Apolipoprotein A-I Mimetic Peptides
Discordance Between In Vitro and In Vivo Properties—Brief Report

Michael Ditiatkovski, Jonatan Palsson, Jaye Chin-Dusting, Alan T. Remaley, Dmitri Sviridov

Objective—Apolipoprotein A-I (apoA-I) mimetic peptides have antiatherogenic properties of high-density lipoprotein in vitro and have been shown to inhibit atherosclerosis in vivo. It is unclear, however, if each in vitro antiatherogenic property of these peptides translates to a corresponding activity in vivo, and if so, which of these contributes most to reduce atherosclerosis.

Approach and Results—The effect of 7 apoA-I mimetic peptides, which were developed to selectively reproduce a specific component of the antiatherogenic properties of apoA-I, on the development of atherosclerosis was investigated in apolipoprotein E—deficient mice fed a high-fat diet for 4 or 12 weeks. The peptides include those that selectively upregulate cholesterol efflux, or are anti-inflammatory, or have antioxidation properties. All the peptides studied effectively inhibited the in vivo development of atherosclerosis in this model to the same extent. However, none of the peptides had the same selective effect in vivo as they had exhibited in vitro. None of the tested peptides affected plasma lipoprotein profile; capacity of plasma to support cholesterol efflux was increased modestly and similarly for all peptides.

Conclusions—There is a discordance between the selective in vitro and in vivo functional properties of apoA-I mimetic peptides, and the in vivo antiatherosclerotic effect of apoA-I-mimetic peptides is independent of their in vitro functional profile. Comparing the properties of apoA-I mimetic peptides in plasma rather than in the lipid-free state is better for predicting their in vivo effects on atherosclerosis.

Visual Overview—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:1301-1306. DOI: 10.1161/ATVBAHA.117.309523.)

Key Words: apolipoprotein A-I ▶ atherosclerosis ▶ cholesterol ▶ high-density lipoprotein ▶ mimetics

Apolipoprotein A-I (apoA-I) is the key structural element of high-density lipoprotein (HDL). While the best studied and probably the most important function of HDL is its role as a specific cholesterol acceptor in the reverse cholesterol transport pathway, HDL has many other functions, including anti-inflammation, antioxidation, antiproliferation, and antithrombosis.1 ApoA-I mimetic peptides were designed to recreate the various beneficial functions of HDL, mostly in the context of protection against atherosclerosis. Most apoA-I mimetics have no homology to the primary structure of apoA-I, instead mimicking a key element of its secondary structure, namely a 22-mer amphipathic α-helix, which is believed to be responsible for lipid-acceptor properties of apoA-I. ApoA-I mimetics have been reported to be effective acceptors of cellular cholesterol and are capable of selectively reproducing functional aspects of HDL in vitro. They have also been shown to be atheroprotective in vivo in animal models (for review see Sviridov and Remaley2). ApoA-I mimetic peptides, thus, offer the opportunity to create new therapeutics with potentially superior properties to that of HDL.

We recently reported on a panel of apoA-I mimetic peptides where we were able to delineate the separate functional properties of each peptide in vitro.3 Some peptides were active as cholesterol acceptors with reduced anti-inflammatory and antioxidation capacity, while others had the inverse properties. In the current study, we tested several peptides from this panel in a mouse model of atherosclerosis to establish if differences in the in vitro capacity of each peptide translates into its corresponding effect in vivo and the ability of this to reduce atherosclerosis.

Materials and Methods
Detailed Methods section is available in the online-only Data Supplement.

Results

Effect of apoA-I Mimetic Peptides on Early Lesion Development

The peptides chosen for study, together with details outlining the functional capacity of each, were previously published3 and are summarized in Table I in the online-only Data Supplement.

Received on: November 28, 2016; final version accepted on: May 5, 2017.

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The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.117.309523/-/DC1.
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Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.117.309523
Supplement. The peptides included ELK-2A2K2E, potent in cholesterol efflux, ELKA-CH2, a selective inhibitor of CD11b expression in macrophages, ELK-2A, a selective inhibitor of VCAM-1 (vascular cell adhesion molecule 1) expression in endothelial cells, and 5A-CH1, which inhibits low-density lipoprotein (LDL) oxidation. In addition, we examined the synergistic effect of ELK-2A2K2E and 5A-C1 (each peptide was added at half the concentration compared with that when tested individually). This peptide combination was selected on the basis of reproducing, at least in vitro, all 4 of the antiatherogenic profiles under study (Figure I in the online-only Data Supplement). Finally, the well-characterized and versatile peptide 5A was also studied under the same conditions.4,5 The effect of each peptide on the development and characteristic of atherosclerotic plaques in apolipoprotein E–deficient mice fed high-fat diet (HFD) was investigated.

