Bcl-x Inactivation in Macrophages Accelerates Progression of Advanced Atherosclerotic Lesions in Apoe<sup>−/−</sup> Mice


Objective—Bcl-x is the most abundantly expressed member of the Bcl-2 gene family in macrophages, but its role in macrophage apoptosis during atherogenesis is unknown.

Methods and Results—We previously reported dual pro- and antiatherogenic effects of macrophage survival in early versus advanced atherosclerotic lesions, respectively, potentially reflecting growing impairment of effecrocytosis during plaque progression. Here, we specifically inactivated Bcl-x in macrophages and evaluated its impact on atherosclerotic lesion formation in Apoe<sup>−/−</sup> mice at various stages of the disease. Bcl-x deficiency in macrophages increased their susceptibility to apoptosis, resulting in the depletion of tissue macrophages in vivo, including its major pool, Kipffer cells in the liver. We also observed increased cholesterol levels that were, however, not associated with any acceleration of early atherosclerotic plaque progression. This observation suggests that the atheroprotective effect of macrophage apoptosis at that stage of disease was counterbalanced by enhanced cholesterol levels. Bcl-x KO<sup>+/−</sup>/Apoe<sup>−/−</sup> mice exhibited significantly larger advanced lesions than control mice. These lesions showed vulnerable traits. Such enhanced lesion size may occur as a result not only of apoptotic cell accumulation but also of elevated cholesterol levels.

Conclusion—Modulation of macrophage resistance to apoptosis through targeted deletion of Bcl-x has a major impact on the entire macrophage cell population in the body, including Kipffer cells. Macrophage survival may, therefore, not only influence atherosclerotic plaque development and vulnerability but also cholesterol metabolism. (Arterioscler Thromb Vasc Biol. 2012;32:1142-1149.)

Key Words: ABC transporter ■ apoptosis ■ atherosclerosis ■ immune system ■ macrophages

Chronic inflammation of the arterial wall is a fundamental feature of atherosclerosis and is characterized by the accumulation of LDL and immune cells driving both innate and adaptive immunity. Monocytes that have been attracted to the inflamed vessel differentiate into macrophages, the major cell type found within atherosclerotic lesions. These macrophages then gorges themselves on cholesterol derived from modified LDL, which they esterify to neutralize the potentially cytotoxic and proapoptotic effects of free cholesterol. However, the accumulation of proinflammatory, prooxidizing, and proapoptotic stimuli favors macrophage death, the formation of apoptotic debris, and supports necrotic core growth. This, in turn, may promote plaque disruption and severe thrombotic events. In support of this hypothesis, experimental evidence shows that vulnerable, necrotic plaques display increased macrophage apoptosis. Given the potential consequences of macrophage apoptosis in advanced atherosclerotic lesions, determining the molecular mechanisms of macrophage death in advanced lesions is therefore a critical goal.

Macrophage death occurs at all stages of disease development. However, it has been suggested that macrophage apoptosis may have a different impact on plaque progression depending on the stage of lesion development during which it occurs. Accordingly, in vivo studies have shown that disruption of the gene coding for the proapoptotic molecule Bax resulted in an increase in early atherosclerotic lesions. It was also shown that disruption of the antiapoptotic factor AIM attenuated early plaque formation. Our previous studies in which macrophage resistance to apoptosis was...
enhanced using macrophage-specific human Bcl-2 overexpression, have also shown accelerated early plaque development. However, enhanced macrophage survival delayed advanced plaque progression, whereas chronic induction of apoptosis in established lesions resulted in the accumulation of apoptotic cells in advanced lesions and enhanced plaque progression.\textsuperscript{13} The relative level of expression of proapoptotic (eg, Bax and Bak) and antiapoptotic (eg, Bcl-2 and Bcl-x) proteins of the Bcl-2 family determines the overall sensitivity of a cell to apoptotic stimuli.\textsuperscript{14} In macrophages from atherosclerotic lesions there is essentially a complete lack of Bcl-2 and little Bcl-x.\textsuperscript{15–17} There are also increased levels of the proapoptotic proteins Bad and Bax.\textsuperscript{15–17} Cell culture studies have shown that Bcl-2 and Bcl-x may be involved in the protection of macrophages against apoptosis.\textsuperscript{18–21} However, 1 study evaluated the in vivo implication of Bcl-2 in the survival of macrophages and its consequences in atherosclerosis.\textsuperscript{22} In this study, deletion of the floxed Bcl-2 gene was mediated by Cre recombinase under the control of the Lysozyme M gene, resulting in the deletion of Bcl-2 in differentiated macrophages, including those present in atherosclerotic lesions.\textsuperscript{22} As Bcl-x may represent an alternative pathway to control macrophage survival, we chose to specifically investigate the impact of Bcl-x deficiency on plaque progression. We evaluated the impact of Bcl-x deletion both at early and advanced stages of the disease as apoptosis exerts different effects on atherogenesis as a function of lesion stage.\textsuperscript{9,13} We addressed the question of the impact of macrophage Bcl-x on atherosclerotic lesion formation by crossing conditional Apoe\textsuperscript{2} /Bcl-x–floxed mice with Apoe\textsuperscript{–/–}/LysM\textsuperscript{Cre/+} mice. Using this model, we provide evidence that Bcl-x expression by macrophages plays an important protective role against apoptosis as its disruption resulted in reduction in these cells in tissues, in elevation in plasma cholesterol levels and in larger and more complicated advanced atherosclerotic lesions.

