Impaired Clearance of Apoptotic Cells Promotes Synergy between Atherogenesis and Autoimmune Disease

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Abstract
To clarify the link between autoimmune disease and hypercholesterolemia, we created the gld.apoE/H11002/H11408/H11002 mouse as a model of accelerated atherosclerosis. Atherosclerotic lesion area was significantly increased in gld.apoE/H11002/H11408/H11002 mice compared with apoE/H11002/H11408/H11002 mice. gld.apoE/H11002/H11408/H11002 mice also displayed increases in lymphadenopathy, splenomegaly, and autoantibodies compared with gld mice, and these effects were exacerbated by high cholesterol diet. gld.apoE/H11002/H11408/H11002 mice exhibited higher levels of apoptotic cells, yet a reduced frequency of engulfed apoptotic nuclei within macrophages. Infusion of lysophosphatidylcholine, a component of oxidized low density lipoprotein, markedly decreased apoptotic cell clearance in gld mice, indicating that hypercholesterolemia promotes autoimmune disease in this background. These data suggest that defects in apoptotic cell clearance promote synergy between atherosclerotic and autoimmune diseases.

Key words: atherosclerosis • autoimmunity • macrophages • lysophosphatidylcholine • lymphoproliferation

Introduction
Autoimmune disorders are characterized by the loss of tolerance to self-antigen and the consequent production of immunoglobulin G autoantibodies (1). The development of autoimmune disease involves a poorly understood interplay of genetic and environmental factors. Recently, it has been found that failure to degrade chromatin or properly clear apoptotic cells contributes to the development of autoimmunity. Impaired apoptotic debris clearance leads to the development of an autoimmune phenotype in mouse models (2, 3). Similarly, in humans, C1q and DNasel deficiencies are associated with the development of SLE (4, 5), and apoptotic bodies and circulating DNA fragments are found in the sera of patients with SLE (6, 7). Furthermore, macrophages from patients with SLE display abnormal morphology and are impaired in their ability to clear apoptotic bodies (8).

Inflammatory cell activity plays an important role in the progression of atherosclerosis. Atherosclerotic lesions are initiated by injury to the endothelium by oxidized low density lipoprotein (oxLDL), inflammatory cytokines, and immune complexes, and its progression involves inflammatory cell interactions with the endothelium and extravasation into the subendothelial space (9). Several lines of genetic evidence in apolipoprotein E–deficient (apoE/H11002/H11408/H11002) or low density lipoprotein receptor-deficient mice suggest that both lymphocytes and macrophages contribute to the early and late stages of atherosclerotic lesion formation (10). Patients with autoimmune diseases, including SLE and rheumatoid arthritis, exhibit a higher incidence of atherosclerosis and are at significant risk of premature cardiovascular disease (11–15). There is considerable controversy regarding the causes of atherosclerosis in patients with autoimmune disease, although chronic inflammation is likely to be a contributing factor. Progress in understanding the mechanisms of accelerated atherosclerosis aso-

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Abbreviations used in this paper: aCL, anticardiolipin antibody; ANA, antinuclear antibody; LDL, low density lipoprotein; LPC, lysophosphatidylcholine; oxLDL, oxidized LDL; TUNEL, TdT-mediated dUTP nick-end labeling; VLDL, very LDL.
associated with autoimmune disease has been impaired by the lack of an appropriate animal model.

Fas is a receptor that initiates an apoptotic signal when bound to its ligand, FasL (16). Fas-mediated apoptosis functions in part to control peripheral lymphoid responses (17) and macrophage survival (18, 19). In humans, diminished Fas-mediated apoptosis gives rise to Canale-Smith syndrome, a disease associated with lymphoproliferation and autoimmunity (20–22). The gld and lpr mouse strains have inactivating mutations in FasL and Fas, respectively, and they suffer from lupuslike autoimmune disorders with autoantibody specificities similar to those seen with the human disease (23–26). In addition, they exhibit lymphadenopathy and splenomegaly. These mice typically do not exhibit signs of atherosclerosis, but they are susceptible to atherosclerotic lesion formation when fed an atherogenic diet containing cholic acid (27).

To develop a better understanding of the link between atherogenesis and autoimmune disease, we generated an atherosclerotic mouse model that lacks functional FasL by crossing apoE−/− with gld mice. The gld apoE−/− mice displayed enhanced atherosclerosis compared with apoE−/− mice reminiscent of accelerated atherosclerosis seen in patients with autoimmune disease. These mice also exhibited increased lymphoproliferation and autoimmunity compared with gld mice, and this phenotype was enhanced by a high-fat diet. The gld apoE−/− mice had high levels of apoptotic material both in tissues and in the circulation. This was due, at least in part, to an impaired ability to scavenge apoptotic debris, suggesting that synergism between atherosclerosis and autoimmune disease can be mediated by impaired apoptotic body clearance.

