W mice and what has been described previously in human lupus EPCs.(8) IFN- α also has been shown to decrease colony formation of hematopoietic progenitor cells,(51) further supporting its potential implication in EPC decreases in SLE. Indeed, similar to what we now report in NZB/W EPCs, increased apoptosis of bone marrow

CD34+ cells has been reported in human SLE, but the precise etiology was not characterized.(52) It is possible that the effect of type I IFNs on NZB/W EPCs could be related to not only the exposure to increased levels of these cytokines, but also to increased sensitivity to type I IFN effects, as recently proposed in human SLE.(53) Among various antiangiogenic effects, type I IFNs may promote enhanced *in situ* bone marrow EPC apoptosis, with a subsequent decrease in total numbers of these cells. This could lead to

decreased mobilization and homing into damaged vascular structures, therefore promoting endothelial dysfunction. The lack of apparent decrease in circulating EPCs despite decreased levels in the bone marrow and spleen requires further investigation but may be related to different kinetics with regards to mobilization and homing between murine and human EPCs. It is also possible that the effects of type I IFNs are enhanced in specific organs such as bone marrow or spleen. Indeed, a recent report indicates expansion of bone marrow IFN- α producing dendritic cells in NZB mice which may potentially indicate that the bone marrow niche could be exposed to even higher levels of this cytokine than other peripheral compartments.(22) This hypothesis needs to be investigated in future studies.

In conclusion, the NZB/W lupus-prone mice is characterized by the development of endothelial dysfunction and abnormal phenotype and function of EPCs, which is associated with *in vivo* exposure of these cells to increased levels of and/or higher sensitivity to type I IFNs. NZB/W mice therefore represent a good model to study the mechanisms leading to endothelial dysfunction and abnormal vasculogenesis in lupus. Future studies specifically examining the role of type I IFNs in premature cardiovascular disease and endothelial dysfunction in murine and human SLE are warranted.

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Abbreviations used

Ach	acetylcholine
ac-LDL	1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (diI)-acetylated LDL
ApoE	apolipoprotein E
BS-1	<i>Bandeiraea (Griffonia) Simplicifolia</i> Lectin I (Isolectin B4)
C57BL/6	C57BL/6J
CV	cardiovascular CV
EPCs	endothelial progenitor cells
IFN-α	interferon α
ISGs	type I-IFN sensitive genes
KPSS	PSS containing 100 mmol/L potassium chloride
LDL	low density lipoprotein
PE	phenylephrine
PSS	physiological salt solution
B6/<i>lpr</i>	B6.MRL-Fas ^{<i>lpr</i>} /J
NZB/W	New Zealand Black/New Zealand White F ₁
SLE	systemic lupus erythematosus
SNP	sodium nitroprusside

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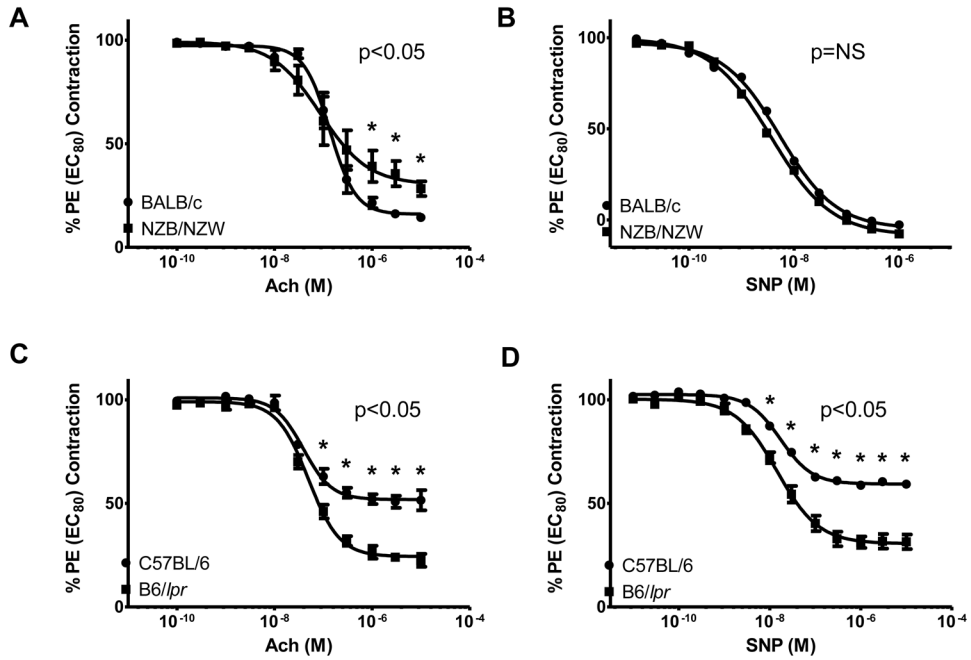


