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/ Mice Lacking Macrophage Bcl-2**

Edward Thorp, Yankun Li, Liping Bao, Pin Mei Yao, George Kuriakose, James Rong,  
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# Brief Report: Increased Apoptosis in Advanced Atherosclerotic Lesions of *ApoE*<sup>-/-</sup> Mice Lacking Macrophage Bcl-2

Edward Thorp, Yankun Li, Liping Bao, Pin Mei Yao, George Kuriakose, James Rong, Edward A. Fisher, Ira Tabas

**Objective**—Macrophage apoptosis plays important roles in atherosclerosis. Bcl-2 is a key cell survival molecule, but its role in macrophage apoptosis in atherosclerosis is not known. The goal herein was to determine the effect of macrophage-targeted deletion of Bcl-2 on macrophage apoptosis in atherosclerotic lesions of *ApoE*<sup>-/-</sup> mice.

**Methods and Results**—*Bcl2*<sub>flox</sub>-*LysMCre* mice were created as a model of macrophage Bcl-2 deficiency. Macrophages from these mice were more susceptible to apoptosis than those from control *Bcl2*<sub>WT</sub>-*LysMCre* mice. The mice were bred onto the *ApoE*<sup>-/-</sup> background and fed a Western-type diet for 4 or 10 weeks. Apoptotic cells were equally very rare in the lesions of both groups of the 4-week-diet mice, and there was no difference in lesion area. However, *Bcl2*<sub>flox</sub>-*LysMCre*;*ApoE*<sup>-/-</sup> plaques from the 10-week-diet protocol had a 40% to 45% increase in apoptotic cells and, in female mice, a ≈25% increase in plaque necrosis ( $P<0.05$ ) compared with *Bcl2*<sub>WT</sub>-*LysMCre* lesions.

**Conclusions**—Macrophage Bcl-2 plays a protective role against macrophage apoptosis specifically in advanced atherosclerotic lesions of *ApoE*<sup>-/-</sup> mice. (*Arterioscler Thromb Vasc Biol.* 2009;29:169-172.)

**Key Words:** atherosclerosis-pathophysiology ■ apoptosis ■ macrophage ■ animal models of human disease

Macrophage apoptosis can be a critical event in atherosclerosis and occurs at all stages of disease development.<sup>1</sup> In early lesions, macrophage apoptosis is associated with a decrease in lesion cellularity and plaque progression,<sup>2,3</sup> which could be attributable to rapid phagocytic clearance or egress of the apoptotic cells or decreased influx of macrophages. In advanced lesions, however, clearance of apoptotic cells is defective, which leads to secondary necrosis of apoptotic cells.<sup>4–6</sup> Accordingly, we have proposed that macrophage apoptosis in advanced lesions leads to plaque necrosis, which is thought to promote plaque disruption and acute clinical events in humans.<sup>7</sup> In support of this idea, vulnerable necrotic human plaques have increased macrophage apoptosis.<sup>8</sup> Moreover, genetic or pharmacological manipulations that decrease macrophage apoptosis in advanced murine lesions decrease plaque necrosis, and vice versa.<sup>9</sup>

## See accompanying article on page 153

Bcl-2 has been on the forefront of cell survival signaling since its discovery more than 20 years ago, yet there is an absence of in vivo causation studies assessing the role of Bcl-2 in atherosclerosis using genetically altered mouse models. This point is critical, because there are a number of other cell survival molecules in lesional cells, including

Bcl-xL, apoptosis inhibitor expressed by macrophages (AIM; SP $\alpha$ ), Mcl-1, and members of the inhibitor-of-apoptosis (IAP) family, and so redundancy might negate the effect of deletion of any one cell survival molecule. Because Bcl-2 is expressed in other lesional cell types and holo-*Bcl2*<sup>-/-</sup> mice have multiple abnormalities at birth,<sup>10</sup> we created macrophage-targeted Bcl-2-deficient mice and studied the effect of this mutation on atherosclerosis in Western diet-fed *ApoE*<sup>-/-</sup> mice. Our studies indicate that macrophage Bcl-2 plays a protective role against macrophage apoptosis specifically in advanced atherosclerotic lesions. Moreover, in the more advanced lesions of female mice, macrophage Bcl-2 also has a modest protective effect against plaque necrosis.

## Materials and Methods

Materials and methods related to cultured macrophages, genetically altered mice, plasma lipid analysis, laser capture microdissection, quantification of atherosclerotic lesions, and statistics appear in the supplemental materials (available online at <http://atvb.ahajournals.org>).

## In Situ TUNEL Assays and Plaque Necrosis

Apoptotic cells in atherosclerotic lesions were detected by the TUNEL (TdT-mediated dUTP nick end labeling) technique using the TMR red in situ cell death detection kit (Roche). Nuclei were stained with DAPI. TUNEL-positive nuclei were counted under an Olympus

