The Interplay of Lipoproteins and Innate Immune Responses in Systemic Lupus Erythematosus and its Role in Premature Cardiovascular Disease.

by

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Dedication

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List of Abbreviations

1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)
22 amino acid sequence in ETC-642 (22A)
3-amino-1,2,4-triazole (3-AT)
3-chlorotyrosine (3-ClY)
3-nitrotyrosine (3-NY)
Activating Transcription Factor 3 (ATF3)
Apolipoprotein (apo)
ATP Binding Cassette A1 (ABCA1)
ATP Binding Cassette G1 (ABCG1)
Body Mass Index (BMI)
Cardiovascular Disease (CVD)
Chloroquine (CQ)
Cholesterol Efflux Capacity (CEC)
Cholesteryl Esters (CE)
Cl-Amidine (Cl-Am)
Control (Ctrl)
Coronary Artery Disease (CAD)
Diphenylene Iodonium (DPI)
Endothelial Nitric Oxide Synthase (eNOS)
Endothelial Progenitor Cell (EPC)
Erythrocyte Sedimentation Rate (ESR)
First Apoptosis Signal (Fas) ligand (FasL)
Genome-wide Association Study (GWAS)
Granular Antimicrobial Peptide (gAMP)
High-density Lipoprotein (HDL)
Histone Deacetylase 1 (HDAC1)
Inducible Nitric Oxide Synthase (iNOS)
Interleukin (IL)
Intermediate-density Lipoprotein (IDL)
Krebs Ringer’s Phosphate Glucose (KRPG)
Lectin-like Oxidized Low-density Lipoprotein Receptor 1 (LOX1R)
Lipoplysaccharide (LPS)
L-NG-monomethyl-L-arginine (L-NMMA)
Low-density Granulocytes (LDG)
Low-density Lipoprotein (LDL)
Myeloperoxidase (MPO)
Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Oxidase (NOX)
Neutrophil Elastase (NE)
Neutrophil Extracellular Trap (NET)
Nitric Oxide (NO)
Normal-density Granulocytes (NDG)
Nuclear Factor Kappa B (NFkB)
Oxidized High-density Lipoprotein (oxHDL)
Oxidized Low-density Lipoprotein (oxLDL)
Paraoxonase (PON)
Pattern Recognition Receptors (PRRs)
Peptidylarginine Deiminase 4 (PAD-4)
Phorbol 12-myristate 13-acetate (PMA)
Plasmacytoid Dendritic Cell (pDC)
Reactive Nitrogen Species (RNS)
Reactive Oxygen Species (ROS)
Reverse Cholesterol Transport (RCT)
Scavenger Receptor (SR)
Single Nucleotide Polymorphism (SNP)
Sphingomyelin (SM)
Sphingosine 1-phosphate (S1P)
Superoxide Dismutase (SOD)
Systemic Lupus Erythematosus (SLE)
Systemic Lupus Erythematosus Disease Activity Index (SLEDAI)
Toll-like Receptor (TLR)
Transforming Growth Factor Beta (TGF-β)
Triglycerides (TG)
Tumor Necrosis Factor (TNF)
Type I Interferon (IFN-I)
Tyrosine (Y)
Vascular Smooth Muscle Cells (VSMC)
Very Low-density Lipoprotein (VLDL)
Abstract

Systemic lupus erythematosus (SLE) is an autoimmune syndrome that primarily affects women and leads to significant internal organ damage. Patients with SLE exhibit a high risk for premature atherosclerotic cardiovascular disease (CVD) which is not fully explained by traditional risk factors (e.g. smoking, age, and hyperlipidemia). As both SLE and CVD are chronic inflammatory diseases associated with abnormal lipoprotein and myeloid cell responses, certain overlapping, not yet elucidated mechanisms may explain SLE susceptibility to CVD. It is therefore necessary to establish specific biomarkers for CVD risk in SLE patients and further elucidate the interplay between the aberrant innate immune system and lipoprotein biology present in SLE atherosclerosis. Recent evidence implicates abnormal lipoprotein activity as an important factor of accelerated atherosclerosis in SLE, but the mechanisms leading to this alteration and the impact that it has on immune responses in the plaque remains to be determined. Oxidation of high-density lipoprotein (oxHDL) could explain this altered activity and act as a biomarker of SLE CVD as it is already associated with CVD in the general population and linked to deviant pro-atherogenic innate immune responses.

We hypothesized that the chronic oxidative environment characteristic of SLE promotes pro-atherogenic alterations to HDL. We further posited that neutrophil extracellular trap (NET) formation, which is enhanced in SLE, significantly contributes to HDL oxidation. Finally, we predicted that oxidized SLE HDL skews macrophages toward pro-inflammatory pathways, thereby promoting vascular damage.
We demonstrated that lupus patients had high levels of oxHDL and impaired HDL-associated vasoprotective activity. The pattern of oxidation was consistent with alterations previously reported to be pro-atherogenic in the general population and with dysfunctional HDL activity. Because SLE plasma was also highly oxidized, this indicated that the site of oxidation was in the circulation, rather than in tissue-resident atherosclerotic plaques as is typically reported in the literature. Indeed, we demonstrated that the oxidative machinery externalized in NETs induces a pattern of HDL oxidation associated with CVD. Furthermore, when we suppressed NET formation in lupus-prone mice in vivo, levels of HDL oxidation significantly decreased. We then assessed the pro-inflammatory effects of lupus HDL on macrophages.

Control macrophages exposed to SLE HDL displayed induction of nuclear factor kappa B (NFκB) activation and synthesis of pro-inflammatory cytokines. While healthy, control HDL blocked toll-like receptor (TLR)-induced inflammatory cytokine production, SLE HDL failed to abrogate this inflammation. This was linked to an impaired ability of SLE HDL to activate the anti-inflammatory, transcriptional repressor activating transcription factor 3 (ATF3), compared to healthy HDL. This effect was dependent upon SLE HDL binding the scavenger receptor lectin-like oxidized low-density lipoprotein receptor (LOX1R). However, when macrophages were cultured with SLE HDL in the presence of the HDL mimetic ETC-642, the anti-inflammatory status of the macrophages was restored. Administration of ETC-642 to lupus-prone mice, in vivo, decreased their inflammatory cytokine profile and enhanced ATF3 mRNA levels.

This novel identification of a specific oxidation pattern in SLE HDL and plasma should be examined as a non-traditional, CVD risk factor in a larger lupus cohort to monitor its association with CVD risk. These studies have identified two innate immune response pathways that may contribute to cardiovascular risk and amplification of inflammation in SLE. Enhanced NET
formation generates the oxidative species required for HDL oxidation. The oxHDL, in turn, stimulates pro-inflammatory responses in macrophages and blocks anti-inflammatory pathways. As such, therapies that target the high NET formation in SLE or that block the interaction of oxHDL with macrophages should be explored.
Chapter 1

Introduction

Systemic lupus erythematosus:

A. Definition, Demographics and Clinical Features:

SLE is an autoimmune syndrome of unclear etiology. It affects approximately 5 million people worldwide, primarily women and people of African, Hispanic and Asian descent\textsuperscript{1,2}. It is a multi-systemic condition that can cause significant tissue damage, primarily to the kidneys, skin, brain, vasculature and joints\textsuperscript{3-5}.

One of the key features of SLE is the presence of auto-antibodies that recognize nuclear components. Indeed, anti-nuclear antibodies are observed in more than 95% of SLE patients\textsuperscript{6}. These include antibodies against double-stranded (ds)DNA, and against RNA-binding proteins such as Sm, Ro, La, and ribonucleoprotein (RNP)\textsuperscript{6-12}. Because these auto-antibodies target intracellular components, it is considered that aberrant cell death and/or defective clearance of debris promotes the modification and externalization of auto-antigens that, in a predisposed individual, leads to loss of tolerance and unregulated inflammation. Indeed, lupus patients exhibit a number of clinical and subclinical markers indicative of a chronic inflammatory environment including elevated levels of various key cytokines, particularly type I interferons (IFN-I)\textsuperscript{4,13-19}.

Patients with SLE have an increased risk of premature CVD. This is particularly striking in young SLE patients (35-44 years old), where the cardiovascular risk is approximately 50-fold\textsuperscript{20,21}. While new medications have improved the quality of life for SLE patients and increase
overall survival rates, cardiovascular disease (CVD) is still one of the major causes of death in SLE, and its incidence and prevalence have not decreased\textsuperscript{22-24}. Importantly, this enhanced CVD risk cannot be explained by traditional risk factors (e.g. dyslipidemia, smoking, obesity), suggesting that other factors characteristic of lupus play a prominent role in CVD and need to be elucidated\textsuperscript{21, 25-27}.

B. Mouse Models of Lupus:

Both spontaneous and genetically modified mouse strains exist which develop a lupus-like phenotype. However, these mice do not completely reflect lupus pathogenesis as mice in general show different antibody isotypes, low levels of peripheral blood neutrophils compared to humans, and a relative protection from CVD\textsuperscript{28, 29}. Nevertheless, mice are useful to explore several putative mechanisms associated with human lupus, with some of the most utilized strains summarized below:

\textit{New Zealand Black/New Zealand White F1 ((NZB×NZW) F1) Hybrid}

The New Zealand Black and New Zealand White mice were one of the first strains to be studied as a spontaneous autoimmunity model\textsuperscript{30}. When these strains were back-crossed, the resulting (NZB×NZW) F1 progeny showed an even stronger lupus-like phenotype. (NZB×NZW) F1 mice develop nephritis, anti-dsDNA antibodies, IFN-I response, with a strong sex-bias\textsuperscript{30}. The disease progresses relatively slowly in these mice, with an average life span of 35 weeks for females and 58 weeks for males.

\textit{NZM2328 (NZM) Mice:}

NZM2328 (NZM) mice, developed from numerous (NZB×NZW) F1 back-crosses, show a prominent lupus phenotype\textsuperscript{31}. NZM mice develop nephritis, anti-DNA antibodies, accelerated
NETosis, a strong sex-bias and IFN-I signature. Proteinuria appears at 24 weeks and most mice die of renal disease by 30 weeks.

Other Models:

First apoptosis signal (Fas) signaling is important for the negative selection of auto-reactive lymphocytes during their development. Mice deficient in Fas (MRL/lpr) or FasL (generalized lymphadenopathy [gld]) are also present in the lupus literature. Though they do possess auto-reactive B and T cells, they are considered less physiologically relevant models. These mice can demonstrate accelerated NETosis and an IFN-I signature, but do not show a strong sex-bias. Other mouse strains with reported lupus-like phenotypes are abundant in the literature, but the mice mentioned above are the most commonly used for SLE-related studies.

C. Pathogenesis:

I. Genetic and Gender Links

Certain genetic factors appear to predispose people to SLE. Indeed, individuals are approximately 24 times more likely to have lupus if they possess a sibling with lupus. A number of genome-wide association studies (GWAS) have been performed on SLE patients of various ethnic backgrounds. Single nucleotide polymorphisms (SNPs) linked to SLE can be found in genes related to antigen presentation, B and T cell activation, pattern recognition receptor (PRR) signaling, DNA repair/digestion mechanisms, NFκB and IFN-I signaling and cell debris clearance. This suggests that both aberrant adaptive and innate immune response pathways are important in SLE pathogenesis.

As with many autoimmune disorders, gender also appears to play a significant role in SLE. While male lupus patients exhibit increased organ damage and mortality compared to lupus
females, women are particularly prone to SLE \textsuperscript{79, 80}. This may be due to aberrant immune cell responses downstream of estrogen signaling \textsuperscript{81-88}. Additionally, a number of SLE susceptibility genes are present on the X chromosome, which is more transcriptionally active in lupus females compared to lupus males and control females \textsuperscript{89, 90}.

\textit{II. Adaptive Immune Responses in Systemic Lupus Erythematosus}

Because of the syndrome’s high association with auto-antibodies, lupus has classically been attributed to abnormal adaptive immune responses. The bone marrow, as a site of B cell genesis and maturation, appears to be significant to lupus pathogenesis. In lupus bone marrow, neutrophils generate high levels of IFN-I, B-cell activating factor (BAFF), and a proliferation-inducing ligand (APRIL) \textsuperscript{16, 91, 92}. These cytokines promote the survival, activation and longevity of auto-reactive immature and memory B cells \textsuperscript{93, 94}. Despite enhanced B cell survival, lupus bone marrow nonetheless shows higher levels of apoptotic cells than healthy bone marrow \textsuperscript{95, 96}. This is due to impaired debris clearance pathways in lupus \textsuperscript{97-101}. As such, lupus B cells are reared in an environment which promotes their survival and exposes them, and other marrow-resident leukocytes, at an early stage to cell debris-derived self-antigens. Once outside the bone marrow, B cells are further exposed to self-antigens and induced, with the help of T cells, to differentiate into pathogenic auto-antibody-producing plasma cells. In addition to B cells, there is ample evidence that T cell subsets are aberrant in SLE and important contributors to lupus pathogenesis \textsuperscript{102, 103}.

Various cytokines enhance T cell survival and activation in SLE \textsuperscript{104, 105}. Estrogen signaling also appears to promote aberrant B and T cell survival in SLE \textsuperscript{84-86}. Lupus T cells demonstrate an altered T cell receptor physiology, leading to stronger T cell signaling \textsuperscript{106-109}. High plasma levels of soluble co-stimulatory factors may also strengthen T cell activity in SLE \textsuperscript{110}. These responses trend towards inflammatory rather than anti-inflammatory pathways due to the high ratio of
interleukin (IL)-17 producing T helper (Th) cells to regulatory Th cells in SLE\textsuperscript{111-114}. On the other hand, there is evidence that lupus pathogenesis is linked to T cell anergy and decreased IL-2 production\textsuperscript{115-118}. More recent evidence implicates abnormalities in other T cell subsets, including T follicular helper cells\textsuperscript{102,103}. Abnormal B and T cell responses seen in SLE not only contribute to idiopathic SLE pathogenesis, but may contribute to the generation of the inflammatory cytokines and auto-antibodies linked to lupus-related atherogenesis, as discussed below.

