2. Expanded materials and methods (e-component)

2.1 Animals, diet and A-II infusion

Forty male LDL receptor-deficient ($LDLR^{-/-}$) in the C57BL/6J genetic background, were obtained from The Jackson Laboratory (Stock number 002207) at 7 weeks of age. After acclimatizing for one week, all the mice were fed a Western-type high-fat diet (0.15% cholesterol, 21% milkfat by weight, 42% calories from fat, TD 88137, Harlan Teklad) [1], with food and water available ad libitum, and with animal care given in accordance with institutional guidelines.

After 4 weeks on the diet, mice were implanted with Alzet osmotic minipumps (Model 2004, Durect Corporation), containing either saline vehicle (0.15 mol/L NaCl, 0.01 N acetic acid)(16 mice) or A-II (catalogue No. A9525, Sigma-Aldrich Corp.) dissolved in saline vehicle to deliver ~ 620 ng • min^{-1} • kg^{-1} (24 mice). These pumps were replaced after 4 weeks, giving totals of 11 weeks on the diet and 7 weeks of infusion before termination of the experiment. Pumps were placed into the dorsal subcutaneous space of anesthetized mice (100 mg/kg ketamine plus 5 mg/kg diazepam) through a small incision on the upper back that was closed with 2 or 3 absorbable sutures and healed quickly.

To create $LDLR^{-/-}$ / $IL\text{-}6^{-/-}$ double-null mice, $IL\text{-}6^{-/-}$ mice were obtained from The Jackson Laboratory (Stock number 002650) and bred with $LDLR^{-/-}$ mice. F2 generation doubly-null mice of both were identified viable at expected Mendelian ratios by PCR genotyping of tail-snip DNAs (standard methods and per Jackson Lab 3 primer genotyping protocols)

2.2 Plasma lipid determinations

At time of first A-II pump implantation and at time of sacrifice, mice were anesthetized (as above), 0.2 mL blood samples were taken from the retroorbital venous plexus. Serum total cholesterol and triglyceride levels were measured using the Total Cholesterol E and Triglyceride TGH R1 and R2 colorimetric reagent kits (as directed, Wako Pure Chemical Industries, Ltd.) along with standards and lipid calibrator (Wako) and cholesterol reference kits (Verichem Laboratories, Inc.)

2.3 Tissue harvest and quantification of atherosclerotic lesions

Methods for aorta preparation and *en face* morphometry to quantitate lesions were modified from established techniques [2]. The heart with attached aorta (extending to the iliac bifurcation) was carefully dissected free, fixed in 10% phosphate-buffered formalin for 24 hours at 4°C, and stored in PBS plus 0.1% NaN_3 at 4°C. Aortae were fixed, cleaned, stained with oil red O (Sigma) to visualize intimal fatty lesions from the proximal arch to the iliac bifurcation, and flattened onto slides [3,4]. Photomicroscopy images (Nikon D-1 camera with a 105 mm Micro-Nikkor lens) were exported into Photoshop 6 (Adobe), atherosclerotic lesion areas were selected by contrast differences, and images were uploaded into the ScionImage (National Institutes of Health/Scion Corp) software for measurement of lesion areas. Total atherosclerotic lesion area (mm^2) is expressed as percent positive staining relative to total aortic area.

2.4 Ex vivo aorta organ culture and cytokine secretion assays

At time of sacrifice and before fixing for oil red O staining, freshly dissected aortae (proximal arch to iliac bifurcation) were placed in 1 mL of Dulbecco's modified Eagle's medium containing 1X ITS (insulin, transferrin and selenium (both, Invitrogen Life Technologies, [GIBCO]) plus 0.1% bovine serum albumin (Sigma) [5]. Explanted aortae were incubated for 4 h at 37°C in a 5% CO_2 incubator.

Duplicate samples of secreted cytokines in 50 μL of explant medium were assayed using a Luminex platform-based sandwich ELISA multiplex bead-array system (Bio-Plex Suspension Array System, Bio-Rad Laboratories, Inc.) with mouse cytokine panel reagent kits (18-plex or 19-plex; Bio-Rad or BioSource International Inc., respectively). Cytokine measurements were normalized to a unique set of standards to minimize inter-assay variation, and results are reported as pg/mL.

2.5 Preparation of aortic cross sections

At time of sacrifice, anterior portions of hearts with proximal aorta were carefully dissected free, OCT-embedded, flash-frozen in liquid nitrogen, and stored at – 80 °C. Aortic cryosections (transverse, 6 μm thick) on slides were prepared as previously described [6]. Slides were fixed in acetone:methanol (1:1) for 10 minutes and dried for *in situ* hybridization and AB-staining procedures.

2.6 In Situ hybridization of IL-6 mRNA in aortic cross sections

Probe generation: First-strand cDNA synthesis was performed using 5 μg of total mouse liver RNA, random nonamers (Sigma) and SuperScript II reverse transcriptase (Life Technologies, as directed). This cDNA was then used as template to PCR amplify a 904 bp mouse IL-6 gene fragment (GenBank accession no. **NM031168**) using forward and reverse primers, 5'-CCTTCTTGGGACTGATGCTG and 5'-CCAAGAAACCATCTGGCTAGG, designed using the Primer3 software (Steve Rozen, Whitehead Institute for Biomedical Research) and standard techniques. The IL-6 fragment was gel purified, cloned into plasmid pCR II-TOPO (Invitrogen Life Technologies, as directed) and the resulting construct (pCRII-TOPO-IL-6) DNA-sequenced (UTMB Genomics Core) for insert orientation. Bacteriophage SP6 and T7 promoter sites flanking IL-6 in this construct were then used in *in vitro* transcription reactions to run off sense (negative control) and antisense digooxigenin-labeled cRNAs to be used as

probes for *in situ* hybridizations with colorimetric detection (DIG RNA Labeling and Colorimetric Detection Kits, Roche Applied Science, as directed). Color photomicroscopy was performed with a Nikon Microphot – FXA System.

2.7 Immunohistochemistry of aortic cross sections

Aortic cryosection slides were prepared as described above and blocked with 2% normal IgG (same species as source of secondary antibody (Ab) for 2 h at room temperature. For immunofluorescent microscopy, slides were incubated with primary antibodies to mouse fibroblasts (1:50, Acris Antibodies GmbH, BM4018), activated macrophages (1:100, Abcam Inc., anti-F4/80, ab16911) or IL-6 (1:100, Santa Cruz Biotechnology, Inc., sc-1265), followed by FITC (fluorescein isothiocyanate)- or Texas Red-conjugated secondary antibodies (1:100, Santa Cruz; Abcam). Cross sections were examined and photographed with a Nikon Eclipse TE 300 inverted UV microscope fitted with a Photometrix CoolSNAP Fx digital camera interfaced with MetaMorph software (Universal Imaging Corp., Downingtown, PA). To qualitatively enable consistent immunofluorescent exposures for each group of Ab-fluorophore exposures, a relatively high signal intensity field was selected for auto exposure with MetaMorph, and then the rest of the photos for that "antibody set" were taken at that same exposure (usually 700 to 1000 ms using the 60X oil immersion microscope lens). Phospho-STAT3 was detected with primary Ab (Santa Cruz, B7, sc-8059) followed by "ABC" staining using a Mouse on Mouse Peroxidase kit (Vector Laboratories). Color photomicroscopy was as above for *in situ* hybridizations.

2.8 Data analysis

Data are reported as mean ± standard deviation (SD). Differences were analyzed by ANOVA (single factor or two-factor with replication) or by Student's t-test (two-sample, two-tail, assuming unequal variances). Values of $P<0.05$ were considered significant.

References

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