Lymphocytes Are Not Required for the Rapid Onset of Coronary Heart Disease in Scavenger Receptor Class B Type I/Apolipoprotein E Double Knockout Mice

Sharon L. Karackattu, Michael H. Picard, Monty Krieger

Objective—Scavenger receptor class B type I (SR-BI)/apolipoprotein E (apoE) double knockout (dKO) mice exhibit many features of human coronary heart disease (CHD), including occlusive coronary atherosclerosis, cardiac hypertrophy, myocardial infarctions, and premature death. Here we determined the influence of B and T lymphocytes, which can contribute to atherosclerosis, ischemia–reperfusion injury, and cardiomyocyte death, on pathology in dKO mice.

Method and Results—The lymphocyte-deficient SR-BI/apoE/recombination activating gene 2 (RAG2) triple knockout mice and corresponding dKO controls generated for this study exhibited essentially identical lipid-rich coronary occlusions, myocardial infarctions, cardiac dysfunction, and premature death (average lifespans 41.6±0.6 and 42.0±0.5 days, respectively).

Conclusions—B and T lymphocytes and associated immunoglobulin-mediated inflammation are not essential for the development and progression of CHD in dKO mice. Strikingly, the dKO mice bred for this study (mixed C57BL/6×SV129×BALB/c background; strain 2) compared with the previously described dKO mice (75:25 C57BL/6:SV129 background; strain 1) had a shorter mean lifespan and steeper survival curve, characteristics especially attractive for studying the effects of environmental, pharmacological, and genetic manipulations on cardiac pathophysiology. (Arterioscler Thromb Vasc Biol. 2005;25:803-808.)

Key Words: atherosclerosis № HDL receptor № myocardial infarction № RAG2 № echocardiography

Murine models of dyslipidemia, such as the apolipoprotein E (apoE) or low-density lipoprotein (LDL) receptor knockout (KO) mice, have been used extensively to study atherosclerosis. However, even when subjected to high-fat/high-cholesterol diets, all but one of these hypercholesterolemia and atherosclerosis systems usually do not result in spontaneous development of occlusive coronary artery disease, myocardial infarction (MI), cardiac dysfunction, or the premature death that are hallmarks of human coronary heart disease (CHD). The exception, mice doubly deficient for the HDL receptor scavenger receptor class B type I (SR-BI) and apoE (double KO [dKO]) not only exhibit extensive aortic sinus and occlusive coronary arterial atherosclerosis (advanced plaques with fibrous caps, fibrin deposition, and cholesterol clefts), but they also experience severe CHD at a very young age (4 to 6 weeks).1–3 The hearts of dKO mice are hypertrophic and exhibit left ventricular (LV) dilation and multiple, large MIs. Severe cardiac dysfunction is demonstrated by multiple ECG abnormalities (ST segment elevation and depression, anesthesia-induced conductance abnormalities [eg, bradyarrhythmias, atrioventricular blocks]), a 70% reduction in ±dP/dT, and 50% reduced ejection fraction. The mice die between 5 and 8 weeks of age (mean 6 weeks). Thus, the many similarities in CHD of dKO mice and humans raise the possibility that these mice may help in the study of the pathophysiology of CHD and genetic, pharmacological, and environmental approaches for its prevention and treatment.

B and T lymphocytes can modulate development of atherosclerotic lesions and damage to cardiac tissue,4–10 and thus may contribute to pathophysiology in dKO mice. Disruption of the recombination activating genes (RAG1 or RAG2)11,12 renders mice B and T cell–deficient and reduces the rate of atherosclerosis development in low-fat chow-fed apoE KO mice. Thus, lymphocytes contribute to but are not essential for murine atherogenesis. The influence of lymphocytes on lesion development in apoE KO mice is dramatically reduced when atherosclerosis is accelerated by a high-fat/high-cholesterol diet that exacerbates hypercholesterolemia.9,13,14 In addition to participating in atherosclerosis in occlusive CHD, lymphocytes may directly contribute to ischemia–reperfusion injury, myocardial damage, and cardiomyocyte death.15–19 For example, anticardiac antibodies and sensitized T lymphocytes have been detected in patients with acute MI.20 Lymphocytes have also been implicated in cardiomyocyte injury during autoimmune myocarditis,21–25 and spontaneous
RAG2-dependent, lymphocyte-mediated immune responses in mice lacking the negative immunoregulatory receptor PD-1 cause dilated cardiomyopathy and death from congestive heart failure.26