\textit{III. Innate Immune Responses in Systemic Lupus Erythematosus}

The contributions of the innate immune system to SLE pathogenesis were long underappreciated, but have surfaced as significant mediators of disease inflammation and promoters of autoimmunity. Most research on SLE innate immune signaling revolves around the aberrant processing and detection of self-nucleic acids within dead cell debris\textsuperscript{42, 47-51, 119, 120}. Numerous mechanisms exist in healthy individuals in order to stimulate the quick, anti-inflammatory phagocytosis and digestion of cellular remnants\textsuperscript{121-126}. This keeps self-components from interacting with PRRs. In lupus patients, however, these pathways are defective. Lupus serum factors and aberrant phagocyte cell surface marker expression is linked to the impaired removal of dead cell debris\textsuperscript{59, 76, 77, 100, 101, 127-130}. Because of this, lupus plasma and serum show high levels of cell fragments\textsuperscript{131-136}. Not only do lupus patients have high levels of exposed self-antigens in the circulation, they also appear to have aberrant means of detecting this material. Immune complexes, for example, form when auto-reactive antibodies bind to complement proteins and undigested nucleic acids. These structures are recognized by highly expressed lupus activating Fc gamma receptors, are internalized, and, rather than being digested, are transported to endosomal, nucleic acid-sensing TLRs\textsuperscript{137-144}. This process initiates a series of highly inflammatory responses that results in tissue damage.
The activation of nucleic acid-binding PRRs, in particular, will induce an anti-viral response typified by strong IFN-I signaling. Indeed, the risk for SLE is enhanced by SNPs in interferon response factor genes, and increased IFN-I signatures are associated with lupus flares and organ damage . When IFN-I is used as a therapy for cancer and chronic infections it can induce a lupus-like disease . Furthermore, NZM lupus-prone mice that lack IFN-I receptor are protected from many lupus characteristics including vascular pathologies, abnormal B cell populations, renal disease, and auto-antibody levels . Lupus innate immune cells appear to be the main producers of this IFN-I and significant to SLE pathogenesis .

Lupus monocytes demonstrate an inflammatory phenotype (CD14+, CD16+), are strong responders to immune complexes, and produce large amounts of oxidative species, IL-6, TNF, and IFN-I . This enhanced cytokine production may be due to the increased histone H4 acetylation within these genes in lupus . SLE monocytes appear to accelerate adaptive immune activity through the enhanced production of CD40 and BAFF . Their capacity as antigen presenting cells, however, increases when cultured with IFN-I or SLE serum, pushing them towards dendritic cell differentiation .

Lupus dendritic cells produce high levels of IFN-I, TNF and IL-6 . Non-myeloid, plasmacytoid dendritic cells (pDCs) are particularly inflammatory, especially when stimulated with immune complexes and NETs (described below) . Exaggerated pDC activity has been linked to both lupus nephritis and skin lesions . Lupus dendritic cells may be potent lymphocyte activators as they express higher levels of MHC II, CD86, APRIL and BAFF . Lupus monocytes which differentiate instead into macrophages also appear to have a pathogenic signature, though by which mechanisms is not clearly understood.
Lupus macrophages have primarily been linked to kidney disease\textsuperscript{186,187}. When the kidneys of MRL/lpr mice were injured via an ischemia/reperfusion model, the mice failed to promote the production of repair-associated macrophages (M2, described below) and instead an inflammatory, damaging macrophage phenotype (M1, discussed below) persisted\textsuperscript{188}. The pro-inflammatory nature of lupus macrophages may be linked to their increased inflammasome activity\textsuperscript{189,190}. Additionally, mutations in the exonuclease TREX1 are associated with lupus and TREX1 deficient macrophages exhibit inflammatory cytokine production and enhanced antigen presentation activity\textsuperscript{191,192}. Though not well characterized, many of these macrophage inflammatory pathways may also promote SLE atherogenesis\textsuperscript{193-198}.

While aberrant nucleic acid-sensing PRR signals are significant to lupus pathogenesis, not a lot of work has demonstrated how the SLE innate immune cells mentioned above respond to lipoproteins. What is known is that of the receptors that appear to interact with lipoproteins, SLE shows altered activity and expression of TLR-2 and TLR-4, and patients possess auto-antibodies against certain scavenger receptors\textsuperscript{119,199-209}. This gap in SLE research is not insignificant given the high association of SLE with CVD.

Due to the apparent relationship between SLE pathogenesis and dead cell debris, extensive research has been conducted on cell death pathways in lupus. Leukocytes and non-immune cells do show a propensity for cell death in SLE, which was initially only attributed to apoptosis\textsuperscript{210-214}. However, as apoptosis is normally considered an anti-inflammatory response, and as not all lupus patients exhibit impaired clearance activity, it was necessary to identify additional mechanisms by which modified self-epitopes became readily exposed to the periphery. Within the last decade, neutrophil extracellular trap (NET) formation was identified as one of the putative mechanisms.
a. Neutrophil Extracellular Trap Formation

NET formation was initially described as an innate immune response performed by granulocytes to kill or inactivate bacteria. The process is distinct from both apoptosis and necrosis, is caspase-independent, and is characterized by the decondensation of chromatin material and mixing of nuclear and cytoplasmic contents (particularly granular antimicrobial peptides [gAMPs]) prior to extrusion of this material from the cell as a meshwork of chromatin and gAMPs. These structures can then surround the pathogenic material, be it a protozoa, bacteria, virus or fungi, and mediate either its destruction, via cytotoxic gAMPs (myeloperoxidase [MPO], LL-37, neutrophil elastase [NE]), or mark the pathogen for phagocytosis. A number of intact pathogens and pathogen associated molecular patterns (lipopolysaccharide [LPS], zymosan) can induce NETosis. Sterile stimuli include activators of calcium signaling (phorbol myristate acetate [PMA] and calcium ionophore [A23187]), certain cytokines (IL-1β, -8, TNF), integrin stimulation (Mac-1) and auto-antibodies or immune complexes. Though the precise mechanisms of NET formation have yet to be fully elucidated and may be stimuli-specific, a few pathways do appear to be necessary for NETosis. Stimuli activate the granulocyte’s oxidative machinery and ROS production. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) and superoxide dismutase (SOD) produce hydrogen peroxide (H₂O₂) to release NE from an azurophilic gAMP complex, then MPO activates NE’s proteolytic activity and migration to the nucleus (Figure 1-1). In the nucleus, NE cleaves histone H4, promoting the decondensation of DNA. Another enzyme that appears important for DNA decondensation in NETosis is peptidylarginine deiminase (PAD)-4. Nuclear PAD-4 modifies histone arginine residues into citrulline residues, resulting in a change from a positive charge to a more neutral charge thereby decreasing the DNA:histone electrostatic interaction.
Once NETs form and serve their function, they should be rapidly degraded as they contain many cytotoxic gAMPs and expose otherwise cryptic self-epitopes to the periphery. In healthy individuals, this is performed by phagocytic macrophages and an abundance of constitutively present serum nucleases. However, each of these mechanisms, from NET activation to clearance, appears to be dysregulated in lupus patients.

A significant amount of work has been performed over the last five years linking aberrant NET formation and lupus. Granulocytes from SLE patients show an increased capacity to form NETs. In particular, lupus patients possess a unique granulocyte population of low-density granulocytes (LDGs), so named as they are identified and isolated from the peripheral blood mononuclear cell (PBMC) layer rather than the denser red blood cell layer after density gradient centrifugation. Compared to healthy and lupus normal density granulocytes (NDGs), lupus LDGs show an enhanced capacity to synthesize inflammatory cytokines, including IFN-I, an increased, spontaneous formation of NETs, decreased phagocytic activity and an enhanced

![Figure 1-1. Mechanisms leading to nuclear decondensation prior to Neutrophil Extracellular Trap (NET) formation.](image-url)
expression of primary gAMPs \(^{214, 231}\). Importantly, NETs are potential sources of nuclear, modified auto-antigens and oxidative species \(^{177, 233-236}\). Both factors are significant to SLE pathogenesis and possibly SLE-related CVD \(^{59, 76, 77, 166, 201, 237-246}\). As NET degradation and anti-oxidant activity are impaired in SLE, this may promote extensive, chronic inflammation \(^{98, 130, 247-250}\). Therapies for blocking NET formation, namely through PAD inhibition, result in decreased IFN-I signatures and tissue inflammation in lupus-prone mouse models \(^{33, 40}\).

Given the association of atherosclerosis with chronic inflammation, oxidative stress, and aberrant NETosis, pDC, monocyte and macrophage immune responses (described later in this thesis), many of the pathways discussed here appear to contribute to SLE-related CVD.

**IV. Appearance of Clinical Features**

Work from various groups indicates that the appearance of pathogenic auto-antibodies can appear in individuals predisposed to lupus many years before the onset of clinical disease \(^{251}\). The evolution from pre-clinical autoimmunity to clinically apparent disease is unclear, but it is likely that stochastic events and both genetic and environmental factors play crucial roles \(^{251-254}\). In addition to auto-antibodies, the lupus milieu is characterized by increased levels of cell death, innate immune cell activation, inflammatory cytokine production (IL-6, TNF, IFN-I), and adaptive immune cell expansion \(^{17-19, 211, 251, 255, 256}\). Acute manifestations of lupus are sometimes initiated after infection, ultra violet light exposure, or other types of environmental stresses \(^{252-254, 257, 258}\).

With the initiation of more effective therapies, patients are managing to live for decades with this disease. Yet, the smoldering clinical presentation as well as the periods of flare and remission exposes patients to prolonged oxidative stress that promotes the development of end-stage chronic complications. Among them, atherosclerotic CVD is of particular concern given the clinical impact. It is believed that vascular damage starts to develop early on during the natural
history of lupus and is not identified in the majority of patients given the lack of established biomarkers\textsuperscript{25, 251, 259}.

D. Treatment:

Therapies for mild SLE typically include anti-malarials (e.g. hydroxychloroquine), non-steroidal anti-inflammatory drugs, or low dose corticosteroids. With increased severity, higher doses of corticosteroids are needed\textsuperscript{260}. Yet, steroids have extensive metabolic effects that may contribute to CVD-risk\textsuperscript{261-263}. Additionally, broad immunosuppressants including mycophenolate mofetil, methotrexate, cyclophosphamide, and azathioprine are often prescribed. Unfortunately, these can cause SLE patients to become severely immunocompromised, leading to death by infections\textsuperscript{22-24, 264}. Targeted biologics are relatively new to SLE, with belimumab (anti-BAFF) as the only currently FDA-approved monoclonal antibody for the treatment of SLE\textsuperscript{265}. The efficacy of anti-T cell, anti-B cell and anti-IFN therapies are under current clinical trial investigations\textsuperscript{266-268}. Statins are also frequently prescribed to SLE patients, but it is unclear that they significantly decrease CVD risk in this patient population\textsuperscript{269}. Indeed, while many of these therapies have improved the quality of life of SLE patients, to date no drug has been shown to decrease CVD risk in SLE. Furthermore, it remains unclear if the mechanisms implicated in premature CVD in lupus are similar to the ones observed in atherosclerosis within the general population (described below).
Atherosclerosis:

A. Definition, Demographics and Clinical Features:

Atherosclerosis is the build-up of plaque, composed of cholesterol and cellular infiltrates, in the sub-endothelial space of various major arterial trees. Atherosclerosis is a main contributor to the development of heart attacks and strokes. Specifically, coronary artery disease (CAD) is now considered the primary cause of mortality in men and women worldwide. The Framingham heart study, initiated in 1948, was instrumental in identifying significant risk factors for heart disease. “Classical” risk factors include sex (higher risk for males), age (higher risk after the age of 45), diabetes, smoking, raised body mass index (BMI), blood pressure, and an abnormal lipid profile (dyslipidemia): elevated total cholesterol, low-density lipoprotein cholesterol (LDL), triglycerides (TG), and low levels of high-density lipoprotein cholesterol (HDL). However, as discussed elsewhere in the text, these factors are largely not applicable to lupus patients as cardiovascular events in this disease typically occur in women, relatively early in life (35-44 years), and as such cannot be predicted by the Framingham risk equation.

Over the years, newer markers of altered lipoprotein composition or function have been linked to “typical” CVD: high apolipoprotein (apo) B:apoA-I ratios, modifications in the protein cargo of both HDL and LDL, and impairments in cholesterol efflux capacity (CEC). Additionally, atherosclerosis is known as a chronic inflammatory condition as inflammatory indicators are significant biomarkers for CVD in the general population: enhanced levels of high-sensitivity C-reactive protein, pro-inflammatory monocytes, IL-6 levels, and low endothelial progenitor cell (EPC) numbers. As discussed later in the text, lupus patients also show an inflammatory signature, but mechanisms and markers of lupus-CVD still need to be established.
B. Mouse Models of Cardiovascular Disease

CVD is difficult to replicate in mouse models. One possible explanation for their protection from atherosclerosis is the high prevalence of HDL in mice compared to humans. Many other mechanisms attributed to atherogenesis in humans are not observed in mice. For example, unlike humans, mice seem to have a bias towards more nitric oxide synthase (NOS) and less MPO activity, which affects the types of oxidative species generated and possibly macrophage polarization. Additionally, most models require the mice to be on a special high fat, “Western” or “Paigen”, diet to see full disease effects. Primary sites of atherogenesis also differ between humans and mice, with plaque development mainly in aortic regions in mice and in the coronary arteries in humans. There are, however, the LDLR-/- and apoE-/- mice which are often used in CVD studies. These mutations and altered diet, however, create models of murine CVD which show extreme dysregulation of lipoprotein activity and composition that are not entirely physiologically relevant to human atherogenesis.