Materials and Methods

Generation of Mice and Study Protocol. apoE−/− and gld mice were obtained from Jackson ImmunoResearch Laboratories on the C57BL/6J background. The gld mutation is a point mutation in the FasL gene that occurred spontaneously in the C3H/HeJ strain (24). Fully congenic C57/B6−/− and BALB/c−/− derived progeny were genotyped by sequence analysis. A 437-bp PCR fragment was generated with primers GLDF1, 5′-AACC-CCCCACTCAAGGTCATCCCCCTG-3′, and GLDR0, 5′-ATTATTCGTGGTGCATGAT-3′. The PCR products were labeled using the PE BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), and sequenced. The area of interest was ~100 bp downstream from the start of the amplified fragment. In this region, the sequence 5′-CTTTTTTT-3′ indicated the wild-type FasL allele, whereas 5′-CCTTTTTT-3′ indicated gld and 5′-CC(C/T)TTTTTTTT-3′ indicated a heterozygote (unpublished data).

Quantitative Analyses of Atherosclerosis, Hyperlipidemia, Splenomegaly, and Lymphadenopathy. After 12 wk on a Western or normal diet, food was removed for an 8-h fast. After the fast, the mice were weighed and sacrificed. Blood was drawn by cardiac puncture for determination of total plasma cholesterol and triglyceride levels (Excell Labs). Heart, spleen, and submandibular lymph nodes were excised and weighed. The vasculature was perfused intracardially with 0.9% sodium chloride, and the aorta was isolated from the aortic arch to the iliac bifurcation. The adventitia was thoroughly stripped, and the aorta opened longitudinally, pinned to a white silicone gel, and fixed in 10% neutral buffered formalin for 24 h. After fixation, aortae were rinsed with PBS, stained with Oil red O solution, and destained in 60% isopropyl alcohol. Aortae were photographed using an Olympus digital camera, and lesion size was measured using a National Institutes of Health image program on a Macintosh computer. Because there was no significant difference in lesion area between males and females (unpublished data), datasets for both sexes were combined.

Cell Isolation and Flow Cytometry. Lymph nodes were harvested, minced, and put through a 70-μm cell strainer. Cells were washed in 2% FBS/PBS and stained for 15 min at 4°C. Flow cytometry analysis was performed using fluorescence-labeled monoclonal antibodies against the following: CD3-CyC, CD4-FITC, CD8-PE (BD Biosciences), or TdT-mediated dUTP nick-end labeling (TUNEL; Roche Diagnostics Corp.).

Immunohistochemistry. Aortae were fixed using 10% neutral buffered formalin and paraffin embedded. 6-μm sections of aorta were cut from the aortic arch to the thoracic aorta. Slides were deparaffinized, treated with 3% hydrogen peroxide for 30 min, and blocked in 10% goat serum using an avidin/biotin block. Samples were incubated overnight at 4°C in rat anti–mouse F4/80 (dilution 1:40; Serotec, Inc.), rat anti–mouse F4/80 (dilution 1:1,000; BD Biosciences), or mouse IgG as a negative control in 5% goat serum/PBS. After washing, samples were incubated for 30 min in goat anti–rat IgG diluted 1:500 (Caltag). Visualization of immune complexes with streptavidin–horseradish peroxidase and aminoethylcarbazole was followed by a hematoxylin counterstain. Macrophage and T cell quantification were determined by scoring samples from at least eight mice per group, choosing every sixth slide from the aortic arch through the thoracic aorta. Analysis was performed by two investigators who were blinded to the sample identity. Lymph nodes were fixed and sectioned according to the aforementioned protocol. TUNEL staining was performed according to the manufacturer’s directions (Roche Diagnostics Corp.). Quantification of all TUNEL staining was performed by examining six randomly selected fields in each lymph node section by two investigators who were blinded to sample identity. To generate the overlapping merged figures, sequential sections were used for fluorescent staining of macrophages and follicular dendritic cells with anti–mouse F4/80 (Caltag) and anti–mouse CD21 (BD Biosciences). Macrophage...
ingestion of TUNEL-positive apoptotic debris was quantified as described previously (3).

Autoantibodies. Serum levels of antinuclear antibodies (ANAs) were measured by immunofluorescence using Hep-2–coated slides (The Binding Site Inc.). Slides were incubated for 20 min with serial dilutions (1:40 to 1:2,560) of mouse serum in PBS, washed in PBS, and incubated with FITC-labeled goat anti-mouse IgG (whole molecule; Sigma-Aldrich). Slides were counterstained with Evan’s blue and viewed using fluorescent microscopy. The titer value is defined as the inverse value of the last positive dilution. Anticardiolipin antibodies were measured using a commercially available ELISA assay that detected IgG (Alpha Diagnostic). Values are reported as the mean absorbance. Serum IgG levels were measured using a commercially available ELISA assay (Bethyl Laboratories).

