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Hematopoietic Fas Deficiency Does Not Affect Experimental Atherosclerotic Lesion Formation despite Inducing a Proatherogenic State

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The Fas death receptor (CD95) is expressed on macrophages, smooth muscle cells, and T cells within atherosclerotic lesions. Given the dual roles of Fas in both apoptotic and nonapoptotic signaling, the aim of the present study was to test the effect of hematopoietic Fas deficiency on experimental atherosclerosis in low-density lipoprotein receptor-null mice ($Ldlr^{-/-}$). Bone marrow from $Fas^{-/-}$ mice was used to reconstitute irradiated $Ldlr^{-/-}$ mice as a model for atherosclerosis. After 16 weeks on an 0.5% cholesterol diet, no differences were noted in brachiocephalic artery lesion size, cellularity, or vessel wall apoptosis. However, $Ldlr^{-/-}$ mice reconstituted with $Fas^{-/-}$ hematopoietic cells had elevated hyperlipidemia [80% increase, relative to wild-type (WT) controls; $P < 0.001$] and showed marked elevation of plasma levels of CXCL1/KC, CCL2/MCP-1, IL-6, IL-10, IL-12 subunit p70, and soluble Fas ligand ($P < 0.01$), as well as systemic microvascular inflammation. It was not possible to assess later stages of atherosclerosis because of increased mortality in $Fas^{-/-}$ bone marrow recipients. Our data indicate that hematopoietic Fas deficiency does not affect early atherosclerotic lesion development in $Ldlr^{-/-}$ mice. ***(Am J Pathol 2011, 178:2931–2937; DOI: 10.1016/j.ajpath.2011.02.011)***

Complications from atherosclerosis remain major causes of morbidity and mortality worldwide.[1] Atherosclerosis is widely accepted as an inflammatory process.[2,3] The development and composition of atherosclerotic lesions reflect a balance between accumulation, proliferation, and apoptosis of cellular components of the vessel wall, including macrophages, T lymphocytes, endothelial cells, and smooth muscle cells.

Fas (Apo-1, CD95), a 45-kDa member of the death receptor family, is known to induce apoptosis in susceptible cells on binding Fas ligand (FasL, CD178).[4] Fas expression has been demonstrated on macrophages, T lymphocytes, and smooth muscle cells within human atherosclerotic lesions.[5] In contrast to lymphocytes and smooth muscle cells, macrophages are relatively resistant to Fas-mediated apoptosis.[6] This correlates with increased expression of cFLIP during monocyte to macrophage differentiation.[7] However, macrophage resistance to Fas-mediated apoptosis can be reversed *in vitro* by Toll-like receptor ligands, including lipopolysaccharide, lipid A, zymosan, poly(I:C), and CpG DNA.[8] Furthermore, there is increasing recognition of nonapoptotic responses to Fas ligation, including cellular proliferation, differentiation, and NF-κB activation in several cell types.[9,10] In macrophages, Fas engagement also triggers proinflammatory cytokine production.[6,11]

Mouse models of Fas or FasL deficiency develop progressive lymphadenopathy and splenomegaly due to dysregulated lymphocyte proliferation.[12,13] Fas-deficient mouse models include $Fas^{lpr/lpr}$ mice (lymphoproliferation, *lpr*), which have an inactivating point mutation in the cytoplasmic domain,[14] and $Fas^{-/-}$ mice, which are complete deficient.[12] When *lpr* mice are crossed with atherosclerosis-prone $Apoe^{-/-}$ mice, the animals developed accelerated atherosclerosis accompanied by lymphoproliferation and a lupus-like syndrome.[15] Autoimmune disorders such as systemic lupus erythematosus, recognized as an independent risk factor for atherosclerosis, are associated with an approximately 7.5-fold in-

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crease in cardiovascular complications.[16] Thus, in the previous model,[15] development of autoimmunity may have confounded the relationship between Fas deficiency and atherogenesis.

