Low-Density Lipoprotein Receptor–Related Protein 1 Prevents Early Atherosclerosis by Limiting Lesional Apoptosis and Inflammatory Ly-6C^{high} Monocytosis

Evidence That the Effects Are Not Apolipoprotein E Dependent

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Background—We previously demonstrated that macrophage low-density lipoprotein receptor (LDLR)–related protein 1 (LRP1) deficiency increases atherosclerosis despite antiatherogenic changes including decreased uptake of remnants and increased secretion of apolipoprotein E (apoE). Thus, our objective was to determine whether the atheroprotective effects of LRP1 require interaction with apoE, one of its ligands with multiple beneficial effects.

Methods and Results—We examined atherosclerosis development in mice with specific deletion of macrophage LRP1 (apoE$^{-/-}$ MΦLRP1$^{-/-}$) and in LDLR$^{-/-}$ mice reconstituted with apoE$^{-/-}$ MΦLRP1$^{-/-}$ bone marrow. The combined absence of apoE and LRP1 promoted atherogenesis more than did macrophage apoE deletion alone in both apoE-producing LDLR$^{-/-}$ mice (+88%) and apoE$^{-/-}$ mice (+163%). The lesions of both mouse models with apoE$^{-/-}$ LRP1$^{-/-}$ macrophages had increased macrophage content. In vitro, apoE and LRP1 additively inhibit macrophage apoptosis. Furthermore, there was excessive accumulation of apoptotic cells in lesions of both LDLR$^{-/-}$ mice (+110%) and apoE$^{-/-}$ MΦLRP1$^{-/-}$ mice (+252%). The apoptotic cell accumulation was partially due to decreased efferocytosis as the ratio of free to cell-associated apoptotic nuclei was 3.5-fold higher in lesions of apoE$^{-/-}$ MΦLRP1$^{-/-}$ versus apoE$^{-/-}$ mice. Lesion necrosis was also increased (6 fold) in apoE$^{-/-}$ MΦLRP1$^{-/-}$ versus apoE$^{-/-}$ mice. Compared with apoE$^{-/-}$ mice, the spleens of apoE$^{-/-}$ MΦLRP1$^{-/-}$ mice contained 1.6- and 2.4-fold more total and Ly6-C^{high} monocytes. Finally, there were 3.6- and 2.4-fold increases in Ly6-C^{high} and CC-chemokine receptor 2–positive cells in lesions of apoE$^{-/-}$ MΦLRP1$^{-/-}$ versus apoE$^{-/-}$ mice, suggesting that accumulation of apoptotic cells enhances lesion development and macrophage content by promoting the recruitment of inflammatory monocytes.

Conclusion—Low-density lipoprotein receptor protein 1 exerts antiatherogenic effects via pathways independent of apoE involving macrophage apoptosis and monocyte recruitment. **(*Circulation*. 2011;124:454-464.)**

Key Words: atherosclerosis ■ apolipoproteins E ■ low-density lipoprotein receptor–related protein 1 ■ Ly6-C antigen, mouse ■ monocytes ■ apoptosis

Macrophages play a critical role in atherogenesis by taking up lipoprotein particles trapped in the arterial intima and activating the inflammatory response, or turning into apoptosis-prone foam cells. Apolipoprotein E (apoE) and the low-density lipoprotein receptor (LDLR)–related protein 1 (LRP1) are critical for these processes.[1–3] Plasma apoE associates with lipoproteins and causes bulk clearance of remnants.[4] Macrophages contribute to <10% of plasma apoE[5] but produce most of the apoE in the atheroma. Evidence supports an antiatherogenic role for both systemic and macrophage apoE.[1,2,6] Low-density lipoprotein receptor–related protein 1 is both a cargo transporter and a signaling receptor. It binds >30 distinct extracellular ligands and various cytoplasmic adaptor proteins.[7] Low-density lipoprotein receptor–related protein 1 protects against atherosclerosis when expressed by vascular smooth muscle cells,[8] hepatocytes,[9] or macrophages.[3,10]

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Clinical Perspective on p 464

Low-density lipoprotein receptor–related protein 1 and apoE may work in common pathways in preventing atherosclerosis. Apolipoprotein E on remnant lipoproteins serves as a ligand for their LRP1-mediated internalization[9] and is one of several triggers of LRP1-activated downstream signaling.[7] Apolipoprotein E signaling via LRP1 has been shown to promote cell survival.[11] Interaction of apoE with macrophage LRP1 initiates calcium mobilization, and both apoE and LRP1 limit the macrophage inflammatory response by preventing nuclear factor $\kappa\beta$ (NF-$\kappa\beta$) activation.[12,13] In addition, our previous studies demonstrated that macrophages lacking LRP1 cause increased atherosclerosis development in LDLR$^{-/-}$ mice despite upregulation of apoE secretion,[3,14] suggesting that LRP1 mediates the beneficial effects of apoE. In these studies, we also showed that the LRP1/apoE axis limits macrophage inflammation and regulates the rate of efferocytosis of apoptotic macrophages.[14]

The objective of the current studies was to investigate whether LRP1 modulates atherosclerosis independently of apoE. We examined atherosclerosis development in mice with specific deletion of macrophage LRP1 (apoE$^{-/-}$ MΦLRP1$^{-/-}$) and in LDLR$^{-/-}$ mice reconstituted with apoE$^{-/-}$ MΦLRP1$^{-/-}$ bone marrow. Our studies demonstrate that the proatherogenic effects of macrophage LRP1 deletion are enhanced in the absence of macrophage apoE and further enhanced in the absence of systemic apoE. The atherogenic effects of macrophage LRP1 deletion in the setting of complete apoE deficiency include enhanced intimal macrophage accumulation, excessive macrophage apoptosis, decreased efferocytosis, increased necrosis, and enhanced content of proinfammatory Ly6-C^{high} monocytes and CC-chemokine receptor 2–positive (CCR2+) macrophages.[15,16]

Methods

A detailed description of all methods is available in the online-only Data Supplement.

Mice

Mice lacking macrophage LRP1 (MΦLRP1$^{-/-}$) were developed as described[3] and mated with apoE$^{-/-}$ mice to obtain apoE$^{-/-}$ MΦLRP1$^{-/-}$ mice. All mice were on C57BL/6 background. Animal procedures were performed in accordance with the Institutional Animal Care and Usage Committee of Vanderbilt University.

Atherosclerosis Analyses

Low-density lipoprotein receptor–deficient mice (12-week old) were lethally irradiated (9.5 Gy) using a cesium gamma source and transplanted with 5×10^6 bone marrow (BM) cells from female apoE$^{-/-}$ (n=10) or apoE$^{-/-}$ MΦLRP1$^{-/-}$ mice (n=10) as described.[1,2] After 4 weeks, the mice were placed on Western-type diet for 8 weeks, and then the extent of atherosclerosis was examined. For experiments comparing atherosclerosis in apoE$^{-/-}$ (n=12) or apoE$^{-/-}$ MΦLRP1$^{-/-}$ mice (n=12), 8- to 10-week old female mice were fed a Western-type diet for 8 weeks. The extent of atherosclerosis was examined both in Oil Red O–stained cross-sections of the proximal aorta (15 alternate 10-μm cryosections) and by en face analysis using the KS300 imaging system (Kontron Elektronik GmbH) as described.[1]

Analyses of Macrophage Apoptosis

Peritoneal macrophages were seeded in chamber slides (Nalge Nunc International) and incubated for 16 hours in serum-free Dulbecco modified Eagles medium alone or containing lipopolysaccharide (50 ng/mL). Cell death was determined by TUNEL (Tdt-mediated dUTP nick end labeling) staining using the in situ detection kit (Roche).