### Materials and Methods

#### Generation of Bcl-x\textsubscript{floxed/+}–LysM-Cre Mice

The floxed Bcl-x mice of mixed genetic background 129SvEv/C57BL6 were obtained from Dr. Edmund B. Rucker (University of Missouri, Columbia).\textsuperscript{23} LysM-Cre mice in which Cre recombinase expression is driven by the lysozyme M promoter via gene targeting into the endogenous lysozyme M locus were obtained from the Jackson Laboratory (Stock Number: 004781; backcrossed 6 times in C57BL6 genetic background). Eight crossings of Bcl-x\textsubscript{floxed/+} mice with LysM\textsubscript{Cre/+} mice and backcrossings with Apoe\textsuperscript{–/–} mice using Apoe\textsuperscript{–/–}/LysM\textsuperscript{Cre/+} mice. Using this model, we provide evidence that Bcl-x expression by macrophages plays an important protective role against apoptosis as its disruption resulted in reduction in these cells in tissues, in elevation in plasma cholesterol levels and in larger and more complicated advanced atherosclerotic lesions.

#### Atherosclerosis Studies

To assess the impact of macrophage Bcl-x on atherosclerosis progression, 7-week-old Bcl-x KO\textsuperscript{Mdr1a}/Apoe\textsuperscript{–/–}, Bcl-x\textsubscript{floxed}/Apoe\textsuperscript{–/–}, LysM\textsubscript{Cre}/Apoe\textsuperscript{–/–} and Apoe\textsuperscript{–/–} mice were fed a 1%-cholesterol rich diet (Harlan Teklad, TD97131) for 10 weeks or a Western diet consisting of 0.15% cholesterol and 20% saturated fat (SAFE, France) for 10 or 27 weeks.

Plasma lipid analyses, quantification of atherosclerotic lesions, TUNEL staining, immunohistochemistry, analysis of gene expression by Q-PCR, isolation of liver leukocytes and of peritoneal macrophages, flow cytometry, and generation of bone marrow-derived macrophages (BMDM). All these procedures were performed as previously described\textsuperscript{14–20} and are detailed in the Methods section in the online-only Data Supplement.

#### Statistical Analysis

Statistical calculations were performed using GraphPad Prism, version 4.03. Results were analyzed by Student’s unpaired t-tests with Welch’s correction if variances were unequal. \( P<0.05 \) was considered significant.

#### Results

**Generation and Characterization of Macrophage-Specific Bcl-x Deficiency**

Mice in which exons 1 and 2 of the Bcl-x gene were flanked with loxP sites were crossed with mice expressing Cre recombinase under the control of the lysozyme M gene to generate mice in which Bcl-x was deleted in macrophages.\textsuperscript{22,24–28} DNA recombination was confirmed in thioglycollate-elicited peritoneal macrophages in which the wild-type allele was almost undetectable. When backcrossed into an Apoe\textsuperscript{+/+} background, Bcl-x KO\textsuperscript{Mdr1a}/Apoe\textsuperscript{–/–} mice and Apoe\textsuperscript{–/–} mice by RT-PCR, mRNA expression of Bcl-x in BMDM of Bcl-x KO\textsuperscript{Mdr1a} mice was 0.002% of that of controls (\( P<0.000001 \)), whereas expression of Bcl-x was increased 2-fold in Bcl-x KO\textsuperscript{Mdr1a} macrophages (\( P<0.01 \)) (Figure IA in the online-only Data Supplement). The mRNA levels of the proapoptotic genes Bad, Bax, and Bid were similar in BMDM from Bcl-x KO\textsuperscript{Mdr1a} mice and controls (Figure IA in the online-only Data Supplement). Despite upregulation of Bcl-2, BMDM derived from Bcl-x KO\textsuperscript{Mdr1a}/Apoe\textsuperscript{–/–} mice were markedly more susceptible either to free-cholesterol- or to oxysterol-induced apoptosis when incubated with Ac-LDL and an ACAT inhibitor (\( P<0.01, \) Figure 1A) or with 7-ketocholesterol (\( P<0.05, \) Figure IB in the online-only Data Supplement), respectively, arguing for a pivotal role of Bcl-x in free-cholesterol induced apoptosis. Similarly, freshly harvested peritoneal macrophages and cultured peritoneal and pulmonary macrophages from Bcl-x KO\textsuperscript{Mdr1a}/Apoe\textsuperscript{–/–} mice were more susceptible to apoptosis induced by serum starvation as compared to Apoe\textsuperscript{–/–} mice (Figure IB in the online-only Data Supplement). We also evaluated in peritoneal macrophages from both Bcl-x KO\textsuperscript{Mdr1a}/Apoe\textsuperscript{–/–} and Apoe\textsuperscript{–/–} genotype, the protein levels of Bcl-x and Bcl-2 (Figure 1B). The Western blot analysis further demonstrates the lack of Bcl-x protein in Bcl-x KO\textsuperscript{Mdr1a}/Apoe\textsuperscript{–/–} versus Apoe\textsuperscript{–/–}, whereas Bcl-2 protein levels were unchanged.

In addition, we assessed leukocyte and monocyte subset counts in the different groups of mice maintained under chow diet or cholesterol-rich diet. In female mice fed a chow diet, we found that such cell counts were not altered in Bcl-x KO\textsuperscript{Mdr1a}/Apoe\textsuperscript{–/–} mice as compared to wild type Apoe\textsuperscript{–/–} mice (Figure 1C and Figure IIA in the online-only Data Supplement). By contrast, under the cholesterol-rich diet, we observed that the leukocyte count was 30% higher in Bcl-x KO\textsuperscript{Mdr1a}/Apoe\textsuperscript{–/–} mice than in Apoe\textsuperscript{–/–} mice (Figure IIA in
the online-only Data Supplement). As observed in other studies, the cholesterol-rich diet-induced mononcytosis (30% higher in controls) that was more severe in Bcl-x KO^Mac/Apoe^-/- mice (150% higher) and was linked to increased in the Gr1^hi^ monocyte subset (also called Ly-6C^hi^ monocytes) (Figure 1C). The possibilities of gender differences related to the effect of Bcl-x deficiency were excluded; indeed, a similar leukocyte and monocyte pattern was observed in males (Figure IIB and IIC in the online-only Data Supplement).