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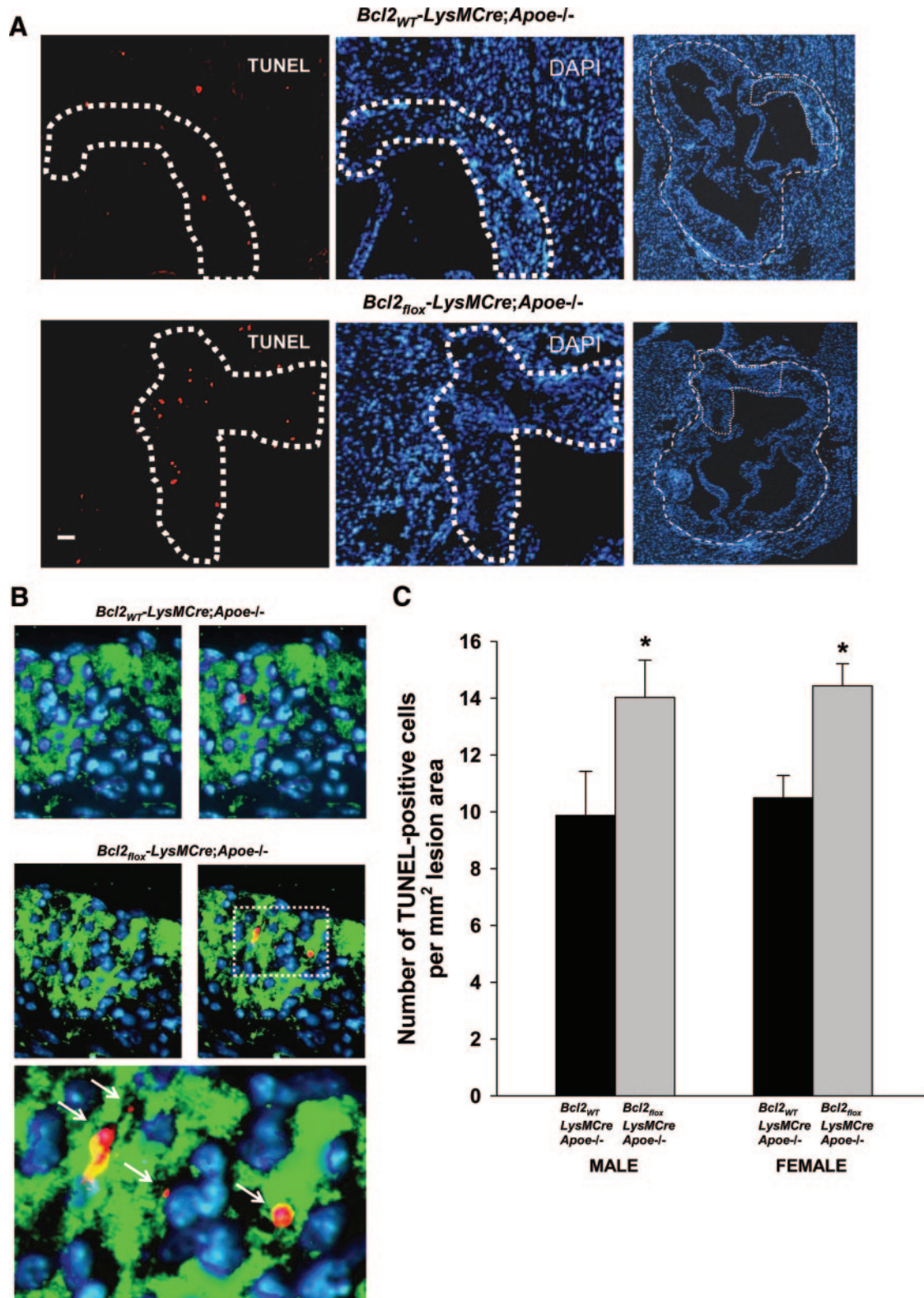
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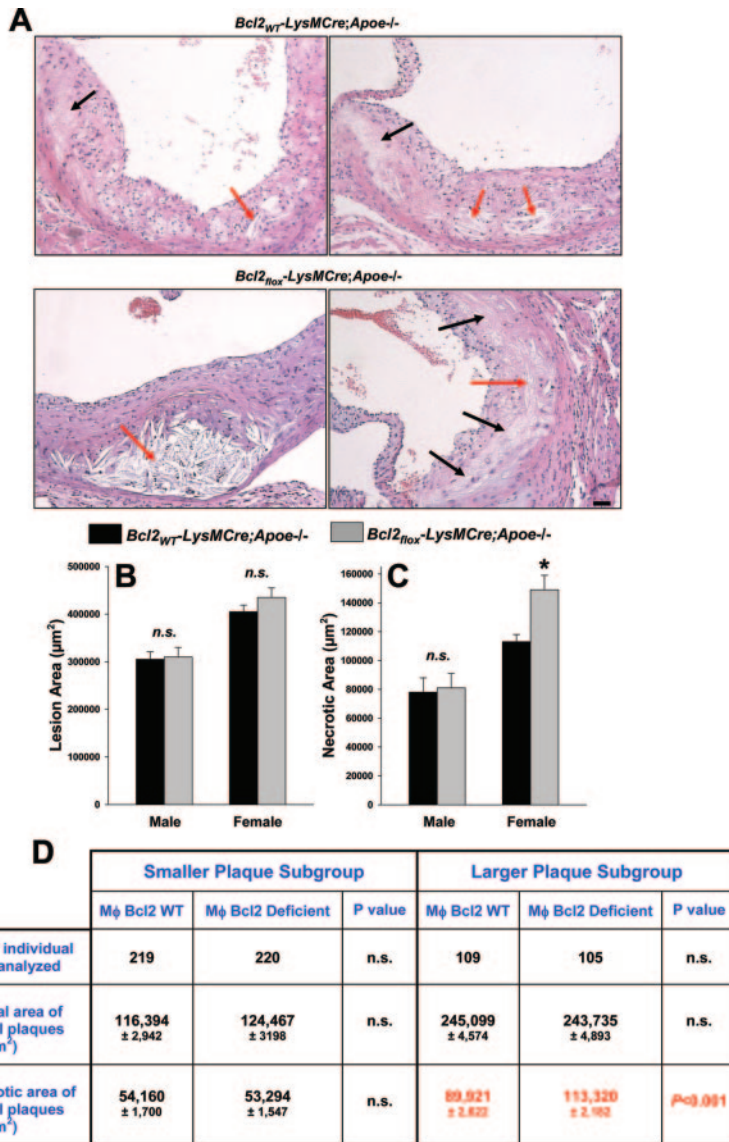
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**Figure 1.** In situ TUNEL staining of aortic root lesions of *Bcl2<sub>WT</sub>-LysMCre;Apoe<sup>-/-</sup>* and *Bcl2<sub>flox</sub>-LysMCre;Apoe<sup>-/-</sup>* mice fed a Western-type diet for 10 weeks. **A**, The left and middle panels show TUNEL- and DAPI-stained aortic root lesions from 10-week-diet-fed female *Bcl2<sub>WT</sub>-LysMCre;Apoe<sup>-/-</sup>* and *Bcl2<sub>flox</sub>-LysMCre;Apoe<sup>-/-</sup>* mice. The full aortic root sections are shown in the right panel (outlined by the dashed lines). Bar, 20  $\mu$ m. **B**, Macrophage (green) and TUNEL (red) staining of female *Bcl2<sub>WT</sub>-LysMCre;Apoe<sup>-/-</sup>* and *Bcl2<sub>flox</sub>-LysMCre;Apoe<sup>-/-</sup>* lesions. Hoechst-stained nuclei are blue. The 2 left images show only macrophage and nuclei. The bottom image is a high-magnification of the area designated by the box in the right image from the *Bcl2<sub>flox</sub>-LysMCre;Apoe<sup>-/-</sup>* lesion. The arrows in this image show apoptotic macrophages. **C**, Quantification of TUNEL-positive nuclei per mm<sup>2</sup> lesion area. The differences between the 2 genotypes for both males and females were statistically significant (asterisks,  $P < 0.05$ ).



