Enhanced Immune System Activation and Arterial Inflammation Accelerates Atherosclerosis in Lupus-Prone Mice

Emmanuel L. Gautier, Thierry Huby, Betty Ouzilleau, Chantal Doucet, Flora Saint-Charles, Guilaine Gremy, M. John Chapman, Philippe Lesnik

Objective—Premature atherosclerosis is a characteristic feature of systemic lupus erythematosus, a prototypic autoimmune disease. The principle cellular and molecular mechanisms which underlie such accelerated atherosclerosis are indeterminate.

Methods and Results—The pathophysiology of lupus-mediated atherogenesis was evaluated in a novel animal model involving transplantation of bone marrow cells from the lupus prone strain gld into Ldl-r−/− mice. Diet-induced atherogenesis in lethally-irradiated Ldl-r−/− mice transplanted with gld bone marrow cells resulted in accelerated atherosclerosis (+65%) as compared with control mice transplanted with wild-type marrow cells. Enhanced atherogenesis was associated with enhanced activation of both B and T lymphocytes and with arterial inflammation involving endothelial cell activation, monocyte recruitment, and accumulation of apoptotic debris, macrophages, and CD4 T cells, but was independent of plasma lipid levels and renal function.

Conclusions—Our data support the contention that despite the absence of both disturbed cholesterol homeostasis and renal dysfunction in autoimmune gld→Ldl-r−/− mice, lupus disease induces enhanced activation of the immune system and acts locally on the vasculature to induce inflammation, together with accumulation of apoptotic debris, macrophages, and CD4 T cells, thereby accelerating plaque progression. (Arterioscler Thromb Vasc Biol. 2007;27:1625-1631.)

Key Words: atherosclerosis • lupus • arterial inflammation • immune system • apoptotic cells

The risk of cardiovascular disease (CVD) is significantly increased in the prototypic autoimmune disease, systemic lupus erythematosus (SLE).1,2 The mechanisms underlying premature CVD in SLE may be related to accelerated atherosclerosis.3,4 Such accelerated atherosclerosis potentially involves a combination of autoimmune-specific mechanisms and traditional cardiovascular risk factors (dyslipidemia, renal failure, and inflammation), although the central mechanisms involved are indeterminate.5 Moreover, as most SLE patients are on active therapy (corticosteroids and other pharmacological agents), such agents might interfere with the atherogenic process5 and lead to confounding findings. Therefore, identification of the mechanisms that contribute to disease progression might allow optimization of therapeutic approaches for prevention of CVD in SLE patients. In this setting, development of murine models may facilitate evaluation of the specific impact of autoimmunity on atherosclerosis progression and of the underlying mechanisms involved.

Genetic studies of various strains of lupus-prone mice have identified at least 30 chromosomal regions of interest reflecting the multifactorial aspect of SLE.6 In this context the use of various strains of lupus-prone mice and the study of their impact on atherosclerosis may highlight the key molecular mechanisms that promote autoimmune-accelerated atherosclerosis. Mouse strains defective for the Fas/Fas L pathway (gld, lpr) present lupus-like autoimmune disorders comparable to those of human SLE.6,7 Indeed, these mice are characterized by a deficit in apoptotic cell clearance8 which induces the production of a repertoire of autoantibodies directed against neoantigens derived from apoptotic cells (nucleosome, dsDNA). In addition, these mice progressively develop renal dysfunction with age.6,7

When gld mice are crossed with atherosclerosis-susceptible Apoε−/− animals, they develop accelerated atherosclerosis associated with defective phagocytosis of apoptotic cells in lymph nodes and glomerulonephritis.8 These findings suggest that defective apoptotic cell clearance, a feature of autoimmunity, might potentiate atherosclerosis. Impaired clearance of apoptotic cells is equally observed in Apoε−/− mice9 and could synergize with the gld mutation to exacerbate both autoimmunity and atherosclerosis. Moreover, the renal dysfunction observed in gld Apoε−/− mice might potentiate atherosclerosis as renal dysfunction is associated with accelerated atherosclerosis in mice and humans.10,11

To further evaluate the relationship between autoimmune disease and accelerated atherosclerosis, we generated an ath-
erosclerosis-susceptible autoimmune mouse model by transfer of bone marrow cells from the gld strain to lethally irradiated Ldl-r−/−. In this new murine model, we demonstrated both an enhanced activation of B and T cells and a vascular inflammatory profile that results in accelerated atherosclerosis. Such enhanced atherogenesis was independent of plasma lipid levels and renal dysfunction.

**Materials and Methods**

Please see the supplemental data section at http://atvb.ahajournals.org for detailed Methods.

**Mice, Bone Marrow Transplantation, and Study Design**

Ldl-r−/− and gld (FasL-deficient mice) on the C57BL/6J background were obtained from Jackson Laboratories. Males Ldl-r−/− mice (8- to 9-week-old) were subjected to medullar aplasia with 10 Gray lethal total body irradiation. The next day, femurs were isolated from donor gld or wt mice and 2.5×10⁶ bone marrow cells were injected via the retroorbital vein into the irradiated mice to rescue their hematopoietic systems. Mice were housed in cages under air-filtered conditions for 4 weeks to allow the hematopoietic system to reconstitute, after which they were fed a diet consisting of 0.15% cholesterol and 20% saturated fat (SAFE) for 12 weeks.

