Type I Interferons and the Development of Impaired Vascular Function and Repair in Human and Murine Lupus

by

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Ach	acetylcholine
ac-LDL	acetylated LDL
AdControl	empty adenovirus
AdIFN-α	adenovirus expressing murine IFN-α
ALPS	autoimmune lymphoproliferative syndrome
ANA	antinuclear antibody
ANCA	anti-neutrophil cytoplasmic antibody
APC	antigen presenting cell
APOE	apolipoprotein E
ApoE$^{-/-}$	B6.129P2-*Apoe*^{tm1Unc}/J
APRIL	a proliferation inducing ligand
B6/*lpr*	B6.MRL-Fas^{lpr}/J
bFGF	basic FGF
BLyS	B Lymphocyte Stimulator
BS-1	*Bandeiraea (Griffonia) Simplicifolia* Lectin I (Isolectin B4)
C57BL/6	C57BL/6J
CAC	myeloid circulating angiogenic cell
CV	cardiovascular
CVD	cardiovascular disease
DC	dendritic cells
dil	1'-dioctadecyl-3, 3, 3', 3'-tetramethylindocarbocyanine perchlorate
DMSO	dimethyl sulfoxide
EBM2	endothelial basal media 2
EC	endothelial cell
EGF	epidermal growth factor
eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cell
EPO	erythropoietin
FACS	fluorescent activated cell sorter
FasL	Fas Ligand
FBS	fetal bovine serum
FGF	fibroblast growth factor
G-CSF	granulocyte colony-stimulating factor
gld	generalized lymphoproliferative disease
GM-CSF	granulocyte-macrophage colony-stimulating factor
GWAS	genome wide association studies
HDL	high density lipoprotein
HGF	hepatocyte growth factor
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA

HuR	Hu antigen R
IC	immune complex
IFI	IFN-induced protein
IFIT	IFI with tetratricopeptide repeats
IFN	interferon
IFN-α	interferon α
IFNAR	IFN-α receptor
IGF-1	insulin-like growth factor-1
IL	interleukin
IL-1R1	IL-1 receptor 1
IL-1RN	IL-1R antagonist
INZM	IFNAR-/- NZM
IP-10	IFN-γ-induced protein-10
IPA	Ingenuity Pathway Analysis
ISGs	type I-IFN sensitive genes
ISG15	ubiquitin-like modifier
ISGF3	interferon stimulatory gene factor 3
IRF	interferon regulatory factor
ISRE	IFN-stimulated response element
JAK	Janus kinase
KPSS	PSS containing 100 mmol/L potassium chloride
LDG	low-density granulocyte
LDL	low density lipoprotein;
LDLR	low density lipoprotein receptor
lpr	lymphoproliferative
MAP	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein 1
mDC	myeloid DC
MHC	major histocompatibilty complex
Mφ	macrophage
MRL	Murphy's recombinant large
MX-1	myxovirus resistance -1
NK	natural killer
NSAID	nonsteroidal anti-inflammatory drugs
NZB/W	New Zealand Black/ New Zealand White F1 hybrid
NZM	New Zealand Mixed
OAS-1	2', 5'-oligoadenylate synthetase
oxLDL	oxidized LDL
PBMC	peripheral blood mononuclear cell
pDC	plasmacytoid DC
PDGF	platelet derived growth factor
PE	phenylephrine
PI3K	phosphatidylinositol 3-kinases
PIAS	protein inhibitor of activated STAT
piHDL	pro-inflammatory HDL
PRKR	protein kinase RNA-activated

PSS	physiological salt solution
RA	rheumatoid arthritis
RNP	ribonucleoprotein
SAM	significance microarray analysis
SDF-1	stromal cell-derived factor-1
SLE	systemic lupus erythematosus
SLEDI	SLE Disease Activity Index
SNP	sodium nitroprusside
SOCS	suppressor of cytokine signaling
SP	specificity protein
SPC	smooth muscle progenitor cell
SRA	scavenger receptor A
STAT	signal transducer and activator of transcription
TLR	Toll-like receptor
TMB	3, 3', 5, 5'-tetramethylbenzidine
TNF-α	tumor necrosis factor alpha
TRAIL	TNF-related apoptosis-inducing ligand
UEA-1	*Ulex europaeus agglutinin-1*
VEGFR	VEGF receptor
VEGF-A	vascular endothelial growth factor A
vWF	von Willebrand factor
vLDL	very low density lipoprotein

Abstract

Patients with systemic lupus erythematosus (SLE), an autoimmune disease of unclear etiology, have a greatly increased risk of developing premature atherosclerotic cardiovascular disease (CVD), independent of traditional vascular risk factors. While CVD is a major cause of mortality and morbidity in these patients, the exact mechanisms behind this increased vascular risk are unknown. SLE patients develop a profound imbalance of vascular damage and repair which is mediated by interferon (IFN)-α and, potentially, by other type I IFNs. This dissertation elucidates the molecular mechanisms by which IFN-α inhibits vascular repair mediated by endothelial progenitor cells (EPCs), and demonstrates that type I IFNs play a key role in the development of endothelial dysfunction in lupus-prone and non-lupus-prone mice *in vivo*. Using a gene expression array approach, we identified that IFN-α promotes antiangiogenic responses in EPCs through repression of IL-1 signaling and of vascular endothelial growth factor. These effects are mediated through the JAK/STAT pathway. These transcriptional abnormalities are operational *in vivo*, as evidenced by vascular rarefaction observed in human lupus tissues. In murine studies, we identified that the lupus prone mouse strain New Zealand Black/New Zealand White F1 displays an abnormal vascular phenotype similar to human SLE. In the closely related NZM 2328 strain, we demonstrate that type I IFNs induce endothelial dysfunction, decreased neoangiogenesis and enhanced platelet activation. These deleterious effects on the vasculature mediated by type I IFNs were not secondary to lupus disease activity, gender or lupus prone phenotype. Furthermore, type I IFNs are also involved in atherosclerosis severity and acceleration of thrombosis in Apolipoprotein-E deficient mice, an atherosclerosis-prone mouse strain. Our results strongly suggest that type I IFNs

play a vital role in the induction of endothelial dysfunction and aberrant vascular repair in lupus through the inhibition of important proangiogenic molecules. These results also indicate that type I IFNs may play a deleterious role in the vasculature in “idiopathic” atherosclerosis not related to systemic autoimmune diseases. These observations could also have important implications in the identification of therapeutic targets to prevent CVD and in the understanding on how microbial infections may trigger vascular damage.

Chapter 1
Introduction

Systemic Lupus Erythematosus:

Definition and demographics:

Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder of unclear etiology, with clear genetic and environmental associations, that can affect almost any organ[1,2]. SLE afflicts predominantly women of childbearing age[3], with an estimated female:male ratio of 9:1[2]. SLE is characterized by acute disease flares, which are initiated by poorly understood triggers. These acute flares are classically followed by periods of relative disease inactivity. The exact prevalence of SLE is unknown, but estimates range from 14.6-100 per 100,000[4,5]. It is thought that 1.5 million Americans suffer from SLE, and approximately 16,000 new cases are diagnosed annually[6]. The prevalence of lupus is higher in certain ethnic groups and African-American and Hispanics tend to develop more severe disease than Caucasians[7]. More than 30 years ago, a bimodal mortality curve was described in patients in SLE. The first peak occurs within 5 years of diagnosis due to acute complications from lupus and/or infections. The second peak occurs at least 10-15 years after diagnosis and is primarily caused by the development of cardiovascular disease (CVD)[8].

Currently, there is no cure for SLE; however, with improvements in immunomodulatory treatments and better control of some of the chronic complications, the 10 year survival for these patients has significantly improved and is now 80-90% in the US[1]. The observed improvements in survival have led to increased accrual of chronic complications, which are the result of prolonged immune dysregulation and drug-induced effects. Indeed, as lupus patients live longer, accelerated development of CVD, predominantly due to premature

atherosclerosis has become a more prominent cause of both morbidity and mortality[9-11].

Clinical Manifestations and Treatment:

SLE is one of the most heterogeneous diseases with regards to clinical manifestations[1]. Almost any organ can be affected in SLE, with the most common targets being the kidney, skin, and joints. A significant proportion of patients develop renal damage, primarily manifested as glomerulonephritis, which can progress to end-stage renal disease. The mechanisms that lead to progression of renal damage in SLE remain poorly characterized. Other organs commonly affected are the central nervous system, the lungs and the heart. The most conserved laboratory finding in SLE is the presence of self-reactive antibodies[1], with the most prevalent being autoantibodies that target nucleic acids (ANA). These ANAs target DNA (both single- stranded and double-stranded), RNA, and nucleic acid-protein complexes (Ro, La, Smith, RNP)[1].

Treatment of SLE relies primarily on the use of immunomodulatory and immunosuppressive drugs. Commonly used are corticosteroids, nonsteroidal anti-inflammatory drugs (NSAIDs), and antimalarials such as hydroxychloroquine[12]. Various immunosuppressive drugs, including azathioprine, mycophenolate mofetil and cyclophosphamide are also commonly used[12]. New biologic agents are currently being tested in SLE and a drug targeting the molecule B lymphocyte stimulator (BLyS) was just approved by the Food and Drug Administration for its specific use in SLE. While significantly effective in controlling disease in most patients, many of the currently used drugs have significant toxicity and can contribute to the accumulated damage and mortality seen in SLE[1,2,7,13].

Cardiovascular manifestations in SLE patients:

As mentioned above, atherosclerotic CVD is more prevalent in SLE than in other diseases and represents one of the most common causes of mortality in this disease[9,14-16]. Further, a significant proportion of individuals with SLE (even those with low disease activity)[17] have evidence of subclinical vascular damage

which may precede the development of atherosclerosis. These subclinical abnormalities include the development of endothelial dysfunction (with preservation of vascular smooth muscle function)[17], increased carotid intima media thickness[18], arterial stiffness[19], and coronary perfusion abnormalities[20]. Both carotid plaque and aortic atherosclerosis are highly prevalent in lupus patients[11,21].

Lupus patients also have an increased risk to develop thrombotic events[22,23]. Predisposing factors include smoking, the presence of autoantibodies against phospholipids and beta2-glycoprotein-I, genetic factors, and medications[24]. While the precise mechanisms predisposing lupus patients to increased thrombosis are still unclear, this procoagulant state may increase the development of acute coronary syndromes in this population.

Pathogenesis of SLE:

While the exact cause of SLE is unknown, there is sufficient evidence to suggest that this disease arises from an unchecked immune response directed against self. When compared to healthy individuals, multiple abnormalities are found at all levels of the immune system of SLE patients. Individuals who develop SLE have inherited or acquired defects in both B and T cell tolerance[25-27]. The development of somatically mutated IgG autoantibodies against chromatin components is a central feature in SLE[2,28,29]. Many detrimental T and B cell abnormalities have been reported[28-34] and are beyond the scope of this work. To generalize, autoantibodies produced by aberrant B cells can target cells through direct recognition[35] or the deposition of immune complexes (ICs)[36], leading to the activation of the complement system and recruitment of other immune cells such T cells, monocytes, macrophages (Mφ) and neutrophils[37]. Presence of autoreactive T cells and activated and dysfunctional Mφ and monocytes have been well described in SLE[38-46]. While significant effort has focused on abnormalities in adaptive immunity, more recent evidence indicates that alterations in innate immunity may also be important in disease development. The alterations in innate immunity may also be crucial in

perpetuation of aberrant immune responses, activation of adaptive immunity and organ damage in SLE. In addition to the defects in Mφ and monocytes, SLE patients have an abnormal population of low density granulocytes (LDGs)[47]. These LDGs are proinflammatory and have been shown to kill endothelial cells[47]. Furthermore, abnormalities in various other components of the innate immune system, including dendritic cells (DC, both myeloid and plasmacytoid), and natural killer (NK) cells have been described[42,48-56]

Many cytokines have been implicated in SLE disease pathogenesis. Cytokines involved in regulating B cell development and maturation including BLyS, a proliferation-inducing ligand (APRIL) and IL-10 have been shown to play a role in disease pathogenesis[57-59]. Additionally, T cell cytokines such as IFNγ and IL-12 are also increased in SLE and may play a pathogenic role[58,60,61]. Recent work has implicated type I IFNs, particularly IFN-α, as major players in SLE pathogenesis[62-64].

Type I Interferons and SLE:

SLE patients have elevated levels of type I IFNs in the circulation[65-67], which correlate with disease flares and disease activity[68]. Additionally, in gene expression array studies, many of the most overexpressed genes in peripheral blood mononuclear cells (PBMCs) are IFN stimulated genes (ISG)[62,63]. Further, genome wide association studies (GWAS) demonstrate that many of the risk variants associated with SLE are related to type I IFN signaling, including *IFN regulatory factor* (IRF*)-7*[69], *IRF-5*[70], and *signal transducer and activator of transcription* (STAT*)4*[71]. Additionally, patients with diseases that require treatment with exogenous recombinant type I IFNs can develop lupus-like symptoms[36,72,73]. Several lupus-prone mouse strains (see below) show improvement in lupus manifestations when type I IFN signaling is abrogated[74-76], and administration of exogenous type I IFNs accelerates lupus progression[77,78]. The potentially vital role for type I IFNs in SLE pathogenesis is currently being tested in a number of clinical trials[12].

While IFN-α (which has < 15 subtypes) is the most commonly type I IFN associated with SLE, the type I IFN family also includes IFN-β, ε, κ, ω, δ, and τ[79]. The type II IFN family consists solely of IFN-γ[64], and the relatively new type III IFN family contains IFN-λ(three family members)[80]. Each IFN family signals through a separate receptor[62-64].

The type I IFN receptor is composed of both IFN-α receptor (IFNAR) 1 and IFNAR2. The type I IFN receptor signals primarily via the Janus kinase (JAK)/STAT pathway[64]. Upon receptor dimerization, TYK2 and JAK1 associate with IFNAR1 and IFNAR2, respectively; this induces autophosphorylation and activation of the JAKs. The phosphorylated receptors serve as docking sites for STAT molecules which are then phosphorylated and activated themselves. After activation, the STAT molecules dimerize and migrate to the nucleus, where they initiate gene transcription. STAT1 and STAT2 are the primary STATs activated by type I IFNs. STAT1 and 2 form a heterodimer and complex with IRF-9 to form a multimeric transcription factor, interferon stimulatory gene factor 3 (ISGF3). In the nucleus, ISGF3 recognizes genes containing an IFN-stimulated response element (ISRE)[81]. The genes activated by type I IFNs include many of the canonical antiviral genes[64]. Other STAT molecules have also been shown to be activated by type I IFN signaling, including STAT4[82] and STAT6[83], and could be important in the regulation of genes not containing a ISRE sequence. Additionally, type I IFNs can activate both the phosphatidylinositol 3-kinases (PI3K) pathway and the mitogen-activated protein (MAP) kinase pathway[84,85]. Type I IFN signaling is negatively regulated via protein inhibitor of activated STAT (PIAS), suppressor of cytokine signaling (SOCS) and dephosphorylation of the receptor at the membrane[86].

Through the expression of genes containing an ISRE, an antiviral program can be initiated in cells. These antiviral genes include *myxovirus resistance* (MX)-*1, 2', 5'-oligoadenylate synthetase* (OAS)-*1, protein kinase RNA-activated* (PRKR), *IFN-induced protein* (IFI) *44, IFI44L, IFI with tetratricopeptide repeats* (IFIT) *1 and IFN-γ-induced protein* (IP)-*10*. This program creates an environment that induces infected cells to either eliminate viral components or to undergo

apoptosis, and induces uninfected cells to become more resistant to viral infection[87]. Interestingly, there are additional genes induced by type I IFNs which do not seem to have a direct role in this viral program[62,63,88-90], and their role is not well understood.

Type I IFNs have profound effects on both the innate and adaptive immune system. They induce the maturation of both myeloid DC (mDC) and Mφ[64]. In mDCs, IFNs up regulate the expression of major histocompatibility complex (MHC) molecules, and of various costimulatory molecules[64]. Additionally, type I IFNs can promote NK and T cell cytotoxicity[58,64] and protect B cells and activated T cells from undergoing apoptosis, while promoting proliferation[64,91]. Further, in CD40-activated B-cells, type I IFNs induce their differentiation into plasma cells[92] and can induce B cells to undergo isotype switching[64]. Interestingly, type I IFNs can also have inhibitory properties on both T[93] and B cells[94].

Type I IFNs have additional effects beyond those on the immune system. It has recently been shown that type I IFNs have potent effects on progenitor cells in the bone marrow, inducing enhanced progenitor cell turnover, but impairing their functional capacity to repopulate the bone marrow niche after bone marrow transplantation[95,96]. Type I IFNs are also known to be antiangiogenic. In endothelial cells, type I IFNs inhibit proliferation, migration, tube formation in Matrigel, and induce downregulation of proangiogenic molecules such as vascular endothelial growth factor (VEGF)[97-100].

Type I IFNs can induce many of the immune abnormalities see in SLE patients, including enhanced DC maturation and increased antigen presentation, increased proliferation and differentiation of T and B cells, induction of BLyS, APRIL, and other cytokines[64]. However, the exact source of increased IFN-α in SLE is unclear. While every nucleated cell in the body has the potential to make IFN-α following viral infection, the main producers of this cytokine are the plasmacytoid DCs (pDCs) via recognition of nucleic acids by TLR 3, 7, and 9[64]. pDCs can produce up to 1000 fold more type I IFNs than other cell types, and this may be due to their constitutive expression of IRF7, a key transcription factor

for IFN-α expression[101,102]. However, mDCs can also synthesize biologically relevant levels of type I IFNs[102,103], and in the pristane-induced lupus model, a monocyte subset appears to be the main source of type I IFNs required for disease progression[104]. Additionally, our group has demonstrated that LDGs are an additional source of type I IFNs in SLE[47].

Relevant mouse models of lupus

There are several genetically-lupus-prone mouse strains available. Only the models relevant to this dissertation will be discussed below.

MRL/*lpr* mice:

One widely used lupus-prone strain is the Murphy's Recombinant Large (MRL) mouse[105]. The immunological abnormalities in these mice are considered secondary to a mutation in Fas, a death receptor, (called the lymphoproliferative (*lpr*) mutation). A mutation in Fas-Ligand (FasL) or generalized lymphoproliferative disease (*gld*) also results in a lupus-prone phenotype very similar to the *lpr* mutation[106]. In MRL/*lpr* mice , lupus-like clinical manifestations develop in both genders and disease progression is rapid, with splenomegaly, lymphadenopathy, detection of ANAs and lethal glomerulonephritis in 50% of mice by 6 months of age[105]. This model's human counterpart, Canale-Smith syndrome, is more consistent with an autoimmune lymphoproliferative syndrome (ALPS) than with human lupus[107]. Further, type I IFNs do not appear to play a pathogenic role in this mouse model (and in fact may protect these mice from developing lupus-like symptoms)[108,109]. Therefore, this model does not appear to be the most representative of the human disease. However, it is widely used by many groups of investigators.

New Zealand Black/New Zealand White F1 hybrid and NZM 2328 mice:

The New Zealand Black/New Zealand White (NZB/W) is another widely used lupus-prone mouse strain[105]. As in human SLE, NZB/W females develop the disease earlier and at a much higher rate than males. These mice develop

ANAs and glomerulonephritis at a later stage than the *lpr* mice, with 50% mortality seen by 8 months of age secondary to immune complex glomerulonephritis[105]. In contrast to the *lpr* mice, NZB/W lupus is polygenic and a complex interplay from the genetic background of both parental strains is required for full disease progression. Further, unlike the *lpr* mice but similar to human SLE, disease progression in NZB/W is dependent on type I IFNs[74,110]. Additionally, several New Zealand Mixed (NZM) strains have been derived from the NZB/W stain, which share many of the characteristics of the NZB/W cross. One of these strains, which is used in this work, is the NZM 2328. Disease progression in this strain is very similar to the parental NZB/W strain. Administration of recombinant IFN-α induces acceleration of disease in both the NZB/W and the NZM 2328 strains. As such, NZB/W and NZM show many similarities to human SLE and are considered some of the best murine models of human SLE disease pathogenesis.

Pathogenesis of atherosclerotic vascular disease.

CVD is the number one cause of death in the US and has become a major epidemic in Western societies[111]. Atherosclerosis and endothelial dysfunction develop in a dyslipidemic environment, at sites of nonlaminar flow in large and medium sized arteries[112,113]. Endothelial dysfunction[114] is a state in which the vasodilatory, anticoagulant or anti-inflammatory properties of the endothelium are impaired. This endothelial dysfunction predisposes individuals to the development of plaque. Accumulation of oxidized low density lipoprotein (LDL) in the vessel wall can activate the endothelium and induce recruitment of immune cells[113]. Monocyte/Mφ are important for lipid removal, and have been shown to play a key role in early lesion development[115]. Mφ recognize the modified lipids via scavenger receptors, such as scavenger receptor-A (SRA) and CD36, which mediate their uptake. Mφ which cannot mobilize their ingested cholesterol begin to collect it in cytoplasmic droplets, and evolve into “foam cells”. These foam cells are found in large number in atherosclerotic lesions and are the first macroscopically visible sign of atheroma development. These structures are

also known as “fatty streaks”[112]. The fatty streaks develop into mature atherosclerotic lesions with further accumulation of oxidized-LDL (oxLDL) and immune cells. Many immune cell subsets are recruited to plaque lesions, including B and T lymphocytes, neutrophils, NK cells, DCs and others[112,113]. These plaques are stabilized by a fibrous cap which is generated by infiltrating vascular smooth muscle cells.

Stable plaques are rendered unstable by various immune mediated mechanisms that compromise the protective fibrous cap. DCs can activate T cells in the plaque[113,116,117], leading to their upregulation of FasL and TNF-related apoptosis inducing ligand (TRAIL), which promotes lymphocyte-mediated cytotoxicity of the vascular smooth muscle cells[118]. Additionally, Mφ can be activated by oxLDL and release many proinflammatory molecules and proteases that can also weaken and destabilize the plaque[113]. Once destabilized, the plaque may eventually rupture and expose the highly prothrombotic necrotic core, promoting the development of atherothrombotic events[112]. A typical atherosclerotic plaque is depicted in Figure 1-1.

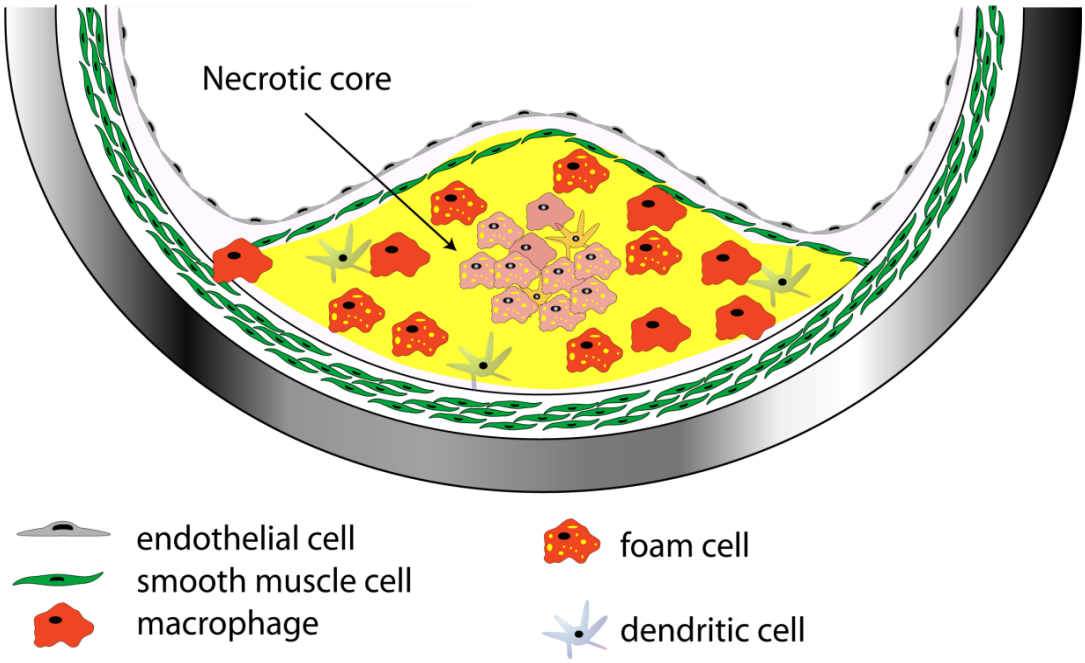


Figure 1-1 Diagram of mature atherosclerotic plaque.
A mature atherosclerotic plaque, as depicted, above contains large numbers of infiltrating macrophages. After uptake of lipid, these macrophages form foam cells and may undergo necrosis. Accumulation of necrotic macrophages and other cells results in formation of a large necrotic core in the atherosclerotic lesion. This necrotic core can be stabilized by the formation of fibrous cap by infiltrating vascular smooth muscle cells. The fibrous cap contains the prothrombotic material in the necrotic core and prevents atherothrombotic events.

Mice have played a vital role in improving our understanding of atherosclerosis pathogenesis. Interestingly, mice are generally resistant to developing atherosclerosis, but several atherosclerosis murine models have been developed. The two most widely used strains are: a) mice lacking apolipoprotein E ($ApoE^{-/-}$) or b) mice deficient in the low density lipoprotein receptor ($LDLR^{-/-}$). $ApoE^{-/-}$ mice cannot clear remnant lipoproteins created from vLDL and chylomicrons, which leads to hyperlipidemia and the development of atherosclerosis[119]. The $LDLR^{-/-}$ mice cannot remove LDL from circulation and also develop hyperlipidemia and significant atherosclerosis[119]. These models

have also allowed us to better understand the role of immune cell subsets in atherosclerosis development and progression[120].

Vascular Repair

Aberrant endothelial repair and maintenance appear to play a key role in atherosclerosis progression, and proper maintenance of the balance of endothelial damage and repair may prevent endothelial dysfunction[121]. Endothelial repair and maintenance of the vascular system typically occur through angiogenesis and vasculogenesis. Angiogenesis is the process by which the vascular network is extended by local proliferation and expansion of resident endothelial cells. In contrast, vasculogenesis is the *de novo* formation of blood vessels by primitive angioblasts or progenitor cells[122]. Until recently, it was believed that vasculogenesis only occurred in embryogenesis, while all expansion and repair occurred via angiogenesis in adults. However, Asahara et al demonstrated in 1997 that there is a population of adult cells that can induce neovasculogenesis[123]. These cells were named endothelial progenitor cells (EPC) and express the surface markers CD34, CD45, CD31, and VEGF receptor (VEGFR)-2[123]. When EPCs are cultured on a fibronectin matrix under proangiogenic stimulation, they develop an endothelial-like morphology and the ability to internalize acetylated LDL (ac-LDL)[123]. These cells also express von Willebrand factor (vWF)[124], endothelial nitric oxide synthase (eNOS)[125], and CD133[126]. CD133 detection provided definitive evidence that these cells were not simply mature endothelial cells detached from the vessel wall, since the mature endothelium does not express this molecule[126]. Subsequently, a second population of cells with angiogenic potential was identified in peripheral blood. These cells have been called myeloid circulating angiogenic cells (CACs) and are characterized by their expression of myeloid markers and low expression of CD34[127-129]. Because these cells have a less defined and stable phenotype, their existence and importance in neovasculogenesis has been controversial. EPCs have high proliferative capacity[130] and are mobilized from the bone marrow in response to various molecules including VEGF, stromal cell-derived factor

(SDF)-1, erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF), as well as in response to estrogen and exercise. Upon mobilization from the bone marrow, EPCs are recruited to sites of vascular damage[122,131-135]. After homing to the damaged site, EPCs and CACs can either directly differentiate into endothelial cells or be a source of many proangiogenic molecules such as VEGF, hepatocyte growth factor (HGF), insulin-like growth factor (IGF)-1, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), fractalkine, GM-CSF, IL-6, IL-8, and platelet derived growth factor (PDGF)-AA[134,136]. Further, CACs have been shown to act in similar ways as EPCs, including their capacity to form tube-like structures in Matrigel, incorporate into newly-formed blood vessels *in vivo*, and secrete proangiogenic factors[122,128,134]. Additionally, *in vitro* expanded EPCs or crude mononuclear bone marrow fractions can increase blood flow, capillary density, improve cardiac function in ischemic heart models, and incorporate into newly formed capillaries[137]. Further, in humans suffering from acute myocardial infarction, injection of EPC or EPC/CACs results in a beneficial anti-ischemic effect[138]. EPCs are also beneficial in ischemic kidney and hind limb models[134,139,140]. Importantly, decreases in EPC/CAC numbers and/or function are associated with enhanced vascular complications and correlate with increased risk of atherosclerosis[121,141,142]. Furthermore, low EPC levels predict the occurrence of CV complications in the general population[143]. Abnormalities in EPCs have been identified in various diseases characterized by an increased CV risk, including diabetes mellitus, hypertension, aging and rheumatoid arthritis[142-146]. Additionally, there is evidence that EPC self renewal can be exhausted in environments of chronic vascular damage, as seen in atherosclerosis[147,148]. Interestingly, cardioprotective drugs such as statins (HMG-CoA reductase inhibitors) increase EPC mobilization and function, implicating an additional mechanism for their beneficial effects on CV risk beyond lowering lipid levels[149].

Atherosclerosis and SLE

While traditional Framingham CV risk factors (including age, lipids and tobacco use) may play a role in the development of premature CVD in SLE, they cannot fully account for the increased prevalence of this complication. Indeed, they appear to be less important predictors of CV events than is the presence of active SLE[15,150,151]. As such, it has been proposed that the immune dysregulation characteristic of SLE likely plays an important role in accelerated atherosclerosis development[152]. On the other hand, typical markers of enhanced inflammation, such as high levels of C-reactive protein, are not typically detected in SLE patients and do not tend to correlate with atherosclerosis development in this patient population. As such, it is quite possible that the immune-mediated mechanisms implicated in vascular damage in lupus are distinct and possibly related to lupus pathogenesis[151].

