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Effects of apoB-derived peptide vaccination in a murine model of systemic lupus erythematosus

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Boston University
EFFECTS OF APOB-DERIVED PEPTIDE VACCINATION IN A MURINE MODEL OF SYSTEMIC LUPUS ERYTHEMATOSUS

by

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EFFECTS OF APOB-DERIVED PEPTIDE VACCINATION IN A MURINE MODEL OF SYSTEMIC LUPUS ERYTHEMATOSUS

BRIAN TUAN SAMUELS\N

ABSTRACT

Objective: Atherosclerotic disease progression is mediated in part, by immunological mechanisms. In recent years, interest has increased towards the prospect of modulating these immune mechanisms through vaccination to ameliorate the course of disease. Patients with lupus are at a significantly higher risk for accelerated atherosclerosis and related complications. The goal of this study was to assess the outcome of immunization in mouse models of lupus, and lupus with accelerated atherosclerosis.

Materials/Methods: Atherosclerosis-prone apoE⁻/⁻ mice and autoimmune gld mice were previously crossed to generate the gld.apoE⁻/⁻ mouse. Mice were treated with an apoB-100-derived vaccine, Alum (adjuvant control), or PBS control. The antibody response was determined by quantifying the amount of circulating anti-apoB100. Serum triglyceride and cholesterol levels were analyzed. Kidney tissue from gld and gld.apoE⁻/⁻ mice was processed and histologically analyzed, using glomerular tuft size as a measure of renal disease and by extension, autoimmune disease severity.

Results: Immunization led to a pronounced initial antibody response that was decreased by the endpoint of the study. No significant differences in serum...
triglyceride or cholesterol were observed regardless of treatment. Similarly, no significant differences were observed in glomerular tuft size.

Conclusion: The data suggests that immunization with an apoB-100-derived vaccine neither improves nor worsens autoimmune disease severity in the gld.aPOE−/− mouse model. It also appears that immunization is tolerated in the autoimmune background. While further study is necessary to determine the efficacy of immunization in reducing atherosclerotic disease in this model, this may be a possible therapy to lower incidence of atherosclerosis in lupus patients.
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LIST OF ABBREVIATIONS

apoB-100 ................................................................. Apolipoprotein B-100
apoE .............................................................................. Apolipoprotein E
cIMT ................................................................. Carotid intima-media thickness
DCs ........................................................................... Dendritic cells
HDL ............................................................................. High-density lipoprotein
IgG ............................................................................ Immunoglobulin G
IgM ........................................................................... Immunoglobulin M
IL-10 .......................................................................... Interleukin-10
LDL ............................................................................. Low-density lipoprotein
MDA ........................................................................ Malondialdehyde-modified
IgM-p210MDA .................................................. MDA-modified apoB-100 Immunoglobulin M
IgG-p210nat ......................................................... Natural apoB-100 p210 Immunoglobulin G
OxLDL ......................................................................... Oxidized LDL
OxPL ........................................................................ Oxidized phospholipids
SLE ........................................................................... Systemic lupus erythematosus
TGF-β ................................................................. Transforming growth factor beta
TNF-α ........................................................................ Tumor necrosis factor alpha
TNF-β ........................................................................ Tumor necrosis factor beta
Treg ...................................................................... Regulatory T cell
VCAM-1 ............................................................... Vascular cell adhesion protein 1
INTRODUCTION

Atherosclerosis

Atherosclerosis is a chronic disease characterized by the development of lesions and plaque on the inner intimal layer of the arterial walls. It is the primary cause of cardiovascular disease, a public health risk of widespread prevalence. Cardiovascular disease is the single largest cause of death among people in industrialized countries (Braunwald, 1997). In 2010, cardiovascular disease was the cause of 25% of deaths globally, increasing from 20% in 1990 (Lozano, 2012).

Certain regions along arteries are at a substantially higher risk of developing atherosclerotic lesions owing to shear stress and the particular hemodynamics of blood flowing along those regions (Dai, 2004). Structurally, these atherosclerotic lesions form a thickening of the arterial intima. Their cores are composed of lipids and foam cells, which are lipid-laden macrophages. Outer layers contain fibrous connective tissue, smooth muscle cells, and immune cells including macrophages, T cells, and dendritic cells (Stary, 1995). Lesion development is chronic in nature and often quiescent for decades. Rupture of the plaque cap is the classical end-disease event and exposes the contents of the lesion to the blood, forming a thrombus that can lead to myocardial infarction (Hansson, 2005). The initiation and progression of lesions are a consequence of both endothelial injury and the immune response of the body to that injury.
High levels of low-density lipoprotein (LDL) cholesterol in the blood are one of the major risk factors for atherosclerosis (Ross, 1999). While wild-type laboratory mice do not tend to develop atherosclerosis, mice deficient for apolipoprotein E (apoE) have pronounced hypercholesterolemia and are highly prone to developing spontaneous atherosclerotic lesions (Hansson, 2005). One of the main initiators of injury, along with aforementioned shear stress, is the response of immune cells to the accumulation of modified LDL phospholipids in the vessel wall (Leitinger, 2003). Regions of the arterial intima at risk of atherosclerosis allow LDL molecules to adhere to and permeate the endothelium (Tabas, 2007). Once retained in the endothelium, the LDL molecules are more vulnerable to oxidation than they were in the bloodstream (Schwenke, 1989). A variety of mechanisms leading to LDL oxidation have been explored, but a complete mechanistic picture has not been definitively established. One candidate enzymatic mechanism is 2/15-lipoxygenase which is expressed on monocytes. Mice deficient in the L-12LO allele of 2/15 lipoxygenase crossed to the atherosclerosis-prone apoE⁻/⁻ background display diminished atherosclerosis (Cyrus, 1999). Another enzyme with a putative role in LDL oxidation is myeloperoxidase, which is present in atherosclerotic plaques and acts to modify LDL molecules to increase their uptake by macrophage scavenger receptors (Daugherty, 1994) (Podrez, 2000).

