Original Research

A new recombinant human apolipoprotein E mimetic peptide with high-density lipoprotein binding and function enhancing activity

Wentao Zhao[1,2], Fen Du[1,3], Michelle Zhang[1], Shengfang Sun[4], Hong Yu[3] and Daping Fan[1]

[1]Department of Cell Biology and Anatomy, School of Medicine, University of South Carolina, Columbia, SC 29209, USA; [2]Department of Chemistry, Tianjin University, Tianjin 300072; [3]Department of Biochemistry and molecular Biology, School of Basic Medicine, Wuhan University, Wuhan 430071, People's Republic of China; [4]Department of Chemistry and Biochemistry, University of South Carolina, Columbia, SC 29208, USA

Corresponding author: Dr Daping Fan, 6439 Garners Ferry Road, Bldg 1, B-12, Columbia, SC 29209, USA. Email: daping.fan@uscmed.sc.edu

Abstract

We generated a novel human apolipoprotein E (apoE)-mimetic peptide, designated EpK. EpK contains an N-terminal cysteine residue, a low-density lipoprotein receptor-binding fragment, a 6 × lysine linker and a lipid-binding fragment. The recombinant peptide was expressed in *Escherichia coli*, and purified with a chitin bead column followed by a Heparin Sepharose CL-6B column to yield pure peptide. EpK displayed high solubility in aqueous solution at neutral pH and adopted a low content of α-helical structure which was significantly increased in 2,2,2-trifluoroethanol or upon lipid binding. EpK retained similar 1,2-dimyristoyl(d54)-sn-glycero-3-phosphocholine binding activity as human apoE3 albeit with slower kinetics. Cell culture studies showed that EpK mediated cholesterol efflux from cholesterol-loaded primary murine macrophages with higher mass-based efficiency than human apoAI and human apoE3, and that EpK inhibited lipopolysaccharide (LPS)-induced proinflammatory cytokine expression in murine macrophages. When injected into apoE$^{-/-}$mice, EpK predominantly associated with high-density lipoprotein (HDL), which was also shown in *in vitro* incubation experiments. Moreover, association of EpK with HDL enhanced the ability of HDL in mediating cholesterol efflux and suppressing LPS-induced proinflammatory cytokine expression in cholesterol-loaded human acute monocytic leukemia cell line (THP-1) macrophages. These data suggest that this novel recombinant apoE mimetic peptide enhances HDL function and harbors antiatherogenic potential.

Keywords: apolipoprotein E, HDL, macrophage, inflammation, cholesterol efflux, atherosclerosis

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Introduction

High-density lipoprotein (HDL) has been demonstrated to protect against atherosclerosis and its related cardiovascular events through multiple mechanisms, including promoting reverse cholesterol transport, suppressing inflammation, antioxidation, antithrombosis and promoting the production of nitric oxide.[1–4] HDL analogs and other HDL function-enhancing agents hold immense promise as antiatherosclerosis therapies,[5,6] in light of the remaining high residual cardiovascular risk even with aggressive low-density lipoprotein (LDL)-cholesterol lowering therapy. There are several lines of HDL-enhancing agents currently in the market or under development, including niacin, cholesterylester transfer protein inhibitors, lecithin cholesterol acyltransferase activators, apoAI expression stimulating agents, endothelial lipase inhibitors, peroxisome proliferator-activated receptor/liver X receptor agonists and HDL apolipoproteins or their mimetics.[6] Among them, niacin is the most consistent one with sound HDL-raising effects; however, its major side-effect (flushing) significantly limits its usage, while the antiatherosclerotic and cardiovascular event reducing effects are yet to be confirmed. The other therapeutic agents are all under various stages of development.

Apolipoprotein AI (ApoAI) is the major HDL apolipoprotein component, and its antiatherogenic role is well documented.[7–9] The use of full-length human apoAI or its mutant, apoAI-milano, in either lipid-free or associated with phospholipid (reconstituted HDL) forms, or shorter apoAI mimetics, as HDL enhancing and antiatherosclerotic agents, has garnered immense interest.[10–12] ApoE is another major apolipoprotein in HDL particles; however, it is also a component of other lipoproteins, making apoE a multifunctional protein with critical roles in lipoprotein metabolism

and in the pathogenesis of atherosclerosis.[13] As a ligand for the low-density lipoprotein receptor (LDLR) family and heparin sulfate proteoglycan (HSPG), apoE mediates the uptake of lipoprotein remnants, thereby contributing to hepatic plasma cholesterol clearance.[14–16] As lipid-free protein or in association with HDL particles, apoE promotes reverse cholesterol transport and exerts antioxidative and anti-inflammatory function, as well as inhibiting vascular smooth muscle cell proliferation.[17,18] ApoE consists of two structural and functional domains: the four-helix-bundle N-terminal domain that is responsible for LDLR binding and HSPG binding, and the C-terminal domain that is responsible for lipid binding and Aβ peptide binding.[13] An amphipathic α-helical motif is the basis for the lipid binding; in particular, the α-helices in the C-terminal domain region (residues 220–260) initiate lipid binding and further trigger the conformational change of the N-terminal domain.[19] This lipid-triggered conformational change of the N-terminal domain ensures the LDLR-binding capability.[20] The LDLR-binding site is comprised of a cluster of positively charged residues in the fourth helix of the N-terminal domain.[21]