Here, we describe the effect of Fas expression in bone marrow-derived cells, using the atherosclerosis-prone low-density lipoprotein receptor-deficient (*Ldlr*$^{-/-}$) mouse (human ortholog, *LDLR*). We find excess mortality with hematopoietic Fas deficiency in this atherosclerosis model, with no evidence of lymphoproliferation. Absence of lymphoproliferation is unexpected, because *Fas*$^{-/-}$ mice develop increased splenomegaly and lymphadenopathy at an earlier age than do *lpr* mice.[17] Surprisingly, we found no significant effect on atherosclerotic lesion development or vascular wall apoptosis at an early stage of lesion development, despite an enhanced atherogenic state characterized by increased plasma lipids and systemic cytokine and chemokine levels.

Materials and Methods

Mice

Ldlr$^{-/-}$ and *Fas*$^{-/-}$ mice (B6.129P2-Fastm1Osa/J, Stock ID 003233)[17] on the C57Bl/6J background were obtained from Jackson Laboratories (Bar Harbor, ME). Female *Ldlr*$^{-/-}$ mice, 12 weeks old, received 11 Gy irradiation for marrow ablation. The next day, bone marrow cells obtained from male WT C57Bl/6J ($n = 16$) or *Fas*$^{-/-}$ ($n = 16$) mice were administered to irradiated recipients via retro-orbital injection. Following a 4-week recovery period, mice were placed on an 0.5% cholesterol diet (Harlan Teklad RD-97234; Harlan Sprague Dawley, Indianapolis, IN) for a planned duration of 24 weeks.[18] Mice were maintained under specific pathogen-free conditions. Blood was obtained by cardiac puncture at the time of sacrifice. Individual complete blood counts were performed on a Hemavet 950 veterinary hematology analyzer (Drew Scientific, Dallas, TX). All experimental procedures were performed with approval from the Animal Care Committee of the University of Washington.

Assessment of Atherosclerotic Lesions

At the time of sacrifice, mice were perfused via the left ventricle with 10 mL PBS containing 1 mmol/L EDTA, followed by 30 mL fixative solution (PBS, 4% paraformaldehyde in PBS). The heart including the aortic root, thoracic aorta, and its branching vessels was dissected and fixed overnight. The brachiocephalic artery from the branch off the aortic arch to the bifurcation of the right subclavian artery and common carotid artery was dissected, embedded in paraffin, and sectioned into 100 5-μm sections. For quantitation of lesion area, six H&E-stained sections (50-μm intervals) per mouse were analyzed using ImageJ software version 1.42 (NIH, Bethesda, MD). The internal and external elastic lamellae and lesion borders were traced and used to derive lesion areas.[19] Terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) staining was performed according to the manufacturer's instructions (*In Situ* Cell Death Detection Kit; Roche Applied Science, Indianapolis, IN) with DAPI as a nuclear counterstain. DAPI-positive and TUNEL-positive cells were counted under fluorescence microscopy (Nikon Eclipse TE300), using 6 to 10 sections of brachiocephalic artery per mouse. Three independent researchers (X.Z., J.T., A.I.) without knowledge of the tissue source quantified lesion area and TUNEL analysis.

Immunostaining of Tissues

Tissue sections from liver, lung, spleen, and kidney were stained with H&E to evaluate histological changes, and adjacent sections were stained with antibodies to macrophages, (Mac-2; ATCC, Manassas, VA), B lymphocytes (B220; BD Biosciences, San Jose, CA), and T lymphocytes (CD3; Dako, Carpinteria, CA), as well as control rabbit and rat IgG.

Cell Isolation and Flow Cytometry

Spleens were harvested, weighed, and dispersed, and cells were passed through a 70-μm strainer. Peripheral blood leukocytes and isolated splenocytes were analyzed after red-cell lysis with multicolor flow cytometry on a FACScan system (BD Biosciences) with CellQuest software version 3.4. For leukocyte characterization the following antibodies were used: Thy1.2, B220, Ly6G, CD11b, CD95 (Fas), and CD178 (FasL). Fluorochrome-conjugated monoclonal antibodies were all purchased from BD Biosciences.

Chemokine and Cytokine Analysis

The plasma concentrations of CXCL1/KC, CXCL2/MIP-2, CCL2/MCP-1, IL-6, IL-10, IL-12, TNF-α, and IFN-γ were determined using a fluorokine MAP multiplex mouse cytokine panel (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The plasma level of soluble FasL was determined using a commercially available enzyme-linked immunosorbent assay kit (R&D Systems).