Investigation into the initial injury events in atherosclerosis has pointed towards oxidized LDL (oxLDL) and a variety of related oxidized phospholipids
(oxPL) as players in proatherogenic signaling pathways. The activation of immune pathways involves the recognition of oxPL and other pathogens by several innate immune system receptors including toll-like receptors and CD36 receptors expressed on macrophages and dendritic cells (Miller, 2003) (Podrez, 2002). The role of these receptors in the progression of atherosclerosis is supported by the amelioration of atherosclerosis in apoE−/− mice with toll-like receptor deficiency (Björkbacka, 2004). Activated macrophages, that are often found in conditions of atherosclerosis, produce cytokines and chemokines, such as interleukin-1β and macrophage colony-stimulating factor, that contribute to the progression of the inflammatory response (Moore, 2011). Additionally, oxLDL induces endothelial cells to increase expression of adhesion molecules such as vascular cell adhesion protein 1 (VCAM-1), resulting in increased recruitment of monocytes and lymphocytes to the site of the lesion (Hansson, 2005) (McMurray, 1993).

Another type of cell important in lesion development is the T cell. Upon the presentation of antigens by the innate immune system, T cells specific to those antigens are recruited and activated. CD4+ T cells preferentially differentiate into Th1 cells after binding to adhesion molecules or antigens presented by major-histocompatibility-complex molecules at the lesion site. Th1 cells have a largely pro-inflammatory cytokine profile and produce interferon-γ (IFN-γ), interleukin-2 (IL-2), tumor necrosis factor alpha (TNF-α), and tumor necrosis factor beta (TNF-β) (Szabo, 2003). In atherosclerotic lesions, IFN-γ and IL-2 in particular act to
spur a downstream inflammatory response (Frostegård, 1999). Anti-inflammatory immune response players also act at lesion sites. One of the mediators of the anti-inflammatory response is the cytokine interleukin-10 (IL-10), released by Th2 type cells. (Mallat, 1999) Another T cell type that may play a role in lesion development is the regulatory T cell population, which are Foxp3\(^+\) CD8\(^+\)CD25\(^+\). In an attempt to determine whether their presence in lesions contributed to atherosclerosis, these cells were introduced into apoE\(^-/-\) mice which led to diminished atherosclerotic disease (Zhou, 2014).

Evidence of beneficial modulation of atherosclerosis has been demonstrated by the immunoglobulin M (IgM) antibody released by B cells. Upon B cell recognition of a specific antigen, the less specific IgM antibody is initially released as a primary response. As time progresses, IgM production wanes and a larger secondary response of the more specific immunoglobulin G (IgG) antibody production begins (Eales, 2005). In a cross of LDL receptor-deficient (Ldlr\(^-/-\)) and IgM deficient mice, considerably worse atherosclerotic disease was observed (Lewis, 2009). A separate study reported that polyclonal IgM injected into apoE\(^-/-\) mice in the final 4 weeks of a hypercholesterolemic diet mitigated lesion formation, while monoclonal IgM did not. This suggests that different B cell populations may have varying capabilities of exerting atheroprotective effects (Cesena, 2009). Both IgM and IgG antibodies against oxLDL have been observed in atherosclerotic disease. It has been reported that neither IgM nor IgG autoantibodies were independent predictors of cardiovascular disease,
although there was some evidence to suggest that higher IgM levels might have a protective effect (Ravandi, 2011). Overall, the progression of atherosclerotic plaques likely represents a shift in balance towards pro-inflammatory and pro-atherogenic factors.

**Modulation of Atherosclerosis with Vaccines**

Because of the crucial role inflammation plays in atherosclerosis, efforts have attempted to modulate the immune system via vaccination to mitigate the development of atherosclerosis. OxLDL particles, with their integral contribution to atherosclerotic lesions, are an obvious candidate for a vaccine target. Early efforts at immunizing rabbits with oxLDL possessing malondialdehyde-modified (MDA) lysine epitopes were successful in decreasing atherosclerosis, (Palinski, 1995). However, more work is required to determine which component of oxLDL, a complex molecule, could potentially be translated into an effective vaccine. Further research has demonstrated that the specific antigen in oxLDL that elicits an immunogenic response is a particular peptide sequence of the Apolipoprotein B-100 (apoB-100) protein (Fredrikson, Söderberg, 2003).

ApoB-100 is a component of LDL, intermediate density lipoproteins, and very low density lipoproteins. ApoB-100 is the critical signaling component between LDL molecules and their receptors (Walldius, 2004), making it integral in the initial permeation of arterial walls. Proteoglycan matrix proteins have been implicated in binding to the apoB-100 component of LDL, which facilitates
subendothelial accumulation of LDL. Transgenic mice generated with impaired apoB-100 proteoglycan binding display reduced atherosclerosis (Skålén, 2002). Taken together, evidence suggests that apoB-100 plays an important role in the development of atherosclerotic lesions.

The antigen component, designated as p210, is a surface epitope of apoB-100. Immunization with p210 in mice resulted in a protective effect against atherosclerosis (Chyu, 2005). The mechanism by which p210 immunization reduces atherosclerosis is unclear but work over the past decade has delivered some insights. An increase of CD8+ T cells in immunized mice has been noted; CD8+ cells taken from immunized mice and administered to non-immunized mice led to a transfer of protective effects of immunization. Additionally, CD8+ T cells were found to lower the count of dendritic cells at lesion sites (Chyu, 2012). Dendritic cells present antigens to lymphocytes, and play a role in lipid uptake and foam cell formation in atherosclerosis-prone regions of arteries, therefore (Paulson, 2009), this may represent a potential mechanism by which p210 modulates atheroprotective effects.