Impact of Macrophage-Specific Bcl-x Deletion on Atherogenesis and Cholesterol Levels

Based on our previous observations showing that increased survival of macrophages results in larger early lesions but smaller advanced lesions, we decided to use a complementary strategy and assessed the impact of macrophages more susceptible to stress-induced death on lesion progression. For this purpose, Bcl-x KO^Mac/Apoe^-/- mice and controls were fed cholesterol-enriched diets for 10 (0.15% and 1% cholesterol) or 27 (0.15% cholesterol) weeks. Unexpectedly, we observed higher plasma cholesterol levels in Bcl-x KO^Mac/Apoe^-/- mice compared to Apoe^-/- controls at all time points and under all diets, including the basal conditions (chow diet fed mice) (Table I in the online-only Data Supplement). However, after 10 weeks of diet, aortic root lesion area was similar in both Bcl-x KO^Mac/Apoe^-/- mice and controls fed a 0.15% (Figure 2A and Figure IIIA in the online-only Data Supplement) or a 1% cholesterol-enriched diet (Figure 2B and Figure IIIB in the online-only Data Supplement). As expected, early lesion area was larger in mice fed a 1% cholesterol-enriched diet compared to those fed a 0.15% cholesterol-enriched diet (Figure 2A and 2B); 128000 µm² versus 292000 µm² in control females (P<0.004). As previously reported, we did not find any TUNEL+ cells in these early lesions (data not shown). This suggests an efficient removal of apoptotic cells at this stage of the disease. To further validate the potential local depletion of Bcl-x in atherosclerotic lesions, we evaluated the expression of Cre recombinase and of lysozyme M in the whole aorta of Bcl-x KO^Mac/Apoe^-/- and Apoe^-/- mice fed a Western diet for 10 weeks (Figure 2C and 2D). Cre recombinase was significantly expressed (ratio of gene expressed by macrophages over reference genes: 0.57 for Cre; 4.4 for ABCG1; 15.7 for SRA) in the aorta of Bcl-x KO^Mac/Apoe^-/- and absent of Apoe^-/- aortas. Consistent with the local expression of Cre, the expression of Lysozyme M was decreased by 45% in Bcl-x KO^Mac/Apoe^-/- mice versus Apoe^-/- mice, consistent with a mono-allelic expression due to its heterozygous form in LysM-Cre positive cells. Furthermore, similar levels of mRNA for macrophages markers (CD68, SRA, ABCG1) in both groups of mice (Figure 2D) suggested that there were no major changes in the number of macrophages in the lesions. This data indirectly supports the absence of significant differences in lesion size in these males, as already observed in their littermate females (Figure 2A) and in male and female groups fed a 1% cholesterol diet for 10 weeks (Figure 2B). In addition, quantification of inflammatory genes revealed higher levels of IL-1B mRNA in atherosclerotic lesions of Bcl-x KO^Mac/Apoe^-/- mice versus Apoe^-/- mice fed a Western diet for 10 weeks (Figure 2E).

Seventeen additional weeks on a 0.15% cholesterol-enriched diet resulted in larger (>10-fold) and more complicated lesions with necrotic core formation. At this time point, en face analysis of the descending aorta showed that lesion areas were 94% larger (P<0.006) in females and 25% larger (P<0.04) in males in Bcl-x KO^Mac/Apoe^-/- mice relative to controls (Figure 3A-B). In contrast to the 10-week time point, the mean area of the aortic root lesion in Bcl-x KO^Mac/Apoe^-/- mice was 27% larger compared to controls after 27 weeks of diet (P<0.02, Figure 3C and 3D).
We next quantified TUNEL-positive cells in advanced lesions (27 weeks) which mainly expressed macrophage markers (Figure IVA–IVD in the online-only Data Supplement). At 27 weeks of diet we observed a 3-fold increase in the number of apoptotic cells per lesion in Bcl-x KO Mac/Apoe−/− mice compared to controls (P<0.006, Figure 3E; Figure IVE–IVF in the online-only Data Supplement), corresponding to a 3-fold increase in apoptotic cell content per mm² of lesion (P<0.04, Figure 3F). As macrophages undergoing apoptosis in advanced plaques might likely contribute to necrotic core formation, an index of plaque instability, we measured acellular areas in our animals and observed that the necrotic area was 60% larger in Bcl-x KO Mac/Apoe−/− mice compared to controls (P<0.0001, Figure 3G). Collagen content, also indicative of plaque stability, was decreased by 35% in the aortic sinus of Bcl-x KO Mac/Apoe−/− compared to Apoe−/− control mice (P<0.02, Figure 3H and 3I). In males, changes in plaque size (Figure 2B, Figure 3A) and in plasma cholesterol (Table II in the online-only Data Supplement) following extended periods of Western-type diet feeding were comparable to that observed in females (Figure 2A and Figure 2B, Figure 3A and 3C, and Table I in the online-only Data Supplement) suggesting that the impact of Bcl-x deficiency was not gender specific, as previously reported for macrophage Bcl-2 deficiency in Bcl-2 KO Mac/Apoe−/− mice. No significant differences in body weight were observed between genotypes at any time point for both males and females (Table and Table I in the online-only Data Supplement).