The average plasma apoE concentration in humans is 5 mg/dL. Animal studies suggest that apoE can significantly protect mice from developing atherosclerosis even at low plasma concentrations that does not correct high plasma cholesterol levels.[22] This allows apoE to be an ideal therapeutic agent for atherosclerosis. However, efforts to generate a high-yield expression system for apoE have been unsuccessful. There are also many other problems regarding the use of apoE clinically, including its low solubility in aqueous solution and its tendency to undergo enzymatic digestion *in vivo*, thus generating cytotoxic fragments. These issues make using full-length apoE as a therapeutic agent unpractical. To overcome these problems, several apoE mimetics have been developed.[23–26] It is well documented that apoE fragments containing the LDLR-binding region have anti-inflammatory activity,[27,28] while mimetics containing lipid-binding regions can promote cholesterol efflux from macrophages, especially when the peptide contains at least two amphipathic helices.[25,29] One elegantly designed and chemically synthesized apoE-derived peptide, Ac-hE18A-NH_2, which couples the LDLR-binding region with an 18-residue amphipathic helical peptide, has the ability to mediate very-low-density lipoprotein (VLDL)/LDL clearance from plasma and promote cholesterol efflux from macrophages, and displays anti-inflammatory and antioxidative activities.[24,26,30] Furthermore, this peptide is recycled to the plasma after it is internalized by the liver, significantly prolonging its biological effects.[31] For these reasons, this peptide has a potential to be developed into a therapeutic agent to treat atherosclerosis and its resulting cardiovascular diseases. However, the foreign nature of the 18-residue amphipathic peptide may potentially provoke immune response in humans and thus diminish the therapeutic efficiency over time.

Here, we report a novel bacterial recombinant apoE-derived peptide (EpK), which contains an N-terminal LDLR-binding region and a C-terminal lipid-binding region linked by a 6 × lysine fragment. A two-step purification procedure yielded pure peptide. Interestingly, we found that this peptide, when injected into mice, did not reduce plasma cholesterol; instead, it primarily associated with HDL. Both EpK and EpK-containing HDL promoted cholesterol efflux from macrophages and inhibited macrophage inflammatory responses induced by lipopolysaccharide (LPS). These data suggest that this peptide holds promise to eventually be developed into an HDL-enhancing agent for the therapy of atherosclerosis.

Materials and methods

Materials and reagents

Oligonucleotides and primers were customer synthesized by Invitrogen (Carlsbad, CA, USA). Gene synthesis polymerase chain reaction (PCR) was performed using a Phusion High-Fidelity PCR kit from New England Biolabs (NEB, Ipswich, MA, USA). Bacterial expression vector pTWIN1 and chitin beads were purchased from NEB. Competent cell BL21(DE3)pLysS was purchased from Novagen (Darmstadt, Germany) and Heparin Sepharose CL-6B resin from GE Healthcare (Piscataway, NJ, USA). A human acute monocytic leukemia cell line (THP-1) was obtained from ATCC (Manassas, VA, USA). Dulbecco's modified Eagle medium (DMEM) and RPMI-1640 Medium (RPMI) were purchased from Invitrogen. Fetal bovine serum (FBS) was purchased from Atlantis (Norcross, GA, USA). Goat anti-human apoE antibody was purchased from Academy Biomedical (Huston, TX, USA) and horseradish peroxidase (HRP)-conjugated rabbit anti-goat immunoglobulin G (IgG) antibody was purchased from Fisher Scientific (Pittsburgh, PA, USA). ^{3}H-labeled cholesterol was purchased from PerkinElmer (Waltham, MA, USA). Acetylated LDL (acLDL) was purchased from Biomedical Technologies (Stoughton, MA, USA).

Peptide expression and purification

EpK was expressed through *Escherichia coli* BL21(DE3)pLysS cells. Luria-Bertani (LB) medium (with 100 mg/L ampicillin) was used for cell culture. Cultures were grown at 37°C until it reached OD at 600 nm of 1.0. Expression was then induced by 0.5 mmol/L isopropyl β-D-1-thiogalactopyranoside and continued at 25°C for eight hours. The cells were harvested by centrifugation. The cell pellet was resuspended with buffer B1 (20 mmol/L Tris-HCl, pH 8.5, 500 mmol/L NaCl and 1 mmol/L ethylenediaminetetraacetic acid [EDTA]) and lysed by sonication. A two-step procedure was then used to purify EpK. First, the cell extract was loaded onto a chitin bead column and washed with buffer B1 to remove unbounded proteins. The on-column cleavage of the intein-tag was induced by equilibrating the chitin beads in buffer B2 (20 mmol/L Tris-HCl, pH 7.0, 500 mmol/L NaCl, 50 mmol/L dithiothreitol and 1 mmol/L EDTA) overnight at room temperature, and the target peptide was then eluted with additional buffer B2. Second, the eluted peptide from the first step purification was diluted 1:10 with 10 mmol/L phosphate binding buffer (pH 7.4) containing 100 mmol/L NaCl and loaded onto a Heparin Sepharose CL-6B column. It was then washed with 15 column volumes of the same binding buffer containing 200 mmol/L NaCl to remove trace

unbound intein and chitin binding domain, as well as other contaminating proteins. The target peptide was eluted by the binding buffer with increasing concentrations of NaCl. The fractions containing 500–600 mmol/L NaCl were pooled and dialyzed against 10 mmol/L NH_4HCO_3, and lyophilized to yield peptide powder.

Circular dichroism

Circular dichroism (CD) measurements were carried out on a J-815 Spectropolarimeter (JASCO, Essex, UK) with a variable temperature capability under computer control within 0.2°C. EpK was prepared at a concentration of 0.25 mg/mL in 10 mmol/L potassium phosphate buffer (KPi, pH 7.0) or with 25% or 50% 2,2,2-trifluoroethanol (TFE). Far-ultraviolet (190–260 nm) spectra were collected with a cuvette of 0.2-cm pathlength at 37°C.

