Apolipoprotein E Induces Antiinflammatory Phenotype in Macrophages


Objective—Apolipoprotein E (apoE) exerts potent antiinflammatory effects. Here, we investigated the effect of apoE on the functional phenotype of macrophages.

Methods and Results—Human apoE receptors very-low-density lipoprotein receptor (VLDL-R) and apoE receptor-2 (apoER2) were stably expressed in RAW264.7 mouse macrophages. In these cells, apoE downregulated markers of the proinflammatory M1 phenotype (inducible nitric oxide synthase, interleukin [IL]-12, macrophage inflammatory protein-1α) but upregulated markers of the antiinflammatory M2 phenotype (arginase I, SOCS3, IL-1 receptor antagonist [IL-1RA]). In addition, M1 macrophage responses (migration, generation of reactive oxygen species, antibody-dependent cell cytotoxicity, phagocytosis), as well as poly(I:C)- or interferon-γ-induced production of proinflammatory cytokines; cyclooxygenase-2 expression; and activation of nuclear factor-κB, IκB, and STAT1, were suppressed in VLDL-R- or apoER2-expressing cells. Conversely, the suppression of the M2 phenotype and the enhanced response to poly(I:C) were observed in apoE-producing bone marrow macrophages derived from VLDL-R-deficient mice but not wild-type or low-density lipoprotein receptor–deficient mice. The modulatory effects of apoE on macrophage polarization were inhibited in apoE receptor-expressing RAW264.7 cells exposed to SB220025, a p38 mitogen-activated protein kinase inhibitor, and PP1, a tyrosine kinase inhibitor. Accordingly, apoE induced tyrosine kinase–dependent activation of p38 mitogen-activated protein kinase in VLDL-R- or apoER2-expressing macrophages. Under in vivo conditions, apoE+/− mice transplanted with apoE-producing wild-type bone marrow showed increased plasma IL-1RA levels, and peritoneal macrophages of transplanted animals were shifted to the M2 phenotype (increased IL-1RA production and CD206 expression).

Conclusion—ApoE signaling via VLDL-R or apoER2 promotes macrophage conversion from the proinflammatory M1 to the antiinflammatory M2 phenotype. This effect may represent a novel antiinflammatory activity of apoE. (Arterioscler Thromb Vasc Biol. 2011;31:1160-1168.)

Key Words: apolipoproteins • macrophages • inflammation

Apolipoprotein E (apoE) is a major protein component of very-low-density lipoproteins (VLDL) and high-density lipoproteins. Observations in human carriers of apoE variants and in apoE-deficient mice suggest an important role of this apolipoprotein in preventing atherosclerosis.1 The antiatherogenic effects of apoE are usually attributed to its ability to regulate VLDL production and to facilitate hepatic clearance of VLDL and chylomicron remnants.5 However, apoE protects against atherosclerosis even in the absence of measurable effects on plasma lipoprotein metabolism. For instance, transgenic expression of apoE in arterial wall reduces the formation of atherosclerotic lesions without affecting plasma lipoprotein profile.2,3 Conversely, enhanced atherosclerosis is observed in apoE+/− animals transplanted with bone marrow (BM) from apoE-deficient mice.4 These results suggest that apoE exerts local antiatherogenic effects in the arterial wall that are independent from regulation of plasma lipoprotein metabolism.

An increasing body of evidence suggests that atherosclerosis is a chronic inflammatory disease. Macrophages assume a critical role in the initiation and the perpetuation of intravascular inflammation through their ability to produce an
array of cytokines and chemokines, to generate reactive oxygen species, and to process and present antigens to CD4+ T cells. Importantly, macrophages represent a heterogeneous cell population that is distinctly activated by various microenviron-mental signals.5–8 Proatherogenic factors such as Th1 cytokines (eg, interferon-γ [IFN-γ], interleukin [IL]-1β [IL-1β]) and Toll-like receptor ligands (eg, poly(I:C), lipopoly-saccharide) induce a classical activation profile (M1) charac-terized by enhanced production of proinflammatory cytokines, expression of major histocompatibility complex (MHC) class II molecules, and generation of free radicals including inducible nitric oxide synthase (iNOS)-derived nitric oxide (NO). In contrast, exposure of macrophages to Th2 cytokines (IL-4, IL-13) promotes the alternative activation profile (M2) comprising secretion of anti-inflammatory factors (eg, IL-1 receptor antagonist [IL-1RA]), expression of the mannose receptor CD206 or hemoglobin receptor CD163, and reduced NO production due to the upregulation of arginase I activity, which converts arginine to ornithine and urea. In addition, alternatively activated macrophages demon-strate higher phagocytic capacity and reduced motility and cytotoxic effects.

Although the presence of both M1- and M2-polarized macrophages in the arterial wall and the progressive M2 to M1 switch during atherosclerotic lesion development have been recently documented,4,5 the mechanisms contributing to the generation of distinct macrophage phenotypes in context of atherosclerosis remain unclear. In the present study, we report that apoE exerts anti-inflammatory activity by switching the macrophage phenotype from M1 to M2 in a process involving signaling via the very-low-density lipoprotein receptor (VLDL-R) or the apoE receptor-2 (apoER2).

Methods

BM Transplantation

Homozygous apoE−/− and low-density lipoprotein receptor-deficient mice, both on a C57BL/6j background, and wild-type (WT) C57BL/6j mice were obtained from Charles River Laboratories (Sulzfeld, Germany). VLDL-R−/− and apoER2−/− mice were generated as described previously.10,11 To induce BM aplasia, apoE−/− mice (12 animals) were exposed to a single dose of 11 Gy of total body irradiation. BM (10^6 cells) isolated by flushing femurs from apoE−/− and WT mice were administered intravenously. Chimerism was assessed in leukocyte DNA by polymerase chain reaction (PCR).

ApoE Receptor Cloning and Stable Transfections

Human endothelial and brain DNA were reverse transcribed and subjected to PCR with human VLDL-R and apoER2 gene-specific primers. PCR products corresponding to VLDL-R and apoER2 were ligated into expression vector pBK-CMV. RAW264.7 cells were stably transfected by electroporation. Positive clones were isolated and identified by Western blot.