**Impact of Macrophage-Specific Bcl-x Deletion on Gene Expression and Leukocytes in the Liver**

To further document mechanisms by which Bcl-x deficiency in macrophages might affect plasma cholesterol levels, we extracted mRNA from liver of mice fed a 0.15% cholesterol atherogenic diet for 10 weeks to perform quantitative RT-PCR. Levels of mRNA for Bcl-x were decreased in the liver of Bcl-x KO Mac/Apoe−/− mice as compared to controls but significant changes were only observed in mice fed the 0.15% cholesterol-enriched diet (Figure 4A and Figure VA in the online-only Data Supplement). As expected, levels of mRNA for Lysozyme M were significantly decreased (−39%) in LysM<sup>Cre/wt</sup>/Apoe−/− mice as compared to control mice that did not express the Cre recombinase (Bcl-x<sup>x flox/flox</sup>/Apoe−/− and Bcl-x<sup>x flox/wt</sup>/Apoe−/− mice) reflecting a decrease in Lysosome M expression due to its heterozygous form in LysM-Cre positive cells. An additional decrease was observed in Bcl-x KO Mac/Apoe−/− mice reflecting both the former mechanism and an additional depletion of macrophages expressing Lysosome M (−78%, P<0.0001, Figure 4A). mRNAs encoding for the mononuclear cell marker CD68 and F4/80 were significantly decreased (−29%, −32%, and −24%, respectively) in the liver of Bcl-x KO Mac/Apoe−/− mice as compared to controls (Figure 4B). A similar pattern of expression was observed in mice maintained on 1% cholesterol-enriched diet (Figure VA in the online-only Data Supplement). We also evaluated the expression of several genes implicated in lipid homeostasis in the liver. As shown in Figure 4C, the expression levels of LDL-R, ABCG1, and SR-A were significantly decreased (−29%, −40%, and −29%, respectively) whereas those of ACAT-2, LXRA, ABCA1, and SR-B1 were similar in Bcl-x KO Mac/Apoe−/− and control mice. A similar pattern of expression was observed in mice maintained on 1% cholesterol-enriched diet (Figure VB in the online-only Data Supplement). We were also able to demonstrate an ongoing
inflammatory process in the liver of Bcl-x KO Mac/Apoe<sup>2/2</sup> mice as there was significant upregulation of MCP-1 mRNA in comparison to controls, indicative of the activation of endothelial cells and macrophages (Figure 4D). Flow cytometric analysis revealed that the Küpffer cell population (resident macrophage population of the liver) was significantly reduced (~27%, \( P<0.01 \)) in Bcl-x KO Mac/Apoe<sup>2/2</sup> mice compared to controls, whereas the dendritic cell (DC) population was unaffected (Figure 4E). Interestingly, a 2.2-fold increase in MHC Class II positive liver leukocytes was observed in Bcl-x KO Mac/Apoe<sup>2/2</sup> mice as compared with controls (Figure 4E; \( P<0.0003 \)). We next isolated the Küpffer cells of mice maintained on a Western diet for 10 weeks and quantified inflammatory gene expression (Figure 4F). We measured significantly higher levels of IL-1\( \beta \) and a trend to higher levels of IL-18 in Küpffer cells of Bcl-x KO Mac/Apoe<sup>2/2</sup> mice as compared to controls, consistent with the transcriptional profile observed in the whole aorta (Figure 2E). In addition, we confirmed expression of the recombinase Cre in Küpffer cells of Bcl-x KO Mac/Apoe<sup>2/2</sup> mice (none detected in controls). This effect was associated with lower expression of Bcl-x (Figure 4H) and mono-allelic expression of lysozyme M (Figure 4G) in Küpffer cells. The levels of other anti- and proapoptotic genes (Bcl-2, Bid, Bad) were not affected. Interestingly, RNA levels for the LXR responsive gene ABCG1 were increased in Küpffer cells of Bcl-x KO Mac/Apoe<sup>2/2</sup> mice versus controls suggesting accumulation of cholesterol-derived LXR ligands in these cells.

**Discussion**

Given the crucial role of macrophages in the regulation of innate immune responses, the regulation of their life span is critical in both physiological and pathological processes. Macrophage survival depends on multiple pro- and anti-apoptotic molecules, among which the antiapoptotic protein Bcl-x is hypothesized to be critically involved in both monocyte/macrophage differentiation and survival, in humans and mice.\(^{18-21}\) Our experimental model, in which macrophages specifically lack Bcl-x, further emphasizes the critical involvement of Bcl-x in the protection of macrophages from apoptosis. Indeed, we demonstrated that expression of Bcl-x is almost abolished in BMDM and peritoneal macrophages from Bcl-x KO Mac/Apoe<sup>2/2</sup> mice resulting in poor resistance to apoptotic stress in vitro. In vivo, we noticed a clear depletion of Küpffer cells, the liver resident macrophages, as shown by the reduction in expression of mRNA encoding genes mainly associated with macrophages (LysM, SR-A, ABCG1, CD68, and F4/80) in the liver of Bcl-x KO Mac/Apoe<sup>2/2</sup> mice compared to controls, whereas the dendritic cell (DC) population was unaffected (Figure 4E). Interestingly, a 2.2-fold increase in MHC Class II positive liver leukocytes was observed in Bcl-x KO Mac/Apoe<sup>2/2</sup> mice as compared with controls (Figure 4E; \( P<0.0003 \)). We next isolated the Küpffer cells of mice maintained on a Western diet for 10 weeks and quantified inflammatory gene expression (Figure 4F). We measured significantly higher levels of IL-1\( \beta \) and a trend to higher levels of IL-18 in Küpffer cells of Bcl-x KO Mac/Apoe<sup>2/2</sup> mice as compared to controls, consistent with the transcriptional profile observed in the whole aorta (Figure 2E). In addition, we confirmed expression of the recombinase Cre in Küpffer cells of Bcl-x KO Mac/Apoe<sup>2/2</sup> mice (none detected in controls). This effect was associated with lower expression of Bcl-x (Figure 4H) and mono-allelic expression of lysozyme M (Figure 4G) in Küpffer cells. The levels of other anti- and proapoptotic genes (Bcl-2, Bid, Bad) were not affected. Interestingly, RNA levels for the LXR responsive gene ABCG1 were increased in Küpffer cells of Bcl-x KO Mac/Apoe<sup>2/2</sup> mice versus controls suggesting accumulation of cholesterol-derived LXR ligands in these cells.

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KO<sup>44</sup>/Apoe<sup>−/−</sup> mice when compared to controls. Of note, the reduction of Bcl-x expression was not compensated for by major changes in the expression of the antiapoptotic gene Bcl-2 in peritoneal macrophages and Küpffer cells.