DPMC-binding assay

The assay was performed as described previously.[32] Briefly, 10 mg of 1,2-dimyristoyl(d54)-sn-glycero-3-phosphocholine (DMPC; Avanti Polar Lipids Inc, Alabaster, AL, USA) was dissolved in a mixture of chloroform and methanol (3:1 v/v) and dried using N_2. One milliliter of prewarmed buffer (20 mmol/L Tris-HCl, pH 7.2, 250 mmol/L NaCl and 1 mmol/L EDTA) was added to a final lipid concentration of 10 mg/mL, and the mixture was vortexed several times for 30 s each. DMPC vesicles (100 μg) and 100 μg of EpK or apoE3 in buffer were added into a thermostated cuvette and mixed for 5–10 s at 24°C, and clearance of the solution was monitored using a SpectraMax M5 spectrometer (Molecular Devices, Sunnyvale, CA, USA) at 490 nm. All solutions were preincubated at 24°C before the reaction.

Cell culture

To elicit mouse macrophages, 3 mL of 3% thioglycollate was injected intraperitoneally into mice. Three days later, 10 mL phosphate-buffered solution (PBS) was injected into the peritoneal cavity to collect cells. The cells were seeded in either 6- or 24-well dishes in DMEM with 10% FBS for one hour and washed with FBS-free DMEM twice to remove unattached cells. Attached macrophages were further cultured in an appropriate medium. THP-1 cells were cultured in RPMI with 10% FBS. To induce THP-1 monocytes to differentiate into macrophages, 30 ng/mL phorbol-12-myristate-13-acetate (PMA) was added to the culture medium for 72 h before the macrophages were used for further experiments, during which 30 ng/mL of PMA was always included in the medium.

Macrophage cholesterol efflux

For mouse macrophages, attached macrophages in 24-well plates were loaded with 100 μg/mL ^{3}H-cholesterol-incorporated acLDL for three days. Subsequently, DMEM containing an indicated amount of EpK was added and the cells were further cultured for 24 h. For THP-1 cell-derived macrophages, the cells were cultured in RPMI with 30 ng/mL PMA for the entire duration of the experiment. The cells were loaded with 100 μg/mL ^{3}H-cholesterol-incorporated acLDL for 48 h, and efflux medium containing an indicated amount of HDL was added to initiate cholesterol efflux. Medium was taken one and four hours after the HDL-containing efflux medium was added to cells. At the four hours time point, the medium was completely removed and the cells were washed twice with PBS before the cells were collected in isopropanol for cellular ^{3}H radioactivity count. ^{3}H radioactivity in the efflux medium and cells were counted using a Beckman LS6500 liquid scintillation counter (Palo Alto, CA, USA). The percentage of effluxed ^{3}H was calculated to determine the efflux rate.

Quantitative realtime PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen) and RNeasy kit (Qiagen, Valencia, CA, USA). Total RNA was reverse transcribed into cDNA using iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). Quantitative realtime PCR (qPCR) measurements of target mRNA levels were performed on an Eppendorf Realplex$_2$ Mastercycler (Eppendorf, Hamburg, Germany) according to the manufacturer's instructions. Mouse genes were normalized with 18srRNA, and human genes were normalized with glyceraldehyde-3-phosphate dehydrogenose (GAPDH), as endogenous controls, respectively. The primers used in qPCR are as follows. Mouse 18s: 5′ CGCGGTTCTAT TTTGTTGGT 3′ (forward) and 5′ AGTCGGCATCGTTTA TGGTC 3′ (reverse); mouse tumor necrosis factor (TNF) α: 5′ CGTCAGCCGATTTGCTATCT 3′ (forward) and 5′ CGGACT CCGCAAAGTCTAAG 3′ (reverse); mouse interleukin (IL)-6: 5′ AGTTGCCTTCTTGGGACTGA 3′ (forward) and 5′ TCC ACGATTTCCCAGAGAAC 3′ (reverse); mouse monocyte chemotactic protein (MCP)-1: 5′ AGGTCCCTGTCATGCTTCTG 3′ (forward) and 5′ TCTGGACCCATTCCTTCTTG 3′ (reverse); human GAPDH: 5′ GAGTCAACGGATTTGGTCGT 3′ (forward) and 5′ TTGATTTTGGAGGGATCTCG 3′ (reverse); human IL-6: TACCCCCAGGAGAAGATTCC 3′ (forward) and 5′ TTTTCTGCCAGTGCCTCTTT 3′ (reverse); human IL-1β: 5′ GGGCCTCAAGGAAAAGAATC 3′ (forward) and 5′ TTCTGCTTGAGAGGTGCTGA 3′ (reverse); and human CCL2: 5′ CCCCAGTCACCTGCTGTTAT 3′ (forward) and 5′ TGGAATCCTGAACCCACTTC 3′ (reverse).

Plasma cholesterol analysis

Mouse plasma cholesterol levels were determined by enzymatic colorimetric assays using Cholesterol Reagent Kit (Raichem, San Diego, CA, USA). Plasma lipoprotein cholesterol profiles were analyzed by fast-performance liquid chromatography (FPLC) with a Superose 6 10/300 GL column on an AKTA purifier (GE Healthcare). One hundred microliters of mouse plasma was loaded onto the column, and 40 continuous 0.5-mL fractions were collected.

Western blotting analysis

Thirty microliter samples from each FPLC fraction were loaded onto 15% sodium dodecyl sulfate polyacrylamide

gel electrophoresis (SDS-PAGE) gels for electrophoresis. The protein was then transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). Goat anti-human apoE primary antibody and HRP-conjugated rabbit anti-goat IgG secondary antibody were used to detect EpK. Signal was detected using an ECL kit (Amersham Biosciences).

Animals

Wild-type C57Bl/6 mice and apoE-deficient (apoE$^{-/-}$) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and housed at the University of South Carolina Animal Research Facility. Animal care procedures and experimental methods were approved by the Institutional Animal Care and Use Committee of the University of South Carolina.