C. Pathogenesis

1. Genetic and Gender Links

In addition to environmental or behavioral risk factors (smoking, diet, exercise), there is a genetic component to CVD risk. Individuals with at least one parent who exhibits premature CVD are 2.6 (for males) to 2.3 (for females) times more likely to develop CVD. GWAS studies have identified SNPs in genes involved in cholesterol metabolism, vascular integrity, and inflammation as markers of CVD risk. There is a significant increased risk for CVD in men and post-menopausal women. This is different from SLE CVD where most cardiovascular events occur in younger, pre-menopausal women.
II. Lipoproteins

Several CVD risk factors relate to plasma lipoprotein composition and activity. The three general plasma lipoprotein classes are chylomicrons, LDL and HDL. Plasma lipoprotein particles are spherical or discoid phospholipid monolayer structures with the primary function of transporting insoluble cholesterols and TG through the body to where they are needed for energy, hormone, vitamin D, and bile salt synthesis \(^\text{320, 321}\). Structurally, the phospholipid surface of lipoproteins is embedded with proteins (apo, complement, lipases) and non-esterified (free) cholesterol that surrounds an internal core of TG and cholesteryl esters (CE).

Chylomicrons are the largest and least dense of the lipoproteins \(^\text{320, 321}\). Their major function is to transport their high TG content to adipose tissue for storage or active tissue for energy \(^\text{320, 321}\). In addition to LDL, two other subclasses of the “LDL family” exist and are differentiated based on their density and size: very low-, and intermediate-density lipoprotein (VLDL and IDL, respectively) \(^\text{320, 321}\). The “LDL family” of lipoproteins are smaller and denser than chylomicrons. LDL, in particular, is highly associated with CVD as it facilitates the removal of CE out of HDL and into cells \(^\text{320, 321}\). This supplies cells with energy, but can also induce inflammatory reactions and plaque formation, detailed below.

HDL is the smallest and densest lipoprotein, with the largest protein content. apoA-I, which makes up 70% of HDL’s protein weight, mediates the vasoprotective pathway known as reverse cholesterol transport (RCT) \(^\text{322}\). In RCT, HDL’s apoA-I binds ATP Binding Cassette (ABC)A1, ABCG1, and scavenger receptor (SR)B-I on cells to promote the removal of free cholesterol out of these cells. The cholesterol is eventually transformed into CE and delivered to the liver where it is excreted as bile acid. This mechanism is important as it prevents plaque and inflammatory macrophage foam cell development. The quantifiable ability of HDL to promote RCT is called the
CEC, and is inversely associated with CVD risk. The interaction of apoA-I with ABCA1/G1 and SRB-I is also key to promoting anti-inflammatory signals in the vasculature. Paraoxonase (PON) and sphingolipid sphingosine-1-phosphate (S1P) carried within HDL stimulate additional anti-oxidative, vasoprotective mechanisms. What is typically referred to as “HDL” can actually be divided into subclasses differentiated by their density and size: HDL₃ or HDL₂ (nomenclature based on density), or small, medium and large HDL (nomenclature based on size). While native, unmodified HDL possesses many vasoprotective functions, oxidative modifications to HDL can transform it into a pro-atherogenic lipoprotein, discussed below.

III. Inflammation and Atherogenesis

In a healthy cardiovascular system, the vascular endothelial cells prevent platelet adhesion by expressing prostacyclin and endothelial nitric oxide synthase (eNOS), which releases constitutively low amounts of nitric oxide (NO) to regulate vascular tone. In atherosclerosis, however, plaque formation begins with injury to the endothelium, leads to unresolved cell death and inflammation, and eventually thrombosis (Figure 1-2).

a. Initial Injury

Initial inflammatory vascular responses can increase adhesion molecule expression on endothelial cells. Adherent platelets will release inflammatory cytokines (IL-1β, TNF), chemokines, coagulants, and adhesion factors. Thinning of the endothelium exposes the intima layer’s collagen and fibrinogen meshwork, which binds and traps platelets and lipids to promote further inflammation and leukocyte recruitment.
b. **Attempt at Repair and Resolution**

Endothelial progenitor cells (EPCs), found in the bone marrow and circulation, travel to sites of endothelium damage to differentiate and cover the exposed intima layer. EPCs also produce eNOS-derived NO, a process aided by HDL. Monocytes are one of the primary leukocytes recruited in an attempt to remove excess LDL and dead cells stuck in the intima layer. Various scavenger receptors (e.g. SRB-I, LDL receptor) on recruited “classical”, monocytes (CD14+, CD16-) will bind to and engulf LDL in an attempt to get rid of the excess lipoprotein. The monocytes then begin to transform into lipid-loaded foam cell macrophages.

Macrophages develop into an M2 phenotype if raised in an anti-inflammatory, Th2 cytokine environment (IL-4, -13, -10, transforming growth factor beta [TGF-β]) appears to promote M2 polarization. The M2 macrophages then produce cytokines associated with vasorepair such as IL-10, TGF-β, platelet-derived growth factor (PDGF), vascular endothelium growth factor (VEGF) and arginase. In lupus-prone MRL-lpr mice, for example, an inability to generate M2 macrophages is linked to defective repair and enhanced disease after ischemia/reperfusion injury. Like monocytes, the macrophages attempt to remove the pathogenic LDL from the extracellular environment through scavenger receptors such as SRA, SRB-I, CD36, and lectin-like oxidized LDL receptor (LOXIR).

HDL is important for vasorepair mechanisms as it blocks foam cell formation through RCT, abrogates inflammatory and apoptosis signals via apoA-I, PON and S1P activity, inhibits reactive oxygen species (ROS), T cell and caspase activation, and induces eNOS- and activating transcription factor 3 (ATF3)-induced anti-inflammatory and re-endothelialization mechanisms. Whether these vasoprotective functions are active in lupus patient is unknown, however.
c. Unresolved Inflammation and Cell Death

One non-traditional CVD risk factor is the high presence of “inflammatory”/intermediate monocytes (CD14+, CD16+), rather than “classical” monocytes \(^{356, 370-376}\). These non-classical monocytes produce higher levels of ROS, TNF, IL-12B, adhesion molecules, and antigen presentation factors \(^{375, 377-381}\). When monocytes differentiate in an inflammatory, Th1 cytokine environment (lipopolysaccharide, TNF, IFN-γ), it gives rise to the “classical”, inflammatory M1 macrophages phenotype \(^{352-355}\). M1 macrophages produce high levels of ROS, reactive nitrogen species (RNS), IL-6, -1β, -12, and TNF \(^{357-359}\). Indeed, M1 macrophages persist in lupus-prone MRL-lpr mice after ischemia/reperfusion injury and have been linked by some groups to increased lupus organ damage \(^{188}\). This M1 macrophage persistence is also associated with unstable atherosclerotic plaques in CVD \(^{353, 382}\).

Foam-cell macrophages are a potent source of oxidative enzymes that can modify apoB-100 in LDL \(^ {194, 383-391}\). This modified LDL is very cytotoxic and inflammatory: it induces macrophage chemokine release, endothelial adhesion molecule expression and NFκB activation \(^{392-394}\). Similarly, certain modifications in HDL or apoA-I convert the lipoprotein into a pro-inflammatory form (oxHDL) \(^{395-400}\). Oxidized HDL is increased in CVD, promotes adhesion molecule expression, and impairs EPC function \(^ {401, 402}\). Of the possible modifications, 3-chlorotyrosine (3-ClY) and 3-nitrotyrosine (3-NY) oxidized apoA-I is present in the plasma and atherosclerotic plaques of CVD patients \(^ {403-405}\). MPO is the only enzyme capable of catalyzing the formation of 3-ClY, which leads to abrogation of RCT and increased inflammatory pathways \(^ {289, 391, 395, 403-410}\). Part of this effect is due to oxHDL’s decreased affinity for SRB-I and ABCA1 \(^{403-405}\). This also results in loss of ABCA1/G1-mediated anti-inflammatory and anti-apoptotic effects \(^{326, 363, 411-413}\). MPO has largely been associated with pro-atherogenic 3-NY oxidation, though NOS
may also play an important role in its formation 289, 384, 391, 395, 398, 404, 405, 407-410, 414-418. While SLE patients exhibit many of these CVD-associated inflammatory pathways, it has been unclear if specific HDL post-translational modifications occur in SLE which modify the function of the lipoprotein and promote atherogenesis.

d. **Role of Neutrophils in Atherosclerosis**

CVD patients show elevated neutrophil counts that positively correlate with lipid core size in human carotid arteries, suggesting that these cells are important for atherogenesis 419-422. Initially, neutrophils were rarely detected in plaques, but advancements in antibody specificity and two-photon excitation microscopy have increased our understanding of the role of these cells in CVD. Neutrophils have been detected in the shoulder, cap, and tunica adventitia layer of murine and human atheromas 421-424. Recent evidence has also shown that luminal neutrophils can be activated by inflammatory platelets to undergo NETosis, visualized *in vivo*, and may play an important role in coagulation, pDC and macrophage inflammation, endothelial damage, and atheroma lesion size 178, 214, 425-432. Additionally, mice deficient in *PAD-4*, an enzyme crucial in NET formation, or treated with PAD inhibitors are protected from thrombosis and show decreased atheroma lesion formation 425, 433, 434. Neutrophils may be a source of HDL modification, as phagosomal-MPO can mediate 3-C1Y and 3-NY oxidation 414, 435-437. Whether NETosis is a significant contributor to HDL oxidation has yet to be explored as an important mechanism leading to SLE atherogenesis.

e. **Late Stage Atherogenesis and Thrombosis**

There is evidence of enhanced cell death occurring in the arterial wall intima layer due, in part, to their prolonged exposure to an inflammatory milieu. Specifically, cholesterol crystal deposited in endothelial cells, neutrophils, monocytes and macrophages can promote
inflammasome activation, cell death, and damage to the endothelium. This leads to the development of a necrotic lipid core in the intima layer (Figure 1-2). Other leukocytes eventually infiltrate into the atheroma, including T cells, dendritic cells, and mast cells. Dendritic cells exposed to oxidized LDL (oxLDL) become potent antigen presenting cells, leading to T and B cell auto-reactive responses against oxLDL and high anti-oxLDL auto-antibody levels in CAD patients. However, the significance of this remains to be better characterized. It should be noted that some auto-antibodies, particular IgM natural antibodies, may have vasoprotective capabilities.

With the build-up of inflammation and apoptotic debris in the atheroma, vascular smooth muscle cells (VSMCs) are recruited from the tunica media to the intima cap layer. These VSMCs produce a fibrous cap of collagen and elastin to “heal” the damaged endothelium and sequester the necrotic core from the vascular lumen. The plaque will bulge towards the lumen, increasing shear stress, promoting further endothelial cell damage and atherogenesis. Inflammatory macrophages, mast cell and particularly neutrophils (including NETs) will release proteases which digest the fibrotic cap, leading to plaque instability. Eventually the cap will rupture, leaking the necrotic core into the lumen. This will activate the platelet coagulation cascade creating a thrombi or blot clot. Either a complete or partial blockage of blood circulation will occur and can lead to stroke or myocardial infarction.
IV. Lupus-related Cardiovascular Disease

The increase in cardiovascular risk in lupus is striking and cannot be predicted by the Framingham risk equation \(20, 21, 25-27\). It has been proposed that SLE may represent the extreme phenotype of what is seen in “typical” atherogenesis, with an exaggerated inflammatory, immune reaction to endogenous substances (dead cell debris and lipoproteins), leading to enhanced cell death, followed by impaired clearance and repair mechanisms. Recently, the role of immune dysregulation in lupus premature CVD has been linked to a number of putative pathways.
a. Cytokine and Cellular Mechanisms of Lupus Atherosclerosis

Atherosclerosis is considered a chronic inflammatory disease, and both SLE and CVD in the general population share some common inflammatory pathways and abnormalities in innate and adaptive immune responses. IFN-I, for example, may be significant to the pathogenesis of both diseases. An imbalance of vascular damage and repair is triggered by IFN-I. These cytokines promote enhanced apoptosis of EPCs and a dysregulated capacity for these cells to differentiate into mature endothelial cells. Furthermore, IFN-I can promote the differentiation of macrophages into foam cells, as well as platelet activation leading to thrombosis enhancement. Indeed, in the NZM lupus model, attenuation of IFN-I signaling improves vasculopathy and thrombosis risk, while administration of IFN-I (mimicking lupus flares) worsens endothelial function and promotes thrombosis, implicating these cytokines as key drivers of vascular damage in this disease. IFN-I signaling also enhances plaque formation in apoE-/- mice. Additionally, in a human lupus cohort, IFN-I were identified as key associates with endothelial dysfunction and subclinical atherosclerosis in univariate and multivariate analyses.

Given the very high IFN-I levels in SLE, what may be potent pro-atherogenic mechanisms in CVD may be even more severe in SLE.

Another putative mechanism implicated in premature lupus and “typical” atherogenesis is NETosis. Lupus NETs are potent mediators of platelet activation, coagulation, endothelial cell damage and atherosclerosis. NETs taken up by pDCs promote high IFN-I production, which may explain the critical role of pDCs in apoE-/- atherosclerosis. NETosis may also contribute to the high levels of oxidative markers in SLE, but how this enhanced externalized oxidative machinery may modify lipoproteins in atherogenesis remains to be determined.
b. **Dysfunctional HDL Activity**

As mentioned, there is a lack of research concerning lipoprotein signaling in SLE and its implications in atherosclerosis. Most literature to date involves clinical reports on the presence of auto-antibodies against lipoprotein components in SLE (anti-oxLDL, apoA-I, cardiolipin, β2-glycoprotein I, and HDL) but their role in mediating vascular disease remains to be determined. There is debate in the literature about whether IFN-I and the immune complexes which form with these auto-antibodies block LDL uptake, degradation and cell debris clearance. What little work that has been done on the role of HDL in SLE inflammatory pathways is largely observational and does not identify specific pro-atherogenic signaling mechanisms.