As a potential mechanism explaining the development of endothelial dysfunction and accelerated atherosclerosis in SLE, our group and others have reported that patients with this disease develop a striking imbalance between vascular damage and repair. The increased damage is characterized by increased numbers of circulating apoptotic endothelial cells[17], which correlate with endothelial dysfunction and tissue factor generation. The aberrant repair observed in SLE is characterized by decreased numbers of circulating EPCs and impaired functional capacity of both EPCs and CACs[153,154]. Importantly, we have previously reported that IFN-α may not only be a major player in SLE disease pathogenesis and severity[155], but it is also the main driver in the promotion of abnormal EPC phenotype and function in human lupus[153]. IFN-α is directly toxic to EPC/CACs. It also skews CACs away from proangiogenic lineages and into mature mDCs[153] Furthermore, IFN-α has been used as adjuvant antiangiogenic therapy for some cancers and conditions characterized by enhanced angiogenesis, such as diabetic retinopathy[98,156-158]. Studies primarily performed in tumor cell lines suggest that IFN-α impairs angiogenesis by repression of VEGF, and/or by down regulation of specificity protein (Sp)1/Sp3 transactivation

activity[159,160]. However, most studies on the role of type I IFNs in angiogenesis have focused on cancer cell lines and little is known about their role in other systems or in normal cells. Recent evidence also indicates that IFN-α can activate Mφs to induce their differentiation into foam cells through an effect on SRA[161]. It has also been demonstrated that the levels of IFN-α correlate with plaque instability[162,163]. SLE patients also show increased signs of platelet activation[164,165], which may be mediated, at least in part, by a type I IFN effect[166]. This suggests that type I IFNs could also have a role in the enhancement of the thrombotic risk.

Ideally, murine models of lupus could be used to clarify the role of abnormal vascular repair and type I IFNs in the development of atherosclerosis in SLE. Unfortunately, in contrast to human SLE, lupus-prone mice are genetically resistant to overt and florid diet-induced atherosclerosis, unless they are crossed to well established murine models of atherosclerosis[167-170]. Interestingly, a recent report indicates that the NZB/W mice may be more prone to develop endothelial dysfunction[171], as measured by their vasodilatory capacity. Further, NZB/W mice display evidence of the metabolic syndrome, with increased insulin resistance and enhanced visceral fat[172]. These findings suggest that this murine model may be prone to develop vascular damage. However, whether an abnormal EPC phenotype is present in lupus-prone mice and its relationship to a type I IFN effect remains unclear.

Concluding Remarks

In conclusion, SLE is a strikingly complex disease in which both environmental and genetic factors play a pathogenic role. As the management of these patients has improved and their life expectancy has increased, CVD is now one of the major causes of morbidity and mortality and cannot be explained by traditional risk factors. Current evidence suggests that immune-mediated mechanisms may play a prominent role in this increased CV risk in SLE. However, the exact pathways implicated in premature vascular damage in lupus remain to be determined. A profound imbalance of vascular damage and repair

induced by type I IFNs may play a prominent role in premature CVD in this disease[153,154]. Further, evidence that type I IFNs may promote foam cell formation and enhance thrombosis risk could modulate CV risk in SLE[161,166]. These observations indicate the need to explore in depth the exact role that type I IFNs play in the development of SLE.

We hypothesize that type I IFNs promote accelerated atherothrombosis in SLE. This dissertation addresses this hypothesis with two specific aims:

Aim 1- To determine the molecular mechanisms by which IFN-α induces aberrant vascular repair in human SLE.

Aim 2- To characterize the effects of type I IFNs on endothelial function, vascular repair, atheroma formation and thrombosis induction in mouse models of lupus and atherosclerosis.

Results from this work will improve our understanding of the mechanisms behind the increased CV risk seen in SLE, and specifically type I IFNs' role in this increased risk. Further, given the increased interest in development of treatments to abrogate type I IFN signaling in SLE the results from this study could potentially increase the rationale for the use of these agents to prevent CVD complications in SLE.

Chapter 2
Material and Methods

Patient selection:

The University of Michigan institutional review board approved this study. Subjects gave informed consent in accordance with the Declaration of Helsinki. To obtain peripheral blood, patients fulfilled the revised American College of Rheumatology criteria for SLE[173] and were enrolled from the University of Michigan outpatient Rheumatology clinic. Age- and gender- matched healthy controls were recruited by advertisement. Lupus disease activity was assessed by the SLE Disease Activity Index (SLEDAI)[174] (Table 2-1).

Cell isolation, culture and fluorescent microscopy:

Human peripheral blood mononuclear cells (PBMCs), known to contain both EPCs and CACs[135,175,176], were isolated and cultured under proangiogenic stimulation as previously described[153]**,** with a few modifications. Briefly, PBMCs ($2.27 \times 10^6/cm^2$) were cultured in endothelial cell-specific enrichment medium (EBM2; Cambrex, East Rutherford, NJ) on fibronectin-coated wells (BD Biosciences, Franklin Lakes, NJ). Typically, after 1-3 weeks in culture, EPC/CACs differentiate into cells that display typical mature endothelial cell markers and display functional endothelium characteristics[135,153,175,176]. For gene expression experiments, media was changed after 72 hours in the presence or absence of human recombinant IFN-α 2b (Schering, Kenilworth, NJ) at a final concentration of 1000 IU/ml for 6 hours, followed by RNA harvesting. Similar experiments were performed with a more purified EPC population which was directly obtained from control bone marrow. In brief, $CD133^+$ cells obtained from healthy control bone marrows (AllCells, Emeryville, CA) were thawed following manufacturer's recommendations and then cultured in StemSpan SFEM

(Stemcell Technologies, Vancouver, BC, Canada) for 48 hours to allow for cell recovery and expansion, then under similar conditions as peripheral blood EPCs/CACs but at a density of $5x10^5$ cells/ml. The concentration of 1000 IU of recombinant IFN-α/ml was chosen as we had previously found that it adequately inhibited EPC and CAC function[153]. Further, this concentration is in the range observed in the serum of SLE patients during disease flares[177].

To assess the capacity of peripheral blood EPCs/CACs to differentiate into mature endothelial cells, media was changed 120 hours after plating, then every 2-3 days. On days 14 to 21, cells were incubated with markers of mature endothelial cells, including 1'-dioctadecyl-3, 3, 3', 3'-tetramethylindocarbocyanine perchlorate (dil)–acetylated LDL (ac-LDL; Biomedical Technologies, Stoughton, MA), and FITC–*Ulex europaeus agglutinin*-1 (UEA-1; Vector Labs, Burlingame, CA). In experiments to assess the effect of exogenous proangiogenic molecules on SLE and control EPC/CAC function, IL-1β (10ng/ml), IL-6 (10ng/ml), TNF-α (10ng/ml) (Peprotech, Rocky Hill, NJ) or VEGF (1ug/ml) (National Cancer Institute, Bethesda, MD) were added to EPC/CAC cultures and replenished every 48 hours.

Table 2-1 Demographic and clinical characteristics of the patients

		Control patients (n=21)	Lupus patients (n=68)
Females, number (%)		16 (76.2)	64 (94.1)
Age, mean (SEM)		33.1 (2.5)	37 (5)
Disease activity (SLEDAI)	Mean (SEM)	*N/A*	3.65 (0.6)
	< 2, number (%)	*N/A*	40 (58.8)
	≥2, number (%)	*N/A*	60 (88.2)
Medications			
Antimalarials, number (%)		*N/A*	44 (64.7)
Azathioprine, number (%)		*N/A*	2 (2.9)
Mycophenolate mofetil, number (%)		*N/A*	20 (29.4)
Cyclophosphamide, number (%)		*N/A*	1 (1.5)
Methotrexate, number (%)		*N/A*	6 (8.8)
Prednisone, number (%)	< 0.5 mg/kg/day	*N/A*	31 (45.6)
	0.5-1 mg/kg/day	*N/A*	1 (1.5)
	> 1 mg/kg/day	*N/A*	0
No immunosuppression, number (%)		*N/A*	6 (8.8)
Past or current lupus nephritis, number (%)		*N/A*	13 (19.1)

SLEDAI: Systemic Lupus Erythematosus Disease Activity Index; *N/A*: not applicable; SEM: standard error of the mean.

To assess expression of endothelial cell markers and morphology, cells were analyzed by fluorescent microscopy using a Leica DMIRB fluorescent inverted microscope (Leica, Bannockburn, IL). Images were acquired at room temperature using live cells in PBS without mounting media. A total of 8 random fields of view were acquired and images were analyzed using the CellC program (http://www.cs.tut.fi/sgn/csb/cellc/)[178] to quantify mature endothelial cells, which were considered as those that coexpress UEA-1 and ac-LDL. Images were acquired with a 100X total magnification. The numeric aperture for the objective lens of the fluorescent microscope was 0.3. Images were acquired with an Olympus DP30BW camera (Olympus Corporation, Tokyo, Japan) using the acquisition software Olympus-BSW (Olympus). Final processing was done with Adobe Photoshop CS2 (Adobe, San Jose, CA).

Phosphorylated STAT detection and inhibition of IFN-α signaling pathways:

EPCs/CACs were cultured under proangiogenic stimulation, as stated above, for 60 hours, then media was removed and changed to EGM without 20% FBS. After overnight incubation in serum-reduced media, cells were stimulated with 1000 IU/ml IFN-α for 30 minutes, followed by fixation with 4% paraformaldehyde. Cells were permeabilized with 90% methanol and incubated with anti-pSTAT2-FITC (R&D Systems, Minneapolis, MN) and anti-pSTAT6-FITC (Cell Signaling Technology, Danvers, MA). Immunofluorescence was detected using flow cytometry (FACS) on a FACSCalibur machine (BD Biosciences), followed by analysis with FlowJo (Treestar, Ashland, OR).

To inhibit IFN-α signaling, the pan-JAK inhibitor 2-(1,1-Dimethylethyl)-9-fluoro-3,6-dihydro-7H-benz[h]-imidaz[4,5-f]isoquinolin-7-one (pyridone 6) or the phosphoinositide-3-kinase (PI3K) inhibitor (5-(4-Fluoro-2-hydroxyphenyl)furan-2-ylmethylene)thiazolidine-2,4-dione (Calbiochem, Gibbstown, NJ) were used. Human EPCs/CACs were cultured under proangiogenic stimulation for 72 hours, followed by change of media containing 50μm of pyridone 6, 50nm of the PI3K inhibitor, or vehicle (DMSO) for 1 hour before addition of 1000 IU/ml of

recombinant IFN-α. EPC/CACs were incubated for 6 additional hours and then total RNA was isolated.

RNA isolation:

Total RNA was isolated with Tri-pure (Roche, Indianapolis, IN) following the manufacturer's recommendations. For gene expression array analysis, RNA was further purified and concentrated using an RNeasy micro (Qiagen, Valencia, CA). RNA samples were processed on an Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA) to assess RNA integrity.

Microarray data processing, analysis and pathway mapping:

Affymetrix Human U133 Plus 2.0 Genechips (Affymetrix, Inc, Santa Clara, CA) were processed at the University of Michigan Microarray Core Facility following the manufacturer's instructions[65]. The samples analyzed and compared were: untreated and IFN-α-treated peripheral blood EPCs/CACs from healthy controls and lupus patients (n=6 in each group); as well as untreated and IFN-α-treated bone marrow EPCs from healthy controls (n=5 in each group). The CEL files were normalized in GenePattern pipeline (www.GenePattern.org) using the RMA (Robust MultiChip Average) method and the Human Entrez Gene custom CDF annotation version 10 (http://brainarray.mbni.med.umich.edu/Brainarray/default.asp). Of the 17527 gene IDs (corresponding to the 54675 Affymetrix probesets), the number of genes expressed above the Poly-A Affymetrix control expression baseline (negative controls) and used for further analyses were respectively: 15700, 15186 and 15909 in the healthy control peripheral blood cells (n=12), the SLE peripheral blood cells (n=12) and the healthy bone-marrow EPCs (n=10). Statistical paired analyses were performed comparing control IFN-α-treated to untreated peripheral blood cells, lupus IFN-α-treated to untreated peripheral blood cells and healthy IFN-α-treated to untreated bone-marrow EPCs. The significantly regulated genes between the groups (q-value <0.05) were analyzed by building biological literature-based networks using Genomatix Bibliosphere Pathway Edition software (www.genomatix.de)[179,180]. A q value was used instead of a p value to limit the number of false discoveries, as

p values indicate the probability of false positives by chance in the entire experiment while the q value gives the probability of false positives of the genes that are significant. For example, using a p value of 0.05 and the number of genes in our dataset above the Poly-A control (15700), one would expect 750 false positives. In contrast, if a q value of 0.05 is used one would anticipate only 142.5 false positives. Canonical pathways were analyzed using the Ingenuity Pathway Analysis Software (www.ingenuity.com). Normalized data files are available on Gene Omnibus Web site, under the reference number GSE23203 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE23203).

Real-time quantitative PCR:

Total RNA was transcribed into cDNA using oligo dT and MMLV (Invitrogen, Carlsbad, CA) with 1μg of RNA using a MyCycler thermocyler (Bio-Rad, Hercules, CA), and levels of expression were analyzed by ABI PRISM 7900HT (Applied Biosystems) with the following cycling conditions: an initial denaturing/activation at 95°C for 10 minutes followed by 40 cycles of denaturing at 95°C for 30 seconds, annealing at 52°C for 30 seconds, and elongation at 72°C for 30 seconds. Primer sequences are displayed in Table 2-2.

Assessment of serum protein levels, EPC/CAC proliferation and apoptosis*:*

Serum IL-1-receptor antagonist (IL-1RN, also known as IL-1Ra) and IL-1β were quantified by ELISA (R&D Systems), following the manufacturer's instructions. EPC/CAC proliferation and caspase 3/7 activation were assessed after 1 day in proangiogenic culture using the XTT cell proliferation Kit (Cayman, Ann Arbor, MI) and the Apo-ONE Caspase 3/7 Assay (Promega, Madison, WI) respectively, following the manufacturers' instructions.

Assessment of myeloid cell phenotype:

After 5 days in proangiogenic culture in the presence or absence of 10ng/ml IL-1β, cells were harvested using Cell Dissociation Buffer (Invitrogen, Carlsbad, CA) and incubated with the following fluorochrome-conjugated anti-

human mAbs: anti-CD14, anti-CD11c, anti-class II MHC (Ancell, Bayport, MN) and anti-CD86 (BD Biosciences). Immunofluorescence was quantified by FACS with a FACSCalibur machine (BD Biosciences), followed by analysis with FlowJo (Treestar).

Table 2-2 Microarray validation primers		
Gene Name		
IL-1β	Forward	ATG TCT GGA ACT TTG GCC ATC TT
	Reverse	AGA CAA TTA CAA AAG GCG AAG AAG ACT
IL-1α	Forward	CCA GAA GAA GAG GAG GTT GGT C
	Reverse	CTG CCC AAG ATG AAG ACC AAC CA
IL-RN	Forward	CCT CAG ATA GAA GGT CTT CTG GTT AAC
	Reverse	ATG CTG ACT CAA AGG AGA CGA TC
IL-1R1	Forward	GAA GCC TGA TGT TTC TCT GAC TAA AAT GA
	Reverse	AGG TCT TGG AAA AAC AGT GTG GAT ATA AG
IL-1R2	Forward	AAA ATT TGC GGG TAT GAG ATG AAC
	Reverse	ACG TCT GCA CTA CTA GAA ATG CTT C
VEGF-A	Forward	GGT CTC GAT TGG ATG GCA GTA
	Reverse	CAC CCA TGG CAG AAG GAG GA
MX-1	Forward	TAC CAG GAC TAC GAG ATT G
	Reverse	TGC CAG GAA GGT CTA TTA G
β actin	Forward	CAT CAC GAT GCC AGT GGT ACG
	Reverse	AAC CGC GAG AAG ATG ACC CAG

Human kidney tissue and immunohistochemistry:

Kidney tissue was obtained from 25 renal biopsies from subjects with clinical and histological diagnosis of lupus glomerulonephritis (5 with class II, 5 with class III, 5 with class IV, and 5 with class V, according to the new SLE nephritis classification[181]); ANCA- associated vasculitis (n=5) and, for comparison, normal samples from 5 control kidneys from tumor nephrectomies. Relevant clinico-histological parameters are given in Table 2-3.

Table 2-3 Renal function and histological parameters of patients supplying kidney sections.			
	SLE (n=15)	ANCA^{+} vasculitis (n=5)	Control (n=5)
Serum creatinine (mg/dL)	2.82 ± 0.73	1.86 ± 0.83	0.88 ± 0.39
Proteinuria (g/day)	2.55 ± 0.66	1.48 ± 0.662	nd
Mesangial proliferation (score 0-3)	1.53 ± 0.40	0 ± 0	0 ± 0
GBM thickness (score 0-2)	0.33 ± 0.09	0 ± 0	0 ± 0
Global glomerulosclerosis (% of all glomeruli)	0.53 ± 0.14	2.60 ± 1.16	0 ± 0
Segmental glomerulosclerosis (% of all glomeruli)	2.00 ± 0.52	0 ± 0	0 ± 0
Glomerular capillary necrosis (% of all glomeruli)	5.00 ± 1.29	19 ± 8.497	0 ± 0
Extracapillary proliferation (% of all glomeruli)	13.73 ± 3.55	21 ± 9.39	0 ± 0
Interstitial infiltration (score 0-3)	0.20 ± 0.05	1.4 ± 0.63	0 ± 0
Interstitial fibrosis (score 0-3)	0 ± 0	0 ± 0	0 ± 0

Data are presented as mean ± standard error of the mean (SEM). nd: not determined.

Tissue samples for light microscopy were fixed in 4% buffered paraformaldehyde and embedded in paraffin. Routine staining was performed according to standard techniques. For immunoperoxidase staining, sections were rehydrated, then immersed in 10 mM citrate buffer (pH 6.0), treated with microwave irradiation at 500W for 10 minutes, and cooled at room temperature. After incubation with 0.5% avidin (Sigma Chimica, Gallarate, Milan, Italy) and 0.01% biotin (Sigma), to suppress endogenous avidin-binding activity, 3% H_2O_2 solution was applied to block endogenous peroxidase. After washing, sections were sequentially incubated with the primary Abs CD31, IL-1β and IL-1RN (Abcam, Cambridge, UK), then with the secondary biotinylated Ab (Zymed, HistoLine, Milan, Italy) and with peroxidase-labeled streptavidin (Zymed).

Peroxidase activity was detected with 3, 5-diaminobenzidine (Sigma), and sections were dehydrated and mounted in Bio Mount (Bio Optica, Milano, Italy). Specificity of Ab labelling was demonstrated by the lack of staining after substituting PBS and proper control immunoglobulins (Zymed) for the primary Ab.

Images were acquired by a Zeiss Axioscope 40FL microscope, equipped with AxioCam MRc5 digital video camera (Carl Zeiss SpA, Arese, Italy). Images were recorded using AxioVision software 4.3 and analyzed by the AxioVision analysis module (Carl Zeiss SpA). A color threshold procedure allowed selective highlighting of the stained areas in gray mode, and the software was programmed to calculate automatically the percentage of the area or the number of particles occupied by staining.

Mice:

Six- to eight- week old NZB/W, BALB/c, B6.MRL-Fas^{lpr}/J (B6/*lpr*), C57BL/6J (C57BL/6), and B6.129P2-*Apoe*^{tm1Unc}/J (ApoE$^{-/-}$) females were obtained from the Jackson Laboratory (Bar Harbor, ME). Breeding pairs of NZM 2328 (NZM) and NZM 2328 IFNAR$^{-/-}$ (INZM) mice were a gift from Dr. Chaim Jacob (University of Southern California)[75,182] and subsequent NZM and INZM mice were bred at the University of Michigan. Lupus-prone mice were euthanized at two time points: early disease (before proteinuria) and active disease (once proteinuria develops). See Table 2-4 for specific age at time of euthanasia for each mouse strain. Proteinuria was assessed using Uristix 4 (Siemens Healthcare Diagnostics Inc., Tarrytown, NY) following manufacturer's instructions.

IFNAR$^{-/-}$ mice (on C57BL/6 background) were a gift from Dr. H.W. Virgin (Washington University)[183,184] and were bred at the University of Michigan. ApoE$^{-/-}$IFNAR$^{-/-}$ mice were generated by backcrossing ApoE$^{-/-}$ (on C57BL/6 background) and IFNAR$^{-/-}$ mice to generate double heterozygotes, followed by backcrossing the offspring back to the ApoE$^{-/-}$ background to generate full double KOs.

ApoE$^{-/-}$ and ApoE$^{-/-}$IFNAR$^{-/-}$ mice were fed a Western diet (Harland, Madison, WI) starting at 8 weeks of age. Mice were euthanized 10 weeks later and thrombosis, endothelial function and atherosclerosis quantification were assessed (see below). Mice were housed in a specific pathogen-free barrier facility. All animal protocols were reviewed and approved by the University of Michigan's Committee on Use and Care of Animals.

Table 2-4. Age in weeks at time of euthanasia		
Strain (Control)	Early Disease (Pre-proteinuria)	Active Disease (with Proteinuria)
Female NZB/W (BALB/c)	20	36
Female B6//*pr* (C57BL/6)	8	16
Female NZM (INZM)	20	32
Male NZM (INZM)	32	40
ApoE$^{-/-}$(ApoE$^{-/-}$IFNAR$^{-/-}$)	22	
Adenovirus injections	Age at injection	Weeks euthanized post-injection
Female NZM	12	3
Male NZM	12	4-5
Female BALB/c	12	4-5
Female ApoE$^{-/-}$	8	10

Genotyping of APOE$^{-/-}$IFNαβR$^{-/-}$ mice:

Genotype of the offspring of ApoE$^{-/-}$(C57BL/6) and IFNAR$^{-/-}$(C57BL/6) mice was confirmed by PCR using the primers displayed in Table 2-5 and the following PCR cycling conditions: ApoE-94^{o}C-4 minutes, then 35 cycles of 94^{o}C-30 seconds, 64^{o}C-30 seconds, 72^{o}C 30 seconds[185,186]; IFNAR-94^{o}C-4 minutes, then 35 cycles of 94^{o}C-45 seconds, 55.5^{o}C-30 seconds, 72^{o}C 30 seconds followed by a final 72^{o}C for 10 minutes[183,184]. All PCR reactions were performed on a MyCycler thermocyler (Bio-Rad, Hercules, CA).

Adenovirus injections:

Murine IFN-α-expressing adenovirus (AdIFN-α) was a gift from Dr. Anne Davidson (Feinstein Institute for Medical Research)[78], and empty control adenovirus (AdControl) was obtained through the Vector Core at the University of Michigan. Both were serotype 5 and gene expression was driven by the cytomegalovirus-IE promoter/enhancer. Adenovirus was administered to 12 week old NZM and BALB/c mice and to 8 week old $ApoE^{-/-}$ mice. With the exception of female NZM mice, all mice were injected with $4x10^9$ particles of AdIFN-α or AdControl. Female NZM received $3x10^9$ particles, as the higher dose resulted in death from active lupus approximately 14 days post injection while the lower dose led to acute proteinuria by 21-28 days post-injection. AdIFN-α or AdControl were administered in 100 µl of PBS via tail vein injection. Proteinuria was monitored weekly in the NZM and BALB/c mice, as mentioned above, starting two weeks post adenovirus injection. Female NZM mice were euthanized at 3-4 weeks post injection, male NZM and female BALB/c mice were euthanized 4-5 weeks post injection and $ApoE^{-/-}$ mice were euthanized 10 weeks post injection.

Table 2-5 Primers for genotyping APOE$^{-/-}$ IFNAR$^{-/-}$ deficient mice.			
Primer Name			Product size
APOE	Forward	GCC TAG CCG AGG GAG AGC CG	
	Reverse	TGT GAC TTG GGA GCT CTG CAG C	155bp wild type band
	Secondary	GCC GCC CCG ACT GCA TCT	245bp band from the targeted allele
IFNAR	Forward	AAG ATG TGC TGT TCC CTT CCT CTG CTC TGA	
	Reverse	ATT ATT AAA AGA AAA GAC GAG GCG AAG TGG	150bp wild type band
	Secondary	GTA GGT CAA ATG AAA CGT CCC GAA G	250bp band from the targeted allele

Assessment of vascular function in mice:

After euthanasia, 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% O_2/5% CO_2) physiological salt solution (PSS). Aortic rings were set at 700 mg passive tension, equilibrated for 1 hour and washed every 20 minutes. Prior to performing concentration response curves, the vessels were contracted with PSS containing 100mmol/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 EC_{80} for NZB/W and B6/*lpr* or EC_{75} for NZM mice 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 EC_{80} 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 EC_{80} or EC_{75} contraction for NZB/W or NZM mice, respectively[187,188].

Quantification of murine endothelial progenitor cells (EPCs):

Spleens and long bones were harvested after euthanasia. 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) to obtain a single cell suspension. Mononuclear cells were obtained from cardiac puncture blood or from single cell suspensions by Histopaque 1083 density gradient (Sigma Aldrich St. Louis, MO) and RBCs were lysed using RBC lysis buffer (Biolegend, San Diego, CA). Approximately 5-10x10^6 cells from spleen and bone marrow or 2-5x10^5 cells from blood were incubated with mAbs against murine CD34, VEGF-R2 (flk-1), CD117 (kit), Sca1, CD45, CD3, CD19, PDGFR-BB (eBioscience, San Diego, CA), and mouse lineage depletion kit (Miltenyi). These mAbs were used to determine the total number of EPCs, as previously described[189]. EPCs were quantified as $CD34^+VEGFR2^+Lin^-$ or $Sca1^+CD117^+Lin^-$. Quantification of the number of vascular smooth muscle progenitor cells (SPCs) was performed as described[190]. SPCs were quantified as $Sca1^+CD117^+PDGFR^-BB^+Lin^-$. EPC and SPC apoptosis was assessed by Annexin-V staining (BD Bioscience) following the manufacturer's recommendations. FACS was carried out using a FACSCalibur or Cyan (Beckman Coulter Miami, FL) machine, followed by analysis with FlowJo (Treestar) . See Figure 2-1 for gating strategy employed for EPC quantification.

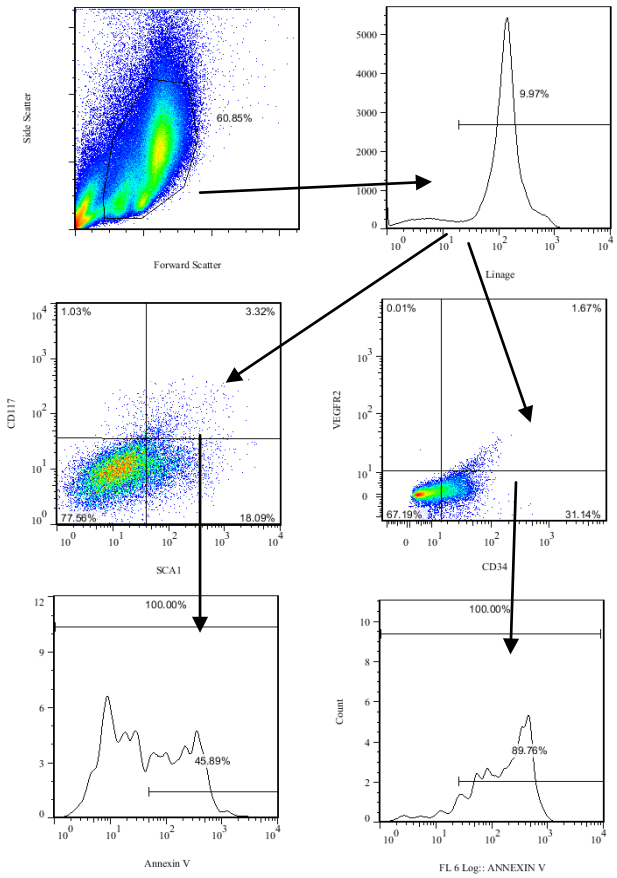


Figure 2-1 Diagram of murine EPC immunofluorescence detection.
A live cell gate was used to exclude dead cells and debris. A lineage negative gate was created using CD45, CD3, CD19 and lineage cocktail. Lineage negative cells were further analyzed for their expression of CD34, VEGFR2 or CD117, Sca1. EPCs were further characterized by examining their expression of Annexin V. Typical staining of murine bone marrow cells is depicted.

Assessment of murine EPC differentiation into mature endothelial cells:

Mononuclear bone marrow or spleen cells isolated by density gradient were plated onto fibronectin coated plates (BD Biosciences) at a density of $1x10^6$ cells/cm^2 in EGM-2 Bulletkit media (Lonza Allendale, NJ) supplemented with 5% heat inactivated FBS. Media was changed after 72 hours in culture, then every 48 hours. When indicated, graded concentrations of recombinant murine IFN-α (PBL InterferonSource, Piscataway, NJ) were added to the freshly plated cells for

the initial 72 hours 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 (dil)–ac-LDL (Biomedical Technologies) for 4 hours. Cells were analyzed by fluorescent microscopy as described above or using a Olympus IX70 inverted microscope (Center Valley, PA), with images acquired using a CoolSNAP HQ2 monochrome camera (Photometrics, Tucson, AZ) and the acquisition software Metamorph Premier v6.3 (Molecular Devices, Downingtown, PA). Images were acquired with an objective magnification of x10, (x100 total magnification). Final processing was performed with Adobe Photoshop CS2 . Endothelial cells were designated as those cells co-stained with BS-1 and ac-LDL, and were quantified in five to six random fields/ well using the CellC software.

Induction of murine carotid thrombosis:

Mice were subjected to photochemical injury of the right carotid artery by the application of Rose Bengal as previously described[191]. Briefly, mice were anesthetized with 1.3 mg intraperitoneal sodium pentobarbital (Butler, Columbus, OH), and placed in a supine position under a dissecting microscope (Nikon SMZ2-T, Mager Scientific, Inc., Dexter, MI). The right carotid artery was isolated and a Doppler flow probe (Model 0.5 VB, Transonic Systems, Ithaca, NY) was applied. Rose Bengal (Fisher Scientific, Fair Lawn, NJ) was diluted to 10 mg/mL in PBS and then injected into the tail vein in a volume of 0.12 mL at a concentration of 50 mg/kg. Just before injection, a 1.5-mW green light laser (540 nm) (Melles Griot, Carlsbad, CA) was applied to the desired site of injury, from a distance of 6 cm, until thrombosis occurred. Flow in the vessel was monitored until occlusive thrombosis occurred, defined as cessation of flow for at least 10 minutes.

Quantification of atherosclerosis in mice:

To quantify atherosclerosis the intact carotid arteries, subclavian arteries, and abdominal aorta with the aortic arch were dissected and stained with Oil Red O (Fisher Scientific). The surface lesion area was analyzed with Image-Pro Plus

software (Media Cybernetics, Bethesda, MD) and expressed as a percentage of total surface area examined, as previously reported[192].