In the first set of experiments, the effect of each peptide on the development of early atherosclerotic lesions in apolipoprotein E–deficient mice after 4 weeks of feeding with HFD was examined. En face analysis of the aorta is presented in Figure 1A through 1D and in Figure II A in the online-only Data Supplement. All peptides have significantly reduced the abundance of atherosclerotic lesions to the same extent in total aorta (Figure 1A) and the aortic arch (Figure 1B). Few lesions were found in the thoracic (Figure 1C) or abdominal (Figure 1D) aorta, which were unaltered by any of the peptides. The development of atherosclerosis was then analyzed in sections of the aortic sinus. Again, all peptides reduced the size of atherosclerotic lesions, and the effect was similar for all tested peptides (Figure 1E; Figure IIIA in the online-only Data Supplement).

To characterize the effect of the peptides on the composition of the plaques, aortic sinus sections were analyzed for the abundance of CD68-positive cells (macrophages; Figure 1F; Figure IIII in the online-only Data Supplement), VCAM-1 (soluble and cellular; Figure 1G; Figure IIIIC in the online-only Data Supplement), collagen (Figure 1H; Figure IIIId in the online-only Data Supplement), and nitrotyrosine (Figure 1I; Figure IIIIE in the online-only Data Supplement). None of these were different to the vehicle control. It is important to recognize that parameters of plaque composition are presented relative to the plaque size. Because treatment with the peptides reduced plaque size, the absolute values of plaque composition parameters were also reduced; however, no difference between the effects of different peptides was found (Figure IV in the online-only Data Supplement).

The effect of each peptide on plasma lipoprotein profile was analyzed after 4 weeks of high-fat feeding. HFD caused a sharp elevation in total cholesterol (Figure VA in the online-only Data Supplement) and LDL cholesterol (Figure VB in the online-only Data Supplement) levels, had no effect on triglyceride levels (Figure VC in the online-only Data Supplement), and caused a sharp reduction of HDL cholesterol level (Figure VD in the online-only Data Supplement). None of the peptides when tested 24 hours after the last dose had a major effect on these plasma lipoprotein levels (Figure VA through VD in the online-only Data Supplement). None of the peptides affected the weight of the animals (Figure VE in the online-only Data Supplement).

To assess the effect of the peptides on systemic inflammation, we measured the levels of plasma cytokines after treatment with the peptides (Table II and Figure VI in the online-only Data Supplement). Across the panel of 13 cytokines measured, only elevation in interferon-β and interleukin-27 reached statistical significance, and overall effects were mild, with no consistent difference between the effects of different peptides.

**Effect of apoA-I Mimetic Peptides on Development of Atherosclerotic Plaque**

The potential synergistic effect of 2 peptides, ELK-2A2K2E and 5A-C1, chosen because they reproduced all the key in vitro antiatherogenic properties, was further examined in an extended 12-week study.

Both peptides individually reduced total aorta abundance of atherosclerotic plaques by 30% to 50%, while the peptide combination showed no significant effect (Figure 2A; Figure IIB in the online-only Data Supplement). A similar pattern was observed when regional differences were analyzed (Figure 2B through 2D). Analysis of atherosclerotic plaques in the sections of the aortic sinus confirmed inhibition of the development of atherosclerosis by the individual peptides but not by their combination (Figure 2E; Figure VIIA in the online-only Data Supplement). When plaque composition was analyzed, we found no effect of the individual peptides nor peptide combination on macrophage infiltration (Figure 2F; Figure VIIB in the online-only Data Supplement), abundance of VCAM-1 (Figure 2G; Figure VIIIC in the online-only Data Supplement), collagen (Figure 2H; Figure VIIID in the online-only Data Supplement), or oxidized proteins (Figure 2I; Figure VIIIE in the online-only Data Supplement).

When the effect of the peptides on plasma lipoprotein profile was assessed, elevation of total cholesterol (Figure VIIIA in the online-only Data Supplement), LDL cholesterol (Figure VIIIB in the online-only Data Supplement), and triglycerides (Figure VIIIC in the online-only Data Supplement) caused by 12 weeks of HFD feeding were inhibited by peptide combination, but was unaffected by the individual peptides. The sharp reduction of HDL cholesterol level (Figure VIIID in the online-only Data Supplement) induced by HFD was unaffected by peptide treatment. We found no effect of the peptides on weight of the animals (Figure VIIIE in the online-only Data Supplement).