**Assessment of Chimerism and Analysis of Gene Expression by Q-Polymerase Chain Reaction (PCR)**

Real time quantitative PCR was performed using a LightCycler PCR System (Roche) as previously described.¹³ The specific primers are described in supplemental Table I available online at http://atvb.ahajournals.org

**Plasma Lipid Analyses and Lipoprotein Profile**

Blood samples were collected and analyzed as previously described.¹³

**Flow Cytometry, Antibody Measurements, and Serum Cytokine Levels**

Please see the supplemental data section at http://atvb.ahajournals.org for details.

**Analysis of Atherosclerotic Plaques, Immunohistochemistry, and Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labeling (TUNEL) Staining**

Atherosclerotic lesions quantification and Immunohistochemistry were performed as previously described.¹³,¹⁴ TUNEL staining was performed according to the manufacturer’s instructions (In situ Cell Death Detection Kit, Roche Applied Science)

**Statistical Analysis**

The statistical significance of the differences between groups was evaluated using the Mann–Whitney U test for unpaired comparisons. *P*<0.05 was considered significant. Values are expressed as mean±SEM.

**Results**

**Generation of Chimeric Mice With gld or wt Bone Marrow-Derived Cells**

Irradiated male Ldl-r−/− mice were reconstituted with bone marrow cells from gld mice (n=18) or wild-type controls (n=14). After 12 weeks of a Western-style diet, Ldl-r−/− mice which had received gld bone marrow cells displayed similar body weight as compared with those receiving wt marrows (21.6±1.0 versus 22.2±1.2 g, respectively). The hematocrit, leukocyte count, and relative levels of T cells, B cells, and monocytes were not significantly different between the two groups (data not shown). The efficacy of transplantation was attested by the significant splenomegaly observed in gld→Ldl-r−/−, a feature of the gld model, and by the fact that we detected less than 5% of Fasl wt alleles in bone marrow cells from these mice (supplemental Figure I, available online at http://atvb.ahajournals.org). Clearly, the chimera for Fasl in our gld→Ldl-r−/− was in the range of 95 to 100%.

**Quantification of Atherosclerotic Lesions**

As shown in Figure 1A, the surface of ORO-positive areas in the aortic root of Ldl-r−/− recipients reconstituted with gld marrow cells was significantly greater (+65%) than that observed for their wt reconstituted controls (170454±64391 versus 104134±31709 μm²; respectively; *P*=0.0005).

**Plasma Lipid and Lipoprotein Profile**

To assess whether the effects of gld marrow cells on atherosclerosis in Ldl-r−/− mice were attributable to changes in lipid or lipoprotein metabolism, we analyzed serum lipids and lipoproteins. Ldl-r−/− mice transplanted with gld marrow cells displayed, as compared with controls, similar plasma total cholesterol (481±67 versus 511±99 mg/dL, respectively), free cholesterol (148±23 versus 150±24 mg/dL, respectively), and triglyceride (206±61 versus 227±64 mg/dL, respectively) levels. Analysis of plasma lipoproteins by gel filtration showed no difference in cholesterol distribution across the VLDL, LDL, and HDL lipoprotein classes between gld→Ldl-r−/− and wt→Ldl-r−/− mice (data not shown).

**Systemic Autoimmunity in gld→Ldl-r−/−**

The spleen/body weight ratio in gld→Ldl-r−/− mice was increased almost 2-fold as compared with wt→Ldl-r−/− mice (Table 1). The enhanced lymphoproliferation and autoimmunity seen both in Fas or Fasl-deficient mice and Fas mutant human subjects is attributable predominantly to the expansion of an unusual T cell population of CD3+ cells that lacks both CD4 and CD8 (double negative T cells, DNTC). Therefore, we performed quantitative analysis of DNTC in spleen and blood samples from each animal. Significant expansion of DNTC was seen in both spleen and blood of gld→Ldl-r−/− animals compared with their wt→Ldl-r−/− controls (Table 1). Lupus is associated with the appearance of specific subsets of autoantibodies directed against nuclear materials. Thus, we measured the levels of antibodies directed against double-strand DNA (dsDNA) and nuclear materials (ANA) and showed that they were significantly elevated in gld→Ldl-r−/− mice as compared with the wt→Ldl-r−/− animals (Table 1). Moreover, autoimmune gld→Ldl-r−/− mice presented a global hypergammaglobulinemia as observed by significantly increased serum IgG levels compared with controls. Therefore, these data demonstrated the development of autoimmune disease in gld→Ldl-r−/− mice.

**Renal Function in Autoimmune Mice**

As autoimmune disease is frequently associated with renal dysfunction, histological analysis was performed on renal
tissue from both groups of mice. Glomerular cellularity in gld→Ldl-r−/− mice was similar to that observed in wt→Ldl-r−/− mice (data not shown). Accordingly, urinary protein and serum and urine creatinine levels were unchanged (Table 1).