Our data clearly show a significant effect of Bcl-x deficiency in macrophages on circulating cholesterol levels of mice maintained on chow- and cholesterol-enriched diets. Interestingly, these observations are consistent with earlier observations in both mice and humans, which also suggest a role for mononuclear phagocytes in cholesterol homeostasis. For example, the hematopoietic growth factor macrophage colony-stimulating factor was reported to lower cholesterol levels in rabbits and nonhuman primate models. At the opposite, op/op mice harboring a mutation in the csf1 gene, which results in a reduction in monocyte and several tissue macrophage populations such as Küpffer cells, present a marked hypercholesterolemia when backcrossed in an Apoe-deficient background. We also previously observed a similar phenotype in CD11c-DTR Apoe<sup>−/−</sup> mice in which a transient elimination of dendritic cells increased plasma cholesterol levels. Taken together, these observations indicate that mononuclear phagocytes may directly or indirectly influence cholesterol homeostasis. The mechanisms responsible for the increase in total plasma cholesterol levels in Bcl-x KO<sup>44</sup>/Apoe<sup>−/−</sup> mice were not explored in the framework of this study, and the deficiency in ABCG1 mRNA in the liver may likely not contribute to the elevation of circulating cholesterol levels as ABCG1 deficiency in bone marrow derived macrophages was not shown to be associated with enhanced cholesterol levels. The lower expression of ABCG1 mRNA may likely reflect the depletion of Küpffer cells, as it is primarily expressed by these cells in the liver and its expression was not downregulated in purified Küpffer cells.

As macrophage apoptosis has been shown to limit the development of early atherosclerotic lesions, we would have expected a protective effect in early lesions in Bcl-x KO<sup>44</sup>/Apoe<sup>−/−</sup> mice. However, because of a concomitant...
elevation in plasma cholesterol in Bcl-x KO<sup>fl/</sup>/Apo<sup>−/−</sup> mice, the protective effect of increased macrophage death may have been overwhelmed by the hypercholesterolemia observed in these mice. The absence of TUNEL positive cells in the lesions of Bcl-x KO<sup>fl/</sup>/Apo<sup>−/−</sup> and control mice may reflect efficient efferocytosis at this stage. Thus, Bcl-x might still play an important role in the control of macrophage survival in early lesions.

In advanced lesions, Bcl-x deficiency in macrophages was associated with larger lesion size in Bcl-x KO<sup>fl/</sup>/Apo<sup>−/−</sup> mice as compared to Apo<sup>−/−</sup> lesions. This finding was associated with a higher frequency of apoptotic cells, a larger necrotic core area and a lower collagen content in the lesions of Bcl-x KO<sup>fl/</sup>/Apo<sup>−/−</sup> mice. Interestingly, all these features are indicative of enhanced plaque vulnerability and further support the concept that apoptosis of macrophages favors necrotic core growth. In this model, we cannot distinguish the respective contribution of higher cholesterol levels or of higher susceptibility of macrophages to apoptosis in lesion development in Bcl-x KO<sup>fl/</sup>/Apo<sup>−/−</sup> mice during the period of advanced plaque growth. However, irrespective of the contribution of cholesterol levels, which are not always correlated to plaque growth, depletion of macrophages is also ongoing in the latter stages of the disease as we observed lower macrophage content in livers and spleens of 1-year-old Bcl-x KO<sup>fl/</sup>/Apo<sup>−/−</sup> mice as compared to controls (data not shown). These results are consistent with our previous observations indicating that macrophage apoptosis in advanced lesions is proatherogenic.

In summary, the present study confirms that macrophage apoptosis appears to be more important in advanced rather than early lesion development, thereby providing a rationale for the association between apoptotic cell accumulation, necrotic core formation, and resulting vulnerable traits. In the quest to identify specific molecular targets and processes that would target the growth of vulnerable lesions, our data suggest that sustaining Bcl-x levels in macrophages would be beneficial both in reducing apoptotic macrophage accumulation in advanced atherosclerotic plaques and maintaining cholesterol homeostasis in the liver, supporting the concept of cross-talk between innate immunity and cholesterol metabolism.

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**Disclosures**

None.

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Supplemental Materials

Bcl-x inactivation in macrophages is associated with accelerated progression of advanced atherosclerotic lesions in Apoe⁻/⁻ mice.


Acronyms.

Bcl-2 : B-cell lymphoma 2
Bcl-x: Bcl-2-like protein 1
Bax : BCL2-interactive cell death susceptibility regulator
Bad : Bcl2 antagonist of cell death
Bid : BH3-interacting domain death agonist
Bak : Bcl-2 homologous antagonist/killer
AIM : Apoptosis inhibitor expressed by macrophages

Supplemental Methods.

Animals. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the European Commission Directive 86/609/EEC. All animal procedures were performed at the Central Animal Facility of the Medical Faculty of La Pitié Hospital with approval from the Direction Départementale des Services Vétérinaires, Paris, France. Mice were housed in a conventional animal facility, weaned at 21 days and fed ad libitum a normal mouse chow diet (RM1, Dietex France).