Analyses of Lesion Apoptosis, Efferocytosis, and Necrosis

TUNEL staining was performed on 5-μm proximal aortic cryosections using the in situ cell death detection kit (Roche). The TUNEL-positive cells were counted in 4 sections per mouse and normalized to Oil-Red-O lesion area. For analysis of efferocytosis, 5-μm proximal aortic cryosections were stained with TUNEL using the TMR red (Roche) detection kit. Nuclei were counterstained with Hoechst, and macrophage cytoplasm was detected using rabbit antimacrophage antibody (Accurate Chemical and Scientific Corp), goat antirabbit Alexa Fluor 488 conjugated secondary antibody (Molecular Probes Inc). The free versus macrophage-associated apoptotic bodies were then counted in 4 sections per mouse. Necrosis was detected using Harris hematoxylin and eosin and quantitated by measuring the hematoxylin and eosin–negative acellular area in the intima versus total intimal area.

Analyses of Lesion MOMA-2, Ly-6C, and CCR2

Macrophage content was detected using rabbit antibody against mouse monocyte/macrophage–specific antibody (MOMA-2; Accurate Chemical & Scientific Corp) as described.[3] Ly-6C- and CCR2-positive cells were detected in 4 cryosections per mouse using rat antimouse Ly6-C biotin (BD Pharmingen No. 557359), rabbit antimouse CCR2 monoclonal (AbCam No. ab32144), either Streptavadin-AlexaFluor 488 (Invitrogen No. S11223) or goat antirabbit AlexaFluor 647 (Invitrogen No. A21244).

Flow Cytometry Analyses of Blood and Spleen Ly-6C^{high} Monocytes

Wild-type (WT, n=10), apoE$^{-/-}$ (n=10), MΦLRP1$^{-/-}$ (n=8), or apoE$^{-/-}$MΦLRP1$^{-/-}$ (n=10) mice were fed a Western diet for 8 weeks, and then blood was collected via cardiac puncture in sodium citrate (10 mmol/L sodium citrate, 13 mmol/L glucose, pH 6.5). Spleens were homogenized by disruption in sterile PBS pH 7.4 through a 70 μmol/L mesh screen. To distinguish monocytes from other blood and spleen cells, FITC fluorochrome-tagged rat antimouse CD90.2, B220, GR1 (Pharmingen) and NK cells (Caltag) were used. Total monocytes were detected using rat antimouse CD11b-PE (Pharmingen), and Ly6-C^{high} monocytes were quantitated using rat antimouse Ly6-C labeled with biotin (Pharmingen) and streptavadin-linked AlexaFluor 647 (Invitrogen).

Statistical Analysis

Differences between mean values were determined by 1-way ANOVA (Bonferroni post test) and Mann-Whitney test using GraphPad PRISM. Before using 1-way ANOVA (Bonferroni post test) to test for significance, the normality of the sample populations was tested by the Kolmogorov-Smirnov test. Significance was set for $P<0.05$.

Results

Impact of Macrophage LRP1 Deletion on Atherosclerosis and Intimal Macrophage Accumulation

Our previous studies have shown that LDLR$^{-/-}$ mice recipient of apoE$^{+/+}$MΦLRP1$^{-/-}$ or apoE$^{-/-}$ MΦLRP1$^{+/+}$ BM develop 40% and 75% larger lesions in the aortic sinus, respectively, compared with LDLR$^{-/-}$

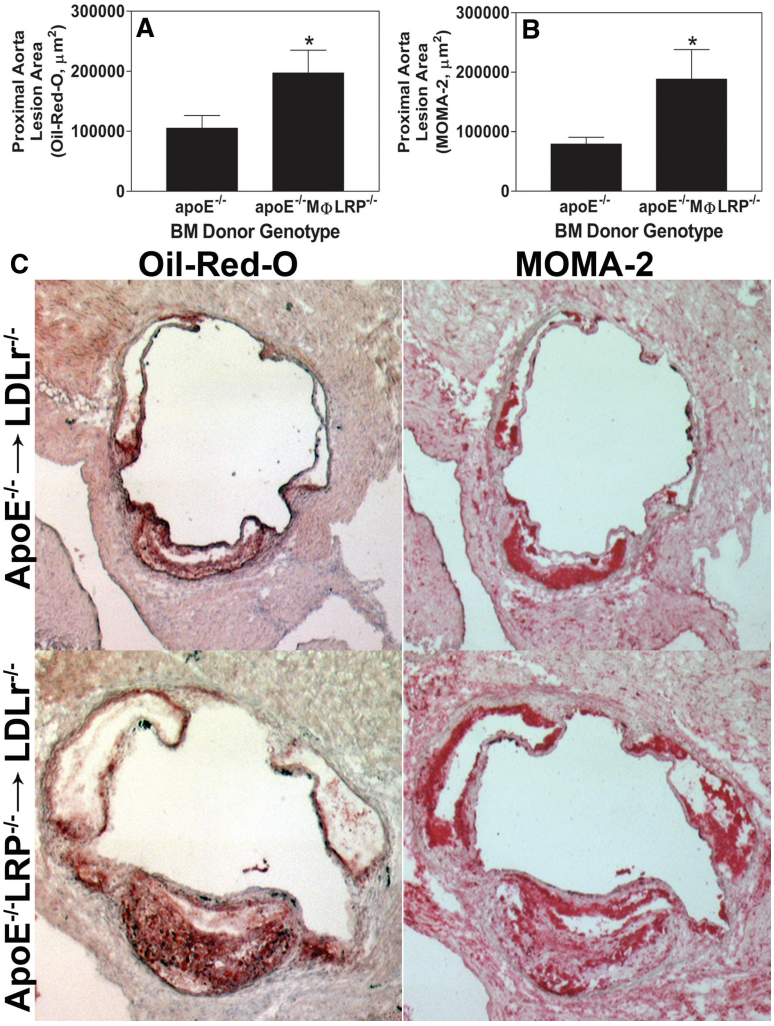

Figure 1. Atherosclerosis in LDLR$^{-/-}$ mice transplanted with BM from apoE$^{-/-}$ or apoE$^{-/-}$ MΦLRP$^{-/-}$ mice. Quantitation of the mean Oil-Red-O (**A**) and MOMA-2 (**B**) stained cross-sectional area of proximal aortas from LDLR$^{-/-}$ mice transplanted with either apoE$^{-/-}$ (n=10) or apoE$^{-/-}$ MΦLRP$^{-/-}$ (n=10) BM. *$P<0.05$, Mann-Whitney test. **C**, Representative images show Oil-Red-O and MOMA-2 stain in aortic root sections. ApoE$^{-/-}$ indicates apolipoprotein E deficient; MΦLRP1$^{-/-}$, macrophage LRP1 deficient; BM, bone marrow; MOMA-2, monocyte/macrophage–specific antibody 2; LRP$^{-/-}$, low-density lipoprotein receptor protein deficient; and LDLr, low-density lipoprotein receptor.