Moreover, at the time of sacrifice, kidney weight to body M, did not reveal differences between the two groups of mice (Table 1). Collectively, these data argue for the absence of specific renal impairment in autoimmune gld→Ldl-r−/− mice.

Figure 1. Increased atherosclerosis, apoptotic material deposition, and macrophage accumulation in gld→Ldl-r−/− mice as compared with wt→Ldl-r−/− mice. A, The degree of atherosclerosis was determined by ORO staining of aortic root sections. Each symbol represents the mean lesion area in a single mouse. The horizontal bar indicates the mean value for the respective group. B, Atherosclerotic lesions were stained by the TUNEL method and the number of apoptotic cells and bodies counted. The ratio of TUNEL-positive cells or bodies to ORO-positive staining was calculated for both groups. Each symbol represents the ratio for a single mouse. C, Atherosclerotic lesions were immunostained for the macrophage CD68 antigen and the degree of macrophage accumulation was determined. Each symbol represents the mean lesion area in a single mouse. Photomicrographs illustrate macrophage abundance in aortic root sections from both groups of mice (magnification ×100). Nuclei are visualized in blue with DAPI staining. *, **, and *** indicate statistically significant differences between the 2 groups: P<0.05, P<0.01, and P<0.0005, respectively.
Table 1. Autoimmune Parameters and Renal Function in wt→Ldl−r−/− and gld→Ldl−r−/− Mice After 12 Weeks of Diet

<table>
<thead>
<tr>
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<th>wt→Ldl−r−/−</th>
<th>gld→Ldl−r−/−</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen/body wt, mg/g</td>
<td>3.8±0.8</td>
<td>7.5±1.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Blood DTNC, % of T cells</td>
<td>8.3±1.8</td>
<td>19.0±6.9</td>
<td>&lt;0.0005</td>
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<tr>
<td>Spleen DTNC, % of T cells</td>
<td>8.3±2.5</td>
<td>16.8±3.7</td>
<td>&lt;0.05</td>
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<td>Serum IgG, μg/mL</td>
<td>15.9±11.8</td>
<td>49.3±28.9</td>
<td>&lt;0.002</td>
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<tr>
<td>Serum ANA, μg/mL</td>
<td>29.7±12.7</td>
<td>58.4±15.0</td>
<td>&lt;0.0001</td>
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<tr>
<td>Serum dsDNA, ng/mL</td>
<td>7.2±3.5</td>
<td>35.3±26.0</td>
<td>&lt;0.0001</td>
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<tr>
<td>Serum creatinine, μmol/L</td>
<td>29.6±1.3</td>
<td>30.3±1.1</td>
<td>NS</td>
</tr>
<tr>
<td>Urinary creatinine, mmol/L</td>
<td>7.7±0.1</td>
<td>7.2±0.6</td>
<td>NS</td>
</tr>
<tr>
<td>Urinary protein, g/L</td>
<td>7.8±0.7</td>
<td>6.5±2.6</td>
<td>NS</td>
</tr>
<tr>
<td>Kidney/body wt, mg/g</td>
<td>6.0±0.3</td>
<td>5.9±0.7</td>
<td>NS</td>
</tr>
</tbody>
</table>

Systemic Inflammatory Cytokines
As immunoinflammatory cytokines play a significant role in atherosclerosis progression, we performed an exhaustive analysis of systemic cytokine profile. Serum levels of cytokines including interleukin (IL)-12, IFNγ, IL-1β, IL-2, IL-4, and IL-6 were significantly decreased (up to 9-fold) in gld→Ldl−r−/− mice as compared with wt→Ldl−r−/− mice (supplemental Table II). No significant changes were observed for IL-10, IL-1α, GM-colony stimulating factor (CSF), and tumor necrosis factor (TNF)-α between the two groups. This particular profile observed in gld→Ldl−r−/− mice was not associated with changes in IL-12p35, IL-1β, IL-4, nor in TNF-α mRNA levels in the spleen of these mice as compared with wt→Ldl−r−/− mice (data not shown), thereby indicating that tissues other than spleen might contribute to the circulating cytokine pool.

Anti-MDA LDL Antibody Levels, IgG2a/IgG1 Ratio, and B Cell Activation in Autoimmune Mice
As levels of antibodies directed against oxidized-LDL are correlated with lesion size,15 we sought to associate the serum titers of anti-MDA-LDL antibodies with the increased extent of lesions in gld→Ldl−r−/− mice. Thus, we observed significantly elevated titers of IgG and IgM to MDA-LDL in gld→Ldl−r−/− mice (Figure 2A and 2B, respectively). Next, as an indicator of Th1 versus Th2 polarization, we measured the serum IgG2a to IgG1 ratio. As this ratio is similar in wt→Ldl−r−/− and gld→Ldl−r−/− mice, it revealed no bias toward a Th1 or a Th2 response (Figure 2C). Finally, B cell activation was revealed by a significant increase in the proportion of CD86-positive cells among the B cell population of gld→Ldl−r−/− mice as compared with controls (Figure 2D).