Blood and tissue analyses. Blood samples were collected in Microvette tubes (Sarstedt) by retro-orbital bleeding under isoflurane anaesthesia (isoflurane (1.5%)/oxygen (0.4 L/min.)). Plasma samples were analysed with an Autoanalyzer using commercial reagent kits (Roche Diagnostics (total cholesterol), Diasys (Free Cholesterol), ThermoElectron (Glucose)) ¹. Bone marrow-derived macrophages were isolated as described earlier ² and cultured in DMEM supplemented with 20% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 2 mM penicillin/streptomycin, and 30% L929 cell–conditioned media.
**Isolation of mouse primary leukocytes.** After perfusion, the liver was dissected out of the abdominal cavity and cut in small pieces. The pieces were incubated in a digestion buffer (PBS/collagenase D (0.4mg/ml, Roche)) at 37°C for 20 minutes. After being digested, the liver was homogenized through a 70-µm cell strainer and centrifuged at 300g for 5 min. The pellet was resuspended into 9ml of 30% Percoll (diluted with PBS) and centrifuged at 1700 rpm for 30 min at room temperature. After removing hepatocytes at the gradient surface, the cells in the pellet were collected and incubated in ACK buffer (ammonium chloride solution) to lyse contaminating red blood cells, washed, and analyzed. Peritoneal macrophages were isolated by peritoneal lavage with ice-cold PBS, 3 days after intraperitoneal injection of 3 ml of 3.0% sterile thioglycolate. The cells were washed with PBS were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 2 mM penicillin/streptomycin. After overnight cultured floating cells were removed by washing with PBS. Adherent cells (peritoneal macrophages) were maintained in the medium and plated onto Lab-TekII Chamber Slide for terminal TUNEL (TdT-mediated dUTP nick end labeling) staining.

**Quantifications of Atherosclerotic Lesions and TUNEL staining.** Atherosclerotic lesions were quantified on serial cross sections (10µm) through the aortic root by oil red O staining as previously described 3, with minor modifications. Mice were sacrificed under isoflurane anaesthesia and perfused with PBS. Hearts were collected, fixed (Accustain 10% formalin solution (Sigma) supplemented with 2 mM EDTA and 20 µM butylated hydroxytoluene at pH 7.4) for 30 minutes followed by overnight incubation in phosphate-buffered 20% sucrose solution at 4°C; hearts were subsequently embedded in Tissue-Tek OCT compound (Sakura Finetek). Approximately 80 sections, 10 µm thick, were cut through the proximal aorta, spanning the three cusps of the aortic valves. Every eighth section was fixed and stained with oil red O (0.3% in triethylphosphate) for 30 minutes and then counterstained with Mayer hematoxylin for 1 minute. Images were captured using a Zeiss Axiovert 135 microscope and analyzed with Adobe Photoshop 7.0 software (Adobe Systems Inc., CA, USA) and the Image Processing Tool Kit (Reindeer Graphics, NC, USA) plug-ins. The extent of atherosclerosis was measured with colour thresholding to delimit areas of oil red O staining and is reported as mean lesion area of the group of the sections analysed. TUNEL staining was performed as previously described 4. Briefly, cryosections (10 µm thickness) 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 Tris-HCl 0.1M pH 7.5 containing 3% BSA and 20% normal bovine serum for 30 min. After washing, TUNEL reaction mixture (TMR Red 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). Plaque necrosis was quantified by measuring the area of hematoxylin and eosin-negative acellular areas in the intima versus total intima.

**Flow cytometry.** Total leukocytes were quantitated by fresh blood dilution in Turk’s solution (Merck). To determine monocyte count, blood was harvested, red blood cells were lysed in ACK
buffer and cells were resuspended in PBS containing 1% BSA and 0.01% sodium azide. Cells were subsequently pre-incubated for 5 min with Fc blocker and incubated for 30 min at 4°C with antibodies directed against CD45 (clone 30F11, Miltenyi), F4/80 (clone BM8, eBiosciences) and CD115 (clone AFS98, eBiosciences) and CD11b (clone M1/70, eBiosciences) and anti-Gr1/Ly-6C antibody (clone RB6-8C5, eBiosciences). Liver leukocytes were stained with Liver leukocytes were stained with anti-CD45 (clone 30F11, Miltenyi), anti-CD11c (clone N418, eBiosciences), anti-F4/80 (clone BM8) and anti-IA\textsuperscript{b} (clone M5:114) all from eBiosciences. The cells were then fixed with 2% paraformaldehyde. Data were acquired on a FC 500 Flow Cytometer (Beckman Coulter) and analyzed with CXP software.

**Assessment of susceptibility of macrophages to apoptosis.** Animals were sacrificed and resident peritoneal and lung macrophages extracted. These were placed in DMEM medium supplemented with 2 mM L-glutamine and 2 mM penicillin/streptomycin, but without FBS, for 4 hours at RT. Cells were then stained for CD45, CD11b, CD11c and F4/80 markers. Cells were also stained with Annexin V, a marker of apoptosis, and analysed by flow cytometry.

**Analysis of gene expression by Q-PCR.** Real-time quantitative RT-PCR was performed as previously described\textsuperscript{1,3,5}. Briefly, RNAs were prepared using TRIzol reagent (Invitrogen) from tissue or cells isolated from mice at sacrifice. Each RNA preparation was hybridized with random hexamer (Promega) and reverse-transcribed using M-MLV reverse transcriptase (Invitrogen). All reactions were performed in duplicate or triplicate and the average expression of three housekeeping genes (beta-glucuronidase, hypoxanthine guanine phosphoribosyl transferase and ribosomal protein S3) was used as a housekeeping gene to account for variability in the initial quantities of cDNA. Expression data were based on the crossing points calculated from LightCycler data analysis software and corrected for PCR efficiencies of both the target and the reference genes. Gene expression analyses were performed on a LightCycler®480 System using the LightCycler480 SYBR green I master (Roche). The primers are listed in Supplemental Table III.
Supplemental Table I. Body weight and lipid parameters.