Plasma Cholesterol and Lipid Profiles

Plasma cholesterol levels were measured at the Northwest Lipid Research Laboratories (Seattle, WA). The lipoprotein cholesterol profiles were determined for three mice per group on mouse plasma separated by fast protein liquid chromatography[20] performed by the University of Washington Clinical Nutrition Research Unit.

Statistical Analysis

Data are reported as means ± SD, unless stated otherwise. Data were analyzed using the GraphPad Prism version 5 software (GraphPad Software, La Jolla, CA), and significance was set at $P < 0.05$. Unpaired two-tailed Student's *t*-test was conducted if variance was normally distributed; otherwise, the Mann-Whitney *U*-test was used. Survival analysis was performed using Kaplan-Meier method with log-rank test to determine differences in survival.

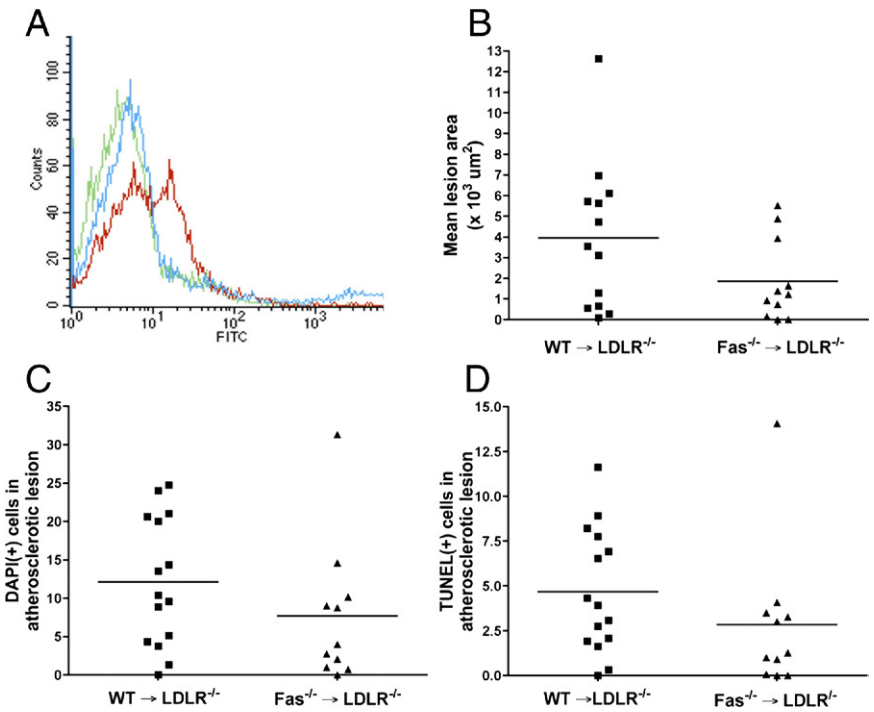

Figure 1. Hematopoietic $Fas^{-/-}$→$Ldlr^{-/-}$ chimeric mice do not show changes in atherosclerotic lesion area, cellularity, or apoptosis. WT→$Ldlr^{-/-}$ (n = 15) and $Fas^{-/-}$→$Ldlr^{-/-}$ mice (n = 11) were generated and fed with an 0.5% cholesterol diet for 16 weeks. **A:** Flow cytometry was performed on peripheral blood of the bone-marrow chimeric mice at the time of sacrifice. Representative profiles are shown. Green indicates background staining with no antibody in $Fas^{-/-}$→$Ldlr^{-/-}$ mice; red indicates positive staining with CD95 antibody in WT→$Ldlr^{-/-}$ chimeric mice; and blue indicates lack of staining with CD95 (Fas) antibody in $Fas^{-/-}$→$Ldlr^{-/-}$ chimeric mice. **B:** Atherosclerotic lesion area was determined from H&E-stained brachiocephalic artery sections (6 per mouse, 50-μm intervals) using ImageJ software. DAPI-positive cells (**C**) and TUNEL-positive cells (**D**) were counted from brachiocephalic artery sections (6 to 10 sections per mouse), using fluorescence microscopy. Each symbol represents an individual mouse; **horizontal lines** indicate the mean value. $P > 0.05$, Mann-Whitney U-test (**B**, **C**, and **D**).