Here we established the presence of T cells in the hearts of dKO mice. We explored the roles of lymphocytes in murine CHD1,3 by generating and analyzing B and T cell–deficient SR-BI/apoE/RAG2 triple KO (tKO) and corresponding dKO control mice. Complete deficiency of B and T lymphocytes had no discernable effects on CHD and premature death. Thus, lymphocytes and associated antibody-driven inflammation are not essential for cardiac pathology in dKO mice.

Materials and Methods
SR-BI(−/−)/apoE(−/−)/RAG2(−/−) tKO mice and control SR-BI(−/−)/apoE(−/−)/RAG2(+/+ ) dKO (strain 2) mice were generated by crossing SR-BI(+/−)/apoE(−/−)/RAG2(+/+) females (75:25 C57BL/6:SV129 background; strain 1) with SR-BI(−/−)/RAG2(−/−) males (mixed C57BL/6×SV129×BALB/c background).27 The offspring SR-BI(+/−)/apoE(+/−)/RAG2(+/−) females were then crossed to sibling SR-BI(−/−)/apoE(+/−)/RAG2(+/−) males to generate littermate isolates of tKO and dKO (strain 2) mice as well as breeder mice that were used to maintain the colonies and generate subsequent experimental animals. dKO (strain 2) animals differed somewhat (see below) from the previously described “strain 1” dKO (75:25 C57BL/6:SV129 background) mice.28 Unless otherwise noted, all dKOs used were from strain 2. For descriptions of all other materials and methods, demonstration of B and T cell deficiencies of the tKO mice, analysis of hematocrits, and a description of lymphoid tissues, please see the online data supplement (available online at http://atvb.ahajournals.org).

Results
At 5 to 6 weeks of age, the hearts of dKO mice exhibit extensive fibrosis around the ventricular outflow tract and patchy MIs in the apex, right ventricular wall, and interventricular septum.3 Figure 1A shows a representative trichrome-stained longitudinal section (41-day-old female). Healthy myocardium is stained red and fibrotic tissue blue. Although hematoxylin and eosin staining suggested extensive inflammatory infiltration,3 the role of B or T lymphocytes in this CHD had not been directly examined.

Immunohistochemical Analysis of Lymphocytes in dKO Hearts
The pan B-cell marker anti-CD19 did not stain heart sections (data not shown), indicating these cells were absent but not eliminating a potential role for immunoglobulin-mediated autoimmune inflammatory heart disease.28,29 However, clusters of cells that tended to coincide with regions of immune-infiltrated and damaged tissue stained with antibodies against the T-cell marker CD4 (Figure 1B and C) but not with isotype control antibodies (data not shown), raising the possibility that T lymphocytes might contribute to myocardial injury. Others have suggested that quantitation of lymphocyte infiltration can be confounded by the antibody and immunohistochemical technique used;30 therefore, no attempt was made to count positively staining cells and correlate them with the extent of tissue damage.

To definitively determine whether lymphocytes play a role in cardiac pathophysiology, we generated and characterized a line of SR-BI(−/−)/apoE(−/−)/RAG2(−/−) tKO mice with total lymphocyte deficiency (see supplemental material).12

Figure 1. Histology of dKO and tKO hearts. Low-magnification (A and D) and high-magnification (B and E) images of longitudinal Masson’s trichrome-stained heart sections (healthy myocardium, red; fibrotic tissue, blue) from 41-day-old dKO (A and D) and tKO (B and E) mice are shown. Sections adjacent to those in B (C) and E (F) were stained with an anti–T-cell antibody (anti-CD4; arrows indicate positive cells). Bars=1 mm (A and D) or 50 μm (B, C, E, and F).