In vivo Matrigel plug angiogenesis assay:

Matrigel plug assays were performed as previously described[193]. In brief, mice were injected subcutaneously with 500 µl growth factor-reduced Matrigel (BD Biosciences) containing 20 nM basic FGF (bFGF) (R&D Systems, Minneapolis, MN). Seven days following injection, mice were euthanized and the plug was excised. Angiogenesis was measured using the 3, 3', 5, 5'-tetramethylbenzidine (TMB) method[194] to quantify hemoglobin levels, as an indirect measure of new blood vessel formation. Hemoglobin quantification was standardized to the weight of the plug. Alternatively, some of the plugs removed were frozen in optimal cutting temperature (O.C.T., Sakura Finetek, Torrance, CA) media for immunohistological examination. Sections were cut and immunohistochemistry was performed. Polyclonal rabbit anti-vWF (Dako, Denmark) was used as a primary antibody, with rabbit IgG serving as a negative control at 10 µg/ml. Fluorescein isothiocyanate (FITC) labeled anti-rabbit IgG (Invitrogen) was used as a secondary antibody at 5 µg/ml, and 4', 6-diamidino-2-phenylindole (DAPI, Invitrogen) was used to observe nuclear staining. Photographs were taken with a fluorescence microscope (Olympus).

RNA isolation and real-time PCR of murine cells:

RNA was isolated from murine bone marrow and spleen EPCs using Tripure following manufacturer's recommendations. cDNA was synthesized using MMLV RT (Invitrogen) and 1µg of RNA using a MyCycler (Bio-Rad). Six ISGs and one house-keeping gene (β-actin) were quantified by real-time PCR using SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) as described[76]. Primer sequences can be found in Table 2-6. Real time PCR was performed 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^{-Ct} method as previously reported[195].

Statistical analysis:

Unless otherwise specified, results represent mean ± 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). Measurements of endothelial function, median effective concentration ($EC_{80\ or\ 75}$), and for agent-induced relaxation in aortic rings were calculated by non-linear regression analysis using GraphPad Prism (La Jolla, CA). Comparisons in the dose response to $EC_{80\ or\ 75}$ contractions were analyzed using a Student's t-test for each concentration of agonist.

For Affymetrix microarray studies, the significance microarray analysis (SAM) was used to define genes significantly differentially regulated between the studied groups; a q-value below 0.05 was considered significant. For all other studies a paired Student's T test or, in the case of the ELISA analysis, a Mann-Whitney T test was performed to determine differences; a p-value below 0.05 was considered significant.

Table 2-6 Primers for type I IFN responsive genes(mouse)		
Gene Name		
Monocyte chemoattractant protein-1 (MCP-1)	Forward	AGG TCC CTG TCA TGC TTC TG
	Reverse	GGA TCA TCT TGC TGG TGA AT
Myxovirus (influenza virus) resistance 1 (Mx1):	Forward	GAT CCG ACT TCA CTT CCA GAT GG
	Reverse	CAT CTC AGT GGT AGT CAA CCC
IFN regulatory factor 7 (IRF-7)	Forward	TGC TGT TTG GAG ACT GGC TAT
	Reverse	TCC AAG CTC CCG GCT AAG T
IFN-inducible protein 10 (IP-10)	Forward	ATC ATC CCT GCG AGC CTA T
	Reverse	ATT CTT GCT TCG GCA GTT AC
ISG15 ubiquitin-like modifier (ISG15)	Forward	CAG AAG CAG ACT CCT TAA TTC
	Reverse	AGA CCT CAT ATA TGT TGC TGT G
IFN-γ	Forward	AGC GGC TGA CTG AAC TCA GAT TGT A
	Reverse	GTC ACA GTT TTC AGC TGT ATA GGG
β-Actin	Forward	TGG AAT CCT GTG GCA TCC TGA AAC
	Reverse	TAA AAC GCA GCT CAG TAA CAG TCC G

Chapter 3

Interferon-α inhibits vasculogenesis in human lupus through modulation of the IL-1 pathway.

IFN-α induces an antiangiogenic signature in control and lupus EPCs/CACs:

In order to determine the effects of IFN-α on vascular repair we utilized an *in vitro* system where peripheral blood EPCs/CACs and bone-marrow EPCs were exposed to proangiogenic stimulation and treated with IFN-α. After treatment with IFN-α, steady state mRNA levels were quantified utilizing Affymetrix GeneChip microarrays. IFN-α treated vs. untreated control and lupus cells exposed to angiogenic stimulation showed no differential expression in IFN-α mRNA but did show a significant up-regulation of many canonical type I ISGs (Table 3-1). For the healthy control EPCs/CACs, a total of 2850 genes (q-value <0.01) were found to be differentially regulated by IFN-α. The associations of these transcripts were assessed with Ingenuity Pathway Analysis (IPA) using canonical pathways. We identified interferon signaling as the top regulated pathway in IFN-α treated cells compared to non-treated (Appendix A, p-value=3.98E-05). Transcriptional network analysis integrating differentially regulated mRNAs with literature mining and automated promoter analysis (Bibliosphere software suite, Genomatix), highlighted IL-1β and VEGF-A among important regulatory nodes (Figure 3-1). IL-1β and VEGF-A were significantly down-regulated in IFN-α-treated control EPCs/CACs when compared to untreated cells (0.7 fold for both genes, q-value < 0.05) (Table 3-1A).

Table 3-1 Affymetrix microarray expression data of studied genes in peripheral blood EPCs/CACs and bone marrow EPCs comparing IFN-α treated to non-treated.

		A. Control peripheral blood EPCs / CACs		B. SLE peripheral blood EPCs/CACs		C. Control bone marrow EPCs	
Entrez gene ID	Gene symbol	Fold-change	q-value[a]	Fold-change	q-value[a]	Fold-change	q-value[a]
Interferon genes							
3439	IFN-α1	1.29	0.243	1.08	0.242	1.06	0.999
3440	IFN-α2	N/A	N/A	N/A	N/A	N/A	N/A
3454	IFNAR1	0.79	**0.005***	0.79	**0.002***	0.80	0.144
3455	IFNAR2	1.05	0.471	1.12	0.079	0.93	0.506
Interferon inducible genes							
4599	MX1	2.74	**0.000***	6.00	**0.000***	6.09	**0.003***
10561	IFI44	1.57	**0.000***	3.42	**0.000***	2.35	**0.021***
10964	IFI44L	1.56	**0.001***	9.92	**0.000***	3.75	**0.020***
3434	IFIT1	2.16	**0.000***	8.44	**0.000***	9.84	**0.000***
Interleukin 1 and receptors							
3552	IL-1α	0.50	**0.003***	0.43	**0.001***	0.64	0.214
3553	IL-1β	0.70	**0.009***	0.62	**0.001***	0.84	0.162
3557	IL-1RN	4.18	**0.000***	10.98	**0.000***	3.37	**0.024***
3554	IL-1R1	0.66	**0.003***	0.76	**0.007***	0.86	0.414
7850	IL-1R2	1.63	**0.002***	1.48	**0.008***	1.27	0.386
3556	IL1RAP	0.75	**0.009***	1.05	0.211	0.98	0.999
Markers of apoptosis							
836	CASP3	1.43	**0.002***	0.95	**0.000***	1.16	0.084
840	CASP7	1.30	**0.001***	2.56	**0.000***	1.58	**0.012***
Other genes							
7422	VEGFA	0.66	**0.002***	0.78	**0.002***	1.04	0.999
5175	PECAM1	1.10	0.091	1.05	0.242	0.88	0.098

[a] A q-value below 0.05 was considered as significant (in bold and with asterisk); N/A: not expressed above the Affymetrix control baseline.

Based on these results, we examined if IL-1β was also regulated in lupus EPCs/CACs after IFN-α exposure. Affymetrix gene expression array analysis defined 1631 genes significantly up and down-regulated by exogenous IFN-α. These genes had a fold-change ≥ 0.15 between the untreated and treated lupus cells. Interferon signaling also appeared as the top regulated pathway (Appendix B, p-value=5.75E-07). Similar, to the IFN-α-treated control cells, the literature-based network analysis of those genes highlighted a major IL-1β node. This IL-1β node and the regulated gene nodes with a direct edge to IL-1β (201 genes in total) are displayed in Figure 3-2 and listed in the Appendix D. IL-1β was down-regulated 0.6-fold in IFN-α-treated SLE cells when compared to untreated SLE cells (Table 3-1B). IL-1RN was up-regulated 11-fold in SLE cells after treatment with IFN-α, while IL-1α was down-regulated (0.43-fold) in the IFN-treated cells (Table 3-1B, q-value <0.05). The expression of IL1-RN gene was doubled in SLE compared to the healthy control EPCs/CACs (fold-change 11.0 vs. fold-change 4.2, respectively) (Table 3-1A and B). Similar to what was found in the control cells, treatment with IFN-α led to decreased expression of the proangiogenic molecule VEGF-A^{131} (fold-change 0.78) in the lupus cells (Table 3-1B). Thus, gene expression array analysis of EPCs/CACs from SLE and controls exposed to proangiogenic stimuli identified an anti-angiogenic signature secondary to IFN-α treatment and a more pronounced antiangiogenic response in SLE than in control cells in response to this cytokine.

Healthy CD133^{+} bone marrow EPCs were found to have 710 genes (q-value <0.05) regulated by IFN-α. Again, the interferon signaling pathway was identified as the top regulated pathway (Appendix C, p-value=6.76E-10, IPA). Although the IFN-α and β mRNA expression were not modified, the ISGs showed significant up-regulation in IFN-α treated compared to untreated bone marrow cells (fold-changes from 2.35 to 9.84) (Table 3-1C). As observed in control and lupus PBMCs, IL-1RN mRNA was 3.4 fold increased when cells were exposed to IFN-α.

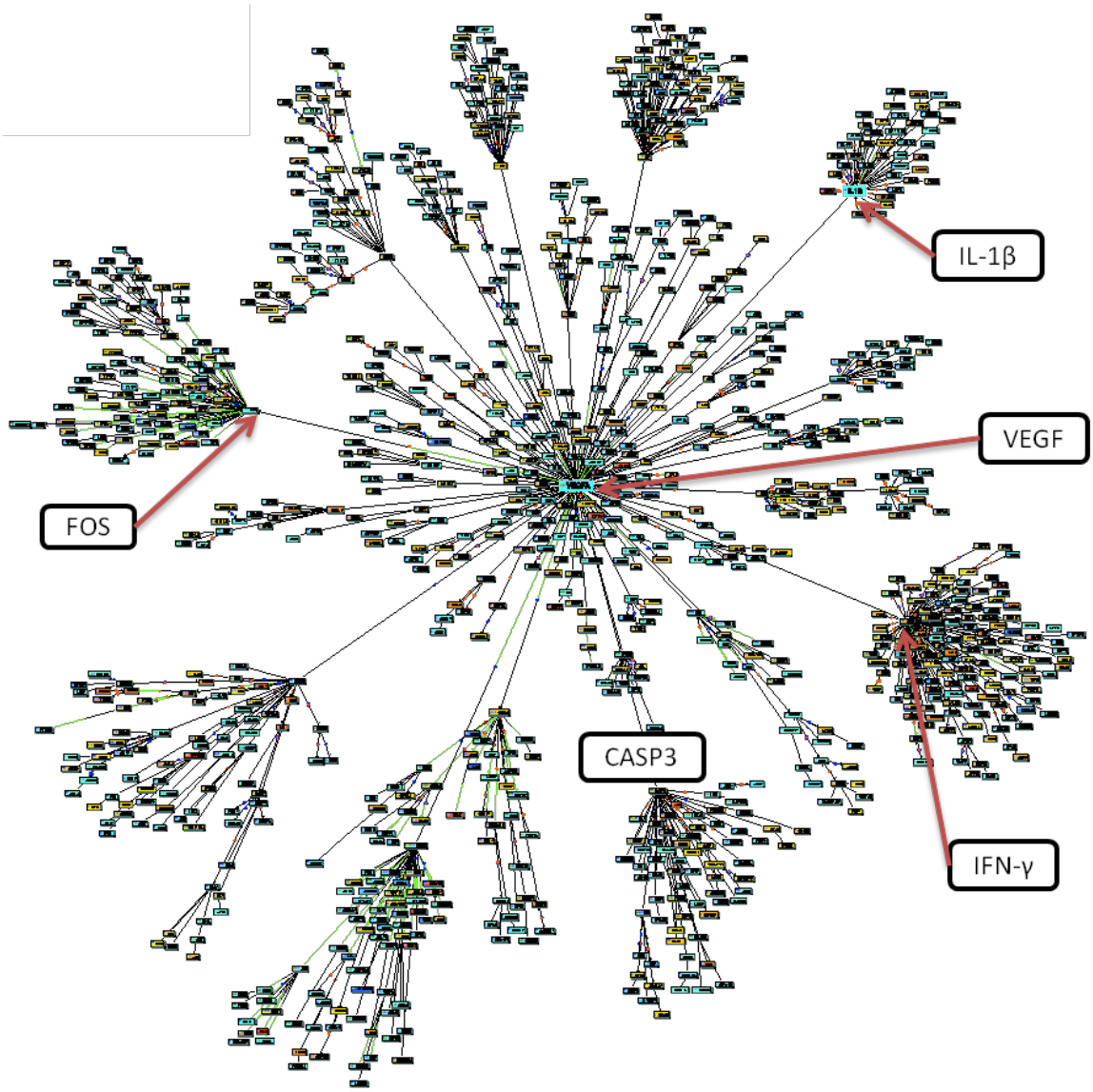


Figure 3-1 Transcriptional network from EPCs/CACs treated with IFN-α.
Literature-based Genomatix Bibliosphere network was performed. The network generated from the regulated gene list between non-treated and IFNα-treated SLE PBMCs (1631 genes with a fold-change ≥ 0.15 and a q-value <0.05) (n=6 in each group). 1208 genes passed the Bibliosphere filter criterion, based on the co-citation of genes in PubMed abstract sentence linked by a function word (B2 filter). The color code from red to blue indicates from up-to down regulation of genes in IFNα-treated SLE PBMCs compared to non-treated SLE PBMCs.

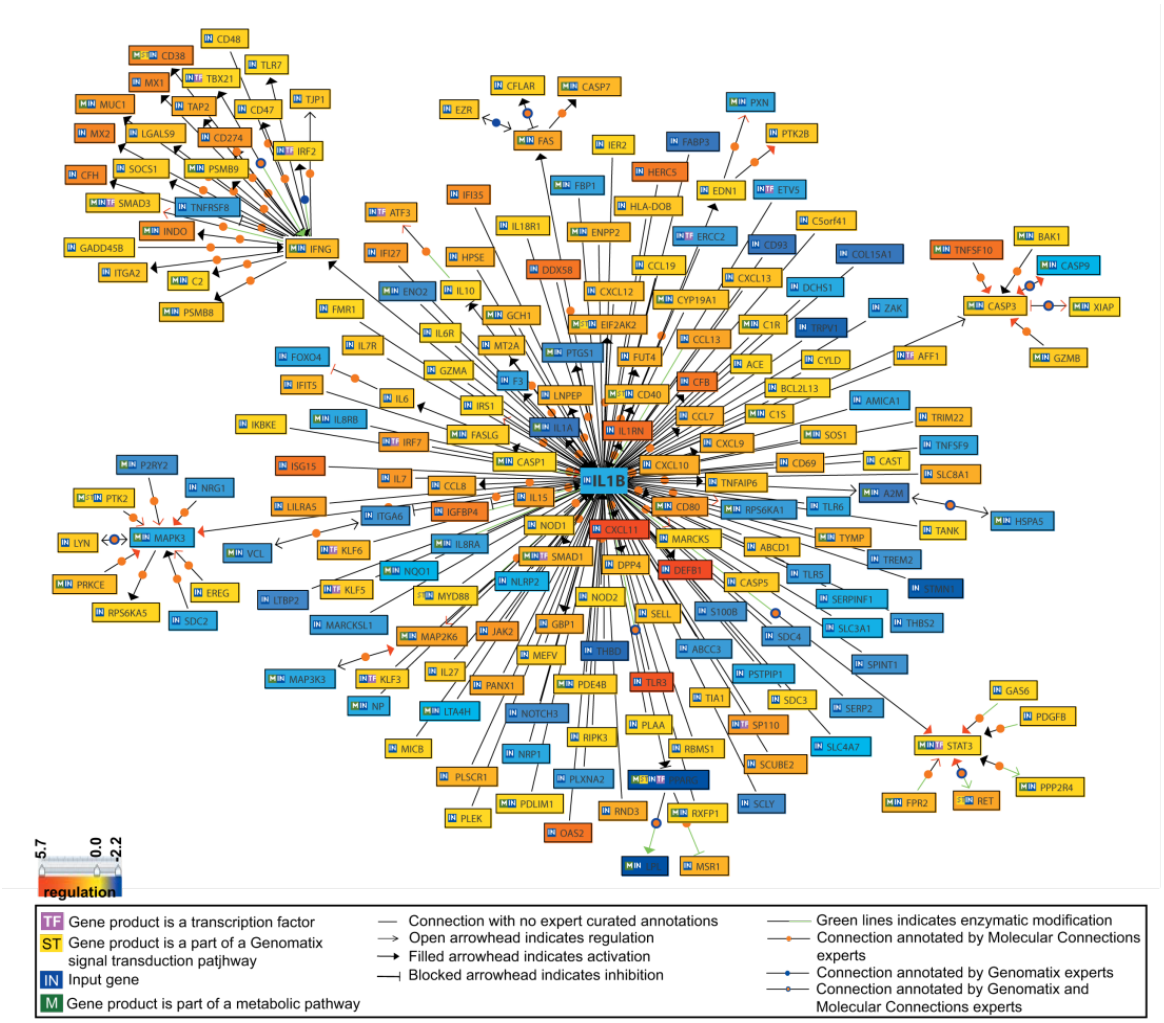


Figure 3-2 Transcriptional network displaying IL-1β as a major node regulated by IFN-α on EPCs/CACs.

Literature-based Genomatix Bibliosphere network was performed. The network generated from the regulated gene list between non-treated and IFNα-treated SLE PBMCs displayed IL-1β as one of the main nodes (1631 genes with a fold-change ≥ 0.15 and a q-value <0.05) (n=6 in each group). 1208 genes passed the Bibliosphere filter criterion, based on the co-citation of genes in PubMed abstract sentence linked by a function word (B2 filter). Only the 201 IL-1β directly connected regulated genes are displayed. The color code from red to blue indicates from up-to down regulation of genes in IFNα-treated SLE PBMCs compared to non-treated SLE PBMCs.

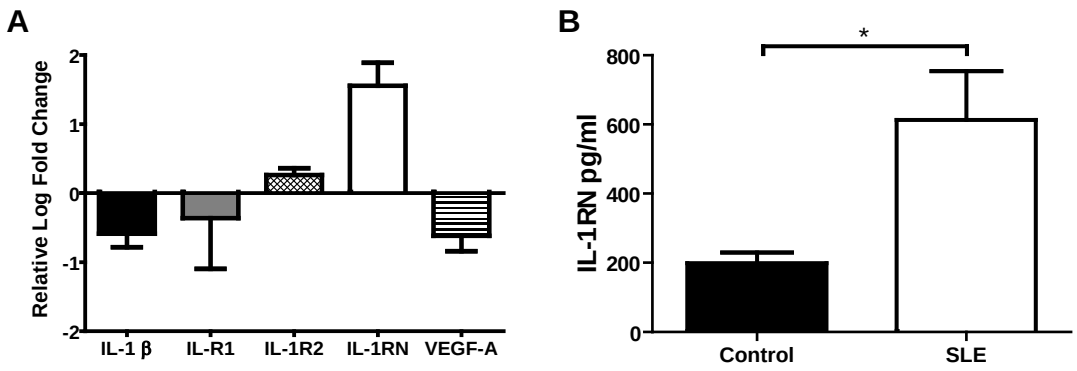


Figure 3-3 IL-1 family members and VEGF-A are regulated by IFN-α in lupus and control EPCs/CACs.

A. Regulation of IL-1β, IL-1R1, IL-1R2, IL-1RN and VEGF-A mRNA levels by IFN-α was confirmed by real-time PCR in EPCs/CACs from healthy controls and SLE patients. Results represent the mean ± SEM log fold change of IFN-α-treated over untreated cells. All treated samples were significantly altered with a $p<0.05$ when compared to untreated samples; (SLE n=11; control=17). **B**. SLE patients display elevated levels of IL-1RN in plasma ($p=0.0116$). Results represent mean± SEM plasma levels of IL-1RN in 35 SLE patients and 10 healthy controls.

Analysis by real time PCR confirmed that IL-1β, IL-1R1, and VEGF-A mRNA levels were significantly down-regulated and IL-1RN and the decoy receptor IL-1R2 significantly up-regulated in IFN-α-treated control and lupus peripheral blood EPCs/CACs when compared to untreated cells (Figure 3-3A, $p<0.05$). Since higher levels and/or increased sensitivity to type I IFNs have been previously reported in SLE[71,196], we then assessed if similar changes in IL-1 related molecules were seen at the protein level *in vivo.* As we expected from our gene expression array results significant increase in circulating IL1-RN (also known as IL-1Ra) levels could be detected in the serum of SLE patients compared to healthy controls (Figure 3-3B, $p<0.02$). Both IL-1β and IL-1α protein levels were below the limit of detection in SLE and control serum (data not shown). Our group had previously demonstrated that SLE patients have decreased serum levels of VEGF-A compared to controls, further supporting the gene expression array and real-time PCR data[153].

IL-1β restores the capacity of lupus EPCs/CACS to differentiate into mature endothelial cells:

We have previously reported that lupus EPCs/CACs, in contrast to control cells, fail to effectively differentiate into endothelial-like cells. Indeed, lupus EPCs/CACs fail to form a mature endothelial monolayer when cultured under proangiogenic conditions on fibronectin-coated wells. We previously demonstrated that IFN-α blockade restores the functional capacity of lupus EPCs/CACs[153]. Given that IFN-α downmodulated IL-1 and VEGF pathways, we addressed if addition of IL-1β and/or VEGF-A could rescue the functional capacity of SLE EPCs/CACs. To this end, recombinant IL-1β and/or VEGF-A was added to proangiogenic media and the numbers of mature endothelial cells were counted. Mature endothelial cells were determined by their ability to uptake ac-LDL and bind the UEA-1 lectin after 14-21 days in culture. We again noted a significant decrease in the capacity of SLE EPCs/CACs to differentiate into mature endothelial cells as well as decrease numbers of EPCs/CACs when compared to control samples. Addition of IL-1β significantly increased the number of mature endothelial cells by 3-fold in lupus cells when compared to media alone (Figure 3-4A $p=0.010$). VEGF-A also increased the number of mature endothelial cells by 2-fold (Figure 3-4 $p=0.045$).

The specificity of the response to molecules regulated by IFN-α was demonstrated, as treatment with other proangiogenic and proinflammatory factors (IL-6 and TNF-α)[197-199] not regulated by IFN-α treatment in the gene expression analysis (data not shown), did not result in any improvement in EPCs/CACs function (Figure 3-4A). In addition, the beneficial effect of IL-1 and VEGF-A were not observed in control cells (Figure 3-4A). Representative images of cells treated with the cytokines indicated above, demonstrate dramatic improvement in response to both IL-1β and VEGFA, but not improvement in response to either IL-6 or TNF-α (Figure 3-4B).

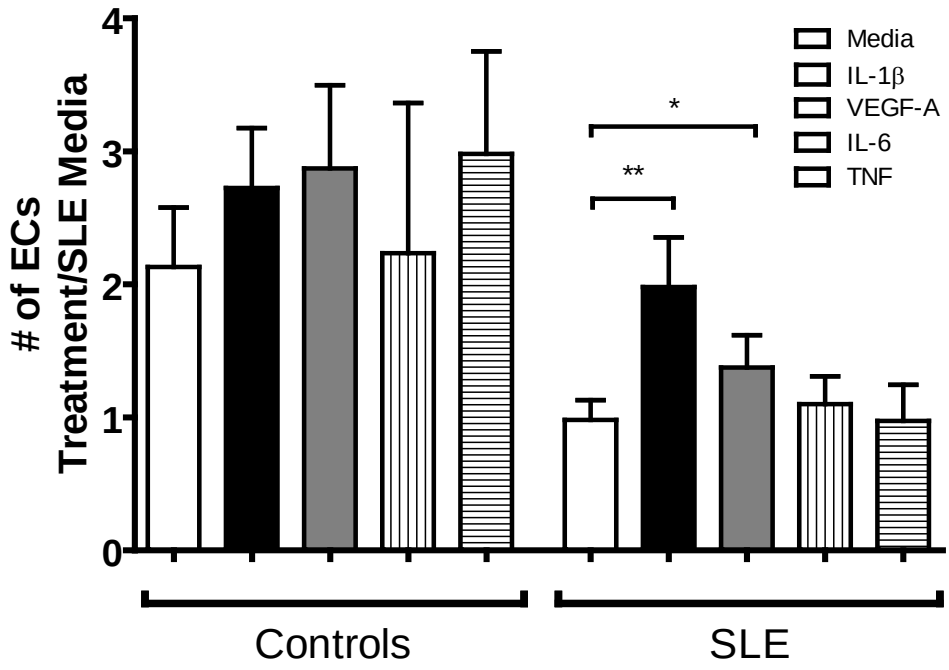


Figure 3-4 IL-1β partially reverses the abnormal phenotype of lupus EPCs/CACs.

A. Addition of recombinant IL-1β to lupus EPC/CAC cultures improves the ability of these cells to differentiate into mature endothelial cells (ECs) to levels comparable to those of healthy controls (**p<0.01, n=24),while addition of recombinant VEGF-A resulted in a more modest but significant improvement (*p<0.05, n=24). Addition of IL-6 or TNF-α to the same cultures had no beneficial effect when compared to untreated cultures (n=9). Control cells showed no significant improvement in their capacity to differentiate into mature endothelial cells with any of the treatments mentioned above (n=7, n=5, n=3, n=3 respectively). Results are expressed as a ratio of the number of endothelial cells/ high power field to the number of untreated lupus endothelial cells/ high power field; *p<0.05.

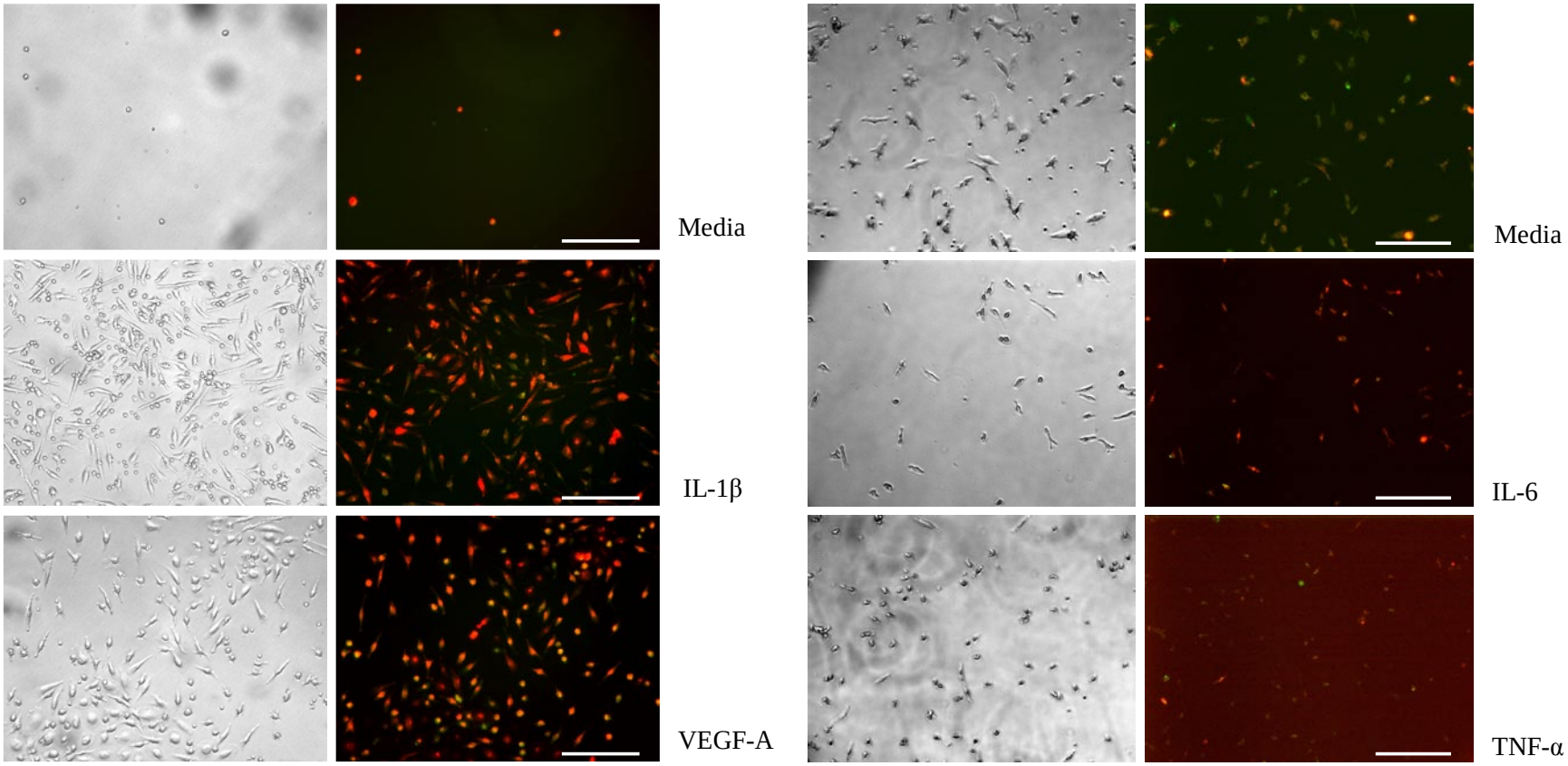


Figure 3-4 IL-1β partially reverses the abnormal phenotype of lupus EPCs/CACs. (Continued)
B. Representative images displaying the effects of IL-1β, VEGF-A, IL-6, and TNF-α on EPC/CAC cultures from 2 SLE patients at 2-3 weeks. Images acquired at 100X total magnification. Bar indicates 200 um. Mature endothelial cells show coexpression of FITC-UEA-1(green) and uptake of DiI-Ac-LDL (red).

IL-1β abrogates the defects in proliferation and viability of lupus EPCs/CACs:

Various mechanisms may lead to improvements in lupus EPC/CAC function by addition of IL-1β, including increased proliferation and/or increased viability through modulation of caspase activity. IL-1β can increase proliferation of murine EPCs[200], rat bone marrow cells[201], and human T cells[202-204], and B cells[203,205]. IL-1β is also able to inhibit apoptosis and promote survival of monocytes[206] and granulocytes[207]. With evidence for increased proliferation and viability potentially playing a role in the improvement in EPC/CAC function, we tested if supplementation with IL-1β and/or VEGF-A promoted increases in proliferation and/or protection from apoptosis. SLE and control cells were cultured with proangiogenic media in the presence or absence of IL-1β and/or VEGF-A for 24 hours. Lupus EPC/CAC proliferation was significantly increased by the addition of recombinant IL-1β ($p=0.023$) or VEGF-A ($p=0.019$) when compared to cells cultured with media alone (Figure 3-5A). Treatment with a combination of IL-1β and VEGF-A did not show a synergistic or additive effect on proliferation compared to cells treated with IL-1β or VEGF-A only (Figure 3-5A). When control EPCs/CACs where treated with IL-1β there was a trend toward increased proliferation ($p=0.084$).

