The role of retinaldehyde and PPARgamma signaling in systemic lupus erythematosus

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THE ROLE OF RETINALDEHYDE AND PPARGAMMA SIGNALING IN SYSTEMIC LUPUS ERYTHEMATOSUS

by

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ABSTRACT

Systemic Lupus Erythematosus (SLE) is an autoimmune disease with chronic inflammation affecting multiple organ systems, as well as accelerated atherosclerosis as a major complication. Prior studies by our lab have shown beneficial effects of PPARγ agonists towards preventing SLE in two different mouse models: the well-established lupus mouse model, MRL/lpr, and the gld.apoE\(^{-/-}\) model of accelerated lupus and atherosclerosis. Retinaldehyde is a retinoic acid precursor that has recently been shown to inhibit PPARγ signaling in adipose tissue. We proposed that abnormal accumulation of retinaldehyde in lupus promotes autoimmunity by inhibition of PPARγ signaling. We measured the serum retinaldehyde levels in both lupus mouse models using reversed-phase high-performance liquid chromatography. We also examined the mRNA expressions of genes involved in retinaldehyde metabolism and PPARγ signaling in white adipose tissues using real-time quantitative PCR. We observed a higher level of circulating retinaldehyde in the MRL/lpr mouse model on a chow diet. The circulating retinaldehyde levels in both gld.apoE\(^{-/-}\) and C57 increased when maintained on a high-cholesterol Western diet. Within visceral and subcutaneous adipose tissue, we saw several changes to expression of the genes responsible for retinaldehyde synthesis and catabolism, however further study is required to definitively assess the role of these genes. Importantly, the expression levels of genes involved in PPARγ signaling
decreased in the subcutaneous fat of gld.apoE⁻/⁻ mice on a Western diet. Our data suggest that retinaldehyde may play a role in SLE pathogenesis and could be a potential therapeutic target for SLE.
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ADH1 ................................................................. Alcohol Dehydrogenase 1
EDTA ................................................................. Ethylenediaminetetraacetic Acid
GAPDH ............................................................... Glyceraldehyde 3-phosphate dehydrogenase
HDL ................................................................. High Density Lipoprotein
mAU ................................................................. Milliabsorbance Unit
MetS ................................................................. Metabolic Syndrome
PPARγ ............................................................... Peroxisome Proliferator-activated Receptor gamma
Rald ................................................................. Retinaldehyde
RALDH1 ............................................................ Retinaldehyde Dehydrogenase 1
RALDH2 ............................................................ Retinaldehyde Dehydrogenase 2
RAR ................................................................. Retinoic Acid Receptor
RBP ................................................................. Retinol Binding Protein
RXR ................................................................. Retinoid X-receptor
SLE ................................................................. Systemic Lupus Erythematosus
SUBQ ............................................................... Subcutaneous Fat
VFAT ............................................................... Visceral Fat
INTRODUCTION

Systemic Lupus Erythematosus

Systemic Lupus Erythematosus (SLE) is an autoimmune disorder that occurs most frequently in women of childbearing age (Bernatsky et al., 2007). The ratio of female and male lupus patients is greater than 8:1 between the ages of 15 to 50 (Kotzin, 1996). The estimated SLE prevalence ranges from about 81 to 103 per 100,000 individuals in the United States every year (Furst et al., 2013). SLE is characterized by the production of autoantibodies against intracellular antigens, with variable clinical features, including rashes, arthritis, nephritis, serositis, cerebritis, and a host of serological abnormalities, including abnormal serum anti-DNA and complement levels (Moser et al., 1998; Lightfoot & Hughes, 1976). The onset and development of SLE may be influenced by genetic and environmental factors, some of which include class II MHC genes, viral or bacterial infections, administration of exogenous hormones, chronic use of certain drugs, and impaired clearance of apoptotic cells (Kotzin, 1996; Baumann, 2002; Aprahamian, 2004).

Lupus mouse model

The Fas system, including the Fas receptor and Fas ligand, is crucial in the immune system, especially against autoimmunity, as it eliminates pathogen-infected cells and potentially dangerous lymphocytes (Strasser et al., 2009). FasL is a death factor that, when it binds to the Fas receptor, induces apoptosis (Nagata & Suda, 1995). Gld and lpr are mouse models with known mutations within genes encoding Fas ligand (FasL) and
Fas receptor, respectively (Nagata & Suda, 1995). Both gld and lpr are autosomal recessive mutations that lead to lymphoproliferation and autoimmunity (Cohen & Einsberg, 1991).

As described by Cohen and Einsberg, the MRL.lpr mouse strain exhibits a proliferative glomerulonephritis, progressive enlargement of lymph nodes, and autoimmune disease in other organs. Purpuric lesions can occur on the ears and tail of the MRL.lpr mice. Since the lpr gene induces abnormal B-cell activation, a higher immunoglobulin level, particularly IgG, is observed in the serum. Manifestations of the lpr defect vary by genetic backgrounds. For instance, MRL.lpr develops the most severe lymphoproliferation and renal disease with the shortest life expectancy, whereas C57BL/6-lpr mice develop a more mild autoimmune disease (Cohen & Einsberg, 1991).