Statistical analysis

Statistical analysis were performed with GraphPad PRISM software (GraphPad Software Inc, La Jolla, CA, USA). Differences between group mean values were determined by one-way analysis of variance (ANOVA) and student *t*-tests. $P < 0.05$ was regarded as statistically significant.

Results

Design, expression and purification of EpK

We designed an apoE mimetic peptide (designated as EpK) containing a human apoE N-terminal domain fragment and a C-terminal domain fragment as depicted in Figure 1a. We synthesized a DNA fragment encoding this peptide through PCR, using oligonucleotides designed with the assistance of the NIH program DNA Works 2.0 (NIH, Bethesda, MD, USA).[33] This peptide contained a cysteine at the N-terminus, an apoE LDLR-binding fragment (10 aa), followed by a 6-lysine cationic peptide link (6 aa) and an apoE lipid-binding fragment (21 aa). The first leucine residue of the 10-residue LDLR-binding region was substituted by an Arginine to optimize the cleavage of the intein during protein purification. We subcloned this DNA fragment into a pTWIN1 vector with an N-terminal intein fusion, and the resulted plasmid was transformed into *E.coli* BL21(DE3)pLysS competent cells. Bacterial expression and purification were performed as described in the Materials and methods section. It is noteworthy that in the second step of the purification procedure, EpK eluted in the 500–600 mmol/L NaCl fractions, while full-length human apoE3 eluted in 400–500 mmol/L NaCl fractions, indicating that EpK has a higher affinity to heparin than full-length apoE3. The final yield is 5–10 mg pure peptide from 1 L LB expression. A 15% reducing SDS-PAGE showed that the peptide is highly pure after the second step purification (Figure 1b). Mass spectroscopy confirmed its correct size (4686.981 versus calculated 4686.69), purity and the partial formation of disulfide bound-mediated dimers (molecular weight 9397.116 versus calculated 9371.38) (Figure 1c). The pI of EpK is estimated as 11.38 due to the high content of positively charged residues. The solubility of EpK in PBS at 37°C and pH 7.0 was as high as 10 mg/mL (~2 mmol/L) after mild sonication, but it decreased as pH increased; at pH 10, it decreased to less than 1 mg/mL in 10 mmol/L phosphate buffer. The peptide powder was dissolved in PBS at 1 mg/mL for cell culture experiments and mouse injection.

EpK adopts an α-helical structure

ApoE adopts a highly α-helical structure which is critical for lipid binding. Web-based secondary structure prediction tool (Network Protein Sequence Analysis) predicted that 34 residues of EpK are in a stretch of α-helix; in other words, EpK may contain 89.47% α-helical structure. To

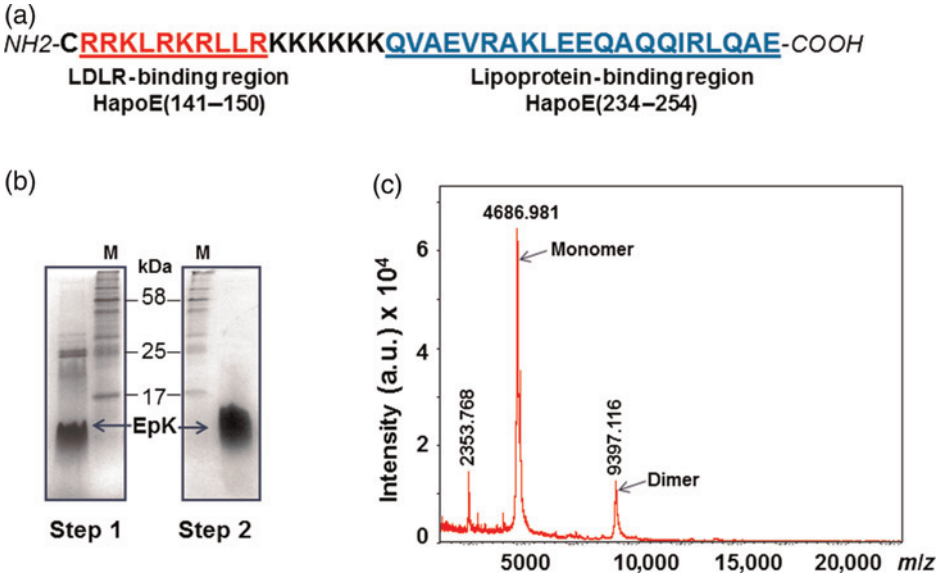

Figure 1 Peptide design, purification and characterization. (a) The sequence of the peptide EpK. (b) Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the purified EpK showing the products after Step 1 and Step 2 purification. (c) Mass spectrometry of purified EpK. (A color version of this figure is available in the online journal)

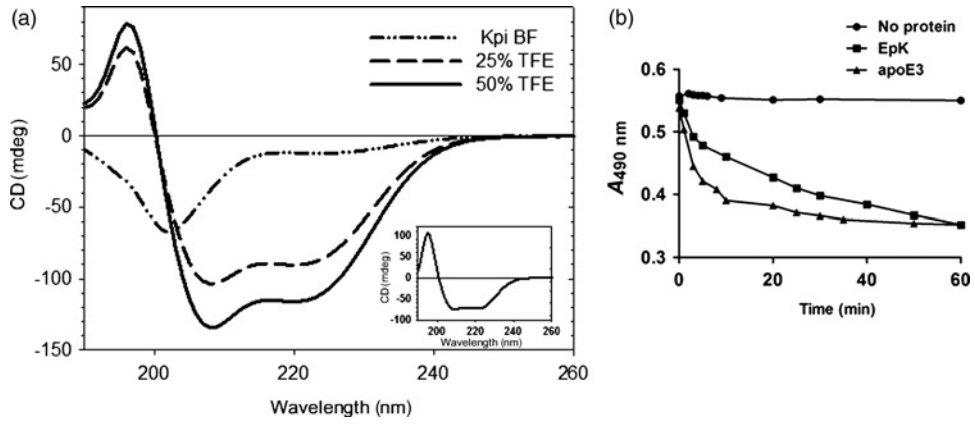

Figure 2 Secondary structure and lipid-binding activity of EpK. (a) Far-ultraviolet circular dichroism (CD) spectrum of EpK in aqueous solution at 37°C. Inset: far-UV circular dichroism spectrum of EpK-1,2-dimyristoyl(d54)-sn-glycero-3-phosphocholine (DMPC) complex. (b) DMPC vesicles were incubated at 24°C with indicated proteins; vesicle clearance as function of time was followed by OD at 490 nm