Finally, we investigated the effect of the peptides on the abundance of immune cells in lymphoid organs. The only effect found was that both individual peptides and the peptide combination caused an increase in the proportion of GR1Lo
monocytes in the blood, with a corresponding reduction in the lymph nodes after 12 weeks (Figure VIIIIF and VIIIG in the online-only Data Supplement).

Pharmacokinetics and Biodistribution of the Peptides

We then examined whether the coadministration of the peptides affected their pharmacokinetics and biodistribution. Peptides ELK-2A2K2E and 5A-C1 were labeled with Alexa 350 and Cascade yellow, respectively, and injected intraperitoneally into apolipoprotein E K/O mice, fed HFD for 2 weeks, either individually or as a 1:1 mixture. The pharmacokinetics of ELK-2A2K2E injected alone was similar to that observed in our previous study6 ($t_{1/2}=2.25$ hours), with 5A-C1 having a longer half-life ($t_{1/2}=3.8$ hours; Figure IXA in the online-only Data Supplement). When the 2 peptides were injected together, the half-life of both peptides was identical ($t_{1/2}=3.8$ hours; Figure IXB in the online-only Data Supplement). Both peptides were stable in vitro at 4°C for at least 7 days.

Next we investigated dynamics of distribution of both peptides among lipoprotein fractions when peptides were injected together. One hour after injection, a relatively large proportion of the peptides was degraded; however, most of the intact ELK-2A2K2E was found as an unbound peptide, with the remainder bound to HDL. Most of 5A-C1 was also found as an unbound peptide, with only minor proportion bound to HDL and LDL fractions (Figure IXC in the online-only Data Supplement). After 2 hours, the distribution of ELK-2A2K2E was mostly unchanged, but relatively more 5A-C1 was found in HDL fraction (Figure IXD in the online-only Data Supplement). After 6 hours, most of the peptides...
were degraded; the intact ELK-2A2K2E was evenly divided between unbound peptide, HDL, and LDL fractions, while intact 5A-C1 remained mainly unbound or in HDL fraction (Figure IXE in the online-only Data Supplement). Thus, despite having been administered together, the 2 peptides distributed between lipoprotein fractions independently of each other.

Functionality of the Peptides in Plasma
We have then compared the cholesterol efflux in vitro to plasma collected from mice treated with peptides in vivo with cholesterol efflux to peptides added in lipid-free form or after adding peptides to hyperlipidemic mouse plasma ex vivo. The capacity of lipid-free peptides to support cholesterol efflux in vitro was consistent with that found in our previous study (Figure XA in the online-only Data Supplement). When the peptides were added to mouse plasma and incubated for 1 hour at 37°C, they statistically significantly, albeit modestly, increased the capacity of the plasma to support cholesterol efflux in vitro (Figure XB and XC in the online-only Data Supplement). Interestingly, the differences between the peptides moderated: the peptides that shown little cholesterol efflux capacity in lipid-free form (5A-C1, ELKA-CH1, and 5A-CH1) were almost as effective as potent peptides when added to plasma. Similar trends were observed when plasma of mice treated with peptides in vivo was used as an acceptor, but the effects weakened, and most of them did not reach statistical significance (Figure XD in the online-only Data Supplement). Thus, supplementation of plasma with peptides both in

Figure 2. The effect of apolipoprotein A-I (apoA-I) mimetic peptides on development of atherosclerotic plaque (12 weeks HFD feeding). The peptides were injected intraperitoneally for 12 weeks into apoE−/− mice fed HFD. The dissected aorta was stained with Sudan IV and analyzed en face for abundance of atherosclerotic lesions in whole aorta (A), aortic arch (B), thoracic aorta (C), and abdominal aorta (D). The heart was dissected and the attached aortic sinus sectioned and stained to examine the lesion size and composition with Oil Red O stain for lesion size (E), for CD68 for macrophage contents (F) anti-whole and soluble VCAM-1 (vascular cell adhesion molecule 1) for lesion inflammation (G), Masson trichrome for collagen contents (H) and for nitrotyrosine (I) to determine oxidative stress of the lesion. Solid lines connect pairs with P<0.05; dashed lines connect pairs with P<0.01.
vitro and in vivo improved plasma cholesterol efflux capacity, but the improvement was modest and similar for all peptides.

Finally, we tested if the peptides are lytic causing hemolysis. No hemolysis of murine red blood cells was caused by any of the peptides added at concentrations up to the maximum level found in plasma (Figure XE in the online-only Data Supplement).