**Figure 2.** Images and quantifications of lesion and necrotic areas of aortic root lesions of *Bcl2<sup>WT</sup>-LysMCre;Apoe<sup>-/-</sup>* and *Bcl2<sup>fllox</sup>-LysMCre;Apoe<sup>-/-</sup>* mice fed a Western-type diet for 10 weeks. A, Images of hematoxylin- and eosin-stained aortic root lesions from 10-week-diet-fed female *Bcl2<sup>WT</sup>-LysMCre;Apoe<sup>-/-</sup>* and *Bcl2<sup>fllox</sup>-LysMCre;Apoe<sup>-/-</sup>* mice. Black arrows, acellular necrotic areas; red arrows, necrotic areas with cholesteryl crystals. Bar, 20 µm. B, Quantification of cross-sectional lesion area. The differences between female vs male lesions in both genotypes are statistically different by Mann-Whitney analysis (*P* < 0.05). C, Quantification of cross-sectional necrotic area. Asterisk, *P* < 0.05. D, Individual plaques in aortic root cross sections from female mice were separated into two groups based on plaque area, <200 000 and >200 000 µm<sup>2</sup>, and then analyzed for plaque necrosis. For each mouse, 6 cross sections were analyzed, and each section had, on average, 3 individual plaques. The differences in necrotic area between larger and smaller plaques for each genotype were statistically significant (*P* < 0.001 by Mann-Whitney).

IX-70 inverted fluorescent microscope. For assessment of macrophage colocalization, macrophages were detected using a rabbit antimacrophage antibody (AIA31240) from Accurate Chemical and Scientific Corporation, and nuclei were stained with Hoechst. Plaque necrosis was quantified by measuring the area of hematoxylin and eosin-negative acellular areas in the intima, as described previously.<sup>11</sup>

**Results**

**Intimal Cell Apoptosis Is Increased in Lesions of *Bcl2<sup>fllox</sup>-LysMCre;Apoe<sup>-/-</sup>* Mice Fed A Western Diet for 10 Weeks But Not 4 Weeks**

Mice with Bcl-2 deficiency in macrophages using the cre-lox strategy (*Bcl2<sup>fllox</sup>-LysMCre*) were created as described in the supplemental materials (supplemental Results and Figure IA and IB). Peritoneal macrophages from these mice were more susceptible than control *Bcl2<sup>fllox</sup>-LysMCre* mice to a variety of apoptosis inducers (supplemental Results and Figure IC and ID). The 2 groups of mice were bred onto the *Apoe<sup>-/-</sup>* background and fed a Western-type diet for 4 or 10 weeks, and the aortic root was examined. The 4-week lesions showed

similar area between the 2 groups of mice, and only rare examples of lesional macrophage apoptosis (supplemental Results).

Background data for the 10-week-diet study are described in supplemental Results and supplemental Figure II. Figure 1A demonstrates and increase in TUNEL-positive nuclei, a measure of apoptosis, in *Bcl2<sup>fllox</sup>-LysMCre;Apoe<sup>-/-</sup>* versus *Bcl2<sup>WT</sup>-LysMCre;Apoe<sup>-/-</sup>* lesions. As expected, the areas of TUNEL staining correlated with macrophage-rich areas in these lesions (Figure 1B). The quantified data for the full cohort of mice is shown in Figure 1C. Examples of hematoxylin and eosin-stained aortic root sections of from female *Bcl2<sup>WT</sup>-LysMCre;Apoe<sup>-/-</sup>* and *Bcl2<sup>fllox</sup>-LysMCre;Apoe<sup>-/-</sup>* mice are shown in Figure 2A. Total lesion area per se was not affected by macrophage Bcl-2 depletion (Figure 2B). Also not affected by macrophage Bcl2 deficiency were plasma levels of tumor necrosis factor (TNF)α and interleukin (IL)-6 (data not shown).

Increases in macrophage apoptosis are translated into increases in plaque necrosis only after further lesion progres-

sion.<sup>12</sup> In the current study, the female mice as a group had larger and more advanced lesions than male mice, and female *Bcl2<sub>flox</sub>-LysMCre;ApoE<sup>-/-</sup>* lesions appeared to have more acellular necrotic areas (black arrows in Figure 2A) as well as necrotic areas with cholesterol crystals (red arrows in Figure 2A). Quantification showed a  $\approx 25\%$  increase in necrotic area in *Bcl2<sub>flox</sub>-LysMCre;ApoE<sup>-/-</sup>* versus *Bcl2<sub>wT</sub>-LysMCre;ApoE<sup>-/-</sup>* female mice ( $P < 0.05$ ) (Figure 2C). Neither fibrous cap thickness nor lesional collagen content were affected by macrophage Bcl-2 deficiency (data not shown).

The difference in the affect of macrophage Bcl-2 deficiency on plaque necrosis could be attributable to a direct effect of sex differences, eg, sex steroids, or to the fact that female lesions were more advanced (see Figure 2B).<sup>12</sup> In an attempt to sort out these possibilities, individual plaques from the female mice were divided into 2 subgroups based on plaque area:  $< 200\,000$  and  $> 200\,000\ \mu\text{m}^2$ . We found a statistically significant difference in necrotic area between the genotypes only in the larger plaque subgroup (Table in Figure 2D). This finding is consistent with the conclusion that macrophage Bcl-2 deficiency has a selective effect on more advanced lesions. There were not enough larger plaques among the male lesions for analysis, and so pending future studies with more advanced male lesions, there is also the possibility of a direct sex effect as well. In summary, macrophage Bcl-2 deficiency is associated with increased advanced lesional macrophage apoptosis and, in large lesions in female mice, an increase in plaque necrosis.

### Discussion

See supplemental materials for a discussion of the findings in this report in relationship to relevant articles in the literature.

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### Disclosures

None.