mice transplanted with WT BM.[2,3] In addition, we have shown that LRP1$^{-/-}$ macrophages express and secrete more apoE compared with WT cells.[14] Because macrophage apoE is a potent antiatherogenic agent,[2] the failure of increased expression of apoE in LRP1$^{-/-}$ macrophages to decrease atherosclerosis[3] is consistent with the possibility that the atherogenic phenotype caused by LRP1$^{-/-}$ macrophages is due to the abrogation of apoE-mediated benefits. Therefore, we set out to study the role of an apoE/LRP1 interaction in atherogenesis by looking at the cellular and vascular consequences of removing either macrophage apoE alone or both systemic and macrophage apoE from mice with LRP1$^{-/-}$ macrophages. We first examined the role of macrophage apoE/LRP1 interaction by transplanting BM from either apoE$^{-/-}$ or apoE$^{-/-}$ MΦLRP1$^{-/-}$ mice into LDLR$^{-/-}$ recipients. We reasoned that, if the atheroprotective effects of macrophage LRP1 were mediated, exclusively or to a large extent, by its interaction with macrophage apoE, then atherosclerosis development would be similar in LDLR$^{-/-}$ mice transplanted with apoE$^{-/-}$ or apoE$^{-/-}$ MΦLRP1$^{-/-}$ bone marrow. Four weeks after BM transplantation, recipient mice were placed on a Western diet for 8 weeks to raise plasma cholesterol levels and induce atherosclerosis. After 8 weeks on Western diet, the plasma cholesterol and triglyceride levels as well as the lipoprotein cholesterol profiles were not different between the 2 groups (online-only Data Supplement Figure IA). Compared with LDLR$^{-/-}$ mice receiving apoE$^{-/-}$ BM, those that received apoE$^{-/-}$MΦLRP1$^{-/-}$ marrow had 88% more lipid-stainable lesion area in the proximal aorta (Figure 1A and 1C) and a 138% increase in MOMA-2 stainable intimal macrophages (Figure 1B and 1C). Aorta en face lesions were minimal and not significantly different between apoE$^{-/-}$ MΦLRP1$^{-/-}$ and apoE$^{-/-}$ BM-recipient mice (0.91% versus 0.73%, $P=0.36$). These results are compatible with independent and additive effects of LRP1 and apoE on atherogenesis despite their cooperation in functional networks.[7,9,11]

Studies have shown that even small amounts of circulating apoE can decrease atherosclerosis without changing plasma cholesterol.[17] Therefore, it is important to bear in mind that in the LDLR$^{-/-}$ mice receiving apoE$^{-/-}$ marrow, systemic apoE can navigate into the vessel wall and affect lesional macrophage function, therefore influencing atherogenesis. To eliminate the effects of plasma apoE, we compared plaque development in apoE$^{-/-}$ and apoE$^{-/-}$ MΦLRP1$^{-/-}$ mice (n=12 each) fed a Western diet for 8

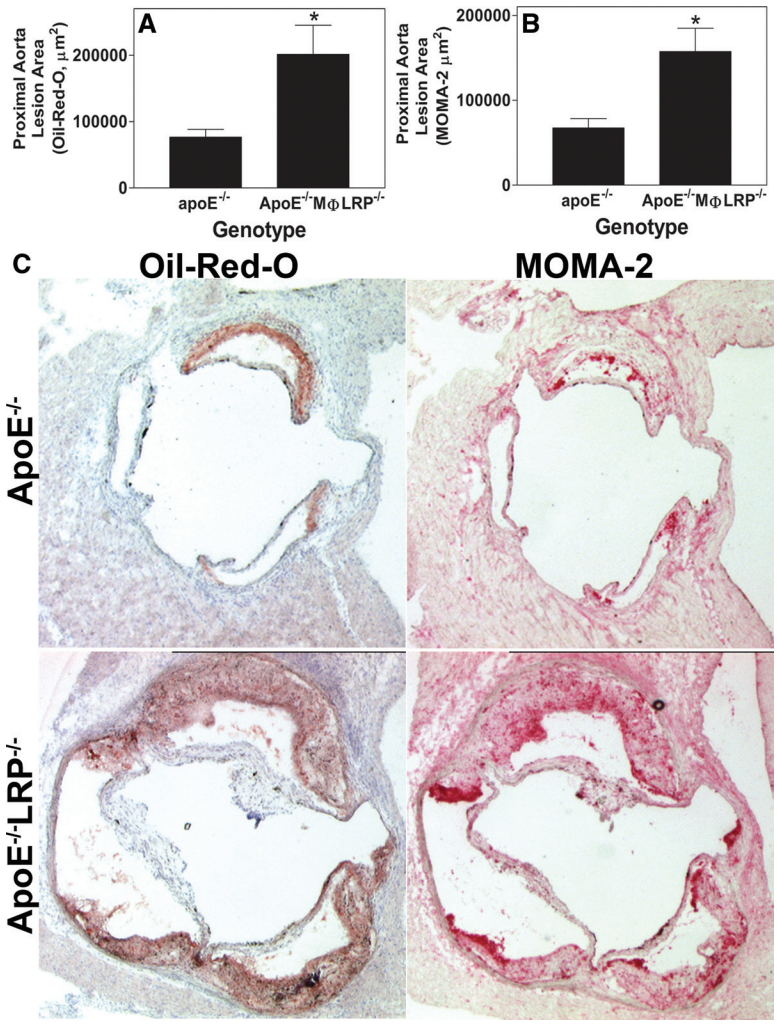

Figure 2. Atherosclerosis in apoE$^{-/-}$ and apoE$^{-/-}$ MΦLRP$^{-/-}$ mice. Quantitation of the mean Oil-Red-O (**A**) and MOMA-2 (**B**) stained cross-sectional area of proximal aortas from apoE$^{-/-}$ and apoE$^{-/-}$ MΦLRP$^{-/-}$ mice (n=12 per group). *$P<0.05$, Mann-Whitney test. **C**, Images show Oil-Red-O and MOMA-2 stain in aortic root sections. ApoE$^{-/-}$ indicates apolipoprotein E deficient; MΦLRP1$^{-/-}$, macrophage LRP1 deficient; MOMA-2, monocyte/macrophage–specific antibody 2; and LRP, low-density lipoprotein receptor protein.

weeks. There were no differences in plasma cholesterol, triglyceride, and lipoprotein fast protein liquid chromatography profiles between the 2 groups (online-only Data Supplement Figure IB). Apolipoprotein E and macrophage LRP1–deficient mice had 163% more Oil-Red-O and 133% more MOMA-2 staining in the proximal aorta compared with apoE$^{-/-}$ mice (Figure 2A through 2C). The fact that LRP1 modulates atherogenesis in the complete absence of apoE proves that LRP1 has apoE-independent effects in the vessel wall.