Results

Hematopoietic $Fas^{-/-}$→$Ldlr^{-/-}$ *Chimeric Mice Show Normal Hematopoietic Reconstitution and No Evidence of Lymphoproliferation*

Bone marrow transplantation was used to generate mice that lacked hematopoietic Fas expression. 12-week-old female $Ldlr^{-/-}$ mice were reconstituted with bone marrow from male donor $Fas^{-/-}$ mice (n = 16) or their respective WT controls (n = 16). After a 4-week recovery period to allow for macrophage repopulation, the mice were then started on an 0.5% cholesterol diet. At 20 weeks after transplantation (16 weeks on the 0.5% cholesterol diet), excess mortality was noted in the $Fas^{-/-}$ bone marrow recipients, relative to WT bone marrow recipients: 5 of 16 dead in $Fas^{-/-}$→$Ldlr^{-/-}$ versus 1 of 16 dead in WT→$Ldlr^{-/-}$ (P = 0.07, log-rank test). The remaining mice were then sacrificed for analysis at 21 weeks after transplantation, 3 weeks ahead of the planned schedule.

Hematopoietic recovery was similar between the two groups. Hematocrit, hemoglobin, total leukocyte, lymphocyte, and monocyte counts were within normal limits and were not statistically different between the two groups (data not shown). Platelet counts were lower in the $Fas^{-/-}$→$Ldlr^{-/-}$ group (541 ± 141, versus 657 ± 89 K/μL in the WT group; P = 0.02) and absolute neutrophil count was higher in the $Fas^{-/-}$→$Ldlr^{-/-}$ group (2.2 ± 1.0, versus 1.3 ± 0.6 K/μL in the WT group; P = 0.01). Flow cytometry of peripheral blood leukocytes confirmed expected phenotypes after bone marrow transplantation (Figure 1A).

Although lymphoproliferation has previously been well described in Fas-deficient mice and bone marrow chimeras using Fas-deficient bone marrow donors,[21] there was no evidence of splenomegaly or lymphadenopathy in the $Fas^{-/-}$→$Ldlr^{-/-}$ or WT→$Ldlr^{-/-}$ mice (Table 1).

Hematopoietic $Fas^{-/-}$→$Ldlr^{-/-}$ *Chimeric Mice Have Hypercholesterolemia, but No Differences Are Observed in Lesion Size, Cellularity, or Apoptosis*

Analysis of brachiocephalic artery atherosclerotic lesions showed no significant difference in lesion area between WT→$Ldlr^{-/-}$ and $Fas^{-/-}$→$Ldlr^{-/-}$ mice (Figure 1B). There were no significant differences in lesion cellularity or apoptosis as assessed by DAPI-positive and TUNEL-positive cells, respectively (Figure 1, C and D).

Total plasma cholesterol levels were increased in $Fas^{-/-}$→$Ldlr^{-/-}$ versus WT→$Ldlr^{-/-}$ chimeric mice (593 ± 129, versus 330 ± 38 mg/dL in WT chimeric; $P < 0.001$) (Figure 2A). Lipoprotein profile analysis on pooled samples showed increased very low density

Table 1. Absence of Lymphoproliferation in Hematopoietic $Fas^{-/-}$→$Ldlr^{-/-}$ Chimeric Mice

	WT→$Ldlr^{-/-}$	$Fas^{-/-}$→$Ldlr^{-/-}$	P value
Body weight (g)	19.7 ± 1.3	20.2 ± 0.6	NS
Spleen weight (mg)	108.9 ± 28.3	108.9 ± 39.8	NS
Spleen/body weight (mg/g)	5.6 ± 1.3	5.4 ± 1.9	NS
Lymphadenopathy	Not detected	Not detected	

Lymphoproliferation parameters were assessed in WT→$Ldlr^{-/-}$ (n = 15) and $Fas^{-/-}$→$Ldlr^{-/-}$ (n = 11) chimeric mice after 16 weeks on an 0.5% cholesterol diet. Data are reported as means ± SD; NS, not significant.