Kidney Analysis. Urine samples were obtained using metabolic cages from 19-wk-old wild-type, apoE−/−, gld, and gld.apoE−/− mice maintained on normal diet. Samples were diluted 1:10 and measured using a protein assay (Bio-Rad Laboratories) at an absorbance of 595 nm. Calculations are shown as milligrams of protein per 24 h. Formalin-fixed kidney sections were stained with hematoxylin and eosin. Stained sections were coded, digitally photographed, and analyzed by an investigator who was blinded to section identity using a stereo microscope (Nikon) fitted with a digital camera (Diagnostic Instruments). Glomerular cross-sectional areas of at least 25 glomeruli from five randomly photographed low-powered fields were measured in each animal using computer-assisted pixel counting (Photopaint 10; Corel Corporation). Mean glomerular tuft area (Aρ) for each animal was calculated from all available glomerular profiles, and tuft volume (Vρ) was calculated as well (28). Vρ = (B/k)(Aρ)^1.3, where B is the shape coefficient for an idealized glomerulus (B = 1.38) and k is a size distribution coefficient (k = 1.10).

Serum Microparticles. Platelet-free plasma was isolated and analyzed as described previously (29). In brief, whole blood was mixed with 0.138 M sodium citrate and centrifuged. After recovery of the plasma, microparticle concentration was determined by capture onto insolubilized annexin V and reported as the nanomolar concentration of phosphatidylserine equivalents.

Intravenous Infusion. 6-mo-old wild-type or gld mice were anesthetized with an intraperitoneal injection of sodium pentobarbital and maintained with supplementary doses as needed. A catheter was inserted into the tail vein for the intravenous infusion of freshly prepared lysophosphatidylcholine (LPC) at rates of 1.5 or 3 pmol/min. Body temperature was maintained by a heating pad. Mice were killed 120 min after the start of infusion. Lymph nodes were harvested, fixed in 10% formalin, and processed for TUNEL staining as described previously.

Results

Lack of FasL in apoE−/− Mice Leads to Greater Atherosclerotic Lesion Area. The aortae of gld.apoE−/− and apoE−/− mice maintained on a 12-wk Western diet demonstrated grossly visible atherosclerotic lesions after Oil red O staining. Lesion area in the apoE−/− mice in this work was in agreement with previously described values (30). Considerably greater lesion area was present in the gld.apoE−/− mice compared with apoE−/− throughout the length of the aorta (Fig. 1 a). Image analysis revealed a threefold increase in plaque area in the aorta of the gld.apoE−/− mice compared with apoE−/− (27.9 ± 2.84 mm² and 9.10 ± 0.9 mm², respectively; P < 0.001) (Fig. 1 c). Lesion area analyses were also performed on age-matched mice that were maintained on normal diet (Fig. 1 b). Again, gld.apoE−/− mice had significantly larger amounts of lesion area when compared with apoE−/−: 6.7 ± 0.5 mm² and 2.1 ± 0.3 mm², respectively (P < 0.001) (Fig. 1 c). In contrast, gld and wild-type mice had virtually no detectable lesions on either Western or normal diet.

Inflammation and Apoptotic Bodies in Vascular Lesions. Histological analysis of aortae of gld and wild-type mice with the macrophage marker F4/80 or the T cell marker CD3 revealed no evidence of macrophage or T cell infiltration (unpublished data), which was consistent with the lack of visible lesions within these vessels. In contrast, the aortae of gld.apoE−/− and apoE−/− displayed evidence of macrophage and T cell extravasation within lesions (Fig. 2, a, b, d, and e). Quantitative analyses revealed approximately threefold more macrophage staining in the vessel cross-sections from the gld.apoE−/− than the apoE−/− mice (Fig. 2 c). Examination of T cells revealed a similar result, showing an increase in T cells within the lesions in the gld.apoE−/− mice relative to the apoE−/− mice (Fig. 2 f). TUNEL immunofluorescence staining of vessel segments revealed apoptotic cells within the atherosclerotic lesions of the apoE−/− mice that can be distinguished from the background autofluorescence of the elastic lamina (Fig. 2 g).

Figure 1. Atherosclerotic lesions in the aortae of gld.apoE−/− and apoE−/− mice are visualized by Oil red O staining. The increase in atherosclerotic lesion area of the gld.apoE−/− compared with the apoE−/−, is macroscopically visible on (a) Western diet, as well as (b) normal diet. The gld and wild-type mice showed no visible lesions (not depicted). (c) Quantification of Oil red O staining area revealed a statistically significant increase in lesion size on Western diet of the gld.apoE−/− (n = 26) compared with the apoE−/− (n = 27), and normal diet gld.apoE−/− (n = 11) compared with the apoE−/− (n = 8) (*, P < 0.001 vs. apoE−/−).
The frequency of TUNEL-positive cells in these lesions was low, consistent with a previous paper (31). In contrast, markedly higher levels of TUNEL-positive fragments were apparent in the vessel wall lesions of the gld-apoE−/− mice (Fig. 2 h). Apoptotic debris typically localized near the luminal surface of the vessel wall, although some positive staining was present within the lesion.