Pertinent to the prospect of modulating the immune response to apoB-100 for the purposes of vaccination, is characterizing the native immune response to apoB-100 in humans. In a population study testing an apoB-100 polypeptide library, native apoB-100 and MDA-modified apoB-100 elicited dichotomous antibody responses. IgM displayed significantly less binding to the MDA-modified apoB-100 compared to native ApoB-100. Administration of the apoB-100 vaccine
demonstrated a more pronounced IgM response than the IgG response against MDA-modified apoB-100 (Fredrikson, Hedblad, 2003). In a more recent study examining a larger population, levels of IgM against MDA-modified apoB-100 (IgM-p210_{MDA}) and levels of IgG against natural apoB-100 p210 (IgG-p210_{nat}) were measured against changes in carotid intima-media thickness (cIMT). IgM-p210_{MDA} was inversely correlated with cIMT progression. IgG-p210_{nat} was initially associated with lower levels of cIMT, but adjusting for traditional atherosclerotic risk factors abolished those associations (McLeod, 2014). Based on these findings, it is reasonable to suggest a native atheroprotective role for IgM against apoB-100 that may be augmented after immunization using apoB-100.

**Systemic Lupus Erythematosus**

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease with a variety of manifestations in various organs and tissues. Autoimmune dysfunction results in a heterogeneous course and range of symptoms, severity, and specific organ system involvement in different patients. Several genes and environmental factors have been implicated in contributing to the risk of SLE but the exact mechanisms of its etiology remain to be elucidated (Tsokos, 2011). Epidemiologically, disease incidence and prevalence is about ten times higher in women than men across most populations. Mortality risk is higher for SLE patients as compared to the general population, with the predominant causes of
death including kidney disease, infection stemming from immunosuppressive therapy, and cardiovascular disease (Bernatsky, 2006).

Mechanistically, several components of the adaptive and innate immune systems interact in the pathology of SLE. T cells in SLE have lower thresholds for activation and experience impaired signaling at their T cell receptor. This leads to the over- and under-expression of various genes which have the potential to increase inflammation (Kyttaris, 2007). Another effect of dysfunctional T cells in SLE is the upregulated production of IgG antibodies by autoreactive B cells (Crispin, 2008). The production of anti-double-stranded DNA antibodies that complex to the patient’s own DNA leads to a deleterious effect on organs and tissues. (Crispin, 2010) Anti-double stranded DNA complexes were found to localize in glomerular basement membranes, suggesting that they may be targeted by complement and lead to lupus nephritis (Van Bruggen, 1997). Defective apoptotic signaling and impaired clearance of necrotic cellular debris in SLE also contributes to immune autoreactivity. Increased exposure to autoantigens diminishes self-tolerance by the immune system and can lead to increased autoreactivity and inflammation (Munoz, 2005).

**Atherosclerosis in Systemic Lupus Erythematosus**

Female patients with SLE have up to a 50-fold higher risk of myocardial infarction than those in comparable populations without SLE (Manzi, 1997). Since the 1970s, survival rates for SLE patients have improved with the notable
exception of deaths due to cardiovascular disease, which has increased over that time (Pons-Estel, 2010). The accelerated progression of atherosclerotic disease in SLE patients, independent of traditional atherosclerotic risk factors except aging, has been identified as the main cause of increased risk of cardiovascular disease events (Roman, 2007). Renal disease has been marked as a potential risk factor for atherosclerosis in SLE patients (Sule, 2011). An association has been identified between SLE disease severity and the extent of plaque development and coronary artery disease (McMahon, 2014). Therefore, the mechanism by which SLE contributes to atherosclerosis is unclear, but it is likely that many of the autoimmune and inflammatory mediators contributing to atherosclerosis may also be involved in SLE.

Mouse models generated by crossing atherosclerosis-prone phenotypes with autoimmune-prone phenotypes have demonstrated that the combination of disease states exacerbates the severity of both. Some of these phenotypes include gld.aapoE^{-/-}, LDLr^{-/-}apoA-I^{-/-}, and Sle.16/LDLr^{-/-} mice (Aprahamian, 2004; Wilhelm, 2009; Lewis, 2012). Of the several proposed components to mechanism related to this thesis, it is possible that the autoimmune and inflammatory environment established by SLE may contribute to proatherogenic processes, potentially by increasing the propensity for endothelial activation or injury. Activation of endothelial cell expression of adhesion molecules has been observed in serum samples of SLE patients with active disease (Janssen, 1994). In addition, a study of otherwise healthy patients post-myocardial infarction
demonstrated increased circulating apoptotic particles compared to patients with non-coronary heart disease (Mallat, 2000).

Other players in SLE autoreactivity have also been investigated in relation to atherosclerosis. High-density lipoprotein (HDL) performs reverse transport of cholesterol throughout the body. In addition to its ability to clear cholesterol, HDL is thought to exert an atheroprotective effect by blocking the oxidation of LDL. It putatively accomplishes this through its antioxidative enzyme paraoxanase, which has lowered activity in SLE patient populations (Alves, 2003). Anti-HDL antibodies have been observed in lupus patients and may represent a pathway by which SLE leads to increased oxidation of LDL and uptake by monocytes (O’Neill, 2010). In addition, states of chronic inflammation can induce HDL to switch to assuming a proatherogenic function and oxidize LDL (Lenten, 1995).

Although anti-oxLDL antibody blood levels also rise with SLE disease activity, a consensus has not been reached on their direct contribution to atherosclerosis (Vaarala, 2000). Along with anti-oxLDL antibodies, several other SLE antibodies including anti-phospholipid antibodies and anti-endothelial cell antibodies are considered markers of atherosclerotic risk but whether they are atheroprotective or atherogenic has not been concluded (Narshi, 2010).