The absence of B- lymphocyte function in tKOs was confirmed by analysis of plasma levels of IgG in dKO and tKO mice. dKO mice had plasma total IgG titers similar to those of SR-BI+/+Apoe−/−/Rag2+/+ control mice (0.51±0.12 versus 0.60±0.089 mg/mL; P=0.608). Plasma total IgG titers for tKO mice and SR-BI+/+Apoe−/−/Rag2−/− control mice fell below the range of detection of the assay kit used, even at sample concentrations that were 5 and 50× higher than that used for the plasma of Rag2+/+ control mice, establishing the absence of detectable IgG in tKO mice as expected.

Plasma Lipids and Lipoprotein Profiles
Disruption of the RAG2 (or RAG1) gene has been shown to lower plasma cholesterol levels in apoE(−/−) or LDL receptor(−/−) mice.9,31,32 Thus, we compared the plasma lipid levels in dKO and tKO mice because dyslipidemia (hypercholesterolemia, abnormally high unesterified cholesterol [UC] to total cholesterol [TC] ratio) is thought to be responsible for occlusive atherosclerosis and CHD in dKO mice.2,3 Consistent with previous reports,9,32 there was a small but significant reduction in plasma UC and phospholipids in tKO animals compared with dKOs (UC [mg/dL] 655±30 versus 767±40, respectively, P=0.03; and phospho-
lipids [mg/dL] 586±28 versus 681±34, respectively, 
P =0.04) but no statistically significant differences in plasma TCs (909±38 versus 1001±54 mg/dL, respectively; 
P =0.17) or UC/TC ratios. Fast protein liquid chromatography (FPLC) analysis of plasma lipoproteins (Figure 2) revealed no major differences in the lipoprotein TC profiles (n=4 for each group). The slightly higher amounts of cholesterol in the very-low-density lipoprotein (VLDL)–size fractions from the dKO mice relative to tKO mice is similar to that reported by Reardon et al for apoE KO mice.32 The minimal alterations in plasma lipoproteins by RAG2 gene disruption appear unlikely to differentially influence atherosclerosis and CHD in dKO and tKO mice.

Cardiac Histopathology
Trichrome staining of ≈6-week-old tKO hearts demonstrated extensive myocardial fibrosis similar to that in dKO mice (Figure 1D; compare with 1A). As in dKO mice,3 neutral lipid deposits (oil red O staining) and macrophage foam cell formation (F4/80 immunohistochemical staining) coincided with regions of fibrosis (data not shown). The absence of CD4+ cells in the hearts of tKO mice (Figure 1E and 1F) confirmed their B and T cell deficiencies (RAG2(−/−) phenotypes). Thus, neither B nor T cells were required for extensive fibrosis/infarction.

Figure 3 shows that lipid-rich occlusive atherosclerotic lesions in coronary arteries of tKO mice were similar to those in dKO mice. There were no significant differences in the numbers of nonoccluded, partially occluded (<50% occluded) and completely occluded (>50% occluded) coronary arteries (n=4 for each group). Severely occluded arteries were prevalent in areas with myocardial fibrosis, especially near the upper ventricular outflow regions. Some occlusions contained significant cellular components; others were predominantly acellular. Thus, lymphocytes did not markedly influence the nature of the occlusive coronary disease and cardiac damage.

Cardiac Structure and Function
Echocardiography was used to assess cardiac function and hypertrophy33–37 in lightly anesthetized (pentobarbital; 25 mg/kg IP) dKO and tKO mice and their SR-BI–positive littermate controls (Table). No significant differences between RAG2(−/−) and RAG2(+/+ ) control mice were observed; therefore, all controls were pooled.

The values of posterior wall thickness (PWT) and LV mass (absolute and normalized to body weight) for the dKO and tKO mice were not significantly different from each other but

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Figure 2. Lipoprotein cholesterol profiles from dKO and tKO mice. Plasma lipoproteins from 39-day-old dKO and tKO mice were size fractionated (Superose 6-FPLC) and TC in each fraction (mg/dL plasma) determined. Chromatograms are representative of multiple, independent determinations. VLDL indicates very-low-density lipoprotein; IDL, intermediate-density lipoprotein; HDL, high-density lipoprotein.