Effects of ApoE and LRP1 Deletion on Lesion and Macrophage Apoptosis

Macrophage apoptosis influences atherosclerosis development,[18] and both LRP1 and apoE play roles in cell survival and in efferocytosis of apoptotic macrophages.[11,14,19] Therefore, we next compared the level of apoptosis induction in peritoneal macrophages of the 4 genotypes under conditions of stress. To induce apoptosis, the cells were incubated for 16 hours in serum-free Dulbeccos modified Eagles medium (nutritional stressor), and cell death was determined by TUNEL staining (Figure 3A and 3B). Compared with WT macrophages, significantly more apoptotic cells were seen in cultures of apoE$^{-/-}$ (3.7 fold), LRP1$^{-/-}$ (2.5 fold), and apoE$^{-/-}$LRP1$^{-/-}$ (6.7 fold) macrophages, suggesting additive effects of LRP1 and apoE on apoptotic susceptibility. Similar differences were observed when apoptosis was stimulated with lipopolysaccharide, where TUNEL-positive cells were 10%, 21%, 24%, and 48% of lesions in WT, apoE$^{-/-}$, LRP1$^{-/-}$, and apoE$^{-/-}$LRP1$^{-/-}$ macrophage cultures, respectively.

We next examined whether the enhanced atherosclerosis that occurs in the absence of macrophage LRP1 can be attributed to increased macrophage apoptosis. TUNEL-staining analyses show that lesions of apoE$^{-/-}$ MΦLRP1$^{-/-}$ →LDLR$^{-/-}$ mice contained more than twice as many apoptotic cells per mm^2 lesion area compared with the lesions of apoE$^{-/-}$→LDLR$^{-/-}$ mice (Figure 3C and 3D). Compared with lesions of apoE$^{-/-}$ mice, there was a 3.5-fold increase in apoptotic cells in lesions of apoE$^{-/-}$ MΦLRP1$^{-/-}$ mice (Figure 4A and 4B), demonstrating that the effects of macrophage LRP1 deletion on lesional apoptotic cell accumulation are more exacerbated in the complete absence of apoE. The apoptotic cells colocalized with macrophage-enriched areas of lesions in both apoE$^{-/-}$ and apoE$^{-/-}$ MΦLRP1$^{-/-}$ mice (Figure 4A). Staining of lesion apoptosis, nuclei, and macrophage cytoplasm enabled the quantitation of free versus macro-

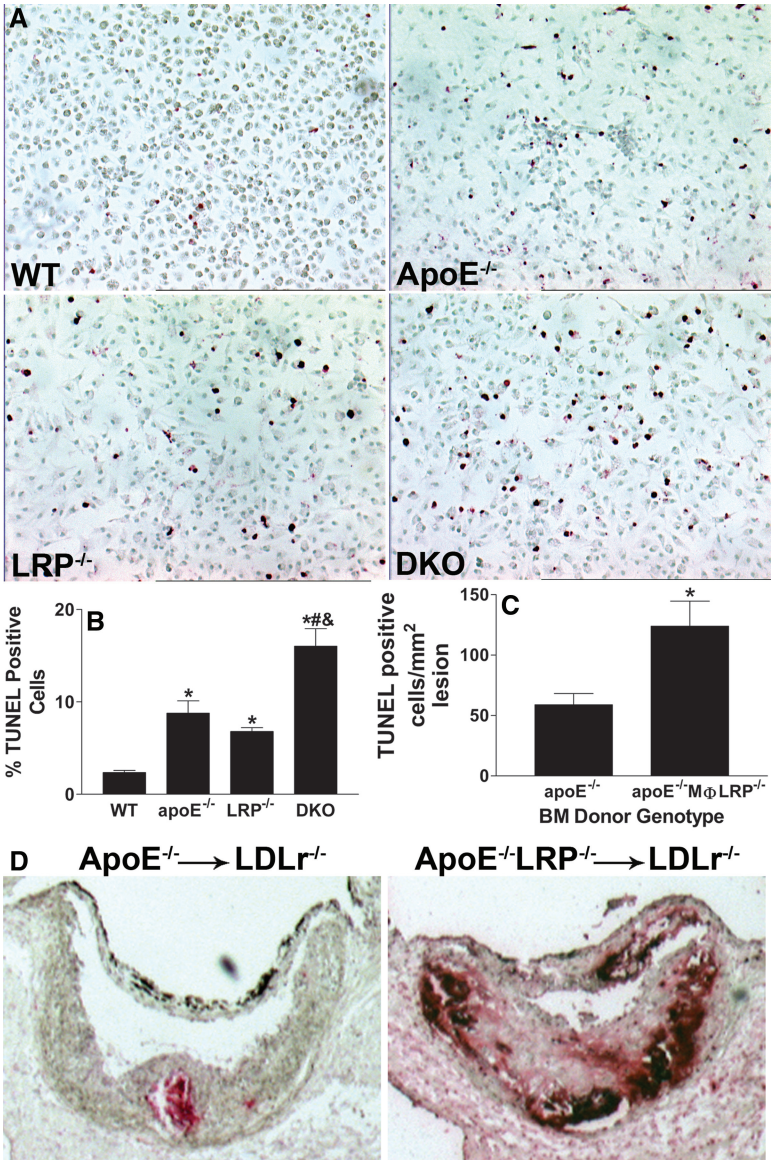

Figure 3. In vitro macrophage apoptosis and atherosclerotic lesion apoptosis**. A**, Images show TUNEL-positive (red) and -negative–stained macrophages after 16 hours with DMEM. **B**, Quantitation of the percentage of TUNEL-positive cells in WT, apoE$^{-/-}$, LRP$^{-/-}$, and apoE$^{-/-}$ LRP$^{-/-}$ cultures (n=6 per group). *, #, and & denote statistical significances compared with WT, apoE$^{-/-}$, and LRP$^{-/-}$ cells, respectively; $P<0.05$, ANOVA with Bonferroni post test. **C**, Quantitation of apoptotic cells in proximal aorta sections from apoE$^{-/-}$ (n=6) or apoE$^{-/-}$ MΦLRP$^{-/-}$ (n=7) BM-recipient LDLR$^{-/-}$ mice. *$P<0.05$, Mann-Whitney test. **D**, Images show TUNEL staining (dark red) of nuclei in aortic root sections. TUNEL indicates Tdt-mediated dUTP nick end labeling; WT, wild type; apoE$^{-/-}$, apolipoprotein E deficient; LRP$^{-/-}$, low-density lipoprotein receptor protein deficient; DKO, double knockout; MΦLRP1$^{-/-}$, macrophage LRP1 deficient; BM, bone marrow; and LDLr$^{-/-}$, low-density lipoprotein receptor deficient.

phage-associated apoptotic bodies as previously described in detail[14] (also see online-only Data Supplement Methods). The ratio of free to macrophage-associated apoptotic nuclei/bodies was 3.8-fold higher in lesions of apoE$^{-/-}$ MΦLRP1$^{-/-}$ mice compared to apoE$^{-/-}$ mice (Figure 4C), indicating that deletion of macrophage LRP1 impairs efferocytosis independently of apoE. Consistent with the excessive accumulation of apoptotic cells and defective lesional efferocytosis, the percentage of necrotic area was 6-fold greater in lesions of apoE$^{-/-}$ MΦLRP1$^{-/-}$ mice (Figure 5A and 5B).