Discussion

ApoA-I mimetic peptides were designed to reproduce key elements of the atheroprotective properties of HDL (such as cholesterol efflux, anti-inflammatory, and antioxidation) and to prevent the development of atherosclerosis in vivo. Because both objectives had been achieved, we sought to define which of those properties, if any, played the larger role. Our major finding was that they were equally potent in preventing the development of atherosclerosis but, more surprisingly, that none of the specific antiatherosclerotic elements observed using in vitro assays were observable in vivo.

There are several ways to interpret this data. It may be that all the elements examined had equal bearing on the ability of each peptide to prevent the development of atherosclerosis. However, given that none of these in vitro elements, on which each peptide was chosen, translated into the corresponding in vivo feature, it is more likely that the atheroprotective effect of these peptides occurs independently of these features by a different mechanism altogether. One such mechanism may be the recently reported atheroprotective effects of apoA-I mimetic peptides that is unrelated to mimicking HDL in the vascular milieu, instead targeting small intestine environment. According to this hypothesis, atheroprotection is achieved through the mitigation of the systemic inflammatory effects of the intestine-derived oxidized lipids or through modulation of intestinal cholesterol efflux. Another possibility is the ability of the peptides to enhance production of natural anti-ox-LDL antibodies. However, it is important to recognize that the peptides tested in this study were not exhaustively characterized in relation to every potential antiatherogenic activity of HDL. For example, the ability of these peptides to stabilize ABCA1 (ATP-binding cassette transported A1) and reduce abundance of lipid rafts or improve vascular reactivity or their potential antiplatelet and antiapoptotic properties were not studied. Furthermore, the differences in key antiatherogenic property of the peptides, enhancing cholesterol efflux, leveled out when peptides were added to plasma ex vivo and in vivo, corresponding to their effect on development of atherosclerosis. This is consistent with findings of Tang et al., indicating that the biological properties of the peptides are affected by their lipidation state.

Another finding of interest was that apoA-I mimetic peptides were more active in reducing the early stages of development of atherosclerosis than in mitigating a developed atherosclerotic plaque. Not only were the overall effects on atherosclerosis more pronounced after 4 weeks compared with 12 weeks of HFD feeding, but the effects were predominantly in thoracic aorta in the longer term study, where initiation of atherosclerosis was delayed when compared with the aortic arch. This suggests that apoA-I mimetic peptides predominantly target early events in the pathogenesis of atherosclerosis, that is, inflammation and accumulation of cholesterol, which is consistent with findings of Wool et al. However, while all the peptides reduced the lesion cholesterol accumulation, no effect on local inflammation was observed. The effects on levels of plasma cytokines were minimal, but increased proportion of anti-inflammatory GR1Lo monocytes in blood and reduced proportion in lymph nodes may be an indirect indication of systemic anti-inflammatory action of the peptides.

We, thus, conclude that while apoA-I mimetic peptides are antiatherogenic, there is a discordance between the in vitro and in vivo functional properties of apoA-I mimetic peptides, which requires us to be cautious in our reliance on some of the standard assay systems commonly used. Determining the various in vitro functional properties of apoA-I mimetic peptide in plasma rather than in the lipid-free state most likely better reflects their physiological effect and better correlates with the observed in vivo effects of the peptides on atherosclerosis. The in vitro assessment of future apoA-I mimetic peptides should, therefore, be based on assays that use plasma as the matrix.

Acknowledgments

We acknowledge the use of facilities of Monash Micro Imaging, AMREP, Victoria, Australia.

Sources of Funding

This study was supported by grants GNT1003106 and GNT1036352 from the National Health and Medical Research Council of Australia.

Disclosures

References


**Highlights**

- Several apolipoprotein A-I mimetic peptides that have different functional properties in vitro were equally effective in reducing atherosclerosis in animal model.
- There is a discordance between the selective antiatherogenic properties of apolipoprotein A-I mimic peptides in vitro and in vivo.
Apolipoprotein A-I Mimetic Peptides: Discordance Between In Vitro and In Vivo Properties—Brief Report

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Arterioscler Thromb Vasc Biol. 2017;37:1301-1306; originally published online May 18, 2017;
doi: 10.1161/ATVBAHA.117.309523

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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SUPPLEMENTAL MATERIALS AND METHODS
Animals studies