**FasL Deficiency Reduces Cholesterol Levels in apoE-deficient Mice.** Total plasma cholesterol was determined for all groups of mice on both Western diet and normal diet (Fig. 3 a). Although the gld-apoE−/− mice became hypercholesterolemic on Western diet, the extent of hypercholesterolemia was less than that of apoE−/− mice. VLDL levels were lower in gld-apoE−/− mice compared with apoE−/− mice. There was also a trend toward lower cholesterol levels in the gld-apoE−/− relative to the apoE−/− in the mice fed normal diet, but this was not statistically significant. With either diet, the gld mice had lipid plasma levels similar to the wild-type controls. Compared with control mice, apoE−/− mice showed a mild increase in plasma triglyceride levels when fed Western diet and normal diet, consistent with previous papers (30) as did the gld-apoE−/− (unpublished data). Fast performance liquid chromatography on each of the four experimental groups was performed on pooled serum samples. The gld-apoE−/− and apoE−/− had profiles containing higher very low density lipoprotein (VLDL) and low density lipoprotein (LDL) levels, and low high density lipoprotein levels compared with the gld and the wild-type mouse on normal diet and Western diet (unpublished data).

**Lack of apoE Exacerbates gld Phenotype.** At the time of killing, body weight and heart weight showed little or no difference in the four strains of mice (unpublished data). In contrast, spleen and lymph nodes from gld and gld-apoE−/− mice showed remarkable increases in weight and size. Although enlargement of these two organs is a characteristic of the gld phenotype, the spleen and lymph nodes of gld-apoE−/− mice were significantly larger when compared with their gld counterparts (Fig. 4, a and b). Spleen weight of gld-apoE−/− mice increased to almost double that found in wild-type or apoE−/− mice (Fig. 4 c). This trend in spleen size was also observed for these strains when maintained on normal diet: 0.33 ± 0.05 g for gld-apoE−/− and 0.25 ± 0.03 g for gld (P < 0.05). Quantification of the submandibular lymph nodes also revealed a

![Image](image_url)

**Figure 2.** Vessel wall inflammation is increased in gld-apoE−/− mice compared with apoE−/− mice. Representative sections of atherosclerotic lesions from the aortic arch of the gld-apoE−/− and apoE−/− mice, maintained on a Western diet for 12 wk. Specimens were stained with F4/80 for macrophages, or (d and e) CD3 for T cells, represented by the brown staining (arrows). The increase of macrophages and T cells is quantified in c and f, respectively. Macrophages and T cell content were significantly higher in the lesions of gld-apoE−/− mice (n = 11) compared with apoE−/− (n = 10) (P < 0.001 vs. apoE−/−). (g) Representative photomicrograph of TUNEL staining in lesions of apoE−/− mice. (h) Representative photomicrograph of TUNEL staining in lesions of gld-apoE−/− mice showing a greater amount of TUNEL-positive fragments than in the apoE−/− lesions. The (−)TUNEL condition indicates sections processed without the terminal deoxynucleotidyl transferase enzyme to assess background autofluorescence of the elastic lamina.
is due predominantly to the expansion of an unusual T cell proliferation seen in Fas or FasL-deficient mice and humans and the pathogenesis of autoimmunity, and the lympho-tom A high-density lipoprotein fraction located on normal diet.

Likewise, a similar pattern was observed for mice maintained on a high-cholesterol Western diet (Fig. 4 d). A statistically significant increase in weight comparing the gld. apoE−/− mice with the gld: 0.07 ± 0.02 g for wild type versus 0.1 ± 0.002 g for apoE−/−, and 0.40 ± 0.07 g for gld versus 1.05 ± 0.08 g for gld. apoE−/− (P < 0.001) when maintained on a high-cholesterol Western diet (Fig. 4 d). A similar pattern was observed for mice maintained on normal diet.