Mice with a gld mutation share a similar phenotype to that of the lpr on the same background, suggesting that these two mutations affect the same pathways of lymphocyte differentiation (Cohen & Einsberg, 1991). The lupus-like phenotypes made the gld and lpr mice important mouse models to give insights to the immunopathology of lupus (Cohen & Einsberg, 1991).

**Atherosclerosis**

Atherosclerosis is a chronic inflammatory condition and a major cause of cardiovascular diseases, a type of disease that affects more than one third of American adults (Frostegard, 2013 & Go et al., 2013). A high concentration of cholesterol, especially low density lipoprotein cholesterol (LDL), leads to accumulations of lipid-rich plaques within the artery wall, forming atherosclerotic lesions (Ross, 1999 & Frostegard,
The development of atherosclerotic lesions can lead to ischemia of the heart or brain, and plaque ruptures of the fibrous cap can trigger myocardial infarction and stroke (Ross, 1999 & Dutta et al., 2012).

**Apolipoprotein E**

Apolipoprotein E (apoE) is an anti-atherogenic protein synthesized in the liver and by macrophages (Elshourbagy et al., 1985). The major function of apoE is to serve as a high affinity ligand for LDL receptors on the membrane, allowing uptake of atherogenic particles, such as chylomicrons and very low density lipoprotein remnants, from circulation (Jawien et al., 2004 & Imaizumi, 2011). Mice deficient in apoE have elevated total circulating cholesterol levels, and a reduction in anti-atherogenic high density lipoprotein (HDL) levels, compared to control mice (Plump et al., 1992). ApoE\(^{-/-}\) mice have been widely used as a mouse model for atherosclerosis studies as they develop extensive arterial lesions on a chow diet, which is markedly accelerated by high cholesterol Western diet (Plump et al., 1992 & Imaizumi, 2011).

**Gld. apoE\(^{-/-}\) mouse model**

Mice that harbor both the gld mutation and apoE deficiency were generated to study accelerated atherosclerosis in systemic lupus erythematosus. This model exhibits larger atherosclerotic lesion area compared with apoE\(^{-/-}\) mice, and increased lymphadenopathy, splenomegaly, and autoantibodies compared with gld mice due in part to impaired clearance of apoptotic bodies (Aprahamian et al., 2004).

**Link between atherosclerosis and SLE**
An increased risk of atherosclerosis is presented in lupus patients, and premature atherosclerosis has been recognized as the major cause of morbidity and mortality in SLE (McMahon & Hahn, 2007 & Nikpour et al., 2005). Patients with SLE have an estimated 50-fold increased risk of myocardial infarction compared to non-SLE controls (McMahon & Hahn, 2007).

Inflammation is an important factor in the initiation and progression of atherosclerosis (Ross, 1993). In SLE patients, the pathogenesis of atherosclerosis is largely contributed by the interplay of inflammation and immune mechanisms, which leads to increased levels of oxidized lipids (such as oxidized LDL and pro-inflammatory HDL) and up-regulation of adhesion molecules and cytokines (McMahon & Hahn, 2007). In addition, impaired clearance of apoptotic bodies has been shown to play a role in the progression of both atherosclerosis and SLE (Aprahamian et al., 2004).

**Retinaldehyde**

Vitamin A is an essential molecule obtained from the diet that plays a key role in immune function as an important modulator of TGFβ, which can suppress several cytokines involved in pro-inflammatory responses (Castellani et al., 2010; Berry & Noy, 2012). Most of the functions of vitamin A are exerted by its active metabolites rather than the parental vitamin A molecule, retinol, due to the tightly regulated biological pathway (Berry & Noy, 2012). Once absorbed in the intestine, vitamin A is transported into bloodstream and enters the liver, converting to retinol, which re-enters bloodstream bound to retinol binding protein (RBP) (Hall et al., 2011). After entering the cells, retinol is reversibly oxidized by alcohol dehydrogenase (ADH) into retinaldehyde, which is then
irreversibly metabolized into retinoic acid (RA) by rate-limiting regulating enzymes, retinal dehydrogenases (RALDH1, 2) (Hall et al., 2011 & Gudas, 2012). Therefore, retinaldehyde is an intermediate metabolite between vitamin A (retinol) and retinoic acid (Kim et al., 1992) (Fig. 1). Retinoic acid serves as a ligand for nuclear receptors such as the retinoic acid receptor (RAR) and the retinoid X-receptor (RXR) (Rudraraju et al., 2014). Besides its biological role in the vision cycle, retinaldehyde has also been found to be present in rodent fat with the capacity of inhibiting adipogenesis and suppressing peroxisome proliferator-activated receptor gamma and RXR responses (Ziouzenkova et al., 2007).

