Macrophage LRP-1 Controls Plaque Cellularity by Regulating Efferocytosis and Akt Activation

Patricia G. Yancey; John Blakemore; Lei Ding; Daping Fan; Cheryl D. Overton; Youmin Zhang; MacRae F. Linton; Sergio Fazio

Objective—The balance between apoptosis susceptibility and efferocytosis of macrophages is central to plaque remodeling and inflammation. LRP-1 and its ligand, apolipoprotein E, have been implicated in efferocytosis and apoptosis in some cell types. We investigated the involvement of the macrophage LRP-1/apolipoprotein E axis in controlling plaque apoptosis and efferocytosis.

Method and Results—LRP-1−/− macrophages displayed nearly 2-fold more TUNEL positivity compared to wild-type cells in the presence of DMEM alone or with either lipopolysaccharide or oxidized low-density lipoprotein. The survival kinase, phosphorylated Akt, was barely detectable in LRP-1−/− cells, causing decreased phosphorylated Bad and increased cleaved caspase-3. Regardless of the apoptotic stimulation and degree of cell death, LRP-1−/− macrophages displayed enhanced inflammation with increased IL-1β, IL-6, and tumor necrosis factor-α expression. Efferocytosis of apoptotic macrophages was reduced by 60% in LRP-1−/− vs wild-type macrophages despite increased apolipoprotein E expression by both LRP-1−/− phagocytes and wild-type apoptotic cells. Compared to wild-type macrophage lesions, LRP-1−/− lesions had 5.7-fold more necrotic core with more dead cells not associated with macrophages.

Conclusion—Macrophage LRP-1 deficiency increases cell death and inflammation by impairing phosphorylated Akt activation and efferocytosis. Increased apolipoprotein E expression in LRP-1−/− macrophages suggests that the LRP-1/apolipoprotein E axis regulates the balance between apoptosis and efferocytosis, thereby preventing necrotic core formation. (Arterioscler Thromb Vasc Biol. 2010;30:00-00.)

Key Words: apolipoprotein E • apoptosis • efferocytosis • inflammation • LRP-1 • necrosis

LRP-1 is a ubiquitous multifunctional receptor, and its systemic expression is essential for embryonic development. LRP-1 is critical for the clearance of plasma remnants, because conditional hepatic LRP-1 deletion results in increased plasma triglyceride and chylomicron levels. We have shown that deletion of macrophage LRP-1 increases atherosclerotic lesion formation in the proximal aorta of low-density lipoprotein receptor (LDLR)−/− mice without affecting serum lipoprotein levels and despite decreased macrophage uptake of apoE-containing very-low-density lipoprotein. The enhanced atherosclerotic lesion formation was associated with increased formation of breaks in the elastic lamina, activated inflammation, and increased macrophage cellularity in the plaque.

Macrophages in atherosclerotic lesions undergo apoptosis and necrosis because of cholesterol toxicity, oxidative stress, and signaling from cytokines and other molecules. A determining factor in lesion formation, remodeling, and progression is the balance between the generation of apoptotic cells and their phagocytosis (efferocytosis), because non-internalized apoptotic cells secrete inflammatory cytokines, thus driving uncontrolled cell death and inducing plaque instability. LRP-1 has been linked to the efferocytosis of apoptotic cells by macrophages and nonprofessional phagocytes through colocalization with ABCA7 and activation of extracellular signal-regulated kinases. However, it remains to be determined whether macrophage LRP-1 plays a significant role in efferocytosis of lesional apoptotic macrophages. Studies have shown that the Merk receptor, not LRP-1, mediates the efferocytosis of macrophages made apoptotic by free cholesterol burden, whereas other studies suggest that the Merk receptor plays a significant role in efferocytosis of lesional apoptotic macrophages. Besides mediating efferocytosis, it is possible that LRP-1 regulates macrophage susceptibility to apoptosis. Studies with cell types, including fibroblasts and neurons, have demonstrated that blockade of LRP-1/ligand interaction via receptor-associated protein enhances cell death under serum-free conditions. In addition, studies have shown that incubation of macrophages with antibodies directed against the ligand binding domain of LRP-1 results in increased diacylglycerol, cAMP, and intracellular calcium mobilization, raising the possibility that...
LRP-1 signaling regulates levels of phosphorylated Akt (pAkt), a critical player in promoting macrophage survival.

Macrophage apolipoprotein E (apoE) is a ligand for LRP-1 and a major determinant of atherosclerosis susceptibility by regulating cholesterol trafficking and reducing oxidative stress. Besides these atheroprotective functions, studies suggest that apoE may reduce inflammation by mediating efficient efferocytosis of apoptotic cells. Compared to wild-type (WT) mice, apoE−/− mice show increased numbers of apoptotic cells and enhanced inflammatory responses in different tissues. Furthermore, in vitro studies have demonstrated that the efferocytosis of apoE−/− apoptotic cells is impaired. Consistent with the notion that apoE plays a role in efferocytosis, the synthesis of apoE is markedly enhanced in macrophages undergoing apoptosis, perhaps as a mechanism to increase recognition and internalization by neighboring phagocytes through LRP-1. In addition, studies have shown that apoE is linked to cell survival in other cell types, including ovarian and neuronal cells. In neurons, apoE signaling reduces susceptibility to apoptosis via interaction with a number of receptors, including apoE receptor 2 (LRP-1).