confirm the ability of EpK to adopt an α-helical structure, we performed CD measurements. As shown in Figure 2a, in 10 mmol/L potassium phosphate (KPi) buffer, the α-helical content of EpK was very low, only 16.9%. However, in TFE, a helix-stabilizing solvent, the α-helical content of EpK increased to 63.1% and 81.5% in 25% and 50% TFE, respectively, confirming that EpK has a high tendency to form an α-helical structure.

EpK binds to DMPC vesicles

To determine the lipid-binding activity of EpK, we performed a DMPC-binding assay using full-length apoE3 as control. As shown in Figure 2b, at 0.25 mg/mL concentration, EpK could as effectively clear the turbidity of DMPC vesicles as full-length apoE of the same concentration over a one-hour period of time. However, the clearance rate of EpK was much slower than that of apoE3; $t_{1/2}$ of EpK was 10 min, while that of apoE3 was 3 min. We further measured the α-helical content of EpK in EpK-DMPC complex. As shown in the inset of Figure 2a, EpK displayed a typical CD spectrum of an α-helical protein and the calculated α-helical content was 53.8%.

EpK enhances macrophage cholesterol efflux and inhibits macrophage inflammation

Using the purified protein, we performed a functional assessment with primary mouse macrophages. First, we tested whether EpK could mediate cholesterol efflux from cholesterol-loaded macrophages. The thioglycollate-elicited peritoneal macrophages from apoE$^{-/-}$ mice were loaded with cholesterol by incubation for three days with 100 μg/mL acLDL. Subsequently, these cholesterol-loaded macrophages were incubated with DMEM in the presence of apolipoprotein or EpK. Compared with cholesterol efflux with DMEM alone (without exogenous cholesterol acceptor), an addition of 10 μg/mL EpK increased the cholesterol efflux from 1.3% to 6.1%, and an addition of 20 μg/mL EpK further increased efflux to 13.4% (Figure 3a). When compared with the same mass of human apoAI and apoE3, EpK displayed a higher potency in mediating cholesterol efflux from macrophages. Next, we examined if EpK inhibits macrophage inflammatory responses to LPS. Freshly isolated thioglycollate-ellicited peritoneal macrophages from apoE$^{-/-}$ mice were allowed to attach for one hour in DMEM with 10% FBS. These cells were washed with DMEM twice and further cultured in serum-free DMEM overnight. The medium was replaced by new DMEM with the addition of 100 ng/mL LPS with or without an indicated amount of EpK (in μg/mL). After six hours of further incubation, the medium was removed, the cells were washed twice with PBS and the cells were lysed in Trizol for total RNA extraction. Quantitative realtime PCR results showed that LPS significantly induced the expression of proinflammatory cytokines, including TNF-α, IL-6 and MCP-1 in primary murine macrophages (Figures 3b–d). The addition of EpK, at both 5 and 10 μg/mL concentrations, significantly reduced the LPS-induced expression of all three cytokines.

EpK associates with HDL

To test if EpK could bind to lipoproteins and mediate plasma cholesterol clearance, EpK (100 μg) or full-length human apoE3 (100 μg) in PBS was retro-orbitally injected into four-month-old apoE$^{-/-}$ mice. After the peptide or the protein was injected, 30-μL blood samples were drawn at 1, 10 and 30 min, and 1, 2, 4 and 24 h for plasma cholesterol measurement. Surprisingly, we found that EpK did not change plasma cholesterol levels in apoE$^{-/-}$ mice, while apoE3 significantly reduced plasma cholesterol immediately after the injection and prolonged for at least four hours (Figure 4a). To investigate why EpK did not mediate plasma lipoprotein clearance, we examined if EpK associates with plasma lipoproteins. We selected six apoE$^{-/-}$ mice with comparable plasma cholesterol and triglyceride levels. Three mice were bled to collect plasma, and 90 μL plasma from each mice was incubated with 10 μL EpK (EpK final concentration: 0.1 mg/mL) for 30 min before the plasma samples were subjected to FPLC. The other three mice were injected with 100 μg EpK each retro-orbitally. Plasma samples were collected 30 min after the injection for FPLC isolation of different lipoprotein fractions. As shown in Figure 4b, EpK injection did not change the