Expression of phenotype-specific markers and phosphorylation of protein kinases p38 mitogen-activated protein kinase and Akt were examined by Western blot. Production of cytokines, prostaglandin E2 (PGE2), and NO were examined with ELISA or enzyme immuno-assay. ApoE binding, CD206 and MHC-II cell surface expression, and fluorescent latex bead uptake for estimation of phagocytosis were investigated by flow cytometry. Transcription factor activity was examined by luciferase reporter assays using gene vectors p(xB)Luc and p(GAS)Luc. NO and H2O2 production were assessed using fluorescence spectroscopy. Production of urea and antibody-dependent cell cytotoxicity were investigated by light spectrometry.

Statistical Analysis

Data are presented as mean±SD from at least 3 separate experiments or as results representative of at least 3 repetitions, unless indicated otherwise. Comparisons were performed with the 2-tailed Student t test. Detailed Supplemental Methods are available online at http://atvb.ahajournals.org.

Results

Generation of Stable Transfectants Expressing Functional VLDL-R or apoER2

Two stable cell lines were generated by transfection of RAW264.7 macrophages with plasmids encoding human variants of VLDL-R or apoER2 expressed in the vasculature. Each cell line produced proteins with expected molecular weights cross-reacting with antibodies against either VLDL-R or apoER2, displayed specific binding of apoE, and produced no endogenous apoE (for details, see Supplemental Methods).

ApoEInduces Typical Characteristics of Alternative Activation in VLDL-R- or apoER2-Expressing RAW264.7 Macrophages

To investigate whether apoE drives macrophages toward the M2 phenotype, RAW264.7 cells expressing VLDL-R or apoER2 were incubated for 24 hours with apoE, and the expression of M1 and M2 polarization markers was investigat-ed. Figure 1A demonstrates that, relative to control cells, the expression of iNOS, a hallmark of classically activated macrophages,5,6 was downregulated in VLDL-R- or apoER2-expressing macrophages in the presence of apoE. By contrast, cells expressing VLDL-R or apoER2 displayed upregulated arginase I, FIZZ1/RELM, and SOCS3, 3 markers of alternative macrophage activation, in response to apoE.6–8 Consistent with these observations, apoE decreased the enzymatic activity of iNOS, as inferred from reduced nitrite/nitrate generation, whereas the production of urea, an arginase I product, was increased (Figure 1B). The latter effect was concentration-dependent and reached the maximum at 5 μg/mL apoE. In addition, preincubation of VLDL-R- or apoER2-expressing macrophages with apoE reduced the steady-state production of M1 cytokines IL-12 and macrophage inflammatory protein-1α, whereas the production of M2 cytokines IL-1RA and granulocyte colony-stimulating factor was enhanced in a concentration-dependent fashion, indicating that apoE shifts the balance from a proinflamma-tory toward an anti-inflammatory cytokine profile (Figure 1C). Endotoxin contamination did not account for the modulatory effects of apoE on macrophage functional phenotype (see Supplemental Materials).

As IL-4 and IL-13, 2 cytokines secreted by macrophages, are typical inducers of the alternative macrophage pheno-
we next examined whether the activation pattern seen in RAW264.7 cells expressing VLDL-R or apoER2 is a direct effect of apoE or whether it is conferred via intermediate products. As shown in Figure 1D, exposure of macrophages to apoE failed to induce IL-4 production but led to a moderate release of IL-13 into cell media. However, recombinant mouse IL-13 receptor α2 (IL-13Ra2), which was previously shown to abrogate cytokine effects under both in vitro and in vivo conditions,12,13 failed to influence apoE-stimulated IL-1RA and G-CSF production in VLDL-R- or apoER2-expressing cells, which argues against the notion that apoE drives the M2 polarization via the IL-13-mediated paracrine loop.

ApoE Alters Functional Phenotype in VLDL-R- or apoER2-Expressing RAW264.7 Macrophages
As alternative macrophage activation is accompanied by changes of cell function such as decreased cell motility and cytotoxicity and increased phagocytosis,5–7 we next investigated the propensity of apoE to modulate the macrophage functional phenotype in RAW264.7 cells expressing VLDL-R or apoER2. As shown in Figure 2A, the induction of chemotaxis by 2 common leukocyte chemoattractants, macrophage colony-stimulating factor and N-formylmethionyl-leucyl-phenylalanine, was equally effective in control and apoE receptor-expressing RAW264.7 macrophages.

Figure 1. ApoE induces macrophage M2 polarization markers in VLDL-R- or apoER2-expressing RAW264.7 cells. VLDL-R, apoER2, or WT RAW264.7 cells were cultured for 24 hours with or without apoE (5 μg/mL) or with increasing concentrations of apoE. A, Total cell lysates were separated by SDS-PAGE and immunoblotted with antibodies against iNOS, arginase I, FIZZ1/RELM, and SOCS3. Data are representative of 3 independent experiments. B, Cell culture media were collected, and concentrations of nitrite and nitrate (products of iNOS) and urea (product of arginase I) were determined by fluorimetric or photometric assays, respectively. Shown are results from 3 independent experiments. C and D, Concentrations of M1 (macrophage inflammatory protein-1α, IL-12) and M2 (IL-1RA, G-CSF, IL-4, IL-13) cytokines were determined by ELISA. IL-13Ra2 was coincubated with apoE for 24 hours at concentration of 0.1 μg/mL. Shown are results from 3 to 5 independent experiments. *P<0.05, §P<0.01, #P<0.001, −apoE vs +apoE).
presence of apoE only in cells expressing VLDL-R or apoER2. Similarly, cytotoxic effects of macrophages, as determined by hemoglobin released from opsonized erythrocytes, were attenuated in the presence of apoE in VLDL-R- or apoER2-expressing cells but not in control cells (Figure 2B).