Our previous studies have demonstrated that deletion of macrophage LRP-1 increases atherosclerosis, plaque instability, inflammation, and macrophage cellularity. Because apoE is a ligand for LRP-1, we postulated that a functional axis exists between macrophage apoE and LRP-1 to minimize inflammation and uncontrolled cell death by maintaining an optimal balance between macrophage survival and efferocytosis of apoptotic cells. To study the effects of macrophage LRP-1 on cellular apoptosis, we used a macrophage-specific LRP-1−/− mouse we developed using a Cre/lox-based approach. Through assessment of annexin V binding of membrane-exposed phosphatidylserine, TUNEL staining, and analyses of activated caspase 3, we show that deletion of LRP-1 increases cellular apoptosis with and without exogenous stimulation of cell death and in the absence or presence of cholesterol overload. The enhanced induction of apoptosis was associated with enhanced inflammation. Furthermore, our studies demonstrate that deletion of macrophage LRP-1 impairs efferocytosis of apoptotic cells and increases susceptibility to apoptosis by suppressing the pAkt pathway. All events occurred under conditions of enhanced apoE expression, thus suggesting that the antiinflammatory, prosurvival, and efferocytosis effects mediated by apoE depend on its interaction with LRP-1. More importantly, we show that macrophage LRP-1−/− deficient lesions have excessive accumulation of TUNEL-positive cells, more dead cells not associated with macrophages, and markedly increased necrotic core formation compared to lesions containing WT macrophages. Taken together, our studies demonstrate an atheroprotective role for the LRP-1/apoE axis in preventing necrotic core formation by regulating the balance between apoptosis susceptibility and efferocytosis.

Materials and Methods
A detailed description of all Materials and Methods is available in the Supplemental materials (available online at http://atvb.ahajournals.org).

In Vivo and In Vitro Analysis of Macrophage Apoptosis
For in vivo analysis, cells taken directly from the peritoneal cavity were subjected to flow cytometry analysis of Annexin V/7aaD (BD Biosciences) and CD11b. For in vitro analysis, WT and LRP-1−/− peritoneal macrophages were incubated for 24 hours in DMEM alone or containing either lipopolysaccharide (LPS; 50 ng/mL) or copper-oxidized LDL (50 g protein/mL). Cell death was determined by TUNEL (Roche). Activated caspase 3 was performed using NucView Caspase Detection kit (Biotium).

In Vitro Measurement of the Efferocytosis of Apoptotic Macrophages
WT, LRP-1−/−, or apoE−/− peritoneal macrophages were labeled with carboxy-fluorescein diacetate succinimidyl ester (Molecular Probes) cell tracer and made apoptotic by incubation with either staurosporine (5 g/mL) for 24 hours or BAY11-7082 (20 g/mL) for 2 hours. Apoptotic cells were then incubated for 2 hours with fresh phagocyte. After vigorous washing with phosphate-buffered saline, the phagocytes were fixed in 4% paraformaldehyde, counterstained with DAPI, and efferocytosis of apoptotic cells was visualized using fluorescence microscopy.

Analysis of Efferocytosis in the Peritoneal Cavity
Carboxy-fluorescein diacetate succinimidyl ester cell tracer green-labeled WT macrophages were made apoptotic by the addition of 20 g/mL Bay 11-7082 in serum-free DMEM for 2 hours. Then, 20×10⁶ cells in 1 mL of phosphate-buffered saline were injected into WT or MφLRP−/− mice that had been injected 2 days previously with 1 mL of 3% thioglycollate. One hour later, the peritoneal cells were harvested and viable cells were labeled by incubation with nonfluorescent C12-resazurin, which metabolizes to red fluorescent C12-resorufin (Invitrogen). The macrophage phagocytes were then labeled using rat anti mouse CD68-biotin antibody (Serotec) and streptavidin-Alexa Fluor 647 conjugate. Flow cytometry was then performed on 5-laser BD LSRII using FACSDiva 6.0 software (BD Biosciences). Cells positive for carboxy-fluorescein diacetate succinimidyl ester plus CD68 plus C12-resorufin vs CD68 plus C12-resorufin only were considered to be phagocytes positive for uptake of apoptotic cells.