Figure 3. Coronary occlusions in dKO and tKO mice. Oil red O (A and B)– and trichrome (C and D)–stained coronary artery sections from 39-day-old dKO (A and C) and 43-day-old tKO (B and D) mice. Bar=20 μm.

Echocardiographic and Gravimetric Analyses

<table>
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<th>FS (%)</th>
<th>PWT (mm)</th>
<th>PWT/BW (mm/g×100)</th>
<th>LV Mass (g)</th>
<th>LV Mass/BW (×100)</th>
<th>HW/BW Ratio (×100)</th>
<th>Heart Rate (bpm)</th>
</tr>
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<td>Control</td>
<td>50.1±5.0 (10)</td>
<td>0.65±0.03 (10)</td>
<td>3.04±0.19 (6)</td>
<td>0.062±0.006 (10)</td>
<td>0.266±0.015 (6)</td>
<td>0.509±0.010 (13)</td>
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<td>dKO</td>
<td>26.1±6.2 (8)</td>
<td>0.80±0.04 (7)</td>
<td>4.96±0.52 (4)</td>
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<td>0.106±0.013 (10)</td>
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<td>P ANOVA</td>
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<td>0.0128</td>
<td>0.0003</td>
<td>0.0019</td>
<td>0.0036</td>
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<td>P (dKO vs tKO)</td>
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<td>0.4615</td>
<td>0.4688</td>
<td>0.6505</td>
<td>0.5054</td>
<td>0.2405</td>
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</table>

BW indicates gravimetric body mass; HW, gravimetric heart mass.

Results for RAG2(+/+) and RAG2(−/−) SR-BI–positive littermates of dKO and tKO mice were combined as controls; n in parentheses.

P ANOVA all 3 groups; P (dKO vs tKO) from unpaired Student t test.

FS indicates fractional shortening; PWT, posterior wall thickness; BW, gravimetric body mass; HW, gravimetric heart mass.
were greater than those of controls. The quantitative effects of CHD on the PWT seen here are similar to those in other forms of cardiac dysfunction. The echocardiographically determined increases in heart size in the dKO and tKO mice were confirmed by gravimetric analysis (Table). The heart-to-body weight ratios of the dKO and tKO mice were similar ($P = 0.2405$) and 1.9- and 1.8-fold larger than for age-matched controls ($P < 0.0001$). The body weights for the dKO and tKO mice were similar (16.8 ± 0.8 g [n = 19] and 16.4 ± 0.4 g [n = 17], respectively) and smaller than that of their littermate controls (18.3 ± 0.6 g [n = 13]). The smaller size of SR-BI(−/−)/apoE(−/−) mice compared with SR-BI–positive controls was reported previously. 

LV fractional shortening (FS; [LV end-diastolic diameter − LV end-systolic diameter/LV end-diastolic dimension]) was used as a measure of the systolic function of the heart. Figure 4 shows representative M-mode and 2D echocardiographic images of control, dKO, and tKO mice. The mean FS for control mice was 50.1% ± 5.0%, corresponding to data reported by several groups for conscious38–40 mice undergoing conscious echocardiography as reported by Takuma et al40 and unrestrained mice undergoing telemetry,39 suggesting that the low dose of anesthetic used did not depress heart rate in the control mice. However, electrocardiography of dKOs has shown these mice to be hypersensitive to certain anesthetic agents. Their rapid deterioration in health and short lifespans prevent these mice from enduring the stress of multiple training sessions required for conscious, unanesthetized echocardiography. Previous electrocardiographic studies have shown that conscious unanesthetized dKO mice exhibit reduced heart rates as they approach the terminal stage of disease (data not shown). Therefore, even if electrocardiographic data were obtained without anesthesia, it is likely that the dKOs and tKOs would still have exhibited heart rates significantly lower than control mice. Given these complications, it is not possible to distinguish with certainty the effects of anesthesia from those of advanced disease on heart rate and echocardiographic data obtained from dKOs and tKOs, although it seems likely that the abnormalities observed were attributable, at least in part, to the underlying pathology and not solely consequences of enhanced sensitivity of these mice to anesthetics.