**Objectives**

Our study set out to assess the impact of immunization with the apoB p210 peptide in murine models of SLE (gld), SLE with associated atherosclerosis
(gld.apoE\(^{-/-}\)), and atherosclerosis (apoE\(^{-/-}\)) as a control. While apoB p210 has been extensively studied in atherosclerosis progression in hyperlipidemic animal models, it has not been explored in the context of lupus or lupus-associated atherosclerosis. It is important to note that immunization of the general populace would not be advisable. However, immunization to ameliorate or limit atherosclerosis in a specialized patient population with accelerated cardiovascular disease risk such as lupus is a treatment worth exploring. It is also possible that beneficial effects on lupus disease could occur secondary to the modulation of the immune response to atherosclerosis. We hypothesized that treatment with the apoB peptide-based vaccine would lessen autoimmune mediated damage. This thesis utilized colorimetric assays to assess the impact of apoB p210 immunization on total serum levels of both cholesterol and triglyceride. ELISA tests for IgM and IgG measured the antibody response to immunization over time. Finally, the effect of immunization on SLE disease severity was assessed through histological observation of kidney health.
METHODS

Animals

ApoE\textsuperscript{-/-} mice with an atherosclerotic phenotype and *gld* mice with a lupus-like phenotype were purchased from Jackson Laboratory. ApoE\textsuperscript{-/-} and *gld* mice were crossed at Boston University School of Medicine to breed *gld*.apoE\textsuperscript{-/-} mice harboring a phenotype of accelerated atherosclerosis and lupus. At 7 weeks of age, mice were administered a primary immunization of 200\(\mu\)L apoB-100 peptide by subcutaneous injection to the dorsal area. Booster shots were subsequently administered at 10 and 12 weeks of age. Control groups were given either PBS or Alum (adjuvant) injections. *Gld* mice were fed a normal chow diet. apoE\textsuperscript{-/-} mice were fed a high cholesterol Western diet (TD 88137, Harlan-Teklad, 0.20% cholesterol, 21% fat). *Gld*.apoE\textsuperscript{-/-} mice received a normal chow diet for the duration of the study or were switched to a high cholesterol Western diet at 13 weeks of age. Small blood samples were collected by tail vein at the midpoint of the study, and all mice were sacrificed at 25 weeks of age. All mouse experiments were performed under protocol approved by the Institutional Animal Care and Use Committee of Boston University School of Medicine.

**Serum Cholesterol and Triglyceride Assays**

Serum cholesterol was quantified by colorimetric assay. Serum samples were diluted 1:5 or 1:10 with PBS. Serial dilutions of Wako cholesterol standard were used to establish a standard concentration curve.
reagent was added to the wells to create an optically active compound. Wells were then heated at 37° C for 5 minutes before being assayed in a Molecular Devices Spectramax M3 using Softmax Pro 6.3 software. Absorbance was measured at 600nm against a background of 700nm. All samples were run in duplicate.

Serum triglyceride levels were assayed in a similar manner as described for the serum cholesterol assay. Infinity triglyceride standard and Infinity reagent were used and the calculation of absorbance was at 500nm against a 600nm background.

Cholesterol and triglyceride absorbance data from Softmax Pro 6.3 were copied into Microsoft Excel. Background absorbance values were subtracted from the active absorbance wavelengths. The duplicate well sample values were averaged and the values of the standard series for each plate were graphed on a scatter plot to calculate a line of best fit. Using the trend line equation of the standard curve, concentration values of each sample were calculated. Sample concentration values obtained in this way were multiplied by the original dilution ratio to return results in units of mg/dL.

**Antibody Assays Following Immunization**

ApoB-100 was diluted with PBS, added to wells, and incubated at 4° C overnight to coat the wells. The plate was washed with PBS-Tween in between steps. Goat serum was used for blocking. Plasma serum samples were added
and incubated for 2 hours. Either goat anti-mouse IgM Ig chain biotinylated antibodies (Jackson) or goat anti-mouse IgG Fc fragment biotinylated (Jackson) were added as detection antibodies. Streptavidin conjugated with alkaline phosphatase was added to bind to the antibodies. P-nitrophenyl phosphate was used as a substrate and NaOH was used to stop the reaction. Absorption was measured at 405nm against a 570nm background.

**Kidney Histology**

Kidneys were harvested from the mice at the time of sacrifice, bisected, stored in tissue cassettes, and fixed in 10% neutral-buffered formalin for at least 24 hours. Kidneys were dehydrated by immersion in a series of ethanol solutions. Ethanol was cleared from the tissues by processing in a series of xylene solutions. Tissues were heated at 58°C and infiltrated with TissuePrep paraffin wax. A Sakura Tissue-Tek embedding machine was used to embed kidney tissues in molten paraffin wax that was then cooled into blocks. A Leica RM2135 microtome was used to cut the blocks into 6 µM sections mounted on ColorFrost Plus charged slides. Slide sections were heated at 58°C, washed in a series of xylene and ethanol to remove paraffin, and then stained using hematoxylin and eosin.

Photomicrographs were captured at 40x objective magnification using an Olympus BX41 microscope and MagnaFire-SP software. Image analysis was performed using Adobe Photoshop CS5 on Windows 7 and Adobe Photoshop
CS6 on Mac OSX. The glomerular tuft size was assessed, (n=30) per mouse, by use of the pixel selection and image analysis tools in Adobe Photoshop. Slides were analyzed by a researcher blinded to their identity.

**Statistical Analyses**

Results are shown as the mean ± SEM. A Student’s T-Test was used to compare the different treatment groups. Results with P < 0.05 were considered statistically significant.
RESULTS

Serum Cholesterol Concentrations Were Calculated from the Absorbance of a Cholesterol Standard Assayed Simultaneously with Samples

Serial dilutions of cholesterol standard were created to establish a dilution curve (Table 1 and Figure 1). Absorbance values from these standards were plotted and the trend line equation was used to calculate the cholesterol concentration in the serum samples.