This was likely a consequence of reduced reactive oxygen species (ROS) generation, as the preincubation of RAW264.7 macrophages with apoE decreased the ROS generation rate in VLDL-R- and apoER2-expressing cells, whereas no inhibitory effects were noted in control cells (Figure 2C). In addition, pretreatment with apoE consistently increased the number of VLDL-R- or apoER2-expressing macrophages phagocytosing fluorescein isothiocyanate–latex beads (Figure 2D). By contrast, apoE failed to affect phagocytosis in control RAW264.7 macrophages.

ApoE Inhibits the Proinflammatory Response of RAW264.7 Macrophages to IFN-γ and Poly(I:C)

Alternatively activated macrophages are characterized by impaired response to M1 stimuli, such as Th1 cytokines and Toll-like receptor ligands.6–8 Therefore, we next assessed the ability of RAW264.7 macrophages to respond to poly(I:C) or IFN-γ stimulation with cyto/chemokine production and activation of transcription factors nuclear factor-κB (NF-κB) or STAT1. ApoE alone failed to induce cytokine or chemokine production. However, following exposure to apoE, VLDL-R- or apoER2-expressing macrophages were significantly suppressed with respect to production of proinflammatory cytokines/chemokines (IL-12, tumor necrosis factor-α, monocyte chemotactic protein-1, IL-6) as compared with control RAW264.7 cells (Figure 3A). Moreover, the expression of cyclooxygenase-2 (COX-2), a marker of proinflammatory macrophage activation, and the release of a COX-2 product, PGE2, in response to IFN-γ and poly(I:C) were both suppressed in the presence of apoE in VLDL-R- or apoER2-expressing but not in control macrophages (Figure 3B). The reduced proinflammatory response to M1 stimuli was further reflected at the transcriptional level by impaired upregulation of poly(I:C)-induced NF-κB activation or IFN-γ-induced STAT1 activation in apoE-pretreated VLDL-R or apoER2-expressing macrophages transiently transfected with reporter plasmids containing either NF-κB- or STAT1-responsive promoters (Figure 3C). At the pretranscriptional level, degradation and phosphorylation of IκB, a component of the NF-κB complex, as well as phosphorylation of STAT1, which both precede the initiation of transcription by NF-κB or STAT1, respectively, were abolished in VLDL-R- or apoER2-expressing macrophages in the presence of apoE (Figure 3D). By contrast, both NF-κB/STAT1 activation and IκB/STAT1 phosphorylation in response to poly(I:C) or IFN-γ remained unaffected by apoE in control RAW264.7 macrophages (not shown).

VLDL-R-Deficient BM Macrophages Display Enhanced Proinflammatory Phenotype

We next examined whether the effects of apoE on macrophage phenotype are also observed in normal primary macrophages, which do not overexpress apoE receptors. Initial studies documented the expression of VLDL-R but not apoER2 in BM-derived murine macrophages (Figure 4A). In contrast to RAW264.7 cells, BM macrophages produced endogenous apoE and the amounts of apolipoprotein released into media within the 24-hour incubation period were comparable in cells derived from WT, VLDL-R−/−, apoER2−/−, and low-density lipoprotein receptor–deficient mice (Figure 4B). As shown in Figure 4B, the expression of M2 polariza-
apoER2
Akt.14 However, RAW264.7 cells do not express Dab1 (not Dab1) and culminates in the activation of protein kinase
the recruitment of intracellular adapter protein disabled-1
Binding of apoE to VLDL-R or apoER2 receptors results in
ApoE Signals via p38 Mitogen-Activated Protein
Kinase and Tyrosine Kinase
Apoe E Signals via p38 Mitogen-Activated Protein Kinase
and Tyrosine Kinase
Binding of apoE to VLDL-R or apoER2 receptors results in
the recruitment of intracellular adapter protein disabled-1
(Dabl) and culminates in the activation of protein kinase
Akt.14 However, RAW264.7 cells do not express Dabl (not shown), and accordingly, apoE failed to produce Akt phosphorylation both in control and apoE receptor-expressing
macrophages (Figure 5A). By contrast, time-dependent increase in p38 mitogen-activated protein kinase (p38MAPK)
phosphorylation on incubation with apoE was observed in
macrophages expressing VLDL-R or apoER2 but not in
control cells (Figure 5A). The apoE-induced p38MAPK phos-
phorylation was substantially reduced after preincubation of
macrophages with PP1A (10.0 μmol/L), a potent inhibitor of
the Src family of tyrosine kinases (Figure 5B). To assess the
involvement of p38MAPK and tyrosine kinase activation in the
modulatory effects of apoE on macrophage M1/M2 pheno-
type, the apoE-induced production of IL-1RA was examined
in macrophages preincubated with PP1A or SB203580, a
specific inhibitor of p38MAPK. As shown in Figure 5C, both
inhibitors abolished apoE-induced production of M2 cyto-
kines in VLDL-R- or apoER2-expressing RAW264.7 cells.

ApoE Directs Macrophages Toward the M2 Phenotype In Vivo
To assess the influence of apoE on macrophage polarization under in vivo condition, chimeric mice were created by
transplanting BM from C57Bl/6 (WT) or apoE-deficient
(apoe−/−) mice into apoE-deficient animals. C57Bl/6 mac-
rophages were previously demonstrated to synthesize and
secrete apoE in a paracrine fashion. As shown in Figure 6A
(left upper panel), genotyping of genomic DNA isolated from
peripheral leukocytes of apoE−/−→apoE−/− transplanted
animals revealed 1 PCR product characteristic for apoE-
deficient mice, whereas an additional WT-specific PCR
product was seen in WT→apoE−/− transplanted animals. In
addition, apoE was detected in supernatants of peritoneal macrophages elicited from WT→apoE−/− but not apoE−/−→apoE−/−
transplanted animals (Figure 6A, left lower panel). Finally,
WT→apoE−/− transplanted animals displayed significantly