Effects of ApoE and LRP1 Deletion on Circulating, Spleen, and Lesion Ly-6C^{high} Monocytes

Studies have shown that apoE$^{-/-}$ mice on a Western diet accumulate more proinflammatory Ly-6C^{high} monocytes (Gr1+CCR2+CX$_3$CR1^{low}) in blood, spleen, and atherosclerotic lesions.[15] In addition, our previous studies have shown that deletion of macrophage LRP1 increases cell migration in response to the CCR2 ligand monocyte chemoattractant protein-1. Furthermore, studies suggest that apoptotic macrophages can enhance lesion development by promoting endothelial cell inflammation and the recruitment of monocytes.[18,20,21] Therefore, we investigated the impact of LRP1 expression on the accumulation of Ly-6C^{high} monocytes. We first examined the effects of either single or combined deletion of apoE and LRP1 on circulating and splenic Ly-6C^{high} monocytes in WT, MΦLRP1$^{-/-}$, apoE$^{-/-}$, and apoE$^{-/-}$ MΦLRP1$^{-/-}$ mice fed a Western diet for 8 weeks, as measured by flow cytometry. The total number of blood monocytes was not significantly different among the 4 strains of mice (Figure 6A). Similar to what was previously reported,[15] Ly-6C^{high} monocyte levels in apoE$^{-/-}$ mice were 2.9-fold higher compared with WT mice (Figure 6B). Interestingly, Ly-6C^{high} monocyte levels were equally increased in MΦLRP1$^{-/-}$ and apoE$^{-/-}$ MΦLRP1$^{-/-}$ mice (Figure 6B), suggesting that an apoE–LRP1 interaction regulates blood Ly-6C^{high} monocytosis. Compared with WT spleens (Fig-

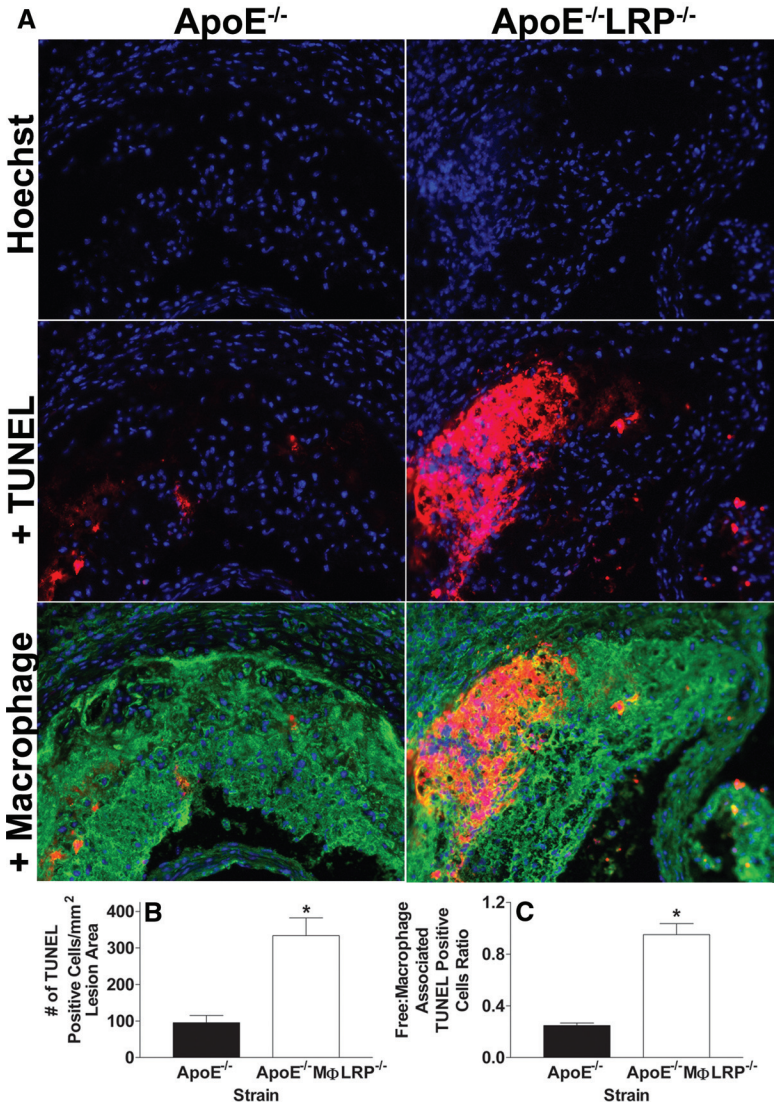

Figure 4. Macrophage apoptosis and efferocytosis in lesions of apoE$^{-/-}$ and apoE$^{-/-}$ MΦLRP$^{-/-}$ mice. **A**, Micrographs show nuclei (Hoechst, blue), nuclei + TUNEL-positive staining (red), and merged images of macrophage cytoplasm (green), nuclei, and TUNEL in aortic root sections from apoE$^{-/-}$ and apoE$^{-/-}$ MΦLRP$^{-/-}$ mice. Quantitation of the number of apoptotic cells (**B**) and of the ratio of free versus macrophage-associated TUNEL-positive cells (**C**) in proximal aorta sections of lesions from apoE$^{-/-}$ and apoE$^{-/-}$ MΦLRP$^{-/-}$ mice (n=6 per group). *P<0.05, Mann-Whitney test. ApoE$^{-/-}$ indicates alipoprotein E deficient; LRP$^{-/-}$, low-density lipoprotein receptor protein deficient; TUNEL, Tdt-mediated dUTP nick end labeling; and MΦLRP1$^{-/-}$, macrophage LRP1 deficient.

ure 6C), the number of total monocytes was 88%, 52%, and 150% higher in MΦLRP1$^{-/-}$, apoE$^{-/-}$, and apoE$^{-/-}$ MΦLRP1$^{-/-}$ spleens, respectively, indicating independent roles for apoE and LRP1 in spleen monocytosis. The number of spleen Ly-6C^{high} monocytes were markedly increased in MΦLRP1$^{-/-}$, apoE$^{-/-}$, and apoE$^{-/-}$ MΦLRP1$^{-/-}$ mice compared with WT mice (Figure 6D), suggesting in large part that the increased monocytosis was due to accumulation of Ly-6C^{high} monocytes. Consistent with independent roles of LRP1 and apoE in spleen Ly-6C^{high} monocytosis, the number of Ly-6C^{high} monocytes in apoE$^{-/-}$ MΦLRP1$^{-/-}$ spleens was 97% higher compared with MΦLRP1$^{-/-}$ spleens and 141% higher compared with apoE$^{-/-}$ spleens (Figure 6D). We next examined the distribution of Ly-6C–positive cells in lesions from apoE$^{-/-}$ and apoE$^{-/-}$ MΦLRP1$^{-/-}$ mice (Figure 7A and 7B). Interestingly, the lesions of apoE$^{-/-}$ MΦLRP1$^{-/-}$ mice contained 3.6-fold more Ly-6C–positive cells compared with lesions of apoE$^{-/-}$ mice, suggesting that LRP1 acts independently of apoE in controlling the accumulation of proinflammatory Ly-6C^{high} monocytes in the plaque. CC chemokine receptor 2 is also a marker for proinflammatory monocytes and macrophages[16] and is critical for the recruitment of Ly-6C^{high} monocytes into the intima.[22] Consistent with the increased Ly-6C^{high} monocyte content in apoE$^{-/-}$ MΦLRP1$^{-/-}$ lesions, these plaques also contained 2.4-fold more CCR2-positive cells compared with lesions of apoE$^{-/-}$ mice (Figure 7C and 7D).