<table>
<thead>
<tr>
<th>Females on Apoe(^{-}/) background</th>
<th>Wild-type</th>
<th>Bcl-x KO(^{Mac})</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>7 week old females on chow diet</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight, g</td>
<td>16.5 ± 0.4</td>
<td>17.4 ± 0.5</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>400 ± 16</td>
<td>523 ± 24</td>
<td>&lt;0.00004</td>
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<tr>
<td>Free cholesterol, mg/dl</td>
<td>135 ± 6</td>
<td>172 ± 7</td>
<td>&lt;0.0002</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>158 ± 16</td>
<td>161 ± 15</td>
<td>NS</td>
</tr>
<tr>
<td><strong>17 week old females maintained for 10 weeks on a 1% cholesterol rich diet</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight at sacrifice, g</td>
<td>18.2 ± 0.4</td>
<td>19.29 ± 0.6</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>1129 ± 83</td>
<td>1701 ± 71</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>Free cholesterol, mg/dl</td>
<td>355 ± 29</td>
<td>493 ± 34</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>80 ± 5</td>
<td>92 ± 7</td>
<td>NS</td>
</tr>
<tr>
<td><strong>17 week old females maintained 10 weeks on a 0.15% cholesterol rich diet</strong></td>
<td>n=13</td>
<td>n=14</td>
<td></td>
</tr>
<tr>
<td>Weight at sacrifice, g</td>
<td>18.7 ± 1.4</td>
<td>19.1 ± 1.6</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>774 ± 116</td>
<td>940 ± 171</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>Free cholesterol, mg/dl</td>
<td>268 ± 41</td>
<td>325 ± 52</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>187 ± 86</td>
<td>161 ± 62</td>
<td>NS</td>
</tr>
<tr>
<td><strong>34 week old females maintained 27 weeks on a 0.15% cholesterol rich diet</strong></td>
<td>n=9</td>
<td>n=8</td>
<td></td>
</tr>
<tr>
<td>Weight at sacrifice, g</td>
<td>24.4 ± 0.9</td>
<td>21.8 ± 1.2</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>717 ± 56</td>
<td>1060 ± 84</td>
<td>&lt;0.002</td>
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<tr>
<td>Free cholesterol, mg/dl</td>
<td>242 ± 19</td>
<td>328 ± 24</td>
<td>&lt;0.01</td>
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<tr>
<td>Triglycerides, mg/dl</td>
<td>164 ± 16</td>
<td>174 ± 28</td>
<td>NS</td>
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Values were determined in overnight fasted age-matched mice. Data represent mean ± SEM. NS indicates not significant.
Supplemental Table II. Body weight and lipid parameters.

<table>
<thead>
<tr>
<th>Males on Apoe&lt;sup&gt;−/−&lt;/sup&gt; background</th>
<th>Wild-type</th>
<th>Bcl-x KO&lt;sup&gt;Mac&lt;/sup&gt;</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 week old males on chow diet</td>
<td>n=16</td>
<td>n= 27</td>
<td></td>
</tr>
<tr>
<td>Weight, g</td>
<td>19.8 ± 0.9</td>
<td>18.5 ± 0.5</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>468 ± 21</td>
<td>595 ± 29</td>
<td>&lt;0.002</td>
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<tr>
<td>Free cholesterol, mg/dl</td>
<td>165 ± 6</td>
<td>197 ± 35</td>
<td>&lt;0.006</td>
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<tr>
<td>Triglycerides, mg/dl</td>
<td>316 ± 17</td>
<td>259 ± 23</td>
<td>NS</td>
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<table>
<thead>
<tr>
<th>17 week old males maintained for 10 weeks on a 1% cholesterol rich diet</th>
<th>n=12</th>
<th>n=23</th>
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<tr>
<td>Weight at sacrifice, g</td>
<td>20.9 ± 0.8</td>
<td>21.7 ± 0.4</td>
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<tr>
<td>Total cholesterol, mg/dl</td>
<td>1195 ± 80</td>
<td>1706 ± 81</td>
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<tr>
<td>Free cholesterol, mg/dl</td>
<td>347 ± 23</td>
<td>457 ± 19</td>
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<tr>
<td>Triglycerides, mg/dl</td>
<td>87 ± 8</td>
<td>88 ± 7</td>
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<table>
<thead>
<tr>
<th>17 week old males maintained for 10 weeks on a 0.15% cholesterol rich diet</th>
<th>n=16</th>
<th>n=24</th>
</tr>
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<tr>
<td>Weight at sacrifice, g</td>
<td>23.0 ± 1.1</td>
<td>22.9 ± 0.7</td>
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<tr>
<td>Total cholesterol, mg/dl</td>
<td>855 ± 99</td>
<td>1047 ± 149</td>
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<tr>
<td>Free cholesterol, mg/dl</td>
<td>309 ± 40</td>
<td>354 ± 40</td>
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<tr>
<td>Triglycerides, mg/dl</td>
<td>286 ± 78</td>
<td>256 ± 103</td>
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<table>
<thead>
<tr>
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<th>n=8</th>
<th>n=12</th>
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<tbody>
<tr>
<td>Weight at sacrifice, g</td>
<td>32.5 ± 1.1</td>
<td>29.9 ± 1.0</td>
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<td>Total cholesterol, mg/dl</td>
<td>873 ± 62</td>
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<tr>
<td>Free cholesterol, mg/dl</td>
<td>277 ± 21</td>
<td>351 ± 23</td>
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<tr>
<td>Triglycerides, mg/dl</td>
<td>208 ± 44</td>
<td>214 ± 20</td>
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</table>

Values were determined in overnight fasted age-matched mice. Data represent mean ± SEM. NS indicates not significant.
# Supplemental Table III. Q-PCR Primers.