Table 1: Cholesterol standard concentrations

<table>
<thead>
<tr>
<th>Cholesterol Standards Plate 1</th>
<th>Concentration (mg/dL)</th>
<th>Assay 1</th>
<th>Assay 2</th>
<th>Average</th>
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<tbody>
<tr>
<td></td>
<td>200</td>
<td>0.71</td>
<td>0.83</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.42</td>
<td>0.41</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.21</td>
<td>0.19</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.11</td>
<td>0.11</td>
<td>0.11</td>
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<tr>
<td></td>
<td>12.5</td>
<td>0.06</td>
<td>0.05</td>
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<tr>
<td></td>
<td>6.25</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>3.125</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Figure 1: Cholesterol standard curve
Treatment with apoB-100 Peptide Vaccine Does Not Affect Serum Cholesterol

A statistically significant difference of serum triglyceride levels was observed in the $gld$.apoE$^{-/-}$ mice between the Alum and the apoB-100 treatment groups. Outside of this finding, neither serum cholesterol nor serum triglyceride levels showed statistically significant differences between treatment groups in apoE$^{-/-}$, $gld$, or $gld$.apoE$^{-/-}$ mice (Table 2 and Figure 2). The hypercholesterolemic apoE$^{-/-}$ phenotype had roughly an order of magnitude higher average serum cholesterol than the $gld$ or the $gld$.apoE$^{-/-}$ mice. $Gld$.apoE$^{-/-}$ mice cholesterol was moderately higher than $gld$ mice as expected.

Within the apoE$^{-/-}$ treatment groups, Alum had the highest cholesterol concentration and the PBS control group had the lowest. In $gld$ mice, Alum and apoB-100 treated mice had similar levels of cholesterol with the PBS group displaying slightly higher levels. The $gld$.apoE$^{-/-}$ apoB-100 treatment group had the highest cholesterol, the alum had the lowest, and the PBS group was between the other two groups. Taken together, while it appears that apoB-100 does not have an effect on apoE$^{-/-}$ or $gld$ mice, the effect on $gld$.apoE$^{-/-}$ could be further delineated in a future study.
Table 2: Serum cholesterol

<table>
<thead>
<tr>
<th>Phenotype/Treatment Group</th>
<th>Serum Cholesterol (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoE−/− PBS</td>
<td>766.8 ± 26.4</td>
</tr>
<tr>
<td>apoE−/− Alum</td>
<td>833.9 ± 33.5</td>
</tr>
<tr>
<td>apoE−/− apoB-100</td>
<td>811.4 ± 24.4</td>
</tr>
<tr>
<td>gld PBS</td>
<td>79.9 ± 20.7</td>
</tr>
<tr>
<td>gld Alum</td>
<td>73.4 ± 11.7</td>
</tr>
<tr>
<td>gld apoB-100</td>
<td>72.3 ± 10.9</td>
</tr>
<tr>
<td>gld apoE−/− PBS</td>
<td>119.2 ± 4.8</td>
</tr>
<tr>
<td>gld apoE−/− Alum</td>
<td>111.1 ± 6.4</td>
</tr>
<tr>
<td>gld apoE−/− apoB-100</td>
<td>133.2 ± 9.1</td>
</tr>
</tbody>
</table>

Figure 2: Serum cholesterol concentration does not change with apoB-100 immunization. Total cholesterol levels were measured by colorimetric assay from serum obtained at study endpoint for (A) apoE−/− (n=19, 19, 20), (B) gld (n=20, 19, 20), and (C) gld.apoE−/− mice (n=15, n=18, n=18) for PBS, Alum, and apoB-100 respectively.
Immunization with apoB-100 Peptide Does Not Affect Serum Triglyceride Levels

The plates used to assay serum triglyceride had serial dilutions of triglyceride standard arranged in a similar fashion as the cholesterol plates. From the standards the concentration of triglyceride in the serum samples was calculated.

ApoE−/− mouse triglyceride levels were about double those of gld mice (Table 3 and Figure 3). The triglyceride concentration of ApoE−/− mice was tightly clustered among the treatment groups, whereas gld mice receiving Alum were slightly higher compared to PBS and apoB-100. Gld.apoE−/− triglyceride averages for the PBS and alum groups were centered halfway between the apoE−/− and gld mice, with the exception of the apoB-100 treatment group, which was close to the levels of the apoE−/− mice. The increase in serum triglyceride levels in gld.apoE−/− mice after immunization was significant compared to Alum treatment (p=0.03) but not when compared to PBS treatment (p=0.11).
### Table 3: Serum triglyceride concentrations

<table>
<thead>
<tr>
<th>Phenotype/Treatment Groups</th>
<th>Serum Triglyceride (mg/dL)</th>
</tr>
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<tbody>
<tr>
<td>apoE&lt;sup&gt;−/−&lt;/sup&gt; PBS</td>
<td>145 ± 20.7</td>
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<td>apoE&lt;sup&gt;−/−&lt;/sup&gt; Alum</td>
<td>141 ± 27.0</td>
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<td>138.5 ± 21.7</td>
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<td>gld Alum</td>
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<td>gld CVX</td>
<td>64.8 ± 19.8</td>
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<td>99.1 ± 5.9</td>
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<td>89.2 ± 6.7 *</td>
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<td>133.6 ± 11.6 *</td>
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* P=0.03 for gld.apoE<sup>−/−</sup> Alum vs. gld.apoE<sup>−/−</sup> apoB-100
Figure 3: Serum triglyceride levels trend towards increase in *gld.a*poE^−/− mice after apoB-100 immunization. Total triglyceride levels (mg/dL) were measured by colorimetric assay from serum obtained at study endpoint for (A) apoE^−/− (n=19, 19, 20), (B) gld (n=20, 19, 20), and (C) gld.a*poE^−/− mice (n=15, 18, 18) for PBS, Alum, and apoB-100 respectively.
Initial Antibody Response to Immunization with apoB-100 Diminishes Over Time

Across phenotypes and treatment groups, antibody titers were significantly higher at the midpoints than at the endpoints. This might be expected, since the midpoint samples were obtained closer to the time of immunization (Table 4). Within specific phenotypes, differences between treatment groups were not statistically significant. At the time of this thesis the gld apoE\textsuperscript{−/−} cohort had not been completed and their antibody response was not able to be included.