**Effects of Immunodeficiency on Survival**

We next determined whether inactivation of the RAG2 gene altered the life expectancies of these mice. Figure 5 shows
These differences are presumably attributable to their different genetic backgrounds. The survival curves from tKO (red line; mixed C57BL/6 × SV129 × BALB/c background; n = 35), dKO (strain 2; black line; C57BL/6 × SV129 × BALB/c background; n = 65), and dKO (strain 1; blue line; 75:25 C57BL/6 × SV129 background; n = 61) mice.

Figure 5. Effect of RAG2 deficiency and genetic background variation on survival of dKO mice. Survival curves from tKO (red line; mixed C57BL/6 × SV129 × BALB/c background; n = 35), dKO (strain 2; black line; C57BL/6 × SV129 × BALB/c background; n = 65), and dKO (strain 1; blue line; 75:25 C57BL/6 × SV129 background; n = 61) mice.

Discussion

Low-fat chow–fed SR-BI/apoE dKO mice rapidly develop fatal occlusive atherosclerotic CHD that closely resembles that in humans.1–3 The simultaneous absence of apoE and the HDL receptor SR-BI, both of which have been shown to protect against murine atherosclerosis,4,5 is responsible for hypercholesterolemic dyslipidemia exceeding that observed in either single KO mouse. Advanced atherosclerotic plaques can be seen in the hearts of these mice as early as 4 to 4.5 weeks of age1,3 and appear to be critically important for CHD pathogenesis.1–3

Here we examined the role of lymphocytes in the CHD of dKO mice by generating immunodeficient tKO mice, which are dKO mice lacking B and T cells because of homozygous disruption of the RAG2 recombinase gene.12 We compared the phenotypes of dKO and tKO mice because previous studies have implicated lymphocytes in atherosclerosis and myocardial damage after ischemic injury (see Introduction). Although occlusive coronary atherosclerosis in dKO mice appears to be the primary cause of CHD and premature death in dKO animals,3 other mechanisms could contribute to pathology. For example, immunoglobulin-mediated inflammatory heart disease can cause murine MI and death, even in the absence of hypercholesterolemia.28,29,44 Thus, analysis of the B and T cell–deficient tKO mice permitted us to determine whether immunoglobulin-mediated or other B and T cell–associated inflammatory heart disease played a role in this CHD model.

We observed that although the immune infiltrate in the damaged myocardium of dKO mice contains T cells, there were apparently no differences in the occlusive coronary atherosclerosis, MI, cardiac dysfunction, and survival of dKO and tKO mice. Thus, immunoglobulin-mediated inflammatory heart disease is not a critical underlying mechanism in CHD in dKO mice, and B and T cells do not play a key role in the onset or progression of disease in this model. These findings are consistent with the previous observations that B and T cells can influence, but are not essential for, murine atherosclerosis and that their influence on atherogenesis is difficult to detect in apoE KO mice with high-fat diet–induced, exceptionally high hypercholesterolemia.9,14 In dKO and high-fat fed apoE KO mice, the extreme hypercholesterolemia appears to eclipse the influence of B- and T-cell deficiency on pathology. Additional studies are necessary to determine the influence on CHD in dKO mice of other immune cells, including macrophages, neutrophils, and natural killer cells, which are present in RAG2-deficient mice.45–47

Interestingly, the strain of dKO mice generated for this study by extensive inbreeding (strain 2) generated animals that exhibited a significantly shorter period over which the most (82%) mice died (38 to 47 days) than that of the mice used for the first report of this model3 (40 to 56 days; strain 1). Thus, these new strains of dKO and tKO mice appear to be especially attractive for evaluating the consequences of environmental, pharmacological, and genetic manipulations on the pathophysiology in this CHD model. Disease progression is rapid, variation in times of death is low (relatively few animals needed to see statistically significant alterations in disease progression), and attempts to alter disease progression in the tKO mice by treatment with biological agents (eg, antibodies and viral vectors) cannot illicit lymphocyte-mediated immune responses that might otherwise confound analysis of the results from immunocompetent animals. Furthermore, additional analysis of the strain 1 and strain 2 mice may possibly help identify modifier genes that influence the rates of disease progression and premature death in these animals.