Discussion

We previously demonstrated that macrophages lacking LRP1 enhance atherogenesis in the setting of increased apoE production.[3,14] These results suggested that the established protective effects of apoE are mediated by its interaction with LRP1. The present studies were designed to determine the effects of macrophage LRP1 deletion in the absence of apoE, either from macrophages only or from all cells, with the aim of evaluating whether the atheroprotective effects of LRP1 are independent of its interaction with apoE. Our results show that LRP1 operates independently of apoE in regulating apoptosis, necrosis, and inflammatory Ly-6C^{high} monocytosis and that deletion

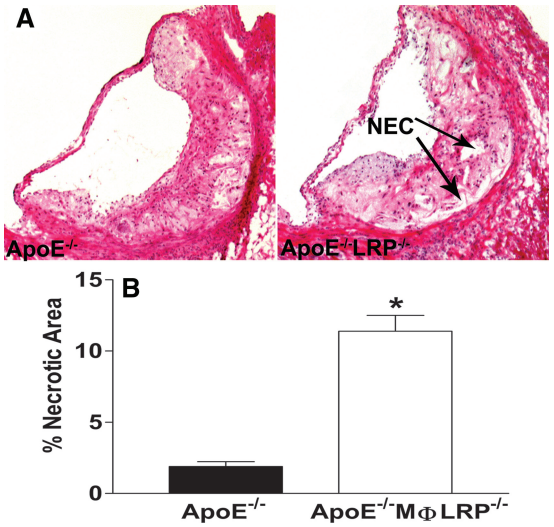

Figure 5. Necrosis in lesions of apoE$^{-/-}$ and apoE$^{-/-}$ MΦLRP$^{-/-}$ mice. **A**, Images show hematoxylin and eosin staining of aortic root sections from apoE$^{-/-}$ and apoE$^{-/-}$ MΦLRP$^{-/-}$ mice. **B**, Quantitation of the percentage of necrotic area in the aortic lesions from apoE$^{-/-}$ and apoE$^{-/-}$ MΦLRP$^{-/-}$ mice (n=6 per group). *P<0.05, Mann-Whitney test. Apoe$^{-/-}$ indicates apolipoprotein E deficient; NEC, necrotic area; LRP$^{-/-}$, low-density lipoprotein receptor protein deficient; and MΦLRP1$^{-/-}$, macrophage LRP1 deficient.

of apoE, locally or systemically, amplifies the atherogenic effects of macrophage LRP1 deletion.

The Atheroprotective Effects of Macrophage LRP1 Are Independent of ApoE

We previously showed that MΦLRP1$^{-/-}$ BM-recipient LDLR$^{-/-}$ mice develop 40% more atherosclerosis in the proximal aorta compared with LDLR$^{-/-}$ mice receiving control BM.[3] Before that, we had shown that deletion of macrophage apoE in LDLR$^{-/-}$ mice increases aortic sinus plaque burden by 75%.[2] These data demonstrate that LRP1 and apoE both exert atheroprotective functions. Given that apoE is a ligand for LRP1, we set out to study whether these proteins work exclusively in an interdependent pathway to maintain vascular integrity. Thus, we examined the effects of combined apoE and LRP1 deletion in macrophages. Our findings that apoE$^{-/-}$ MΦLRP1$^{-/-}$ BM→LDLR$^{-/-}$ mice develop 88% more atherosclerosis than recipients of apoE$^{-/-}$ BM proves that the combined deletion of these proteins has additive effects, thus favoring the hypothesis that the atheroprotective effects of macrophage LRP1 are largely independent of its interaction with apoE. In addition, the increased atherosclerotic effect of combined macrophage apoE/LRP deletion relative to that of single deletion of macrophage LRP1[3,14] may be attributed to the fact that apoE$^{-/-}$ LRP$^{-/-}$ macrophages lack the compensatory benefit of increased macrophage apoE expression caused by isolated LRP1 deletion.

Because in LDLR$^{-/-}$ mice systemic apoE may interact with macrophage LRP1, we also compared atherosclerosis development in apoE$^{-/-}$ and apoE$^{-/-}$ MΦLRP1$^{-/-}$ mice, the latter lacking both systemic and macrophage apoE. Interestingly, apoE$^{-/-}$ MΦLRP1$^{-/-}$ mice developed 163% larger lesions than apoE$^{-/-}$-only mice. This result is consistent with recent studies of Hu and colleagues[10] that showed a 114% increase in apoE$^{-/-}$ LDLR$^{-/-}$ MΦLRP1$^{-/-}$ mice compared with apoE$^{-/-}$ LDLR$^{-/-}$ mice. On the basis of all these studies, we conclude that macrophage LRP1 is atheroprotective regardless of the absence or presence of systemic or macrophage apoE and that other LRP1 ligands and/or signaling events must in part mediate the antiatherogenic functions of macrophage LRP1. Conversely, apoE influences the process of plaque formation even in the absence of macrophage LRP1, as evidenced by the observations that the effects of LRP deletion on atherosclerosis and lesion apoptosis were much more pronounced in the setting of complete apoE deficiency. This is consistent with studies showing that even small amounts of circulating apoE can decrease atherosclerosis development[17] and that exogenous apoE limits inflammation, apoptosis, and foam cell formation.[19,23,24]

LRP1 Expression Limits Ly-6C^{high} Monocytosis

Studies of Swirski and colleagues have shown that apoE$^{-/-}$ mice on a Western diet develop enhanced Ly-6C^{high} mono-

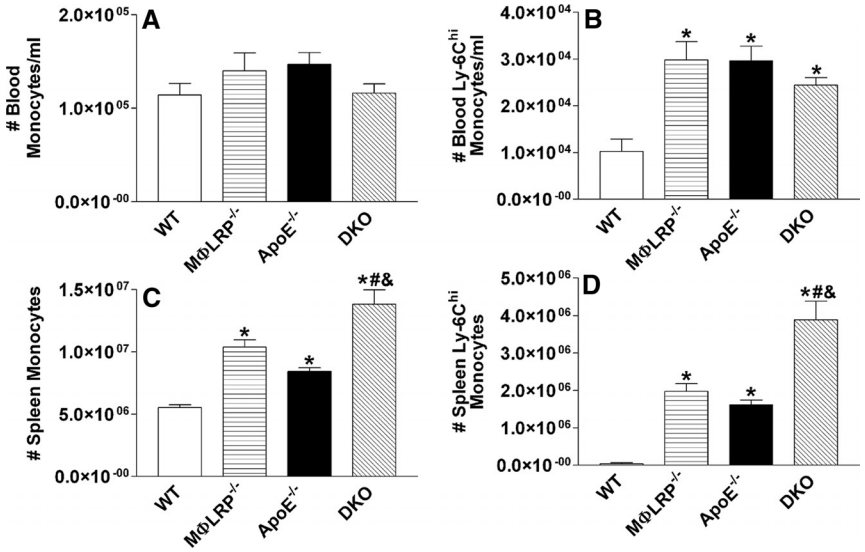

Figure 6. Total and Ly-6C^{high} monocytes in blood and spleens of WT, MΦLRP$^{-/-}$, apoE$^{-/-}$, and apoE$^{-/-}$ MΦLRP$^{-/-}$ mice. **A** through **D,** Wild-type (n=10), MΦLRP$^{-/-}$ (n=8), apoE$^{-/-}$ (n=10), and apoE$^{-/-}$ MΦLRP$^{-/-}$ (n=10) mice were fed a Western-type diet for 8 weeks, and then blood (**A** through **B**) and spleen (**C** through **D**), total (**A** and **C**) and Ly-6C^{high} monocytes (**B** and **D**) were measured by flow cytometry analysis. In **A** through **D**, *, #, and & denote statistical significance compared with WT, apoE$^{-/-}$, and MΦLRP$^{-/-}$ mice, respectively; P<0.05, ANOVA with Bonferroni post test. WT indicates wild type; MΦLRP1$^{-/-}$, macrophage LRP1 deficient; Apoe$^{-/-}$, apolipoprotein E deficient; and DKO, double knockout.