Figure 3. ApoE suppresses poly(I:C)- and IFN-γ-
induced inflammatory response in VLDL-R- or
apoER2-expressing RAW264.7 cells. VLDL-R,
apoER2, or WT RAW264.7 macrophages were
incubated with apoE (5 μg/mL) for 24 hours. A and
B, Cells were stimulated for further 24 hours with
poly(I:C) (10 ng/mL) and IFN-γ (50 ng/mL). Supernat-
"nts were collected and analyzed by ELISA for
cyto/chemokine content (C) or by enzyme immu-
noassay for PGE2 concentration (D, lower panel).
Shown are results from stimulated cells represent-
tive of 3 to 5 independent experiments. *P<0.05,
§P<0.01, #P<0.001, stimulated vs unstimulated
macrophages. ApoE alone did not affect cyto/chemokine or
PGE2 concentrations. Cell lysates were immuno-
botted and probed with anti-COX-2 antibody (B, upper panel). Shown is 1 representative result of
3. Lower panel: densitometric analysis of blots. C,
Cells were transfected with p(B)x-Luc or p(Gas)-
Luc reporter plasmids; stimulated for 24 hours
with, respectively, poly(I:C) (10 ng/mL) or IFN-γ (50
ng/mL) with or without apoE; and analyzed for
luciferase. Luminescence level in unstimulated
cells was set as 1. ApoE alone did not change
luminescence levels. Shown are results from 3 to 4
independent experiments. *P<0.05, §P<0.01, −apoE vs +apoE. D, Cells were exposed to
poly(I:C) or IFN-γ for the indicated times, and
lysates were probed with antibodies against the
total (t) and phosphorylated (p) isoforms of 1xB
and STAT1. Shown are blots representative of 3 to
5 independent experiments.
reduced cholesterol levels and improved plasma lipoprotein profile (Figure 6A, right panel). Collectively, these data demonstrate a successful repopulation of BM-ablated apoE<sup>−/−</sup>/H<sup>11002</sup>/H<sup>11002</sup> mice with macrophages producing functional apoE.

No significant differences were observed between apoE<sup>−/−</sup>- and WT-transplanted animals with regard to plasma levels of proinflammatory cytokines (not shown). However, concentrations of the antiinflammatory IL-1RA were significantly reduced and elevated in animals, which received BM without and with apoE-secreting macrophages, respectively (Figure 6B). To characterize the functional macrophage phenotype in transplanted animals, peritoneal macrophages were examined for the expression of M1/M2 polarization markers. As shown in Figure 6C, macrophages obtained from WT apoE<sup>−/−</sup>-transplanted animals were characterized by reduced production of M1 cytokine IL-12, whereas the production of M2 cytokine IL-1RA was increased. In addition, these cells demonstrated reduced surface expression of MHC-II, an M1 polarization marker (WT: 112±28; apoE<sup>−/−</sup> 78±24 arbitrary units, n=6, P<0.05), whereas the expression of CD206, an M2 polarization marker, was increased (WT: 106±30; apoE<sup>−/−</sup> 147±34 arbitrary units, n=6, P<0.05) (Figure 6D).

**Discussion**

Previous studies support a role of apoE as an immunomodulatory agent. Initial observations demonstrated that the infection of apoE knockout mice with *Listeria monocytogenes* or *Klebsiella pneumoniae* results in an increased susceptibility to death, as well as elevated serum levels of proinflammatory (M1) cytokines, such as tumor necrosis factor-α, as compared with WT animals. Moreover, apoE-deficient mice injected with microbial stimuli (ie, poly(I:C), lipopolysaccharide), which drive macrophages into the M1 inflammatory state, presented with exaggerated tumor necrosis factor-α, IL-6, IL-12, and IFN-γ production that could be reversed by the administration of exogenous apoE or by the adenoviral
reconstitution of apoE expression in the liver. Although these data provide evidence that apoE can suppress the proinflammatory response in vivo, none of the previous studies have assessed the ability of this apolipoprotein to modulate the functional polarization of macrophages. Our present study provides several pieces of evidence documenting that apoE primes macrophages into alternative M2 phenotype with antiinflammatory properties. First, the exposure of apoE receptor-expressing macrophages to apoE led to the expression or the liberation of several markers (ie, arginase I, Fizz1/Relm, SOCS3, IL-1RA) widely recognized as attributes of M2 polarization. Second, the functional characteristics of macrophages exposed to apoE, including reduced migration and attenuated ROS generation and cytotoxicity, as well as upregulated phagocytic activity, were congruent with a typical M2 phenotype. Third, the pretreatment of macrophages with apoE attenuated the proinflammatory activation profile in mouse and human macrophages. Taken together, these observations suggest that several antiatherogenic factors known to suppress NF-κB and STAT1 activity have been demonstrated to favor M1 skewing macrophage toward the M2 functional phenotype and thereby generates cell population with enhanced antiinflammatory properties.

Monocytes/macrophages assume a critical role in the development of atherosclerosis by infiltrating the arterial wall, where they produce oxidative stress and contribute to perpetuation of proinflammatory processes. Several functional properties of macrophages exposed to apoE are consistent with the notion that these cells exhibit attenuated proinflammatory potential. For instance, reduced motility and propensity to produce ROS would be expected to both reduce the ingress of monocytes into the intima and to locally restrict the oxidative stress and the in situ generation of modified lipoproteins. More importantly, the acquisition of M2 phenotype due to interaction with apoE confers on macrophages the resistance to induction of NF-κB and STAT1, 2 transcription factors activated by proinflammatory agents commonly encountered in the atherosclerotic environment. Both activated NF-κB and STAT1 have been detected within arterial lesions, where they colocalize with macrophages, and the increased activity of NF-κB has been actually observed in aortas from apoE-deficient mice. In this context, it is worth noticing that several antiatherogenic factors known to suppress NF-κB or STAT1 activity have been demonstrated to favor M1→M2 phenotypic switching in monocytes and macrophages. For instance, agonists of peroxisome proliferation–activated receptor-γ (a ligand-activated nuclear receptor with potent antiinflammatory properties), sphingosine 1-phosphatase (a lipid constituent of high-density lipoprotein), and FK506 (an immunosuppressive drug) were all shown to induce an antiinflammatory activation profile in mouse and human macrophages.