Analysis of Atherosclerotic Lesion Apoptosis, Efferocytosis, and Necrosis
Recipient LDLR−/− mice (female, 6-weeks-old) were lethally irradiated and transplanted with bone marrow (BM) cells from female WT or MφLRP−/− mice. Four weeks later, the mice were placed on a western-type diet for 16 weeks. Apoptotic cells were detected in 5–μM proximal aortic cryosections by TUNEL using the in situ cell death detection kit, TMR red (Roche). Nuclei were counterstained with Hoechst, and images of 5 serial sections from each mouse were taken using fluorescence microscopy. The efferocytosis in lesions after the procedure was as described by Schrijvers et al and as modified by Thorp et al. The same sections that were stained with TUNEL and Hoechst were stained for macrophages using a rabbit antiactin antibody (A1A31240; Accurate Chemical and Scientific), goat antiactin antibody and conjugated secondary antibody, and Alexa Fluor 488 (Molecular Probes). The free vs macrophage-associated apoptotic cells or bodies were then counted. Lesion necrosis was detected by staining with Harris hematoxylin and eosin (H&E) and quantitated by measuring the hematoxylin and eosin-negative acellular area in the intima vs total intimal area.

Macrophage Survival and Apoptotic Proteins
Total Akt, pAkt, and phosphorylated Bad were detected by Western blot using rabbit polyclonal antibodies to Akt, pAkt (serine 473), and phosphorylated Bad (Cell Signaling Technology). Protein signal was detected using goat antirabbit IgG secondary antibody...
conjugated to horseradish peroxidase and the ECL plus chemiluminescence kit.

**ApoE Secretion and Immunocytochemistry**

Medium apoE was detected by Western blot using rabbit antiserum against mouse apoE and goat antirabbit horseradish peroxidase-conjugated IgG. Immunocytochemistry of cell apoE was performed using rabbit antiserum against mouse apoE and FITC-conjugated goat antirabbit IgG. Cells were counterstained using Vectashield with DAPI to visualize nuclei (Vector Labs).

**Statistical Analysis**

In vitro data are expressed as mean±SD of triplicate determinations. In vivo data are expressed as mean±SEM. Differences between 2 mean values were determined by 2-tailed Student t test, 1-way ANOVA (Bonferroni post test), and Mann-Whitney test. \( P<0.05 \) was considered to be significant.

**Results**

**Effects of Macrophage LRP-1 Deletion on ApoE Synthesis and Secretion**

Because apoE is a ligand for LRP-1 and may function in inflammatory signaling, regulation of efferocytosis, and apoptosis susceptibility, we first examined the effects of LRP-1 deletion on macrophage apoE synthesis and secretion. Western blot of 24-hour-conditioned media from unstimulated LRP-1\(^{-/-}\) macrophages (serum-free DMEM) showed a 3-fold increase in apoE accumulation compared to WT macrophages (Figure 1A). Because LRP-1 is an internalizing apoE receptor, the accumulation of apoE could simply represent inefficient local clearance. However, our findings that apoE mRNA levels increased by 5-fold in the absence of LRP-1 actually suggests the presence of a counter-regulatory loop (Figure 1B). Furthermore, immunohistochemical analyses showed that intracellular apoE was also increased in LRP-1\(^{-/-}\) macrophages after 24-hour incubation in DMEM alone (Figure 1C).

**Deletion of LRP-1 Increases Macrophage Death and Inflammation**

To identify the influence of LRP-1 deletion on apoptosis susceptibility in vitro, WT or LRP-1\(^{-/-}\) macrophages were exposed to 3 apoptotic stimuli (nutrient deprivation with serum-free DMEM, inflammatory LPS, and cholesterol burden with oxidized LDL), and cell death was determined by TUNEL staining (Figure 2A, B). After 24 hours of incubation in serum-free DMEM, the number of TUNEL-positive cells was 2.6-fold greater in cultures of LRP-1\(^{-/-}\) vs WT macrophages. In agreement with studies demonstrating that LPS enhances apoptosis of WT macrophages,\(^{33,34}\) stimulation for 24 hours with LPS vs DMEM alone resulted in increased numbers of TUNEL-positive cells in cultures of both WT and LRP-1\(^{-/-}\) cells. However, positivity for TUNEL staining was 1.8-fold more in LRP-1\(^{-/-}\) macrophages compared to WT.
cells (Figure 2A, B). Similarly, incubation with oxidized LDL increased the TUNEL staining of both cell types, but the number of TUNEL-positive cells was 1.9-fold more in LRP-1−/− macrophages. Under proapoptotic conditions, the inflammatory status of the LRP-1−/− macrophages was heightened compared to WT cells. Incubation with serum-free DMEM resulted in 6.1-fold and 2.3-fold higher IL-1β and IL-6 mRNA levels in LRP-1−/− macrophages compared to WT cells (Supplemental Figure I). Whereas incubation with LPS increased the IL-1β and IL-6 mRNA levels in both cell types, this effect was enhanced in LRP-1−/− macrophages (by 3-fold and 1.7-fold, respectively) compared to WT cells (Supplemental Figure I). Similar to our previous results,3 this effect was enhanced in LRP-1−/− positive cells compared to mice transplanted with WT (n = 4) BM (Figure 3C, D). In the lesions of both WT and LRP-1−/− cells, as determined by annexin V binding (Figure 2C). Despite similar numbers of apoptotic cells under these conditions, LRP-1−/− mice had markedly decreased activated caspase 3 by LRP-1−/− macrophages, even

Figure 3. Peritoneal macrophages positive for annexin V alone (A) or annexin V and 7AAD (B). TUNEL analyses of aortic sections from WT (n = 5) and MΦLRP-1−/− (n = 4) BM recipient LDLR−/− mice fed western diet for 16 weeks (C). Micrographs show nuclei (DAPI; blue), TUNEL-positive staining (red), and merged images. Quantitation of TUNEL results (D) and colocalization of macrophage stain (green) in TUNEL-positive regions of arteries from mice with LRP-1−/− BM (E). *P<0.05, Mann-Whitney test.