6-7 week old B6.129P2-ApoE tm1Unc/JArc (ApoE\(^{-/-}\)) male mice were purchased from Animal Resources Centre (Canning Vale, WA, Australia) and housed at the Alfred Medical Research & Education Precinct animal centre. Male mice were used to enable comparison of the findings with those in our previous studies. At 8 weeks of age mice were placed on a high fat diet (HFD,SF00-219, Specialty Feeds, Glen Forrest, WA) containing 21% fat and 0.15% cholesterol available \textit{ad libitum} and separated into treatment groups (n=10-14 mice per group). Following three days of acclimatization to the diet, the mice commenced treatment three times a week with 30 mg/kg of apoA-I mimetic peptide via intraperitoneal injection. In the combined 5A-C1 and ELK-2A2K2E study, 15 mg/kg of each peptide was delivered. Mice in the control group (n= 13) were injected with the same volume of sterile PBS. In a pilot study we did not find any appreciable changes in myeloid cell numbers in peritoneal cavity after a similar series of intraperitoneal injections of PBS (not shown). After 4 (single peptide) or 12 weeks (combination study) and 24 h post final treatment administration the mice were euthanized by CO\(_2\) inhalation followed by a cervical dislocation. Blood was collected by cardiac puncture, and the vasculature was perfused with PBS containing 1 mM EDTA. The heart was removed for histologic analysis of atherosclerotic plaque development in the aortic sinus region. The aorta was collected from brachiocephalic artery up to the inguinal bifurcation for \textit{en face} analysis. Inguinal lymph nodes and spleen were collected from mice in the 12 week cohort for flow cytometry analysis. All animal experiments were approved by the Animal Ethics Committee of the Alfred Medical Research and Education Precinct and complied with the Australian Code for the Care and use of Animals for Scientific Purposes.

Peptides used in this study were synthesised by Mimotopes (Melbourne, VIC, Australia) and confirmed to be 98% pure. Prior to use, peptides were resuspended in sterile PBS.

Antibodies

Primary antibodies used for immunohistochemistry

1. Polyclonal Rabbit Anti-Nitrotyrosine Antibody (Merck Millipore, #06-284). This antibody was characterized by manufacturer.
2. Monoclonal Rat Anti-Mouse CD106 (VCAM-1) Clone 429 (BD Biosciences, #550547). This antibody characterized in our previous studies.\(^1\,2\)
3. Rat Anti-Mouse CD68 clone FA-11 (Bio-Rad, #MCA1957). This antibody characterized in our previous studies.\(^1\,2\)

Secondary antibodies

2. Biotinylated Rabbit Anti-Rat IgG Antibody, mouse adsorbed (Vector Laboratories #BA-4001).

Control Antibodies:

1. Rabbit IgG (Control Antibody) (Vector Laboratories #I-1000).
2. Rat IgG (Control Antibody) (Vector Laboratories #I-4000).

Antibodies used for flow cytometry

1. Pacific Blue rat anti mouse CD4 clone RM4-5 (BD Biosciences, #558107). This antibody characterized in our previous studies.\(^1\)
2. FITC rat anti-mouse CD206 (MMR) clone MR5D3 (Bio-Rad MCA2235F). This antibody characterized in our previous studies.\(^1\)
3. RPE rat anti-mouse F4/80 Clone CI:A3-1 (Bio-Rad, #MCA497PE). This antibody characterized in our previous studies.\(^1\)
4. PerCP-Cy5.5 rat anti-mouse Ly6G and 6C, clone RB6-8C5 (BD Biosciences, #552093). This antibody characterized in our previous studies. ¹
5. APC rat anti mouse CD11b clone M1/70 (BD Biosciences, #553312). This antibody characterized in our previous studies. ¹
6. APC-H7 rat anti-mouse CD19 clone 1D3 (BD Biosciences, #560143). This antibody characterized in our previous studies. ¹
7. Pe-Cy7 rat anti-mouse CD8a clone 53-6.7 (BD Biosciences, #552877). This antibody characterized in our previous studies. ¹

Histology

For en face analysis, perfused and formalin fixed aortae were stained with Sudan IV and, following removal of stained periaortic fat, imaged and analysed for proportion of stained area using Fiji as described previously. ¹

Aortic sinus sections were prepared as previously described ¹ with minor modifications. Briefly, heart tissue was trimmed and the aortic sinus was embedded in optimal cutting temperature compound (Sakura Finetek, Tokyo, Japan) frozen and cut on 8 µm sections spanning 360 µm of the aortic sinus were collected once all three valves were apparent. Three sections per mouse separated by 120 µm were stained with Oil Red-O or Masson’s trichrome to determine lesion size and lesion collagen content. Lesion macrophage content, oxidative stress and inflammatory state were determined by immunohistochemistry as previously described. ² Staining was analysed with ImagePro Plus and Fiji software packages.