Control cells treated with VEGF-A alone did not show any improvement in proliferation, but the combination of IL-1β and VEGF-A did significantly ($p=0.016$) improve proliferative capacity (Figure 3-5A).

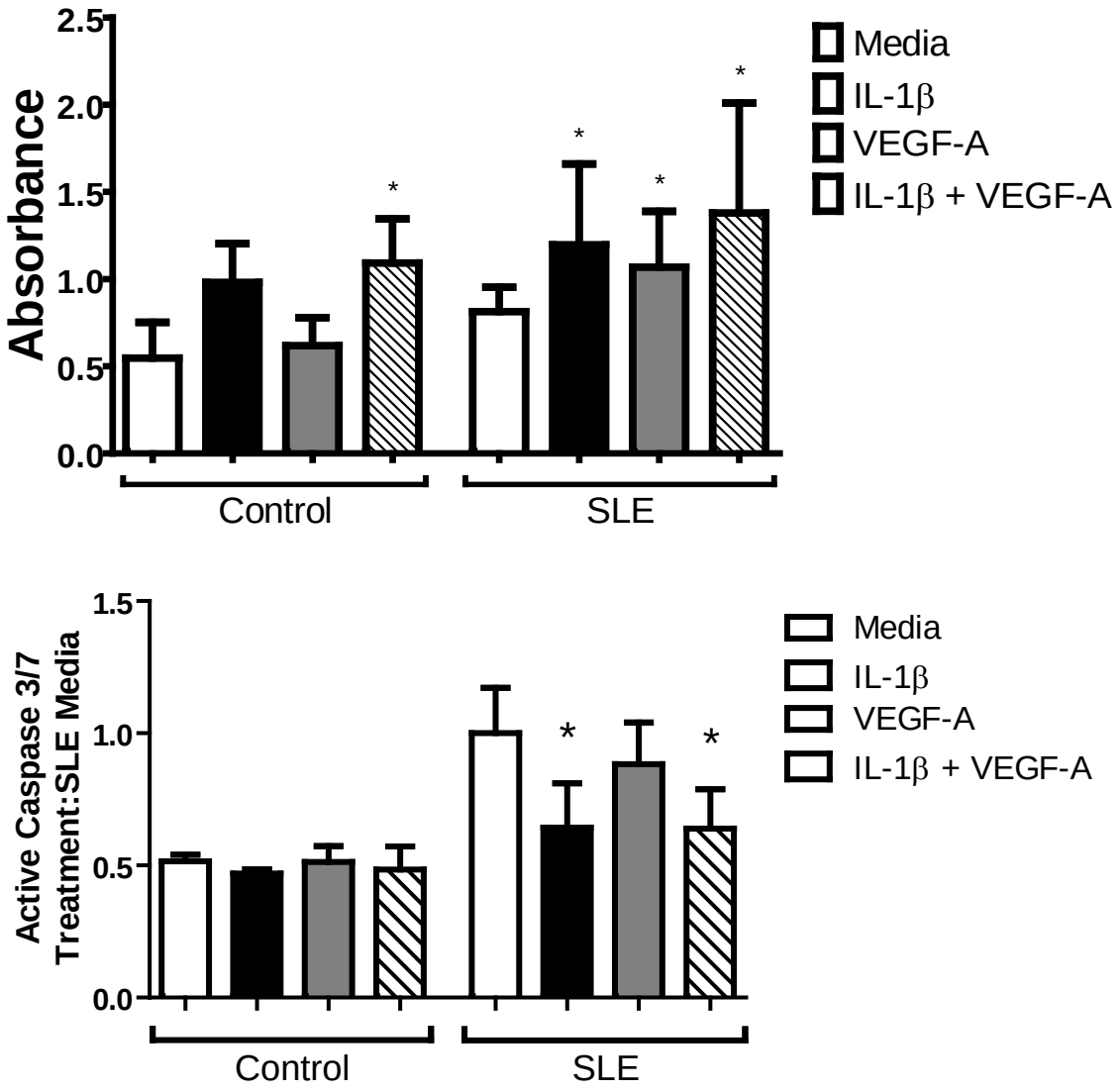


Figure 3-5 IL-1β increases proliferation and inhibits apoptosis of lupus EPCs/CACs.

A. Improvements in EPC/CAC proliferation by IL-1β and VEGF-A supplementation. Results represent mean ± SEM absorbance in duplicate wells; * $p<0.05$ (SLE: n=5 and controls: n=8). **B**. IL-1β inhibits activation of caspase 3 and 7 in lupus but not control EPCs/CACs. Results represent mean fluorescent value of treated samples/mean fluorescent value of SLE media alone ± SEM; (SLE=5; control=8); * $p<0.05$.

Caspase 3 and 7 play a central role in the execution-phase of apoptosis[208] and activation of these molecules is used as a marker of programmed cell death. The mRNA levels of these caspases were significantly upregulated by IFN-α treatment (Table 3-1B) and caspase 3 was also a subnode in the literature based-Bibliosphere network (Figure 3-1 and Figure 3-2). Untreated lupus EPCs/CACs displayed significant increases in caspase 3/7 activation in the presence of media alone, when compared to control cells (Figure 3-5B) indicating increased apoptosis**.** Treatment of lupus EPC/CAC cultures with recombinant IL-1β significantly inhibited caspase 3/7 activation ($p=0.045$) (Figure 3-5B), while treatment with VEGF-A did not have any effect. We also observed a combination of recombinant IL-1β and VEGF-A decreased caspase 3/7 activity to similar levels seen in IL-1β treatment alone. These results indicate that both IL-1β and VEGF-A can improve EPC/CAC proliferation but only IL-1β is effective in inhibiting enhanced apoptosis on these cells. In contrast, neither IL-1β nor VEGF-A addition had any effect on healthy control EPC/CAC apoptosis (Figure 3-5B).

IL-1β decreases lupus DC differentiation:

We previously reported that, in EPC/CAC cultures, IFN-α alters the expression of surface developmental markers on myelomonocytic cells, consistent with skewing the differentiation of these cells from angiogenic CACs to IFN-α–derived DCs. Indeed, IFN-α induces a significant reduction in DC-SIGN^{+}CD14^{+} cells, CD86 up-regulation, and overall down-regulation of CD14[153]. To address if addition of IL-1β could inhibit this phenomenon and allow myeloid lupus cells to favor proangiogenic differentiation rather than DC differentiation, surface markers of mature DCs were examined in 5-day cultures of lupus PBMCs subjected to proangiogenic stimulation in the presence or absence of IL-1β. Addition of IL-1β led to a significant downregulation of the mature DC markers CD86 and MHC class II on SLE patients and a similar trend was observed in controls (Table 3-2). There was also a decrease in CD11c^{+}CD14^{-} cell population, indicating a decrease in total DCs. Additionally,

expression of CD14 was increased in cultures, suggesting an increase in myeloid CACs. These results indicate that the skewing of myeloid cells into nonangiogenic pathways by IFN-α is mediated, at least in part, by downregulation of IL-1 pathways.

IFN-α regulates IL-1 pathways in EPCs/CACs through JAK-STAT modulation:

Various pathways potentially involved in the regulation of IL-1 family members and VEGF were investigated. Hypoxia-inducible factor (HIF)-1α is the main regulator of VEGF-A expression, mainly by posttranslational regulation *via* proteasomal degradation and stabilization by von Hippel-Lindau factor[209]. HIF-1α is a transcription factor and 117 genes, including IL1-β and VEGF-A, of the 2850 in SLE EPCs/CACs have an ischemia response element contained in their promoter. A significant decrease of HIF-1α mRNA expression was detected in lupus EPCs/CACs treated with IFN-α; the significance was not reached in control cells (Table 3-3). However, there was no significant correlation between mRNA levels of HIF-1α and VEGF-A in each group studied (data not shown) indicating that it is unlikely that the changes in HIF-1α steady state mRNA levels induced by IFN-α were the main determinants of VEGF-A downregulation in EPCs/CACs.

Table 3-2 Effect of IL-1β on myelomonocytic cell differentiation.

	Controls			Lupus		
	Untreated	+ IL1β	p-value[a]	Untreated	+ IL1β	p-value[a]
CD14	61.4 ± 6.2	75.9 ± 6.6	**0.018**	51.7 ± 6.8	75.3 ± 4.7	**0.002**
CD11c	51.8 ± 4.7	50.3 ± 6.6	0.779	46.6 ± 6.6	41.7 ± 7.6	0.492
DC-SIGN	21.7 ± 7.0	12.2 ± 1.9	0.318	20.9 ± 6.7	5.8 ± 1.8	**0.033**
CD86	57.5 ± 3.3	36.1 ± 7.2	0.117	40.5 ± 5.9	28.4 ± 5.6	0.079
MHC class II	55.2 ± 12.3	35.7 ± 16.3	**0.037**	22.0 ± 4.8	7.7 ± 3.2	**0.001**
$CD11c^{+}CD86^{+}$	44.8 ± 4.2	19.9 ± 1.7	**0.011**	32.1 ± 4.9	14.1 ± 3.5	**0.006**
$CD14^{+}$DC-$SIGN^{+}$	20.3 ± 6.7	11.1 ± 0.8	0.282	18.8 ± 7.2	5.7 ± 2.0	0.065
$CD11c^{+}CD14^{-}$	1.03 ± 0.3	0.18 ± 0.1	**0.024**	1.8 ± 0.5	0.6 ± 0.3	**0.002**

Numbers indicate mean percentage of positive cells ± standard error of the mean; [a] p-value < 0.05 is considered significant (in bold).

Regulation of VEGF-A occurs not only at the transcriptional level but also postranscriptionally[210] and includes mRNA stabilization by the Hu antigen R (HuR; or ELAV [embryonic lethal, abnormal vision]), a ubiquitously expressed RNA-binding protein[210]. To determine whether HuR mRNA stabilization may play a role in VEGF-A changes observed secondary to IFN-α, we analyzed mRNA expression of known HuR targets[211] in the microarray expression profiles. Of the 14 HuR targets examined, only 4 (including VEGF-A mRNA) were significantly reduced in lupus EPCs/CACs treated with IFN-α, while 3 were reduced in the IFN-treated control EPCs/CACs (Table 3-3). While HuR mRNA was significantly reduced in both control and SLE EPCs/CACs upon exposure to IFN-α, several HuR targets were either upregulated or unchanged after IFN-α treatment. Overall, these results suggest that HuR-dependent RNA degradation is unlikely to be an important mechanism for VEGF-A reduction induced by IFN-α. Further, control bone marrow $CD133^+$ EPCs did not show any significant HIF1A or HuR mRNA expression changes in response to IFN-α (Table 3-3).

From the microarray analyses, the JAK/STAT pathway was one of the most significantly regulated canonical pathways in control and SLE EPCs/CACs after IFN-α treatment (p-value = 0.023 and 0.017 respectively, Appendix A and B). Indeed, SLE peripheral blood EPCs/CACs exposed to IFN-α significantly upregulated STAT1, 2, 3, 4, 5A and 6 while control EPCs/CACs significantly upregulated STAT2,4,5A and 6 (Table 3-3A and B) and control bone marrow $CD133^+$ EPCs significantly upregulated STAT1 and 2 in response to IFN-α treatment (Table 3-3C). Confirming the array data, when control and SLE peripheral blood EPCs/CACs allowed to differentiate for 3 days were stimulated with IFN-α for 30 minutes, there were significant increases in phosphorylation of both STAT2 and 6 (Figure 3-6, $p<0.05$). We then performed subanalysis on EPCs and CACs; in the EPCs, STAT 2 and 6 were significantly activated upon IFN-α stimulation ($p=0.04$ and 0.02, respectively, Figure 3-6A), while in CACs there was a significant activation of STAT2, (Figure 3-6B, $p<0.05$) and a trend towards STAT6 activation. We then tested if JAK inhibition would abrogate the downregulation of proangiogenic molecules seen in IFN-α treated cells.

The pan-JAK inhibitor pyridone 6 induced significant upregulation of IL-1α, IL-1β, IL-1R1 and VEGF-A ($p=0.02$, 0.001, 0.01 and 0.01, respectively, Figure 3-6C) and downregulation of IL-1RN and the canonical type I IFN-inducible gene MX1 ($p=0.06$ and 0.05, respectively, Figure 3-6D) in both IFN-α-treated control and lupus EPCs/CACs. In contrast, there was no change in level of expression of IL-1R2 mRNA after JAK inhibition. The PI3 kinase pathway is also activated by IFN-α in various cell types and PI3K interacts with the JAK-STAT signaling pathway and aids in the activation of a limited number of genes[85]. To assess if the PI3K pathway was also involved in the induction of an antiangiogenic signature in IFN-treated EPCs/CACs, we performed similar experiments using a PI3K inhibitor. In contrast to what was observed with a JAK inhibitor, PI3K inhibition did not induce significant changes in any of the genes examined. Overall, these results indicate that the antiangiogenic signature induced by IFN-α on EPCs/CACs is mediated via JAK-STAT pathways.

Table 3-3 Affymetrix microarray expression data of STAT, HIF1A, HuR and HuR target genes in peripheral blood EPCs/CACs and bone marrow EPCs.

		A. IFN-α-treated compared to non treated *healthy control peripheral blood EPCs/CACs*		B. IFN-α-treated compared to non treated *SLE peripheral blood EPCs/CACs*		C. IFN-α-treated compared to non treated *healthy control bone marrow EPCs*	
Entrez gene ID	Gene symbol	Fold-Change	q-value[a]	Fold-change	q-value[a]	Fold-change	q-value[a]
STAT genes							
6772	STAT1	1.06	0.198	1.45	0.031*	2.61	0.000*
6773	STAT2	1.43	0.000*	1.69	0.000*	1.86	0.000*
6774	STAT3	1.02	0.480	1.54	0.001*	1.45	0.123
6775	STAT4	1.10	0.025*	1.49	0.036*	1.23	0.445
6776	STAT5A	1.25	0.001*	1.21	0.003*	1.04	0.619
6777	STAT5B	0.97	0.329	1.04	0.217	1.01	0.999
6778	STAT6	1.19	0.014*	1.22	0.000*	1.06	0.623
HIF1-α, HuR and HuR target genes							
3091	HIF1A	0.96	0.243	0.96	0.036*	0.94	0.456
1994	ELAVL1	0.88	0.026*	0.85	0.002*	0.84	0.070
153	ADRB1	1.08	0.347	1.05	0.228	1.08	0.627
8900	CCNA1	4.50	0.000*	8.32	0.000*	6.12	0.000*
891	CCNB1	0.91	0.076	0.89	0.041*	0.90	0.150
595	CCND1	0.67	0.001*	1.46	0.012*	1.04	0.999
2353	FOS	0.74	0.009*	0.67	0.006*	1.16	0.627
4609	MYC	0.86	0.102	1.07	0.999	0.88	0.999
3562	IL3	0.91	0.295	0.79	0.999	1.03	0.999
4613	MYCN	1.03	0.451	1.06	0.201	1.01	0.999
4763	NF1	1.05	0.368	0.86	0.016*	1.23	0.480
1026	CDKN1A	1.002	0.502	1.27	0.002*	1.07	0.548
5055	SERPINB2	1.07	0.283	1.18	0.193	0.78	0.232
7124	TNF	1.03	0.476	1.49	0.001*	1.09	0.651
7422	VEGFA	0.66	0.002*	0.78	0.002*	1.04	0.999
2596	GAP43	N/A	N/A	1.03	0.211	0.97	0.548

[a] A q-value below 0.05 was considered as significant (in bold and with asterisk); N/A: not expressed above the Affymetrix control baseline.

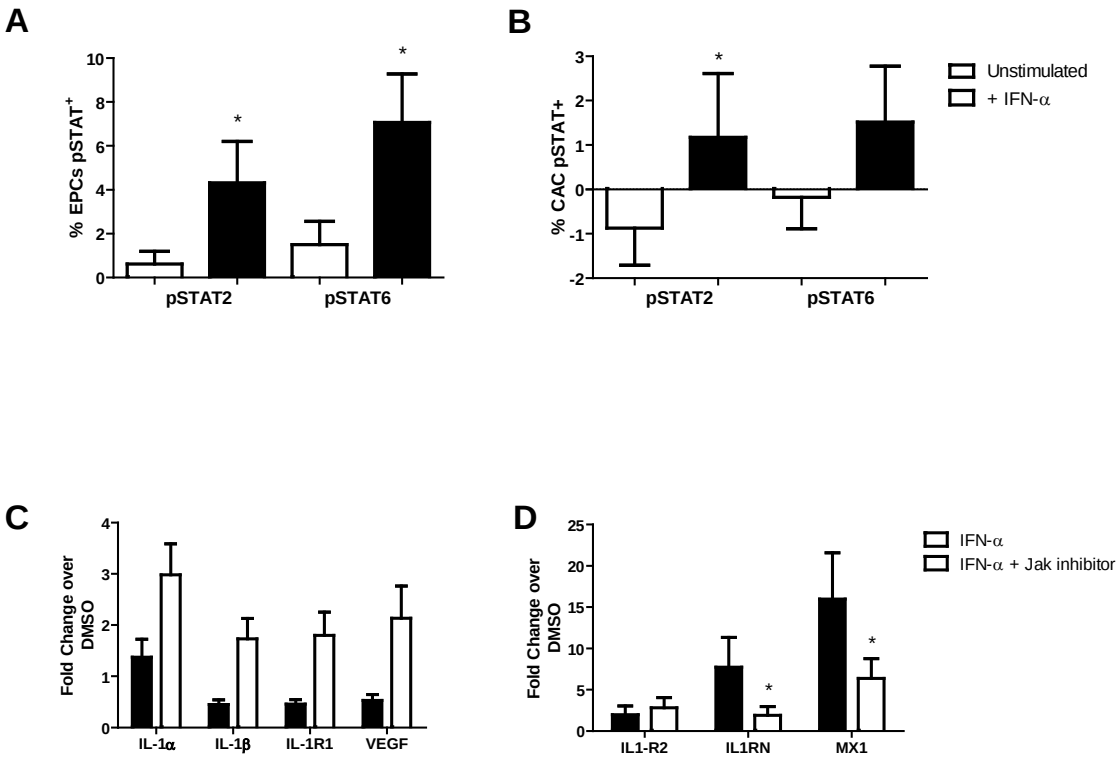


Figure 3-6 IFN-α activates STAT2 and 6 on EPCs/CACs and JAK inhibition leads to downregulation of the antiangiogenic signature in SLE.
Phosphorylation of STAT2 and STAT6 was detected by FACS in EPCs and CACs. Stimulation with IFN-α leads to STAT2 and STAT6 activation in EPCs **(A)** and STAT2 activation in CACs. **(B).** Results represent mean % ± SEM of cells positive for STAT phosphorylation in 7 SLE and 4 control subjects. **C.** JAK inhibition induces upregulation of IL-1α, IL-1β, IL-1R1 and VEGF-A mRNA and decreases mRNA levels of IL-1RN and MX1 **(D).** Results represent average fold-change ± SEM over vehicle-treated cells; SLE n=10 and control n=5; *p<0.05.

Decreased endothelial density and altered IL-1 pathways are observed in SLE *in vivo*:

While our group and others have proposed that decreased vasculogenesis occurs in human SLE[153,154] and murine lupus models also show similar abnormalities *in vivo*, there was no previous evidence on the occurrence of this phenomenon *in vivo* in humans. To this end, we examined the glomeruli and renal blood vessels of lupus nephritis biopsies and

compared blood vessel density and protein expression of IL-1RN and VEGF-A in these compartments to those of control kidneys as well as renal biopsies from patients with another immune-mediated disease, $ANCA^+$ vasculitis. Immunohistochemistry analysis revealed that the glomerular compartment of patients with lupus nephritis had a significant decrease in VEGF-A expression when compared with control biopsies or biopsies from patients with $ANCA^+$ vasculitis and similar degree of kidney function (Table 2-3). This was observed in classes II-V lupus nephritis but was most pronounced in classes IV and V (Figure 3-7). Confirming the *in vitro* data on IFN-treated EPCs/CACs and the serum findings in SLE, IL-1RN was detected in the renal blood vessels of patients with lupus nephritis (particularly class V) but not in the blood vessels of controls or patients with $ANCA^+$ vasculitis. IL-1β was not detected by IHC in any of the tissues examined. The density of the peritubular and interstitial vessels was evaluated. CD31 expression, used as a marker of endothelial cell density, was significantly decreased in the glomerular compartment of lupus nephritis biopsies (particularly class V), when compared to control biopsies, and the levels of this molecule in the renal blood vessels were significantly decreased in all subsets of lupus nephritis compared to control or $ANCA^+$ vasculitis (Figure 3-7). These results indicate that decreased vasculogenesis is also occurring *in vivo* in SLE and that changes in molecules involved in IL-1 signaling and VEGF-A are also observed at the tissue level in this disease.

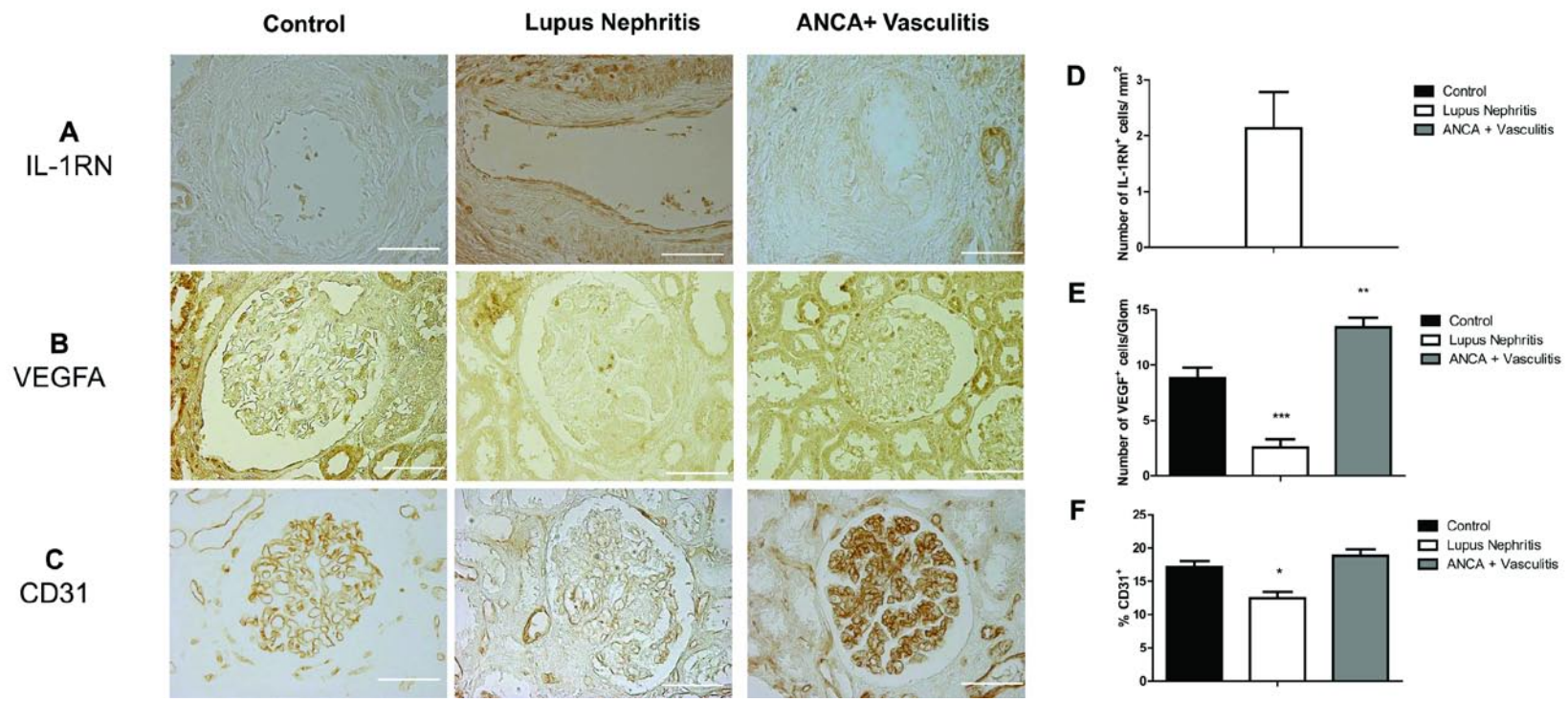


Figure 3-7 Kidneys from patients with lupus nephritis display decreased VEGF-A, increased IL-1RN and decreased glomerular and capillary density.

A and **D**. Representative photographs show that expression of IL-1RN is negative in the endothelium and vessel wall of a control kidney biopsy. In contrast, the vascular endothelium is clearly stained in a case of class V SLE nephritis and numerous positive cells are present in the vessel wall, particularly in the external layers. A mild vascular positivity for IL1RN can be detected in a medium sized vessel from a case of renal $ANCA^+$ vasculitis (Immunoperoxidase, 400X). **B** and **E**. Evident loss of podocyte staining for VEGF-A in glomeruli from class IV lupus nephritis, as compared with control kidneys and $ANCA^+$ vasculitis. (Immunoperoxidase, 200X). **C** and **F**. Compared to glomeruli from a control kidney and a case of vasculitis, glomerular expression of CD31 is significantly reduced in a case of SLE class III lupus nephritis. (Immunoperoxidase, 400X).

Summary

Our results demonstrate that IFN-α exerts its antiangiogenic effects on human EPCs/CACs through the modulation of molecules relevant to IL-1 function and signaling and downregulation of VEGF-A. This modulation includes transcriptional repression of IL-1β, IL-1α, and IL-1R1 and upregulation of the IL-1RN and the IL-1 decoy receptor. In peripheral blood EPCs/CACs, this modulation may lead to a drastic impairment in the downstream effects of IL-1 signaling by decreasing the availability of IL-1α and β and increasing the endogenous inhibitors of IL-1 signaling. We found evidence for this in SLE patients *in vivo*; while IL-1β levels were undetectable in the serum or kidney biopsies of SLE patients and controls, we did observe significantly increased levels of IL-1RN in SLE serum and tissue. We demonstrated that the abnormal lupus EPC/CAC phenotype and function could be abrogated by replacement of exogenous IL-1β and, to a lesser extent, VEGF-A. Previous studies on hepatocytes have found that exposure to IFN-α or IFN-β leads to STAT activation and stimulation of the formation of STAT2:STAT6 complexes, subsequently leading to enhanced IL-1RN synthesis[83]. In this study, we confirmed that IFN-α did in fact lead to STAT2 and STAT6 phosphorylation in control and lupus EPCs/CACs and that inhibition of JAK-STAT signaling leads to abrogation of the antiangiogenic responses.

To address if the *in vitro* findings reflect what is occurring *in vivo* in SLE, we examined kidney sections obtained from patients with lupus nephritis and compared expression of VEGF-A and IL-1 related proteins, as well as capillary density, to control kidney or kidneys from individuals with another autoimmune disease ($ANCA^+$ vasculitis) with similar degrees of renal damage and dysfunction. The blood vessels and glomeruli from SLE kidney biopsies show decreased expression of VEGF-A and increased level of the IL-1RN compared to both controls. We then confirmed that kidneys from SLE patients display decreased renal vascular density and vascular rarefaction, as assessed by CD31 staining.

Overall, our results suggest that enhanced type I IFN synthesis and/or sensitivity to these molecules in SLE leads to the development of abnormalities in the IL-1 pathways that result in a strong antiangiogenic response affecting cells involved in vascular repair. These abnormalities may lead to defective repair mechanisms in ongoing vascular damage in SLE and therefore may contribute to atherosclerosis development and to loss of renal function in this disease.

Chapter 4

Type I IFNs Induce Endothelial Cell Dysfunction, Aberrant Vascular Repair, and Acceleration of Thrombosis and Atherosclerosis.

NZB/W mice have impaired endothelium-dependent vasorelaxation:

As we are interested in the mechanisms that govern the accelerated atherosclerosis seen in SLE we would ideally examine atherosclerosis lesion formation in our murine *in vivo* studies. However, lupus–prone mice and mice in general are resistant to developing atherosclerosis. We chose to examine endothelial dysfunction, as it precedes atherosclerosis and can be used as a surrogate marker of vascular damage[114]. To this end, we examined endothelial function in lupus-prone mice to test our hypotheses that type I IFNs play an important role in accelerated vascular damage and atherosclerosis observed in SLE.

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[212] and in a previous study in these mice[171], 36 week old NZB/W mice display impaired endothelial-dependent vasorelaxation of pre-contracted thoracic aortas, as assessed by blunted responses to acetylcholine (Ach), when compared to age-matched BALB/c mice (Figure 4-1A). Endothelium-independent vasorelaxation in the NZB/W mice, assessed by response of pre-contracted thoracic aortas to sodium nitroprusside (SNP), was not impaired at the same time-point (Figure 4-1B).

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

4-1C and D and not shown). These improvements were seen even in those mice with well established renal disease, as assessed by proteinuria development. 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.