Activation of CD4 T Cells and Accumulation in Atherosclerotic Plaques
CD4-positive T cells are key players in both atherosclerosis and autoimmunity, and therefore we analyzed both their activation status and presence in atherosclerotic plaques. Analysis of splenic CD4 T cells demonstrated that gld→Ldl−r−/− mice displayed increased proportions of both activated CD69-positive T cells (Figure 3A, P<0.005) and memory CD44-positive T cells (Figure 3B, P<0.005); by contrast there was no change in regulatory CD25/Foxp3 double positive T cells (12.1±1.4 in gld→Ldl−r−/− mice versus 11.0±1.5 in wt→Ldl−r−/− control mice). The increase in the activated and memory phenotype of CD4 T cells observed in lupus-prone mice was associated with their increased accumulation within the lesions (Figure 3C, P<0.05).

Apoptotic Structures in Atherosclerotic Lesions
As defective apoptotic cell clearance is observed in lymphoid organs of autoimmune mice, we quantified the number of cells in terminal stages of apoptosis in the aortic sinuses of both groups of mice. Late stage apoptotic cells and apoptotic bodies were stained by fluorescein isothiocyanate (FITC)-dUTP TUNEL whereas nuclei were counterstained with DAPI. Discrimination of apoptotic cells and apoptotic bodies by DAPI staining was based on nuclear size and morphology (normal or fragmented nuclei respectively). The frequencies of apoptotic cells and apoptotic bodies in aortic tissue were 15-fold and 3-fold higher in gld→Ldl−r−/− mice as compared with wt→Ldl−r−/− animals respectively (Figure 1B, P<0.05).
Macrophage Accumulation and Recruitment of Monocytes in Atherosclerotic Lesions.

Aortic root sections from mice on the Western diet were stained with a macrophage-specific antibody directed against CD68. Quantitative analysis revealed a significant 2-fold increase in CD68-staining lesions of gl<sup>dl</sup>r<sup>r<sup>3</sup> mice as compared with wt<sup>Ldl-r<sup>r<sup>3</sup> controls (Figure 1C, P<0.01). Macrophages were abundant in the subendothelial space of gl<sup>dl</sup>r<sup>r<sup>3</sup> mice and constituted the majority of the cells in the lesion (Figure 1C). To further document macrophage accumulation in atherosclerotic lesions, we extracted mRNA from descending aortas of mice to perform quantitative real-time PCR. Levels of mRNA for CD68 and F4/80 were significantly elevated (2- and 4-fold respectively) in the arch and abdominal areas of the aorta in gl<sup>dl</sup>r<sup>r<sup>3</sup> mice as compared with wt<sup>Ldl-r<sup>r<sup>3</sup> controls (Table 2). To investigate the potential molecular mechanisms underlying macrophage accumulation, we next evaluated expression of adhesion molecules and chemokines in the descending aorta. As shown in Table 2, the expression levels of MCP-1, P-selectin, and intercellular adhesion molecule-1 (ICAM-1) mRNA were significantly increased (3.5-, 1.5-, and 2.5-fold, respectively) in gl<sup>dl</sup>r<sup>r<sup>3</sup> mice as compared with their wt<sup>Ldl-r<sup>r<sup>3</sup> controls, while the levels of vascular cell adhesion molecule (VCAM)-1 and CX3CL1 mRNA were comparable in both groups (Table 2). Concomitantly, we observed that expression of the monocyte-associated genes CD115, CD11b, and CCR2 was elevated (1.5-, 2.5-, and 4-fold, respectively) in gl<sup>dl</sup>r<sup>r<sup>3</sup> aortas as compared with their wt<sup>Ldl-r<sup>r<sup>3</sup> controls, whereas CX3CR1 expression was also increased but did not reach statistical significance (Table 2).

Discussion

We have developed a new animal model susceptible to concomitant development of SLE and atherosclerosis. SLE prone-mice exhibited accelerated progression of atherosclerosis, although plasma lipid levels, lipoprotein-cholesterol distribution, and renal function were similar to wild-type controls. Aortic lesions in autoimmune mice were characterized by accumulation of apoptotic cells, consistent with the defective apoptotic cell clearance associated with SLE. In addition, enhanced immune system activation, arterial inflammation, and recruitment of both lymphocytes and monocytes were prominent aspects of accelerated atherosclerosis in mice, but equally represents an independent risk factor for atherogenesis in man. However, as we did not observe renal dysfunction within the time frame of our study (creatinine levels and proteinuria were unchanged in gl<sup>dl</sup>r<sup>r<sup>3</sup> mice), this mechanism does not appear to account for the accelerated atherosclerosis observed in our murine model.