The HDL of lupus patients is considered dysfunctional and “pro-inflammatory.” To date, lupus “pro-inflammatory” HDL has been quantified by the inability of HDL to block LDL oxidation. This inability is higher in SLE than rheumatoid arthritis, in SLE patients with plaque, and correlates with inflammatory markers. Monocytes treated with SLE “pro-inflammatory” HDL show increased chemotaxis and TNF production. In both lupus-prone mouse models and human patients, “pro-inflammatory” HDL activity correlates with low levels of the anti-oxidant enzyme PON. Decreased PON activity also correlates with high anti-apoA-I antibody titers, which increase with disease flares. SLE HDL also demonstrates lower ABCA1/G1-mediated CEC than healthy controls or rheumatoid arthritis patients. Hydroxychloroquine use has been associated with improved lipid profiles and decreased plaque thickness, but it may impair RCT. Interestingly, abrogation of IFN-I decreases levels of oxidized HDL in NZM lupus-prone mice.
D. Treatment:

Preventive measures are important in patients at risk for CVD or in patients that have experienced atherosclerotic events. These include smoking cessation, diet, exercise and control of blood pressure. In addition, many drugs are currently being used or explored as primary or secondary CVD prevention. Statins are frequently used in CVD. Medications that increase HDL levels are being tested and it remains unclear whether they will be safe and useful in primary and secondary CVD prevention. HDL mimetics are synthetic constructs made of apolipoproteins and lipids. As in vitro and in vivo animal models have shown an ability of mimetics to promote anti-atherosclerotic mechanisms, various HDL mimetics are in current clinical trials or were previously tested. In an animal model of lupus atherosclerosis (apoE/-, Fas/- mice), treatment with an apoA-I mimetic showed lower auto-antibody production, indicating a potential therapeutic application in lupus CVD. While many of these therapies have improved the lifespan of CVD patients, to date no drug has been shown to decrease CVD risk in SLE. There is a great need to further characterize the main pathways promoting atherogenesis in lupus as well as the most effective modulators of vascular risk in this disease.
Chapter 2

Mechanisms Leading to Enhanced HDL Oxidation in SLE

A. Introduction:

Lupus patients have an enhanced predisposition for early CVD, but neither clinical markers nor a well-defined innate immune pathway have truly been associated with this risk. As mentioned above, while others have indicated that HDL is dysfunctional in SLE and associated with subclinical atherosclerosis, it remains to be determined what specific modifications occur in lupus HDL to modify its function and what are the cellular sources of this modification.

We hypothesized that SLE patients possess increased levels of modified, dysfunctional HDL and that this modification would render the lipoprotein pro-atherogenic. To this effect, we quantified levels of 3-CIY and 3-NY in lupus HDL and plasma. We focused on these modifications as in previous studies they have been highly linked to the abrogation of HDL’s vasoprotective capabilities such as CEC, anti-oxidative activity, and abrogation of inflammatory pathways.\textsuperscript{395,397,399,401,404} Furthermore, we posited that NET formation, which is enhanced in SLE, exposes the active oxidative machinery (NOS, MPO, NOX) required for 3-CIY and 3-NY oxidation in humans and mice (Figure 2-1)\textsuperscript{414,416,515-520}.
B. Materials and methods

Subject Recruitment

Plasma samples were collected from SLE patients fulfilling the American College of Rheumatology diagnostic criteria for the disease. Healthy controls (Ctrl) were recruited by advertisement. Study was approved by the University of Michigan and NIH IRBs. Lupus disease activity was quantified by the SLE Disease Activity Index (SLEDAI). Pregnant or lactating women and individuals with recent or current infections or liver dysfunction were excluded.

Mice

NZM2328 (NZM) breeding pairs were a gift from Dr. Chaim Jacob. Balb/c mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were bred and housed in a specific pathogen-free barrier facility at the University of Michigan. Female mice were euthanized at 26-weeks of age, before overt development of renal disease. Protocol was approved by University of Michigan’s Committee on Use and Care of Animals.

Figure 2-1. Enzymatic pathways leading to tyrosine oxidation. NADPH oxidase and superoxide dismutase generate the substrates used by myeloperoxidase and nitric oxide synthase. These enzymes then generate the reactive oxygen and nitrogen species (in red) capable of 3-chlorotyrosine and 3-nitrotyrosine modifications to apolipoprotein A-I, which are associated with cardiovascular disease.
Plasma HDL Isolation

HDL was isolated from human and murine plasma by buoyant-density sequential ultracentrifugation \(^{521}\). Protein concentration was estimated with Coomassie (Thermo Scientific, Rockford, IL). Samples were stored at -80 °C until analysis.

Quantification of Oxidized Amino Acids in Plasma, HDL, and apoA-I Peptides

Plasma proteins were precipitated and delipidated \(^{521}\); oxidized amino acids were quantified using isotopically labeled internal standards, \(^{13}\)C\(_6\) tyrosine, \(^{13}\)C\(_6\) 3-chlorotyrosine and \(^{13}\)C\(_6\) 3-nitrotyrosine, by liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) with multiple reaction monitoring (MRM) MS/MS positive ion acquisition mode \(^{522}\).

MRM Analysis of Oxidized Tyrosine-Containing apoA-I Peptides with LC/ESI/MS/MS

Plasma HDL samples were delipidated, and reduced with dithiothreitol (5 μM, Sigma-Aldrich, St. Louis, MO) before alkylation with iodoacetamide, (15 mM, Sigma-Aldrich) \(^{521}\). Samples were trypsin-digested and purified using solid-phase extraction C18 Sep-Pak columns (Waters Corporation, Milford, MA). Isotopically labeled oxidized (nitrated and chlorinated) peptides and native apoA-I peptides were spiked into samples following trypsin digestion. MRM analysis was performed with an Agilent 6490 Triple Quadrupole MS system equipped with an Agilent 1200 Infinity UPLC (Agilent Technologies, New Castle, DE) in positive ion mode \(^{521}\).

Cholesterol efflux capacity (CEC) and MPO Quantification

CEC assay and MPO quantification were performed as described \(^{521}\). J774 cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/10% fetal bovine serum (FBS, Life Technologies, Carlsbad, CA) \(^{323}\). To radiolabel cellular free cholesterol pool, cells were incubated with 1 μCi/ml \(^{3}\)H cholesterol (Perkin Elmer, Waltham, MA) in DMEM/ 1 mg/ml fatty acid-free
bovine serum albumin (FAFA, BSA) and 5 µg/ml ACAT inhibitor Sandoz 58-035 (Sigma-Aldrich) overnight. Cells were incubated with 0.5 mM 8-Br-cAMP to induce ABCA1 expression for 20 hours, then incubated with DMEM/FAFA with or without 2.8% apoB-depleted plasma for 4 hour at 37 °C and chilled on ice. Medium was collected and filtered, [³H] cholesterol content of medium and cells was quantified, and fraction of total [³H] cholesterol released into the medium was calculated ⁵²¹.

HDL Proteomic Analysis

Blood was obtained in heparinized tubes and plasma was collected from 10 Ctrl and 10 SLE patients by Ficoll-Paque (GE Healthcare) density centrifugation. Plasma was separated by gel filtration chromatography using two Superose 6 (GE Healthcare) columns arranged in series on an AKTA fast protein liquid chromatography (FPLC) system (GE Healthcare) as described previously ⁵²³. Fractions within the HDL peak were combined to generate pools representing large and small HDL. Pooled fractions were applied to a phospholipid binding resin to isolate HDL from abundant co-migrating plasma proteins and, after washing twice with 50 mM ammonium bicarbonate, lipoprotein bound proteins were digested with sequencing grade trypsin (1.5 µg/sample) overnight. Resulting peptides were reduced with dithiothreitol and carbamidomethylated with iodoacetamide prior to drying in a speedvac. Dried peptides were suspended in 0.1% trifluoroacetic acid and desalted using ZipTips (Millipore). Desalted peptides were dried and suspended in 20 µL of 0.1% formic acid. Samples were analyzed by ESI-MS/MS on an Orbitrap Elite mass spectrometer (Thermo Scientific) ⁵²³. Peak lists were searched against the UniProtKB/Swiss-Prot database using the Mascot search engine and Scaffold (version Scaffold_2_04_00, Proteome Software, Portland, OR) was used to validate protein identifications.
Statistical significance of group comparisons, by spectral counting, was determined in GraphPad Prism (GraphPad, La Jolla, CA).

Induction and Purification of NETs and Co-culture Assays

Human Ctrl NDG and lupus LDGs were purified from peripheral blood, and murine neutrophils from bone marrow. Cells were plated at a density of 0.25 x 10^6 cells/cm² on tissue-culture plates in RPMI-1640 without phenol red (Life Technologies) for 3-ClY quantification, or in Krebs Ringer’s Phosphate Glucose (KRPG) buffer (Sigma-Aldrich) for 3-NY quantification. NDG were cultured in the presence or absence of 20 nM PMA (Sigma-Aldrich) for 3 hours (human), or 100 nM PMA for 5 hours (mouse) to induce NET formation. As LDGs form NETs spontaneously, they were left unstimulated. To inhibit NET formation, 200 µM Cl-Amidine was added for the full incubation. As NOX and MPO are required for NET formation but are also targets for the conditions where oxidation was inhibited, cells were allowed to form NETs for 1 hour before adding the following inhibitors: diphenylene iodonium (DPI, blocks NOX activity, 100 µM, Tocris, Bristol, UK), L-NG-monomethyl-L-arginine (L-NMMA, blocks NOS activity, 200 µM, Abcam, Cambridge, UK) and 3-amino-1,2,4-triazole (3-AT, blocks MPO activity, 10 mM, Sigma-Aldrich). Inhibitors were replenished hourly during NET formation. NETs were isolated as described, using 100 U/mL DNase I (Roche, Branchburg, New Jersey). Supernatants resulting from the final spin contain NET-bound proteins and DNA. Ctrl, (negligibly oxidized) HDL (50 µg/mL) was incubated with these NETs in the presence or absence of L-NMMA, 3-AT and DPI for 30 minutes at 37° C. Relative abundance of HDL oxidation was calculated as fold change of each condition relative to HDL oxidation with NET formation.
Quantification of NOS and NOX in Human and Murine NETs

Immunoblot: To detect NOS and NOX externalization during NET formation, digested human and murine NETs and whole neutrophil pellets were harvested. NET proteins were precipitated with acetone; 50 µg of NET or whole cell pellet proteins were separated on a 10% SDS-PAGE gel. Proteins were transferred onto a nitrocellulose membrane, incubated in 5% bovine serum albumin (BSA) / phosphate buffered saline (PBS) 0.1% Tween-20 for 1 hour, and stained with goat anti-mouse p47 or p22 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-eNOS or iNOS (1:100, Abcam) or anti-tubulin (negative control, 1:500, Sigma-Aldrich), followed by horseradish peroxidase-conjugated rabbit anti-goat (1:1000, Millipore, Temecula, CA) or goat anti-rabbit (1:5000, Jackson ImmunoResearch, West Grove, PA) secondary antibodies. Densitometry was performed with ImageJ (Bethesda, MD).

Fluorescence microscopy: Human NDG and lupus LDGs and mouse neutrophils were seeded onto poly-L-lysine (Sigma-Aldrich) coated coverslips, stimulated with PMA as above, or left unstimulated (for LDGs) before fixing in 4% paraformaldehyde and staining with goat anti-p47 or p22 (both 1:50 dilution, Santa Cruz), or rabbit anti-iNOS or eNOS (both 1:20 dilution, Abcam) for 1 hour at 4 °C, followed by secondary fluorochrome-conjugated antibodies (Jackson ImmunoResearch) and Hoechst 33342 (Life Technologies) 33,214. Coverslips were mounted with ProLong Gold Antifade (Life Technologies). Images were acquired on a Zeiss LSM510 META confocal laser-scanning microscope (Carl Zeiss Microimaging, Thornwood, NY) with a ×63 lens and quantified 33,214.

Effect of Chloroquine on Neutrophils

Ctrl or lupus whole blood was incubated with 250 ng/mL chloroquine (Sigma-Aldrich, CQ) for two hours before neutrophils were purified as above 527. Effect of CQ on NET formation was
quantified using SYTOX assay (Life Technologies), immunofluorescent staining and immunoblot

To determine the effect of CQ on MPO, NOS and NOX activity, 1 x 10^6 neutrophils/mL, in

either RPMI 1640 or KRPG with protease inhibitor cocktail (Roche), were homogenized at 35,000
rpm (Omni International, Kennesaw, GA) for three 10 second intervals on ice before treatment
with or without 20 nM PMA for 30 minutes, then in the absence or presence of 250 ng/mL CQ for
30 minutes, and finally with or without 50 µg/mL HDL for 30 minutes. This solution was frozen
and analyzed for 3-CIY and 3-NY oxidation.

**In vivo Cl-Amidine Administration**

PAD inhibitor Cl-amidine (Cl-Am) was synthesized. Twelve-week old NZM female

mice were administered daily subcutaneous injections of Cl-Am (10 mg/kg/day) or PBS (Life
Technologies) for 14 weeks. Isolated plasma and HDL were analyzed for 3-CIY and 3-NY
oxidation.