Acknowledgments

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Methods:

_Housing and Genotyping of Mice:_ Animals housed in micro-isolater cages in a Virus Antibody Free Facility were fed a standard chow diet ad libitum. Experiments followed MIT and NIH Animal Care guidelines. Experiments other than survival studies were conducted on mice 36 to 45 days of age. No significant differences were observed between males and females. Genotypes were determined by PCR as previously described\(^1\)\(^-\)\(^3\).

_Confirmation of the lymphocyte deficiency of RAG2(-/-) animals and qualitative assessment of the lymphoid tissues:_

Animals were phenotyped by flow cytometry (Becton Dickinson, FACScan, CellQuest software) of isolated splenocytes using the following antibodies (BD Pharmingen, La Jolla, CA): anti-b220-FITC (B-cells); anti-cd11b-FITC (monocyte/macrophages); and anti-TCRβ-PE, anti-CD8-FITC, and anti-CD4-PE (T-cells).

Splenocytes derived from all animals tested positive for monocyte/macrophages (not shown). However RAG2(-/-), but not RAG2(+/-) animals, lacked mature B- and T-lymphocytes (data not shown).

_Analysis of circulating leukocytes:_ Flow cytometric analyses of circulating leukocytes with markers for CD4, CD8, TCR-β and B220 confirmed the presence of B- and T-cells in dKOs and the absence of these cells in tKOs. As expected, both dKOs and tKOs had
cells staining positive for the monocyte/macrophage marker CD11b. Whole blood collected from mice was gently pelleted (1500 rpm for 5 minutes) and erythrocytes lysed using 144 mM NH₄Cl/17 mM Tris-HCl pH 7.4. Cells were resuspended in 500 µL PBS/0.5%BSA and blocked with rat anti-mouse CD16/CD32 (Mouse BD Fc Block™, BD Pharmingen 553141) for 10 minutes (1 µL of blocking antibody to 50 µL of cell suspension). Cells were then stained with complimentary combinations of the following antibodies, all from BD Pharmingen: PE-conjugated hamster-anti-mouse TCR-β (#553172), FITC-conjugated rat-anti-mouse CD45R/B220 (#553087), FITC-conjugated rat-anti-mouse CD11b (#557396), FITC-conjugated rat-anti-mouse CD4 (L3T4, #553046) and PE-conjugated rat-anti-mouse CD8a (Ly-2, #553032) for 20 minutes at a final dilution of 1:200 in PBS/0.5%BSA. All antibody incubations were carried out on ice. Cells were washed 1x with PBS/0.25% BSA, resuspended in 300 µL PBS/0.5%BSA and analyzed on the BD FacScan flow cytometry system.

**Analysis of plasma total IgG concentrations**: Plasma total IgG concentrations of dKO and tKO mice were measured using the Easy-Titer® Mouse IgG assay kit (Pierce #23300) according to kit instructions.

**Hematocrits**: Hematocrits of tKO and dKO mice aged 34 – 37 days did not differ significantly from each other but both differed significantly from control values. The effects of the dKO genotype on hematocrit has been reported elsewhere⁴.
<table>
<thead>
<tr>
<th>Control (Percent)</th>
<th>tKO (Percent)</th>
<th>dKO (Percent)</th>
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<tbody>
<tr>
<td>44.4 ± 1.9 (n=6)</td>
<td>30.0 ± 0.8 (n=7)</td>
<td>26.5 ± 1.8 (n=6)</td>
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</table>

*P (tKO vs. dKO) = 0.0929

**Controls are pooled SR-BI+/+ApoE-/-Rag2/- and SR-BI+/+ApoE-/-Rag2+/+ mice (hematocrit values of Rag2/- and Rag2+/+ controls were not significantly different from each other and these values were pooled together).

**Lymphoid tissue:** The tKO, but not the dKO, mice did not have a fully developed thymus or normal sized lymph nodes (as judged by visual inspection, data not shown). Qualitative and preliminary quantitative (gravimetric) analysis indicated that there was little or no difference in the sizes or weights of the spleens of the dKO and tKO mice.