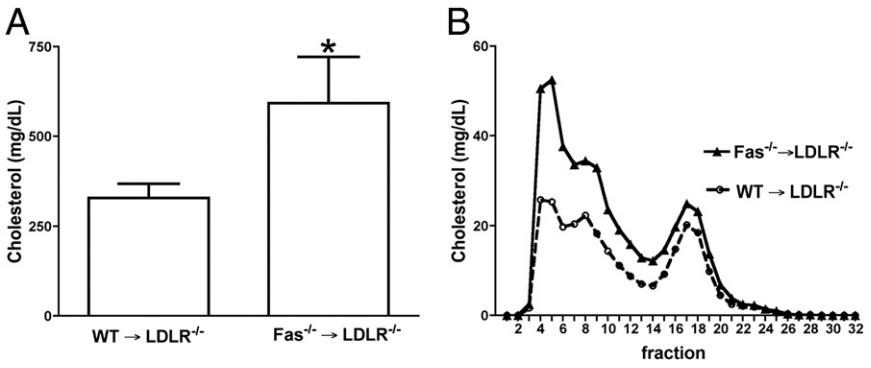

Figure 2. Hypercholesterolemia is increased in hematopoietic $Fas^{-/-}$→$Ldlr^{-/-}$ chimeric mice. WT→$Ldlr^{-/-}$ (n = 15) and $Fas^{-/-}$→$Ldlr^{-/-}$ mice (n = 11) were generated and fed with an 0.5% cholesterol diet for 16 weeks. **A:** Total plasma cholesterol levels. *P < 0.001, Student's t-test. Data are reported as means ± SD. **B:** Fast protein liquid chromatography profiles were performed on pooled plasma samples from WT→$Ldlr^{-/-}$ and $Fas^{-/-}$→$Ldlr^{-/-}$ mice.

lipoprotein (VLDL) and low density lipoprotein (LDL) levels in $Fas^{-/-}$→$Ldlr^{-/-}$ chimeric mice, compared with WT→$Ldlr^{-/-}$ mice (Figure 2B).

Markers of Systemic Inflammation Are Elevated in Hematopoietic Fas$^{-/-}$→Ldlr$^{-/-}$ *Chimeric Mice*

Plasma levels of CXCL1/KC, CCL2/MCP-1, IL-6, IL-10, IL-12 subunit p70 (IL-12p70), and soluble FasL were significantly elevated in $Fas^{-/-}$→$Ldlr^{-/-}$ versus WT→$Ldlr^{-/-}$ mice (Table 2). No significant differences between the two groups were observed for CXCL2/MIP-2, TNF-α, and IFN-γ. Plasma levels of CXLCL1/KC, CCL2/MCP-1, and IL-12p70 were also determined in $Fas^{-/-}$ (n = 4) and WT (n = 3) mice, which were not subjected to transplantation. In these untreated mice, IL-12p70 was below limits of detection in both $Fas^{-/-}$ and WT, in contrast to the marked elevation observed in the $Fas^{-/-}$→$Ldlr^{-/-}$ chimeric mice (Table 2). Similarly, levels of CXCL1/KC and CCL2/MCP-1 were not significantly different between untreated $Fas^{-/-}$ and WT mice (CXCL1/KC: 791 ± 103 pg/mL in $Fas^{-/-}$ versus 1275 ± 229 pg/mL in WT; CCL2/MCP-1: 105 ± 53 pg/mL in $Fas^{-/-}$ versus 142 ± 38 in WT; means ± SEM), whereas levels of these two chemokines were markedly elevated in $Fas^{-/-}$→$Ldlr^{-/-}$ versus WT→$Ldlr^{-/-}$ chimeric mice (Table 2).

The H&E stained sections of lungs, kidneys, liver, and spleen showed inflammatory cell infiltrates surrounding parenchymal microvasculature in the $Fas^{-/-}$→$Ldlr^{-/-}$ group. As shown by immunocytochemistry analysis, the cellular infiltrate was rich in T lymphocytes and B lymphocytes, as well as macrophages (Figure 3). Similar foci of microvascular inflammation were also observed in the spleen and kidney (data not shown).