**Figure 1: Retinaldehyde is an intermediate metabolite of vitamin A.**

**Peroxisome proliferator-activated receptor-γ**

Peroxisome proliferator-activated receptor gamma (PPARγ) is a member of the PPAR subfamily of nuclear hormone receptors (Tontonoz & Spiegelman, 2008). PPARγ is involved in the control of lipid metabolism and inflammation and is expressed in the major cell types that make up atherosclerotic lesions, including macrophage foam cells, smooth muscle cells, lymphocytes, and endothelial cells (Ricote et al., 1998; Sueyoshi et
al., 2001). In vivo studies have shown that PPARγ strongly inhibits atherosclerotic lesion formation (Li et al., 2004). Several clinical studies also supported that PPARγ agonists improve atherosclerotic markers in human subjects (Brown & Plutzky, 2007). The activation of PPARγ induces CD36, a class B scavenger receptor that enhances the uptake of the oxidized LDL into cells, as well as adiponectin, a fat-specific hormone that exerts anti-inflammatory effects (Brown & Plutzky, 2007). In addition, PPARγ activation is able to upregulate RALDH2, the enzyme responsible for converting retinaldehyde to retinoic acid (Szatmari et al., 2006). However, PPARγ activation is inhibited by the presence of retinaldehyde in fat (Ziouzenkova & Plutzky, 2008).

Thiazolidinediones are PPARγ agonists used as anti-diabetic agents due to their ability to increase the insulin sensitivity of type 2 diabetes mellitus. Recently, thiazolidinediones have been shown to reduce autoantibody production, renal disease, and atherosclerosis in mouse models of SLE, by inducing adiponectin (Aprahamian, 2009, 2014). Taken together, these data demonstrate that activation of PPARγ provides benefits towards ameliorating disease, and inhibition of PPARγ is detrimental to disease progression.

**SLE and metabolic syndrome**

Several lines of clinical evidence suggest that SLE is also associated with an increased prevalence of the metabolic syndrome (MetS), which is defined as a combination of cardiovascular risk factors with increased insulin resistance and a high risk of developing type 2 diabetes (Parker et al., 2010). NZBWF1 lupus mice maintained on a high fat diet exhibit visceral obesity and peripheral vascular dysfunction (Gilbert & Ryan, 2011). It has also been demonstrated that metabolic dysfunction can occur in a
mouse strain susceptible to lupus, defined as impaired glucose tolerance (Gabriel et al., 2012). These findings in mice extend to humans, demonstrated by clinical evidence showing that SLE is associated with an increased prevalence of the MetS (Parker & Bruce, 2013; Parker et al., 2013; Chung et al., 2007).

Patients with MetS often present with central adiposity, a main contributor to insulin resistance (Parker & Bruce, 2013). Adiponectin is an adipose-derived protein that decreases with increasing adiposity and has been shown to increase insulin sensitivity (Yang & Chuang, 2006). Since low concentrations of adiponectin have been observed in patients with MetS, adiponectin is proposed by some researchers to be a possible link connecting MetS and SLE (Ryo et al., 2004; Matsuzawa et al., 2004; Parker & Bruce, 2013). Therefore, it stands to reason that retinaldehyde and its role in PPARγ signaling could be crucial to the downregulation of adiponectin and progression of SLE.

**Summary and goals of the thesis**

In summary, since the activation of PPARγ signaling has been shown to have an effect on SLE prevention, and suppressed PPARγ signaling is presented in the mouse model lacking retinaldehyde catabolizing enzyme, we hypothesized that a high level of circulating retinaldehyde may play a role in SLE pathogenesis by inhibiting PPARγ signaling. Therefore, the goals of the thesis are:

1. To assess the body composition of the MRL.lpr lupus mouse model on chow diet, and the gld.apoE−/− mouse model of accelerated atherosclerosis and lupus on both chow diet and high-cholesterol Western diet, in comparison with their wild type controls (MRL and C57, respectively).
2. To develop a reliable assay using high performance liquid chromatography to quantify circulating retinaldehyde levels in our mouse cohorts.

3. To measure the mRNA expression of genes involved in retinaldehyde metabolism, as well as PPARγ signaling, in two white adipose tissue depots from our mouse cohorts.
METHODS

Animals and Diet

We obtained female C57BL/6 (#000664) (wild type), MRL (#000486), and MRL.lpr mice (#000485) from Jackson Laboratories. The female gld.apoE-/- mice were originally generated by crossing gld and apoE-/- mice on a C57BL/6 background, and have been established as a mouse model of accelerated atherosclerosis and lupus (Aprahamian, 2004, 2009).

Starting at 7 weeks of age, the wild type and gld.apoE-/- mice were maintained on a chow diet (Teklad Global 18% Protein Rodent Diet) (n=12 per genotype) or a high-cholesterol Western Diet (Harlan Adjusted Calories Diet, 42% from fat; TD.88137) (n=12 per genotype) for 12 weeks. The chow diet contained, as a percent of total kilocalories, 24% protein (crude protein), 18% total fat (ether extract) and 58% carbohydrate (calculated by subtracting neutral detergent fiber from total carbohydrate). The Western diet contained, as a percent of total kilocalories, 15.2% protein (casein plus additional DL-Methaionine), 42.0% total fat (andydrous milkfat plus cholesterol) and 42.7% carbohydrate (sucrose plus corn starch). The animal experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of Boston University School of Medicine.

High Performance Liquid Chromatography (HPLC)

-Retinaldehyde Oxime Standard

Retinaldehyde (Rald) oxime standard was generated by reducing 100 µM all-trans retinal (Santa Cruz Biotechnology) with 1M hydroxylamine (Sigma H2391) and 25 µM
ethylenediaminetetraacetic acid (EDTA) in PBS under argon and protected from light for two hours at room temperature.