**Statistical Analysis**

Pearson correlation coefficients were calculated between outcomes studied and patient
characteristics. Multivariable linear models were used to explore significant predictors of the
outcomes of interest. The method of best subsets with the R-squared selection criterion guided
model selection process. These models were also used to estimate and test differences between
Ctrl and SLE groups. Skewed variables were logarithm base 10 (log10) or natural log (ln)
transformed to satisfy statistical assumptions. Normally distributed variables were not
transformed. A p value <0.05 was considered significant. Analyses were conducted using SAS
V.9.2 (SAS Institute Inc., Cary, North Carolina, USA) or GraphPad Prism (GraphPad). For Table
2-2, variables indicated with an “**” were transformed (natural log-scale) prior to correlation
calculation. Values indicated with “***” are adjusted as follows: CEC (%): Adjusted for HDL
MPO (fmol/ml): Adjusted for Age, BMI, and LDL (n=49, \( R^2 = 0.08 \)); HDL-3-C1Y: Adjusted for BMI (n=59, \( R^2 = 0.10 \)); HDL-3-NY: Adjusted for cholesterol (n=54, \( R^2 = 0.34 \)); Plasma-3-3-C1Y: Adjusted for LDL (n=50, \( R^2 = 0.21 \)); Plasma-3-NY: Adjusted for LDL (n=50, \( R^2 = 0.07 \)).
Table 2-1. Demographics of patients used for oxidation study. Statistically significant differences between groups noted with “*” (p<0.05). Normally distributed variables are displayed as mean ± standard deviation. Skewed variables are displayed as median (IQR). HDL= high-density lipoprotein; LDL= low-density lipoprotein; ESR= Erythrocyte Sedimentation Rate; SLEDAI= Systemic Lupus Erythematosus Disease Activity Index; ACE= Angiotensin-converting enzyme; MPO= myeloperoxidase. Duplicated from Smith et al. (2014).
C. Results

Patients Characteristics

Ctrl and lupus patients did not differ in most demographic characteristics (Table 2-1). SLE patients also exhibited total cholesterol, BMI, TG, LDL, and HDL levels within the normal range for CVD risk factors mentioned previously. Levels of LDL were significantly lower in SLE compared to Ctrl, possibly associated with the prevalent use of statins (22.5%). SLE statin use correlated with elevated plasma 3-CIY levels, whereas antimalarial use was associated with higher plasma 3-NY content (Table 2-2). SLE patients displayed significantly higher levels of plasma MPO compared to Ctrl (Table 2-1). Elevated MPO levels significantly correlated with a higher ESR and low LDL levels (Table 2-2). Oxidation levels did not correlate with SLEDAI (Table 2-2).

CEC is Impaired in SLE

The ability of HDL to promote cholesterol efflux from macrophages (CEC) is a metric of HDL function and has a strong inverse association with CVD. Plasma from patients with SLE displayed significantly diminished CEC when compared to Ctrl plasma (Table 2-1, Figure 2-2A). These results persisted after adjustment (listed in the Statistical Analysis section) for significant predictors of CEC (Table 2-2) and support previous indications that dysfunctional HDL present in SLE leads to impaired CEC and may promote pro-atherogenic responses.

Chlorinated and Nitrated HDL is Increased in SLE

Impaired CEC has been associated with HDL modifications such as 3-CIY and 3-NY. We quantified levels of 3-CIY, a highly specific product of MPO, and 3-NY, a product of MPO and other RNS-producing enzymes, in SLE and Ctrl isolated HDL and in total plasma. SLE HDL displayed 2-fold higher median levels of 3-NY (Figure 2-2B) and 121-fold higher
median levels of 3-CIY (Figure 2-2C), compared to Ctrl HDL even after adjustment (listed in the Statistical Analysis section). As previous studies have identified that chlorination of tyrosine (Y) residue 192 (Y-192) within the apoA-I protein most directly associates with impaired CEC, with six other tyrosine residues in apoA-I (Y-18, -29, -100, -115, -166, and -236) as other potential sites of oxidation, we determined if regiospecific nitration and chlorination patterns occur in SLE. Levels of 3-NY HDL oxidation were highest at Y-192, 115, 100, 29 and 18 in SLE (Figure 2-2D). The highest levels of MPO-dependent 3-CIY HDL oxidation in SLE samples were observed at Y-192, -115 and -18 (Figure 2-2E), when compared to Ctrl samples. Taken together, this data suggests that regiospecific modifications to HDL by MPO and RNS-producing enzymes, specifically at Y-18, -115, and -192, may be of particular interest in the context of SLE-associated CVD.

When we examined correlations between levels of 3-NY and 3-CIY in SLE HDL, they significantly positively correlated with each other (Figure 2-2F), suggesting that these oxidative modifications are generated at similar sites in the body. There was no significant correlation between 3-NY and MPO levels (Figure 2-2G), but the levels of 3-CIY significantly correlated with plasma MPO levels (Figure 2-2H). This suggests that the RNS leading to HDL nitration are not primarily derived from MPO, but from another oxidative source. While Y-115 and -18 displayed the highest levels of oxidation in lupus, 3-CIY Y-192 showed the most significant positive correlation with plasma MPO levels (Figure 2-2I). Overall, these results support the notion that SLE patients possess high levels of oxidative species leading to HDL oxidation and impairment of lipoprotein function.
Table 2.2. Correlations of lipoprotein characteristics and plasma oxidation with clinical features in all subjects and in patients with SLE only. Shaded boxes reach statistical significance (p<0.05). HDL = high-density lipoprotein; LDL= low-density lipoprotein; CRP= C-reactive protein; ESR = erythrocyte sedimentation rate; SLEDAI = SLE Disease Activity Index; ACE = angiotensin-converting enzyme; CEC= cholesterol efflux capacity; MPO = myeloperoxidase. Variables with “*” were transformed (natural log-scale) prior to correlation calculation. Values indicated with “**” are adjusted as stated in the Statistical Analysis section. Duplicated from Smith et al. (2014)
Figure 2-2. Plasma and HDL of SLE patients exhibit high levels of dysfunction and oxidation. (A) The cholesterol efflux capacity of plasma samples from Ctrl and lupus patients were examined in J774 cells. Levels of HDL (B) 3-NY and (C) 3-CIY were examined in control and lupus patients. The regiospecific tyrosine (Y) oxidation patterns of (D) 3-NY and (E) 3-CIY were determined for control and lupus HDL. Correlations between lupus and control (F) 3-NY and 3-CIY, (G) plasma MPO and 3-NY, (H) plasma MPO and 3-CIY, and (I) plasma MPO and Y192 3-CIY oxidation were determined. Modified from Smith et al. (2014). (N= 20 Ctrl and N=40 SLE donors, *p<0.05, **p<0.006, ***p<0.0001)
**SLE HDL Exhibits an Altered Proteome**

In addition to examining differences in oxidation levels, we quantified protein content variations by FPLC in HDL collected from the plasma of 10 Ctrl and 10 SLE donors. Compared to Ctrl HDL, SLE HDL showed significantly lower complement C4-B, apoA-II and higher levels of vasorin, plasma protease C1 inhibitor, protein Z-dependent protease inhibitor, immunoglobulin fragments, and complement factor I (Figure 2-3A). Vasorin is rarely reported on in the literature. It is largely expressed by VSMCs. When cleaved by ADAM17 protease, it binds to TGF-β to prevent its signaling. It appears to have a role in vascular remodeling, though whether it leads to repair or damaging fibrosis is not clear.

![Figure 2-3. SLE HDL demonstrates a different protein content than Ctrl HDL. Plasma HDL proteomics was determined by FPLC. (N=10 Ctrl and SLE donor samples, p<0.05)](image)

**NET-derived MPO, NOX and NOS Promote HDL Oxidation In Vitro**

As levels of RNS- and MPO-derived oxidative modifications are markedly elevated in lupus and correlate with functional impairment of CEC, we attempted to identify the putative sources of enhanced RNS and MPO activity in SLE. MPO HDL oxidation is classically attributed to plaque macrophages and phagosome activity. Another possible source is peripheral blood MPO, reported enhanced in SLE and which we were able to confirm (Tables 2-1 and 2-2). As a subset of peripheral blood lupus granulocytes (LDGs) have a significantly enhanced capacity to form NETs, a source of externalized NOX and MPO, we examined if the
NETs could induce HDL oxidation\textsuperscript{214,216,525}. Additionally, because we found that MPO levels did not correlate with 3-NY HDL levels (Figure 2-2G), we examined if an alternative producer of RNS (NOS and NOX formation of peroxynitrite, Figure 2-1) could be present in NETs and serve as a source of HDL nitration.

MPO (not shown), NOX (p22 and p47 subunits) and NOS (eNOS and iNOS) were all detected on both Ctrl NDG and LDG NETs by fluorescence microscopy and immunoblot (Figure 2-4A). Next, we exposed healthy HDL to NETs isolated from PMA-stimulated Ctrl NDG or spontaneously by lupus LDGs, in the absence or presence of MPO, NOX and NOS inhibitors (3-AT, DPI and L-NMMA, respectively), and examined HDL oxidation profile. As shown in Figures 2-4B and 2-4C, NETs from both lupus LDGs and Ctrl NDG significantly enhanced HDL oxidation. Hypochlorous acid (HOCl), which is synthesized by MPO downstream of NOX activity, is the major source of HDL chlorination in humans. When NETting Ctrl NDGs and lupus LDGs were treated with 3-AT and DPI to block MPO and NOX activity, respectively, HDL 3-ClY oxidation was abrogated (Figure 2-4B). These results support the hypothesis that MPO and NOX externalized on NETs can induce 3-ClY HDL oxidation in circulation.

Because both NOS and MPO are capable of producing the RNS required for HDL nitration, we used a KRPG solution with no added nitrite to examine the role of NOS alone in 3-NY oxidation. Under these conditions, only NOS could generate the nitric oxide radical (NO\textsuperscript{•}) required for peroxynitrite formation that could lead to HDL nitration (Figure 2-1). MPO, under these conditions, could not be the source of RNS. Indeed, the MPO inhibitor 3-AT was ineffective at blocking HDL nitration, while the NOS inhibitor L-NMMA significantly abrogated HDL 3-NY oxidation for both lupus LDGs and Ctrl NDG (Figure 2-4C). These results suggest that NET-bound
NOS is a source of the RNS causing HDL nitration, and may explain why the levels of MPO did not correlate with the levels of 3-NY oxidized HDL in SLE.

*Chloroquine (CQ) Blocks NET Formation In Vitro*

As a majority (72.5%, Table 2-1) of the SLE patients studied were on anti-malarials, we determined the effect of physiologically relevant concentrations of CQ (250 ng/mL) on NET formation. CQ significantly inhibited NET formation in Ctrl NDGs and lupus LDGs (Figure 2-5A-C). To assess if CQ modified MPO, NOS and NOX activity, neutrophils were homogenized, treated with CQ, and incubated with HDL. CQ did not significantly block 3-ClY or 3-NY oxidation in lupus LDGs or Ctrl NDGs (data not shown). This indicates that though CQ may block NET formation, this is not accomplished by inhibiting oxidative enzyme activity.
Figure 2-4. Human Ctrl NDGs and lupus LDGs contain the oxidative machinery and capacity to cause 3-CIY and 3-NY oxidation. (A) NOX (p22 and p47) and NOS were detected in NETs by immunofluorescence. The ability of NETs from NDGs and LDGs to induce (B) 3-CIY and (C) 3-NY HDL oxidation was examined. The activity of NET formation, MPO, NOS or NOX was inhibited by Cl-Am or lack of PMA, by 3-AT, by NMMA, or by DPI, respectively. Duplicated from Smith et al. (2014). (Data are displayed as mean ± SEM, N= 6/group, *p<0.03, ** p < 0.009, *** p < 0.0009)
Figure 2-5. Chloroquine (CQ) impairs NET formation. Both blood NDGs and LDGs were incubated with CQ and examined for NET formation by (A) SYTOX fluorescent assay and (B) immunofluorescence. (C) Representative images of NET formation with CQ treatment, staining for MPO, iNOS and NOX (p22) components. Duplicated from Smith et al. (2014). (N=4/group, Data are displayed as mean ± SEM, *p<0.03, ** p < 0.0009, **** p < 0.0001)
NET-derived NOS and NOX are Sources of HDL Oxidation in Murine Systems

While present in murine NETs, MPO is not associated with murine atherosclerosis. Lupus-prone NZM mice display striking elevations of 3-NY HDL oxidation, but not 3-CiY, when compared to Ctrl mice, possibly because murine leukocytes contain less MPO, the sole known source of 3-CiY oxidation, than human leukocytes. We therefore examined if NOX and NOS were present in murine NETs and could produce RNS to form 3-NY oxHDL. We identified NOS and NOX (p22 and p47) machinery present in PMA-induced murine NETs (Figure 2-6A). To verify that these NET-bound enzymes could cause HDL oxidation, we exposed native HDL (purified from Ctrl Balb/c mice) to NZM or Balb/c NETs in the absence or presence of MPO, NOX and NOS inhibitors (3-AT, DPI and L-NMMA, respectively) and examined HDL oxidation profiles. Consistent with our previous report, we found no significant 3-CiY oxHDL patterns after HDL incubation with murine NETs (Figure 2-6B). However, a significant oxidation pattern for 3-NY oxHDL was observed (Figure 2-6C). While experiments were performed in nitrite containing media (RPMI) so that either NOS or MPO could potentially promote HDL nitration, only DPI and L-NMMA significantly blocked NET-induced 3-NY HDL oxidation. These results support the idea that MPO is less active in mice and that NOX and NOS are the primary sources of the oxidative species required for HDL nitration in murine systems.

Finally, to verify that NETs are an important source of HDL oxidation in the periphery, we inhibited NET formation in NZM mice in vivo and examined their oxidative profile. NZM mice received the PAD inhibitor Cl-Am daily for 14 weeks, as PAD activity is necessary for NET formation. Cl-Am-treated mice displayed significantly decreased 3-NY content in HDL, but not total plasma, when compared to vehicle treated mice (Figure 2-6D). These results indicate that in vivo inhibition of NET formation in lupus-prone mice decreases HDL oxidation.
D. Summary

We sought to determine if lupus HDL and plasma were oxidized and displayed impaired CEC, factors known to promote atherogenesis. We also pursued a putative innate immune mechanism that would explain the modified HDL in SLE. We found that compared to controls, SLE plasma demonstrated impaired CEC and SLE plasma proteins, including apolipoprotein A-I, showed high 3-CIY and 3-NY oxidation levels. Because 3-CIY and 3-NY oxHDL levels correlated with each other, but 3-NY did not correlate with MPO levels, this suggested that both modifications occur at similar sites in the vasculature, though not through the same enzymes. As the plasma oxidation levels were so high in SLE, the results also suggested that the site of oxidation was largely in the circulation. Most of the literature to date has focused on macrophages in the atherosclerotic plaque as sources of MPO-driven HDL oxidation, yet circulating neutrophils contain higher levels of MPO than tissue-resident macrophages, and both neutrophils and NETs are present around thrombotic and atheroma plaques. Indeed, NOS, MPO and NOX externalized in human and murine NETs were capable of inducing a CVD risk pattern of HDL oxidation. Finally, we demonstrated that in vivo NET inhibition potently decreased levels of 3-NY oxHDL in lupus-prone mice. Therefore, NETs may play a crucial role in the transformation of HDL into its pro-atherosclerotic form.

