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 *ad libitum* and separated into treatment groups (n=10-14 mice per group). Following three days of acclimatisation 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 *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

1. Biotinylated Goat anti-Rabbit IgG (H+L) Secondary Antibody (ThermoFisher Scientific #A16114).
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 Cl: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 Fintek, 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 [^{3}H]-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 [^{3}H]-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 $CuSO_4$ 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

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