<table>
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<th>Gene</th>
<th>Accession Number</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
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<tbody>
<tr>
<td>Bcl-xl</td>
<td>NT_039207.7</td>
<td>5’-CCCCACCAACACTCTCTCTTT-3’</td>
<td>5’-TCCCTACACACCCCCCTCTCTG-3’</td>
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<tr>
<td>LysM</td>
<td>NT_039500</td>
<td>5’-AAAAACCCCAAGAGCTGTAAT-3’</td>
<td>5’-GAATGCCCCTGGGATCTCTC-3’</td>
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<tr>
<td>CD68</td>
<td>NT_096135</td>
<td>5’-TTGGGAACTACACGTCGGG-3’</td>
<td>5’-CGGATTGAATTTGGGCTTG-3’</td>
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<tr>
<td>F4/80</td>
<td>NT_039649</td>
<td>5’-AGTACGATGTGGGGCTTTTG-3’</td>
<td>5’-CCCCATCTGTACATCCCACT-3’</td>
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<tr>
<td>LDL-R</td>
<td>NT_093472</td>
<td>5’-AACTTGCAACCGAGGAGG-3’</td>
<td>5’-CCCAAGGGAGTCACATT3’</td>
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<tr>
<td>ACAT-2</td>
<td>NT_039472</td>
<td>5’-GGCTGTCCAATTTTGTCTGG-3’</td>
<td>5’-ATGCCATCGAGGATCTGGG-3’</td>
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<tr>
<td>LDL</td>
<td>NT_039472</td>
<td>5’-CACTCACTGGTGACCGAAA-3’</td>
<td>5’-CATTGACATCGAGTGGAG-3’</td>
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<tr>
<td>ACAT-2</td>
<td>NT_039472</td>
<td>5’-ATGGAGGTACCTGTTCTCGG-3’</td>
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<td>ABCA1</td>
<td>NT_109315</td>
<td>5’-CTGGACAAACTGGTCCACCT-3’</td>
<td>5’-GAGGAACTGGAATGAGGATG-3’</td>
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<td>ABCG1</td>
<td>NT_039649</td>
<td>5’-CTGGCAACCTCTTAAATGGTCTCG-3’</td>
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<tr>
<td>SRA</td>
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<td>5’-GGCCTGACCTTTGTCTCGG-3’</td>
<td>5’-TAGAAACCGGAGGTGGTAG-3’</td>
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<td>SRBI</td>
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<td>5’-AGTGTCAGGCGCTTCAAATGGTCTCG-3’</td>
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<td>MCP-1</td>
<td>NT_096135</td>
<td>5’-AGAGCGTCAAACGGGAGATG-3’</td>
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<td>Bcl-2</td>
<td>NT_078297</td>
<td>5’-GAGGAACTGGCAAAAGGATG-3’</td>
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<td>IFNγ</td>
<td>NT_039500</td>
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<td>IL-1β</td>
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<td>IL-12p40</td>
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<td>HPRT</td>
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<tr>
<td>β-gus</td>
<td>NT_039314</td>
<td>5’-CTGGCAACCTCTTAAATGGTCTCGG-3’</td>
<td>5’-TAGAAACCGGAGGTGGTAG-3’</td>
</tr>
<tr>
<td>RPS3</td>
<td>NT_039433</td>
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<tr>
<td>Bid</td>
<td>NT_039353.7</td>
<td>5’-AGTGTCAGGCGCTTCAAATGGTCTCGG-3’</td>
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<td>Bad</td>
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<td>Bax</td>
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<td>5’-AGTGTCAGGCGCTTCAAATGGTCTCGG-3’</td>
<td>5’-TCTCTCGAAGTGTAATGAAATTTATCG-3’</td>
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Supplemental References

Supplemental Fig. I. Characterization of mononuclear cells in Bcl-x KO^{Mac}/Apoe^{-/-} mice. Levels of anti-apoptotic genes measured in bone marrow-derived macrophages (BMDM) from Bcl-x KOMac/Apoe^{-/-} mice and Apoe^{-/-} mice by RT-PCR (A). Fresh peritoneal macrophages isolated from control Apoe^{-/-} and Bcl-x KOMac/Apoe^{-/-} mice were serum starved for 4 hours and analyzed for apoptosis by flow cytometry. Peritoneal and pulmonary macrophages isolated from control Apoe^{-/-} and Bcl-x KOMac/Apoe^{-/-} mice were cultured for 6 days, then serum-starved for 24 hours and analyzed for apoptosis by TUNEL staining. Bone marrow derived macrophages prepared from control Apoe^{-/-} and Bcl-x KOMac/Apoe^{-/-} mice were incubated in the presence of 7-ketocholesterol (10µg/ml) in the presence of 10% fetal bovine serum for 24 hours and analyzed for apoptosis by TUNEL staining (B). Values represent mean ± SEM of 6-8 mice per group. Statistically significant differences: * P<0.05, ** P<0.01, *** P<0.001, **** P<0.00001.
Supplemental Fig. II. Quantitative analysis of total circulating leucocytes (A,B), monocytes and individual monocyte subsets (C) in females (A) and males (B,C) mice maintained under both non-inflammatory (chow diet) and atherosclerotic conditions (cholesterol-rich diet). Values represent the mean ± SEM of a minimum of 15-20 mice per group. Statistically significant differences: * P<0.05, ** P<0.01 and *** P<0.001.
Supplemental Fig. III. Representative pictures of early atherosclerotic lesions in aortic roots of mice maintained on 0.15% (A) or 1% cholesterol-rich diet (B) for 10 weeks.
Supplemental Fig. IV. Colocalization of TUNEL cells with Macrophages in advanced atherosclerotic lesions. Nuclei were stained with DAPI (blue, A), staining of macrophages (F4/80) were visualized as green (B) and apoptotic cell (TUNEL staining in red, C). The yellow staining in merged images (D) illustrates that most of the TUNEL positive cells are of macrophage origin. Representative pictures of TUNEL positive cells in advanced atherosclerotic lesion of Apoe-/- (E) and Bcl-x KOMac/Apoe-/- mice (F).
Supplemental Fig. V. Quantification of hepatic mRNA in female mice fed a 1% cholesterol-enriched diet. Levels of mRNA expression of functional markers of the genetic ablation (A), mononuclear phagocytes (A), cholesterol metabolism (B) were evaluated by qPCR in the livers of Bcl-x KOMac/Apo e−/− and Apoe−/− mice fed a cholesterol diet for 10 weeks. Relative expression of each gene was normalized to the average expression of mRNA for three housekeeping genes (beta-glucuronidase, hypoxanthine guanine phosphoribosyl transferase and ribosomal protein S3. Values represent the mean ± SEM of 10 mice per group. Statistically significant differences: * P<0.01, ** P<0.001.