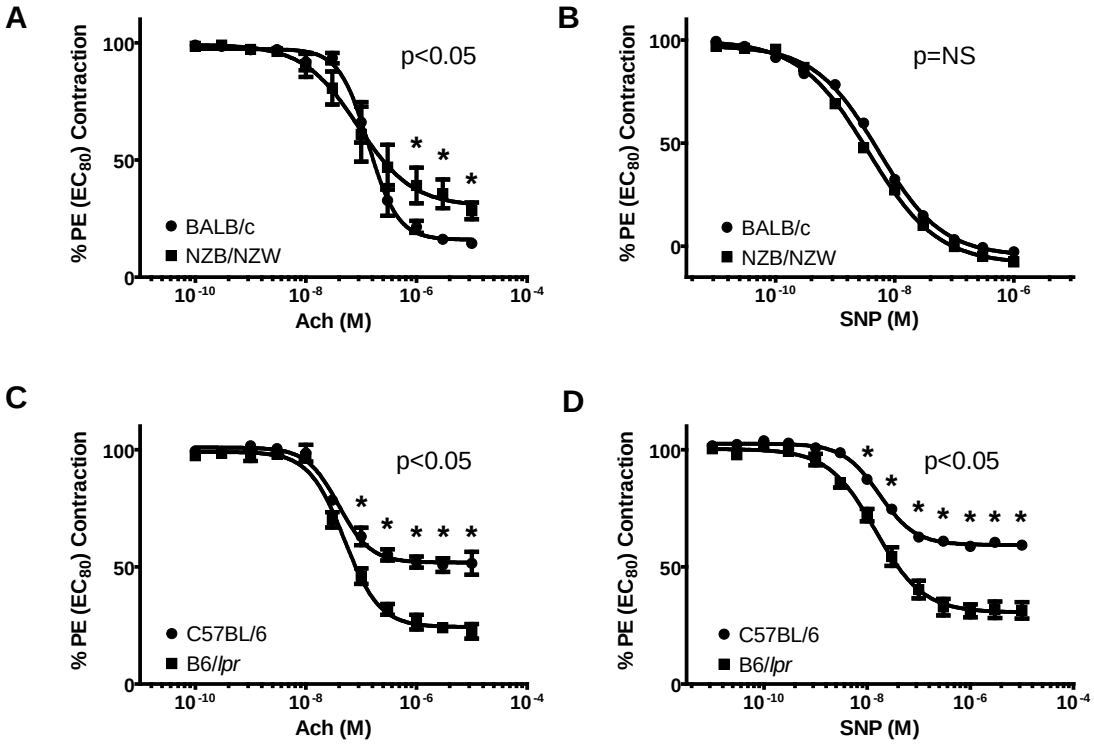


Figure 4-1 Decreased endothelial-mediated vasorelaxation in NZB/W mice.
Results assess acetylcholine (Ach)-mediated endothelium-dependent relaxation and sodium nitroprusside (SNP)-mediated endothelium-independent relaxation in aortic rings from (**A** and **B**) 36 week old NZB/W and BALB/c mice and (**C** and **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 ± SEM % phenylephrine (PE) EC_{80} contraction (n= 5-8 mice/group). * p<0.05; NS=not significant.

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 4-2A). 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 4-2B). 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 to BALB/c mice ($p=0.004$ and $p=0.005$, respectively, Figure 4-2C). At 20 weeks of age, apoptotic EPCs 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 4-2D). Similar results were observed when EPCs were quantified as lineage$^-$/ Sca-1$^+$/ CD117$^+$ cells (not shown).

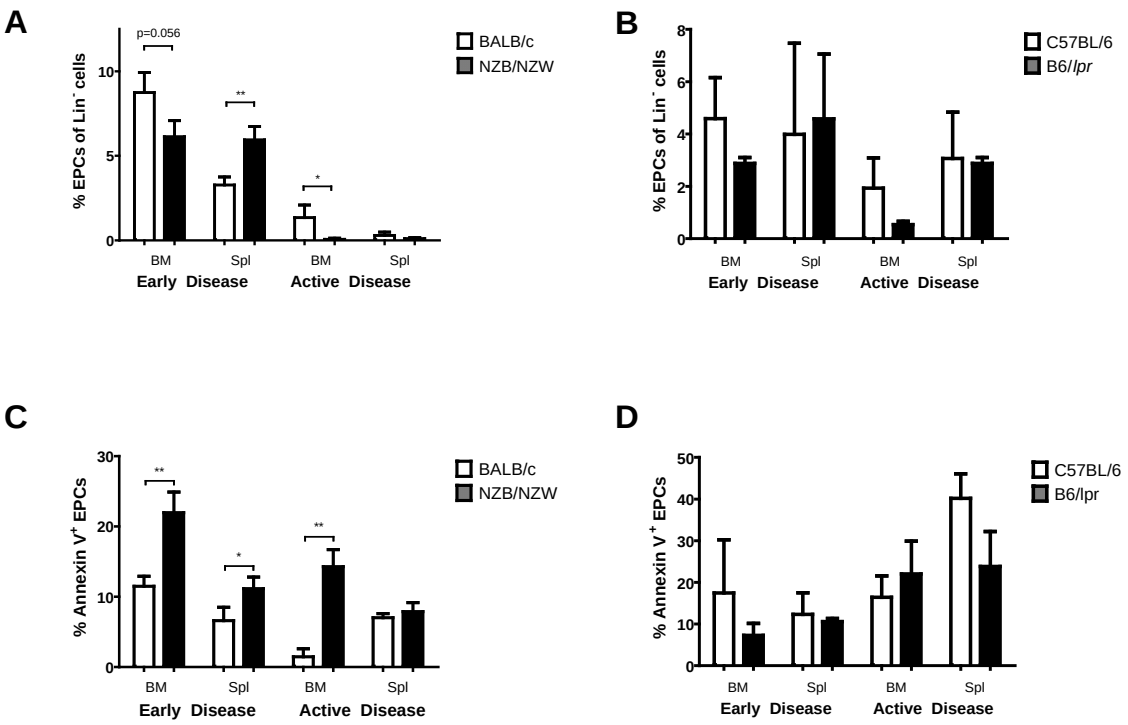


Figure 4-2 EPCs are decreased in NZB/W mice and show higher levels of apoptosis.

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 ± SEM % lineage-negative EPCs (n= 5-8 mice/group). * P<0.05; ** p<0.01.

NZB/W EPCs are impaired in their capacity to differentiate into mature endothelial cells:

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 endothelial cell markers, both at 20 weeks and at 36 weeks (Figure 4-3), when compared to BALB/c mice. Similar to what we previously reported in human SLE[153], 7 day NZB/W cultures typically displayed scattered or clustered endothelial cells but no evidence of endothelial cell monolayer formation; in contrast, BALB/c EPCs clearly differentiated into mature endothelial cells with significant ac-LDL uptake and co-expression of the lectin BS-1. B6*/lpr* bone marrow EPCs displayed no significant impairment in their capacity to differentiate into mature endothelial cells, when compared to control C57BL/6 mice (not shown).

These results indicate that NZB/W mice display clear impairment of EPC numbers and function. This observation suggests that these mice could represent a good model of impaired vascular repair and endothelial dysfunction which are similar to the findings observed in human lupus.

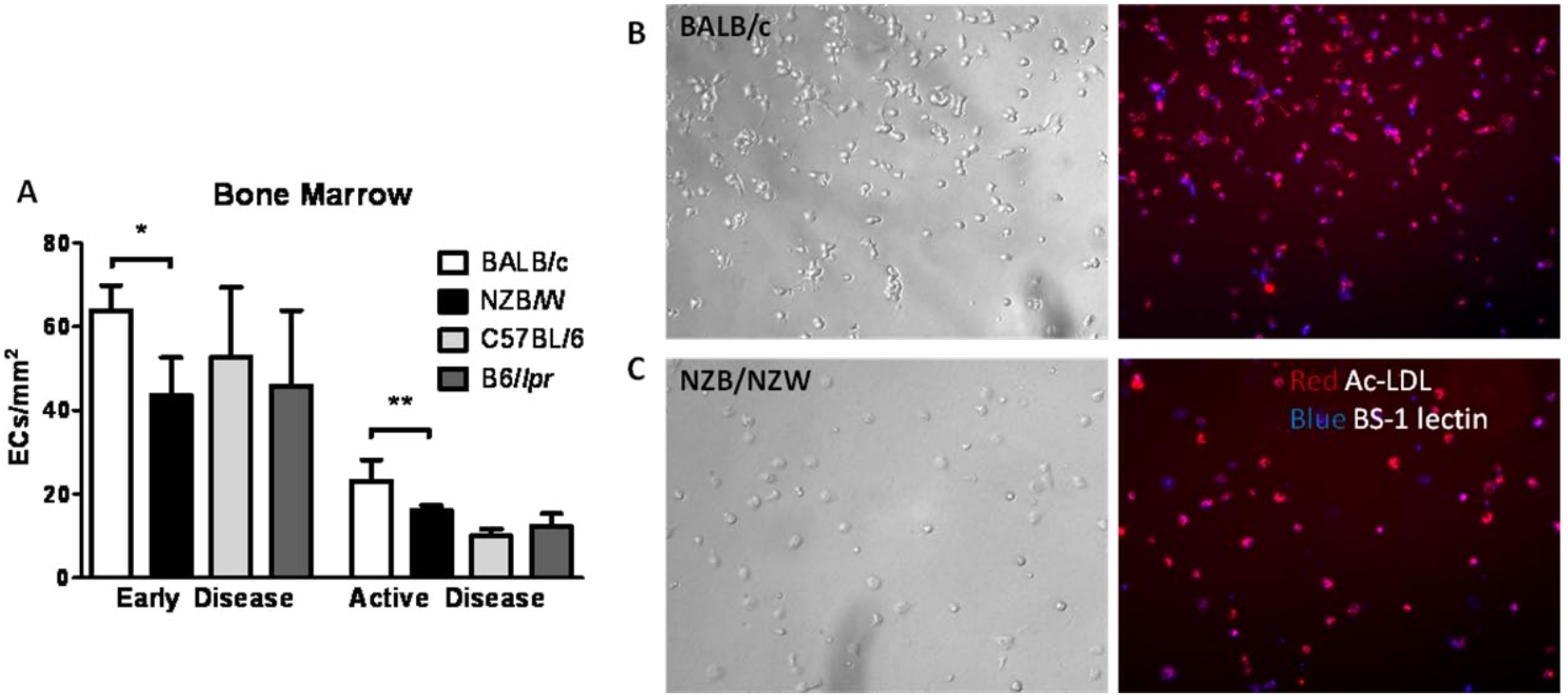


Figure 4-3 NZB/W EPCs exhibit impaired capacity to differentiate into mature endothelial cells. .

Bone marrow-derived EPCs were cultured under proangiogenic conditions and incubated at different time-points during culture with dil-ac-LDL and BS-1-FITC. Mature endothelial cells were identified by co-staining of BS-1 and ac-LDL. **(A)** Bar graphs represent number of mature endothelial cells per cm_2 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 ± SEM of 3-4 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 100X

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

To assess if the increased apoptosis and decreased numbers of EPCs in NZB/W mice could be associated to 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 4-4, 20-week and 36-week old NZB/W mice display significant increases in expression of type I IFN inducible genes in both compartments, when compared to 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 to control C57BL/6 mice (Figure 4-5). 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 4-6A, $p<0.0081$) and IP-10 (Figure 4-6B, $p<0.0113$) was detected in these mice, but not in B6/*lpr* mice, when compared with controls (Figure 4-6). 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 with control mice.

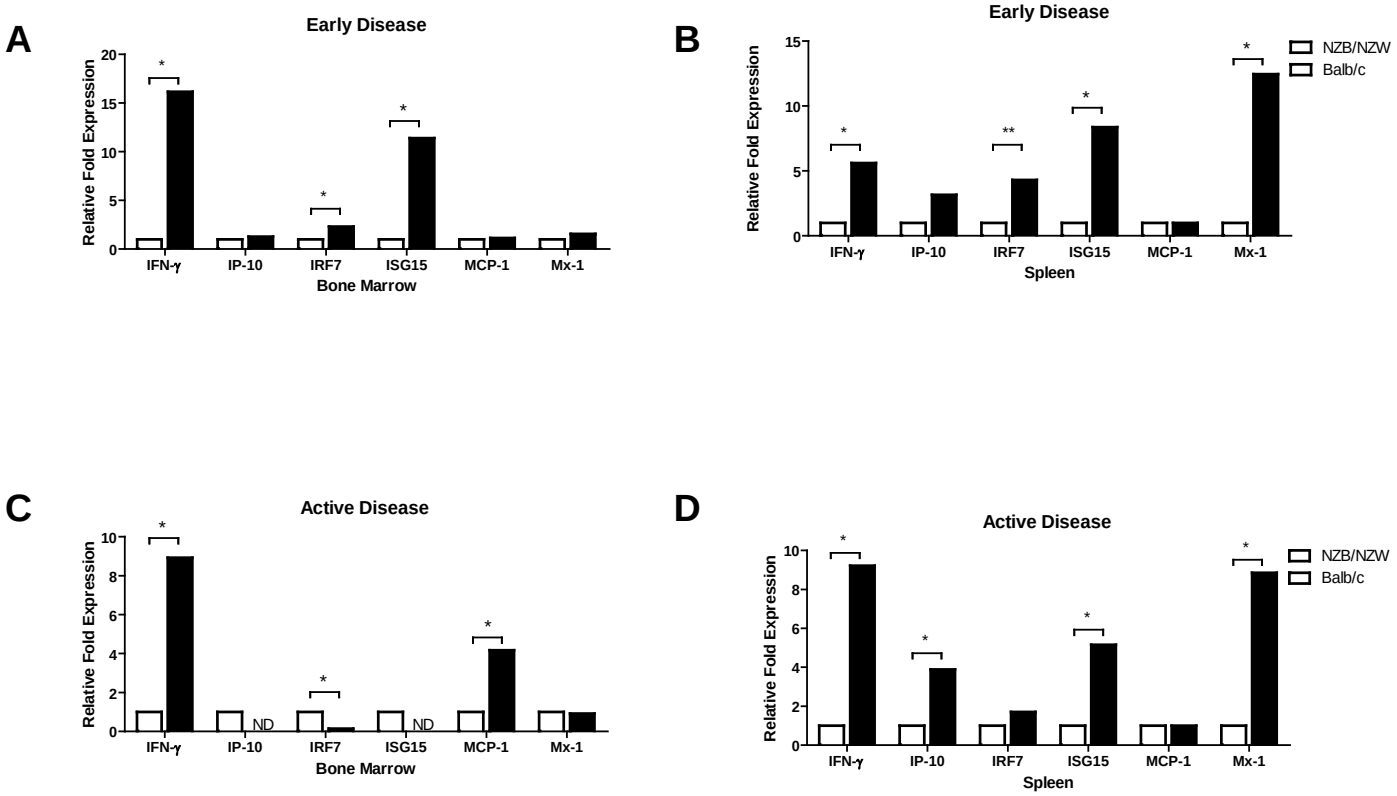


Figure 4-4 Type I IFN-inducible genes are increased in NZB/W EPC compartments.
Results are displayed as relative fold change of type I IFN-inducible genes over control BALB/c mice (n=5 mice/group) in bone marrow and spleen EPC compartments during **(A and B)** early and **(C and D)** active disease stage.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

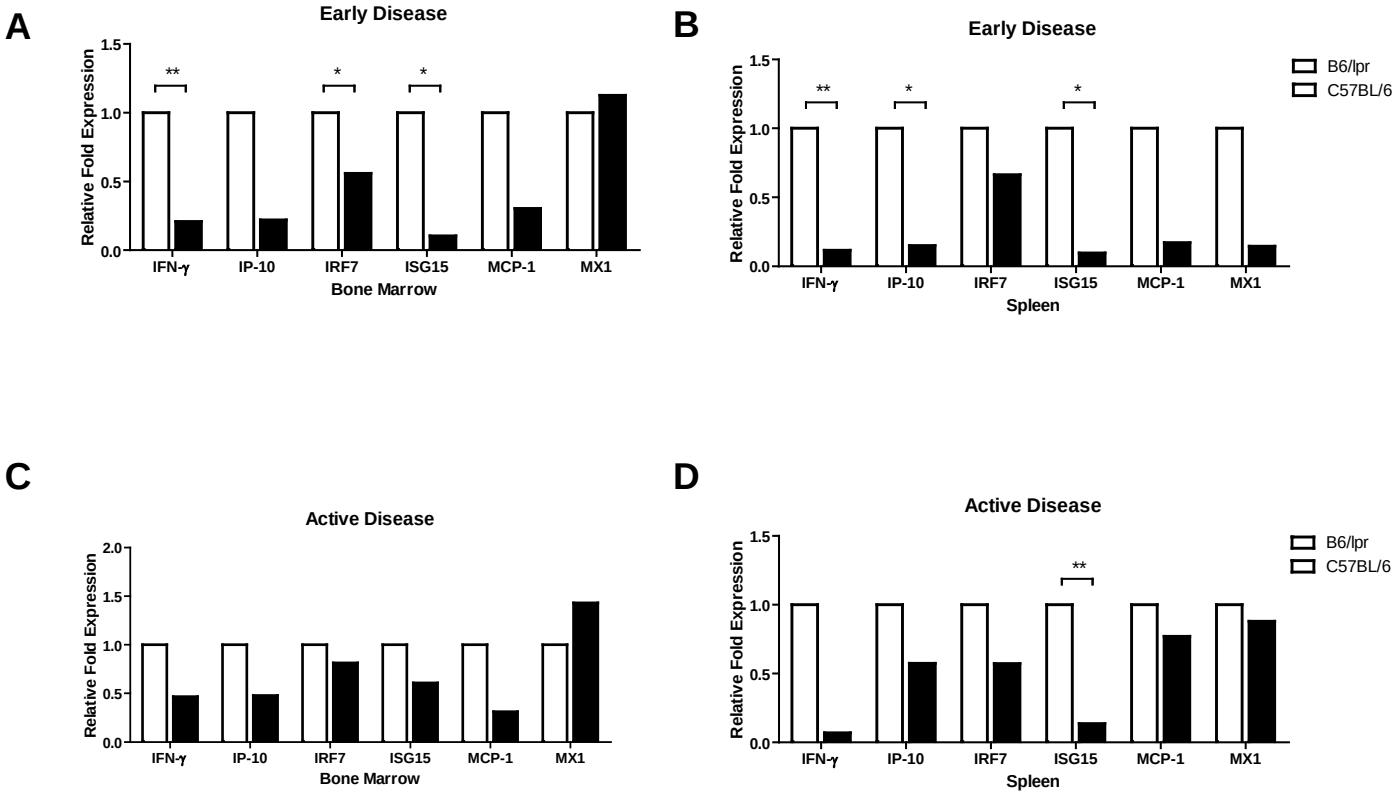


Figure 4-5 B6/lpr EPCs display decreased expression of type I IFN-inducible genes.
Results are displayed as relative fold change of type I IFN-inducible genes over control C57BL/6 mice (n=3 mice/group) in bone marrow and spleen EPC compartments during **(A and B)** early and **(C and D)** active disease stage. Transcripts were normalized to β-actin. *p<0.05, **p<0.01

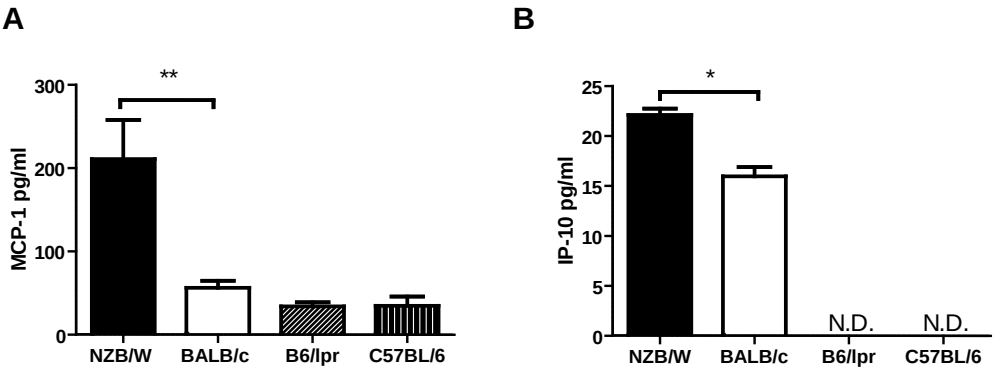


Figure 4-6 Increased circulating levels of type I-IFN inducible proteins in NZB/W mice.

Levels of the type I IFN-sensitive chemokines **(A)** MCP-1 and **(B)** IP-10 were quantified in the plasma of lupus-prone mice with active disease. Results represent mean ± SEM (n=3–4 mice per group, N.D. not detected); *p<0.05.

IFN-α induces cytotoxicity of murine EPCs:

As type I IFNs are toxic to human EPCs and induce significant apoptosis of these cells[153], we examined if murine EPCs showed a similar sensitivity. Murine bone marrow (Figure 4-7) and spleen (not shown) EPCs from control BALB/c mice showed loss in their capacity to differentiate into mature endothelial cells and increased death after *in vitro* exposure to recombinant IFN-α, recapitulating the phenotype observed in NZB/W EPCs (Figure 4-7).

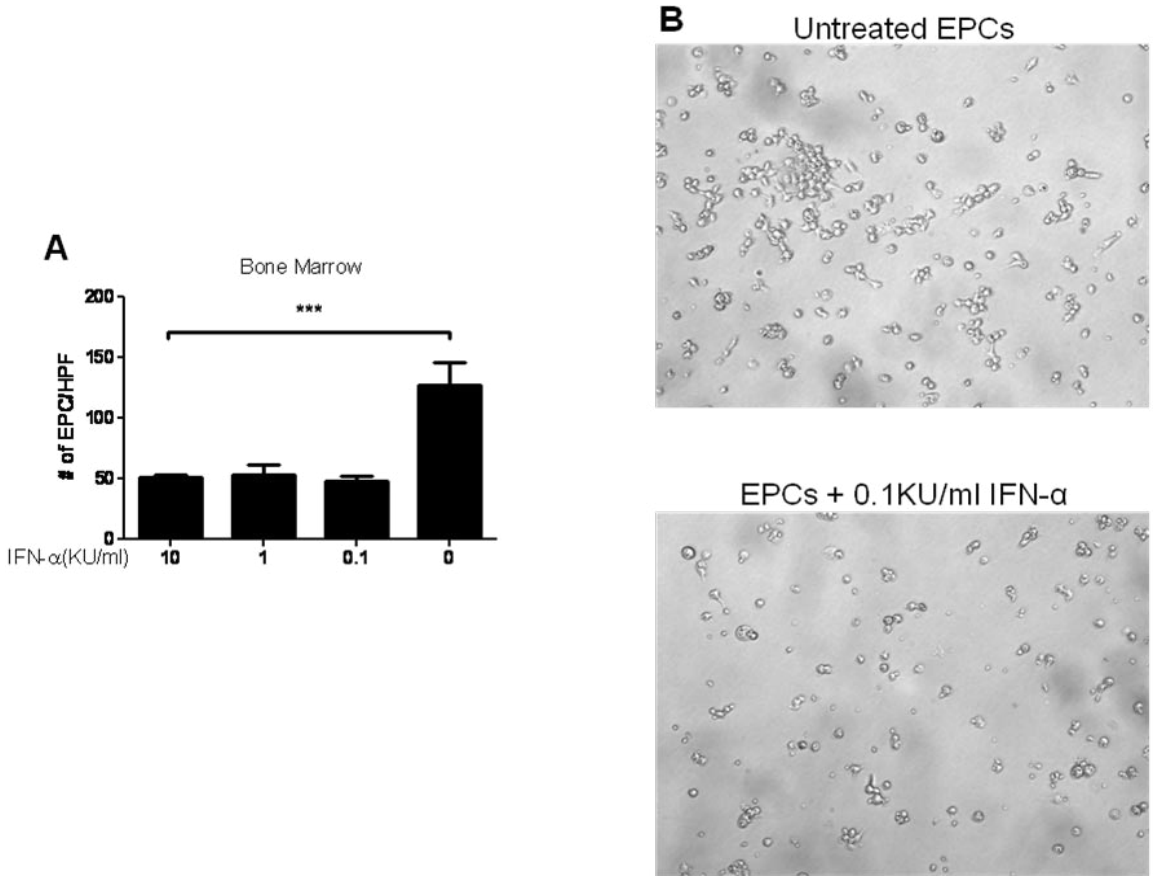


Figure 4-7 IFN-α is toxic to murine EPCs.
Bone marrow EPCs from BALB/c mice were cultured in proangiogenic media in the presence or absence of graded concentrations of recombinant IFN-α for 3 days. Numbers of mature endothelial cells were quantified at 7 days in culture. Exposure to IFN-α resulted in a significant loss of EPC ability to differentiate into mature endothelial cells. **(A)** Bar graph displays 1 representative experiment and represent mean ± 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.

Loss of type I IFN signaling in NZM 2328 mice improves endothelial function and increases EPC numbers:

To address the putative role of type I IFNs on endothelial cell and EPC function *in vivo* in lupus, we utilized the NZM 2328 strain, originally derived from NZB/W mice[213,214]. We compared the wild type NZM 2328 to NZM 2328 lacking the type I IFN receptor (IFNAR; INZM). Female NZM 2328 mice have similar disease progression to NZB/W mice and die from renal failure[213], while INZM mice do not develop lupus features[75]. At 30 weeks of age, female INZM mice displayed significant improvement in endothelial-dependent vascular function (Figure 4-8A $p<0.05$), when compared to age and gender-matched NZM 2328 mice. In contrast, there were no significant differences in endothelial function in younger mice (20 weeks old, not shown) before clinically active lupus developed. Female INZM mice also showed increased EPC numbers in the bone marrow and blood (Figure 4-8B $p<0.01$, $p<0.05$, respectively) at 30 weeks of age, but no significant differences at the earlier time point. Additionally, similar increases in EPC numbers were observed in $Sca1^{+}CD117^{+}Lin^{-}$ EPCs (data not shown). To address if the beneficial effects on endothelial function and EPC numbers observed in INZM mice were a result of blocking type IFN signaling or secondary to the abrogation of lupus development and renal disease[215], we compared male NZM 2328 with male INZM mice, as the male NZM 2328 mice do not develop SLE features. Similar to the female mice, male INZM mice demonstrated a significant improvement in endothelial-dependent vascular responses when compared to age and gender–matched NZM 2328 mice (40 weeks old, Figure 4-8C $p<0.001$). Furthermore, male INZM mice also exhibited a significant increase in EPC numbers in the spleen when compared to male NZM 2328 mice (Figure 4-8D $p<0.05$). It is unclear why the male INZM mice only displayed improvements in EPC numbers in the spleen but not in the bone marrow or peripheral blood like the females. One explanation could be differences in disease progression. Even though the male NZM 2328 mice do not develop full lupus disease symptoms, such as nephritis, they do show signs of abnormal immune activation such as splenomegaly (data not shown), and this local

immune dysregulation could be altering the microenvironment of the spleen resulting in the changes seen in the male. It is also possible that the differences observed in improvements in EPC numbers in different organs could be gender-related. There were no significant differences between the male NZM 2328 or INZM mice at earlier time points (20 and 30 weeks, data not shown). In contrast to our previous results in NZB/W, we did not observe any difference in apoptosis of EPCs with the loss of type I IFN signaling in either the female or male mice (data not shown). Overall, these results indicate that type I IFNs modulate endothelial function and EPC numbers in lupus-prone mice independent of disease activity or gender.

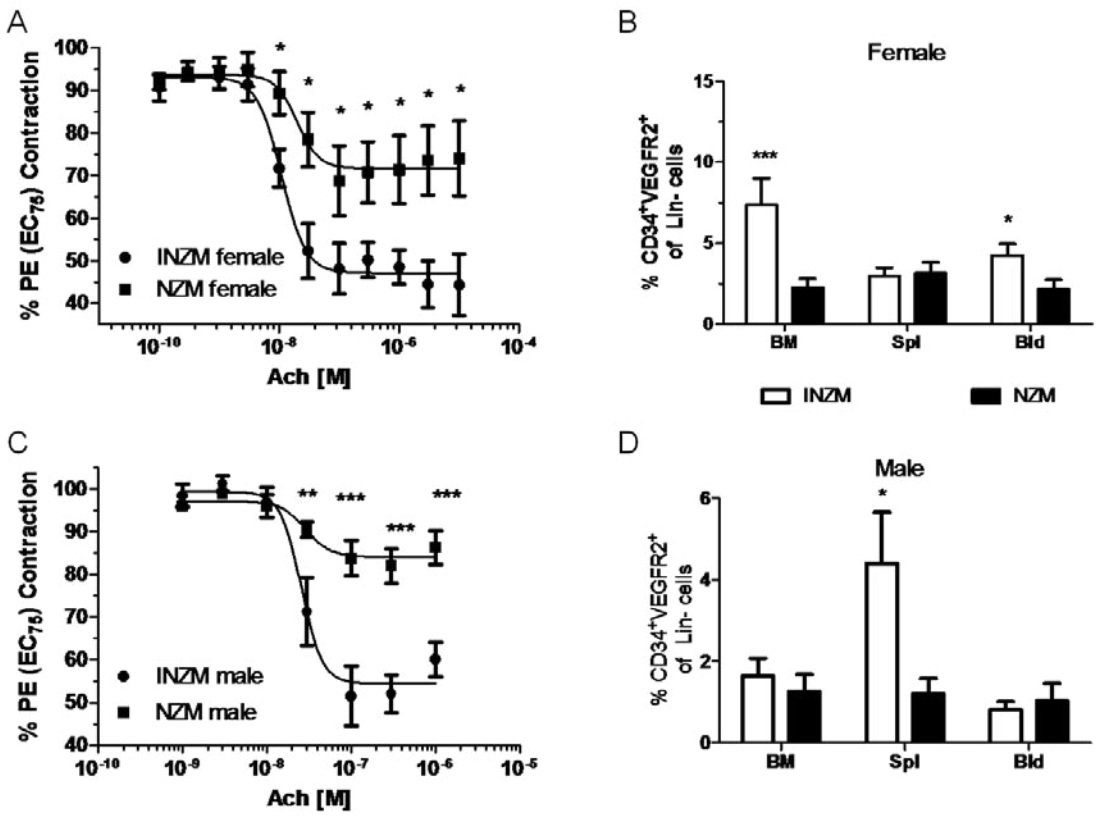


Figure 4-8 Loss of type I IFN signaling results in improved endothelial-dependent function and EPC numbers.

Female and male NZM 2328 mice display improvements in endothelial function and EPC numbers when deficient in type I IFN receptor. **(A)** Female NZM 2328 IFNAR$^{-/-}$ (INZM) mice show improved endothelial-dependent function at 30 weeks of age, when compared to NZM 2328 mice (n=8), **(B)** and increased numbers of EPCs in the bone marrow and in the peripheral blood (n=13). **(C)** Male INZM mice at 40 weeks of age also show improved endothelial-dependent function when compared to NZM 2328 mice (n=6). **(D)** Male INZM mice display increases in EPC numbers in the spleen when compared to NZM 2328 mice (n=6); *p<0.05, **p<0.01, ***p<0.001.

Type I IFN signaling inhibits EPC function and neoangiogenesis in lupus prone mice:

We previously showed that EPCs/CACs from lupus-prone mice have a decreased capacity to differentiate into mature endothelial cells when exposed to proangiogenic stimulation[216]. EPCs/CACs from female INZM displayed increased ability to differentiate into mature endothelial cells, when compared to female NZM 2328. This was observed when either the bone marrow or spleen EPCs were cultured. The improvement in EPC/CAC differentiation was apparent before clinical symptoms of the disease developed (evident by 20 weeks of age) and was maintained after florid disease development at 30 weeks of age (Figure 4-9A, B $p<0.05$). These observations were also confirmed in male mice, where INZM mice displayed significant increases in EPC differentiation when compared to NZM 2328 mice at 40 weeks of age (Figure 4-9C, $p<0.05$). No significant changes were observed at 30 weeks of age in the male mice (data not shown). To address if the changes observed in the *in vitro* assay were also occurring *in vivo*, a Matrigel plug assay was performed to assess the capacity of lupus-prone mice to form new blood vessels. Both female (25-30 weeks) and male INZM mice (30-40 weeks) displayed significantly improved neoangiogenesis when compared to age- and gender-matched NZM 2328 mice (Figure 4-9D $p<0.05$). These results indicate that type I IFNs are involved in the decreased angiogenic response observed in lupus-prone mice, regardless of disease activity or gender.