These results indicate a novel mechanism of HDL oxidation, through NET activity which may occur primarily in the circulation rather than in sub-endothelial plaques. Given the high rate of spontaneous NET activity exhibited by LDGs, our results also contribute to the supposition that these granulocytes may be particularly pathogenic to lupus patients. Indeed, it would be interesting to see if LDGs can be detected in lupus atherosclerotic plaques or if levels of blood LDGs correlate with HDL oxidation levels. The increased presence of these cells in the blood of SLE patients...
combined with their extensive NET activity may explain why the circulating plasma in SLE showed higher levels of oxidation and impaired CEC than seen even in “typical” CVD, found in the general population.409

Given the high levels of circulating plasma oxidation, we focused more on HDL than LDL, which is a less mobile lipoprotein and is generally found stuck in plaques. However, as NET structures have also been detected in tissue, whether NETs in SLE also contribute to LDL oxidation should be explored in the future.33, 40, 214, 425, 540. This may prove a potential biomarker for SLE cardiovascular health along with 3-ClY and 3-NY HDL oxidation. However, a larger cohort of patients than was used in this study would be needed to determine if these biomarkers are sensitive and specific to SLE CVD. Such markers are needed given the lack of association between Framingham risk factors and lupus atherosclerosis.

Pharmacological inhibition of NET formation, by Cl-Am e.g., has been proposed as a possible lupus therapy. Indeed, lupus-prone mice treated with such inhibitors show reduced tissue damage, levels of auto-reactive antibodies and inflammatory cytokines.33, 40, 425. Our murine results indicate that such therapies may also be beneficial for SLE CVD. However, our study was limited by the lack of well-defined lupus-prone mouse models which also develop atherosclerosis. As mentioned previously, mice in general do not develop CVD. NZM mice do show endothelial cell dysfunction and high levels of HDL 3-NY oxidation, but they fail to develop atherosclerotic plaques.34. As such, we did not measure the effect of Cl-Am on plaque formation in lupus mice. Additionally, as 3-NY oxidized HDL (predominant in mice) is not associated with impaired CEC, we did not measure the effect of Cl-Am treated on murine CEC.408. However, as the HDL from lupus patients and mice does possess the hallmarks of a dysfunctional, pro-atherosclerotic lipoprotein, we asked whether these modifications would, in turn, amplify inflammatory responses.
Figure 2-6. Murine granulocytes contain the oxidative machinery and capacity to cause 3-NY oxidation and abrogation of NET formation blocks 3-NY HDL levels. (A) NETs from mice were stained for NOX components (p22 and p47) and NOS. (B) The ability of NETs from Balb/c and NZM mice to induce 3-C1Y HDL oxidation was examined (N=4/group). (C) The ability of NETs from Ctrl Balb/c and NZM mice to induce 3-NY HDL oxidation was quantified. The activity of NET formation, MPO, NOS or NOX was inhibited by lack of PMA, by DPI, or by NMMA, respectively (N=8/group). (D) Levels of plasma and HDL 3-NY in NZM mice that received daily s.c. injections of PBS (N=10) or Cl-Am (N=10) for 14 weeks. Duplicated from Smith et al. (2014). (Data are displayed as mean ± SEM, ** p < 0.01, **** p < 0.0001)
Chapter 3
Lupus HDL Promotes Pro-inflammatory Responses in Macrophages by Binding LOX1R and Failing to Promote ATF3 Activity

A. Introduction:

“Healthy” HDL has a strong inverse correlation to CVD risk, and promotes numerous vasoprotective mechanisms: RCT, and inhibition of LDL oxidation, adhesion molecule expression, TLR-induced inflammatory responses and NFκB activation\(^\text{319, 367, 368, 541, 542}\). When HDL is oxidized, however, it loses many of these vasoprotective effects\(^\text{239, 401, 408, 409, 501, 503, 543}\). As both SLE and CVD appear to share high levels of HDL oxidation and altered monocyte and macrophage immune responses, we hypothesized that these pathways work together to induce enhanced atherosclerosis in SLE\(^\text{197, 333}\).

We predicted that SLE HDL would have an impaired ability to block TLR-induced inflammation, and may even promote inflammatory cytokine production in macrophages. We further examined the role of SLE HDL on macrophage polarization and induction of anti-inflammatory activity through transcription factor regulation. As various scavenger receptors expressed on macrophages (LOX1R, CD36, SR-BI) interact with HDL to modify cholesterol and inflammation homeostasis, we analyzed the role of these receptors on SLE HDL-induced responses\(^\text{197, 544}\). Finally, we hypothesized that a recently described HDL mimetic (ETC-642) could abrogate inflammatory responses induced by oxidized HDL both in vitro human macrophages and in vivo lupus-prone NZM mice\(^\text{512, 545}\). These findings would enable us to
elucidate a novel pathway by which the highly oxidized HDL in SLE transforms healthy macrophages into pro-inflammatory, pro-atherogenic cells.

B. Materials and methods

Subject Recruitment

The same cohort of lupus patients were used as in chapter 2.

HDL and ETC-642 Preparation

HDL was purified as described above. ETC-642 was prepared using ESP24218 apolipoprotein A-I mimetic peptide, sphingomyelin (SM) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) at 1:1:1 weight/weight ratios. All components were dissolved in organic solvent, lyophilized and hydrated with sterile PBS (Life Technologies). The purity and size distribution of the resulting ETC-642 particles were examined by gel permeation chromatography and dynamic light scattering, respectively. ESP24218 was prepared by custom synthesis (GenScript Corp, Piscataway, NJ) and phospholipids were purchased (Avanti Polar Lipids, Alabaster, AL). Experimental controls for ETC-642 components, suspension of DPPC-SM mixture and ESP2418 solution, were prepared by sonication of lipid powders in PBS.

Macrophage Culture

SLE and Ctrl PBMCs were isolated by Ficoll-Paque (GE Healthcare Life Sciences) density gradient. Cells were plated at a density of 0.5x10^6 cell/cm^2 and allowed to adhere for 2 hours; non-adherent cells were removed and attached cells cultured in fresh X-Vivo-15 media (Lonza, Basel, Switzerland) with 10% FBS (Gemini, Sacramento, CA). Monocytes were differentiated into macrophages over the course of 1 week, with media changes every 3 days, and purity was determined by FACS analysis.
Culture Conditions, RNA Isolation and RT-qPCR

When determining the effect of HDL alone on inflammatory cytokine production, Ctrl macrophages were cultured in the presence or absence of Ctrl or SLE HDL (50 µg/mL) for 4 hours before adding TriPure Isolation Reagent (Roche, Indianapolis, IN). When determining the effect of HDL on responses to TLR agonists, the macrophages were incubated in the presence or absence of ETC-642, Ctrl or SLE HDL, or S1P1-3 (50 µg/mL) for 4 hours before media was removed, then incubated with Pam3CSK4 (300 ng/mL, InvivoGen, San Diego, CA), R848 (1 µg/mL, InvivoGen), or LPS (100 ng/mL, Sigma-Aldrich) plus 2% lipoprotein deficient serum (LPDS, Millipore) in DMEM (Life Technologies) for 4 hours. For macrophage polarization assessment, Ctrl and SLE macrophages were cultured in the presence or absence of Ctrl or SLE HDL (50 µg/mL) with 2% LPDS for 3 days. For human ETC-642 studies, various SLE HDL: ETC-642 ratios (1:1, 1:2, 1:4, 2:1, 4:1) were tested on Ctrl macrophages for 4 hours.

RNA was isolated using a Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA) and purity determined by NanoDrop 1000 (Thermo Scientific). Total RNA (1 µg) was reverse transcribed using iScript Reverse Transcriptase (BioRad, Hercules, CA) and RT-qPCR was performed using SSoAdvanced Universal SYBR Green (BioRad). Primer sequences are included in Table 3-1 and 3-2. Samples were run in duplicate using a CFX96 C1000 Touch Real Touch Thermal Cycler (Bio-Rad). Data was analyzed using Bio-Rad CFX Manager software.
Macrophages from 5 Ctrl donors were incubated with or without azide-free goat IgG isotype control (5 µg/mL, SouthernBiotech, Birmingham, AL) or goat IgG anti-LOX1R blocking antibody (5 µg/mL, R&D Systems, Minneapolis, MN) in DMEM for 30 minutes, before addition of HDL or Pam3CSK4 in DMEM with 2% LPDS for 18 hours. Commercially available ELISAs were performed to quantify human IL-6 and TNF in supernatants following manufacturer’s instructions (ALPCO, Salem, NH). For murine in vivo studies, serum IL-6 was quantified by ELISA (eBioscience, San Diego, CA).

![Image](Image)

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Table 3-1. Primers used for human RT-qPCR.
**Immunofluorescence Microscopy**

For TLR studies, Ctrl macrophages were cultured with LPS (100 ng/mL, Sigma-Aldrich), Pam3CSK4 (300 ng/mL, InvivoGen), no, Ctrl or SLE HDL (50 µg/mL) for 2 hours. For scavenger receptor studies, macrophages were cultured with isotype control, anti-LOX1R blocking antibody (see above), rabbit anti-CD36 blocking antibody (1:100, Abcam), or rat anti-SR-BI/II blocking antibody (1:100, Abcam) before incubation with HDL in DMEM/2% LPDS for 30 minutes (for ATF3 experiments), followed by fixation with 4% PFA (Santa Cruz). Fixed cells were permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) for 10 minutes, washed, and then blocked with 0.2% gelatin for 30 minutes. Cells were incubated for 1.5 hours with rabbit anti-p65 (1:500, FIVEphoton Biochemicals, San Diego, CA), mouse anti-ATF3 (1:100, Abcam), mouse anti-TLR-4 (1:250, Abcam), or mouse anti-TLR-2 (1:100, Abcam) followed by incubation for 1 hour with donkey anti-rabbit, or donkey anti-mouse Alexa Fluor 555 (1:500, Life Technologies), then with Hoechst 33342 (1:1000, Life Technologies) for 5 minutes. Coverslips were mounted with ProLong Gold Antifade (Life Technologies). Images were acquired on a Zeiss LSM 510 META laser scanning confocal microscope (Carl Zeiss Microscopy) with a x63 lens and quantified using the Zeiss Zen 2012 SP1 software’s weighted co-localization coefficient analysis.

**Flow Cytometry**

Ctrl or SLE PBMCs were isolated by Ficoll-Paque (GE Healthcare Life Sciences) density gradient. Cells were blocked in FACS solution (Biolegend) for 30 minutes. Surface markers were stained for 20 minutes at room temperature: Pacific Blue anti-human CD16, PE anti-human CD14, APC anti-human CD86 (Biolegend). Monocytes were classified as either non-classical (CD14⁺, CD16++), intermediate/“inflammatory” (CD14++,CD16+), and classical (CD14++, CD16⁻) as described [376]. For M1/M2 studies, cultured SLE or Ctrl macrophages were cultured in the presence
or absence of Ctrl or SLE HDL (50 µg/mL) with 2% LPDS in DMEM for 3 days. Cells were harvested using StemPro Accutase Reagent (Life Technologies), blocked in FACS solution (Biolegend, San Diego, CA) for 30 minutes, and incubated with anti-human CD206 APC and anti-human CD40 Pacific Blue for 20 minutes at room temperature. For ATF3 quantification, cells were incubated in permeabilization buffer (eBioscience) and stained according to manufacturer’s instructions with rabbit FITC anti-ATF3 (1:100, Bioss, Woburn, MA). For human ETC-642 studies, monocytes were isolated by negative selection from Ctrl PBMCs using MACS Monocyte Isolation Kit II (Miltenyi, San Diego, CA). Monocytes were then incubated in DMEM (Life Technologies) with 2% LPDS for 18 hours with Ctrl or SLE HDL (50 µg/mL). Cells were stained for intracellular ATF3 as stated above.

Immunoblot

For studies on HDL-induced total ATF3 production, Ctrl macrophages were incubated in the presence or absence of Ctrl or SLE HDL for 18 hours. In additional experiments, Ctrl macrophages were incubated with isotype control or anti-LOX1R blocking antibody (see above) prior to adding HDL for 30 minutes (for ATF3) or 2 hours (for p65). Nuclear fractions were then isolated using a Nuclear Extraction Kit (Active Motif, Carlsbad, CA). Protein concentrations were determined by BCA analysis (Thermo Scientific). Total cell lysate (10 µg) or nuclear fractions (5 µg) were run on 4-12% NuPAGE Bis-Tris gels (Life Technologies). Blots were blocked (10% BSA) and incubated overnight with rabbit anti-p65 (1:500, FIVEphoton Biochemicals), mouse anti-ATF3 (1:250, Abcam) or rabbit anti-histone 3 (1:2000, Abcam), followed by 1:10,000 donkey anti-rabbit or anti-mouse IRDye 680RD or 800CW (Li-cor, Lincoln, NE) for 1 hour at room temperature. Blots were imaged on an Odyssey CLx (Li-cor).
In Vivo Administration of ETC-642

The protocol was approved by the University of Michigan's Committee on Use and Care of Animals. NZM breeding pairs were obtained from Dr. Chaim Jacob (University of Southern California). Balb/c mice were purchased from the Jackson Laboratory. Mice were bred and housed in a specific pathogen–free barrier facility at the University of Michigan. Starting at 10 weeks of age, NZM female mice were treated with ETC-642 (15 mg/kg, N=8) or equal volume PBS (N=8) for 13 weeks, three times/week via tail vein injection. Mice were euthanized at 23 weeks of age, before clinical development of renal disease became apparent. At euthanasia, spleens were homogenized with an Omni TH Motor and probes (Omni International, Kennesaw, GA) and resulting splenocyte RNA used for RT-qPCR (see above). Serum was collected from terminal bleeds.