Table 4: apoB-100 ELISA performed at midpoint and endpoint

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Glomerular Tuft Size as a Measure of Autoimmune Renal Disease

Renal disease is a common consequence of lupus pathogenesis. By examining morphologic changes in the kidney in an autoimmune model, the extent of autoimmune disease can be assessed. Increased glomerular tuft size is indicative of more severe renal disease in the *gld* and *gld*apoE−/− autoimmune phenotypes.

Among *gld* mice, there was no significant difference in glomerular tuft size regardless of treatment. Average glomerular tuft area was 28,470 ± 1585; 25,514 ± 1373; and 28,391 ± 1664 pixels for PBS, Alum, and apoB-100 treatment groups, respectively (Figure 4). The lack of a statistically significant difference between treatment groups and controls in this study suggests that treatment with apoB-100 neither improved nor worsened autoimmune-related renal disease in these mice.
Representative photomicrographs of H&E stained kidney sections from gld mice receiving subcutaneous injections of (A) PBS (n=20), (B) Alum (n=19), or (C) apoB-100 (n=20). 400x magnification.

Among gld.apoE⁻/⁻ mice, Alum treatment to mice receiving either normal chow trended towards an increase in glomerular tuft size (Figure 5). Gld.apoE⁻/⁻ mice fed a normal diet had an average glomerular tuft area of 36713.5 ± 1746; 40,095 ± 2263; and 37,092 ± 1912 pixels, for PBS, Alum, and apoB-100 treatment respectively. Western diet groups in general had larger tuft areas with the exception of the apoB-100 treatment group, which displayed a smaller average tuft area when fed Western diet. Gld.apoE⁻/⁻ mice fed a Western diet had an average glomerular tuft area of 40,574± 1536; 44,933 ± 2004; and 35,482 ± 1909 pixels, for PBS, Alum, and apoB-100 treatment respectively. None of the differences between these groups were statistically significant.

Overall, average glomerular tuft sizes reflected the severity of autoimmune disease in each phenotype, with gld.apoE⁻/⁻ tufts being larger on average than gld
tufts, but no statistically significant differences were observed among treatment
groups within phenotypes.

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Fig 5: Glomerular tuft size of gld.aapoE<sup>-/-</sup> mice maintained on normal chow or Western diet. Arrows indicate glomerular tufts. Representative photomicrographs of H&E stained kidney sections from gld.aapoE<sup>-/-</sup> mice maintained on (A-C) normal chow or (D-F) Western diet, and receiving subcutaneous injections of PBS (A,D; n= 8, 4), Alum (B,E; n= 10, 5), or apoB-100 (C,F; n= 9, 6). 400x magnification
DISCUSSION

ApoB-100 Immunization Did Not Adversely Affect *gld* or *gld.apoE<sup>−/−</sup>* Mice

In toto, the procedures performed during this study indicated that apoB-100 immunization neither improved nor worsened autoimmune sequela. The antibody response to immunization was gauged via ELISA for specific IgG and IgM antibodies. Serum cholesterol and triglyceride absorbance did not show significant differences between treatment groups, with the single exception of *gld.apoE<sup>−/−</sup>* Alum and apoB-100 treatment groups. Autoimmune disease severity was examined through measurement of glomerular tuft size, and no significant differences were found between treatment groups. The paucity of statistically significant changes either in a positive or a negative direction following immunization suggest that the modulation of the immune response using apoB-100 has neither a beneficial nor an adverse effect in the *gld* or *gld.apoE<sup>−/−</sup>* models. This is promising insofar as it does not invalidate the possibility of utilizing such a vaccination therapy in a lupus setting.

The observation of lower serum cholesterol in *gld.apoE<sup>−/−</sup>* mice compared to *apoE<sup>−/−</sup>* mice reflects results from previous papers using the phenotypes (Aprahamian, 2004) (Aprahamian, 2006). The lack of statistically significant differences between serum cholesterol and serum triglyceride in *apoE<sup>−/−</sup>* is in line with previous studies investigating apoB-100 immunization (Chyu, 2005) (Wigren, 2011). In the 2005 study, both the highest serum cholesterol and the
greatest reduction in atherosclerotic disease were seen in the apoE<sup>−/−</sup> apoB-100 treatment group.

Of note, the cholesterol and triglyceride values for the gld mice were not significantly different between treatment groups. It is a viable concern that immunization could have an adverse effect on serum levels of either in the autoimmune model. Taking into consideration that oxLDL is elevated in SLE patients, this is a promising indicator for the tolerance of this particular immunization in an autoimmune environment. Similarly, as increased glomerular tuft size is indicative of more severe renal disease in the gld and gld.apoE<sup>−/−</sup> autoimmune phenotypes, the lack of a statistically significant difference between treatment groups and controls suggests that treatment with apoB-100 neither improved nor worsened autoimmune-related renal disease in these mice. Taken together, these findings do not demonstrate immediately obvious problems with apoB-100 immunization in a lupus model, which leaves the door open for further exploration of its applicability for SLE patients.

**Potential Consequences of Anti-Atherosclerosis Immunization in SLE Patients**

One potentially complicating aspect of the prospective immunization using apoB-100 related peptide in the context of an SLE disease background is the preexisting presence of anti-oxLDL antibodies in SLE patients. OxLDL levels are elevated in SLE patients and are associated with cardiovascular and renal
disease (Frostegård, 2005). SLE patients also have elevated levels of oxLDL-β2GPI complexes (β2GPI binds to oxLDL but not its non-oxidized form) and the IgG antibodies against these complexes have been suggested to contribute to atherosclerotic risk in SLE (Lopez, 2003).