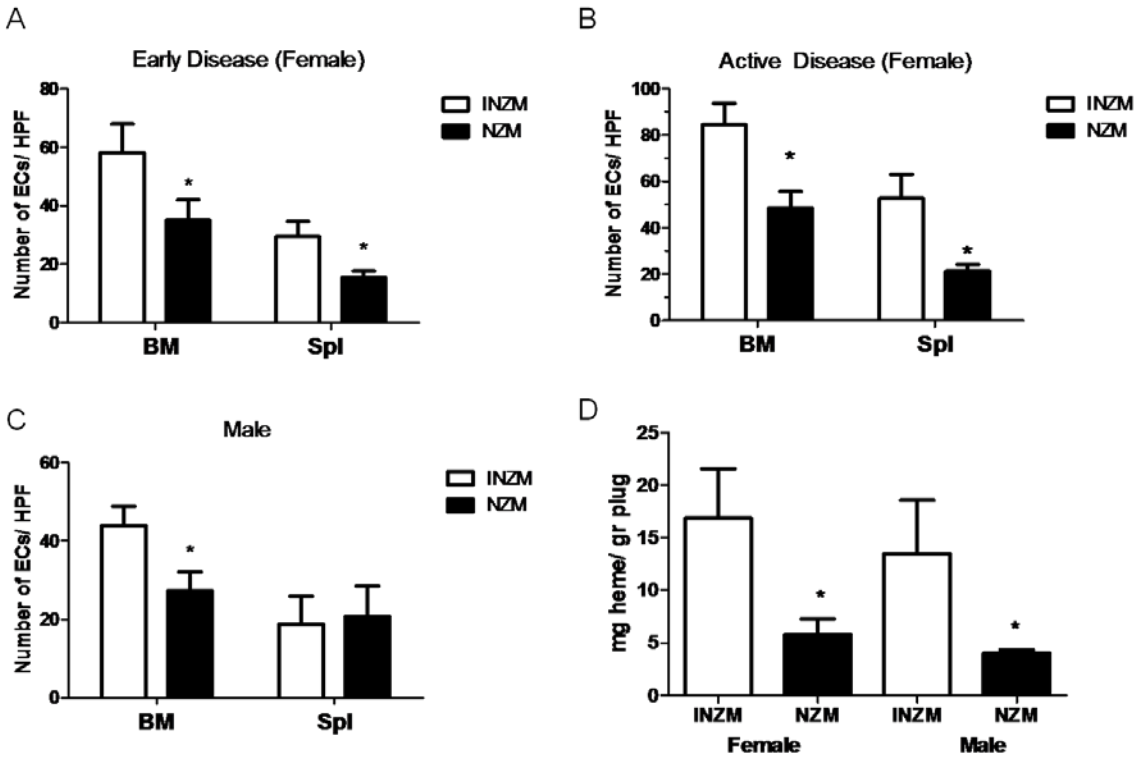


Figure 4-9 Type I IFNs inhibit EPC ability to differentiate into mature endothelial cells and undergo neoangiogenesis.

INZM mice show significantly improved EPC function when compared to NZM 2328 mice. **(A)** Improvement in EPC differentiation into mature endothelial cells occurred in female INZM mice at 20 weeks of age, before active disease development (n=8), **(B)** and was maintained after disease onset by 30 weeks of (n=6). **(C)** Male INZM also showed significant improvement in bone marrow EPC differentiation into mature endothelial cells at 40 weeks of age (n=9). **(D)** Female and male INZM mice display enhanced neoangiogenesis, as measured by the amount of total mg hemoglobin (heme)/g of Matrigel plug (n=16, 25-30 weeks in females; n=14, 30-40 weeks in males).*$p<0.05$.

Acute IFN-α administration induces endothelial and EPC dysfunction:

As lack of type I IFN signaling resulted in significant improvements in endothelial and EPC function in INZM vs. NZM 2328 mice, we explored if acute exposure to enhanced levels of IFN-α, such as those seen in acute infections and/or lupus flares, could promote endothelial and/or EPC dysfunction. NZM 2328 mice underwent systemic administration of an IFN-α-expressing adenovirus (AdIFN-α) or empty adenovirus (AdControl). Efficacy of adenovirus injection was assessed by spleen size at the time of euthanasia as this strongly correlated with presence of type I IFN regulated genes (Appendix E), and IFN-α levels were unable to be reliably detected in the serum by ELISA. Female NZM 2328 mice that received AdIFN-α at 12 weeks of age (before proteinuria onset) displayed a significant worsening of endothelial-dependent function of the thoracic aortas, when compared to recipients of AdControl by 3 weeks post-exposure (Figure 4-10A, $p<0.05$). As expected, AdIFN-α treatment also led to worsening of disease activity and acceleration in the development of nephritis (not shown). In contrast to what was observed in INZM mice or NZB/W mice, acute exposure to enhanced levels of IFN-α did not have a significant effect on either EPC numbers or EPC apoptosis (data not shown). However, EPC/CAC function, as assessed by their capacity to differentiate into mature endothelial cells, was significantly reduced following exposure to AdIFN-α (Figure 4-10C, $p<0.05$).

In order to establish if the defects observed by acute IFN administration were secondary to exacerbation of lupus disease activity, male NZM 2328 mice were also exposed to AdIFN-α or AdControl, as male NZM 2328 mice are spared from lupus development upon exposure to IFN-α. At 12 weeks of age, male NZM 2328 mice also exhibited significantly decreased endothelial-dependent function of the thoracic aortas after acute exposure to AdIFN-α (Figure 4-10B $p<0.01$). Similar to female NZM 2328 mice, acute IFN-α exposure led to no significant changes in EPC numbers or apoptosis (data not shown). In contrast to female NZM 2328 mice, male mice did not display impaired EPC/CAC function

(Figure 4-10D) upon exposure to AdIFN-α. These results indicate that the effects of IFN-α on the vasculature cannot be fully explained by lupus disease activity.

To assess if a lupus-prone background is needed for the deleterious effects of IFN-α on endothelial cell and EPC function, female BALB/c mice were similarly administered AdIFN-α or AdControl. BALB/c mice also displayed decreased endothelium-dependent function of thoracic aortas following acute exposure to IFN-α (Figure 4-10E $p<0.01$). As in the case of NZM 2328 females, evidence of EPC dysfunction was also observed after acute exposure to IFN-α (Figure 4-10F $p<0.05$) while no changes were observed in EPC numbers or apoptosis (data not shown). These results demonstrate that type I IFNs can cause endothelial cell and EPC dysfunction independent of lupus disease and autoimmune background.

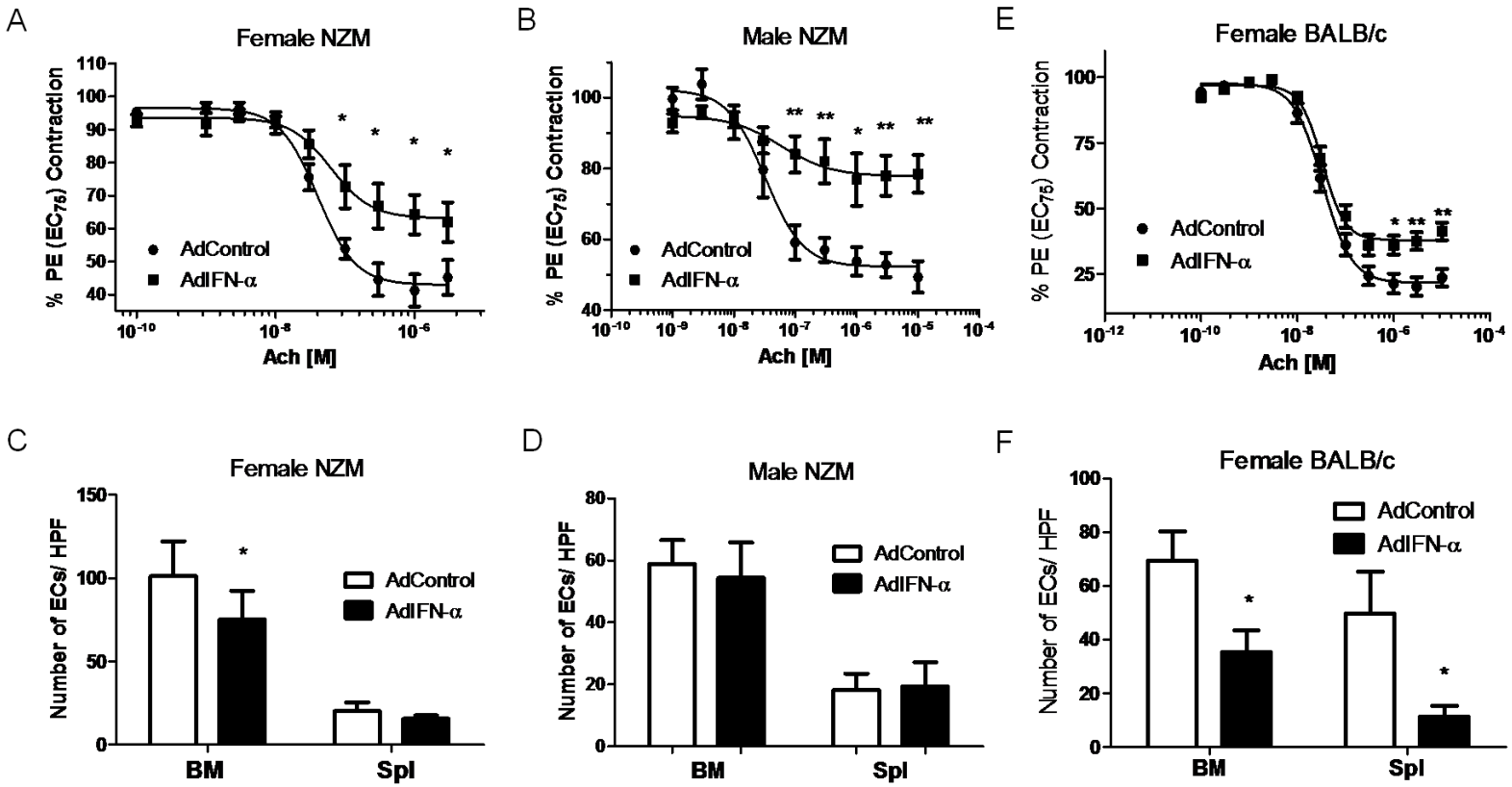


Figure 4-10 Acute exposure to IFN-α induces decreased endothelial cell and EPC function.
(A) Pre-nephritic female NZM 2328 mice exposed to IFN-α adenovirus (AdIFN-α) display decreased endothelial function (15 weeks, n=10), **(C)** and reduced EPC capacity to differentiate into mature endothelial cells, when compared to age matched NZM 2328 mice treated with control adenovirus (AdControl) (n=11). **(B)** Male NZM 2328 mice similarly exposed to AdIFN-α also demonstrate decreased endothelial cell function (15 weeks, n=4), **(D)** while EPC function was not significantly altered (n=5). **(E)** Non autoimmune BALB/c mice also exhibit a decrease in endothelial cell function (15 weeks, n=12) **(F)** and EPC function (n=12) in response to acute IFN-α exposure; *p<0.05, **p<0.01, ***p<0.001.

Type I IFNs promote atherosclerosis development:

As endothelial cell dysfunction can be a predictor of plaque development[114], and type IFN-α has been shown to increase foam cell formation[161], we determined if type I IFNs could directly affect atherosclerosis severity. To this end ApoE$^{-/-}$ mice, a widely used mouse model of atherosclerosis[120], were studied, as both NZB/W and NZM 2328 mice are genetically resistant to developing plaque. The effects of type I IFNs on atherosclerosis were tested in two complimentary ways, either through acute exposure to AdIFN-α at the time of Western diet initiation or through genetic abrogation of type I IFN signaling. After 12 weeks on a Western diet, female and male ApoE$^{-/-}$IFNAR$^{-/-}$ mice showed significantly reduced atherosclerotic lesion size (Figure 4-11A, p=0.016). Additionally there was a trend for ApoE$^{-/-}$ mice exposed to IFN-α to have larger lesions size, although this did not reach significance (Figure 4-11A, p=0.16). Representative images of atherosclerotic lesions in ApoE$^{-/-}$IFNAR$^{-/-}$ and ApoE$^{-/-}$ mice are shown in Figure 4-11B.

Aberrant vascular repair has been proposed to play a role atherosclerosis development in ApoE$^{-/-}$ mice[148]. Therefore, the angiogenic potential of these mice was also assessed. When compared to ApoE$^{-/-}$ mice, ApoE$^{-/-}$IFNAR$^{-/-}$ displayed increased angiogenic capacity in an *in vivo* Matrigel plug assay. These results indicate that lack of the IFNAR promotes enhanced capacity for neoangiogenesis and vascular repair (Figure 4-11C, $p<0.05$).

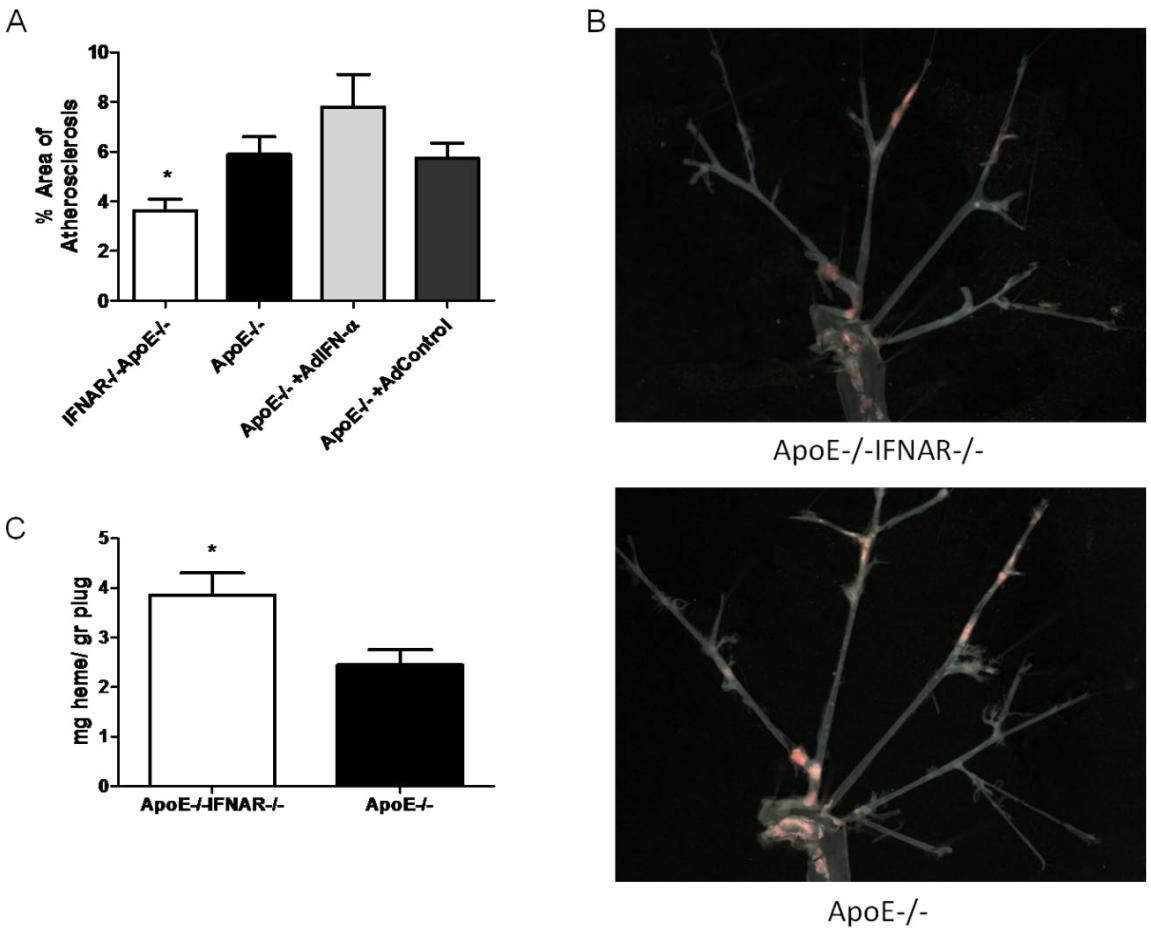


Figure 4-11 Type I IFNs promote atherosclerosis lesion progression in ApoE$^{-/-}$ mice.

ApoE$^{-/-}$ atherosclerotic lesion formation is sensitive to perturbations in type I IFN signaling. **(A)** ApoE$^{-/-}$IFNAR$^{-/-}$ displayed reduced atherosclerotic lesion size, while ApoE$^{-/-}$ mice administered IFN-α displayed a non significant trend in increased lesion size ($p<0.05$, n=9 or $p<0.077$, n=13 respectively). **(B)** A representative image of aortic atherosclerosis in ApoE$^{-/-}$ and ApoE$^{-/-}$IFNAR$^{-/-}$ visualized with Oil Red O. **(C)** ApoE$^{-/-}$IFNAR$^{-/-}$ mice display increased neoangiogenesis, as assessed by amount of hemoglobin (heme)/Matrigel plug (n=11); *$p<0.05$.

Effects of type I IFNs on thrombosis:

In both idiopathic atherosclerosis and in SLE, thrombosis can have serious adverse effects and promote development of acute coronary syndromes. SLE patients are at increased risk of thrombosis development which appears to be multifactorial. Additionally, type I IFNs can affect the platelet transcriptome[166]. Therefore, to address if type I IFNs could promote this potentially lethal complication, a model of arterial thrombosis following photochemical injury was used. After acute exposure to AdIFN-α, ApoE mice displayed arterial thrombosis at an accelerated rate (Figure 4-12A, $p<0.05$); in contrast, no significant effect was seen when $ApoE^{-/-}IFNAR^{-/-}$mice were compared to $ApoE^{-/-}$ mice (Figure 4-12A). The increased thrombosis in $ApoE^{-/-}$ mice upon acute exposure to AdIFN-α was associated with an increase in endothelial and platelet activation, as measured by serum P-selectin levels (Figure 4-12 B, $p<0.05$). Elevated levels of soluble P-selectin were also increased in $ApoE^{-/-}IFNAR^{-/-}$ (Figure 4-12B, $p<0.05$); however, $ApoE^{-/-}IFNAR^{-/-}$ mice had a trend of decreased amount of monocyte platelet aggregates, another marker of platelet activation, which did not reach significance (data not shown). Further, acute exposure to IFN-α resulted in endothelial and platelet activation in both BALB/c and NZM 2328 mice (Figure 4-12C, $p<0.05$). In contrast to what was observed for $ApoE^{-/-}$ mice, levels of soluble P-selectin were decreased in 30 week old female INZM mice (Figure 4-12C, $p<0.05$). These results indicate a possible dual role for type I IFNs in both activation and downregulation of both endothelial cells and platelets, depending on mouse strain, that requires further investigation.

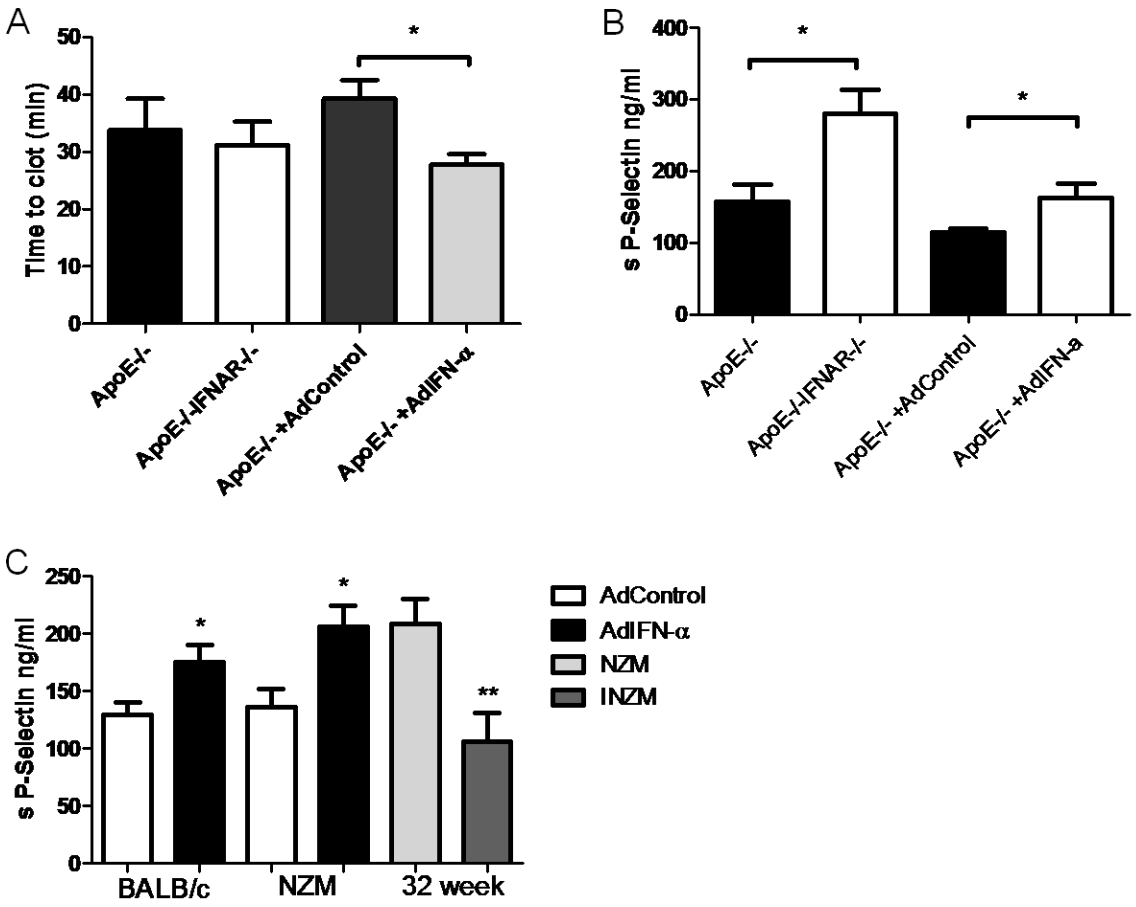


Figure 4-12 Type I IFNs accelerate thrombosis and endothelial and platelet activation.

ApoE$^{-/-}$ mice administered AdIFN-α have accelerated clotting. **(A)** ApoE$^{-/-}$ mice administered IFN-α displayed significantly faster clotting times, while lack of type I IFN signaling had no effect on clotting time (n=14 and 10, respectively). **(B)** Both lack of type I IFN signaling and acute exposure to IFN-α induced increased levels of soluble P-selectin in the serum of ApoE$^{-/-}$ (n=4 and 8, respectively). **(C)** In non-atherosclerotic prone mice administration of IFN-α increased soluble P-selectin levels, while abrogation of type I IFN signaling in NZM 2328 mice reduced levels of soluble P-selectin (n=6, n=6 and n=7, respectively). *p<0.05, **p<0.01.

Summary

The results from this study suggest that the NZB/W murine model recapitulates the abnormal vascular phenotype in human SLE, which includes endothelial cell dysfunction, decreased EPC numbers and impaired EPC function[153,154,217,218]. 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 endothelial cells. Decreases in EPCs in NZB/W mice were already apparent in the bone marrow before overt clinical disease developed. The significant decreases in bone marrow and splenic EPCs that ensues as lupus 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[148]. Further, this vascular defect is dependent on type I IFN signaling. NZM 2328 mice lacking the IFNAR displayed improved endothelial cell function and improvements in EPC numbers, but did not display any improvement in degree of EPC apoptosis. The changes seen were not secondary to lupus disease activity, as similar improvements were also observed in male NZM 2328 which are protected from clinical disease. These results demonstrate that type I IFNs play a role in endothelial and EPC/CAC function which is separate from their effects on SLE disease progression. Further, acute IFN-α exposure accelerated the degree of endothelial cell dysfunction in both male and female NZM 2328 mice, and decreased EPC function in female mice. IFN-α also significantly impaired endothelial cell and EPC function in non-autoimmune BALB/c mice, suggesting that the effects observed in lupus-prone mice are not solely due to their autoimmune genetic background. Interestingly, the acute exposure to IFN-α was not sufficient to deplete EPC numbers in the time-frame examined in this study.

Type I IFNs have previously been implicated in atherosclerosis lesion instability[162,163], and this study extends those findings to demonstrate that type I IFNs play a role in plaque lesion development. Further, the decreased atherosclerosis progression observed in mice lacking type I IFN signaling is associated with an increased angiogenic potential. This could be directly linked

to modulation of vascular repair, as these mice also displayed increased angiogenic capacity in *in vivo* and *in vitro* studies.

Previous work has indirectly linked type I IFNs to platelet activation and thrombosis in SLE[166]. In the studies describe above, ApoE$^{-/-}$ mice administered IFN-α had an increased rate of thrombosis and this observation was associated with increased levels of soluble P-selectin a marker of platelet activation; however recent work has illustrated that the main producer of soluble P-selectin in mice are endothelial cells[219,220]. This indicates that acute exposure to type I IFNs results in increased endothelium activation/dysfunction which then leads to a prothrombotic environment, but our experiment can also not rule out a role of platelet activation in the generation of this prothrombotic environment. This increased activation of both endothelial cells and platelets was also observed in both BALB/c and NZM 2328 mice that received acute administration of exogenous IFN-α. It is unclear why the lack of the IFNAR on the ApoE$^{-/-}$ background would increase soluble P-selectin levels but decrease monocyte platelet aggregates, while an opposite effect was seen on the NZM 2328 lupus background. One possible explanation for these differences in the dyslipidemia typically observed in the ApoE$^{-/-}$, as this is known to activate platelets and impair endothelial function[221]. Additional studies are needed to determine how type I IFNs modulate endothelial and platelet activation in autoimmune and nonautoimmune background and the interplay of these molecules, plasma lipids and other factors.

Overall, these results indicate that type I IFNs play a pivotal role not only in general disease pathogenesis in SLE, but specifically in the increased CV risk that affects individuals with this disease. Indeed, our results indicate pleiotropic effects of this group of cytokines on the vasculature from early endothelial cell damage and dysregulated repair of the damaged vasculature, to the development of atherosclerotic plaque, its destabilization, and the development of acute coronary syndromes through thrombosis enhancement.

Chapter 5
Discussion and Conclusions

Type I IFNs modulate angiogenesis and vasculogenesis through global effects on IL-1 pathways:

While it is widely accepted that SLE patients exhibit a greater propensity to develop CV complications[10,14], the mechanisms leading to this enhanced risk remain unclear. Our group and others have previously proposed that IFN-α (and potentially other type I IFNs) may play a prominent role in promoting vascular damage in SLE, at least in part, by interfering with the balance between vascular damage and repair, thereby promoting the development of a dysfunctional endothelium[153,154]. Indeed, abnormalities in vasculogenesis have been proposed to play an important role in the development of atherosclerosis and organ damage (including renal failure) in various diseases[121,143,144,222,223].

This work focused on IFN-α given previous evidence for its antiangiogenic effects. Up to this date, the exact mechanisms by which IFN-α exerts its antiangiogenic effect have been unclear and work has primarily focused on cancer cell lines. The first aim of this work addressed the molecular mechanisms by which IFN-α induces aberrant vascular repair in EPCs/CACs. While there was a heterogeneous set of genes induced by IFN-α, with modulation of both angiogenic and antiangiogenic genes, the balance was strongly in favor of the induction of an antiangiogenic profile . We demonstrated that IFN-α exerts its antiangiogenic effects on EPCs/CACs through the modulation of molecules relevant to IL-1 function and signaling, as well as through downregulation of the proangiogenic molecule VEGF-A. In peripheral blood EPCs/CACs, this significant modulation may lead to a drastic impairment in the downstream

effects of IL-1 signaling, by decreasing the availability of IL-1α and β, and by increasing the endogenous inhibitors of IL-1 signaling. Further, previous studies have suggested that lupus PBMCs are less capable of releasing IL-1β in response to stimulation[224]. However, a link between these abnormalities and IFN-α, and its potential role in lupus vascular damage, had not been previously reported. The results reported above support studies performed in other patient populations, where exogenous type I IFNs were found to downregulate IL-1 and upregulate IL-1RN. In addition, these results also support previous reports on the effect of IFN-α on healthy control myeloid cells *in vitro,* where IL-1RN is upregulated without inducing an increase in IL-1[225-227]. It also appears that the effects of type I IFNs on IL-1 may vary significantly depending on the dose used to treat the cells[228]. As such, the high levels of type IFNs observed in SLE may lead to a severe downregulation of IL-1 signaling pathways that promote a strong antiangiogenic response. Supporting the hypothesis that this antiangiogenic effect is general to type I IFNs, IFN-β can inhibit the stimulatory capacity of T cells on monocytes to synthesize IL-1β. IFN-β can also induce upregulation of IL-1RN in monocytes[229,230].

Our results demonstrate that the antiangiogenic response induced by type I IFNs is mediated primarily through the JAK/STAT pathway. Indeed, inhibition of JAK signaling resulted in suppression of both the antiviral and the antiangiogenic gene responses. The effects of JAK inhibition on EPCs/CACs is similar to what has been seen in other angiogenesis models[231] and on different cell types including Mφ[232].

While IL-1β is predominantly considered a proinflammatory molecule produced in response to infection or cellular damage, it also has strong proangiogenic properties[233,234]. Further, recombinant IL-1RN (anakinra) has been used as antiangiogenic therapy in various diseases[235]. IL-1α and β have strong proangiogenic effects on murine and human EPCs[200,236] and IL-1β promotes tumor angiogenesis[233]. Part of the effects mediated by IL-1β on angiogenesis is through regulation of various proangiogenic factors, including VEGF-A[237,238]. Indeed, IL-1 promotes VEGF-A synthesis[239] and genes in a

cluster of IL 1-related molecules (*Il1f9*, *Il1f10*, *Il1rn*), including IL-1RN, have been proposed to regulate VEGF-induced angiogenesis[240]. This may explain, at least in part, why the strongest responses in improvement of EPC/CAC function in the SLE cultures were observed with addition of IL-1β, as this molecule may have upregulated endogenous VEGF-A expression in addition to other proangiogenic effects. IL-1β also plays a key role in ischemia-induced neovascularization by mobilizing EPCs in a VEGF-dependent manner as well as by upregulating expression of VEGF, VEGFR-2 and adhesion molecules on endothelial cells[241]. IFN-α also downregulates VEGF-A, either directly through potential effects on its promoter, and/or indirectly through changes in IL-1 signaling. All these abnormalities may lead to defective repair mechanisms and ongoing vascular damage in SLE and therefore may contribute to atherosclerosis development.