Discussion

In the present study, mice were generated to examine the effect of hematopoietic Fas deficiency on atherosclerotic lesion development. Whether the apoptotic or the nonapoptotic function of macrophage Fas expression predominates *in vivo* is unknown. No differences were found in brachiocephalic artery lesion area, cellularity, or vessel wall apoptosis between $Fas^{-/-}$→$Ldlr^{-/-}$ versus WT→$Ldlr^{-/-}$ chimeric mice. We focused our analysis on brachiocephalic artery lesions, because the brachiocephalic artery is a highly reproducible site of lesion formation throughout various stages of lesion development.[22] Although many studies use the aortic root or *en face* staining with Oil Red O, both of these approaches have major limitations. A site with comparable flow disturbance to the aortic root of the mouse does not exist in humans, and effects of gene deletion on lesions in the aortic root are often distinct from other aortic sites.[23] Also, *en face* staining of the aorta with Oil Red O is limited to evaluation of lipid accumulation and does not allow evaluation of cell type distribution within lesions or other potential molecular mediators. Most importantly, the brachiocephalic vessel has been shown to model the characteristics of advanced atherosclerotic lesions in humans.[24]

Table 2. Hematopoietic $Fas^{-/-}$→$Ldlr^{-/-}$ Chimeric Mice Have Elevated Systemic Cytokine and Chemokine Levels

	WT→>$Ldlr^{-/-}$ (pg/mL)	$Fas^{-/-}$→$Ldlr^{-/-}$ (pg/mL)	P value*	Lower limit of detection (pg/mL)
CXCL1/KC	541 ± 311	1434 ± 832	<0.01	10.8
CXCL2/MIP-2	46 ± 17	37 ± 6	NS	40.4
CCL2/MCP-1	Not detected	2112 ± 1153	<0.01	148.4
IL-6	Not detected	119 ± 86	<0.01	4.2
IL-10	Not detected	262 ± 209	<0.01	5.1
IL-12 subunit p70	Not detected	4701 ± 3541	<0.01	89.7
TNF-α	Not detected	Not detected	NS	5.6
IFN-γ	Not detected	Not detected	NS	30.4
Fas ligand	13 ± 7	71 ± 51	<0.01	7.2

Plasma cytokines, chemokines, and FasL levels were determined in WT→$Ldlr^{-/-}$ (n = 15) and $Fas^{-/-}$→$Ldlr^{-/-}$ (n = 11) chimeric mice after 16 weeks on an 0.5% cholesterol diet. WT, wild type. Data are reported as means ± SD.

*P values were determined by Student's t-test (MIP-2, FasL) or Mann-Whitney U-test (remainder). NS, not significant.