In addition, enhancement of M2 functional phenotype was observed in macrophages cocultured with CD4+CD25+ regulatory T cells—a lymphocyte subset with potent inflammation-suppressing and antiatherogenic properties. Finally, deficient CD40-TRAF6 signaling was recently shown to drive macrophages toward antiinflammatory M2 signature and to protect against atherosclerosis in apoE-deficient mice. Collectively, these observations suggest that skewing macrophage toward the M2 functional phenotype may represent a universal mechanism used by various protective factors to counteract the development of intravascular inflammation and atherosclerosis.

The molecular mechanism underlying the immunomodulatory effects of apoE on macrophages remains unclear. As apoE absorbs cholesterol from cell membranes, the possibility cannot be excluded that the cholesterol depletion-induced perturbation of membrane microenvironment triggers the
signaling cascade required for functional polarization of macrophages. However, apoA-I—another apolipoprotein depleting membranes of cholesterol—failed to affect macrophage phenotype (Baitsch D, Nofer JR, unpublished data, 2008), suggesting that the observed effects were specific for apoE. The results of the present study argue against the role of intermediate macrophage products, such as IL-4 and IL-13, and suggest that the direct effect of apoE mediated via specific receptors, together with the ensuing activation of the intracellular signaling machinery, is required for effective switching of the functional macrophage phenotype. Both VLDL-R and apoER2 were previously demonstrated to bind (via a NPXY tetra–amino acid motif) and to phosphorylate an intracellular adaptor protein Dab1, which in turn mobilizes a common set of signaling modules, including protein tyrosine kinase Fyn, as well as phosphatidylinositol-3–kinase and protein kinase Akt.14,20 However, Dab1 is expressed neither in RAW267.4 nor in primary murine macrophages (Bock HH, unpublished data, 2010), and consequently, apoE failed to activate Akt in cells overexpressing VLDL-R or apoER2. Our results suggest rather that activation of p38MAPK represents a centerpiece of intracellular signaling steering the M1→M2 phenotypic conversion. This possibility is supported by the observation that pharmacological p38MAPK inhibition fully abolished the production of M2 polarization marker IL-1RA. In this context, it is of note that in human platelets, engagement of apoER2 leads to a potent activation of p38MAPK in a process dependent on the Src family kinase Fgr and blocked in the presence of Src kinase inhibitor PP1.30 In our hands, PP1 inhibited both the apoE-induced p38MAPK activation and the IL-1RA production, suggesting the involvement of a protein tyrosine kinase of as yet unknown identity. Taken together, these data point to common signaling mechanisms used by apoER2 (and possibly also by VLDL-R) to modulate various functional aspects of cells of hematopoietic origin.

The present data do not allow any conclusion with regard to the role played by VLDL-R or apoER2 in the pathogenesis of atherosclerosis. Various isoforms of both receptors were previously found to be expressed in the vasculature, and VLDL-R appears to be particularly abundant in atherosclerotic lesions, where it colocalizes with macrophages and likely facilitates foam cell formation.31–34 Actually, transplantation of VLDL-R+ macrophages into VLDL-R-deficient mice accelerated the development of atherosclerotic lesions.35 However, neither generalized nor macrophage-specific VLDL-R deficiency conferred protection against atherosclerosis, and increased intimal thickening after injury has even been observed in VLDL-R-deficient mice.35–37 These observations indicate that the proatherogenic effect of VLDL-R arising from the enhanced cholesterol uptake by macrophages may be counterbalanced by some as yet unidentified atheroprotective effects. On the basis of the present results, it is tempting to speculate that the antiinflammatory macrophage polarization triggered by VLDL-R signaling mitigates proatherogenic effects related to VLDL-R-mediated ingestion of cholesterol-rich lipoproteins.

In conclusion, our results demonstrate that apoE signaling via VLDL-R or apoER2 promotes phenotypic switching of macrophages to a novel, antiinflammatory phenotype. These macrophages show decreased activation of transcription factors NF-kB and STAT1 and reduced motility and production of free radicals and proinflammatory cytokines. Macrophage conversion from proinflammatory M1 to antiinflammatory M2 phenotype represents a novel antiatherogenic activity of apoE and may be part of a universal mechanism used by various protective agents to counteract the development of atherosclerotic lesions.

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Disclosures

None.

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SUPPLEMENTARY MATERIALS

METHODS

Materials – Human apoE isolated from pooled human plasma was obtained from Biodesign Int., Saco, MN, and contained more than 90% of E3 isoform. Human-specific and human and mouse double-specific antibodies against apoE were obtained from Acris Antibodies, Hiddenhausen, Germany and Epitomics Inc., Burlingate, CA, respectively. Antibodies against apoE-specific receptors apoER2 and VLDL-R were from LifeSpan Biosciences, Seattle, WA, and SantaCruz Biotechnology, Santa Cruz, CA. Antibodies against phosphorylated and native isoforms of mitogen-activated protein kinase p38 and protein kinase Akt as well as against IκB and STAT1 were obtained from Cell Signaling Technology, Beverly, MA. Antibodies against inducible nitric oxide synthase (iNOS) and arginase-1 were from BD Biosciences, Heidelberg, Germany. Antibodies against cyclooxygenase-2 (COX-2), FIZZ1/RELM, and suppressor of cytokine signaling 3 (SOCS3) were from Abcam, Cambridge, UK and LabVision Corp., Fremont, CA. Fluorescence-labeled antibodies against MHC-II (I-A/I-E) and CD206, isotype-matched irrelevant immunoglobulins and recombinant mouse IL-13 Rα2 Fc chimera were purchased from BD Biosciences and LifeSpan Biosciences. Interferon-γ (IFN-γ) and monocyte-colony stimulating factor (M-CSF) was purchased from PeproTech EC, London, UK. Pharmacological kinase inhibitors PP1A and SB203580 as well as the fluorescence dye H$_2$DCF-DA were from MERCK Biosciences, Darmstadt, Germany. Erythrocytes opsonized with IgG (Coombs cells E) were obtained from Biotest, Dreieich, Germany. ELISAs for determination of MCP-1, IL-4, IL-6, IL-12, IL-13, MIP-1α, TNFα, G-CSF, and IL-1RA were provided by R&D Systems, (Minneapolis, MN). QCL-1000 Chromogenic LAL
endpoint assay was purchased by Cambrex (Vervier, Belgium). 2,3-diaminofluoren (DAF), poly(cytidylic-inosinic) acid (poly(I:C)) fluoresceine isothiocyanate (FITC)-coupled carboxylate-modified polystyrene latex beads and other chemicals were from Sigma, Deisenhofen, Germany, and were of highest purity available.