-Serum Sample Preparation

After euthanasia by CO₂, blood was drawn from the mice by cardiac puncture. Plasma was separated by immediately centrifuging at 4000 rpm for 5 minutes at 4°C (Eppendorf, 5430R). Plasma (minimum of 250μL; maximum of 400μL) was added to a solution containing 1M hydroxylamine (Sigma H2391) and 25μM EDTA in methanol under argon and protected from light for two hours at room temperature. This allows the retinaldehyde in the blood to be converted to its oxime derivative, a more stable form of retinaldehyde, for HPLC analysis. The samples were stored at -80 °C until further processing.

-HPLC Sample Preparation

Before HPLC analysis, 10μl of 20 ng/μl Vitamin D₂ solution was added to each serum sample, to serve as an internal standard for recovery calculation. After 1 hour at 4°C in the dark, the retinaldehyde oxime was extracted from the prepared retinaldehyde oxime standard or serum samples by adding 4000μl Hexane and centrifuging at 1000xg for 3 minutes at 4°C (Sorvall RT6000B). The hexane extracts were evaporated under a gentle stream of nitrogen. The residues were reconstituted in 120μl acetonitrile and transferred to a 350μL Fused Insert MacroVial (Thermo Scientific) for HPLC analysis.

-HPLC System

The HPLC system (Agilent 1200 Series) included a quaternary pump (G1311A) operating at a flow of 1.0ml/min, a 3μm Pecosphere C-18 column (83X4.6mm,
PerkinElmer), a thermoregulated autosampler (G1329A), a column thermostat (G1316A),
and a Diode Array Detector (G1315D). The mobile phase was composed of 60%
acetonitrile, 39.9% water and 0.1% formic acid. Using a reverse-phase HPLC method,
analytes were separated at 1ml/min with a linear gradient of acetonitrile:water:formic
acid from 60%:39.9%:0.1% to 94.9%:5%:0.1% over a duration of 5 minutes.

-Peak Identification, Quantification and Internal Standard Validation

The analyte peaks were identified by retention times and UV spectra, according to
those observed with the external standards, retinaldehyde oxime and vitamin D₂, in each
run. The serum retinaldehyde concentrations were quantified by using the standard curve
of external retinaldehyde oxime standard, adjusted by the % recovery obtained from the
added vitamin D₂ standard. The vitamin D₂ standard was validated by three test runs
substituting mouse serum with 5mg/ml of Bovine Serum Albumin (BSA) (AmericanBio,
Natick, MA) solution in water. All testing samples were run in triplicate.

Tissue Collection

Mice were euthanized using carbon dioxide at endpoint. The spleen, cervical
lymph nodes, visceral fat, subcutaneous fat and heart were removed from the body, snap
frozen in liquid nitrogen, and stored at -80 °C for RNA analysis and histology. The
weights of the spleen and cervical lymph nodes, as well as total body weight were
recorded.

Reverse Transcriptase PCR (RT-PCR) and Quantitative Real Time PCR (qPCR)

The spleen, lymph node, visceral fat and subcutaneous fat tissues from the mice
were homogenized in QIAzol Lysis Reagent (for adipose tissues) (QIAGEN), or Buffer
RLT (for non-adipose tissues) (QIAGEN), using the Qiagen TissueLyzer II System. Total RNA was isolated using an RNeasy tissue kit (Qiagen) and assayed using a Nanodrop spectrophotometer (ThermoScientific, Wilmington, DE) to assess purity and concentration. RT-PCR was performed with 1 µg of RNA using the QuantiTect Reverse Transcription Kit (QIAGEN). Gene expression analysis was performed using T100™ Thermal Cycler (Bio-Rad). 2µL of diluted cDNA (diluted 1 10) generated from RT-PCR was then used as template for qPCR amplification using TaqMan® Real-Time PCR Master Mix (Life Technologies) in a total reaction volume of 20 µL. The Comparative Ct method (ΔΔCt) was employed in ViiA™ 7 Real-Time PCR System (Life Technologies) to determine relative fold changes in mRNA expression. GAPDH was used as the endogenous control. The mRNA expression of, ADH1, RALDH1, RALDH2, PPARγ, adiponectin, and CD36, were examined using TaqMan® expression assays. All samples were run in duplicate.
RESULTS

Reference standards of HPLC developed using Bovine Serum Albumin.

Due to the within-run and between-run variations of the sample preparation prior to HPLC separation, it was important to establish a reliable internal standard in each sample. The Bovine Serum Albumin (BSA) solution shares a high physiological similarity with mouse serum samples (Hamidi & Zarei, 2009). Therefore, we used a BSA solution with a fixed concentration (5mg/ml) to test the recovery efficiencies of the internal vitamin D$_2$ standard and the external oxime standard.