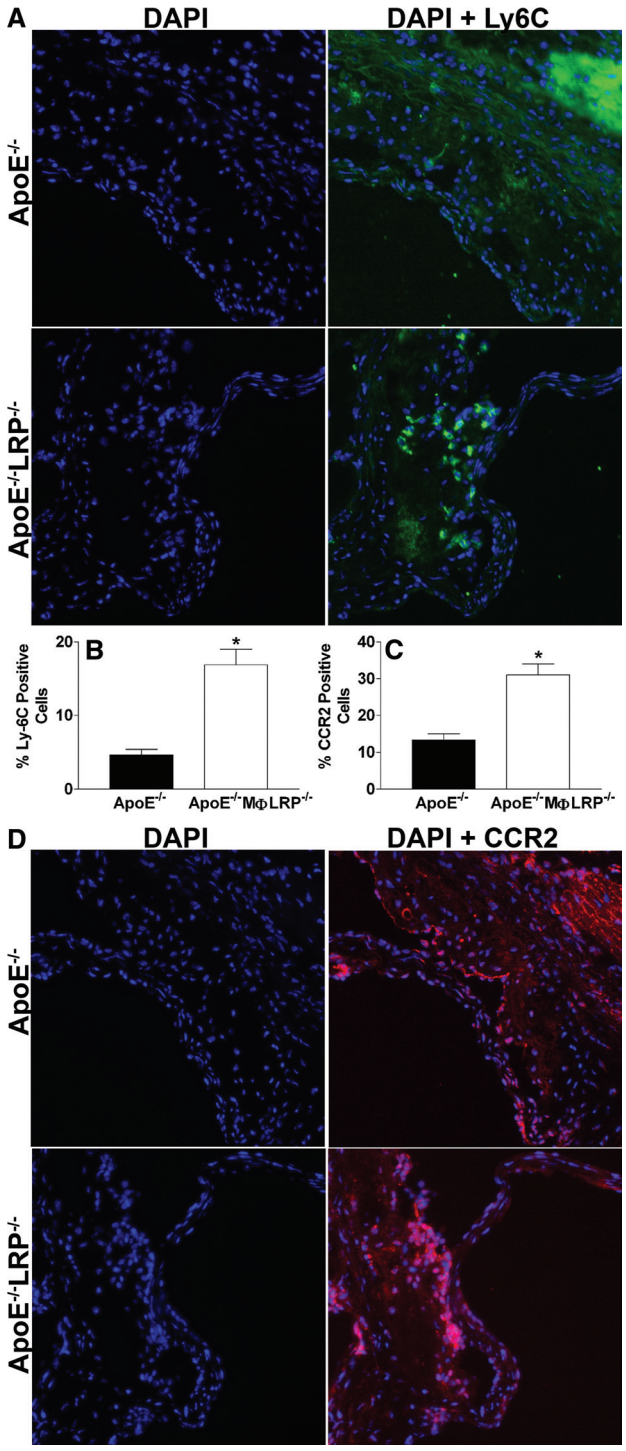

Figure 7. Ly-6C- and CCR2-positive cells in lesions of apoE$^{-/-}$ and apoE$^{-/-}$ MΦLRP$^{-/-}$ mice. **A** and **D**, Representative images show nuclei (blue, Dapi) and merged images of Ly-6C (**A**, green) and CCR-positive staining (**D**, red) and nuclei. **B** and **C**, Quantitation of the percentage of Ly-6C- (**B**) and CCR2- (**C**) positive cells in aortic root sections from apoE$^{-/-}$ and apoE$^{-/-}$ –MΦLRP$^{-/-}$ mice (n=6 per group). *$P<0.05$, Mann-Whitney test. Apoe$^{-/-}$ indicates apolipoprotein E deficient; LRP$^{-/-}$, low-density lipoprotein receptor protein deficient; MΦLRP1$^{-/-}$, macrophage LRP1 deficient; and CCR2, CC-chemokine receptor 2.

cytosis that results from increased survival and proliferation as well as lower conversion of Ly-6C^{high} monocytes to Ly-6C^{low} monocytes.[25] A role for lipid loading in this process was suggested by the fact that statins prevented the Ly-6C^{high} monocytosis whereas incubation with LDL promoted Ly-6C^{high} monocyte survival.[25] Our studies demonstrate that deletion of macrophage LRP1 enhances circulating, splenic, and atheroma Ly-6C^{high} monocytosis (Figures 6B, 6D, and 7B). The observation that Ly-6C^{high} monocytosis in blood and spleen is similar in MΦLRP1$^{-/-}$ versus apoE$^{-/-}$ mice suggests that LRP1 plays a direct role in limiting Ly-6C^{high} monocytosis, as plasma cholesterol levels were much lower in MΦLRP1$^{-/-}$ mice compared to apoE$^{-/-}$ mice (101±12 versus 817±40 mg/dL). Consistent with this concept is the observation that blood and spleen Ly-6C^{high} monocytosis was markedly enhanced in MΦLRP1$^{-/-}$ mice compared with WT mice, which had similar plasma cholesterol levels (Figure 6B and 6D). The finding that blood Ly-6C^{high} monocytosis was similar in MΦLRP1$^{-/-}$, apoE$^{-/-}$, and apoE$^{-/-}$ MΦLRP1$^{-/-}$ mice (Figure 6B) could indicate that an interdependent LRP1/apoE interaction regulates circulating Ly-6C^{high} monocyte levels, where the absence of the ligand causes an equivalent effect to the absence of its receptor. However, it is worth noting that circulating Ly-6C^{high} monocytes are maintained at low levels because of their rapid deployment to sites of inflammation[26–28] and their shuttling back to the bone marrow compartment for conversion to Ly-6C^{low} monocytes,[26] raising the possibility that the lack of a difference in blood Ly-6C^{high} monocyte levels is merely a reflection of increased recruitment of monocytes to inflammatory areas in apoE$^{-/-}$ MΦLRP1$^{-/-}$ mice. Spleen and lesion Ly-6C^{high} monocyte levels were indeed markedly increased in apoE$^{-/-}$ MΦLRP1$^{-/-}$ mice compared with apoE$^{-/-}$ mice (Figure 6B), supporting an independent role for LRP1 in controlling Ly-6C^{high} monocytosis. Splenic Ly-6C^{high} monocytosis is increased during inflammation,[29] and the spleen has been recently proven to serve as the source of Ly-6C^{high} monocytes for recruitment to acute inflammatory sites, such as ischemic heart muscle.[28] Our previous studies showed that deletion of macrophage LRP1 enhances the expression of inflammatory cytokines including monocyte chemoattractant protein-1, TNF-α, and interleukin 1β,[14] which could enhance recruitment of Ly-6C^{high} monocytes to the spleen. In addition, the enhanced Ly-6C^{high} monocytosis is likely due in part to activation of NF-$\kappa\beta$ because increases in monocyte/macrophage survival and proliferation result from NF-$\kappa\beta$ activation,[30–32] and our studies demonstrated that expression of LRP1 reduces NF-$\kappa\beta$ activation.[12] Another possibility is that LRP1 regulates Ly-6C^{high} monocytosis by influencing cholesterol homeostasis, given that cholesterol efflux reduces monocytosis[33] and deletion of LRP1 decreases ABCA1 expression and cholesterol efflux in smooth muscle cells.[34]