Another prominent feature of autoimmunity in SLE patients concerns abnormal T and B-cell responses manifested by their activation status and the dysregulation in systemic cytokine profile. In our mouse SLE model, serum levels of IL-12, IFN-γ (Th1 cytokines), IL-4 (Th2 cytokine), IL-1β,
IL-6 (inflammatory cytokines), and the T-cell growth factor IL-2 were significantly decreased whereas those of IL-10, TNF-α, and GM-CSF remained unchanged. These findings are consistent with clinical investigations in SLE patients, in which attenuated expression of IL-12,19,20,21 IFN-γ,19,22 IL-4,19 and IL-22 have been documented. Similarly, in the lupus-prone autoimmune strain NZB/WF1, IL-12, IL-1, IL-2, IL-4, and IL-6 cytokine levels were decreased, whereas those of the antiinflammatory cytokine IL-10 and the proinflammatory cytokine TNF-α18,23,24 were unchanged as equally observed in our model. Overall, the impact of reduced cytokine levels (ie, Th1 and inflammatory cytokines) might appear primarily antiatherogenic. Indeed, in mouse models of atherosclerosis, substantial data support a proatherogenic role for IFN-γ25,26 IL-12,27,28 IL-4,27,29 and IL-1β30, whereas IL-10 plays an antiatherogenic role.31

Surprisingly, we observed an enhanced activation of the immune system consistent with increased B and T cell activation, increased memory phenotype of CD4 T cells, and concomitant elevation of anti–MDA-LDL IgG and IgM levels in gld→Ldl-r−/− mice as compared with controls. Similar findings concerning T cell activation were reported in a recent study based on transplantation of bone marrow cells from congenic mice expressing a susceptibility locus for SLE in Ldl-r−/− mice.17 In addition, it is important to note that the proportion of atheroprotective type 1 regulatory T cells did not differ between gld→Ldl-r−/− and wt→Ldl-r−/− mice. In our model, despite lower proinflammatory cytokine levels, we report higher activation of both B and T cells. These results are not contradictory as a diminished threshold of activation for both T and B cells could favor the onset of autoimmunity. These defects appear to be key players in the accelerated atherosclerosis typical of lupus. In this setting, the elevated degree of CD4 T cell activation, and memory status in gld→Ldl-r−/− mice, which is associated with increased lesional T cell numbers could accelerate atherogenesis and is consistent with a highly inflammatory plaque phenotype.

Marked accumulation of apoptotic cells and bodies occurred in aortic sinus tissue of gld→Ldl-r−/− mice, thereby corroborating the observation that increased amounts of apoptotic material were deposited in the lymph nodes of gld Apoe−/− mice.8 Several studies have demonstrated that the impaired clearance of apoptotic cells by macrophages is a feature of SLE,16 and that a defective complement system may play an active role in this process.32 However, we did not observe decreased mRNA expression of the main complement components C1q, C2, C3, and C4 in the liver of gld→Ldl-r−/− animals (data not shown). Additionally, the observed elevation in titers of autoantibodies to dsDNA and ANA directed against nuclear material suggest that elevated levels of circulating nucleosomes may occur in gld→Ldl-r−/− mice. Such nucleosomes may partly account for defective apoptotic cell clearance as suggested by Laderach and colleagues.33 In this context, the accumulation of TUNEL-positive bodies in the aortic sinus is indicative of an ineffective clearance of primary apoptotic cells, ie, cells exposing phosphatidylserine in the outer plasma membrane leaflet, rather than the result of increased rates of apoptosis. Such defective apoptotic cell clearance observed in atherosclerotic lesions suggests that apoptotic cells evolve to necrotic cells, the latter triggering proinflammatory and immunostimulatory responses.34,35 In turn, impaired clearance of dying cells may promote arterial inflammation and contribute to lesion development.3,34,36

In our model, plaque progression was potentiated by local inflammation in the arterial wall of gld→Ldl-r−/− mice as demonstrated by significant upregulation of mRNAs indicative of activation of endothelial cells (MCP-1, P-selectin, and VCAM-1) and macrophages (MCP-1) as compared with controls. This finding was associated with significantly elevated expression of mRNAs encoding genes associated with monocytes (CCR2, CD11b, and CD115) and macrophages (CD68 and F4/80) in the descending aorta of gld→Ldl-r−/− mice. Endothelial cell activation might be induced by elevated circulating IgG levels among which specific IgGs and/or specific immune complexes might interact with the endothelium to activate endothelial cells inducing endothelial dysfunction.37,38,39,40 Elevated endothelial permeability typical of endothelial dysfunction facilitates enhanced lipoprotein access, retention, and deposition in the intimal space, a classical feature of fatty streak formation. In this setting, the adhesion molecules and chemokines whose expression is upregulated in the aortas of gld→Ldl-r−/− mice are known to be key players in the recruitment of monocytes into the subendothelial space, thus favoring lesion progression.41,42 Indeed, increase in the expression of CD11b may reflect the entry of newly recruited monocytes,9,14 thereby leading to the accumulation of macrophages in the arterial wall. However, we cannot exclude the possibility that macrophages with the gld mutation could display higher resistance to apoptosis, a feature equally reported in another mouse model for SLE,43 and which, in turn, favors their accumulation in the aortic sinus of gld→Ldl-r−/− mice. Nevertheless, the similar proportions of splenic F4/80+ macrophages observed in wt→Ldl-r−/− and gld→Ldl-r−/− mice (4.8±1.1 versus 3.3±0.5%, respectively) suggest that the half life of gld macrophages is not affected and argues that higher resistance to apoptosis may not be operative in aortic lesions.