Animals – Homozygous apoE−/− mice on a C57BL/6J background and wild-type (WT) C57BL/6J mice (6 - 8 weeks, 20-25 g, female) were obtained from the Charles River Laboratories (Sulzfeld, Germany). ApoER2 and VLDL receptor knockout mice have been generated in the laboratory of J. Herz as described [1,2] previously. All animals were housed under pathogen-free conditions at the Center for Animal Studies of the University Hospital Münster, were maintained on a sterilized regular chow diet and had unlimited access to food and water. Bone marrow-transplanted animals received drinking water supplied with cotrimoxazol (Ratiopharm, Ulm, Germany; 96 mg/L). All animal experiments were performed according to national regulations and approved by government authorities in charge of animal protection (LANUV).

Bone marrow (BM) transplantation and assessment of chimerism – to induce bone marrow aplasia, apoE−/− mice (12 animals) were exposed to a single dose of 11 Gy total body irradiation using a Universa Cobalt 60 Roentgen ray source (Philips, The Netherlands) 1 day before the transplantation. Bone marrow was isolated by flushing femurs and tibias from female apoE−/− and WT mice with phosphate-buffered saline (PBS) and single-cell suspensions were prepared by passing the cells through a 70 µm cell strainer. Irradiated recipients received 1.0 x 10^6 cells by intravenous injection into the tail vein. The hematological chimerism of transplanted animals was determined in genomic DNA from blood leukocytes 6 weeks after transplantation. The presence of the targeted and wild-type apoE alleles was assessed simultaneously by PCR amplification using the following oligonucleotide primers: 5′-
GCCTAGCGAGGGAGAGCGG-3', 5'-TGTGACTTGGGAGCTCTGCAGC-3', and 5'-GCGCCCCAGACTGCATCT-3'.

**Cell culture** – RAW264.7 cells were obtained from American Tissue Cell Collection (ATCC, Manassas, VA) and incubated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS, Biochrom, Berlin, Germany), 100 U/mL penicillin G, 100 µg/mL streptomycin and 2 mmol/L L-glutamine. Cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Resident peritoneal macrophages were harvested by peritoneal lavage with 10 ml of sterile PBS containing 2.0 U/mL heparin, collected by centrifugation (400 x g; 10 min; 4°C), washed, plated on 12-well plates at a cell density of 0.5 x 10⁶, and maintained in RPMI-1640 medium supplemented with 10% FCS and antibiotics. After 4h, plates were washed three times with PBS to remove non-adherent cells and used for further experiments. Bone marrow-derived macrophages were isolated from femoral bone marrows of mice. After erythrocyte lysis cell suspensions were plated on 90 mm cell culture dishes in DMEM supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin, and macrophage colony-stimulating factor (M-CSF, 50 ng/mL) and cultured for a week. After detachment of macrophages with 10 mmol/L EDTA, cells were resuspended in DMEM containing 10% FCS and 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin, seeded in 12-well plates at a cell density of 0.5 x 10⁶, and used for further experiments.

**ApoE receptor cloning and stable transfections** – Human endothelial and brain RNA were obtained from BD Biosciences and reverse transcribed with oligo(dT) primers using iScript cDNA Synthesis Kit (Bio-Rad, München, Germany). The resulting cDNA was subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) with human apoER and VLDL-R gene-specific primers containing endonuclease restriction sites for BamHI and XhoI. PCR products corresponding to apoER2 variant
lacking exon 5 and exon 18 and VLDL-R variant 2 were purified, cut and ligated into expression vector pBK-CMV. RAW264.7 cells were cultured in DMEM with FCS (10% v/v) and antibiotics up to 90 % confluency. The transfection was performed by electroporation using Amxa Nucleofector-I device (Kit V, program D-32, Lonza AG, Köln, Germany). Transfected cells were grown in the presence of G418 (0.75 g/L) as a selection marker. Positive clones were isolated and identified by Western blot using apoER2- and VLDL-R-specific antibodies.

**ApoE binding** – ApoE was labeled with 5-carboxyfluorescein (FLUOS) using fluorescence protein labeling kit (Roche, Mannheim, Germany). Briefly, apoE (0.1 mg) was dissolved in 0.2 mL PBS and mixed with FLUOS solution (20 mg/mL) to reach the molar ratio of 1:5. The mixture was incubated for 2h at room temperature and intensely dialyzed against PBS using Slide-A-Lyzer dialysis cassette (ThermoFisher Scientific, Rockford, IL). RAW264.7 cells (1 x 10^6/mL) were suspended in PBS containing Ca^{2+} and Mg^{2+} (1.0 mmol/L) and incubated for 1h at 4°C with increasing apoE concentrations as indicated in figure legends. Thereafter, cells were washed with PBS and analyzed using Coulter Epics XL flow cytometer as described below.