Our test runs showed that 1µmol of oxime standard has a retention time of 6.5min and a peak area around 200 units (Fig 2a). Varying concentrations of oxime solutions were evaluated by comparing to the oxime peak areas in mouse serum. The oxime area of mouse serum ranged from 100 to 300 units. Therefore, the oxime solution with a concentration of 0.1 µmol/µl was selected as an external standard as 10µl of the solution provided a peak area around 200 units. Vitamin D$_2$ standard was added at a concentration of 200ng (10 µl of 20ng/ µl) and had a retention time of 17min and a peak area around 450 units (Fig 2b). When mixed with BSA solution, the oxime standard has an average peak area around 110 units, and the vitamin D$_2$ has an average peak area around 270 units, showing a recovery rate of 55%, 59%, respectively. Based on the testing results, we were able to obtain a factor close to 1 for the recovery rate ratio of oxime and vitamin D$_2$, indicating that the two compounds have similar yield and that vitamin D$_2$ can be used as an internal standard. We identified the oxime peaks in the serum samples of our mouse
models using the retention time of the external oxime standard in each run (Fig 2c-f). We quantified the retinaldehyde concentrations in our serum samples based on the recovery rate ratio of oxime and vitamin D₂.

**Figure 2: Chromatograms of oxime standard, vitamin D₂ standard, and serum samples.** Values in milliabsorbance units (mAU) of (a) oxime standard (at 325nm). (b) Vitamin D₂ standard (at 265nm). Oxime peak in serum samples of (c) wild type mouse on Western diet; (d) gld.apoE⁻/⁻ mouse on Western diet; (e) MRL mouse; (f) MRL.lpr mouse.

**Analysis of MRL.lpr lupus mouse model**

Because lupus is an inflammatory disorder accompanied by a high oxidative stress state, we hypothesized that elevated retinaldehyde (Rald) levels might be present in SLE. The female MRL.lpr mouse develops severe lymphadenopathy and splenomegaly
and has an average lifespan of 17 weeks. MRL.\textit{lpr} mice, and its control, MRL, were fed with chow diet. At 16 weeks of age, MRL.\textit{lpr} mice showed no difference in body weight compared with their controls (Fig. 3a), but presented larger lymph nodes and spleens. Interestingly, the MRL.\textit{lpr} mice displayed decreased weight of visceral and subcutaneous adipose depots (Fig. 3b-d).

**Figure 3: Body and organ weights of MRL.\textit{lpr} mice.** (a) Endpoint body weight. (b) Lymph node, (b) spleen, and (c) visceral (VFAT) and subcutaneous (SUBQ) adipose tissues were excised and weighed (n=8 per group; *, p< 0.05).

To determine if retinaldehyde may be playing a role in lupus pathogenesis, we first measured circulating retinaldehyde levels. MRL.\textit{lpr} mouse displayed increased serum retinaldehyde levels compared with the MRL control on a chow diet ([Rald]=5.00± 0.36, 3.78± 0.24 nmol/μl, respectively) (Fig. 4).
Figure 4: Circulating retinaldehyde (Rald) is increased in MRL/lpr mice. Serum was collected and analyzed by HPLC from 16 week old mice maintained on a chow diet. (MRL, n=8; MRL/lpr, n=7; p<0.05).

To examine a potential cause for the observed increase in retinaldehyde levels, mRNA expression levels were determined for genes involved in retinaldehyde synthesis and catabolism, alcohol dehydrogenase 1 (ADH1) and retinaldehyde dehydrogenase 1 & 2 (RALDH1, 2), respectively. In visceral fat, MRL/lpr mice revealed a significantly higher expression of the gene encoding the enzyme that produces retinaldehyde, ADH1, but a trend towards increasing RALDH2 as well (p=0.06) (Fig. 5a). No changes to PPARγ, adiponectin or CD36 were observed in visceral adipose tissue (Fig. 5b). In subcutaneous adipose tissue, MRL/lpr showed a significantly lower expression of RALDH1, and a trend towards lower levels of PPARγ and adiponectin (Fig. 5c-d).
Figure 5: Altered retinaldehyde metabolism and PPARγ signaling gene expression in adipose tissue of MRL./lpr mice. Quantification by RT-PCR of genes involved in retinaldehyde production and breakdown, and PPARγ, adiponectin (adipoQ), and CD36 in (a-b) visceral fat and (c-d) subcutaneous fat of MRL./lpr lupus mice vs. MRL controls maintained on chow diet (n=6 per group; *, p<0.05).

Analysis of gld.apoE−/− mouse model of lupus and accelerated atherosclerosis.

To determine if the results we observed in MRL./lpr mice are specific to one lupus model, we examined the gld.apoE−/− mouse model of accelerated atherosclerosis and lupus. After 12 weeks of Western diet, no change was observed in body weight of gld.apoE−/− mice compared to the wild type controls (Fig. 6a). However, as expected, the gld.apoE−/− mice presented significantly larger lymph nodes and spleens (Fig. 6b-c). Similar to our observations in the MRL./lpr mice, visceral and subcutaneous fat depots had significantly less mass in the gld.apoE−/− mice compared to wild type controls (Fig. 6d).
Figure 6: Body and organ weights of *gld.aapoE<sup>−/−</sup>* mice. (a) Endpoint body weight of *gld.aapoE<sup>−/−</sup>* vs. wild type after 12 weeks of Western diet. (b) Lymph nodes and (c) spleen were harvested from *gld.aapoE<sup>−/−</sup>* and wild type mice and weighed wet. (d) The visceral fat (VFAT) and subcutaneous fat (SUBQ) weight were collected and weighed from *gld.aapoE<sup>−/−</sup>* vs. wild type (n=10 per group; *, p<0.01).