Plasma analysis

Blood was collected every four weeks by sub-mandibular bleeding or cardiac puncture into EDTA containing tubes (BD Pharmingen). Plasma total cholesterol and triglycerides were assessed by commercial colorimetric kits (T-Cho E and TG E kits, Wako Japan). HDL-C levels were assessed following dextran sulphate MG2 precipitation.

Plasma cytokine levels were assessed with a commercial multiplex bead immune assay kit (LEGENDplex Mouse Inflammation panel, Biolegend) as per the manufacturer’s instructions and analysed on FACSCantoII (BD Pharmingen).

The capacity of plasma from treated mice to accept cholesterol was examined in [³H]-cholesterol labelled, TO-901317 activated RAW 264.7 cells as described previously ². Individual plasmas for each treatment were combined and the labelled cells were incubated for 4 hours in presence of 1% plasma. This was compared to the ability of plasma from ApoE⁻/⁻ mice fed HFD for 4 weeks incubated with apoA-I mimetic peptides for 1 hour at 37°C (1:1 plasma and 1mg/ml peptides in sterile PBS) to accept cholesterol when added at a final concentration of 1% plasma and 10 µg/ml peptide.

Peptide pharmacokinetics

ApoE⁻/⁻ mice fed HFD for 2 weeks were injected intraperitoneally with 1 mg fluorescently labelled peptides (Alexa 350 ELK-2A2K2E and Cascade Yellow 5A-C1) alone or in a 1:1 combination as previously described. ² Peptide plasma concentration was determined on Viktor plate reader for each time point by comparing fluorescence in plasma to a standard curve with known peptide concentrations. 20 µl of plasma was separated by FPLC using two consecutive Superose 6 PC3.2/30 columns (GE Healthcare) to elucidate peptide distribution in lipoprotein fractions.
**Immune cell quantitation**

Inguinal lymph nodes, spleen and blood from 12 week old mice were analysed for immune cell differences as previously described. 1 Briefly, single cell suspensions created with gentleMACs were labelled with anti-CD19 (1D3), -CD4 (RM4-5), -CD8a (53-6.7), -Gr-1 (RB6-8C5), -CD11b (M1/70), and F4/80 and analysed with Weasel software (WEHI) following data collection on FACSCanto II (BD).

**Assessment of peptide functionality in vitro**

Properties of the individual peptides and peptide combination *in vitro* were assessed as previously described. 3 Briefly, cholesterol efflux was measured using [3H]-cholesterol labelled, TO-901317 activated RAW 264.7 cells for 4 hours in the presence of 10 µg of the individual peptides or ELK-2A2K2E / 5A-C1 combination (5 µg each). CD11b expression was measured by flow cytometry on FACSCalibur using anti-CD11b (BD Pharmingen) in resting human monocytes activated with PMA in the presence of 40 µg/ml of HDL or 40 µg/ml of 1:1 combination of ELK-2A2K2E and 5A-C1. Effect of peptide on expression of VCAM-1 in endothelial cells was assessed using stably transfected SVEC4-10 cells with luciferase under VCAM-1 promoter 3 that were incubated with 0.75 mg/ml of ELK-2A2K2E, 5A-C1 or 1:1 combination of the two peptides. LDL oxidation by CuSO4 was measured in presence of 100 µg/ml ELK-2A2K2E, 5A-C1 or 1:1 combination of the two peptides as previously described. 3 Murine red blood cell lysis assay was carried out as described previously with minor modifications. 4 A percentage of haemolysis was calculated relative to complete lysis control (1% Triton X-100) following subtraction of a PBS only negative control.

**Statistics**

Mean ± SEM are shown. Statistical significance of differences between groups was assessed with ANOVA; post hoc analysis was performed with Tukey’s test.

**References**

SUPPLEMENTAL TABLES AND FIGURES
Table I. Anti-atherogenic properties of the peptides *in vitro*

- inactive, +, ++, +++; low, medium or high levels of activity

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Cholesterol efflux from macrophages</th>
<th>Inhibition of CD11b expression on monocytes</th>
<th>Inhibition of VCAM-1 expression on EC</th>
<th>Inhibition of LDL oxidation</th>
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<tr>
<td>ELK-2A2K2E*</td>
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<tr>
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<td>+</td>
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*Data derived from findings presented in reference 3.
†Data derived from findings presented in Supplemental Fig. I.
Table II. Effect of 4-weeks peptide treatment on plasma cytokine levels