Whether the role of anti-oxLDL antibodies is atherogenic or atheroprotective is incompletely understood. Studies of SLE patients examining the relationship between anti-oxLDL antibody levels and carotid intima-media thickness (cIMT) have been further complicated by the multifarious types of oxLDL. Antibodies binding to MDA-LDL are inversely correlated between IgM antibodies to MDA-LDL and cIMT (Karvonen, 2003). Further research has shown that antibodies binding to oxLDL-β2GPI-complexes are positively correlated between cIMT and IgM and IgG anti-oxLDL-β2-GPI antibodies (Nowak, 2012). That SLE patients have higher levels of IgG antibodies to the oxLDL-β2-GPI complexes than healthy controls (Lopez, 2003) speaks to the complications and risks that lupus poses to the prospect of immunomodulatory treatment. It is conceivable that immunization with apoB-100 could be well tolerated by the general population yet some antibody-mediated aspect of its mechanism of action might hold risks unique to SLE patients.

Regulatory T Cells as Potential Mechanism of Action of apoB Peptide Based Vaccination
The observation that immunization with an apoB p210 peptide led to a reduction of atherosclerosis without mounting an antibody response (Fredrikson, 2008) prompted further exploration of other components of the immune system that could potentially be involved in its immunomodulatory mechanism. One such component is the regulatory T cell (Treg) population. Specifically, Treg cells are FoxP3+ CD4+ CD25+ T cells, with the expression of FoxP3+ transcription factor delineating them from CD4+ CD25+ effector T cells. T cell autoreactivity is necessary for the body to combat pathogens that display similarity to self-antigens, but this must be balanced. Control over the extent of self-reactivity is exerted at several stages of T cell maturation including during development in the thymus where they are tested against MHC and self-antigens. This selects for T cells with a “goldilocks” amount of self-recognition (not too much, not too little) but is not an error-proof process. After maturation and release from the thymus into the periphery, T cells are subject to downregulation by Treg cells (Sakaguchi, 2000).

Regarding their potential atheroprotective role, depletion of Treg cells in mice was shown to worsen atherosclerotic lesions (Ait-Oufella, 2006). Deletion of FoxP3+ also worsens atherosclerosis as well as hypercholesterolemia (Klingenberg, Gerdes, 2013). In contrast, Treg cells transferred into mice lessened the development of atherosclerotic lesions (Mallat, 2003). There are also some incipient findings that Treg cells might have analogous functionality in humans. Human patients with acute coronary syndrome were found to have
markedly decreased levels of FoxP3+ Tregs (Cheng, 2008). Tissue samples from patients undergoing vascular surgery and at autopsy showed that FoxP3+ cells were less prevalent at sites of atherosclerotic lesions (De Boer, 2007). However, no clinical studies have linked Tregs to future atherosclerotic risk, making the results of the animal studies promising yet not conclusive.

In a study investigating the possible involvement of Tregs in the apoB p210 vaccine mechanism, the T cell population of immunized mice had a higher proportion of CD4+ spleen cells expressing FoxP3+ than control mice (Wigren, 2011). Immunized mice were also found to have higher levels of IL-10 (an anti-inflammatory cytokine) as well as a lower proportion of CD25+ effector cells. Crucially, the administration of CD25+ antibodies lowered the FoxP3+ count and reversed the atheroprotective effects of immunization in mice treated with apoB p210. (The possibility that a CD25+ effector response was responsible for the efficacy of apoB p210, and that this was inhibited by the antibodies, was discounted by the authors on the basis of multiple prior studies unambiguously identifying Th1 CD25+ cells as proatherogenic.) That the administration of CD25+ antibodies also lowered IL-10 levels raises the possibility that the secretion of that cytokine may be part of the mechanism by which FoxP3+ modulates the immune response in atherosclerosis. An increase in FoxP3+ cells in lymph nodes, as well as reductions in atherosclerosis, proatherogenic T cells and their cytokines, was observed in response to apoB p210 as well as other apoB related peptides administered via continuous subcutaneous delivery (Herbin 2012).
Dendritic Cells as Potential Mechanism of Action of apoB Peptide Based Vaccination

Dendritic cells (DCs) are involved in both the innate and adaptive immune systems. In their capacity as agents of the adaptive immune system, DCs are responsible for presenting antigens to T cells. They permeate the body in “immature” form, unable to activate T cells. After taking up antigens and forming a MHC complex, they travel to lymph nodes and the spleen where they complete the maturation process. Once there, DCs are able to influence T cells to launch immune responses or to maintain self-tolerance. When upregulating an immune response, DCs direct the expansion of Th1 populations as well as natural killer T cells (Maldonado-López, 1999) (Homann, 2002). In addition, DCs upregulate transforming growth factor beta (TGF-β) and IL-10 to induce T cell tolerance, (Akbari, 2002). The control that dendritic cells wield over the fate of T cells has made them a target of investigation in immunomodulatory therapy.

Treatment of DCs with the immunosuppressive cytokine IL-10 has shown promise in terms of altering the immune response of treated animals. Pulsing DCs with IL-10 in vitro was found to reduce several inflammatory cytokines, including IFN-γ, IL-4, and IL-5 (Bellinghausen, 2001). Susceptibility to IL-10 treatment is specific only to immature DCs (Jonuleit, 2000). Intriguingly, a similar pulsing technique with DCs using TGF-β was able to upregulate FoxP3+ Treg cells (Luo, 2007).
Narrowing the focus to the treatment of atherosclerosis, DCs specialized to the arterial intima and belonging to the overall immunological milieu of T cells and other monocytes have been identified (Waltner-Romen, 1998). Atherosclerosis-prone regions of the intima were found to have more DCs than atherosclerosis-resistant areas (Lord, 1999). The introduction of DCs pulsed with MDA-LDL into apoE−/− mice worsened atherosclerotic disease, implying that their response to oxLDL may help mediate the inflammatory response in atherosclerosis (Hjerpe, 2010).