**Western Blotting** - Macrophages were lysed in a buffer containing 50 mmol/L Tris-HCl, 0.15 mol/L NaCl, 1% (v/v) Nonidet P-40, 5% (v/v) sodium deoxycholate, 0.1% (v/v) SDS, 300 mmol/L NaF, 1 mmol/L EGTA, 1 mmol/L orthovanadate, and protease inhibitors (Complete, Roche). Cell lysates (50 µg/lane) were subjected to SDS-polyacrylamide gel electrophoresis. Thereafter, proteins were transferred to PVDF membranes, which were blocked overnight in Tris-buffered saline containing 5% non-fat dry milk prior to incubations with antibodies. For each blot with anti-phosphospecific antibodies loading controls were performed, using an antibody against the non-phosphorylated isoform of the respective antigen.
**Determination of mRNA levels** - Total RNA from bone marrow macrophages was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized from 2 µg of total RNA using Superscript II reverse transcriptase and Oligo(dT) from Invitrogen (Karlsruhe, Germany). The resulting cDNA was subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) with gene-specific primers for murine VLDL-R and apoER genes and mouse/rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) obtained from R&D Systems or Invitrogen. For PCR reaction, 2 µg of cDNA was incubated at standard conditions with 2.5 U of HotStarTaq DNA Polymerase (Qiagen) and 7.5 µmol/L of each primer pair. After an initial denaturation at 94°C for 15 min, the reaction was carried out in 30 cycles (94°C, 1 min; 60°C, 0.5 min; 72°C, 0.5 min) followed by a final extension at 72 °C for 7 min. PCR products were visualized by agarose gel electrophoresis and visualized using the AlphaEase FC software system (Alpha Innotech, San Leandro, CA).

**Transient transfections and reporter assays** – Transcription factor-specific reporter gene vectors p(κB)$_5$-Luc and p(GAS)$_4$-Luc were obtained from Stratagene (La Jolla, CA) and Invitrogen, respectively. These vectors contain five or four concatamerized consensus binding sites for NF-κB and STAT-1, respectively, linked to a SV40 minimal promoter in front of the firefly luciferase (LUC) reporter gene. An eucaryotic expression vector for galactosidase (pCMV-SPORT-βgal) was obtained from Invitrogen. RAW264.7 cells were seeded in 24-well plates at a density of 0.5 x 10$^6$ cells/well. 24h prior to transfection cells were incubated in serum-free DMEM. 2.0 µl LipofectAmine 2000 transfection reagent (Invitrogen) and 0.8 µg of pNF-κB-Luc or pGAS-Luc plasmids were added for 6h to cells in serum-free DMEM (0.6 mL final volume) according to manufacturer’s recommendations. Thereafter, cells were incubated with poly(I:C) or IFN-γ in the absence or presence of apoE as indicated in
figure legends. To determine luciferase activity, cells were lysed using passive reporter lysis buffer (Promega, Madison, WI), transferred to polystyrene tubes, and assayed for luminescence intensity using Luciferase Assay System (Promega, Mannheim, Germany) and Sirius Luminometer (Berthold Detection Systems, Bad Wildbad, Germany). β-Galactosidase activity was determined by standard protocol and used to normalize firefly luciferase activity in relation to transfection efficiency.

**Estimation of NO synthase and arginase activities** – Enzymatic activities of NO synthase and arginase were evaluated indirectly by assaying concentrations of their respective products in cell culture supernatants. Total nitrate/nitrite (NOx) concentrations were determined after reduction of nitrate to nitrite with nitrate reductase. A commercially available fluorimetric assay (Alexis, Gruenberg, Germany) was used according to the instruction of the manufacturer. The detection limit for nitrite was 0.1 µmol/L. Urea was quantified photometrically in 10 fold-concentrated supernatants using commercially available kinetic UV assay (Roche). The detection limit for urea was 80 µmol/L.

**Migration assay** - RAW264.7 were incubated for 24h in FCS-free DMEM with or without 5 µg/ml ApoE, harvested, washed twice with PBS and were resuspended to a final concentration of 5 x 10^5 cells/mL. Assays were performed in an 12-well plate using ThinCert™ inserts with pore size 8 µm (Greiner Bio-One, Solingen, Germany). Chemottractants (M-CSF, fMLP) were added to wells containing DMEM (1.5 mL) in concentrations indicated in figure legends. 2.5 x 10^5 cells per cell insert were incubated for 2h at 37°C. Thereafter, media were removed from inserts, which were washed twice with PBS. Insert membranes were fixed for 1h in methanol (4°C) and dried overnight. Cells fixed on the insert membrane were stained with crystal violet for 2h and intensely washed, the dye was eluted with SDS (10%, w/v) and its concentration was estimated by measuring the optical density at 560 nm.
Assessment of reactive oxygen species (ROS) production – ROS generated intracellularly decompose to hydrogen peroxide. Therefore, intracellular hydrogen peroxide levels were taken as an estimation of ROS production. Briefly, RAW264.7 cells in suspension (5.0 x 10^5/mL) were incubated with 10 µmol/L H_2DCF-DA for 30 min and then washed and resuspended in PBS. H_2DCF-DA is a nonpolar compound that readily diffuses into cells, where it is hydrolyzed and oxidized to the highly fluorescent 2',7'-dichlorofluorescein (DCF). The level of DCF fluorescence reflecting the H_2O_2 concentration was monitored using spectral fluorimeter (F-2000 Hitachi, Tokyo, Japan) at 534 nm after excitation at 488 nm. Following exposure to apoE (5µg/mL), the percent fluorescence increase was determined every 1 min. ApoE neither showed autofluorescence nor quenched the DCF fluorescence.

Antibody-Dependent-Cell-Cytotoxicity (ADCC) - RAW264.7 were incubated for 24h in FCS-free DMEM with or without 5 µg/ml apoE, harvested, washed twice with PBS and resuspended to a final concentration of 2 x 10^5/mL. The assay was performed in a 12-well plate. Macrophages (1 x 10^6/well) were allowed to adhere for 3 h. Opsonized erythrocytes (5 x 10^5 cells) were added to each well and incubated for 2 h at 37°C. Thereafter, 0.1 mL supernatant was carefully withdrawn and used for determination of extracellular erythrocyte lysis. A stock solution of 2,7-diaminofluoren (DAF) was prepared by solving 100 mg of DAF in 10 mL of glacial acetic acid (90%; v/v). 1 mL of DAF stock solution and 0.1 mL of H_2O_2 (30%; v/v) were added to 10 mL Tris-buffer (0.2 mol/L) containing 6 mol/L urea. 0.1 mL of this solution was mixed with 0.1 mL of the supernatant. The reaction mixture was incubated for 5 min and the optical density was determined at 595 nm.