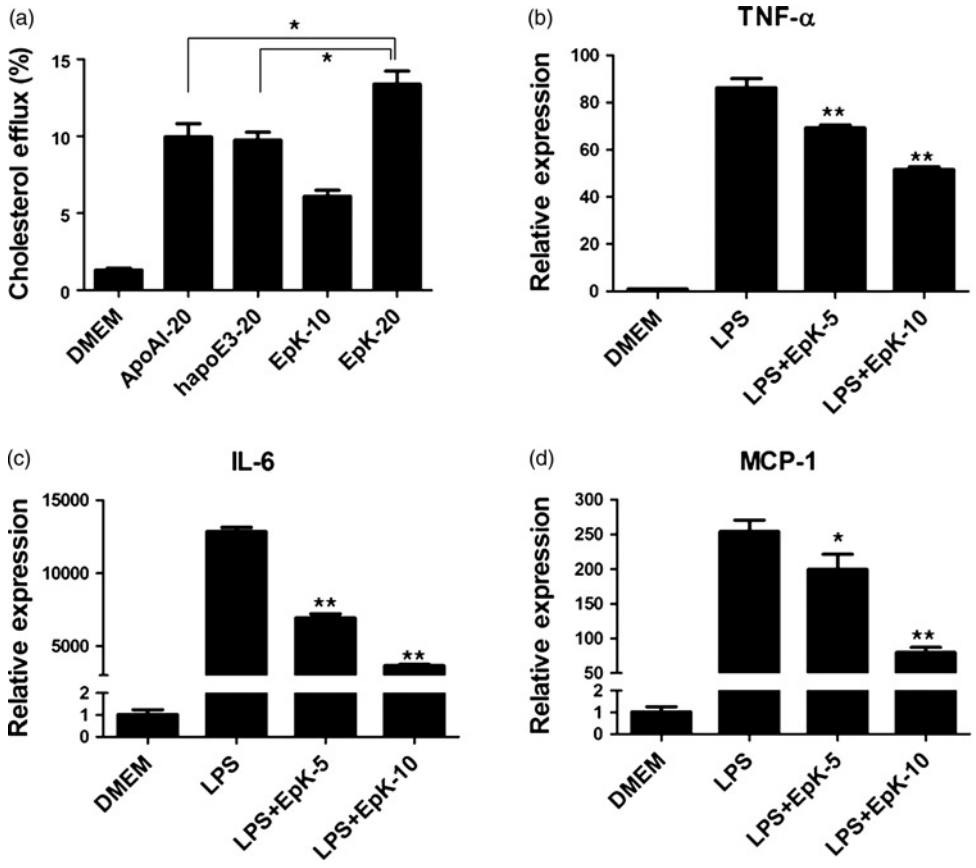

Figure 3 EpK enhances macrophage cholesterol efflux and inhibits macrophage inflammatory response to lipopolysaccharide (LPS). (a) Peritoneal macrophages from apoE$^{-/-}$ mice were loaded with ace-LDL for three days and efflux to Dulbecco's modified Eagle's medium (DMEM), human apoAI, human apoE3 and EpK at indicated concentrations (μg/mL) was measured as described in Materials and Methods. $^{*}P < 0.01$. Vertical bars represent mean $\pm$ SEM. For each treatment, $n = 3$. (b–d). Peritoneal macrophages from apoE$^{-/-}$ mice were treated with LPS (100 ng/mL) and EpK at indicated concentration (μg/mL) for six hours in DMEM. The expression levels of indicated cytokines were measured by quantitative realtime polymerase chain reaction. $^{*}P < 0.05$, $^{**}P < 0.01$, versus LPS group. Vertical bars represent mean +/– SEM. For each treatment, $n = 3$

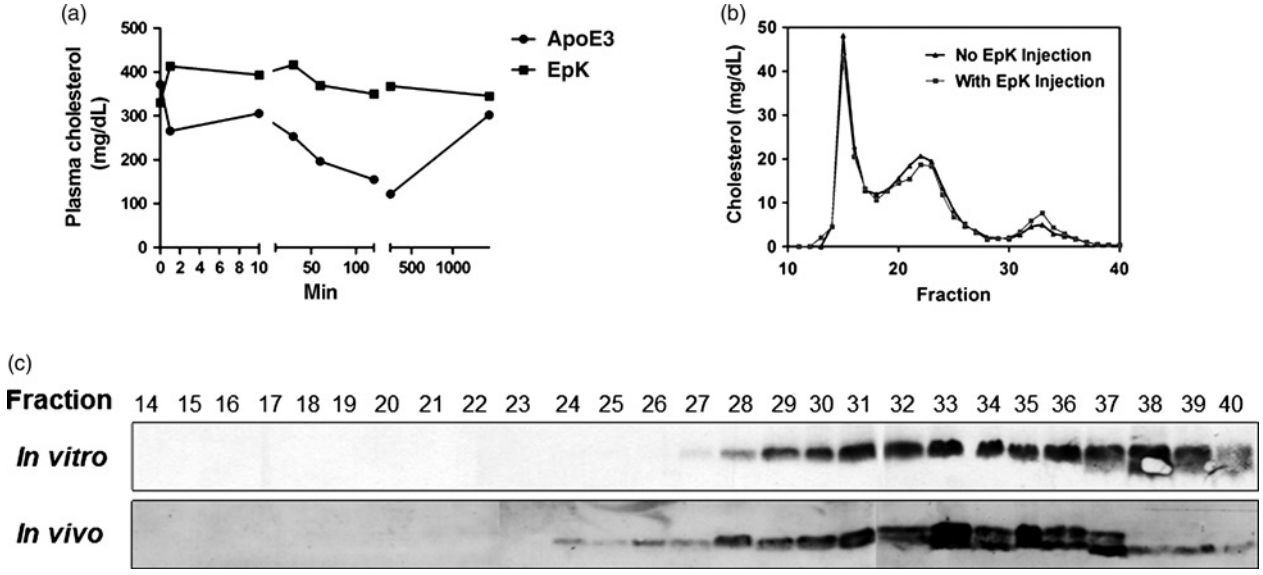

Figure 4 EpK does not mediate cholesterol clearance from plasma but primarily associates with HDL. (a) ApoE$^{-/-}$ mice were retro-orbitally injected with apoE3 or EpK (100 μg/mouse); plasma cholesterol levels were followed at indicated time points. The figure shows a representative of three pairs of mice. (b) Pooled plasma samples from three mice without EpK injection or from three mice injected with EpK (100 μg/mouse; plasma collected 30 min after injection) were subjected to fast-performance liquid chromatography (FPLC) to obtain plasma lipoprotein profiles. (c) EpK was detected by an anti-human apoE antibody in FPLC lipoprotein fractions from the apoE$^{-/-}$ mouse plasma incubated for 30 min with EpK *in vitro* or in the FPLC fractions of the plasma from apoE$^{-/-}$ mice collected 30 min after the injection of EpK