Macrophage LRP-1 Deletion Increases Susceptibility to Apoptosis by Impairing the pAkt Survival Pathway

A number of studies, including ours, have demonstrated that the pAkt pathway is fundamental in reducing WT macrophage susceptibility to apoptosis.15,17,18 Because earlier studies demonstrated that antibody ligation to macrophage LRP-1 increases intracellular calcium mobilization, cAMP, and diacylglycerol,13,14 we next determined whether deletion of macrophage LRP-1 affects activation of Akt. Compared to WT macrophages, LRP-1−/− cells had markedly decreased levels of pAkt [serine 473] when incubated with either serum-free DMEM alone or with LPS (Figure 4A). Similar differences were observed with pAkt [serine 308] levels (data not shown). Interestingly, total Akt levels were also decreased in LRP-1−/− macrophages vs WT cells in the presence of DMEM alone or with LPS (Figure 4A). This effect was likely attributable to decreased levels of Akt1 mRNA (Figure 4B). One mechanism by which pAkt prevents apoptosis is by phosphorylating Bad.38 When Bad is phosphorylated, it loses its ability to complex with both Bcl-2 and Bcl-XL, thus allowing these 2 antiapoptotic proteins to prevent the activation of caspase 9 and caspase 3.38,39 In keeping with the decreased pAkt, levels of phosphorylated Bad [serine 156] were decreased in LRP-1−/− macrophages treated with either DMEM alone or LPS when compared to WT cells (Figure 4A). Consistent with the changes in pAkt and phosphorylated Bad [serine 156], immunohistochemical analyses showed increased activated caspase 3 by LRP-1−/− macrophages, even in the absence of exogenous cell death stimulation (Figure 4C). Thus, suppression of the pAkt survival pathway likely

Figure 4. Western blot analysis of caspase 9 and caspase 3 expression from WT (A) and MΦLRP-1−/− (B) BM recipient LDLR−/− mice fed western diet for 16 weeks. Western blots show antiactive caspase 9 (C), antiactive caspase 3 (D), and anti-bad (E). Results are expressed as arbitrary units (AU) and show significantly reduced caspase 9 (C) and caspase 3 (D) levels in MΦLRP-1−/− mice compared to WT (E). *P<0.05, Mann-Whitney test.
contributes to the increased numbers of apoptotic cells in cultures of LRP-1/H11002/macrophages (Figure 2) and in lesions of M/H9021/LRP-1/H11002/recipient LDLR/H11002/mice (Figure 3D).

Effects of Macrophage LRP-1 Deletion on Efferocytosis of Apoptotic Cells

Because LRP-1 has been suggested to play a role in the uptake of apoptotic cells, we next determined whether deletion of macrophage LRP-1 reduced the uptake of apoptotic WT macrophages. To examine this possibility, carboxy-fluorescein diacetate succinimidyl ester-labeled WT cells were made apoptotic by incubation with staurosporine and then added to WT or LRP-1/H11002/macrophages (Figure 5A, B). Eighty-three percent of the WT cells treated with staurosporine were apoptotic, as determined by annexin V binding. Deletion of macrophage LRP-1 decreased the uptake of WT apoptotic cells by 60% (Figure 5C), suggesting that the increased numbers of apoptotic/nonviable cells in LRP-1/H11002/cultures (Figure 2) result in part from impaired efferocytosis.

Because ABCA7 is linked to efficient phagocytosis of apoptotic bodies via LRP-1 signaling and interacts with the amphipathic helical apoproteins, we also determined the effects of LRP-1 deletion on ABCA7 synthesis. Deletion of macrophage LRP-1 resulted in a 4-fold increase in ABCA7 mRNA synthesis compared to WT cells (Figure 5D), suggesting that the lack of efferocytosis via LRP-1 results in compensatory upregulation of its partner, ABCA7.