CD4+ T cells are important for autoantibody production and the pathogenesis of autoimmunity, and the lymphoproliferation seen in Fas or FasL-deficient mice and humans is due predominantly to the expansion of an unusual T cell population that is CD3+, but lacks both CD4 and CD8 (double negative T cells; reference 32). We performed a phenotypic analysis of lymph node T cells from gld and gld. apoE−/− mice to determine whether the enhanced lymphoproliferation and autoimmunity seen in the gld. apoE−/− mice could be accounted for by obvious alterations in the ratio of the different T cell subsets (Table I). No significant differences in percentages of CD4+, CD8+, or double negative CD3+CD4−CD8− T cells were seen. The total number of all T cell subsets was increased in the gld. apoE−/− mice (unpublished data), reflecting their larger lymph node

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**Figure 3.** Lipid profiles of apoE−/− and gld. apoE−/− mice maintained on normal diet and Western diet. (a) Total cholesterol levels were determined in gld. apoE−/− (n = 32), apoE−/− (n = 31), gld (n = 26), and wild type (n = 31) on Western diet and in gld. apoE−/− (n = 11), apoE−/− (n = 8), gld (n = 5), and wild type (n = 5) on normal diet (*, P < 0.001 vs. gld, wild type; †, P < 0.001 vs. apoE−/−). (b) Fast performance liquid chromatography profiles of sera from gld. apoE−/− and apoE−/− mice maintained on normal and Western diet. VLDL, LDL, and high density lipoprotein fraction locations are indicated.

**Figure 4.** Lymphadenopathy and splenomegaly in gld and gld. apoE−/− mice. (a) Representative spleens excised from paired littermates of wild-type, gld, and gld. apoE−/− mice. (b) Representative submandibular lymph nodes (arrows) dissected from paired littermates of wild-type, gld, and gld. apoE−/− mice. Inset shows higher magnification image of lymph node from wild-type mouse. Diagram illustrates dissected area. (c) Spleen weights in the different strains of mice fed normal diet: gld. apoE−/− (n = 11), apoE−/− (n = 8), gld (n = 5), and wild type (n = 5); or Western diet: gld. apoE−/− (n = 26), apoE−/− (n = 31), gld (n = 26), and wild type (n = 31) (*, P < 0.001 vs. gld; †, P < 0.05 vs. gld; ‡, P < 0.001 vs. apoE−/−, wild type). (d) Lymph node weights in the different strains of mice fed normal diet: gld. apoE−/− (n = 11), apoE−/− (n = 8), gld (n = 5), and wild type (n = 5); or Western diet: gld. apoE−/− (n = 26), apoE−/− (n = 31), gld (n = 26), and wild type (n = 31) (*, P < 0.001 vs. gld; †, P < 0.001 vs. apoE−/−, wild type).
size, which was most likely due to an overall enhancement of the autoimmune process in these mice.

ANA titers in gld mice exhibited high titers on normal diet and Western diet (Table II). ANA titers in gld mice on normal diet and Western diet (Table II). The levels of aCL were higher in gld mice than in apoE<sup>-/-</sup> mice and the Western diet led to a further elevation of aCL in gld mice. The gld.<apoE<sup>-/-</sup> mice displayed the highest levels of aCL, which were approximately fourfold higher than that seen in gld mice on either normal or Western diets. The gld.<apoE<sup>-/-</sup> mice also displayed elevated levels of IgG on both normal and Western diet, indicative of a polyclonal B cell activation (Table II).

Because autoimmune disease is frequently associated with renal dysfunction, a histological analysis was performed on the kidneys from the four strains of mice. Kidneys from gld.<apoE<sup>-/-</sup> mice had larger, more cellular glomeruli compared with all other strains (Fig. 5 a). Quantification revealed that, whereas the wild-type, apoE<sup>-/-</sup>, gld mice had glomerular tuft volumes of 1.6 × 10<sup>6</sup> μm<sup>3</sup>, 1.3 × 10<sup>6</sup> μm<sup>3</sup>, and 2.1 × 10<sup>6</sup> μm<sup>3</sup>, respectively, the gld.<apoE<sup>-/-</sup> volumes were significantly increased to 5.0 × 10<sup>6</sup> μm<sup>3</sup> (Fig. 5 b). Correspondingly, proteinuria was observed in the gld.<apoE<sup>-/-</sup> mice (Fig. 5 c), a further indication of renal impairment.