The role of proinflammatory stimuli on EPC's viability and function is conflicting as both enhanced EPC death[242] and promotion of endothelial differentiation[201] have been reported. Our experiments do not address the effects of IL-1β on EPC/CAC function in a proinflammatory setting but, rather, in a noninflammatory proangiogenic context. In a proinflammatory setting IL-1β may indeed have different effects to those observed in our study. When SLE EPC/CACs were treated with IL-1β in a proangiogenic setting, there was a substantial improvement in EPC/CAC differentiation and survival. This response was unique to IL-1β, as treatment with IL-6 and TNF-α did not result in a similar restoration and VEGF-A had a much less pronounced beneficial effect. These findings indicate that the abnormal EPC/CAC phenotype and function observed in SLE cannot be attributed solely to a general loss of proangiogenic molecules[197]. Further, IL-1β was also able to inhibit the skewing of myeloid cells present in the proangiogenic culture from CACs to mature DCs[153,243] a phenomenon previously reported to be induced by IFN-α. While this may seem counterintuitive as IL-1β is widely used to promote DC maturation, this can be at least partially explained by the fact that it is generally used in combination with other proinflammatory cytokines such as

TNF-α and CD40L[244-247]. A previous study has reported that IL-1β can impair maturation in DCs treated with rapamycin[248], but to our knowledge no other studies have examined the isolated effects of IL-1β treatment on DCs or differentiating monocytes.

Decreased IL-1 and increased IL-1RN may theoretically support a phenotype that protects the vasculature, given the anti-inflammatory effects, but in a disease like SLE where there is evidence of ongoing endothelial cell damage and death, a cytokine profile that promotes an antiangiogenic response would be overall deleterious. Further this antiangiogenic environment could synergize with enhanced vascular death (due to putative vascular insults which are present in lupus including autoreactive and/or activated T-cells[249,250], neutrophils[47], autoantibodies[251], and/or type I IFNs) to accelerate atherosclerosis development.

To our knowledge, our study reports for the first time that vasculogenesis is impaired *in vivo* in human lupus. This was evident by the observations that kidneys from SLE patients display decreased renal vascular density and prominent vascular rarefaction, coupled to an antiangiogenic signature in blood vessels. These results are consistent with our hypothesis that patients with SLE have impaired vascular repair. These findings also have potential to increase our understanding of the mechanisms leading to loss of renal function in patients with SLE. A vascular component appears to be a key driver in the pathogenesis of renal scarring in various conditions. Progressive interstitial fibrosis is associated with the loss of peritubular capillaries[252,253]. VEGF-A is an important endothelial cell angiogenic, survival, and trophic factor and is constitutively expressed in human and rodent kidneys[254,255]. The kidney is a highly vascularized organ and has two sequential microvasculature beds: glomerular and peritubular capillaries. Loss of these capillaries is strongly associated with the progression of chronic kidney disease to end-stage renal disease in other populations and animal models[256]. Rodent models of progressive renal failure show progressive loss in glomerular and tubular VEGF-A coinciding with loss of glomerular and

peritubular capillaries and the subsequent development of glomerulosclerosis and interstitial fibrosis[257]. In humans, pharmacologic inhibition of VEGF-A during cancer treatment can lead to proteinuria and worsening kidney function[258]. Further, glomerular and peritubular capillary rarefaction is an important feature of disease progression in other conditions including diabetic nephropathy[259]. Recently, a role for both glomerular and tubular VEGF-A contributing to the maintenance of glomerular or peritubular capillaries and renal tissue survival has been proposed in diabetes[260], as the decrease of renal VEGF-A expression in diabetic kidneys was associated with a reduction in peritubular capillary densities, similar to what was observed in our study of lupus nephritis biopsies. Interestingly, other renal diseases including minimal change disease or idiopathic membranous nephritis have not shown the reduction of tubulointerstitial VEGF-A, indicating that renal damage or proteinuria *per se* does not lead to downregulation of this proangiogenic molecule[260]. This was confirmed in our study, where patients with $ANCA^+$ vasculitis and renal damage did not display downregulation of VEGF-A or renal capillary drop-out.

Several studies have also linked progression of renal disease to certain IL-1RN polymorphisms which are associated to higher IL-1RN levels in the general population[261,262]. However, the exact mechanisms linking renal failure progression to high levels of this anti-inflammatory molecule had not been characterized. Given the observations made in our study, it is possible that the antiangiogenic effect of increased IL-1RN further promotes a reduction in renal capillaries which could hamper renal perfusion. Indeed, in our study, decreased VEGF-A and increased IL-1RN observed in lupus nephritis biopsies correlated with the development of vascular rarefaction. As such, this antiangiogenic response triggered by type I IFNs in SLE kidneys could contribute to progression of kidney damage. This hypothesis is also supported by recent observations that type I IFNs produced by resident renal cells promote end-organ disease in autoantibody-mediated glomerulonephritis[263] and that systemic administration of IFN-α to lupus murine models worsens

nephritis[264]. While the effects of enhanced IFN-α in lupus nephritis are likely multifactorial, the potential role of this cytokine in leading to microvascular rarefaction and progression of renal failure may play an important role.

To summarize, we have identified a novel pathway by which IFN-α interferes with EPC/CAC function in SLE and may lead to abnormal vascular repair, atherosclerosis progression and loss of renal function in this disease.

Type I IFNs accelerate atherothrombosis, endothelial and EPC dysfunction *in vivo:*

Prior to our studies, it was unclear if any lupus-prone mouse strain displayed a similar phenotype to human SLE with regards to vascular dysfunction and aberrant vascular repair. The results of the experiments described above demonstrate that the NZB/W murine model recapitulates the abnormal findings in EPC phenotype and function and in aberrant endothelial reactivity recently reported in human SLE[153,154,217,218]. These abnormalities include decreases in numbers of EPCs in spleen and bone marrow, increased EPC apoptosis and decreased functional capacity of these cells to differentiate into mature endothelial cells. Importantly, decreases in EPCs in NZB/W mice are apparent in the bone marrow before overt clinical disease develops, perhaps as a result of ongoing vascular damage mobilizing the EPCs from the bone marrow. 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 are apoptotic. The significant decreases in bone marrow and splenic EPCs that ensues as lupus disease progresses could indicate exhaustion of the EPC pool in NZB/W mice, similar to previously reported murine models of atherosclerosis[148]. Interestingly, this defect could not solely be attributed to lupus disease activity or renal failure, as B6/*lpr* mice, with similar levels of renal dysfunction, do not display any impairment in endothelial cell or EPC function.

The mechanisms that lead to abnormal endothelial cell and EPC function in the NZB/W mice, but not in the B6/*lpr* mice, remain to be defined and are likely to be multifactorial. Indeed, genetic differences, autoantibodies, immune complexes and other immunological abnormalities could all play differential roles in vascular damage in both models. One possible explanation for the differences seen in endothelial cell and EPC function in these mice are differential responses

to type I IFNs. These cytokines have been proposed to play an important role in disease development and progression in NZB/W mice and derived congenic strains[110], while the B6/*lpr* mice do not depend on type I IFNs for their disease progression and may actually be protected by their effects[265]. Additionally, our group has shown that, exposure of control EPCs to recombinant IFN-α recapitulates the phenotype observed in human SLE EPCs[153]. Furthermore, neutralization of type I IFNs *in vitro* results in restoration of the normal capacity of human lupus EPCs to differentiate into mature endothelial cells[153]. While the precise mechanisms leading to aberrant EPC phenotype and function and endothelial dysfunction in the NZB/W mice remain to be determined, an attractive hypothesis is that, similar to human lupus, type I IFNs play a major role 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 various NZB/W EPC compartments, including bone marrow and spleen, 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*, mice at the preautoimmune stage were found to have increased expression of many known type I IFN regulated genes[266]. Further, nonautoimmune BALB/c bone marrow murine EPCs lose their 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 previously in human lupus EPCs[153].

IFN-α can decrease colony formation of hematopoietic progenitor cells[267], 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[268]. 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, or result in loss of functional capacity of progenitor cells in the bone marrow compartment[95,96]. This could lead to decreased mobilization and homing into damaged vascular structures, therefore

promoting endothelial dysfunction. The variation in EPC levels in different mouse organs requires further investigation; differences may be related to variations in 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 suggest that the bone marrow niche could be exposed to even higher levels of this cytokine than other peripheral compartments[49].

To directly address the role of type I IFNs in the aberrant vascular phenotype observed in both human and murine lupus, the NZM 2328 mouse model was selected. This mouse strain is derived from NZB/W mice, and female NZM 2328 mice have a very similar lupus disease progression to the parental strain. Further the direct effects of type I IFNs could be assessed by comparing wild-type NZM 2328 mice to NZM 2328 mice that lack the type I IFN receptor. The results strikingly indicate that female NZM 2328 mice that lack type I IFN signaling display improved endothelium-dependent responses, when compared to wild-type NZM2328 mice. This improvement in endothelium-dependent function is associated with increases in the number of bone marrow and peripheral blood EPCs. In addition, female INZM mice show improved bone marrow and spleen EPC differentiation. These observations were not primarily related to an improvement in lupus disease activity in the mice lacking type I IFN signaling, as similar differences were found when comparing male NZM 2328 and INZM mice, both of which are devoid of lupus characteristics.

In contrast to what was seen in NZB/W mice, neither female nor male NZM 2328 mice displayed differences in the degree of EPC apoptosis when compared to mice with impaired type I IFN signaling. These results suggest that in this mouse model, contrary to what has been observed in humans, the deleterious effects of type I IFNs on the vasculature are not secondary to enhanced EPC apoptosis. Future studies should address if this observation is characteristic of various lupus mouse models or more specific to the NZM 2328 model.

As abrogation of type I IFN signaling resulted in improved endothelial cell and EPC function in lupus-prone mice, we also addressed if administration of exogenous IFN-α using an adenovirus vector would be sufficient to exacerbate endothelial cell and EPC dysfunction in NZM 2328 mice. Additionally, age-matched male NZM 2328 mice were used to control for effects due to lupus immune dysregulation and/or to renal disease, as exogenous IFN-α accelerates disease progression in female, but not in male mice. Furthermore, the effect of IFN administration on female BALB/c mice was also examined to determine if the effect of IFN-α were dependent on a lupus prone genetic background. Female NZM 2328 are exquisitely sensitive to IFN-α and succumb rapidly to renal disease; therefore, a lower dose of virus was administered to these mice. Acute exposure to IFN-α resulted in significantly impaired endothelial-dependent vascular responses in female and male NZM 2328 mice and in BALB/c mice, when compared to mice treated with control adenovirus. These results convincingly demonstrate that type I IFNs can induce endothelial dysfunction, irrespective of lupus disease activity, gender or genetic background. Furthermore, female NZM 2328 and BALB/c mice displayed decreased function of EPCs following IFN-α exposure, while male NZM 2328 mice did not show significant impairment in EPC function. This interesting finding suggests that sexual hormones may modulate EPC responses to type I IFNs. Further, estrogen is known to mobilize EPCs from the bone marrow[269,270] and may modify EPC behavior in other yet uncharacterized ways.

While we did not evaluate atherosclerosis as an outcome in lupus prone mice, given their resistance to the development of this complication, NZB/W and NZM 2328 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[212]. This was observed without exposing the mice to a Western diet which could have further enhanced these abnormalities.

Unlike observations seen in our previous studies, acute IFN-α exposure did not modulate EPC numbers or apoptosis. This observation may be explained

by the recent finding that type I IFNs have the ability to increase progenitor cell proliferation and turnover[95,96]. This could counteract, at least in the short term, any decrease in EPCs due to toxicity or to consumption. It is also possible that the time-points at which we chose to examine EPC numbers and cell death after IFN exposure may not have been the ideal to detect enhanced apoptosis. While no changes in EPC numbers and viability were observed upon acute exposure to IFN-α, this cytokine did lead to impairment in their functional capacity to differentiate into mature endothelial cells. Furthermore, these findings correlated with evidence of significant enhancement in new blood vessel formation *in vivo* in female and male lupus-prone mice that lack type I IFN receptor. These observations support the hypothesis that type I IFNs significantly interfere with vascular repair and new blood vessel formation, a phenomenon that could play an important role in atherosclerosis development and progression in SLE and in other conditions associated to accelerated atherosclerosis[143].

Our results support previous findings that EPCs play a key role in vascular health[143,144,271,272], as the development of endothelial dysfunction in the NZB/W and NZM 2328 mice correlated with decreased EPC numbers and function. Further, in NZB/W mice, similar to what has been reported in human SLE, endothelial dysfunction also correlates with enhanced EPC death.

Endothelial dysfunction is considered to be one of the earliest events in the development of atherosclerosis and a promoter of disease progression and trigger for CV events[114,273,274]. 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[19]. While renal dysfunction has been associated with the development of endothelial dysfunction and abnormal EPC phenotype and function[275-278], it is unlikely that lupus nephritis significantly contributed to these abnormalities in NZB/W and NZM 2328 mice, as B6/*lpr* mice with similar degrees of renal dysfunction and established SLE features did not display these vascular abnormalities. Furthermore, the differences in endothelial function in the

presence or absence of type I IFN signaling were observed in male NZM 2328, which do not develop lupus or renal involvement. While the B6/*lpr* phenotype can enhance the development of atherosclerosis when compounded by ApoE deficiency[169], 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[153,212]. The lack of endothelial and EPC dysfunction in B6/*lpr* mice in the active disease stage, and the improvements seen in male INZM mice, support that the abnormalities observed in NZB/W and NZM 2328 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[153,212].

While similar vascular phenotypes were observed in both the absence of type I IFN signaling and acute exposure to IFN-α, it is unclear if the mechanisms generating this shared phenotype are completely shared. For example, improvements in endothelial function are not seen in INZM mice until 30 weeks of age, while acute exposure to type I IFNs results in endothelial dysfunction within 3-5 weeks post-exposure. Further work is warranted to better understand the mechanisms resulting in the vascular changes induced by acute type I IFN exposure vs. chronic lack of type I IFN signaling.

As acute administration of IFN-α promotes endothelial dysfunction in both autoimmune and nonautoimmune backgrounds and, given evidence that type I IFN levels correlate with atherosclerotic plaque instability in humans[162,163], we examined the putative role of type I IFNs in atherosclerosis development in non-lupus-prone mice. Atherosclerosis-prone ApoE$^{-/-}$ mice that were crossed to mice lacking IFNAR had significantly reduced atherosclerotic lesion development, and increased vascular repair, as demonstrated by improved *in vivo* angiogenesis. These results provide support for the possible role of aberrant vascular repair in the progression of atherosclerosis. Furthermore, our observations support a putative role for type I IFNs in altering this vascular repair and promoting plaque formation. ApoE$^{-/-}$ mice acutely exposed to IFN-α did have a trend toward

increased lesion size, but this did not reach significance. However, the exposure to acute exogenous IFN-α did significantly increase the prothrombotic potential, as indicated by reduced time to clot after photochemical injury and by enhanced endothelial and platelet activation. Increased endothelial and platelet activation was also observed in BALB/c and NZM2328 mice that received IFN-α, while female INZM mice had evidence of decreased endothelial and platelet activation. The exact mechanisms behind the increased platelet activation and increased thrombotic potential seen in our experiment are not known. Further research is need to elucidate if platelet activation is secondary to a direct action of IFN-α on platelets or on megakaryocytes, as has been recently reported in human studies[166], and/or to endothelial activation and damage triggered by IFN-α. Further complicating the analysis of these results are recent observations that platelets from patients with lupus that are activated by circulating immune complexes form aggregates with antigen presenting cells (APC), leading to enhancement of IFN-α secretion. One can speculate that a feed-forward loop could develop, wherein type I IFNs would induce platelet activation, which would trigger more platelets APC aggregates and further secretion IFN-α and enhance the thrombosis risk.[279]

In summary, type I IFNs induce an aberrant vascular phenotype in both lupus-prone mice and nonautoimmune mice and in atherosclerosis-prone mice. This aberrant vascular phenotype is characterized by the development of endothelial dysfunction and abnormal numbers and function of EPCs. NZB/W and NZM 2328 mice therefore represent good models to study the mechanisms leading to endothelial dysfunction and abnormal vasculogenesis in lupus. Further, we have provided evidence that type I IFNs play a significant role in atherothrombosis progression. These results provide additional insights into the mechanisms behind the increased CV risk in SLE, and on potential immune-mediated mechanisms leading to atheroma formation in the general population.

Concluding Remarks, Future Direction, Implications:

Our work has demonstrated a novel pathway by which IFN-α exerts its antiangiogenic effects on EPCs. This previously unrecognized effect on IL-1 pathway modulation opens new insights into how type I IFNs may interfere with vascular repair. As such, these results increase our understanding of the mechanisms by which SLE patients are at greater risk of developing CVD, and enhance the prominent role of type I IFNs in organ damage in SLE.

Some additional aspects that require further clarification deal with the downstream pathways by which type I IFNs cause transcriptional repression of proangiogenic factors. Indeed, while JAKs most commonly signal through STATs, there is very little evidence that the latter mediate repression of gene transcription. STATs mediate the upregulation of antiviral genes predominantly through ISGF3, but it is possible that transcriptional repression of angiogenic molecules occurs through a different mechanism. Further dissecting the signaling pathways by which IFN-α modulates the antiangiogenic response will allow a more rapid identification of therapies that target the antiangiogenic properties of type I IFNs, while leaving its antiviral properties intact. Better understanding of these mechanisms may have benefits beyond SLE; for example, in the cancer field it may lead to a maximization of the antiangiogenic properties of type I IFNs to limit tumor growth.

A limitation of our work was that we did not address if IL-1β functions in angiogenesis are differently altered in a proinflammatory environment. Nevertheless, our findings have implication beyond lupus and give a note of caution to those treating patients with medical conditions that require treatment with exogenous type I IFNs, including multiple sclerosis and viral hepatitis. It is possible that CV risk could be enhanced in these patients and future studies should address if this merits clinical monitoring during and after treatment. Additionally, as mentioned above, even though recombinant IL-1RN has been successfully used in various inflammatory conditions including rheumatoid arthritis and various autoinflammatory diseases[280,281], the exact role that

exogenous IL-1RN could play in overall CV risk in these diseases is unclear. While small numbers of SLE patients have been treated with recombinant IL-1RN without major adverse effects[282,283], no studies have systematically addressed the effect of this compound on vascular risk in SLE or other systemic autoimmune diseases. Our study adds a note of caution that should be further investigated with regards to the role of IL-1 blockade in conditions with increased vascular damage or abnormal vascular repair, including SLE and RA[144,153].

Our results also have implications in the larger field of atherosclerosis. While type I IFNs have been implicated in plaque destabilization[162,163], no other studies to this point had linked type I IFNs to development of atherosclerosis through endothelial dysfunction and impaired vasculogenesis. This has broad implications, as type I IFNs are induced by a variety of pathogens. Therefore, we could speculate that in susceptible individuals, pathogen induced type I IFNs could play an important role in “idiopathic” atherosclerosis development and in increased atherothrombotic risk and acute coronary syndromes due to plaque destabilization. Further work is needed to elucidate the role of IL-1 modulation and vascular repair in the setting of atherosclerosis, as mice deficient in IL-1 have reduced atherosclerotic lesions[284,285]; however, the exact cause of the reduction is not well understood and may be secondary to global changes in lipid metabolism or inflammation.

Our work has identified two murine lupus models, the NZB/W and NZM 2328 strains, which recapitulate the aberrant vascular phenotype reported in SLE patients. As in human SLE, disease progression in these mice is dependent on type I IFNs. This identification of lupus-prone mice which have similar vascular defects as seen in human SLE has the potential to greatly increase our understanding of the mechanisms that are enhancing the CV risk in SLE *in vivo*. These models will allow future studies to further explore the mechanisms by which type I IFNs promote enhanced CV risk in SLE, and may provide further rationale for the treatment of SLE with agents that target type I IFNs for the prevention or amelioration of CVD. Additionally, bone marrow chimeras utilizing the NZM 2328 and INZM mouse strains will allow for the potential identification of

the cell types responsible for the enhanced type I IFN effect leading to increases in CV risk in SLE. Furthermore, these studies will allow us to identify if type I IFNs act directly on the endothelium to induce endothelial dysfunction or if secondary effects via immune and progenitor cells are also required to develop accelerated vascular damage. Additional work is needed to understand how chronic vs. acute exposure to type I IFNs modulates EPC survival, migration, homing and incorporation into newly formed vascular structures.

One limitation of murine lupus models is they do not display a pattern of disease flares followed by remission. A possible model to better mimic the type I IFN exposure seen in human SLE is to administer poly:IC to induce global induction of these molecules. Further, this method exposes bone marrow cells to enhanced levels of type I IFNs[96], thereby assuring exposure of EPCs to these molecules. This experiment could address if cycles of chronic exposure to type I IFNs, and hence chronic vascular damage, are needed for depletion/consumption and induction of apoptosis of EPCs. Further, these experiments could address the mechanisms leading to differences observed in EPC numbers in mice lacking the IFNAR, when compared to those acutely exposed to IFN-α. One limitation of this approach is that only males or nonautoimmune mice could be used, as this treatment would be expected to accelerate disease progression in female NZM 2328 mice and result in accelerated death due to glomerulonephritis.

The murine models of lupus described in this work, could allow us to test *in vivo* the role of IL-1 and IL-1-associated molecules in endothelial cell and EPC dysfunction, and if the modulation of this pathway could have a beneficial effect on vascular health and renal function in lupus. A possible role of IL-1RN in renal health has been implicated in NZM 2328 mice deficient in STAT6[286], which show improved renal function. This improvement could at least be partially explained by the expected decreases in IL-1RN expression, given the crucial role that STAT6 plays in this phenomenon in other cell types[83]. While the improvement in kidney function in these mice was likely meditated by multiple factors, it is

possible that the predicted decreased levels of IL1RN could create a more proangiogenic environment in the kidney conducive to improved renal function.

Type I IFNs may also play a role in the increased risk of developing thrombosis seen in SLE[166]. Our work supports recent observations that type I IFNs may have a role in platelet activation and induction of accelerated thrombosis. The potential mechanisms and pathways through which type I IFNs mediate this prothrombotic environment warrant further examination and could allow the identification of downstream targets amenable to pharmacologic intervention.

The work presented in this dissertation expands our understanding of how type I IFNs affect vascular damage and repair in SLE patients. Our findings, as summarized in Figure 5-1, demonstrate that type I IFNs play a direct role at many levels of vascular damage and atherosclerosis progression and CV risk in these patients. The effects of type I IFNs range from the initial dysfunction in both EPCs and endothelial cells to the induction of foam cells and development of plaque, to the final stages of plaque destabilization and increasing thrombotic risk. Our work therefore indicates a vital role for type I IFNs not only in disease pathogenesis, but in vascular injury and the development of organ damage in SLE and, potentially in other conditions associated to vascular injury. As such, type I IFNs should be regarded as a risk factor for the development of atherosclerosis and atherothrombotic events in SLE and, perhaps, in the general population.

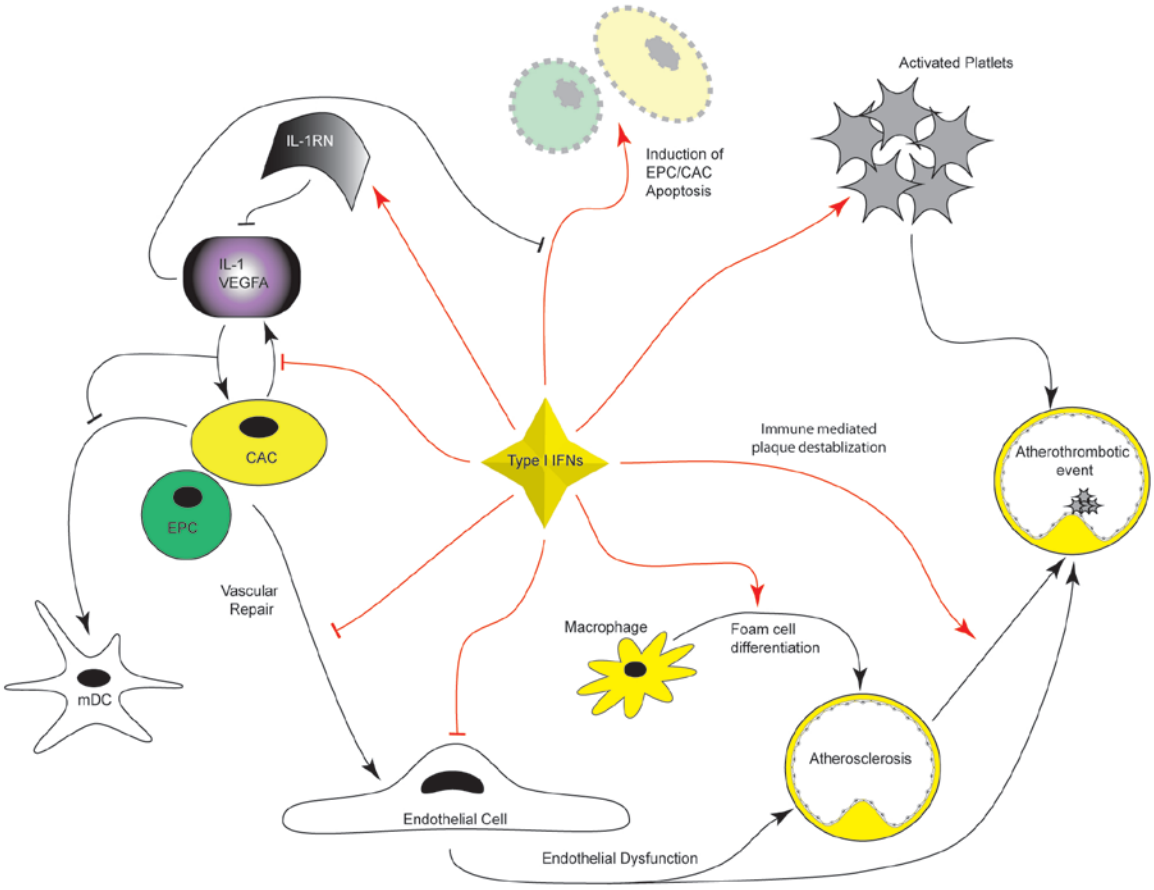
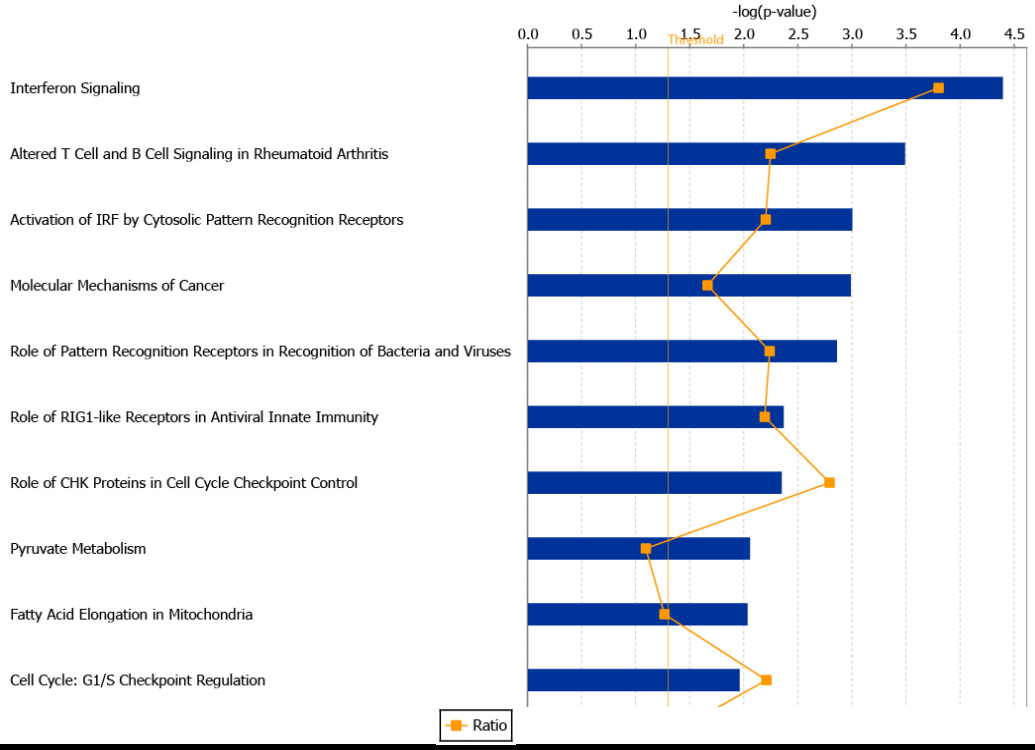


Figure 5-1 Proposed mechanisms by which type I IFNs increase cardiovascular risk and increase atherosclerosis.

Appendices

Appendix A Top 10 Canonical pathways regulated by IFN-α in healthy control peripheral blood EPCs/CACs

Top 10 canonical pathways as assessed by Ingenuity Pathway Analysis software from the 2850 genes differentially regulated in the healthy control EPCs/CACs treated by IFN-α compared to non-treated (q-value < 0.01).