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## Thorp *et al.*-Macrophage Bcl-2 in Advanced Atherosclerosis

### Online Supplementary Material

## MATERIALS AND METHODS

### Materials

Cell culture media and reagents were from Invitrogen. Low-density lipoprotein (LDL;  $d$  1.020-1.063 g/ml) was isolated from fresh human plasma by preparative ultracentrifugation as previously described.<sup>1</sup> Acetyl-LDL was prepared by reaction of LDL with acetic anhydride.<sup>2</sup> Compound 58035 (3-[decyldimethylsilyl]-N-[2-(4-methylphenyl)-1-phenylethyl] propanamide), an inhibitor of acyl-CoA:cholesterol O-acyltransferase (ACAT), was generously provided by Dr. John Heider, formerly of Sandoz, Inc. (East Hanover, NJ).<sup>3</sup> Antibodies against Bcl-2, Bcl-xl and  $\beta$ -actin were from Santa Cruz Biotechnologies, Inc.

### Eliciting and culturing mouse peritoneal macrophages and assay for macrophage apoptosis

8–10-week old mice were used. Macrophages were harvested by peritoneal lavage three days after intraperitoneal (i.p.) injection of concanavalin A.<sup>4</sup> The cells were cultured in DMEM medium supplemented with 10% FBS and 20% L-cell conditioned medium for 48-72 h, at which point they were typically at ~90% confluence. The cells were incubated with a variety of apoptosis inducers: FC accumulation, 100  $\mu$ g/ml acetyl-LDL + 10  $\mu$ g/ml 58035 for 20 h; oxidized LDL, 100  $\mu$ g/ml copper-oxidized-LDL for 20 h; UV radiation, 15 min at 254 nm, 20 J/cm<sup>2</sup>; and staurosporine, 100 nM staurosporine for 11 h. Early-mid-stage apoptosis was assayed by staining with annexin V and propidium iodine (PI), respectively, using the Vybrant Apoptosis Assay kit #2 (Molecular Probes).<sup>5</sup> The cells were examined immediately using an Olympus IX-70 inverted fluorescent microscope equipped with filters appropriate for fluorescein and rhodamine, and images were obtained using a Cool Snap CCD camera (RS Photometrics) equipped with imaging software from Roper Scientific. Three fields of cells (~650 cells/field) were photographed for each condition, and the number of

annexin V/PI-positive cells in each field were counted and expressed as a percent of the total number of cells.

### **Construction of Bcl-2 targeting vector and generation of ES clones (Figure 1A)**

A 12.5-kb mouse genomic DNA fragment containing exon 2 of the *Bcl2* gene was obtained from a mouse 129 lambda genomic library. A Neo cassette flanked by two *loxP* ("floxed" Neo) sites was inserted as shown in Figure 1A. A 3.5-kb *EcoRI*-*XbaI* fragment was cloned to serve as the short and middle arms. A third *loxP* site along with a new *EcoRI* site was inserted into the *NcoI* site of this fragment, and the modified 3.5-kb fragment was then inserted at the 3' end of the floxed Neo cassette. The long arm was a 6 KB *BglII*-*BglII* fragment, which was inserted at 5' end of the floxed Neo cassette. Ten micrograms of targeting vector, referred to as *Bcl2<sup>flox</sup>*, was linearized by *AscI* and then transfected by electroporation into 129 embryonic stem cells.

### **Generation of *Bcl2<sup>flox</sup>* and *Bcl2<sup>flox</sup>-LysMCre* Mice**

Cells from an ES cell colony that contained homologously recombinant *Bcl2<sup>flox</sup>* were injected into C57BL6/J host blastocysts, which were then implanted into pseudopregnant female mice. Male offspring with 75-90% agouti color, contributed by the ES cells, were bred with C57BL6/J females. Pups that were 100% agouti, indicating germ line transmission, were screened by PCR of the genomic DNA extracted from tail clips. The PCR primers are: *forward 1*: CAGAGCTACCAAAGGGCAAG, *forward 2*: TCGCCTTCTTGACGAGTTCTTC; and *reverse*: TCCAGTGGTCTTCTCCCATC. Heterozygous *Bcl2<sup>flox</sup>* mice were identified and sibmated, and homozygous *Bcl2<sup>flox</sup>* mice resulting from this mating (~25% of the offspring) were bred with homozygous *LysMCre* mice, which were obtained from Dr. Irmgard Förster, Technical University of Munich. In *LysMcre* mice, Cre recombinase is driven by the lysozyme promoter via gene targeting into the lysozyme locus.<sup>6</sup> The pups, which were heterozygous for both *Bcl2<sup>flox</sup>* and *LysMCre*, were then bred with homozygous *Bcl2<sup>flox</sup>* mice to generate the mice used for this study. For the studies described herein, *Bcl2<sup>flox</sup>-LysMCre* and *Bcl2<sup>WT</sup>-LysMCre* were used as experimental and control mice, respectively. For the experimental mice, the *Bcl2<sup>flox</sup>* genotype was homozygous, and for both groups, the *LysMCre* genotype was

heterozygous. For the atherosclerosis studies, *Bcl2<sub>fllox</sub>-LysMCre* and *Bcl2<sub>WT</sub>-LysMCre* mice were bred onto the *Apoe<sup>-/-</sup>* C57BL/6 background. The pups, at 8 weeks of age, were fed a high-fat (21.2%), high-cholesterol (0.2%) Western diet (Harlan Teklad, Madison WI) for 4 or 10 weeks. The number of mice for the atherosclerosis study was 28 for *Bcl2<sub>WT</sub>-LysMCre;Apoe<sup>-/-</sup>* and 23 for *Bcl2<sub>fllox</sub>-LysMCre;Apoe<sup>-/-</sup>*, split approximately equally between males and females.

### **Plasma Lipid Analysis**

Terminal fasting plasma samples were collected via exsanguination after left-ventricular puncture. Plasma lipoprotein profiles were determined by fast performance liquid chromatography (FPLC) on a Superose 6 column at a flow rate of 0.2 ml per minute. Total cholesterol in the plasma and in the FPLC fractions were assayed (Wako).