Circulating retinaldehyde levels were quantified in both wild type and *gld.aapoE<sup>−/−</sup>* mice maintained on a chow diet or a high-cholesterol Western diet. Our data show that the circulating retinaldehyde levels in mice were largely influenced by Western diet (Fig. 7). In a comparison between wild type and *gld.aapoE<sup>−/−</sup>* mice, circulating retinaldehyde did not change significantly, regardless of diet. However, wild type mice fed with Western diet had significantly higher retinaldehyde levels compared to wild type that received chow diet ([Rald]=6.99±0.82, 2.30±0.44nmol/µl, respectively). Similarly, retinaldehyde levels in *gld.aapoE<sup>−/−</sup>* mice increased with Western diet ([Rald]=2.91±0.66, 5.43±0.71nmol/µl, respectively).
Figure 7: Circulating retinaldehyde (Rald) is increased by Western diet. Serum retinaldehyde concentration was measured by HPLC, chow diet (C57, n=16; gld.apoE\(^{-/-}\), n=7) vs. Western diet (C57, n=10; gld.apoE\(^{-/-}\), n=8) (*, p<0.05).

Since our most significant observation arose from mice maintained on Western diet, we continued our studies with those cohorts. We next examined the mRNA expression levels of genes involved in retinaldehyde synthesis and catabolism. We observed no change in the expression of these genes in the visceral fat of gld.apoE\(^{-/-}\) mice compared to wild type when maintained on Western diet (Fig. 8a). In addition, no change was observed to expression of PPAR\(\gamma\) or its target genes in visceral fat of gld.apoE\(^{-/-}\) compared to control (Fig. 8b). However, in subcutaneous fat, gld.apoE\(^{-/-}\) showed significantly less expression of the enzymes responsible for retinaldehyde metabolism as well as PPAR\(\gamma\), adiponectin, and CD36 when compared to wild type mice (Fig. 8d).
Figure 8: Differing expression of retinaldehyde and PPARγ genes in adipose tissue of wild type and gld.aapoE⁻/⁻ mice. Quantification by RT-PCR of genes involved in retinaldehyde production and breakdown, and PPARγ, adiponectin (adipoQ), and CD36 in (a-b) visceral fat and (c-d) subcutaneous fat after Western diet (n=6 per group; *, p<0.01).

Since the serum retinaldehyde levels in both wild type and gld.aapoE⁻/⁻ mice models were affected by diet, we questioned whether these expression changes would be further affected by lupus and accelerated atherosclerosis. We therefore measured the mRNA expression of the genes in adipose tissues of gld.aapoE⁻/⁻ fed with a chow diet compared to its counterpart fed with a Western diet. Analysis in visceral adipose tissue of mRNA levels of ADH1, tended to increase (p=0.052), while RALDH1 trended to decrease (Fig. 9a). Western diet resulted in significantly decreased adiponectin mRNA expression in visceral fat (Fig. 9b). The results for enzyme expression were slightly different when examined in subcutaneous fat, trending to decrease ADH1 and RALDH1, while RALDH2 trended to be higher (Fig. 9c). Overall, the most dramatic change to PPARγ and its target genes occurred in subcutaneous adipose tissue, in which we observed robust decreases in both PPARγ and adiponectin (Fig. 9b-d).
Figure 9: Western diet inhibits PPARγ signaling in gld.apoE−/− mice. Quantification by RT-PCR of genes involved in retinaldehyde production and breakdown, and PPARγ, adiponectin (adipoQ), and CD36 in (a-b) visceral fat and (c-d) subcutaneous fat in a mouse model of accelerated atherosclerosis and lupus on chow diet vs. on Western diet (n=4 per group; *, p<0.05).

Taken together, it appears that amount of circulating retinaldehyde is not only affected by lupus, but also by Western diet. Changes to the components of the retinoic acid pathway, as well as downstream PPARγ signaling can be observed in both the MRL.lpr mouse model of lupus and the gld.apoE−/− mouse model of accelerated atherosclerosis and lupus. This suggests a potential pro-inflammatory role of retinaldehyde in lupus.
DISCUSSION

Countless scientific efforts have been devoted to the search for new therapeutic target of SLE from various mechanistic perspectives. The beneficial role of PPARγ agonist treatment has recently come to light, however, while adiponectin signaling is a proposed mechanism, the exact pathway to target has not been elucidated. With the link of metabolic syndrome and SLE recently reported, and a role for retinaldehyde in PPARγ signaling inhibition, the relationship between retinaldehyde and lupus pathogenesis should be brought to scientific attention. The main goal of our current research was to study the biological role of retinaldehyde in the development of SLE.