LRP1 Prevents Macrophage Apoptosis Independently of ApoE

Macrophage apoptosis plays a role in atherosclerosis development.[18] Studies have shown that LRP1 mediates the phagocytosis of apoptotic macrophages and promotes

cell survival.[11,14] ApoE has also been implicated in mediating phagocytic[14,19] and antiapoptotic pathways in cells.[35] In some cell types, the antiapoptotic effects of apoE are mediated by LRP1/apoE interaction.[11] Our in vitro studies show that both LRP1 and apoE deletion increase macrophage apoptosis and that the combined deletion of LRP1 and apoE produces an additive effect in increasing the number of apoptotic macrophages (Figure 3A and 3B). This observation suggests that apoE and LRP1 have independent roles in macrophage apoptosis. Thus, apoE may protect macrophages from apoptosis by interacting with proteins other than LRP1. As an example, scavenger receptor class B, type 1, has been shown to mediate the phagocytic clearance of apoptotic cells and bind with high affinity to apoE/phospholipid complexes.[36] However, our recent studies demonstrate that macrophage LRP1 deletion in LDLR$^{-/-}$ mice promotes the accumulation of lesion apoptotic macrophages by decreasing efferocytosis despite increased apoE secretion compared with WT cells.[14] This suggests that in vivo the independent antiapoptotic mechanisms of apoE cannot compensate for the absence of LRP1. Consistent with the in vitro studies, we demonstrate that combined deletion of macrophage LRP1 and apoE in vivo results in markedly increased numbers of lesion apoptotic cells both in the presence of systemic apoE (Figure 3C) and in the complete absence of apoE (Figure 4B). Furthermore, the accumulation of apoptotic macrophages resulting from combined deletion of LRP1 and apoE was associated with impaired efferocytosis (Figure 4C). Low-density lipoprotein receptor protein 1 ligands that can regulate apoptotic-cell homeostasis in vivo include prosaposin,[37] α_2-macroglobulin,[38] β_2 glycoprotein-1,[39] and calreticulin.[40]

In recent years, it has become clear that macrophage apoptosis has divergent effects on atherosclerosis[18,41] depending on the balance between generation of apoptotic cells and efficient phagocytic clearance. Studies by our group and others have shown that in the early fatty streak phase,[18,42,43] modest increases in apoptotic macrophages coincide with decreased atherosclerosis because of efficient efferocytosis. In later lesion stages, the accumulation of apoptotic cells has been proposed to promote plaque instability.[41] The noninternalized apoptotic cells secrete inflammatory cytokines,[14,44] driving more postapoptotic necrosis resulting in plaque instability.[14,41] Consistent with the accumulation of inflammatory apoptotic macrophages promoting plaque instability, the necrotic area was markedly increased in lesions of apoE$^{-/-}$ MΦLRP1$^{-/-}$ mice compared with lesions in apoE$^{-/-}$ mice (Figure 5) after only 8 weeks of consuming a Western diet.

Besides impacting plaque stability, the excessive accumulation of apoptotic macrophages in lesions of mice with combined deficiency of apoE and LRP likely contributes to the accelerated atherosclerosis development by facilitating the recruitment of monocytes. Studies have demonstrated that apoptotic macrophages contain oxidized phospholipid, which promote secretion of inflammatory cytokines, endothelial cell activation, and recruitment of monocytes.[18,21] The current studies show that the combined deficiency of macrophage apoE and LRP1 enhance the macrophage content in lesions of both LDLR$^{-/-}$ and apoE$^{-/-}$ mice. Consistent with this possibility also is the finding that the lesions of apoE$^{-/-}$ MΦLRP1$^{-/-}$ contained more Ly-6C^{high} monocytes (Figure 7B). Compared with Ly-6C^{low} monocytes, Ly-6C^{high} monocytes preferentially bind to activated endothelium and migrate into atherosclerotic lesions.[15] CC chemokine receptor 2 is a marker for proinflammatory monocytes and macrophages[16] and is critical for the recruitment of Ly-6C^{high} monocytes into the intima.[15] Thus, the increased content of CCR2-positive cells (Figure 7C) in apoE$^{-/-}$ MΦLRP1$^{-/-}$ lesions is also consistent with enhanced recruitment of proinflammatory Ly-6C^{high} monocytes. Other studies have shown that accumulation of lesion apoptotic macrophages is associated with enhanced atherosclerosis progression and monocyte recruitment,[18,45,46] particularly in the presence of inflammation resulting from defective efferocytosis.[20,47,48]

In conclusion, our study demonstrates that macrophage LRP1 slows atherosclerosis development by limiting lesion macrophage death and Ly-6C^{high} monocytosis. These atheroprotective functions occur via mechanisms that are independent of its interaction with apoE. Thus, apoE and LRP1, 2 abundant atheroma proteins that physically interact with each other to regulate cell survival, remnant uptake, and activate downstream signaling[7,9,11] actually influence atherogenesis through nonredundant pathways.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Apolipoprotein E (apoE) is a plasma protein that regulates both clearance of very low–density lipoprotein and maturation of high-density lipoprotein. It is also expressed at high levels by macrophages and has been found to have strong anti-atherogenic effects in mouse models. In humans, high-density lipoprotein–associated apoE correlates with presence of coronary artery disease and may become a biomarker for this common disease. Apoliprotein E binds to multiple receptors, including low-density lipoprotein receptor protein 1 (LRP1), a member of the low-density lipoprotein receptor family. Low-density lipoprotein receptor protein 1 binds multiple ligands and can both internalize cargo and trigger signaling-mediated downstream effects. Both proteins control cellular cholesterol trafficking and plaque volume via regulation of cell death. These functions are key targets for the development of therapeutic strategies aiming at inducing plaque regression, an elusive and highly prized objective. We previously determined that macrophages lacking LRP1 cause accelerated atherosclerosis, a paradoxical finding given that in these cells (a) atherogenic lipoproteins are internalized with reduced efficiency and (b) secretion of apoE is significantly upregulated. Because this observation was made in mice expressing normal amounts of systemic and macrophage apoE, it was not possible to determine whether the negative effect of LRP1 removal was either caused by the interruption of an apoE–LRP1 axis or attenuated by the overexpression of apoE. The current studies clearly show that most functions of apoE and LRP1 in the artery wall occur through mutually independent pathways and that the absence of apoE greatly magnifies the effects of LRP1 deficiency on cell death. Our results help understand the forces controlling plaque volume expansion or contraction and may inform development of regression-inducing agents.