### **Laser Capture Microdissection (LCM) Analysis of Bcl-2 Expression in Macrophages from Atherosclerotic Lesions**

LCM and RNA extraction was performed as previously described.<sup>7,8</sup> Briefly, frozen proximal aortic sections were fixed in 70% ethanol for 15 s followed by cold acetone for 5 min and then subjected to rapid immunostaining for macrophage marker CD68. Sections were subsequently dehydrated in graded ethanol solutions and cleared in xylene. After air-drying for 30 min, laser capture was performed under direct microscopic visualization on the CD68-positive stained areas by melting of selected regions onto a thermoplastic film mounted on optically transparent LCM caps (Arcturus Engineering, Mountain View, CA). The PixCell II LCM System (Arcturus Engineering) was set to the following parameters: 15- $\mu$ m laser spot size, 40-mW power, 3.0-ms duration. The thermoplastic film containing the microdissected cells was incubated with 200  $\mu$ l of 4 M guanidinium isothiocyanate and 1.6  $\mu$ l of 2-mercaptoethanol for  $\approx$ 10 min on ice. Total RNA was extracted from the LCM samples or from the whole sections using a MicroRNA Isolation Kit (Stratagene) following the manufacturer's instructions. For measurement of macrophage enrichment by LCM, RT-QPCR was used to determine the level of *Cd68* mRNA and the standard control gene encoding cyclophilin A. The level of *Bcl2* mRNA in the LCM samples was then measured by RT-QPCR. The



forward primer and reverse primers for Bcl-2 were: CATCTTCTCCTTCCAGCCTGA and ACGTCCTGGCAGCCATCTC, and the probe was 6FAM-GCAACCCAATGCCCGCTGTG. The reactions were run on a MX4000 multiplex quantitative PCR system (Stratagene), the thermal profile settings were 50°C for 2 min, 95°C for 10 min, then 45 cycles at 95°C for 15 sec and 60°C for 1 min.

### **Quantification of Atherosclerotic Lesions**

At the end of the Western diet feeding period, the mice were fasted overnight, weighed, and anesthetized with isoflurane. The hearts were then perfused *in-situ* with saline and removed. Aortic roots were fixed in 10% formalin, placed in a biopsy cassette, and processed in a Leica tissue-processing machine followed by embedment in paraffin blocks. Paraffin-embedded sections were cut serially at 6- $\mu$ m intervals from the aortic sinus and mounted on slides. Prior to section staining, sections were deparaffinized in xylene and rehydrated in graded series of ethanol. For morphometric lesion analysis, sections were stained with Harris' hematoxylin and eosin. Total intimal lesional area was quantified by averaging six sections which were spaced 30  $\mu$ m apart, from the base of the aortic root. Images were viewed and captured with a Nikon Labophot 2 microscope and analyzed using Image Pro Plus software.

### **Statistical Analysis**

Data are presented as mean  $\pm$  S.E.M. Non-parametric Mann–Whitney test was used to measure the statistical differences in lesion analyses. Student t-test assuming two samples with equal variances was used in other analyses.  $P < 0.05$  was considered statistically significant.

## **RESULTS**

### **Creation of Mice With Bcl-2 Deficiency in Macrophages**

Exon 2 of the murine *Bcl2* gene was flanked by *loxP* sites (*Bcl2*<sub>fllox</sub>) using the scheme depicted in **Supplementary Figure IA**, and the targeting vector was inserted into ES cells through homologous recombination to create *Bcl2*<sub>fllox</sub> mice. Homozygous *Bcl2*<sub>fllox</sub>

mice were crossed with mice in which Cre recombinase is driven by the lysozyme promoter (*LysMCre*) to effect deletion of Bcl-2 in fully differentiated macrophages, but not monocytes.<sup>6</sup> *Bcl2<sup>flox</sup>-LysMCre* mice appeared healthy and active and gained weight similar to wild-type and *Bcl2<sup>WT</sup>-LysMCre* mice (below). As shown in **Supplementary Figure IB**, peritoneal macrophages from these *Bcl2<sup>flox</sup>-LysMCre* showed no detectable Bcl-2 by immunoblot, whereas *Bcl2<sup>WT</sup>-LysMCre* mice expressed the same level of Bcl-2 as in *Bcl2<sup>WT</sup>* mice. Also note that macrophages from *Bcl2<sup>flox</sup>* mice without *LysMCre* showed no decrease in Bcl-2 expression (*lower blot* in Suppl. Fig. IB).

### **Peritoneal Macrophages from *Bcl2<sup>flox</sup>-LysMCre* Mice Show Increased Susceptibility to a Variety of Apoptosis Inducers**

To determine the effect of Bcl2 deficiency on models of macrophage apoptosis that may be relevant to advanced atherosclerosis, we compared macrophages from *Bcl2<sup>WT</sup>-LysMCre* and *Bcl2<sup>flox</sup>-LysMCre* mice for their susceptibility to apoptosis by free cholesterol loading and oxidized LDL, two proposed inducers of lesional apoptosis.<sup>9, 10</sup> As shown in **Supplementary Figure IC**, with quantification displayed in the first pair of bars in **Supplementary Figure ID**, FC-induced apoptosis was enhanced in macrophages lacking Bcl-2. This particular experiment was conducted using a time point in which FC-induced apoptosis was not yet maximal in wild-type macrophages, but enhancement of apoptosis in Bcl2-deficient macrophages was also observed with longer periods of cholesterol loading. The higher susceptibility to apoptosis induced by cholesterol loading was not due to increased lipoprotein uptake or cholesterol accumulation in the Bcl-2-deficient cells. *Bcl2<sup>flox</sup>-LysMCre* macrophages were also more susceptible to apoptosis induced by oxidized LDL (Suppl. Fig. ID, 2<sup>nd</sup> pair of bars) as well as by two more general inducers of apoptosis, UV irradiation and the phosphatase inhibitor staurosporine (Suppl. Fig. ID, 3<sup>rd</sup> and 4<sup>th</sup> pair of bars). Thus, Bcl-2 plays a critical role in protecting macrophages against a variety of apoptosis stimuli, including two of those thought to function in advanced atherosclerosis.