**Table I. Percent T Cell Composition of Lymph Nodes**

<table>
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<tr>
<th></th>
<th>CD4&lt;sup&gt;+&lt;/sup&gt;</th>
<th>CD8&lt;sup&gt;+&lt;/sup&gt;</th>
<th>CD3&lt;sup&gt;+&lt;/sup&gt;CD4&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;-&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>gld</td>
<td>13.0 ± 4.8</td>
<td>5.9 ± 2.1</td>
<td>43.3 ± 9.7</td>
</tr>
<tr>
<td>gld.&lt;apoE&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>16.4 ± 4.1</td>
<td>7.7 ± 3.6</td>
<td>33.5 ± 11.2</td>
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Figure 5. Evidence of renal dysfunction in the gld.<apoE<sup>-/-</sup> mouse. (a) Representative hematoxylin and eosin stained sections of renal tissue showing typical glomeruli in gld.<apoE<sup>-/-</sup> mice compared with wild-type, apoE<sup>-/-</sup>, and gld mice when maintained on Western diet. (top) Magnification, 100. (bottom) Magnification, 400. (b) Quantification of glomerular tuft volume revealed a statistically significant increase in glomerular size in the gld.<apoE<sup>-/-</sup> mice compared with the control groups (*, P < 0.001). (c) The output of urinary protein by gld.<apoE<sup>-/-</sup> mice maintained on normal diet was significantly increased compared with wild type, apoE<sup>-/-</sup>, and gld (*, P < 0.001).
Increased Apoptotic Material and Impaired Clearance in gld, apoE<sup>−/−</sup> Mice. Apoptotic material was analyzed in the sera and lymph nodes of the different groups of mice. Apoptotic fragments within the lymph nodes were visualized using TUNEL immunofluorescent staining. Very few TUNEL-positive fragments were observed in the wild-type and apoE<sup>−/−</sup>/H<sup>11002/H<sup>11408/H<sup>11002</sup></sup> mice (Fig. 6 a and not depicted). The gld mice showed an increase in the frequency of TUNEL-positive fragments compared with wild-type or apoE<sup>−/−</sup>/H<sup>11002/H<sup>11408/H<sup>11002</sup></sup> (Fig. 6 b). However, the frequency of TUNEL-positive fragments was 40% higher in the gld.apoE<sup>−/−</sup>/H<sup>11002/H<sup>11408/H<sup>11002</sup></sup> mice than in the gld (P < 0.01), and higher levels of apoptotic nuclei were seen when gld.apoE<sup>−/−</sup>/H<sup>11002/H<sup>11408/H<sup>11002</sup></sup> mice were fed a Western diet (P < 0.05) (Fig. 6, c and d). These histological results were corroborated by FACS® analysis of TUNEL-positive fragments (Fig. 6 e).

A microparticle analysis was performed on platelet-free plasma using annexin V capture to assess the levels of apoptotic microparticles in the circulation (29). Although the differences between experimental groups was not as strik-

### Table II. Circulating Autoantibody Levels

<table>
<thead>
<tr>
<th></th>
<th>ANA (titer)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>aCL</th>
<th>Serum IgG</th>
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<tr>
<td></td>
<td>Normal diet</td>
<td>Western diet</td>
<td>Normal diet</td>
</tr>
<tr>
<td>WT</td>
<td>ND</td>
<td>ND</td>
<td>0.056 ± 0.03</td>
</tr>
<tr>
<td>apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>0.084 ± 0.02</td>
</tr>
<tr>
<td>gld</td>
<td>104 ± 24</td>
<td>120 ± 25</td>
<td>0.164 ± 0.02</td>
</tr>
<tr>
<td>gld.apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>400 ± 80&lt;sup&gt;b&lt;/sup&gt;</td>
<td>823 ± 118&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.658 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup>Inverse titer of last positive dilution.
<sup>b</sup>P < 0.001 versus WT, apoE<sup>−/−</sup>, gld.
<sup>c</sup>P < 0.05 versus gld.apoE<sup>−/−</sup> on normal diet.
<sup>d</sup>P < 0.01 versus WT, apoE<sup>−/−</sup>, gld.

A, absorbance; aCL, anticardiolipin; ANA, antinuclear antibody.

**Figure 6.** Elevated apoptotic fragments in lymph node and circulation of gld.apoE<sup>−/−</sup> mice. (a–c) Lymph node sections from animals fed Western diet were stained with TUNEL (FITC) and (d) quantified showing number of TUNEL-positive cells in the different strains of mice maintained on normal or Western diet (*, P < 0.05 vs. gld.apoE<sup>−/−</sup> on normal diet). gld.apoE<sup>−/−</sup> also showed a significant increase in TUNEL-positive cells compared with gld mice on normal or Western diet, respectively. Insets in a–c show higher magnification views. (e) FACS® analysis of TUNEL-positive cells in freshly isolated lymph node from Western diet-fed mice corroborates the histological findings (*, P < 0.05 vs. gld; P < 0.01 vs. WT, apoE<sup>−/−</sup>; †, P < 0.05 vs. WT, apoE<sup>−/−</sup>). (f) Platelet-free plasma isolated from different strains of mice on Western diet and assayed for apoptotic fragments by annexin V capture (*, P < 0.05 vs. gld; P < 0.01 vs. WT, apoE<sup>−/−</sup>).
ing as that found with TUNEL analyses of lymph node sections, the gld.apoE<sup>−/−</sup> mice showed significant increase in total microparticle concentration, relative to wild-type and apoE<sup>−/−</sup> mice (Fig. 6 f). The microparticle population in gld.apoE<sup>−/−</sup> mice was >30% endothelial origin based on an anti-CD31 colocalization procedure (reference 29 and unpublished data).