<table>
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<tr>
<th>Cytokine</th>
<th>Vehicle</th>
<th>5A</th>
<th>ELK-2A2K2E</th>
<th>5A-C1</th>
<th>ELK-2A2K2E+5A-C1</th>
<th>ELKA-CH1</th>
<th>ELK-2A</th>
<th>5A-CH1</th>
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<tr>
<td>IL-23 (pg/ml)</td>
<td>53.22 ± 2.76</td>
<td>261.82 ± 69.08</td>
<td>116.82 ± 43.75</td>
<td>391.48 ± 178.68</td>
<td>103.22 ± 54.39</td>
<td>223.80 ± 66.17</td>
<td>342.77 ± 116.48</td>
<td>401.67 ± 133.16</td>
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<td>IL-1α (pg/ml)</td>
<td>8.00 ± 1.00</td>
<td>15.75 ± 2.39</td>
<td>8.52 ± 1.32</td>
<td>10.22 ± 1.54</td>
<td>12.05 ± 3.42</td>
<td>9.88 ± 1.46</td>
<td>14.93 ± 2.05</td>
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<td>IFN-γ (pg/ml)</td>
<td>6.06 ± 0.65</td>
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<td>17.91 ± 6.01</td>
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<td>TNF-α (pg/ml)</td>
<td>10.48 ± 0.54</td>
<td>13.15 ± 1.96</td>
<td>10.74 ± 0.97</td>
<td>11.10 ± 0.95</td>
<td>32.87 ± 23.10</td>
<td>11.65 ± 1.20</td>
<td>16.07 ± 2.15</td>
<td>12.68 ± 1.90</td>
</tr>
<tr>
<td>MCP-1 (pg/ml)</td>
<td>12.95 ± 2.09</td>
<td>17.88 ± 3.41</td>
<td>12.31 ± 2.26</td>
<td>13.69 ± 2.34</td>
<td>27.91 ± 17.93</td>
<td>13.83 ± 1.95</td>
<td>19.77 ± 3.02</td>
<td>17.68 ± 3.32</td>
</tr>
<tr>
<td>IL-12p70 (pg/ml)</td>
<td>3.70 ± 0.22</td>
<td>9.81 ± 2.51</td>
<td>4.42 ± 0.67</td>
<td>5.84 ± 1.60</td>
<td>5.95 ± 1.99</td>
<td>8.19 ± 1.76</td>
<td>8.23 ± 1.72</td>
<td>9.21 ± 3.66</td>
</tr>
<tr>
<td>IL-1α (pg/ml)</td>
<td>30.29 ± 1.02</td>
<td>55.94 ± 12.44</td>
<td>50.61 ± 15.77</td>
<td>73.31 ± 26.09</td>
<td>71.72 ± 28.53</td>
<td>49.69 ± 14.81</td>
<td>71.41 ± 15.35</td>
<td>37.00 ± 3.05</td>
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<tr>
<td>IL-10 (pg/ml)</td>
<td>157.23 ± 19.58</td>
<td>355 ± 83.44</td>
<td>318.27 ± 75.18</td>
<td>249.77 ± 62.53</td>
<td>216.61 ± 57.69</td>
<td>240.98 ± 45.34</td>
<td>333.9 ± 76.13</td>
<td>300.21 ± 53.62</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>4.65 ± 0.5</td>
<td>10.34 ± 2.37</td>
<td>8.84 ± 3.30</td>
<td>12.35 ± 5.65</td>
<td>14.62 ± 7.02</td>
<td>7.49 ± 2.47</td>
<td>11.06 ± 3.05</td>
<td>5.91 ± 0.69</td>
</tr>
<tr>
<td>IL-27 (pg/ml)</td>
<td>458.57 ± 124.20</td>
<td>1595.89 ± 531.88**</td>
<td>927.24 ± 369.21</td>
<td>1551.31 ± 658.89**</td>
<td>814.07 ± 333.50†</td>
<td>691.92 ± 177.07†</td>
<td>1370.75 ± 633.25**</td>
<td>837.27 ± 148.51†</td>
</tr>
<tr>
<td>IL-17A (pg/ml)</td>
<td>8.05 ± 1.54</td>
<td>27.00 ± 8.30</td>
<td>13.57 ± 3.51</td>
<td>75.64 ± 55.06</td>
<td>46.6 ± 25.81</td>
<td>20.81 ± 7.29</td>
<td>27.55 ± 6.16</td>
<td>14.67 ± 2.91</td>
</tr>
<tr>
<td>IFN-β (pg/ml)</td>
<td>725.56 ± 346.23</td>
<td>1398.61 ± 424.03*</td>
<td>1157.65 ± 458.75</td>
<td>1246.15 ± 409.40</td>
<td>1137.20 ± 680.83</td>
<td>1138.70 ± 431.56**</td>
<td>1609.33 ± 439.17</td>
<td>977.57 ± 283.20</td>
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<tr>
<td>GM-CSF (pg/ml)</td>
<td>10.72 ± 0.48</td>
<td>27.59 ± 7.98</td>
<td>20.70 ± 7.38</td>
<td>34.96 ± 13.69</td>
<td>24.22 ± 10.92</td>
<td>23.54 ± 6.85</td>
<td>29.27 ± 8.43</td>
<td>19.77 ± 3.61</td>
</tr>
</tbody>
</table>