Figure 1. Decreased endothelial-mediated vasorelaxation in NZB/W mice

Results assess acetylcholine (ACh)-mediated endothelium-dependent relaxation and sodium nitroprusside (SNP)-mediated endothelium-independent relaxation in aortic rings from (A), (B) 36-week-old NZB/W and BALB/c mice and (C), (D) 16-week-old B6/lpr and C57BL/6 mice. NZB/W mice have impaired ACh-mediated responses, while B6/lpr mice showed enhanced ACh-mediated and SNP-mediated responses. Results are mean \pm SEM % phenylephrine (PE) EC₈₀ contraction (n= 5–8 mice/group). * $p < 0.05$; NS=not significant.

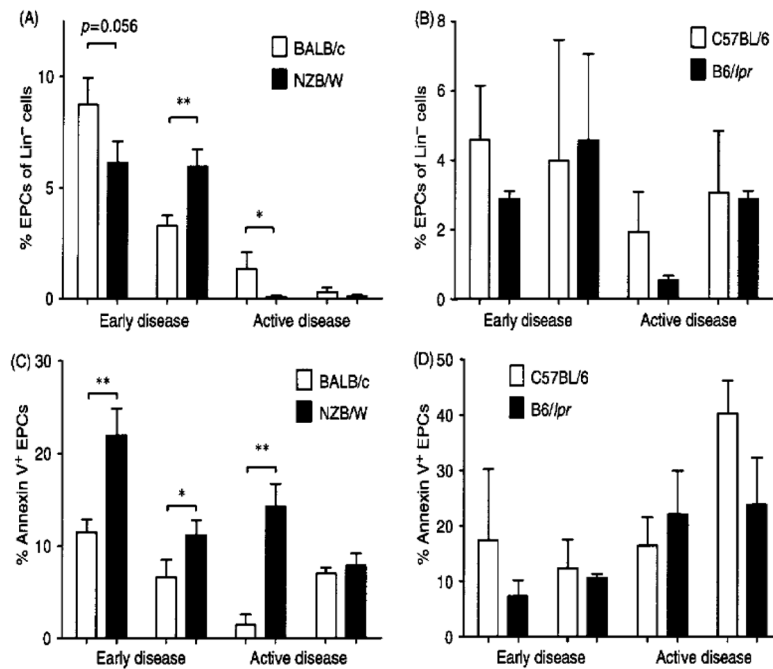


Figure 2. EPCs are decreased in NZB/W mice and show higher levels of apoptosis

EPCs were quantified in the bone marrow and spleen in pre-nephritic (early disease) and nephritic (active disease) NZB/W (A) and B6/lpr mice (B), as well as control mice. A significantly higher number of NZB/W EPCs were apoptotic, as determined by Annexin V expression at (C) pre-nephritic (early) time-point, both in the bone marrow and the spleen and at the nephritic (late) time point in the bone marrow. The B6/lpr mice showed no significant increases in EPC apoptosis at (D) the early or active disease time points. Results are mean \pm SEM % lineage-negative EPCs (n= 5–8 mice/group). * p <0.05; ** p <0.01.