Assessment of phagocytosis – RAW264.7 were seeded in a 24 well-plate (3 x 10^5 cells/well) and incubated for 24h in DMEM (0.5 mL) with or without apoE (5 µg/mL). FITC-coupled carboxylate-modified polystyren latex beads (0.33 µL equivalent to ~50
beads/cell) were added for last 18h of incubation. Thereafter, cells were harvested, washed twice with PBS and analyzed for the uptake of FITC-coupled beads on CoulterEpics XL flow cytometer as described below.

Flow cytometric analysis of cell surface molecule expression – peritoneal macrophages (1 x 10^5/mL) were washed and incubated for 1h at 4°C in a blocking solution (PBS containing 0.5% BSA, 5mmol/L EDTA, 2 mmol/L NaN_3 and heat-inactivated normal rabbit serum (10% v/v)). Thereafter, cells were washed, resuspended in blocking solution without rabbit serum, incubated for 1h at 4°C with FITC-coupled monoclonal antibodies against CD206 (1.0 µg/10^5 cells) and MHC-II (0.4 µg/10^5 cells), washed again and analyzed for mean fluorescence using CoulterEpics XL flow cytometer equipped with a 488 nm argon laser. Viable cells were gated using forward and side light-scatter patterns. 5000 events were registered for analysis.

Determination of cytokine, chemokine, and prostaglandin E_2 (PGE_2) levels – RAW264.7 were incubated for 24h in FCS-free DMEM with or without 5 µg/ml ApoE, harvested, washed twice with PBS. In some experiments cells were additionally stimulated with agonists in serum-free DMEM for indicated times. Peritoneal macrophages were incubated for 24h in FCS-free DMEM. Media were collected and cytokine (IL-1RA, IL-12, and TNF_α) or chemokine (MCP-1 and MIP-1_α) levels in incubation solutions were determined using commercially available ELISA kits according to manufacturers’ instructions. PGE_2 levels were determined using an enzyme-linked immunoassay (EIA) kit according to the manufacturer instructions (Cayman Chemical, Ann Arbor, MI).

Plasma cholesterol and triglyceride concentration and distribution - Plasma total cholesterol (TC), HDL-cholesterol (HDL-C) and triglycerides (TG) were determined enzymatically using commercially available kits (Roche). Plasma lipoproteins were
fractionated using Smart™ chromatographic system (Pharmacia, Uppsala, Sweden) equipped with a Superose 6 column (3.2 mm × 30 mm). Plasma was fractionated at a constant flow rate of 50 µL/min, using a buffer containing 150 mmol/L NaCl and 1 mmol/L EDTA, pH 8.0. In total, 24 fractions with a volume of 50 µL each were collected. Total cholesterol content of effluent was determined using enzymatic colorimetric assay (Roche).

**Determination of endotoxin concentration** – Endotoxin content in apoE was determined using limulus amebocyte lysate (LAL) endpoint assay according to manufacturer’s instruction.

**General Procedures** - Data are presented as means ± S.D. form three separate experiments or as results representative for at least three repetitions, unless indicated otherwise. Comparisons between the groups were performed with two-tailed Student t-test using MedCalc 6.0 statistical software. p values less than 0.05 were considered significant.

**RESULTS**

**Generation of stable transfectants expressing functional VLDL-R- or ApoER2** – Two stable cell lines, RAW-VLDL-R and RAW-apoER2 were generated by transduction of RAW264.7 macrophages with respective plasmids encoding human variants of VLDL-R- or apoER2 expressed in the vasculature. The schematic representation of VLDL-R- or apoER2 variants used in this study is shown in Fig. I-A. Each transduced cell line produced a 143/161 kDa or a 97 kDa proteins that cross-reacted with rabbit polyclonal antibodies raised against VLDL-R- or apoER2, respectively (Fig. I-B). In addition, both cell lines displayed a specific binding of FITC-labeled apoE (Fig. I-C). Mouse macrophages were previously demonstrated to produce apoE in a paracrine
fashion. However, neither RAW264.7 cells nor RAW-VLDL-R and RAW-apoER2 transfectants released appreciable amounts of apoE (Fig. I-D).

**Modulatory effects of apoE on macrophage functional phenotype are not due to endotoxin contamination** - We were concerned that the observed modulatory effects of apoE on macrophage functional phenotype could be attributed to the contamination with endotoxin, which may bind to apoE [3]. However, the plasma-isolated apoE used in the present study contained <20 pg endotoxin/µg of apoprotein as determined by the LAL method. In addition, the stimulatory effects of apoE on IL-1RA production by VLDL-R- or apoER2-expressing RAW264.7 cells were retained in the presence of polymyxin B (10 µg/mL), which is known to neutralize endotoxin activity.
Figure I. Characterization of ApoE receptor-expressing RAW264.7 macrophages –

Wild-type RAW264.7 cells were transfected with pBK-CMV expression vectors encoding vasculature-expressed VLDL-R variant 1 or apoER2 variant lacking exon 5 and exon 18. A. Schematic representation of apoE receptors expressed in RAW264.7 cells. Ligand-binding repeats are numbered and growth factor repeats are indicated with capital letters. Arrowheads mark positions of introns. B. Total lysates from apoE receptor-expressing or wild-type cells were blotted and probed with antibodies against VLDL-R (left panel) or apoER2 (right panel). C. ApoE receptor-expressing or wild-type cells were incubated with increasing concentrations of 5-carboxyfluorescein-labeled apoE for 60 min at 4°C and binding was quantified by flow cytometry. Left panel: representative histogram (5 µg/mL apoE). Right panel: concentration-dependent apoE binding. Shown are means from 2 to 3 independent determinations. D. Media from RAW264.7 cells expressing VLDL-R or apoER2 were electrophoresed along with increasing amounts of recombinant apoE as a concentration standard. Blots were probed with antibody against apoE with double anti-mouse and anti-human specificity.

REFERENCES