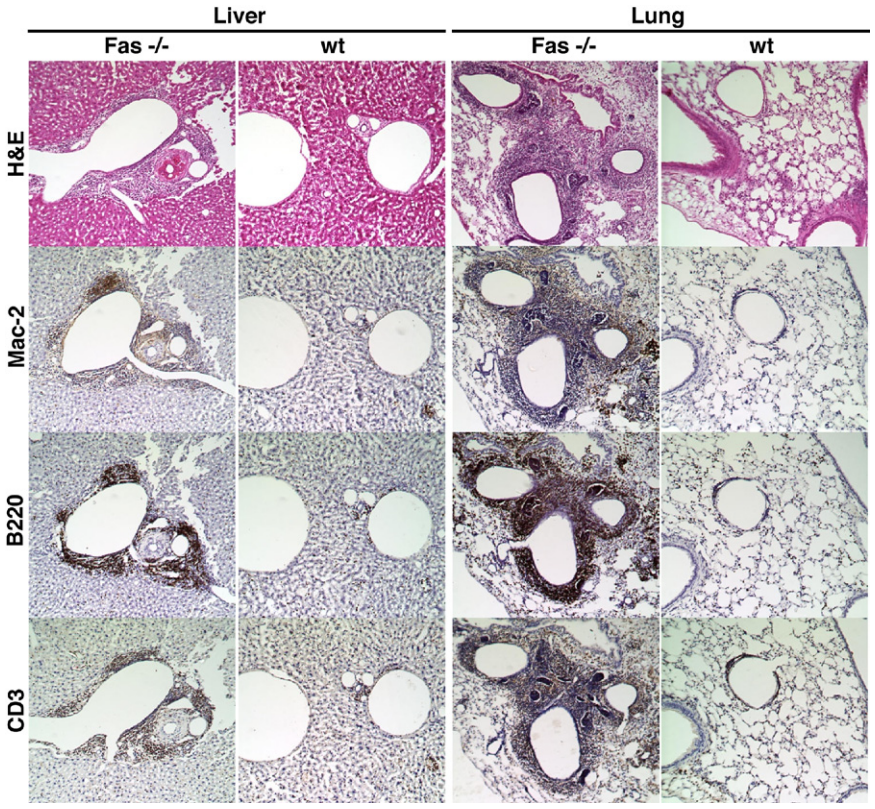

Figure 3. Microvascular inflammation is observed in hematopoietic $Fas^{-/-}\rightarrow Ldlr^{-/-}$ chimeric mice. Liver and lung tissue from $Fas^{-/-}$ and WT hematopoietic chimeras generated on $Ldlr^{-/-}$ background were evaluated after 16 weeks on an 0.5% cholesterol diet. Adjacent sections were stained with H&E, the macrophage antibody Mac-2, the B lymphocyte antibody B220, or the T lymphocyte antibody CD3. Original micrographs, ×20.

Although it would have been desirable to analyze lesions at later time points, it was not possible to keep $Fas^{-/-}\rightarrow Ldlr^{-/-}$ chimeric mice on the atherogenic diet for more than 16 weeks, because of excess mortality in this group. In the original description of $Fas^{-/-}$ mice, Adachi et al[12] reported 50% mortality by 5 months, in keeping with the present results. Thus, the increased mortality we observed is unrelated to the transplantation procedure or the $Ldlr^{-/-}$ background, but rather is intrinsic to complete absence of Fas. The reasons for the increased mortality in the $Fas^{-/-}$ mice are not understood.

Our findings extend the results of previous studies examining the role of dysregulated FasL/Fas signaling in atherosclerosis (Table 3).[15,25–27] Global deficiency of Fas signaling leads to a modest increase in aortic root lesion area in $Apoe^{-/-}$ mice, and ineffective apoptosis was the hypothesized mechanism for accelerated atherogenesis.[15] Another study showed an approximately 20% increase in aortic root lesion size (cross-sectional) and *en face* lesion area.[25] Both of these studies used *lpr* mice, which carry a leaky Fas mutation that may result in expression of small amounts of intact Fas mRNA or pro-

Table 3. Comparison of Global and Hematopoietic Fas-Deficient and FasL-Deficient Models and Their Effect in Mouse Models of Atherosclerosis

	$Fas^{-/-}\rightarrow Ldlr^{-/-}$ hematopoietic Fas-deficiency	*lpr*/$Apoe^{-/-}$ global Fas-deficiency	*gld*$\rightarrow Ldlr^{-/-}$ hematopoietic FasL-deficiency	*gld*/$Apoe^{-/-}$ global FasL-deficiency
Atherosclerotic lesion area	Unchanged	Up	Up	Up
Site of lesion analysis	Brachiocephalic artery cross-section	Aortic root cross-section (Refs. 17 and 25) and *en face* aorta (Ref. 25)	Aortic root cross-section	*en face* aorta
Age of lesion (weeks)	16	20 (Ref. 17) to 24 (Ref. 25)	12	12
Diet	0.5% cholesterol	Normal chow	Western	Western
Cholesterol levels, compared with control	Up	Down	Unchanged	Down
Lymphoproliferation (splenomegaly, adenopathy)	None	+/+	+/Not reported	+/+
Systemic chemokine and cytokine levels	Up	Not reported	Down	Not reported
References	Present study	Adachi et al[17]; Ma et al[25]	Gautier et al[26]	Aprahamian et al[27]

Unchanged, unchanged compared to control; up, increased compared to control; down, decreased compared to control.

tein.[28,29] In the present study, we used the *Fas*$^{-/-}$ strain generated by Adachi et al,[12] and we confirmed complete absence of hematopoietic Fas expression in the hematopoietic *Fas*$^{-/-}$→*Ldlr*$^{-/-}$ chimeric mice by flow cytometry.