In order to investigate the relationship between circulating retinaldehyde level and SLE, we compared the serum retinaldehyde levels in a lupus mouse model (MRL.\textit{lpr}) and its control (MRL). MRL.\textit{lpr} mice showed a higher circulating retinaldehyde level than its control on a chow diet. In addition, MRL.\textit{lpr} expressed more alcohol dehydrogenase (ADH1) mRNA in the visceral fat. To our surprise, circulating retinaldehyde levels did not differ between \textit{gld}.apoE\textsuperscript{-/-} mice and wild type on a chow diet or on a Western diet. However, both \textit{gld}.apoE\textsuperscript{-/-} and wild type on a Western diet showed higher serum retinaldehyde concentrations than their counterparts on a chow diet. These results suggest that the circulating retinaldehyde level is influenced by both lupus and high cholesterol Western diet.

Interestingly, circulating retinaldehyde levels in male \textit{gld}.apoE\textsuperscript{-/-} mice did not change after feeding with a Western diet (n=3 on a chow diet, n=11 on a Western diet; [Rald]=2.96±1.00, 4.29±0.90 nmol/μl, respectively; p=0.51) (data not shown). This
result suggests that there might be a sex-specific response to the high cholesterol Western diet in the lupus mouse model. A caveat to these results is the small number of male mice analyzed after chow diet. However, this is still of potential interest since there is a higher prevalence of SLE in females than in males, and our results could suggest a potential gender specific relationship between circulating retinaldehyde and SLE.

There are several enzymes involved in the synthesis and catabolism of retinaldehyde as well as PPARγ signaling (Fig. 1). It has been described that retinoic acid is produced primarily by RALDH1 in visceral fat and by RALDH1 and RALDH3 in subcutaneous fat (Reichert et al., 2011). Therefore, we measured the mRNA expression of these enzymes in two different depots of white adipose tissues (visceral and subcutaneous) in order to determine which, if any, enzyme expression in this pathway is altered in SLE. Despite similar circulating retinaldehyde levels between gld.apoE-/- and wild type mice, we still observed differences in gene expression. While no change was observed between gld.apoE+/- and wild type on a chow diet, gld.apoE+/- on a Western diet showed a significant decrease in the mRNA expression of ADH1 in subcutaneous fat compared to wild type controls. A slightly larger magnitude of decrease was observed in RALDH1 compared with wild type on the same diet. It is interesting to note that a decrease of RALDH1 mRNA expression was only found in the subcutaneous fat of both of the lupus models (gld.apoE-/- and MRL.lpr), indicating that the enzyme expression may be altered in a depot-specific manner in lupus.

An interesting observation was that RALDH2 tended to be higher in some instances. This would suggest that retinaldehyde could be more efficiently catabolized,
however, this in contrast to our observations on retinaldehyde accumulation in lupus models. One possibility could lend from the study demonstrating that RALDH2 is inefficient at retinaldehyde oxidation (Duester, 2008). Another possible explanation is that RALDH2 is expressed specifically in mitochondria (Kitabatake et al., 1981). It has been shown that T lymphocytes of proteins with SLE have a disrupted mitochondrial membrane potential, resulting in constant hyperpolarization (Perl et al., 2012). This causes a shift from apoptosis induction, to a necrosis pathway, thus, leading to inflammation in SLE. Therefore, it is possible that dysfunctional mitochondrial biogenesis under conditions of SLE may disrupt proper signaling of RALDH2, thereby still leading to an increased buildup of retinaldehyde. This would be an area of future study.

Exposure of wild type mice to high fat diet increases PPARγ mRNA expression in adipose tissues (Vidal-Puig et al., 1996). In our study, the gld.apoE-/- mice showed decreased PPARγ mRNA expression in subcutaneous fat, and decreased adiponectin mRNA expression in both visceral fat and subcutaneous fat after being fed with high-cholesterol Western diet. Even though both gld.apoE-/- and C57 presented increased circulating retinaldehyde after 12 weeks of Western diet, only gld.apoE-/- had decreased mRNA expression of the genes involved in PPARγ signaling pathway, indicating that lupus models may be more vulnerable to the increased level of circulating retinaldehyde induced by Western diet.

Western diet accelerated the body weight gain to a similar extent in both gld.apoE-/- and wild type mice, but the weight gain in gld.apoE-/- mice was due to
enlarged lymph nodes and spleens, whereas the weight gain in wild type mostly came from increased fat mass. It would be beneficial to subject the mice to metabolic phenotyping to determine the lean mass to fat mass ratio for confirmation. Both lupus models showed a significant less white adipose tissue mass compared with their controls, suggesting that adipogenesis is impaired in lupus. In addition, the white adipose tissue mass in glld. apoE+/− mice fed Western diet trended to be lower than the glld. apoE−/− fed with a chow diet (p=0.07). The disrupted expression of retinoic acid pathway components and downstream signaling targets known to be involved in adipogenesis is further evidence that this area of research merits future study. Potential experiments could include administration of high-fat/high-sucrose diet to lupus models and analysis of adipose tissue inflammation.

Although adipose tissue plays a role in retinoid metabolism, and fat is the second largest storage site for retinoid in the body, it is important to investigate the mRNA expression of genes involved in retinaldehyde metabolism pathway as well as PPARγ signaling in the liver, given the fact that liver is the largest storage site for retinoid (Blomhoff et al., 1991). As we observed a significantly decreased white adipose tissue mass in lupus models, the contribution of the retinaldehyde metabolism enzymes in white adipose tissue to the circulating retinaldehyde level is questionable. It is possible that the lipids accumulated in the liver, rather than by the adipose tissues, contribute to the observed increase in circulating retinaldehyde.