### **Intimal Cell Apoptosis is Not Increased in Lesions of *Bcl2<sup>flox</sup>-LysMCre;ApoE<sup>-/-</sup>* Mice Fed A Western Diet for 4 Weeks**

In preparation for atherosclerosis studies, the *Bcl2<sup>flox</sup>* and *LysMCre* mice were backcrossed into the C57BL6/J background and then crossed with *Apoe<sup>-/-</sup>* C57BL6/J mice. To assess the role of macrophage Bcl-2 in early atherosclerosis, *Bcl2<sup>WT</sup>-LysMCre;Apoe<sup>-/-</sup>* and *Bcl2<sup>flox</sup>-LysMCre;Apoe<sup>-/-</sup>* mice were fed the Western-type diet for 4 weeks. The two groups of mice had similar weights, plasma lipoproteins, and lesion areas in the proximal aorta ( $111,853 \pm 19,894$  vs.  $113,420 \pm 28,842 \mu\text{m}^2$  in *Bcl2<sup>WT</sup>-LysMCre;Apoe<sup>-/-</sup>* and *Bcl2<sup>flox</sup>-LysMCre;Apoe<sup>-/-</sup>*, respectively; n = 10 per group). Macrophage apoptosis in these small lesions was equally very rare or not detectable in either group of mice. Thus, macrophage Bcl-2, unlike the cell survival molecule AIM,<sup>11</sup> does not play an important role in atherogenesis or macrophage apoptosis in early lesions of *Apoe<sup>-/-</sup>* mice.

## DISCUSSION

Macrophage apoptosis is emerging as a key event in atherosclerosis. As such, it is important to understand the role of key cell survival and pro-apoptotic molecules in this process *in vivo*. Bcl-2 is the prototypical cell survival protein,<sup>12</sup> yet its role in macrophage apoptosis *in vivo* has been very difficult to study due to sickness of holo-*bcl2<sup>-/-</sup>* mice and the fact that Bcl-2 may function in multiple cell types in atheromata. In that context, the strategic advance brought forth here is the creation and use of macrophage-targeted Bcl-2-deficient mice to study the role of this key molecule in advanced atherosclerosis. The advantage of this model over bone marrow transplantation is the avoidance of lethal irradiation, which may affect atherosclerosis.<sup>13</sup> With regard to cell specificity, *LysMCre* also deletes floxed genes in neutrophils,<sup>6</sup> but analysis of cells in the 10-week lesions by nuclear morphology and by immunostaining revealed no detectable neutrophils (data not shown). Moreover, Förster and colleagues<sup>6</sup> found that *LysMCre* expression leads to only minor deletion of floxed genes in dendritic cells and does not delete floxed genes in T cells or B cells.<sup>6</sup> However, Chong *et al.*<sup>14</sup> found evidence of partial *LysMCre*-induced deletion of a floxed gene in these cell types in spleen and blood. While it is conceivable that Bcl-2 deletion in these cells could affect atherogenesis, the increase in TUNEL-positive cells observed in our study occurred in macrophage-rich regions of the lesions.

In the first phase of the project, we were able to show definitively that macrophages in advanced atherosclerotic lesions express *Bcl2* mRNA, a point that has been confusing in the literature.<sup>15-17</sup> In terms of its role in advanced lesional macrophage apoptosis, we wondered whether overlapping roles of other cell survival molecules and/or dominant effects of pro-apoptotic molecules would dilute the effect of Bcl-2 deficiency. However, the data show a significant effect of macrophage Bcl-2 deficiency on advanced lesional macrophage apoptosis. The mechanism behind this observation, and perhaps behind the finding that Bcl-2 did not increase macrophage apoptosis in early lesions (below), may be centered on the role of endoplasmic reticulum (ER) stress in advanced lesional macrophage apoptosis.<sup>8</sup> Mechanistic studies with cultured primary macrophages, molecular-genetic causation results in mouse models of advanced atherosclerosis, and immunohistochemistry studies of human coronary and carotid vulnerable plaques have formed a strong base of evidence to support the role of ER stress in advanced lesional macrophage apoptosis<sup>8, 18-23</sup>. In this regard, there are a number of studies demonstrating how Bcl-2 may be particularly important in balancing the pro-apoptotic stimulus of ER stress. For example, Bim is a key pro-apoptotic molecule in ER stress-induced apoptosis,<sup>24</sup> and the pro-apoptotic activity Bim would be expected to be more robust in the absence of Bcl-2, which sequesters and inhibits Bim.<sup>25</sup>

The most important question surrounding macrophage apoptosis during atherosclerosis is its effect on lesion progression. Arai *et al.*<sup>11</sup> used a holo-knockout model to assess the role of another cell survival molecule, AIM ( $SP\alpha$ ), in early atherosclerosis in *Ldlr*<sup>-/-</sup> mice. The authors found that AIM deficiency was associated with increased lesional macrophage apoptosis and decreased lesion area. This important finding, along with other early lesional studies in which pro-apoptotic genes were manipulated,<sup>26-29</sup> established the principle that early lesional macrophage apoptosis decreases lesion cellularity. The mechanism of this effect is likely linked to the process of efficient phagocytic clearance of the apoptotic macrophages in early lesions.<sup>30, 31</sup> In this regard, we found no effect on macrophage apoptosis or lesion area in early atherosclerotic lesions. This finding is consistent with the result that overall

lesion area was not affected in our advanced lesional model. Thus, in early lesional macrophages, AIM appears to play a more important survival role than Bcl-2.

In advanced atherosclerotic lesions, evidence is mounting that macrophage apoptosis is causative for plaque necrosis, an important feature of vulnerable plaques in humans (see Introduction). The mechanism for this effect is likely linked to the observation that phagocytic clearance of apoptotic cells is defective in advanced lesions, because when apoptotic cells are not efficiently cleared, they become secondarily necrotic.<sup>30, 31</sup> Although other mouse studies have established this principle through the manipulation of pro-apoptotic genes<sup>18, 23, 32-34</sup>, the current study is the first in which a cell survival molecule has been knocked out and studied in advanced lesions. The lack of an effect on overall lesion area shown in this study is consistent with the concept that the dead macrophages accumulate as debris in necrotic areas rather than being cleared by phagocytic uptake (efferocytosis) or leaving the lesion altogether.<sup>30</sup> This concept is consistent with the finding that lesion morphology rather than lesion size is more predictive of acute coronary artery events in humans.<sup>35, 36</sup> Regarding the necrotic area data herein, the data show that the increase in advanced lesional macrophage apoptosis in female *Bcl2<sub>flox</sub>-LysMCre;ApoE<sup>-/-</sup>* mice was, as hypothesized, associated with an increase in lesional necrosis. The quantitatively modest nature of this effect, and its absence in male mice, may be due to the fact that overall lesion development was not as high as in other studies of this nature, particularly in the male mice. As noted above, one of our recent studies in this area, in which we followed macrophage apoptosis and plaque necrosis from moderately advanced to very advanced lesions, showed that changes in macrophage apoptosis in lesions preceded changes in plaque necrosis.<sup>34</sup> This finding is consistent with a lag period between the onset of macrophage apoptosis and the subsequent collection of post-apoptotic necrotic cells into necrotic areas. Although our subgroup analysis of larger vs. smaller lesions (Figure 2D) supports the possibility that the smaller lesions of male mice might account for the lack of an effect on necrosis between the two genotypes, there is also the interesting possibility that a direct sex effect, e.g., due to effects of sex steroids, could influence the response of plaques to increased macrophage apoptosis due to Bcl-2

deficiency. Future studies with more advanced atherosclerosis will be needed to test this possibility.