In our innovative gld→Ldl-r−/− mouse model, we provide new insight into the molecular mechanisms that may underlie accelerated atherosclerosis in autoimmune disease. Indeed, our experimental findings highlight enhanced activation of the immune system, aortic inflammation, and endothelial cell activation, together with lesional accumulation of apoptotic cells, macrophages, and CD4 T cells as being central to the development and progression of atherosclerosis in autoimmune mice. These findings may lead to development of innovative therapeutic strategies in SLE patients at high cardiovascular risk.

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Disclosures

None.

References

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Supplementary Material For:

Enhanced immune system activation and arterial inflammation accelerates atherosclerosis in lupus-prone mice

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Supplemental Methods

Mice, bone marrow transplantation and study design. 

*Ldl-r<sup>-/-</sup>* and *gld* mice (*FasL*-deficient mice) on the C57BL/6J background were obtained from Jackson Laboratories. The animals were housed in a conventional animal facility with a 6 AM to 6 PM dark/light cycle. Mice were weaned at 21 days and fed a chow diet ad libitum (RM1, Dietex). Males *Ldl-r<sup>-/-</sup>* mice (8-9-week-old) were subjected to medullar aplasia with 10 Gray lethal total body irradiation. The next day, femurs were isolated from donor *gld* or *wt* mice and 2.5 x 10<sup>6</sup> bone marrow cells were injected via the retororbital vein into the irradiated mice to rescue their haematopoietic systems. Mice were housed in cages under air-filtered conditions for 4 weeks to allow the haematopoietic system to reconstitute, after which they were fed a diet consisting of 0.15% cholesterol and 20% saturated fat (SAFE, France) for 12 weeks. All animal
procedures were performed with accreditation from the French government and under strict compliance with Animal Welfare Regulations.

**Assessment of chimerism.**

Genomic DNA from bone marrow cells was isolated using the Nucleospin DNA Kit (Macherey Nagel). Chimerism was evaluated by quantifying the amount of *FasL* *wt* DNA in *gld→Ldl-r<sup>−/−</sup>* mice. Data obtained after amplification were standardized for the amount of input DNA by quantification of a nonrelevant gene (*Sry*). Real time quantitative PCR was performed using a LightCycler PCR System (Roche) as previously described<sup>1</sup>. The specific primers used were as followed: *FasL* *wt* forward primer 5′-GTCCATCCCTCTGGGTAATGG-3′ and *FasL* *wt* reverse primer 5′-GCTTATACAGACGGGAAAA-3′ for *FasL* *wt* allele, and *Sry* forward primer : 5′- GCTTTCGGACTGCGAACATGG-3′ and *Sry* reverse primer 5′- GTCTTTCGCTGTATGTGAGTTG-3′ for *Sry* gene . Expression data were based on the crossing points calculated from the LightCycler data analysis software and corrected for PCR efficiencies of both the target and the reference gene. The mean value from the analysis of 5 *wt→Ldl-r<sup>−/−</sup>* mice was set to 100% and use to determine the percentage of *wt* alleles in *gld→Ldl-r<sup>−/−</sup>* mice.

**Plasma lipid analyses and lipoprotein profiles.**

Blood samples were collected following an overnight fast in Microvette tubes by retro-orbital bleeding (Sarstedt) after 11 weeks of high-fat diet. Plasma total cholesterol (Roche Diagnostics), free cholesterol (Wako) and triglyceride (Biomérieux) concentrations were measured by enzymatic colorimetric assays using an automatic system (Konelab). Plasma lipoproteins were fractioned by gel filtration on two Superose 6 (Amersham Biosciences) columns connected in series using a BioLogic DuoFlow Chromatography System (BioRad)<sup>1</sup>. 
**Flow cytometry analysis and antibodies.**

After 11 weeks of high-fat diet, blood was collected by retro-orbital bleeding and red blood cells were lysed by ammonium chloride (ACK) treatment. Leucocytes were then resuspended in PBS/1% BSA/0.01% sodium azide, pre-incubated for 5 min with Fc blocker (BD Pharmingen) and then incubated for 30 min at 4°C with antibodies directed against CD45 (clone 30F11, Miltenyi), CD3 (clone 145-2C11, BD Pharmingen), CD4 (clone GK1.5, Miltenyi or clone RM4-5, BD Pharmingen and eBiosciences), CD8 (clone 53-6.7, eBiosciences), F4/80 (clone CI:A3-1, Serotec) and CD19 (clone 6D5, eBiosciences) to analyse the proportion of individual leukocyte subpopulations. To assess the peripheral blood count in mice, leucocytes were incubated with an anti-CD45 antibody, washed and 100 µL of calibrated fluorospheres (FlowCount, Beckman Coulter) was added to each sample. Data were acquired until 1000 beads were counted according to the manufacturer’s instructions. Splenocytes were harvested at sacrifice, minced and filtered through a 70-µm cell strainer. After ACK treatment, cells were resuspended in PBS/1% BSA/0.01% sodium azide, pre-incubated for 5 min with Fc blocker (BD Pharmingen) and incubated for 30 min at 4°C with antibodies directed against CD45, CD3, CD4, CD8, F4/80, CD19, CD69 (clone H1.2F3), CD44 (IM7, eBiosciences), CD25 (clone 7D4, Beckman Coulter), CD86 (clone GL1, eBiosciences) and Foxp-3 (clone FJK-16s, eBiosciences) in order to analyse the proportions of splenocyte subpopulations. Cells were analysed on a Coulter Epics XL flow cytometer (Beckman Coulter) using Epics XL32 software.
**Antibody measurements.**