As retinaldehyde is the sole precursor of retinoic acid, decreased expression of the retinaldehyde catabolizing enzymes lead to a decreased amount of retinoic acid
production. It has been shown that retinoic acid blocks adipogenesis by upregulating the expression of the adipogenesis inhibitors Pref-1, Sox9, and Kruppel-like factor 2 (Berry et al., 2012). Similar results have been found in RALDH1−/− mice, including smaller lipid cell size and less lipid accumulation (Ziouzenkova et al., 2007). Our study has observed a higher circulating retinaldehyde level in MRL./lpr lupus model, which presented impaired adipogenesis compared with its control. However, whether this is a causal relationship requires further investigation.

Our data suggest that there might be a strain-specific association between serum retinaldehyde concentration and lupus pathogenesis. There might also be a gene-diet interaction involved. This is an area worth further investigation, and could be extended to include other mouse models of lupus such as the NZB/W mouse strain, or better yet, examined in human serum from SLE patients and healthy controls.

-Limitations

There are some limitations in our study. One major limitation regarding our diet comparison is that we used chow diet as a control diet for the Western diet. The plant-derived ingredients in a chow diet show a high variability in nutrient contents (Sasidharan et al., 2013). Therefore, the difference between a chow diet and a high-cholesterol Western diet is not limited to the fat content. The chow diet used in this study contained 15IU/g of vitamin A. Whereas the Western diet contained 10g/kg vitamin mix, which was composed of 3.96g/kg of 500,000IU/g vitamin A palmitate. Therefore, the vitamin A content in the Western diet was 19.8IU/g. The different amount of vitamin A obtained
from these two diets would also contribute to a differential change in the circulating retinaldehyde levels in our mouse models.

More importantly, clear evidence has shown that most commercial chow diets contain high levels of phytoestrogens due to their soy-based ingredients that would influence the hormone levels, especially circulating isoflavone level, in the mouse model (Brown & Setchell, 2001). The prevalence of SLE differs largely between sexes, indicating a huge impact of sex hormones in the disease pathogenesis. Even though we focused on female mice in our current study, this diet choice would limit our capacity to expand our research on the male mice. This would also challenge our extra finding about the non-differential circulating retinaldehyde levels in the male gld.apoE⁻/⁻ mouse models on chow and Western diet. Therefore, the use of a chow diet as a control diet is a major confounding factor in our study. An ideal control diet for a high-cholesterol Western diet should be a semi-purified research diet with intentionally modified ingredients that only varies in fat content (Sasidharan et al., 2013). If chow diet had to be used, comparisons should only be made regarding the response of different genotypes fed with the same diet.

GAPDH was used as an endogenous control gene when to measure the mRNA expressions of genes of interest in the adipose tissues using RT-PCR. However, it has been shown that GAPDH is not the best choice as its expressions vary in adipose tissues (Suzuki et al., 2000). 18S rRNA would be a better control due to its relatively invariant expression level in different tissues (Bookout & Mengelsdorf, 2003). In addition, the mRNA expression we observed might not be accurate information for the enzyme levels
in certain tissues because the mRNA may not be fully transcribed into protein. Western blotting could be employed for a higher accuracy in enzyme level measurement.

We used vitamin D$_2$ as an internal standard for retinaldehyde HPLC analysis, since the rodent diets we used in our study contained only vitamin D$_3$ to meet the vitamin D requirement. In addition, our standard testing results suggested vitamin D$_2$ is a better choice for internal standard and the ratio of the extraction efficiencies of vitamin D$_2$ and retinaldehyde oxime are close to 1:1. However, since there are still some variations among each run, using radiolabelled tracers in each sample is a more accurate method to measure the recovered oxime level.

Since we observed a significant impact of Western diet on circulating retinaldehyde concentrations in both $gld$.apoE$^{+/}$ and C57, as well as the mRNA expression of adiponectin and PPAR$\gamma$ in adipose tissues of $gld$.apoE$^{+/}$, it is important to distinguish whether the effects were caused by the quality or the quantity of the food intake. However, we did not measure the food consumption of the mice, leaving this question unanswered.

**Summary**

In conclusion, we observed a high circulating retinaldehyde level in the MRL./$pr$ lupus model. In addition, our study reveals that a high-cholesterol Western diet can increase circulating retinaldehyde, and this can affect PPAR$\gamma$ signaling on a lupus background. This proposal has begun to elucidate the hypothesis that retinaldehyde buildup in lupus is crucial to the progression of SLE and metabolic disease. In addition, we observe changes in PPAR$\gamma$ target genes in adipose tissue, which is understudied in
terms of signaling and adipogenesis in SLE. However, further investigation remains to determine the biological role of retinaldehyde in SLE. Therefore, our future proposed studies would allow a molecular dissection of retinaldehyde-induced inhibition of PPARγ signaling in adipose tissue of lupus mouse models to determine a potential therapeutic target to benefit SLE and its metabolic complications.
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CERTIFICATIONS
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