In summary, we have used a new and valuable mouse model to explore the role of the prototypical cell survival molecule—Bcl-2—in macrophage apoptosis in advanced atherosclerotic lesions. Our data indicate that macrophage Bcl-2 expression provides a dampening effect on macrophage apoptosis specifically in advanced atherosclerotic lesions. Given the effect of this genetic manipulation on plaque necrosis in female mice and the likely role of plaque necrosis in lesion disruption and subsequent acute clinical atherothrombotic vascular events in humans, the findings herein add to our understanding of the molecular mechanisms involved in the critical process of benign-to-vulnerable plaque transformation.

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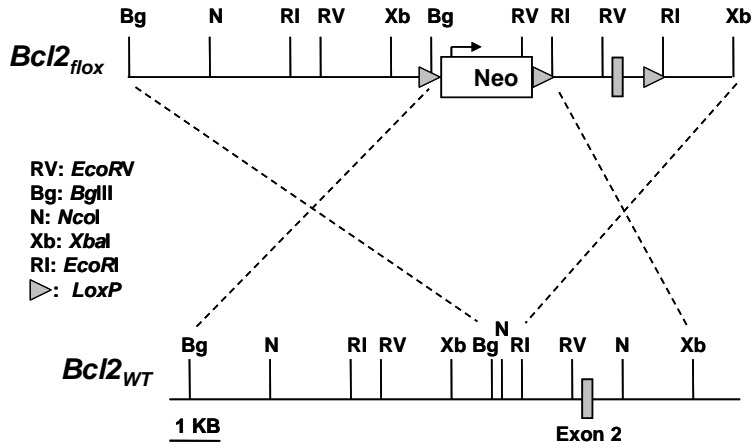
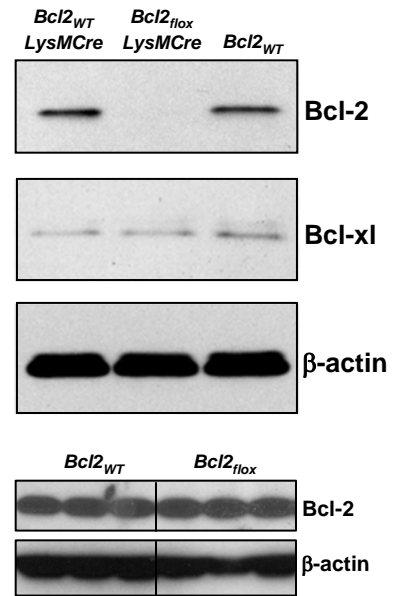
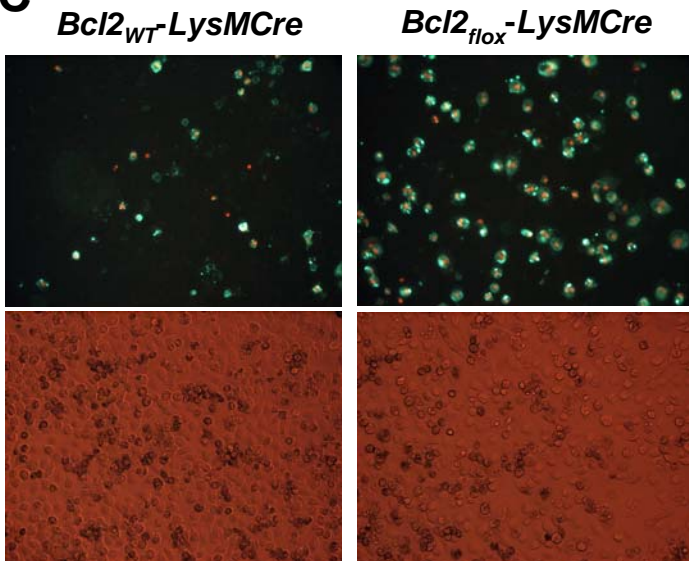
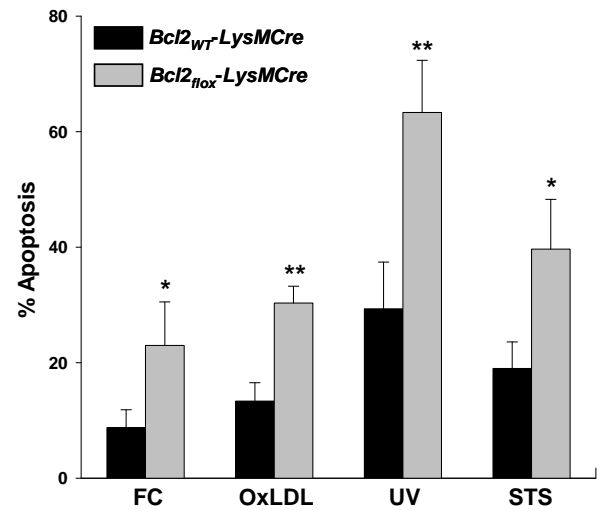
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## FIGURE LEGENDS