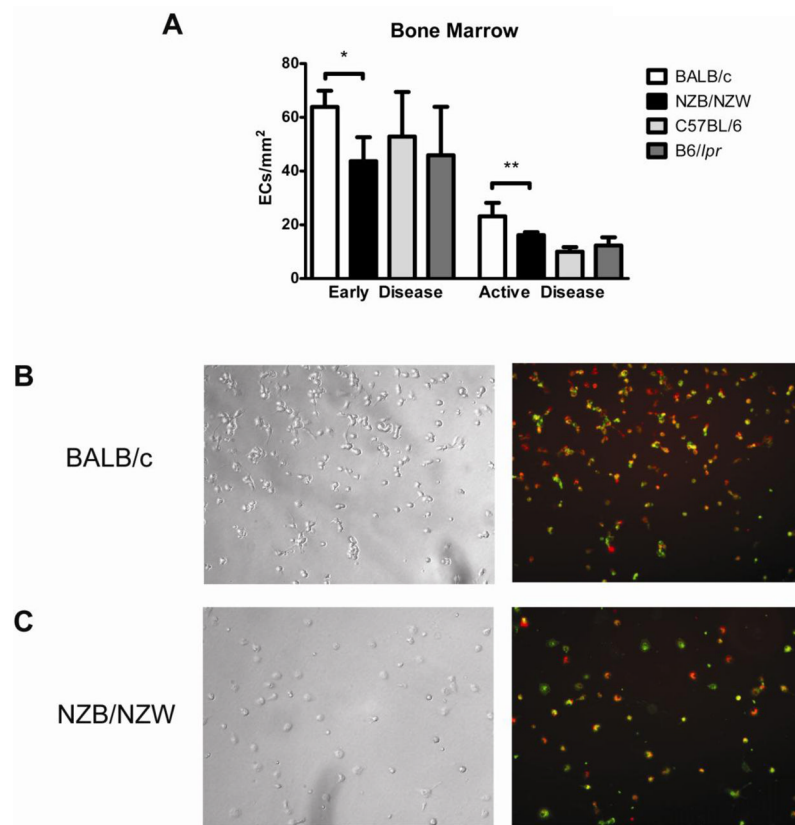


Figure 3. NZB/W EPCs exhibit impaired capacity to differentiate into mature ECs
 Bone marrow-derived EPCs were cultured under proangiogenic conditions and incubated at different time-points during culture with DiI-ac-LDL and BS-1-FITC. Mature endothelial cells were identified by co-staining of BS-1 and ac-LDL. (A) Bar graphs represent the number of mature endothelial cells per centimeter at day 7, when comparing NZB/W and B6/lpr bone marrow-derived EPCs with control EPCs, at early and active disease time-points. Results are mean \pm SEM of 3 or 4 independent experiments; * $p < 0.05$, ** $p < 0.01$. (B), (C) Results are representative images obtained from BALB/c (B) and NZB/W (C) EPCs cultured under proangiogenic conditions for 7 days. Left panels show bright field images and right panels show images obtained by fluorescent microscopy. NZB/W EPCs show decreased ability to differentiate into endothelial cells, when compared to BALB/c mice. DiI-Ac-LDL is red while BS-1 lectin is green. Total magnification is $\times 100$.

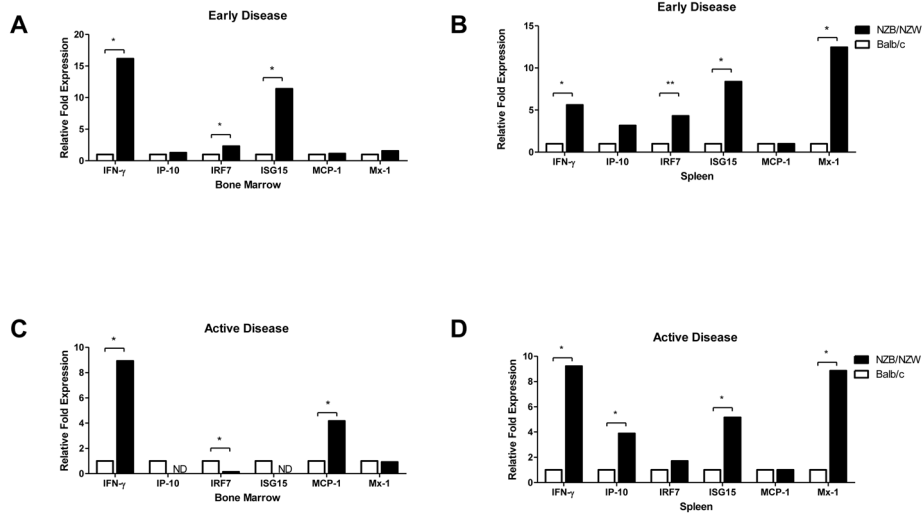


Figure 4. Type I IFN-inducible genes are increased in NZB/W EPC compartments
 Results are displayed as relative fold change of type I IFN-inducible genes over control BALB/c mice ($n=5$ mice/group) in bone marrow and spleen EPC compartments during (A), (B) early and (C), (D) active disease stage. Transcripts were normalized to β -actin; * $p<0.05$; ** $p<0.01$; ND = not detected.