**Plasma Lipid Composition and FPLC Lipoprotein Total Cholesterol Profiles:**

Plasma from nonfasting animals was obtained from blood drawn at sacrifice by centrifugation at 14,000 rpm (Spectrafuge 16M) for 10 minutes at 4°C. Lipid concentrations were determined by enzymatic assays on plasma diluted 1:4 in phosphate buffered saline (PBS) using kits (Cholesterol C-II, Free Cholesterol E and Phospholipids B) from Wako Chemical USA Inc., (Richmond, Virginia, USA). Plasma was diluted 1:4 in elution buffer (154 mM NaCl, 1 mM EDTA, pH 8) and subjected to FPLC analysis (total cholesterol determined for each fraction) either immediately following collection or after storage at 4°C as previously described.³

**Echocardiography:**
M-mode and two-dimensional transthoracic echocardiography were performed on lightly sedated mice (pentobarbital, 25 mg/kg IP) using a 13-MHz linear array ultrasound transducer with the instrument adjusted for maximal frame rate (Acuson Sequoia, Siemens Medical, Mountain View, CA). M-mode echocardiograms were obtained at mid-left ventricle level. Two-dimensional (2D) images consisted of long-axis views of the left ventricle (LV) and short-axis images at basal, mid and apical portions of the left ventricle. The long axis length of the LV was measured from the 2D long axis view and all other measurements were performed on the short axis at the mid LV level. The fractional shortening was calculated as a measure of LV systolic function and the LV mass was calculated by the D' method.

**Gravimetry:**

Mice were weighed and euthanized by Avertin overdose. Intact hearts were removed and rinsed clean of blood with heparin/PBS (10 units/ml) (heparin sodium salt, Sigma). Whole hearts were then blotted dry and weighed using an analytical balance.

**Histology:**

Mice were anesthetized with 2.5% Avertin. Blood was drawn via heart puncture with a heparinized syringe or from the retro-orbital plexus with a heparinized capillary tube. Hearts were collected and rinsed in heparin/PBS, immersed in cold Kreb’s buffer (120 mM NaCl/25 mM NaHCO₃/3.3 mM KH₂PO₄/0.8 mM K₂HPO₄/1.2 mM MgCl₂/1.2 mM CaCl₂/10 mM glucose, pH 7.4) for 30 minutes, embedded in Tissue-TeK OCT compound (Sakura Finetek) and fresh frozen using dry ice/isopentane. Serial cryosections
(10µm) cut onto Fisher MicroProbe Plus slides (Fisher Scientific) were stained with Masson’s Trichrome (Sigma) or Oil red O and hematoxylin.

**Immunohistochemistry:** Cryosections (10µm) fixed in ice-cold acetone for 10 minutes were stained for macrophages (F4/80 (Serotec, diluted 1:10)), B-cells (anti-CD19, clone ID3 Pharmingen diluted 1:10)) and T cells ((anti-CD3, clone 145-2C11 Pharmingen diluted 1:10) [Anti-lymphocyte antibodies stained positive control spleen sections.]). Sections were blocked for 30 minutes at room temperature with 10% non-immune serum in PBS from the host animal of the secondary antibody and incubated with the primary antibody in PBS containing 2% serum at 4°C overnight. Negative controls omitted primary antibodies. Biotinylated secondary antibodies (Pharmingen diluted 1:50) goat-anti-rat Ig (GAR, multiple adsorption) or mouse-anti-hamster were visualized with alkaline phosphatase Vectastain ABC kit and Vector Red substrate (Vector). T-lymphocytes were also detected in 4% paraformaldehyde fixed (6 minutes) cryosections blocked (1 hour, room temperature, 10% normal goat serum (NGS) in PBS) and immunostained with rat-anti-mouse CD4 L3T4 monoclonal antibody (Pharmingen, diluted 1:40) in PBS containing 1% NGS at 4°C overnight followed by GAR and visualization with Vectastain Elite ABC kit (diaminobenzidine substrate,Vector). Negative controls employed isotype-matched control Rat IgG

**Statistical Analysis:**

$P \leq 0.05$ was considered significant (2-tailed, unpaired student’s $t$ test or ANOVA test, GraphPad Prism 4.0). Survival curves employed the Kaplan-Meier function with the logrank test (GraphPad Prism 4.0). Values are expressed as mean ± SEM.