Interestingly, the impaired efferocytosis occurs under conditions of a marked increase in apoE secretion by LRP-1/H11002/macrophages (Figure 1A) and despite a 9-fold increase in apoE synthesis in WT cells made apoptotic by treatment with staurosporine (data not shown). This suggests that apoE depends on LRP-1 for its effects on efferocytosis. To more directly examine a role for the LRP-1/apoE in efferocytosis, we compared the phagocytosis of WT, apoE/H11002/macrophages, and LRP-1/H11002/macrophages by WT, apoE/H11002/, and LRP-1/H11002/efferocytes (Figure 5E). Compared to the uptake by WT phagocytes, phagocytosis of WT apoptotic cells by LRP-1/H11002/phagocytes was decreased by 64%, and that by apoE/H11002/phagocytes was decreased by 70%. These data demonstrate that phagocyte-
Deletion of Macrophage LRP-1 Impairs Efferocytosis and Promotes Necrosis In Vivo

We examined the in vivo phagocytosis of WT apoptotic cells by peritoneal macrophages of both WT (n=4) and MφLRP-1<sup>−/−</sup> (n=5) mice (Figure 6A). Compared to the efferocytosis of WT apoptotic macrophages by peritoneal phagocytes in WT mice, the phagocytosis in MφLRP-1<sup>−/−</sup> mice was decreased by 53%. We next determined whether lesional phagocytosis is defective in MφLRP-1<sup>−/−</sup> (n=4) vs WT (n=5) BM recipient LDLR<sup>−/−</sup> mice fed a western diet for 16 weeks by following established procedures by Schrijvers et al<sup>2</sup> as modified by Thorp et al.<sup>10</sup> The number of TUNEL-positive cells that were associated with viable macrophages vs the free TUNEL-positive cells (Figure 6C, D) was quantified (Figure 6B). As depicted in the images (Figure 6C, D), apoptotic cells or bodies were counted as free when they were not associated with or found in close proximity to viable macrophages (detected as Alexa Fluor 488-stained macrophage cytoplasm surrounding a Hoechst-stained nucleus). Apoptotic cells or bodies that were associated with macrophage cytoplasmic debris but not in contact or close proximity with viable macrophages were counted as free. Compared to lesions in WT BM recipient LDLR<sup>−/−</sup> mice, the ratio of free to macrophage-associated TUNEL-positive cells was 6.8-fold greater in lesions of MφLRP-1<sup>−/−</sup> recipient mice (Figure 6B). Consistent with the defective lesional efferocytosis, the percent necrotic area was 5.7-fold greater in lesions of MφLRP-1<sup>−/−</sup> vs WT (n=5) BM recipient LDLR<sup>−/−</sup> mice (Figure 6E).

**Discussion**

Our previous studies demonstrated that macrophage LRP-1 expression paradoxically slows the development of atherosclerosis even though it increases uptake of remnant lipoproteins. The enhanced atherosclerotic lesion formation that occurs with macrophage LRP-1 deletion is associated with disruption of the elastic lamina, heightened inflammation, and increased macrophage cellularity. An imbalance between the rate of apoptosis and efferocytosis of dying cells by macrophages accelerates atherosclerosis by promoting post-apoptotic necrosis, inflammation, and plaque instability. The present studies show that deletion of macrophage LRP-1 increases cell death in vitro by impairing efferocytosis and by increasing susceptibility to apoptosis via suppression of Akt activation. More importantly, a critical in vivo role for LRP-1 is demonstrated by the findings that deletion of macrophage LRP-1 promoted marked increases in lesional TUNEL-positive cells, free apoptotic cells, and necrotic area. In addition to causing an imbalance between efferocytosis and apoptosis susceptibility, absence of LRP-1 induced inflammation and increased apoE secretion. The increased apoE secretion by LRP-1<sup>−/−</sup> macrophages is apparently at odds with the finding of increased atherosclerosis that occurs with macrophage LRP-1 deficiency, because increased expression of macrophage apoE in the vessel wall consistently has been linked to reduced atherogenesis in the mouse. This suggests that some or most of the beneficial effects of apoE in the artery wall involve interaction with LRP-1. The current studies provide evidence that atheroprotective functions of the LRP-1/apoE axis include regulation of macrophage inflammation, apoptosis susceptibility, and efferocytosis.

**Role of the LRP-1/ApoE Axis in Efferocytosis**

The present studies demonstrate that deletion of macrophage LRP causes marked reductions in efferocytosis of apoptotic cells in cultures of macrophages (Figure 5C) and in vivo in the peritoneal cavity (Figure 6A) and atherosclerotic lesions.
Thus, suppression of the pAkt pathway likely contributes to the increased cell death in LRP-1−/− macrophage cultures and in LRP-1−/− lesions, which is substantiated by studies showing that Akt activation is a critical determinant of macrophage survival.15,17,18 The impairment in Akt activation is consistent with studies showing that macrophage LRP-1 signaling increases intracellular cAMP, diacylglycerol, and calcium mobilization, which are important second messengers leading to Akt phosphorylation.15,16 Furthermore, recent studies demonstrated that knockdown of LRP-1 in Schwann cells decreases pAkt levels.44 Decreased synthesis of Akt1 mRNA contributed to the reduction in Akt activation in LRP-1−/− macrophages (Figure 4), suggesting that the multiple downstream effects of LRP-1 signaling also regulate transcription of Akt1. A critical determinant of Akt1 transcription in macrophages is the transcription factor CREB, which is activated downstream from cAMP production.45 It is of interest to note that consistent with LRP signaling stimulating cAMP production, recent studies demonstrated that antibodies to LRP increased the levels of phosphorylated CREB in neuronal cells.46 Like LRP-1, apoE has been implicated in cell survival. ApoE stimulates activation of Akt in neuronal cells in a calcium- and cAMP-dependent manner.24 In addition, apoE interaction with macrophages increases intracellular calcium mobilization and diacylglycerol production. Interestingly, this effect is inhibited by receptor-associated protein.14 Thus, it is likely that interaction of endogenous apoE with macrophage LRP-1 promotes activation of Akt and reduces apoptosis susceptibility. Although a number of LDL receptor family members have been implicated in the survival effects of apoE on neuronal cells,28–30 our studies suggest that in macrophages, the prosurvival effects of apoE depend on interaction with LRP-1.