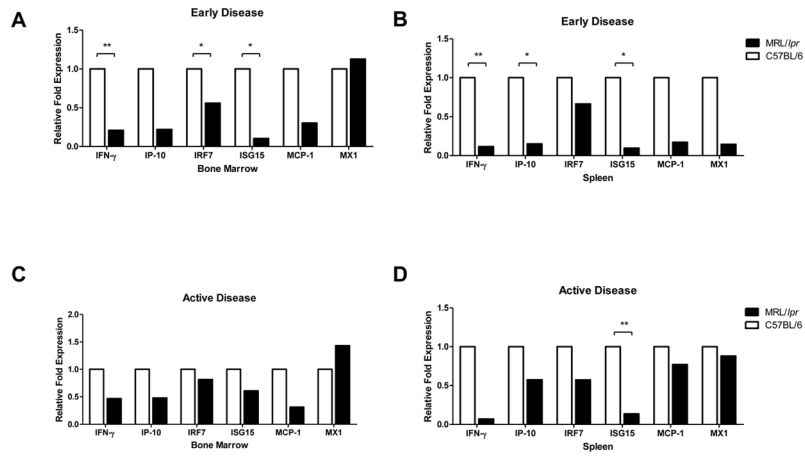


Figure 5. B6/lpr EPCs display decreased expression of type I IFN-inducible genes
 Results are displayed as relative fold change of type I IFN-inducible genes over control C57BL/6 mice ($n=3$ mice/group) in bone marrow and spleen EPC compartments during (A), (B) early and (C), (D) active disease stage. Transcripts were normalized to β -actin; * $p<0.05$; ** $p<0.01$.

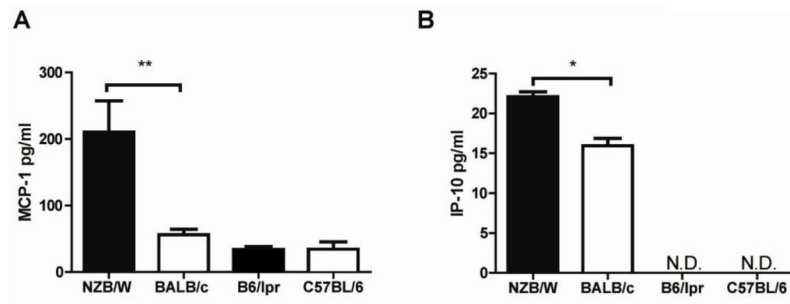


Figure 6. Increased circulating levels of type I-IFN inducible proteins in NZB/W mice
Levels of the type I IFN-sensitive chemokines MCP-1 and IP-10 were quantified in the plasma of lupus-prone mice with active disease. Results represent mean \pm SEM ($n = 3-4$ mice per group). * $p < 0.05$.

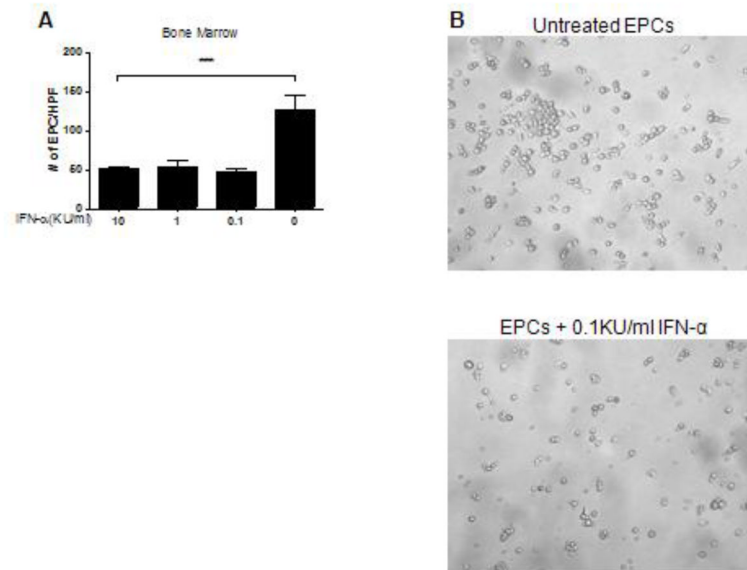


Figure 7. IFN- α is toxic to murine EPCs

Bone marrow EPCs from BALB/c mice were cultured in proangiogenic media in the presence or absence of graded concentrations of recombinant IFN- α for 3 days. Numbers of mature ECs were quantified at 7 days in culture. Exposure to IFN- α resulted in a significant loss of EPC ability to differentiate into mature endothelial cells. (A) Bar graph displays 1 representative experiment and represent mean \pm SEM of 5 high-power fields; $n=3$, *** $p<0.001$. (B) Representative bright field images of untreated BALB/c bone marrow EPCs (top) and BALB/c bone marrow EPCs treated with 0.1KU/ml IFN- α . IFN- α -treated EPCs from BALB/c controls acquired the phenotype of lupus cells and were unable to form an endothelial cell monolayer.