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Table 3-2. Primers used for murine RT-qPCR.
Statistical Analysis

Significance was determined as stated in chapter 2.

C. Results

*SLE HDL Induces Pro-inflammatory Responses in Macrophages and Fails to Abrogate Inflammatory Cytokine Synthesis Triggered by TLR Agonists.*

We showed that SLE HDL is enriched in 3-NY and 3-C1Y HDL oxidation and displays impaired CEC when compared to Ctrl HDL, yet we wished to determine the inflammatory nature of this SLE HDL (Table 2-1, 2-2, and Figure 2-2) 259, 501, 503, 543. Ctrl macrophages exposed to SLE HDL synthesized significantly higher levels of IL-6 and TNF than macrophages exposed to Ctrl HDL, both at the mRNA (Figure 3-1A) and protein levels (Figure 3-1B).

Ctrl HDL has recently been reported to block TLR-induced inflammatory cytokine production in macrophages 367, 368. We confirmed that Ctrl HDL significantly decreased the synthesis of IL-6, TNF, IL-1β and IL-12B mRNA following TLR-4, -1/2, or -7/8 stimulation (Figure 3-1C). In contrast, SLE HDL was impaired in its ability to block TLR-induced inflammatory cytokine synthesis in macrophages. The differences in the inhibitory effects of Ctrl versus lupus HDL on TLR signaling could be due to TLR sequestration or activation, either resulting in TLR internalization. To address this, we treated Ctrl macrophages with either Ctrl or SLE HDL and examined TLR-2 and -4 surface expression. Neither Ctrl nor SLE HDL affected TLR-2 or -4 surface expression or internalization (Figure 3-1D). Because of the inflammatory nature of SLE HDL, we quantified the effect of each HDL cohort on NFκB activation, as measured by p65 nuclear translocation. SLE HDL promoted p65 nuclear translocation in Ctrl macrophages, compared to Ctrl HDL (Figure 3-1E). Overall, these results indicate that SLE HDL promotes
inflammatory responses in macrophages and has an impaired ability to block TLR-induced inflammatory signals, without modifying TLR cellular trafficking.
SLE Patients Exhibit an Enhanced Inflammatory Monocyte and M1 Macrophage Bias, which is Increased by SLE HDL.

As inflammatory monocytes are linked to CVD risk, we wanted to quantify inflammatory monocyte presence in our cohort of SLE patients. Based on two different gating strategies (Figure 3-2A), SLE patients exhibit a higher level of non-classical and intermediate (inflammatory) monocytes than Ctrl donors (Figure 3-2 B and C). It should be noted that the SLE LDGs (granulocyte population visible in the SLE SSC-A vs. FSC-A in Figure 3-2A) do not contribute to these monocyte percentages as LDGs are CD86−.

M1 macrophages are associated with atherosclerosis and pro-inflammatory cytokine production, similar to what was induced in vitro by lupus HDL (Figure 3-1A). We therefore quantified M1 and M2 markers in monocyte-derived SLE and Ctrl macrophages. At the protein level, Ctrl macrophages had significantly higher CD206 (an M2 marker) expression compared to SLE macrophages when both were cultured in media alone (Figure 3-2D). The addition of Ctrl HDL to the SLE and Ctrl macrophages increased CD206 expression, but Ctrl HDL-treated SLE macrophages still showed significantly lower CD206 expression than Ctrl macrophages. Addition of SLE HDL to SLE or Ctrl macrophages did not increase CD206 expression. Additionally, Ctrl macrophages cultured in media alone showed lower CD40 (an M1 marker) expression compared to SLE macrophages cultured in media alone (Figure 3-2D). Ctrl macrophages did not
significantly modify CD40 expression with the addition of SLE or Ctrl HDL. SLE macrophages, however, showed decreased CD40 expression following exposure to Ctrl HDL (Figure 3-2D). At the mRNA level, Ctrl macrophages exposed to Ctrl HDL displayed enhancement of M2 markers (CCL22 and CD206), while SLE HDL up-regulated the M1 marker SOCS3 and down-regulated M2 markers (Figure 3-2E)\textsuperscript{195,548}. These results indicate that SLE monocytes and macrophages are biased towards an inflammatory signature, which is exacerbated by SLE HDL and dampened by exposure to Ctrl HDL.
Figure 3-2. SLE patients show an enhanced inflammatory monocyte and M1 macrophage phenotype. (A) PBMCs from SLE and Ctrl donors were stained for CD86, CD14, and CD16 to quantify non-classical, intermediate (inflammatory), and classical monocyte populations, based on two gating strategies (B and C, N=14). (D) Ctrl and SLE macrophages were treated with SLE and Ctrl HDL and CD206 and CD40 surface expression quantified by flow cytometry (N=5). (E) Ctrl macrophages were treated with Ctrl or SLE HDL. M1 (SOCS3) and M2 (CCL22, CD206) genes were quantified by RT-qPCR (N=5). (Data are displayed as mean ± SEM, * p<0.05, ** p<0.01, *** p<0.0005, **** p<0.0001)
**Lupus HDL is Impaired in its Capacity to Promote ATF3 Synthesis and Anti-inflammatory Activity.**

ATF3 is ubiquitously expressed at low levels, and is up-regulated in response to inflammatory signals (IFN-γ, IL-1β, LPS), cell stress and calcium signaling\(^\text{549,550}\). The 3' UTR of ATF3 mRNA contains many AUUUA sites, indicating that it is an immediate-early response gene\(^\text{551}\). Through an interaction with histone deacetylase 1 (HDAC1), ATF3 blocks IL-6, TNF, and IL-12B production downstream of inflammatory signals\(^\text{365,552}\). Recent evidence indicates that HDL from healthy individuals modulates inflammatory responses in macrophages through induction of ATF3\(^\text{365,367}\). Given that lupus HDL promoted inflammatory cytokine production and showed an impaired capacity to down-regulate TLR-induced cytokines, we examined the effect of this lipoprotein on ATF3 nuclear translocation and synthesis.

When exposed to lupus HDL, Ctrl macrophages failed to up-regulate ATF3 mRNA by 2 hours when compared to Ctrl HDL treatment (Figure 3-3A). Furthermore, after 6 hours of exposure, Ctrl macrophages exposed to lupus HDL expressed significantly lower ATF3 mRNA levels compared to macrophages exposed to Ctrl HDL. At the protein level, Ctrl monocytes or monocyte-derived macrophages exposed to lupus HDL for 18 hours showed significantly lower ATF3 protein levels compared to Ctrl HDL, as demonstrated by immunoblot (Figure 3-3B) and flow cytometry (Figure 3-3C). Additionally, monocyte-derived SLE macrophages expressed lower levels of ATF3 than Ctrl macrophages (Figure 3-3D). These results suggest that lupus HDL fails to induce the expression of the inflammatory repressor ATF3 and this abnormality may play a role in the induction of macrophage pro-inflammatory responses observed in this disease.
The Binding of Lupus HDL to LOX1R Prevents the Nuclear Translocation of ATF3 and Promotes Inflammatory Responses in Macrophages.

As ATF3 nuclear translocation is key to its activity, we examined the effect of lupus HDL on ATF3 intracellular mobilization. While Ctrl HDL-treated Ctrl macrophages demonstrated significant ATF3 nuclear translocation, SLE HDL treatment failed to promote ATF3 activation...
One possible explanation for how SLE HDL blocks ATF3 nuclear translocation is the degradation of ATF3 protein and mRNA. However, as differences in ATF3 protein nuclear translocation can be detected as early as 30 minutes after challenging with HDL, this explanation is less likely.

Previous work demonstrated that HDL oxidized by MPO or purified from patients with established CAD acquires the ability to bind LOX1R, and CuSO₄-oxidized HDL increases NFκB activation, and both TNF and LOX1R production. While lectin-like oxidized LDL receptor 1 (LOX1R) expression has primarily been reported on endothelial cells, we and others find that it is also expressed on monocytes and its levels increase during differentiation into macrophages (data not shown). As lupus HDL contains enhanced levels of MPO-catalyzed 3-ClY and 3-NY, we assessed if the pro-inflammatory effects of lupus HDL required its binding to LOX1R. When we incubated healthy macrophages with blocking anti-LOX1R or isotype control antibodies prior to addition of SLE or Ctrl HDL and examined ATF3 nuclear translocation, we found that by preventing the binding of SLE oxHDL to LOX1R we restored ATF3 nuclear translocation (Figure 3-4A). Ctrl HDL-induced ATF3 activation was not affected by anti-LOX1R blocking antibody treatment. It is possible that SLE HDL binding to LOX1R activates an immediate signaling event which results in the inactivation of ATF3. Little is known about which post-translational modifications occur to ATF3, but future work should focus on how these modifications may affect the protein’s activity.

When we tried blocking other scavenger receptors previously implicated in binding oxidized HDL (CD36 and SRB-I) we saw no significant change in SLE HDL’s effect on ATF3 activity (Figure 3-4B). It remains unclear which scavenger receptor binds healthy HDL to promote
ATF3 and block inflammation. Given that blocking CD36 decreased the induction of ATF3 by Ctrl HDL, however, it is possible that this scavenger receptor may play a role in the immunoregulatory roles of normal HDL function (Figure 3-4B). Overall, these results suggest that SLE HDL preferentially binds to LOX1R and initiates a pathway that leads to blockade of ATF3 activity.

Finally, Ctrl macrophages were incubated with blocking anti-LOX1R or isotype control antibody followed by SLE or Ctrl HDL for 18 hours, and supernatant IL-6 and TNF levels were measured by ELISA. LOX1R blockade significantly decreased the amount of IL-6 and TNF released due to lupus HDL treatment (Figure 3-4C). Blocking LOX1R prior to addition of SLE also decreased NFκB activation (p65 nuclear translocation, Figure 3-4D). These results indicate that lupus HDL engagement with LOX1R blocks the activity of ATF3, enhances NFκB activation, and promotes inflammatory cytokine synthesis by macrophages.
The HDL Mimetic ETC-642 Abrogates the Inflammatory Effects Induced by Lupus HDL.

ETC-642 is a HDL mimetic compound composed of a 22 amino acid sequence (22A) derived from apoA-I, the most abundant protein in HDL, and the phospholipids 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and sphingomyelin (SM). This compound improves cardiovascular parameters in rabbit models and has been shown to mobilize cholesterol in plasma.
compartments following infusion in dyslipidemic patients. Given that this compound mimics some of the anti-inflammatory effects of HDL, we assessed its role in down-regulating the deleterious effects of lupus HDL.

ETC-642 blocked TLR-induced inflammatory cytokine production in Ctrl macrophages (Figure 3-5A). In contrast, the individual protein or lipid components of ETC-642 did not block IL-6 or TNF mRNA induction by TLR agonists (Figure 3-5B). Similarly, ATF3 nuclear translocation was induced by ETC-642, but not by its individual components (Figure 3-5C). To address if ETC-642 could reverse the pro-inflammatory effects of SLE HDL, Ctrl macrophages were exposed to various ratios of ETC-642 and SLE HDL, then ATF3 nuclear translocation was assessed. The 1:4 SLE HDL:ETC-642 ratio significantly enhanced ATF3 nuclear translocation (Figure 3-5D) and blocked SLE HDL-induced IL-6 and TNF mRNA up-regulation (Figure 3-5E). Similarly, SLE HDL:ETC-642 1:4 increased ATF3 at the mRNA (Figure 3-5E) and protein levels (Figure 3-5F). ETC-642 and the SLE HDL:ETC-642 1:4 mixture also blocked NFκB activation (Figure 3-5G). Overall, these results indicate that ETC-642 can hamper macrophage inflammation induced by lupus HDL by mimicking the effects of healthy HDL on ATF3 and NFκB.
Sphingosine-1-phosphate Abrogates TLR-1/2 Induced Inflammation.

The HDL₃ subclass has been identified as one of the most vasoprotective versions of HDL. This may be attributed to its high concentration of the sphingolipid sphingosine-1-phosphate (S1P). This molecule has been linked to M2 macrophage polarization, endothelial NO production and its levels inversely correlated with ischemic heart disease risk and CAD. We therefore examined the effect of increasing concentrations of S1P (S1P-1 0.05 nmol/mg, S1P-2 0.5 nmol/mg, S1P-3 5 nmol/mg) mixed with DPPC and 22A on inflammatory cytokine production. Macrophages pre-incubated with the highest concentration of S1P (S1P-3) prior to Pam3CSK4 challenge showed significant abrogation of IL-6 (Figure 3-6A) and TNF (Figure 3-6B) mRNA levels. S1P-3 did not abrogate the inflammatory cytokine mRNA levels as extensively as ETC-642 however. This data indicates that S1P may be beneficial for regulating inflammatory cytokine production in SLE.
ETC-642 Dampens Inflammatory Cytokine Production in Lupus-prone NZM Mice.