Serum IgG, IgG1, IgG2a, double strand-DNA (dsDNA) autoantibodies and antinuclear (ANA) autoantibodies were measured using commercial ELISA kits (Alpha Diagnostic) according to the manufacturer’s instructions.

**Assessment of renal function.**

Kidneys were collected at sacrifice and paraffin embedded. Serum creatinine, urinary protein and urinary creatinine levels were measured using commercial kits.

**Analysis of atherosclerotic plaques and immunohistochemistry.**

Mice were sacrificed under isoflurane anesthesia and perfused with sterile ice-cold PBS. Hearts were collected, fixed in 10% formalin solution for 30 minutes followed by overnight incubation in phosphate-buffered 20% sucrose solution at 4°C. Hearts were then embedded in Tissue-Tek OCT compound (Sakura Finetek). Atherosclerotic lesions were quantified through the aortic root using oil red O (ORO) staining as previously described\(^2\). Briefly, approximately 60 sections, 10 \(\mu\)m thick, were cut through the proximal aorta. Every tenth section was stained with oil red O (0.5% in propylene glycol) for 4 hours and then counterstained with Mayer haematoxylin for 1 minute. Images were captured using a Zeiss Axiovision microscope and a Canon numerical camera. The extent of atherosclerosis was measured with color thresholding to delimit areas of oil red O staining.

For immunohistochemistry, aortic root cryosections (10 \(\mu\)m) were air-dried and fixed in 10% formalin for 30 minutes. Sections were then blocked for 60 minutes with 3% BSA in PBS and then incubated with anti-CD68 antibody (Serotec, 1:300) or anti-CD4 antibody (BD Biosciences, 1:100) or control antibody overnight at 4°C. After washing, a biotinylated goat anti–rat Ig
secondary antibody (PharMingen, 1:300) was added, followed by streptavidin–horseradish peroxidase (PharMingen). The signal was enhanced using the tyramide signal amplification (TSA) kit (Du Pont NEN Research Products) according to the manufacturer's protocol and the sections were counterstained for nuclei with DAPI (Vector Laboratories).

**Serum cytokine profile.**

The serum concentrations of IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12 p70, GM-CSF and TNF-α were determined using the Proteoplex murine cytokine array kit (Novagen) according to the manufacturer’s instructions. The serum level of IFN-γ was determined using an ELISA kit (‘Femto-HS’ High Sensitivity ELISA, eBiosciences).

**TUNEL staining.**

Cryosections (10 µm) were air-dried and fixed in 10% formalin for 30 minutes. Sections were then placed in 0.1M Citrate buffer (pH 6.0) and submitted to 750W microwave irradiation for 1 min. Slides were washed in PBS and blocked by immersion in 0.1M Tris-HCl pH 7.5 containing 3% BSA and 20% normal bovine serum for 30 min. After washing, TUNEL reaction mixture (*In situ* Cell Death Detection Kit, Roche Applied Science) was added and slides were incubated for 60 min at 37°C. Sections were washed in PBS and counterstained for nuclei with DAPI (Vector Laboratories). Nuclear size discriminates apoptotic cells (normal size) and apoptotic bodies (fragmented nuclei).
Measurement of anti-MDA LDL antibodies.

MDA-LDL (10μg/mL) dissolved in PBS were coated overnight at 4°C into 96-well ELISA plates (Nunc) and then plates were blocked with 3% BSA in PBS. Serum samples were diluted in PBS containing 1% BSA and added at room temperature for 2 hours followed by anti- mouse IgG-HRP or anti-mouse IgM-HRP and revelation was achieved by addition of the substrate. Plates were read at 405 nm.

Analysis of gene expression by Q-PCR.

RNAs were prepared using TRIzol reagent (Invitrogen) from frozen tissue specimens isolated from mice at sacrifice. Each RNA preparation (n=8 per group) was hybridized with random hexamer (Promega) and reverse-transcribed using M-MLV reverse transcriptase (Invitrogen). Real time quantitative PCR was performed using a LightCycler PCR System (Roche) as previously described\(^1\). The specific primers are described in supplemental Table I available online at http://atvb.ahajournals.org. All reactions were performed in duplicate or triplicate and HPRT (hypoxanthine guanine phosphoribosyl transferase) was used as a housekeeping gene in order to account for variability in the initial quantities of cDNA. In all PCR assays and for each primer set, expression of a control cDNA (pool of reverse transcribed-RNA prepared from different mouse tissues) was included and used as an inter-run calibrator. Expression data were based on the crossing points calculated from the LightCycler data analysis software and corrected for PCR efficiencies of both the target and the reference gene.