Role of the LRP-1/ApoE Axis in Macrophage Inflammation

Studies show that macrophage apoptosis attributable to either oxidative stress47 or free cholesterol stimulation48 is associated with increased inflammation. Our results show that heightened inflammation occurs in apoptotic LRP-1−/− macrophages even without exogenous stimulation or cholesterol burden (Supplemental Figure 1A, B).3,49 Furthermore, even when comparable numbers of apoptotic cells are induced in cultures of WT vs LRP-1−/− macrophages by incubation with a nonspecific protein kinase inhibitor (Figure 2C), the LRP-1−/− macrophages exhibit markedly increased secretion of inflammatory cytokines compared to WT cells (Figure 2D), thus demonstrating that regardless of either the degree or the mode of apoptosis, the deletion of LRP results in enhanced inflammation. This suggests that LRP-1 is a critical player in controlling macrophage inflammation. Besides impaired effecrosis, other mechanisms may mediate the LRP-1 effects on inflammation. Addition of exogenous apoE50,51 or expression of endogenous apoE52,53 reduces inflammation in macrophages stimulated with LPS and interferon-γ. Furthermore, our previous studies demonstrated that deletion of macrophage LRP increases nuclear factor (NF)-κB activation.49 Interestingly, the antiinflammatory effects of apoE involve...
decreased NF-κB activation during LPS stimulation of macrophages.52

It is also likely that the LRP-1/apoE interaction regulates inflammation by impairing the TNF receptor-1 (TNFR-1) pathway, because we previously showed that deletion of LRP-1 results in increased cell surface TNFR-1, which binds TNF-α and causes enhanced NF-κB activation and inflammation.49 Because NF-κB activation can also promote cell survival,54 the enhanced NF-κB activation that occurs with LRP-1 deficiency is somewhat at odds with the simultaneous increase in cell death. However, in some cell types, NF-κB activation can promote either cell survival or apoptosis via selective gene regulation depending on the pathway of stimulation (ie, growth factor vs etoposide).55,56 Thus, it is plausible that NF-κB activation via the TNF-α-TNFR-1 pathway is proapoptotic and contributes to the enhanced cell death in LRP-1/−/− macrophages. Consistent with this possibility are studies demonstrating that TNF-α stimulates apoptosis via nitric oxide production.6 In addition, we have obtained evidence that the expression of proapoptotic death receptor-5 and its ligand TRAIL57,58 is increased, whereas that of antiapoptotic Bcl-xL is decreased in LRP-1/−/− macrophages (Yancey et al, unpublished observations).

In summary, our studies demonstrate that deletion of macrophage LRP-1 creates an imbalance between effec-
trosis and apoptosis susceptibility resulting in enhanced inflammation, lesion cell death, and plaque necrosis. Because these effects were accompanied by increased apoE secretion in LRP-1/−/− macrophages, a functional axis between these 2 proteins may play a unique and dominant role in atherogenesis.

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Disclosures
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Supplement Material

Supplemental Methods

In vivo and In vitro Analysis of Macrophage Apoptosis

WT and LRP1−/− peritoneal macrophages were collected in PBS 4 days after peritoneal injection of 3% thioglycollate. For in vivo analysis of macrophage apoptosis, cells taken directly from the peritoneal cavity were subjected to flow cytometry analysis using AnnexinV/7aaD kit from BD Biosciences, San Diego CA. Macrophages were distinguished from other cell types by co-staining with FITC-conjugated antibody directed to CD11B. For in vitro analysis of apoptosis, WT and LRP1−/− macrophages were seeded in Laboratory-Tek chamber slides (Nalge Nunc International) at 0.5x10⁶ cells in DMEM with 10% FBS. The cells were then incubated for 24h in serum-free DMEM alone or containing either lipopolysaccharide (LPS, 50ng/ml) or copper oxidized LDL (50μg protein/ml, Intracel, Frederick, MD). Cell death was then determined by TUNEL (Tdt-mediated dUTP nick end labeling) staining using the in situ cell death detection kit (Roche), according to the manufacturer’s instructions. TUNEL-positive cells were quantitated in triplicate chamber slide wells per treatment with cells being counted under light microscope (∗400) in 10 fields per well. Apoptosis was also determined by immunohistochemical analysis of activated caspase 3 after WT or LRP1−/− macrophages were incubated for 24h in serum-free DMEM alone. Detection of activated caspase 3 was carried out according to manufacturer’s protocol using NucView Caspase Detection kit (Biotium, Hayward CA).