Similar to human SLE, we previously showed that lupus-prone NZM mice display endothelial dysfunction and enhanced levels of oxidized HDL $^{34,543}$. We then assessed if in vivo systemic administration of ETC-642 could dampen inflammatory responses in female lupus-prone NZM mice. Compared to PBS-treated NZM mice, splenocytes from ETC-642-treated NZM mice displayed significant increases in ATF3 mRNA levels as well as significant decreases in mRNA levels of IL-6, TNF, IL-1β, IL-12B, and IFN stimulated genes (ISGs: MX1, ISG15, IRF-7) (Figure 3-7A). ETC-642-treated mice also showed lower IL-6 serum levels (Figure 3-7B). In contrast, ETC-642 treatment did not modify auto-antibodies or proteinuria (data not shown). Overall, these results indicate that in vivo administration of the HDL mimetic ETC-642 can dampen inflammatory responses in lupus.
D. Summary

We sought to determine if the enhanced oxidation status of SLE HDL affects macrophage innate inflammatory responses related to CVD. We found that, in contrast to the anti-inflammatory effects of Ctrl HDL, Ctrl macrophage exposure to SLE HDL enhanced pro-inflammatory cytokine production (in the absence of TLR agonists) and failed to block TLR-induced cytokine production. As the SLE HDL proved to be pro-inflammatory in the absence of a TLR agonist, did not show any effect on TLR mobilization, but directly promoted NFκB activation, we explored TLR-independent signaling pathways involved in the pro-inflammatory effects of lupus HDL. We examined LOX1R’s influence on SLE HDL-induced inflammation as it is one scavenger receptor linked to the pro-inflammatory properties of oxidized lipoproteins. Indeed, the SLE HDL-induced NFκB activation, cytokine production, and ATF3 repression was largely dependent on SLE HDL binding to LOX1R.

ETC-642 was able to overcome the inflammatory effects of SLE HDL in human macrophages. The individual components of ETC-642 (22A, DPPC and SM) had no significant effect on ATF3 or NFκB activation. In vivo treatment of lupus-prone mice with ETC-642 also

\[\text{Figure 3-7. ETC-642 blocks in vivo inflammatory cytokine production in lupus-prone NZM2328 (NZM) mice.} \] 

Lupus-prone NZM mice were treated with PBS or ETC-642 (15 mg/kg, 3x per week, i.v.) by tail vein injection for 13 weeks. (A) Splenocytes were examined for ATF3, inflammatory cytokines and interferon stimulated genes by RT-qPCR. (B) Serum IL-6 levels were quantified by ELISA. (N=8 mice, Data are displayed as mean ± SEM, * p<0.05, ** p<0.009, *** p<0.0008)
decreased systemic inflammatory markers. In conclusion, modified lupus HDL binds to LOX1R in macrophages, leading to induction of NFκB activity and failure to induce ATF3 synthesis and activation, thereby promoting pro-inflammatory responses.

In these studies, we focused on how SLE versus Ctrl HDL affects macrophage polarization and inflammatory responses in the context of CVD. However, aberrant macrophage function in lupus may also be linked to skin flares, kidney damage and pulmonary problems\textsuperscript{188, 567-569}. Future studies could explore whether infusion of lupus-prone mice with ETC-642 or Ctrl murine HDL dampens inflammation in each of these organ systems through altered macrophage function or phenotype. Such studies may be hard to translate to human macrophage responses, however, given the differences in macrophage polarization markers and gene activity exhibited between humans and mice\textsuperscript{195, 354, 544}. Nonetheless, these studies may identify added benefits of ETC-642 or other apoA-I mimetics as therapies for SLE.

In our hands, ETC-642 can prevent the pro-inflammatory effects of lupus HDL using \textit{in vitro} models and general inflammation via an \textit{in vivo} model. Whether ETC-642 treatment also improves vascular health in lupus-prone mice has yet to be determined. Again, the lack of well-defined lupus-prone mouse models which also develop atherosclerosis means that the effect of ETC-642 on atherogenesis could not be examined. It would be possible, however, to examine its effect on endothelial cell differentiation, angiogenesis, oxidative stress, or clotting following photochemical-induced thrombosis in lupus-prone mice\textsuperscript{34, 468}. What affect lupus oxidized HDL has on LOX1R signaling in mice is also not known.

\textit{LOX1R/-} mice, especially when crossed with \textit{apoE/-} or \textit{LDLR/-} mice, do show improved vasorelaxation, and reduced inflammatory cytokine levels, intima thickening, and plaque macrophage accumulation\textsuperscript{570-573}. Whether LOX1R has a higher affinity for 3-CIY or 3-NY
oxidized HDL has yet to be determined. This is worth establishing given that our lupus patients’
HDL showed more 3-C1Y than 3-NY, yet lupus-prone NZM mice show more 3-NY oxidized HDL.
This difference in HDL oxidation patterns and how LOX1R responds to each may demonstrate
another atherogenic mechanism which varies between mice and humans. Yet, it would be
interesting to see if lupus-prone mice treated with LOX1R neutralizing antibody demonstrate
improved vascular health. Such therapies, then, could be explored for SLE patients.
Chapter 4

Conclusions

A. Summary

The aims of our study were to characterize the oxidation status of SLE plasma and HDL, and examine aberrant lupus and CVD-related innate immune mechanisms up- and downstream of this lipoprotein. We found that lupus patients have dysfunctional and highly oxidized HDL and plasma, which fits patterns linked to CVD in the general population. These measurements, therefore, should be explored as biomarkers of CVD risk in SLE. Our data indicates that the high level of NETosis in SLE can contribute to these pro-atherosclerotic modifications, especially as abrogation of NET formation in lupus-prone mice led to decreased HDL oxidation. Finally, we showed that SLE oxHDL induces pro-inflammatory and defective anti-inflammatory characteristics in cultured control macrophages. Importantly, these inflammatory pathways can be blocked in mice by treatment with an HDL mimetic. These results highlight the significance of multiple aberrant innate immune mechanisms leading to lupus atherosclerosis, and could be applicable to other chronic inflammatory diseases.

To date, most research on SLE CVD has focused on its subclinical nature and association with IFN-I, TNF, NETosis, defective anti-oxidant activity, lupus-associated medications, anti-phospholipid antibodies, and endothelial cell death. The CVD literature includes reports demonstrating that NETs can exert direct damage to the endothelium and contribute to coagulation cascades. Other groups have shown how oxidized lipoproteins can contribute to atherogenesis. By demonstrating how lupus NET activity contributes to specific pro-
inflammatory and pro-atherosclerotic modifications to HDL, our results not only fit in with these previously proposed atherosclerotic mechanisms but also combine heretofore separated pathways and build upon known mechanisms of SLE and CVD. Our results also have special clinical significance. By demonstrating the interplay between neutrophils, macrophages and lipoprotein innate signaling pathways, we confirmed that therapies which block NET activity, such as Cl-Am, or alter the activity of SLE HDL, such as ETC-642, may both be beneficial to lupus patients. The limitations that we faced in these studies, namely patient cohort size and available lupus-CVD mouse models, may be addressed by future studies and should elaborate on our findings.

B. Future Directions

I. SLE HDL’s Effect on Neutrophil Activity

Given the potent effects of lipoproteins on macrophage function and the putative important contribution of NETs and neutrophils to SLE and CVD, it will be important to determine if lipoproteins also modify neutrophil biology. Indeed, healthy HDL can affect cell death pathways, ROS generation, and gAMP activity

As these pathways are associated with NET formation, it is conceivable that HDL could alter the predisposition of a neutrophil to undergo NETosis. Whether these effects are lost upon oxidative modifications to HDL, such as seen in SLE, is also unclear. Additionally, the effect of ETC-642 or other mimetics on NETosis should be further characterized. The response of other neutrophil-related functions (degranulation, phagocytosis, ROS generation, etc.) to modified and unmodified lipoproteins could be further examined. As lipoproteins in general and HDL in particular are carriers not only of plasma proteins but also of miRNAs, these molecules may hold putative “messenger” capabilities.
II. SLE HDL Composition

The initial proteomics analysis of lupus HDL revealed an enrichment of vasorin, a protein that still requires significant characterization. Whether SLE HDL has a dampening effect on TGF-β signaling should be explored given that vasorin has been proposed to down-modulate this pathway \(^{530-532}\). This may be relevant to anti-inflammatory signaling, repair mechanisms, macrophage polarization and VSMC activity relating to SLE atherogenesis. However, validation of these findings in a larger cohort of SLE patients should be studied to verify our results and identify other potential immunomodulatory proteins in HDL. For example, previous groups have stated that the circulation is not the site of HDL oxidation because of the high presence of anti-oxidative compounds in the plasma \(^{578}\). Yet, it appears that SLE patients possess less anti-oxidant activity, including low PON levels in HDL \(^{238, 239, 247-250}\). Further proteomic analysis could demonstrate low anti-oxidative enzyme levels in SLE and, if performed in conjunction with NETosis activity assays, could strongly indicate the abnormally high circulating oxidative environment in SLE patients. In addition, lipidomic analysis of HDL should be explored in SLE to assess whether some of the differences in SLE versus control signaling pathways could be explained by altered lipoprotein lipid content.

For example, previous work indicates that S1P can block TLR-induced inflammation, and promote “classical”, anti-inflammatory monocyte activity \(^{579-581}\). Whether S1P levels differ between SLE and Ctrl HDL should be validated. As lipids can also be oxidized, exploring differences in lipid oxidation levels between controls and SLE, which receptors they bind, and any downstream pro- or anti-inflammatory pathways may prove revealing. This could explain why, while blockade of LOX1R significantly abrogated the inflammatory cytokine production and NFκB activation induced by SLE HDL, it did not reduce levels down to that of Ctrl HDL.
treatment (Figure 3-4C and D). This lipidomic analysis and comparisons could also reveal putative therapeutic targets. Additionally, as mentioned above, HDL’s effect on target cells may be affected not only by receptor binding and signaling, but through the delivery of miRNAs to target cells.

HDL is a known carrier of miRNAs, with different miRNA “libraries” found in control versus CVD donors. If these HDL-associated miRNAs are delivered into target cells, this could direct cholesterol homeostasis, macrophage polarization, and SLE pathogenesis mechanisms. It is possible that some of the differential inflammatory and anti-inflammatory effects seen between control and lupus HDL are due to the transfer of miRNAs from the HDL to the macrophages.

Future studies in larger cohorts should therefore explore the association of the protein, lipid, and perhaps mRNA cargo of lipoproteins with anti-atherogenic effects and disease activity in lupus and other inflammatory diseases. Indeed, it remains to be determined whether our findings are applicable to most chronic inflammatory diseases, are distinct to SLE, or even distinct to humans.

III. Of Mice Versus Men

Previous groups have demonstrated the importance of IFN-I signaling to SLE and CVD pathogenesis. Suzuki et al. demonstrated the ability of HDL to block IFN-I responses. Importantly, this study was performed in mouse models and with murine cell lines. In our hands, we did not see a consistent effect of healthy human HDL on the blockade of ISGs in human macrophages. As with De Nardo et al., we found that IL-6, TNF and IL-12B showed the largest effects with HDL treatment. Nevertheless, lupus-prone mice exposed to ETC-642 displayed down-regulation of ISGs (Figure 3-7). As such, the effect of HDL on IFN-I responses may be
species specific. It will be important to better understand the differences in lipoprotein modulation between human and murine systems to identify the best experimental design to test novel therapies that modulate and signaling abnormal lipoprotein phenotype and function in chronic inflammatory conditions. For example, it is possible that this is due to differences in scavenger receptor expression in mice versus humans, which leads to altered immune responses to HDL treatment. This would not be entirely surprising given the already known significant differences between mouse and human mechanisms of atherogenesis and macrophage polarization.

C. Implications of Studies

The results of the studies presented above highlight the important role of innate immune responses, namely aberrant interactions between neutrophils, macrophages, the oxidative machinery, and lipoproteins in the promotion of accelerated atherogenesis and increased CVD risk in chronic inflammatory diseases such as lupus (Figure 3-8). These results may also be relevant to individuals with “typical” atherosclerosis, where innate immune responses may trigger and amplify vascular damage and abnormal lipoprotein biology.

Neutrophils, in particular, were largely ignored as mediators of atherogenic inflammation and oxidation until very recently. This was mostly due to an inability to detect neutrophils in plaques. With improved techniques, not only are neutrophil markers readily detectable in atheromas, but NETs too are visible in plaques, thrombi and in circulation. NETs have already been linked to coagulation pathways and endothelial cell death, but our results demonstrate an additional mechanism by which they promote atherogenesis: oxidation of lipoproteins thereby affecting their CEC and anti-inflammatory function.
Lipoproteins too have been underappreciated as mediators of immune signaling. Previous groups have shown that unmodified HDL can block TLR-4 signaling and NFκB activation, while oxLDL and oxHDL increase NFκB signaling and adhesion molecule expression. Our findings on the anti-inflammatory nature of healthy HDL, especially with S1P, in addition to the pro-inflammatory effect of SLE HDL support the theory of lipoprotein-mediated immune cell signaling. Furthermore, we show that lipoproteins not only affect cytokine production but also alter macrophage polarization, an idea supported by other groups.

A number of other diseases have been recently linked to altered neutrophil and lipoprotein activity in the context of enhanced CVD risk: rheumatoid arthritis, psoriasis, vasculitis, liver disease and renal dysfunction. The mechanisms elucidated here may be relevant to those disorders as well and should be explored in future experiments. Additionally, therapies which target NET activity and lipoprotein composition should be further examined with regards to their potential beneficial athero-protective and immunomodulatory role in SLE and CVD.
Figure 3-8. Proposed mechanism for NETosis-induced HDL oxidation and macrophage inflammation in SLE-related CVD. NET formation (which is enhanced in SLE) causes the exposure of the oxidative enzymes NOS, MPO and NOX to the periphery, where they cause 3-Ciy and 3-Ny HDL oxidation. This modified HDL then promotes an M1 macrophage phenotype with enhanced IL-6 and TNF production, matched with decreased ATF3 production and activation. This is largely dependent on oxidized HDL binding LOX1R. The toxic NETs, dysfunctional oxidized HDL and macrophage inflammatory cytokine production all contribute to SLE-related CVD.
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