Statistical analysis.
The statistical significance of the differences between groups was evaluated using the Mann-Whitney U test for unpaired comparisons. P<0.05 was considered significant. Values are expressed as mean ± SEM.

**Supplementary Reference**


**Supplementary Figure Legends**

**Supplementary Figure 1.** Assessment of chimerism in *gld→Ldl-r/-* mice. The amount of FasL *wt* DNA was determined in DNA from bone marrow cells of *wt→Ldl-r/-* and *gld→Ldl-r/-* mice and the proportion of wt allele was determined. More than 95% of the FasL alleles in *gld→Ldl-r/-* mice were of donor origin.
Supplemental Figure I.
## Supplemental Table I. Specific primer pairs

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Sense primer</th>
<th>Antisense primer</th>
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<tbody>
<tr>
<td>MCP-1</td>
<td>NM_011333</td>
<td>5'-CCCTGTCATGCTTCTGGG-3'</td>
<td>5'-GGATCATCTTGCTGGTGGAATG-3'</td>
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<tr>
<td>ICAM-1</td>
<td>NM_010493</td>
<td>5'-AACGAGGACCAAGAGATGGA-3'</td>
<td>5'-AGCTGTCTGCGGTATGTCT-3'</td>
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<td>VCAM-1</td>
<td>NM_011693</td>
<td>5'-CCCAAACAGAGGCAGAGTGT-3'</td>
<td>5'-TGAGCAGGTCCAGGTTCACAG-3'</td>
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<td>P-selectin</td>
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<td>5'-AACGAGGCAGAGCTCAAA-3'</td>
<td>5'-TTTACAGGTGAATCTCTGGG-3'</td>
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<tr>
<td>CX3CL1</td>
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<td>5'-CGACAAGATGACCTCAACGA-3'</td>
<td>5'-CTGTGTCTGCTCCAGGACAA-3'</td>
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<td>CCR2</td>
<td>NM_009915</td>
<td>5'-CATAGAAATCAGAGCAGAAATGGA-3'</td>
<td>5'-TGAGGAGGCAGAAAAATAGCA-3'</td>
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<tr>
<td>CX3CR1</td>
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<td>5'-GTCAGTGATGCTTTGCGCT-3'</td>
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<td>CD11b</td>
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<td>CD115/CSF-1R</td>
<td>NM_001037859</td>
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<td>5'-CAGGACATCAAGCCATTCACAG-3'</td>
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<td>F4/80</td>
<td>NM_010130</td>
<td>5'-CTTGCGATGAGTTCCAGTCC-3'</td>
<td>5'-GCAAGGAGGACAGATGTTATCGTG-3'</td>
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<td>CD68</td>
<td>NM_009853</td>
<td>5'-TTGGGAACTACACAGTGCCG-3'</td>
<td>5'-CGGATTTGAATTTGCGCTTG-3'</td>
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<td>HPRT</td>
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<td>5'-TGACACTTGAAAACAATGC-3'</td>
<td>5'-AACAATCTCGAGAGGTCTTT-3'</td>
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</table>
Supplemental Table II. Systemic cytokine profile in *wt* → *Ldl-r<sup>-/-</sup>* and *gld* → *Ldl-r<sup>-/-</sup>* mice after 12 weeks of diet.

<table>
<thead>
<tr>
<th></th>
<th><em>wt</em> → <em>Ldl-r&lt;sup&gt;-/-&lt;/sup&gt;</em></th>
<th><em>gld</em> → <em>Ldl-r&lt;sup&gt;-/-&lt;/sup&gt;</em></th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>317 ± 145</td>
<td>42 ± 51</td>
<td>=0.06</td>
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<tr>
<td>IL-1β</td>
<td>408 ± 196</td>
<td>ND</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IL-2</td>
<td>659 ± 379</td>
<td>69 ± 48</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>IL-4</td>
<td>1432 ± 661</td>
<td>370 ± 444</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IL-6</td>
<td>554 ± 182</td>
<td>194 ± 174</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IL-10</td>
<td>237 ± 230</td>
<td>195 ± 188</td>
<td>NS</td>
</tr>
<tr>
<td>IL-12</td>
<td>354 ± 117</td>
<td>86 ± 84</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>TNFα</td>
<td>314 ± 187</td>
<td>210 ± 188</td>
<td>NS</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>159 ± 124</td>
<td>48 ± 47</td>
<td>NS</td>
</tr>
<tr>
<td>IFNγ</td>
<td>6.8 ± 2.7</td>
<td>3.8 ± 1.4</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Serum cytokines levels are expressed as pg/mL
ND : Not Detected