In Vitro Measurement of the Efferocytosis of Apoptotic Macrophages

WT, LRP1−/−, or apoE−/− macrophages were made apoptotic and used for in vitro measurement of efferocytosis. Macrophages were collected from the peritoneal cavity four days
post injection with 3% thioglycollate and seeded in 100mm wells at 20x10^6 cells in DMEM with 10% FBS. The viable macrophages (WT, apoE^-/-, or LRP1^-/-) were labeled with 5μM carboxyfluorescein diacetate, succinimidyl ester (CFDA SE) cell tracer (Molecular Probes, Eugene, OR) in DPBS for 30min at 37ºC in an air atmosphere. The cells were then washed and incubated overnight in serum-free DMEM. To induce apoptosis, the labeled macrophages were incubated with either staurosporine (5μg/mL) for 24h or BAY11-7082 (20μM, Calbiochem) for 2h. Either treatment made >80% of the cells apoptotic as determined by annexinV binding and uptake by WT macrophages was similar using WT cells made apoptotic with the two stimuli (42±4% versus 37±2% positive for uptake. The phagocytes were seeded at 0.5x10^6 cells/well in 2-well chamber slides. The phagocytes were washed in DPBS, and then 1.5x10^6 CFDA-SE cell tracer labeled apoptotic cells in DMEM were added to WT, LRP1^-/-, or apoE^-/- efferocytes. After 2h incubation, the phagocytes were washed vigorously four times with PBS to remove noningested apoptotic macrophages. The phagocytes were fixed in 4% paraformaldehyde and counterstained with DAPI for visualization of cell nuclei (Vector Labs, Burlingame CA) and then the efferocytosis of the apoptotic cells was visualized using fluorescence microscopy. CFDA-SE cell tracer positive phagocytes versus total phagocytes were quantitated in triplicate chamber slide wells per treatment with cells being counted in 10 fields per well. The validity of the phagocytosis assay was confirmed by preincubating the WT phagocytes for 24h with the phagocytosis inhibitor, 3-methylchoannotrene, which reduced the uptake of apoptotic WT macrophages by 97%.

Analysis of Efferocytosis in the Peritoneal Cavity

WT macrophages were seeded onto 100 mm tissue culture wells at a density of 20x10^6 cells in 10%FBS/DMEM. Non-adherent cells were then washed away and the macrophages
were incubated for 48h in 1%FBS/DMEM. The macrophages were then washed three times and labeled with 5µM Vybrant CFDA SE Cell Tracer Green for 30 minutes as described above. The macrophages were then made apoptotic by the addition of 20µM Bay 11-7082 (Calbiochem) in serum-free DMEM for 2h. The CFDA SE -labeled, apoptotic WT macrophages were then harvested by scraping in ice cold PBS, combined with the media supernatant and pooled for centrifugation. Cells were resuspended in PBS, counted and diluted to a concentration of 20x10^6 per ml. One ml of the WT apoptotic cells was injected into WT or MΦLRP^- mice that had been injected two days prior with 1ml of 3.0% thioglycollate. Typically, 7 to 10 million cells are recovered in the peritoneal lavage 2 days post injection of WT mice with 1ml of 3% thioglycollate making the apoptotic cell to phagocyte ratio approximately 2 to 3. One hour later the mice were sacrificed and the peritoneal cells harvested by lavage with PBS. Harvested cells were counted, centrifuged and resuspended at concentration of 1x10^6 per ml in PBS. Three million cells from each mouse were then labeled with 250nM Vybrant Cell Metabolic Assay C12-resazurin solution (Invitrogen) in PBS to label viable cells. Cells were incubated for 15 minutes at 37° in an open-air incubator. In viable cells, nonfluorescent C12-resazurin is metabolized to red fluorescent C12-resorufin. The cells were then washed with PBS, pelleted by centrifugation and resuspended in 100µl PBS per 1x10^6 cells. These cells were then labeled with rat anti-mouse CD68-biotin antibody (Serotec) and streptavidin-Alexa Fluor 647 conjugate for 20 minutes before being washed, pelleted and resuspended in 1ml PBS for flow cytometric analysis. Flow cytometry was performed on a 5-laser BD LSRII using FACSDiva 6.0 software (BD Biosciences). Cells positive for CFDA SE + CD68 + C12-resorufin versus CD68 + C12-resorufin only were considered to be phagocytes positive for uptake of apoptotic cells.