lipoprotein profiles in mice. We next examined the distribution of EpK in different FPLC fractions using Western blot, taking advantage of the fact that EpK was recognized by the goat anti-human apoE antibody (50A-G1b; Academy Biomedical, Houston, TX, USA). Figure 4c shows that in the *in vitro* incubation experiments, EpK was eluted in the fractions 28–40 (HDL and lipid-free fractions), and in the *in vivo* injection experiment, EpK was mainly eluted in fractions 28–37 (HDL) and much less but still detectable in fractions 24–27 (LDL) and 38–40 (lipid-free). These data indicated that EpK mainly associated with HDL, but not with VLDL and only very minor with LDL, explaining the finding that this peptide did not reduce plasma cholesterol levels after being injected into apoE$^{-/-}$ mice.

EpK-containing HDL has enhanced function

We further investigated whether the association of EpK enhances HDL's function in promoting macrophage cholesterol efflux and inhibiting macrophage inflammatory responses. We pooled the FPLC fractions 30–37 (HDL) from the plasma of the apoE$^{-/-}$ mice with or without injection of EpK, respectively. The pooled HDL fractions were concentrated using Centricon concentrators (Millipore, Billerica, MA, USA) and filtered with a 0.45-μm filter. We used human monocyte cell line THP-1-derived macrophages to perform cholesterol efflux experiments with HDL as a cholesterol acceptor. After cholesterol loading for three days, THP-1 macrophages were washed and RPMI with the addition of HDL at 100 μg cholesterol/mL concentrations was added to the cells. Medium was collected one and four hours after the HDL-containing efflux medium was added to cells for the ^{3}H radioactivity count and cholesterol efflux measurements. At the one hour time point, both HDL from apoE$^{-/-}$ mice with or without EpK injection significantly enhanced cholesterol efflux to the culture medium compared with plain RPMI medium (5.78% and 4.86% versus 0.93%; one-way ANOVA $P < 0.01$) (Figure 5a); but the efflux mediated by EpK-containing HDL was not significantly different from that mediated by HDL without EpK. However, at the four hours time point, EpK-containing HDL mediated significantly more cholesterol efflux than control HDL (10.58% versus 7.42%, $P < 0.01$). We next tested if EpK-containing HDL inhibits macrophage inflammatory cytokine expression induced by LPS more profoundly than control HDL. THP-1-derived macrophages were stimulated with LPS (50 ng/mL), with the addition of HDL (100 μg/mL cholesterol) prepared from apoE$^{-/-}$ mice with or without injection of EpK. Six hours after the treatment, the cells were lysed in Trizol for total RNA extraction. Quantitative realtime PCR was used to detect IL-6, IL-1β and CCL2 expression from THP-1 derived macrophages. Data showed that LPS significantly increased the expression of all three cytokines from THP-1 macrophages (Figures 5b–d). Both control HDL and EpK-containing HDL significantly suppressed the expression of the three cytokines, while EpK-containing HDL was significantly more potent compared with control HDL ($P < 0.01$), indicating that EpK association confers HDL with more potent anti-inflammatory activity.

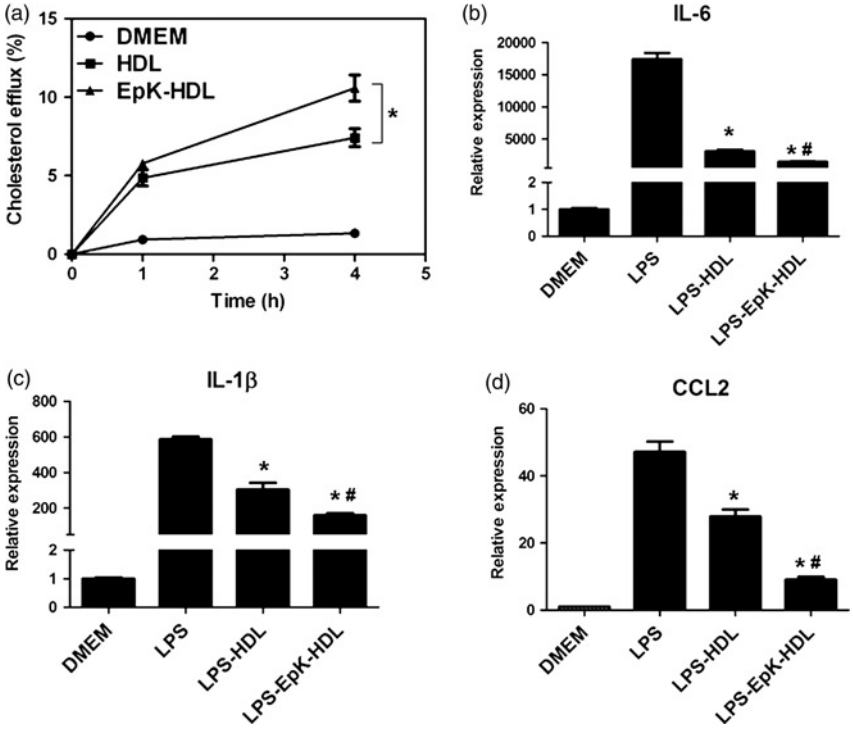

Figure 5 EpK-containing HDL promotes cholesterol efflux from macrophages and inhibits inflammatory response to lipopolysaccharide (LPS). (a) Cholesterol efflux from THP-1 macrophages to HDL (100 μg/mL cholesterol) isolated from apoE$^{-/-}$ mice with or without EpK injection. $^*P < 0.01$. (b–d) EpK-containing HDL inhibits inflammatory cytokine expression from LPS-stimulated THP-1 macrophages. $^*P < 0.01$ versus LPS group; $^\#P < 0.01$ versus LPS-HDL group. Vertical bars represent mean $\pm$ SEM. For each treatment, $n = 3$