Given the studies elucidating the changes apoB-100 immunization makes on T cell populations and considering the role DCs play on T cell fate, investigating DCs in relation to the mechanism of apoB-100 could be fruitful. A recent study exploring the use of DCs to deliver apoB-100 p210 peptide minus any adjuvant found some differences in immune response compared to prior studies. Injected DCs loaded with various forms of apoB-100 peptide were tracked as they migrated to lymph nodes and were found to remain in an immature state, suggesting that these peptides work towards immune tolerance. Further study showed variations in ability to control Treg count, IL-10 release and IgG antibody response. Also, DC loaded with the p45 and p210 forms of apoB-100 led to an IgG antibody response. (Pieride, 2013) While differences between peptides with and without DC loading were observed, there are a host of other factors unrelated to DCs such as the timing of antigen availability that may be responsible for those differences.
Alternative Methods and Limitations

Administration of the Alum adjuvant alone to apoE<sup>−/−</sup> mice has been demonstrated to reduce atherosclerosis (Khallou-Laschet, 2006). However, although increased p210 IgG antibody and Treg level have been measured in apoE<sup>−/−</sup> mice injected with Alum, no similar changes were seen when wild type C57 mice were injected (Wigren, 2011). To further elucidate the specific immune modulation occurring with apoB-100 immunization, methods of administration other than injection could be useful.

One such method recently tested was the intranasal administration of apoB-100 p210 protein combined with cholera toxin (CTB), which contains an antigen useful for respiratory tract uptake of the complex (Klingenberg, Lebens, 2010). Immunization in this manner led to a 35% decrease in atherosclerotic lesion size as compared to ovalbumin complexed to CTB or controls. As a mechanism, the authors suggest that IL-10 secreted by Tregs suppresses T effector cells specific for apoB-100. The two candidate Treg populations suspected were FoxP3<sup>+</sup> induced Treg and Tr1 cells. Tr1 cells are a population of Treg cells distinguished by their pronounced secretion of the anti-inflammatory cytokines IL-10 and TGF-β as well as their maturation outside the thymus in an IL-10-dependent process (Roncarolo, 2006). FoxP3<sup>+</sup> Treg cells were ruled out as the atheroprotective agent because of the reliance of FoxP3<sup>+</sup> on TGF-β signaling. Even when researchers eliminated TGF-β signaling, the atheroprotective effect of
immunization was maintained, although they note this does not exclude FoxP3+ cells from playing a part in activating Tr1 cells.

The wealth of information from animal studies investigating the relationship between oxLDL and atherosclerosis has made strides in establishing how oxidatively modified LDL and antibodies against it contribute to atherosclerotic disease. However, a clear understanding of the specific mechanism by which oxLDL contributes to atherosclerosis in humans remains elusive. Animal and in vivo studies have illustrated several pathways by which oxLDL can drive towards a pathogenic state, posing the challenge of identifying which are active in the progression of human disease. The incomplete understanding is partially due to uncertainty over the extent of homology in oxidative modifications to LDL between animals and humans. Previous studies on the apoB-100 vaccine have found that the antibodies generated against the p210 peptide do not cross react with human native or modified LDL (Chyu, 2005). This reflects the uncertainty about which specific modified LDL epitope generates an immune response in the natural course of the disease.

Further uncertainty stems from the genetic diversity inherent in the immune responses to modified LDL, first observed in animals and subsequently explored in humans (Shi, 2000) (Gargalovic, 2006). Over a thousand genes expressed by endothelial cells in humans have been found to be upregulated by oxLDL. Systems genetics testing of the endothelial cells of heart transplant donors revealed widely differential sensitivity among individuals to the effects of
oxidation by one particular modified LDL epitope (Romanoski, 2011). The range of genetic variation in immune response to just one epitope underscores the amount of work needed to untangle the atherosclerotic immune response pathway, especially in the context of attempting to modulate the pathway through immunization.

In conclusion, although the hypothesized reduction in autoimmune disease severity in immunized mice was not observed in this study, neither was there an observed worsening in autoimmune disease severity. The characteristics of the mouse phenotypes tested, namely hypercholesterolemia and lupus-like autoimmunity, were not significantly impacted by the immunization therapy. Assessments of atherosclerotic disease markers were not performed, but if similar reductions in atherosclerotic disease progression were seen after immunization in these models as seen in prior studies, it would show promise for the potential future treatment of atherosclerotic disease in SLE patients. Clinical efficacy of immunization against atherosclerosis is yet to be tested. If immunization is found to be clinically feasible, it may represent a novel treatment for atherosclerosis and cardiovascular disease in SLE patients.
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REFERENCES


CURRICULUM VITAE

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• Thesis title: Effects of apoB-Derived Peptide Vaccination in a Murine Model of Systemic Lupus Erythematos

• Responsible for serum analysis by ELISA, and histological data collection throughout the processes of tissue collection, fixation, embedding, operation of microtomes and cryostats, slide staining, and image analysis
Palomar Health - Escondido, CA 2011–2013

Clinical scribe, Emergency Department

• Assisted emergency medicine physicians throughout the entirety of their shifts while documenting clinical encounters

• Managed and created electronic medical records for emergency department patients including medical histories, physical exam findings, imaging studies, diagnostic workup, medications administered, and procedures performed

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Volunteer - Rosie’s Place – Boston, MA 2014 – Present

• Serve food and distributes food pantry items at Rosie’s Place, a shelter for poor and homeless women in Boston

Independent Music Production 2008 – Present

• Combines a background in piano and guitar playing with an aptitude for computers to compose and edit original music

SKILLS

Computer Software: Adobe Photoshop, Cerner Powerchart Firstnet, Microsoft Office, Apple Logic