Fasl$^{gld/gld}$ mice (generalized lymphoproliferative disease; *gld*) carry a point mutation near the carboxy terminal of the FasL gene. This results in expression of a nonfunctional protein, incapable of inducing Fas-mediated apoptosis.[30] The effect of nonfunctional FasL on atherosclerosis was previously examined by generation of *gld*→*Apoe*$^{-/-}$ chimeric mice; *en face* plaque area in the aorta of *gld*→*Apoe*$^{-/-}$ mice was increased threefold, relative to *Apoe*$^{-/-}$ controls.[27] Similar findings were noted when *gld* expression was restricted to the hematopoietic compartment using bone marrow transplants into *Ldlr*$^{-/-}$ recipient mice; approximately a 60% increase in lesion area was observed in the aortic root.[26] The use of Western-type diets for both the *gld*→*Apoe*$^{-/-}$[27] and *gld*→*Ldlr*$^{-/-}$ models[26] should be noted, because results may be confounded by effects of metabolic syndrome and TH2 versus TH1 immune response.[31] The high-cholesterol diet used in the present study had been shown to induce hypercholesterolemia and extensive atherosclerosis in *Ldlr*$^{-/-}$ mice without the features of metabolic syndrome (obesity, hyperglycemia, hypertriglyceridemia, insulin resistance).[18] In the present study, restriction of *Fas*$^{-/-}$ deficiency to hematopoietic elements was not associated with enhanced atherosclerotic lesion formation. Nonetheless, differences in mouse models, diet, lesion age, and site of lesion analysis among the relevant studies (Table 3) limit the extent to which study results can be directly compared.

Fas-mutant or FasL-mutant chimeric mice have also been used to study other models of inflammation, with varying outcomes. In an experimental autoimmune uveitis model, WT mice reconstituted with *lpr* or *gld* bone marrow had improved ocular inflammation scores.[32] In a model of Fas-mediated acute lung injury, WT mice reconstituted with *lpr* bone marrow were not protected from alveolar damage, as evidenced by increases in tissue caspase 3 activation, total lung neutrophil content, and alveolar permeability.[33]

A notable feature in our model is the marked increase in the level of several circulating proatherogenic and antiatherogenic cytokines or chemokines in the *Fas*$^{-/-}$→*Ldlr*$^{-/-}$ chimeric mice, compared with WT→*Ldlr*$^{-/-}$ chimeric mice. We tested several of the mediators that were markedly elevated in the *Fas*$^{-/-}$→*Ldlr*$^{-/-}$ chimeric mice (CXCL1/KC, CCL2/MCP-1, and IL-12p70) and found no difference in levels between *Fas*$^{-/-}$ mice and WT mice that had not been subjected to transplantation, suggesting that the elevation of cytokines and chemokines in the *Fas*$^{-/-}$→*Ldlr*$^{-/-}$ chimeric mice is a response to transplantation into the *Ldlr*$^{-/-}$ background. There is ample evidence on the proatherogenic effects (CXCL1/KC,[34] CCL2/MCP-1,[35,36] and IL-12[37]) and antiatherogenic effects (IL-6[38,39] and IL-10[40]) of these mediators, as assessed by knockout mouse models. Soluble FasL is capable of activating NF-κB and promotes autoimmunity and tumorigenesis through nonapoptotic mechanisms.[41,42]

As already noted, the excess mortality in *Fas*$^{-/-}$→*Ldlr*$^{-/-}$ group is consistent with observations by Adachi et al,[12] who reported a 50% mortality rate at 5 months in *Fas*$^{-/-}$ mice. Because no other causes for excess mortality in *Fas*$^{-/-}$→*Ldlr*$^{-/-}$ group were found (no graft failure, infection, or lymphoproliferation), the increased mortality in the *Fas*$^{-/-}$→*Ldlr*$^{-/-}$ mice is likely due to this intrinsic effect of Fas deficiency, compounded by the proinflammatory milieu generated in the *Ldlr*$^{-/-}$ background, as evidenced by the development of microvascular inflammation, neutrophilia, and thrombocytopenia in this group. The absence of lymphadenopathy or splenomegaly in the *Fas*$^{-/-}$→*Ldlr*$^{-/-}$ mice, compared with the *Fas*$^{-/-}$ mice, is also notable. The most likely explanation is that the altered immune milieu with elevation of multiple cytokines and chemokines acts to suppress lymphoproliferation.

In summary, hematopoietic Fas deficiency is not associated with enhanced early atherosclerosis, despite worse hypercholesterolemia and systemic inflammation. Differences in mouse strains (*Fas*$^{-/-}$ versus *lpr*), site of lesion analysis, lymphoproliferation, and systemic inflammation may explain the discord between the present results and previously published findings of worse atherosclerosis associated with dysregulated FasL/Fas signaling. Recognition of these confounding factors increases our understanding of the role of FasL/Fas signaling in atherosclerotic lesion development.

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