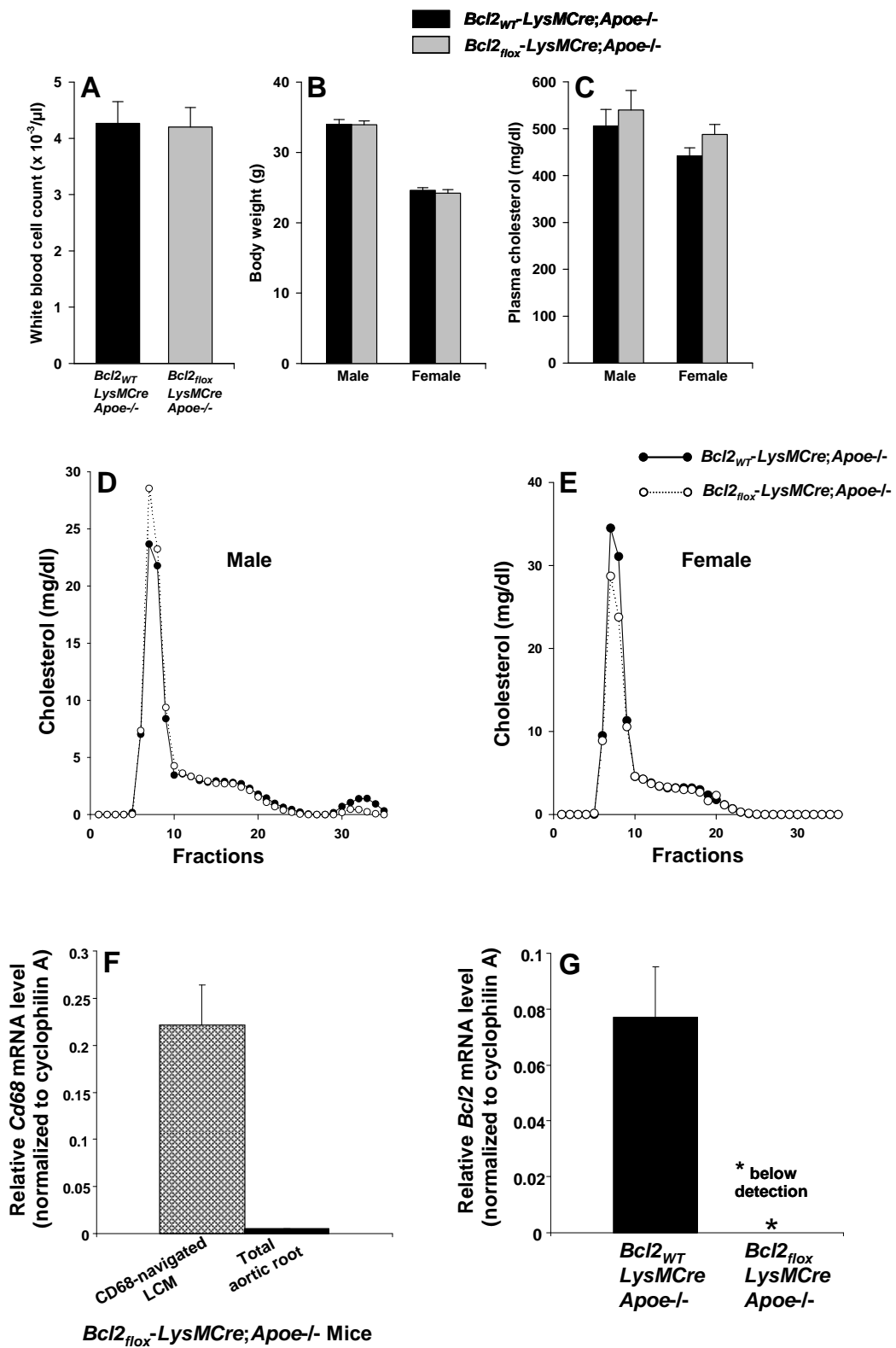
**Online Supplementary Figure I: Design of floxed *Bcl2* gene construct and apoptotic susceptibility of macrophages from *Bcl2<sup>flox</sup>-LysMCre* mice.** **A**, The *Bcl2<sup>flox</sup>* gene construct, in which the *Bcl2* gene was engineered to contain *LoxP* sites (*gray triangles*) flanking exon 2 (*gray rectangle*). *Neo*, cassette encoding gene for neomycin (G418) resistance; *Bcl2<sub>WT</sub>*, wild-type gene. **B**, Immunoblot of Bcl-2, Bcl-xL, and  $\beta$ -actin in peritoneal macrophages from *Bcl2<sub>WT</sub>-LysMCre*, *Bcl2<sup>flox</sup>-LysMCre*, and *Bcl2<sub>WT</sub>* mice. **C**, Macrophages from *Bcl2<sub>WT</sub>-LysMCre* and *Bcl2<sup>flox</sup>-LysMCre* mice were incubated under FC-loading conditions and were then assayed for apoptosis by staining with Alexa-488-labeled annexin V (*green*) and propidium iodide (*red*). Phase images are shown underneath the fluorescence images. **D**, Quantification of percent of apoptosis by various stimuli including FC accumulation (*FC*), oxidized LDL (*oxLDL*), UV radiation (*UV*), and staurosporine (*STS*). The differences between the two genotypes in each case were statistically significant (*asterisks*,  $P < 0.05$ ; *double asterisks*,  $P < 0.01$ ).

**Online Supplementary Figure II: Weight, plasma lipids, and lesional *Bcl2* mRNA expression of *Bcl2<sub>WT</sub>-LysMCre; Apoe<sup>-/-</sup>* and *Bcl2<sup>flox</sup>-LysMCre; Apoe<sup>-/-</sup>* mice fed a**

**Western-type diet for 10 wks.** **A**, Peripheral white blood cell count; **B**, Body weight; **C**, Plasma total cholesterol; **D-E**, Plasma lipoprotein-cholesterol profile; **F**, RT-QPCR quantification of *Cd68* mRNA, relative to cyclophilin A mRNA, in RNA obtained by LCM of intimal foam cells of aortic root lesions from *Bcl2<sup>flox</sup>-LysMCre;Apoe<sup>-/-</sup>* mice fed a Western-type diet for 10 wks. **G**, RT-QPCR quantification of *Bcl2* mRNA, relative to cyclophilin A mRNA, in RNA obtained by LCM of intimal foam cells of aortic root lesions from *Bcl2<sup>WT</sup>-LysMCre;Apoe<sup>-/-</sup>* and *Bcl2<sup>flox</sup>-LysMCre;Apoe<sup>-/-</sup>* mice fed a Western-type diet for 10 wks.

**A****B****C****D**

Online Supplementary Figure I



**Online Supplementary Figure II**