*Analysis of Atherosclerotic Lesion Apoptosis, Efferocytosis, and Necrosis*
Recipient LDLR^{-/-} mice (female, 6-weeks old) were lethally irradiated (9 Gy) using a cesium gamma source and transplanted with $5 \times 10^6$ bone marrow cells from female WT or MΦLRP^{-/-} mice through injection into the retro-orbital venous plexus. Four weeks post bone marrow transplantation, the mice were placed on a western-type diet for 16 weeks, and then the extent of atherosclerosis was examined in oil red O-stained cross-sections of the proximal aorta (15 alternate, 10-µm cryosections) using the KS300 imaging system (Kontron Elektronik GmbH). Lesion apoptotic cells were detected in five-micron proximal aortic cryosections by TUNEL after Triton X-100 treatment using the in situ cell death detection kit, TMR red (Roche, Mannheim, Germany) following the manufacturer’s instructions. The nuclei were counterstained with Hoechst, and images of the sections were taken using fluorescence microscopy. Five serial sections from each mouse were stained, and the number of apoptotic cells per section were quantitated and normalized to the lesion area as determined by oil red-O staining. We analyzed the efferocytosis in lesions following the procedure as described by Schrijvers et al. and as modified by Thorp and colleagues. The same sections that were stained with TMR red TUNEL and Hoechst were also stained for macrophage cytoplasm using a rabbit antimacrophage antibody (AIA31240, Accurate Chemical and Scientific Corp.), goat anti-rabbit biotinylated conjugated secondary antibody, and Alexa Fluor 488 (Molecular Probes, Inc.). The free versus macrophage associated apoptotic cells or bodies were counted in five sections per mouse. Apoptotic cells or bodies were counted as free when they were not associated with or in close proximity to viable macrophages that were detected as Alexa Fluor 488 stained macrophage cytoplasm surrounding a Hoeschst-stained nucleus. Apoptotic cells or bodies that were associated with macrophage cytoplasmic debris, but not in contact or close proximity with viable macrophages were counted as free. To determine lesion necrosis, five serial sections from each
mouse were stained with Harris’s hematoxylin and eosin (H&E). Necrosis was quantitated by measuring the H&E negative acellular area in the intima versus total intimal area.

ApoE Secretion and Immunocytochemistry

Macrophages were collected as stated above and 2x10⁶ cells were seeded in DMEM with 10%FBS. Cells were washed twice with cold PBS, and fresh DMEM was added for 24h. Media samples were separated using 10% SDS PAGE. After protein transfer to nitrocellulose, apoE was detected using rabbit anti-serum against mouse apoE (1:1000 dilution) and goat anti-rabbit IgG (1:5000 dilution) conjugated to horseradish peroxidase. Protein was visualized using ECL reagents (Amersham, Piscataway, NJ). For immunocytochemistry studies, cells were seeded at a density of 50x10⁵ per well in DMEM with 10% FBS, and after adherence, the cells were washed and incubated for 24h in serum-free DMEM. For apoE detection, the cells were fixed in 4% paraformaldehyde for 30 min at room temperature, permeabilized with 0.1% Triton X in PBS, and blocked with PBS infused with 10% FBS for 30 min at room temperature. Rabbit anti-serum against mouse apoE (1:1000 dilution) was added to the cells and incubated at 37° C for 1h. The cells were washed 3 times in PBS and incubated with FITC-conjugated goat anti-rabbit for 1h at room temperature. The cells were washed with PBS and cover slips were mounted using Vectashield with DAPI to visualize cell nuclei (Vector Labs, Burlingame CA).

Macrophage Survival and Apoptotic Proteins

Cells were incubated for the indicated times with serum-DMEM alone or containing LPS (50ng/ml) and then lysed in RIPA buffer containing the Sigma protease inhibitor cocktail. The cell proteins were separated using NuPage 10% Bis-Tris gels and then transferred onto nitrocellulose membranes using NuPage transfer buffer for 3h at 26V. Total Akt, phosphorylated Akt (pAkt), and phosphorylated Bad (pBad) were detected using rabbit
polyclonal antibodies to Akt, pAkt{superscript}(serine 473), and pBad{superscript}(serine 136) (Cell Signaling Technology, Inc., Beverly, MA). The protein signal was then detected using goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase and the ECL plus chemiluminescence kit (Amersham, Piscataway, NJ).

**Real-time RT PCR and ELISA Analyses**

Total RNA was isolated from thioglycollate-elicited peritoneal macrophages using Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. Relative quantitations of the target mRNA were performed with the ABI Prism 7700 Sequence detection system and normalized with 18S ribosomal RNA and an internal control. Relative mRNA levels for individual genes were determined using TaqMan Assays-on-Demand kits (Applied Biosystems, ABI Foster City, CA). The data were analyzed using comparative C_T methods and are representative of multiple experiments, each with triplicate determinations. Medium cytokine levels were detected using ELISA kits (BD Pharminogen).

**Statistical Analysis**

In vitro data are expressed as mean ± standard deviation of triplicate determinations. In vivo data are expressed as mean ± SEM. Differences between two mean values were determined by two-tailed Student’s t-test, one-way ANOVA (Bonferroni’s post test), and Mann-Whitney test. p<0.05 was considered to be significant.

**References**


Supplemental Figure I. IL-1β (A) and IL-6 (B) mRNA levels after 6h in serum-free DMEM alone or with LPS (50ng/ml). Data represent 2 experiments. *p<0.05, Student’s t test.