Data shown as mean ± SEM. * p<0.05, ** p<0.01 versus vehicle. † p<0.05 versus 5A, ‡ p<0.05 versus 5A-C1
Supplemental Figure I. The effect of combination of the peptides on anti-atherogenic properties in vitro.

In “combination” the peptides were added at proportion 1:1 (w/w); each peptide was added at half the concentration compared to that when tested individually.  

A – Cholesterol efflux from RAW 264.7 cells. Peptide concentration 10 µg/ml; B – VCAM-1 expression in endothelial cells. Peptide concentration 0.75 mg/ml; C – LDL oxidation. Peptide concentration 100 µg/ml; D – CD11b expression in human monocytes. Peptide concentration 40 µg/ml. Methodology of each of the assay is described in the Materials and Method section. Dashed lines connect pairs with p<0.01.
Supplemental Figure II. Representative *en face* images of the aortae of mice treated with the peptides.

A- Early lesions from 4 weeks study. B – Atherosclerotic lesions from 12 weeks study. The background around the vessels was replaced with uniform black to assist with visual assessment.
Supplemental Figure III. Morphology and immunostaining of aortic sinus sections after 4 weeks treatment with the peptides.
Supplemental Figure IV: Absolute values of the abundance of CD68+ cells (A), VCAM-1 (B), collagen (C) and nitrotyrosine (D) in early lesions; the effect of apoA-I mimetic peptides.

Solid lines connect pairs with \( p < 0.05 \); dashed lines connect pairs with \( p < 0.01 \).
Supplemental Figure V. Plasma lipoprotein concentration and body weight after 4 weeks treatment with the peptides.

Effect of 4 week HFD feeding and peptide treatment on total cholesterol (A), LDL-C (B), triglycerides (C), HDL-C (D) and weight (E). Lipoprotein concentrations were determined as described in Materials and Methods section. Dashed lines connect pairs with p<0.01. “Vehicle T0” refers to time-point prior to commencement of HFD.
Supplemental Figure VI. Heat Diagram of the effect of 4-weeks peptide treatment on plasma cytokine levels.

This figure is representation of the data shown in Table II.
Supplemental Figure VII. Morphology and immunostaining of aortic sinus sections after 12 weeks treatment with the peptides.
Supplemental Figure VIII. Plasma lipoprotein concentration and body weight after 12 weeks HFD feeding and treatment with the peptides (A-E). Proportion of GR-Lo monocytes in blood and lymph nodes (F,G).

A – total cholesterol; B – LDL-C; C – triglycerides; D – HDL-C; E – body weight; F - GR1lo monocytes in blood; G – GR1lo monocytes in lymph nodes. Lipoprotein concentrations were determined and immune cells quantitated as described in Materials and Methods section. Solid lines connect pairs with p<0.05, dashed lines connect pairs with p<0.01. “Vehicle T0” refers to time-point prior to commencement of HFD.
Supplemental Figure IX. Pharmacokinetics and biodistribution of the peptides

A, B - 1mg of fluorescently labelled 5A-C1 (green) and ELK-2A2K2E (red) were administered one at a time (A) or as 1:1 combination (B) via intraperitoneal injection to ApoE⁻/⁻ mice fed HFD for 2 weeks. Vertical dashed lines indicate the calculated half-life of each peptide in plasma.

C-E – To elucidate peptide distribution in lipoprotein fractions plasma was separated by FPLC and individual fractions analysed for fluorescence. Fluorescently labelled peptide lipoprotein distribution one (C), two (D) and six (E) hours post injection are shown.
**Supplemental Figure X. Functionality of the peptides in plasma.**

A – Cholesterol efflux to the lipid-free peptides. B – Cholesterol efflux to the peptides added to mouse plasma in vitro and incubated for 1 h prior to the efflux assay. C – Values of cholesterol efflux to the peptides added to mouse plasma after subtraction of the efflux to plasma without peptides. D – Cholesterol efflux to plasma from mice treated with the peptides. E – Haemolysis following a 2 hour incubation of murine RBC with peptides.