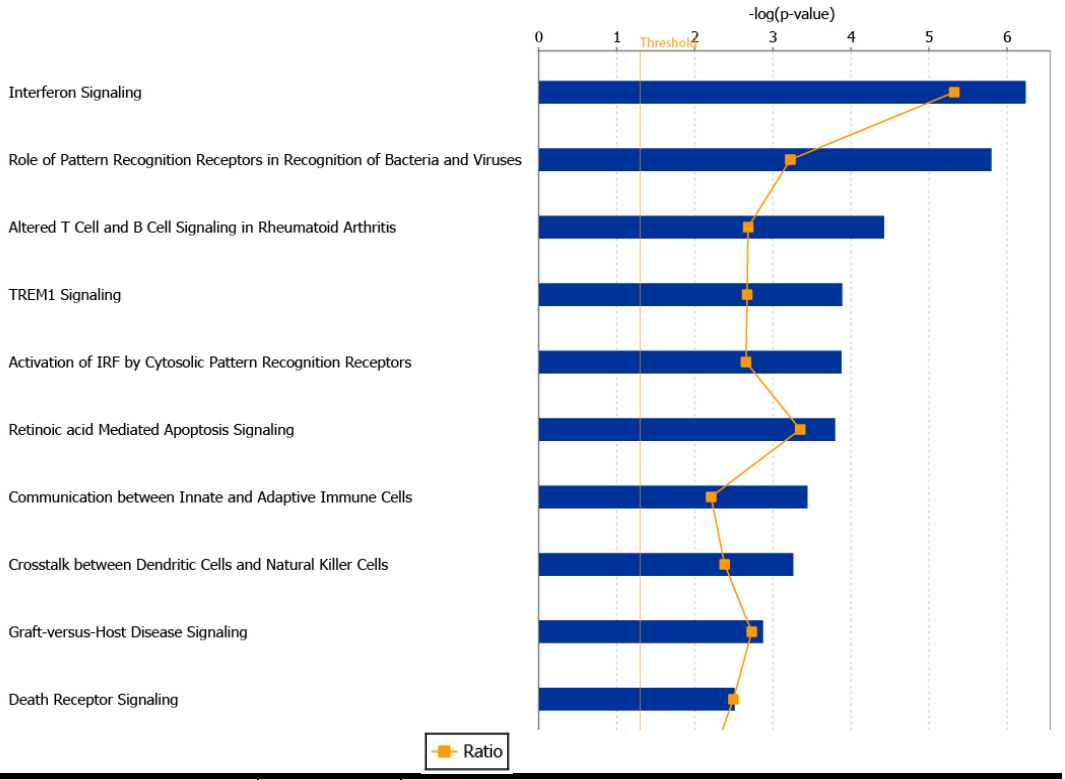


Ingenuity Canonical Pathways	p-value	Molecules
Interferon Signaling	3.98E-05	FNG, IFIT3, OAS1, MX1, IFNGR1, IFI35, IRF9, PSMB8, JAK2, TAP1, IFIT1, IFITM1, STAT2, IFNAR1
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	3.24E-04	IFNG, RELA, TNFSF11, TRAF3, IL1A, IL10, RELB, IL15, IL1F6, HLA-DMB, FAS, TNFRSF17, CD80, CXCL13, TGFB1, IL1RN, IL2, TLR7, HLA-DOB, IL1B, TLR3, IL23A, TNFSF13B, FASLG
Activation of IRF by Cytosolic Pattern Recognition Receptors	1.00E-03	DHX58, RELA, TRAF3, IL10, NFKBIE, ZBP1, IRF9, IKBKE, ADAR, ISG15, TANK, IFIH1, IRF7, RIPK1, IFNA7, DDX58, STAT2, IKBKAP, IFIT2, IFNAR1

Molecular Mechanisms of Cancer	1.02E-03	PLCB2, TGFBR1, BAD, APH1B, SMAD3, CCND1, TGFB1, ARHGEF11, BRCA1, FZD2, SMAD1, FASLG, CASP10, TP53, PIK3C2B, CASP3, TFDP1, RALB, CDK6, GNAZ, AURKA, RALBP1, DAXX, CCND3, MAX, RND3, RABIF, PTPN11, PRKCD, IRS1, CDK4, E2F1, PRKACA, CFLAR, FNBP1, CAMK2G, RAP2A, MAP2K6, RELA, PA2G4, NFKBIE, GNA11, CTNNA1, BMPR2, PSEN2, PSENEN, SMAD5, JAK2, FAS, ARHGEF19, HHAT, NLK, SOS1, SMAD4, PRKCE, PIK3R2, ARHGEF3, MAP2K1, CHEK2, PRKDC, HAT1, RHOC, GNA12, DVL1, ADCY3, SMAD7, BAK1, NBN, FOS, PLCB4, PRKCI, PRKAG2, RBPJ, CASP7, PRKAR1A, PRKCB
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	1.38E-03	RELA, PIK3C2B, OAS1, OAS2, IL10, MYD88, C5, OAS3 (includes EG:4940), IFIH1, IRF7, NOD2, TICAM1, IL2, DDX58, CASP1, TLR7, NOD1, IL1B, PIK3R2, EIF2AK2, TLR3, NLRC4
Role of RIG1-like Receptors in Antiviral Innate Immunity	4.27E-03	DHX58, RELA, TRAF3, NFKBIE, IKBKE, TANK, IFIH1, TRAF2, IRF7, RIPK1, DDX58, IFNA7, TRIM25, CASP10
Role of CHK Proteins in Cell Cycle Checkpoint Control	4.47E-03	TP53, RFC4, E2F1, HUS1, RAD9A, SLC19A1, RPA1, TLK2, BRCA1, CHEK2, RFC3, NBN
Pyruvate Metabolism	8.71E-03	AKR7A2, ACACB, ACAA1, IL21R, ACOT9, ALDH9A1, GLO1, ACSM5, ALDH1A3, BCKDHA, ALDH3A2, DLAT, ACSS2, ACYP1, ACACA, RWDD2B, PDHB, ACSL1, RWDD2A, ALDH7A1
Fatty Acid Elongation in Mitochondria	9.33E-03	ACAA1, PPT1, AUH, PECR, SDS, HSD17B4, HADH
Cell Cycle: G1/S Checkpoint Regulation	1.10E-02	TP53, PA2G4, TFDP1, SMAD3, CUL1, HDAC1, CDK6, SKP1, CCND1, HDAC6, MAX, CCND3, TGFB1, CDK4, E2F1, SMAD4

Appendix B Top 10 Canonical pathways regulated by IFN-α in SLE peripheral blood EPCs/CACs

Top 10 canonical pathways as assessed by Ingenuity Pathway Analysis software from the 1631 genes differentially regulated in the lupus EPCs/CACs exposed to IFN-α compared to non-exposed (q-value < 0.05, fold-change ≥ 0.15).

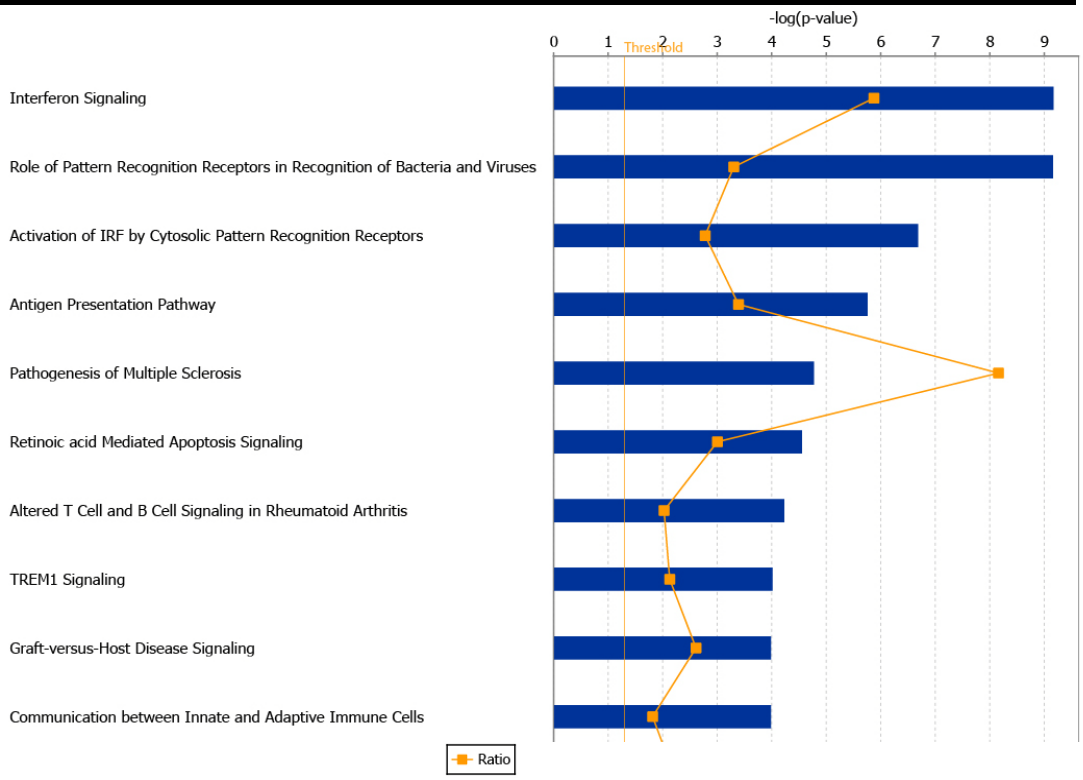


Ingenuity Canonical Pathways	p-value	Molecules
Interferon Signaling	5.75E-07	IFIT3, SOCS1, IFNG, OAS1, MX1, IFI35, IRF9, PSMB8, JAK2, TAP1, IFIT1, IFITM1, STAT2
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	1.58E-06	PIK3C2B, OAS1, OAS2, IL10, MYD88, MAPK3, C5, IL6, OAS3 (includes EG:4940), IFIH1, IRF7, NOD2, TLR5, DDX58, TLR6, CASP1, TLR7, NOD1, IL1B, EIF2AK2, TLR3
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	3.72E-05	IFNG, IL1A, IL10, IL15, IL6, FAS, TNFRSF17, CD40, TLR5, CD80, CXCL13, IL1RN, TLR6, TLR7, HLA-DOB, IL1B, TLR3, TNFSF13B, FASLG
TREM1 Signaling	1.29E-04	IL10, MAPK3, STAT3, JAK2, IL6, NOD2, TLR5, CCL7, CD40, TLR6, TLR7, CASP1, IL1B, TLR3, CASP5
Activation of IRF by Cytosolic Pattern Recognition Receptors	1.32E-04	DHX58, IL10, ZBP1, IKBKE, IRF9, IL6, ISG15, TANK, IFIH1, IRF7, RIPK1, CD40, DDX58, IKBKAP, STAT2, IFIT2
Retinoic acid Mediated	1.58E-04	CASP9, PARP10, PARP15, CASP3, PARP8,

Apoptosis Signaling		TNFSF10, PARP11, CFLAR, PARP12, TNFRSF10A (includes EG:8797), PARP9, PARP14
Communication between Innate and Adaptive Immune Cells	3.63E-04	IFNG, IL1A, IL10, IL15, IL6, TNFRSF17, CXCL10, TLR5, CD80, CD40, IL1RN, TLR6, TLR7, IL1B, TLR3, TNFSF13B
Crosstalk between Dendritic Cells and Natural Killer Cells	5.50E-04	IFNG, IL15RA, KLRD1, CD69, ACTA2, IL15, TNFSF10, IL6, TREM2, FAS, CAMK2D, CD40, CD80, MICB, TLR7, TLR3, FASLG, CAMK2G, PVRL2
Graft-versus-Host Disease Signaling	1.35E-03	IFNG, IL1A, CD80, IL1RN, HLA-DOB, IL1B, IL6, FAS, FASLG, GZMB
Death Receptor Signaling	3.09E-03	CASP3, TNFSF10, IKBKE, XIAP, FAS, TANK, TRADD, RIPK1, CASP9, CFLAR, TNFRSF10A (includes EG:8797), CASP7, FASLG

Appendix C Top 10 Canonical pathways regulated by IFN-α in Bone Marrow EPCs

Top 10 canonical pathways as assessed by Ingenuity Pathway Analysis software from the 710 genes differentially regulated in the healthy CD133^{+} bone marrow EPCs treated with IFN-α compared to non-treated (q-value < 0.05).



Ingenuity Canonical Pathways	p-value	Molecules
Interferon Signaling	6.76E-10	IFIT3, IFIT1, OAS1, IFITM1, MX1, IFI35, STAT2, IRF9, PSMB8, JAK2, STAT1, TAP1
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	6.92E-10	PIK3C2B, OAS1, OAS2, IL10, MYD88, C1QC, IL6, OAS3 (includes EG:4940), TLR2, IFIH1, IRF7, DDX58, CASP1, TLR7, NOD1, EIF2AK2, TLR3, C3AR1
Activation of IRF by Cytosolic Pattern Recognition Receptors	2.09E-07	DHX58, IL10, ZBP1, IRF9, IL6, ADAR, ISG15, IFIH1, IRF7, RIPK1, DDX58, STAT2, IFIT2, STAT1
Antigen Presentation Pathway	1.74E-06	PSMB9, HLA-E, HLA-DOB, PSMB8, TAP1, TAP2, TAPBP, MR1, HLA-F
Pathogenesis of Multiple Sclerosis	1.70E-05	CXCL10, CCR1, CCR5, CXCL9, CXCL11
Retinoic acid Mediated Apoptosis Signaling	2.82E-05	PARP10, PARP15, PARP8, TNFSF10, PARP11, CFLAR, PARP12, PARP9, PARP14
Altered T Cell and B Cell Signaling in Rheumatoid	5.89E-05	TLR2, CD80, IL10, IL1RN, IL15, TLR7, CD86, HLA-DOB, IL6, TLR3, TNFSF13B, FAS

Arthritis		
TREM1 Signaling	9.55E-05	TLR2, CCL7, IL10, TLR7, CASP1, CD86, JAK2, IL6, TLR3, FCGR2B
Graft-versus-Host Disease Signaling	1.02E-04	CD80, HLA-E, IL1RN, CD86, HLA-DOB, IL6, FAS, HLA-F
Communication between Innate and Adaptive Immune Cells	1.02E-04	CXCL10, TLR2, CD80, IL10, IL1RN, IL15, TLR7, CD86, IL6, TLR3, TNFSF13B

Appendix D Genes directly connected to IL-1β
List and regulation of the 201 genes directly connected to IL-1β displayed in Figure 3-2 from the IFN-α treated EPCs/CACs in SLE compared to non-treated study.

Entrez Gene ID	Gene symbol	Gene name	Fold-change	q-value[a]
3553	IL1B	Interleukin 1, beta	0.62	0.001
10855	HPSE	Heparanase	2.99	0.000
2358	FPR2	Formyl peptide receptor 2	2.58	0.003
23786	BCL2L13	BCL2-like 13	1.63	0.000
1728	NQO1	NAD(P)H dehydrogenase, quinone 1	0.65	0.000
120425	AMICA1	Adhesion molecule antigen 1	0.60	0.000
1890	TYMP	Thymidine phosphorylase	2.98	0.000
9497	SLC4A7	Solute carrier family 4 sodium bicarbonate cotransporter, member 7	0.66	0.000
10333	TLR6	Toll-like receptor 6	0.61	0.001
3600	IL15	Interleukin 15	3.86	0.001
24145	PANX1	Pannexin 1	3.19	0.000
3665	IRF7	Interferon regulatory factor 7	4.04	0.000
3660	IRF2	Interferon regulatory factor 2	1.64	0.000
3667	IRS1	Insulin receptor substrate 1	1.60	0.036
3673	ITGA2	Integrin, alpha 2	2.06	0.001
3655	ITGA6	Integrin, alpha 6	0.54	0.008
3620	INDO	Indoleamine-pyrrole 2,3 dioxygenase	5.25	0.000
3627	CXCL10	Chemokine (C-X-C motif) ligand 10	3.07	0.013
3001	GZMA	Granzyme A	1.65	0.001
1672	DEFB1	Defensin, beta 1	53.22	0.000
3586	IL10	Interleukin 10	1.71	0.000
9592	IER2	Immediate early response 2	1.61	0.001
9641	IKBKE	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	1.61	0.000
9672	SDC3	Syndecan 3	1.62	0.006
9636	ISG15	ISG15 ubiquitin-like modifier	9.07	0.000
3557	IL1RN	Interleukin 1 receptor antagonist	10.98	0.000
3569	IL6	Interleukin 6	2.12	0.001
3570	IL6R	Interleukin 6 receptor	1.53	0.000
3577	IL8RA	Interleukin 8 receptor, alpha	0.58	0.003
3574	IL7	Interleukin 7	4.54	0.000
3575	IL7R	Interleukin 7 receptor	1.99	0.002
3579	IL8RB	Interleukin 8 receptor, beta	0.61	0.002
10392	NOD1	Nucleotide-binding oligomerization domain containing 1	1.89	0.000
29126	CD274	CD274 molecule	4.56	0.001
9252	RPS6KA5	Ribosomal protein S6 kinase, 90kDa, polypeptide 5	1.71	0.002
55655	NLRP2	NLR family, pyrin domain containing 2	0.64	0.003
1803	DPP4	Dipeptidyl-peptidase 4	2.42	0.002
30009	TBX21	T-box 21	1.53	0.020
9373	PLAA	Phospholipase A2-activating protein	1.59	0.000

57758	SCUBE2	Signal peptide, CUB domain, EGF-like 2	3.36	0.000
3717	JAK2	Janus kinase 2	4.56	0.000
838	CASP5	Caspase 5	1.93	0.001
831	CAST	Calpastatin	1.53	0.000
834	CASP1	Caspase 1	1.57	0.000
836	CASP3	Caspase 3	1.95	0.000
840	CASP7	Caspase 7	2.56	0.000
842	CASP9	Caspase 9	0.65	0.001
3431	SP110	SP110 nuclear body protein	4.23	0.000
2633	GBP1	Guanylate binding protein 1	3.24	0.004
9051	PSTPIP1	Proline-serine-threonine phosphatase interacting protein 1	0.62	0.002
952	CD38	CD38 molecule	6.27	0.000
958	CD40	CD40 molecule	2.55	0.001
962	CD48	CD48 molecule	1.79	0.001
969	CD69	CD69 molecule	2.82	0.001
961	CD47	CD47 molecule	1.90	0.000
943	TNFRSF8	Tumor necrosis factor receptor superfamily, member 8	0.58	0.001
941	CD80	CD80 molecule	4.41	0.000
9124	PDLIM1	PDZ and LIM domain 1	1.55	0.001
64127	NOD2	Nucleotide-binding oligomerization domain containing 2	1.59	0.001
629	CFB	Complement factor B	8.80	0.000
688	KLF5	Kruppel-like factor 5	1.92	0.001
715	C1R	Complement component 1, r subcomponent	1.93	0.003
716	C1S	Complement component 1, s subcomponent	1.97	0.004
717	C2	Complement component 2	1.60	0.018
7414	VCL	Vinculin	0.55	0.000
10346	TRIM22	Tripartite motif-containing 22	2.49	0.000
3084	NRG1	Neuregulin 1	0.57	0.008
7430	EZR	Ezrin	1.95	0.000
7442	TRPV1	Transient receptor potential cation channel, subfamily V, member 1	0.36	0.000
2643	GCH1	GTP cyclohydrolase 1	2.52	0.001
2621	GAS6	Growth arrest-specific 6	1.65	0.001
7130	TNFAIP6	Tumor necrosis factor, alpha-induced protein 6	2.21	0.001
7100	TLR5	Toll-like receptor 5	0.57	0.002
3429	IFI27	Interferon, alpha-inducible protein 27	4.30	0.002
2526	FUT4	Fucosyltransferase 4	2.85	0.000
7056	THBD	Thrombomodulin	0.39	0.000
7058	THBS2	Thrombospondin 2	0.57	0.002
7098	TLR3	Toll-like receptor 3	24.22	0.000
7082	TJP1	Tight junction protein 1	2.04	0.000
7072	TIA1	TIA1 cytotoxic granule-associated RNA binding protein	1.95	0.000
5524	PPP2R4	Protein phosphatase 2A activator, regulatory subunit 4	1.70	0.000
5581	PRKCE	Protein kinase C, epsilon	2.40	0.000
5595	MAPK3	Mitogen-activated protein kinase 3	0.63	0.000
3002	GZMB	Granzyme B	1.94	0.002
3552	IL1A	Interleukin 1, alpha	0.43	0.001
5698	PSMB9	Proteasome (prosome, macropain) subunit, beta type, 9	1.72	0.002

5696	PSMB8	Proteasome (prosome, macropain) subunit, beta type, 8	2.0	0.000
2119	ETV5	Ets variant gene 5	0.60	0.000
5610	EIF2AK2	Eukaryotic translation initiation factor 2-alpha kinase 2	3.16	0.000
2068	ERCC2	Excision repair cross-complementing rodent repair deficiency, complementation group 2	0.57	0.001
5608	MAP2K6	Mitogen-activated protein kinase kinase 6	3.62	0.000
5341	PLEK	Pleckstrin	1.54	0.000
5359	PLSCR1	Phospholipid scramblase 1	2.81	0.000
5362	PLXNA2	Plexin A2	0.57	0.004
5468	PPARG	Peroxisome proliferator-activated receptor gamma	0.25	0.000
23586	DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	9.03	0.000
5155	PDGFB	Platelet-derived growth factor beta polypeptide	1.86	0.000
5142	PDE4B	Phosphodiesterase 4B, cAMP-specific	1.91	0.001
5176	SERPINF1	Serpin peptidase inhibitor, clade F, member 1	0.64	0.004
5168	ENPP2	Ectonucleotide pyrophosphatase/phosphodiesterase 2	2.52	0.001
2185	PTK2B	PTK2B protein tyrosine kinase 2 beta	1.96	0.000
65108	MARCKSL1	MARCKS-like 1	0.57	0.000
3430	IFI35	Interferon-induced protein 35	5.24	0.000
5029	P2RY2	Purinergic receptor P2Y, G-protein coupled, 2	0.52	0.000
215	ABCD1	ATP-binding cassette, sub-family D (ALD), member 1	2.03	0.000
1316	KLF6	Kruppel-like factor 6	2.48	0.000
11035	RIPK3	Receptor-interacting serine-threonine kinase 3	1.57	0.000
10010	TANK	TRAF family member-associated NFKB activator	1.55	0.002
5937	RBMS1	RNA binding motif, single stranded interacting protein 1	1.81	0.000
5979	RET	Ret proto-oncogene	3.51	0.009
5747	PTK2	PTK2 protein tyrosine kinase 2	1.74	0.002
5742	PTGS1	Prostaglandin-endoperoxide synthase 1	0.53	0.000
5829	PXN	Paxillin	0.60	0.000
1540	CYLD	Cylindromatosis	1.67	0.001
3458	IFNG	Interferon, gamma	2.42	0.010
22918	CD93	CD93 molecule	0.43	0.000
355	FAS	Fas	3.50	0.000
331	XIAP	X-linked inhibitor of apoptosis	1.53	0.000
8651	SOCS1	Suppressor of cytokine signaling 1	1.88	0.001
8642	DCHS1	Dachsous 1	0.62	0.002
2332	FMR1	Fragile X mental retardation 1	1.60	0.000
8714	ABCC3	ATP-binding cassette, sub-family C, member 3	0.56	0.000
8744	TNFSF9	Tumor necrosis factor superfamily, member 9	0.60	0.001
8743	TNFSF10	Tumor necrosis factor superfamily, member 10	9.79	0.000
2069	EREG	Epiregulin	1.55	0.018
246778	IL27	Interleukin 27	1.90	0.000
3075	CFH	Complement factor H	6.17	0.000
3925	STMN1	Stathmin 1	0.33	0.000
3965	LGALS9	Lectin, galactoside-binding, soluble, 9	2.31	0.000
54209	TREM2	Triggering receptor expressed on myeloid cells 2	0.54	0.000
59350	RXFP1	Relaxin/insulin-like family peptide receptor 1	1.70	0.004
3309	HSPA5	Heat shock 70kDa protein 5	0.58	0.000
1306	COL15A1	Collagen, type XV, alpha 1	0.44	0.001

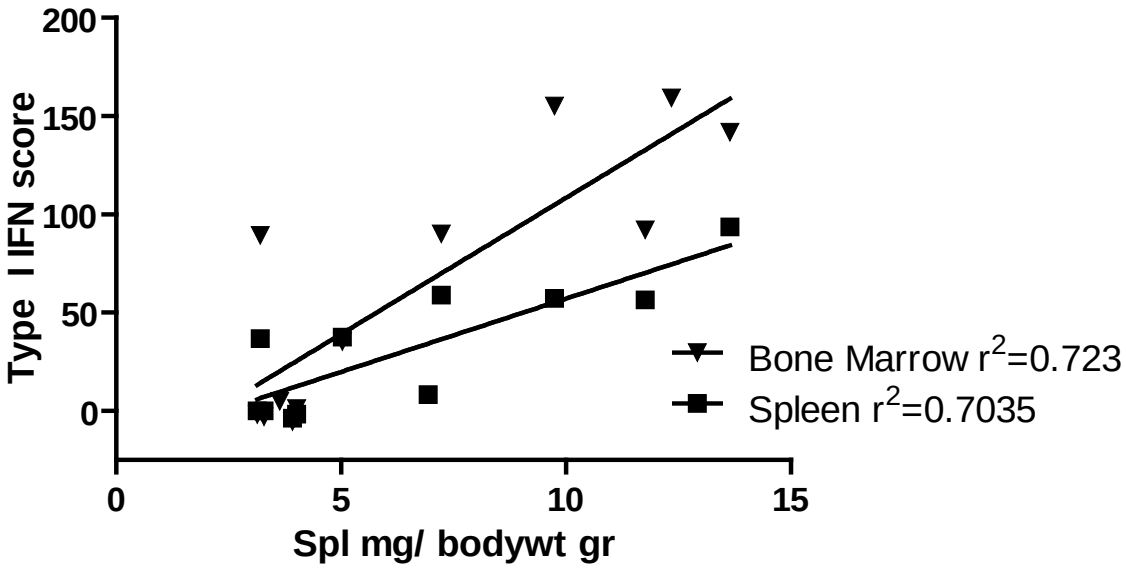
2026	ENO2	Enolase 2	0.52	0.000
2152	F3	Coagulation factor III	0.59	0.001
1588	CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1	2.11	0.006
4086	SMAD1	SMAD family member 1	2.67	0.000
4082	MARCKS	Myristoylated alanine-rich protein kinase C substrate	1.68	0.000
4088	SMAD3	SMAD family member 3	1.88	0.000
4048	LTA4H	Leukotriene A4 hydrolase	0.65	0.000
6195	RPS6KA1	Ribosomal protein S6 kinase, 90kDa, polypeptide 1	0.57	0.000
4053	LTBP2	Latent transforming growth factor beta binding protein 2	0.50	0.000
4067	LYN	V-yes-1 Yamaguchi sarcoma viral related oncogene homolog	1.93	0.000
4012	LNPEP	Leucyl/cystinyl aminopeptidase	2.80	0.000
4023	LPL	Lipoprotein lipase	0.24	0.000
390	RND3	Rho family GTPase 3	3.17	0.000
356	FASLG	Fas ligand	1.89	0.006
6285	S100B	S100 calcium binding protein B	0.53	0.000
2	A2M	Alpha-2-macroglobulin	0.46	0.002
6387	CXCL12	Chemokine (C-X-C motif) ligand 12	2.41	0.000
6385	SDC4	Syndecan 4	0.55	0.000
6383	SDC2	Syndecan 2	0.62	0.000
6373	CXCL11	Chemokine (C-X-C motif) ligand 11	45.97	0.000
6363	CCL19	Chemokine (C-C motif) ligand 19	1.80	0.001
24138	IFIT5	Interferon-induced protein with tetratricopeptide repeats 5	2.61	0.000
6354	CCL7	Chemokine (C-C motif) ligand 7	3.00	0.002
6355	CCL8	Chemokine (C-C motif) ligand 8	3.09	0.003
6357	CCL13	Chemokine (C-C motif) ligand 13	3.52	0.004
3112	HLA-DOB	Major histocompatibility complex, class II, DO beta	2.05	0.002
4277	MICB	MHC class I polypeptide-related sequence B	1.65	0.000
4283	CXCL9	Chemokine (C-X-C motif) ligand 9	2.96	0.010
4299	AFF1	AF4/FMR2 family, member 1	1.96	0.000
4210	MEFV	Mediterranean fever	1.89	0.001
4215	MAP3K3	Mitogen-activated protein kinase kinase kinase 3	0.62	0.000
578	BAK1	BCL2-antagonist/killer 1	1.66	0.001
6402	SELL	Selectin L	2.18	0.000
10563	CXCL13	Chemokine (C-X-C motif) ligand 13	2.23	0.000
6519	SLC3A1	Solute carrier family 3, member 1	0.64	0.024
467	ATF3	Activating transcription factor 3	2.82	0.000
4303	FOXO4	Forkhead box O4	0.61	0.000
6546	SLC8A1	Solute carrier family 8, member 1	3.07	0.000
387923	SERP2	Stress-associated endoplasmic reticulum protein family member 2	0.58	0.006
51284	TLR7	Toll-like receptor 7	1.51	0.008
6692	SPINT1	Serine peptidase inhibitor, Kunitz type 1	0.54	0.000
6654	SOS1	Son of sevenless homolog 1	2.08	0.000
51274	KLF3	Kruppel-like factor 3	1.77	0.000
51191	HERC5	Hect domain and RLD 5	8.07	0.000
6774	STAT3	Signal transducer and activator of transcription 3	1.54	0.001
6891	TAP2	Transporter 2, ATP-binding cassette, sub-family B	3.26	0.001

4860	NP	Nucleoside phosphorylase	0.62	0.000
4854	NOTCH3	Notch homolog 3	0.52	0.001
153222	C5orf41	Chromosome 5 open reading frame 41	1.94	0.001
4939	OAS2	2'-5'-oligoadenylate synthetase 2	9.37	0.000
2170	FABP3	Fatty acid binding protein 3	0.43	0.000
51540	SCLY	Selenocysteine lyase	0.51	0.000
3487	IGFBP4	Insulin-like growth factor binding protein 4	7.44	0.000
1636	ACE	Angiotensin I converting enzyme	1.61	0.000
51776	ZAK	Sterile alpha motif and leucine zipper containing kinase AZK	0.60	0.000
4481	MSR1	Macrophage scavenger receptor 1	2.90	0.000
8837	CFLAR	CASP8 and FADD-like apoptosis regulator	1.80	0.000
8809	IL18R1	Interleukin 18 receptor 1	1.78	0.010
8829	NRP1	Neuropilin 1	0.61	0.000
4502	MT2A	Metallothionein 2A	2.14	0.004
4599	MX1	Myxovirus resistance 1	6.00	0.000
4582	MUC1	Mucin 1, cell surface associated	4.32	0.000
4616	GADD45B	Growth arrest and DNA-damage-inducible, beta	1.70	0.000
4615	MYD88	Myeloid differentiation primary response gene 88	1.63	0.000
4600	MX2	Myxovirus resistance 2	6.30	0.000
2203	FBP1	Fructose-1,6-bisphosphatase 1	0.59	0.000
1906	EDN1	Endothelin 1	1.61	0.007
353514	LILRA5	Leukocyte immunoglobulin-like receptor, subfamily A, member 5	3.67	0.001

[a] A q-value below 0.05 was considered as significant (all the 201 genes are significantly differentially regulated).

Appendix E. Positive correlation of spleen weight to IFN score

After injection with AdIFN-α or AdControl spleen: body weight ratio strongly correlated with the type I IFN score. The IFN score is derived from 6 IFN responsive genes. The number of standard deviations from the mean of the AdControl treatment for each gene is calculated. Then the sum of the 6 individual scores is used to generate the total IFN score.



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