Further histological analyses of lymph node sections from gld.apoE<sup>−/−</sup> mice were performed to assess the uptake of apoptotic nuclear material by macrophages. Lymph node sections from gld and gld.apoE<sup>−/−</sup> mice displayed reduced frequencies of macrophages with evidence of ingested TUNEL-positive material compared with wild-type or apoE<sup>−/−</sup> mice (Fig. 7, a and b). However, the decrease in frequency of macrophages with engulfed apoptotic nuclei was most evident in gld.apoE<sup>−/−</sup> mice (P < 0.05 relative to gld) (Fig. 7 c). Of note, the uptake of apoptotic nuclei by macrophages in gld.apoE<sup>−/−</sup> mice was significantly reduced when animals were fed Western diet compared with normal diet (P < 0.05). Therefore, lymph nodes from gld.apoE<sup>−/−</sup> mice displayed greater accumulation of apoptotic debris, whereas macrophages in these mice displayed a greater deficit in their ability to engulf this material, and both of these effects were more pronounced by a high cholesterol diet.

**LPC Infusion Impairs Apoptotic Cell Clearance.** LPC is the predominant phospholipid component of oxLDL that is believed to mediate many of the proatherogenic activities associated with hyperlipidemia (34). To test whether LPC could interfere with the clearance of apoptotic bodies, wild-type and gld mice were infused with LPC, and apoptotic material accumulation in lymph nodes was assessed (Fig. 8). For this experiment, two doses of LPC were chosen that are known to have vasoactive properties in vivo (35). LPC infusion led to a small, dose-dependent increase in the frequency of TUNEL-positive fragments in lymph nodes of wild-type mice. In contrast, infusion of LPC in gld mice led to markedly greater accumulation of apoptotic debris in lymph nodes. The level of apoptotic debris in lymph node sections from gld mice infused with LPC was similar to that seen in gld.apoE<sup>−/−</sup> mice fed Western diet (Fig. 6).

**Discussion**

Although it is widely accepted that inflammation plays a role in atherosclerosis, the etiology of accelerated atherosclerosis associated with autoimmune disease is not well understood. In this work, a mouse model was created by crossing a strain that is susceptible to atherosclerotic lesion formation (apoE<sup>−/−</sup>) with a strain that develops lymphoproliferation and autoimmunity (gld). The gld.apoE<sup>−/−</sup> mice displayed threefold more atherosclerotic lesion area in the aorta than their apoE<sup>−/−</sup> counterparts on a Western diet. Surprisingly, the gld.apoE<sup>−/−</sup> mice exhibited exacerbated lymphoproliferation and autoimmunity relative to the gld phenotype, an effect that was further enhanced by Western diet. These data demonstrate synergistic interactions between the pathogenesis of atherosclerotic and autoimmune/lymphoproliferative diseases in the gld.apoE<sup>−/−</sup> mouse.

**Figure 7.** Evidence of impaired macrophage function in gld.apoE<sup>−/−</sup> mice. (a) Lymph node sections of wild-type mice stained with TUNEL (FITC) and F480 (PE) show colocalization of TUNEL-positive cells and macrophages (arrows, yellow merge). (b) Lymph node sections of gld.apoE<sup>−/−</sup> mice stained with TUNEL (FITC) and F480 (PE) show an abnormal morphology of macrophages and diminished colocalization. (c) Quantified results revealing a statistically significant decrease of macrophage ingestion of TUNEL-positive fragments in the gld and gld.apoE<sup>−/−</sup> mice, with gld.apoE<sup>−/−</sup> mice decreasing further than gld on both normal diet and Western diet (*, P < 0.05 vs. gld).

**Figure 8.** LPC promotes apoptotic body accumulation. LPC solutions or saline were infused for 120 min via tail vein in wild-type or gld mice. Lymph node sections were stained for TUNEL–positive cells. Quantified results reveal a significant increase in TUNEL–positive cells in gld mice after receiving LPC infusion (*, P < 0.001).
The role of Fas-mediated apoptosis in the genesis of vascular lesions is incompletely understood and controversial (36). Deficiencies in Fas-mediated apoptosis will produce vasculitis in some strains of mice (37) and promote intimal hyperplasia in a flow-restricted model of vascular injury (38). These data suggest that the Fas/FasL system is atheroprotective through its ability to inhibit vascular inflammation. However, acute overexpression of FasL or downstream effector molecules in wild-type animals was found to activate a proinflammatory program and promote atherosclerosis in some studies (39, 40), but not others (41, 42). The results of the current analysis show that, in toto, the Fas/FasL system has an atheroprotective function and that it serves to minimize vascular inflammation. In the context of the apoE−/− background, a deficiency in Fas-mediated apoptosis led to larger and more complex lesions, containing increased amounts of inflammatory cells and apoptotic debris. These features, in combination with the autoimmune phenotype, make the gld apoE−/− mouse strain a relevant model system to study the pathophysiology of accelerated atherosclerosis that occurs in patients with autoimmune disease (13–15).