Discussion

EpK was designed to contain some important features: (1) it has both an LDLR-binding region and a lipid-binding region; while the LDLR-binding region renders it anti-inflammatory, the lipid-binding region is required for its association with lipoproteins; (2) the cysteine residue at the N-terminus mediates dimer formation, doubling the length of the peptide to enhance its function; (3) there is a positively charged 6-lysine linker region between the LDLR-binding and lipid-binding regions to enhance its solubility and to increase phospholipid-binding affinity through hydrophobic interactions between lysine side chains and phospholipid acyl tails, as well as through the hydrophilic interactions between the positively charged lysine amide groups and negatively charged phospholipid head groups.

Compared with other apoE mimetic peptides under investigation, which are all chemically synthesized, EpK is produced by recombinant bacterial expression. The purification procedure is simple and yields highly pure protein without any extra tags. The final yield of highly pure peptide is 5–10 mg from 1 L of LB medium. The cost is significantly lower than chemical synthesis, and it is easy to scale up expression and purification in order to obtain a large amount of the peptide for further animal studies to investigate the effects on atherosclerosis.

As mentioned previously, a well-studied synthetic apoE mimetic peptide, Ac-hE18A-NH_2, which is composed of the LDLR-binding region of apoE (residues 141–150: LRKLRKRLLR) covalently linked to an 18-residue Class A amphipathic peptide 18A, was found to have multiple functions. It binds LDL and VLDL, and mediates their uptake via HSPG.[24] Thus, it can lower plasma LDL and VLDL in hyperlipidemic rabbits and mice.[26,30] Although it did not increase plasma HDL levels, it improves HDL function by increasing plasma PON-1 activity.[26] It also can mediate macrophage cholesterol efflux and inhibit macrophage and endothelial cell inflammatory responses to LPS stimulation.[31] Furthermore, its recycling property makes the beneficial effects of this peptide extend to over 24 h.[31] We designed EpK partially based on this Ac-hE18A-NH_2 peptide; however, we elected to use human apoE lipid-binding region as the C-terminal part of the peptide to eliminate any potential immunogenicity to humans.

Secondary structure measurements showed EpK adopted an α-helical structure in the presence of TFE, representing its conformation in a lipid-binding state. The tendency to form an amphipathic α-helix ensures the lipid-binding activity of the peptide. A DMPC-binding assay further confirmed its lipid-binding ability by demonstrating that EpK can bind to DMPC vesicles and transform them into smaller particles. EpK adopts an α-helical structure in the EpK-DMPC complex.

Cell culture studies showed that EpK mediated cholesterol efflux from cholesterol-loaded primary murine macrophages and inhibited murine macrophage inflammatory responses to LPS stimulation. These functions are consistent with the properties of other apoE mimetic peptides.[24,25,27,29] The C-terminal lipid-binding region may confer this cholesterol efflux property, and the LDLR-binding region may result in its anti-inflammatory function.

Although EpK is equipped with an LDLR-binding region which also has strong heparin-binding activity as shown in the purification procedure, it did not reduce plasma cholesterol when injected into apoE$^{-/-}$ mice. This may be explained by its preferential binding to HDL instead of VLDL and LDL. The reason why EpK does not bind to VLDL and LDL is currently unknown; we speculate that EpK preferentially binds to smaller lipoprotein particles like HDL with a more curved surface enriched with phospholipids. As shown in the DMPC-binding assay, compared with full-length apoE3, EpK bound to large DMPC vesicles with much slower kinetics. Nevertheless, the HDL binding preference confers this peptide with some advantages: (1) it will not be quickly cleared from plasma by LDLR because it does not bind VLDL and LDL. Thus, EpK's half-life in circulation should be prolonged; (2) it predominantly binds to HDL, greatly potentiating its HDL-enhancing function, which has been shown in the current study.

Using EpK-containing HDL from apoE$^{-/-}$ mice injected with EpK, we found that EpK association significantly enhanced HDL function in both mediating macrophage cholesterol efflux and inhibiting macrophage inflammatory responses to LPS stimulation. Among the many merits of HDL, mediation of reverse cholesterol transport and anti-inflammation are the two most important ones, and these two properties may be intrinsically linked to each other. Recent evidence suggests that the anti-inflammatory effects of HDL in macrophages are likely mediated in part by cholesterol efflux via ABCA1 and ABCG1.[34] Most of the beneficial effects of HDL are attributable to its major apolipoprotein components, apoAI and apoE. This has prompted researchers to develop apoAI, apoE or their mimetics to enhance HDL functions. Currently, full-length apoAI in lipid-free or lipid-associated form is in clinical trials with promising results.[10,35] Several apoAI-derived peptides are also under development. We believe EpK is another promising HDL functional enhancer with unique features.

In summary, we report here the generation and functional characterization of a novel apoE mimetic peptide, EpK. It was obtained by recombinant bacterial expression with high yield and high purity. It adopted an α-helical structure and was able to bind to lipid. EpK could mediate macrophage cholesterol efflux and inhibit macrophage inflammatory response to LPS. EpK bound to HDL both *in vitro* and *in vivo*. Association of EpK enhanced HDL functions, including promoting macrophage cholesterol efflux and attenuating macrophage inflammation. These data suggest that EpK has the potential to be developed into a new antiatherogenic agent.

Author contributions: DF, WZ and HY designed the study; WZ, FD, MZ and SS conducted the experiments; WZ, HY and DF analyzed the data; and HY and DF wrote the manuscript.

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