The gld apoE−/− mice displayed increases in lymphadenopathy, splenomegaly, and autoimmune antibodies against cardiolipin and nuclear antigens compared with gld mice. The gld apoE−/− mice also exhibited immune-mediated glomerular injury. In this mouse model, abnormal glomeruli and increased urinary protein excretion rates were observed. This is of particular interest because the strain of gld mouse (B6) that was used in this work does not normally exhibit appreciable renal dysfunction (43).

The interrelationship between vascular and autoimmune diseases in this mouse model may be mediated by perturbations in the mechanisms that clear apoptotic bodies. Recent studies in patients with SLE and lupus-prone animal models suggest that autoimmunity can result from defects in the ability to scavenge apoptotic bodies (3, 8). A key finding of our analysis is the observation that gld apoE−/− mice contain significantly more TUNEL-positive nuclear fragments in their lymph nodes and higher levels of circulating apoptotic microparticles than the gld mice, consistent with the aggravated autoimmune phenotype in these animals. Despite increases in apoptotic material, macrophages in gld apoE−/− mice contained less apoptotic debris. The accumulation of apoptotic cells is of significance because delays in clearance will allow cells to enter late stages of apoptosis and secondary necrosis, and thereby promote inflammation (44). In addition, autoantigens become available for uptake by dendritic cells (2, 8). This process can facilitate the breakdown of peripheral tolerance through the effective presentation of autoantigens to the immune system.

The impaired clearance of apoptotic bodies may also have consequences for atherosclerotic disease. Patients with acute coronary syndromes have elevated levels of circulating apoptotic microparticles (29). These particles are highly thrombogenic and can account for the majority of the tissue factor activity in the lipid core of vascular plaques (45). In this regard, apoptotic fragments were found to be more abundant in the sera and atherosclerotic plaques of the gld apoE−/− mice than the apoE−/− mice, and their accumulation in the vessel wall could contribute to lesion progression. In addition, aCL was markedly elevated in the gld apoE−/− mice compared with gld or apoE−/− mice. aCL is a marker of autoimmune diseases and a risk factor for atherosclerosis and vascular complications (46, 47). Antiphospholipid antibodies may promote vascular inflammation by binding to apoptotic cells, thereby enabling their recognition by macrophage Fc receptors (48). Although macrophage phagocytosis of apoptotic bodies through scavenger receptors is normally antiinflammatory, uptake through Fc receptors can promote a proinflammatory state that is associated with cytokine secretion (48). Finally, immune complexes containing cardiolipin and other autoantigens can directly trigger endothelial cell expression of inflammatory cytokines and surface adhesion molecules, leading to endothelial dysfunction and their conversion to a prothrombotic phenotype (49).

It is interesting to speculate on how the process of atherogenesis can promote the accumulation of apoptotic bodies. OxLDL is a proatherogenic component of LDL cholesterol. OxLDL and antibodies against oxLDL can interfere with the recognition of the phosphatidylcholine moieties on apoptotic bodies by macrophage scavenger receptors and inhibit their phagocytosis (50, 51). Thus, the accumulation of apoptotic bodies in gld apoE−/− mice could result from oxLDL-mediated interference of macrophage function. Consistent with this hypothesis is the observation that gld apoE−/− mice maintained on Western versus normal diet displayed higher levels of apoptotic bodies in lymph nodes and a lower frequency of macrophages with ingested apoptotic debris. Furthermore, Western diet also increased circulating autoantibody levels as well as lymphadenopathy and splenomegaly. In this regard, we found that the continuous infusion of LPC, a major component of oxLDL, impairs apoptotic cell clearance. Although these effects could be seen in wild-type mice, the effects of LPC on apoptotic cell clearance were much more robust in gld mice. Recent data have suggested that LPC is a component of the phagocyte recruitment signals that are secreted by cells undergoing apoptosis (52). Therefore, high levels of LPC in hypercholesterolemic gld apoE−/− mice might interfere with signal gradients that are required for the normal recruitment of phagocytes to dying cells.

In summary, the gld apoE−/− model reflects the accelerated atherosclerosis that occurs in patients with autoimmune disorders. These data suggest that the immune dysregulation associated with SLE and similar disorders plays a key role in promoting atherogenesis. Our findings also indicate a positive feedback interaction between atherogenesis and autoimmune disease. In this regard, hyperlipidemic conditions may contribute to the severity of autoimmune disease by promoting the accumulation of apoptotic debris. Although it is difficult to ascertain the relationship between serum lipid levels and disease severity in patient populations.
due to the prevalence of corticosteroid therapy, an analysis of untreated pediatric SLE patients concluded that hyperlipidemia was associated with the active disease (53). Furthermore, it has recently been reported that a cholesteryl-lowering diet improves quality of life in patients with SLE (54). Therefore, further application of the gld. apoE−/− model could be valuable in delineating the interplay between autoimmunity and vascular disease progression.

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