Materials and Methods

Peripheral Blood Mononuclear Cell (PBMC) Isolation from Blood

Peripheral blood from mice was collected by cardiac puncture in presence of heparin as anticoagulant. Blood (~ 1ml per mouse) was pooled according with experimental requirements and diluted with Dulbecco's Phosphate Buffered Saline (PBS) without calcium and magnesium (Lonza) 1x (ratio 1:1). The diluted samples were subjected to density gradient separation on Ficoll Paque Plus (ratio 1:1) (GE Healthcare Life Sciences, Buckinghamshire, UK) and centrifuged. After centrifugation the PBMC layer was collected and washed in Hank’s Balanced Salt Sodium (HBSS) with phenol red without calcium and magnesium (Lonza).

Monocyte Isolation and Macrophage maturation

PBMC were re-suspended in RPMI 1640 supplemented with Gentamicin (8μg/ml), Penicillin and Streptomycin (100 UI/ml), 1% L-Glutamine and 10% Foetal Calf Serum FCS (referred to here after as RPMI/FCS media) and placed in 6 well culture plates (surface area 5x10^5 per well), in 12 well culture plates (2.5x10^5 per well) or in 8-well multi-chamber Millicell® EZ slides (Millipore, Watford, UK) (5x10^4 per well) depending on the experimental protocol. After 2 hours, cells were washed in RPMI/FCS media to remove non-adherent cells and incubated at 37 °C at 5% CO₂. Alternatively, mouse monocytes were positively selected from PBMC preparations using magnetic beads conjugated with a mouse antibody for the monocyte/macrophage cell marker, CD11b (Miltenyi Biotec, Surrey, UK). After density gradient isolation, PBMC were re-suspended in 90μl of Macs Rinsing buffer (PBS pH 7.2, 0.5% bovine serum albumin (BSA), 2mM EDTA) (Miltenyi Biotec, Surrey, UK) and 10μl of rat anti-mouse CD11b MicroBeads (Miltenyi Biotec, Surrey, UK ) were added to each sample (per 10^7 total cells). Cells were gently mixed and incubated for 20 minutes on ice. Subsequently samples were washed in Macs Rinsing buffer (Miltenyi Biotec, Surrey, UK)
and magnetically sorted using MS Columns (Miltenyi Biotec, Surrey, UK) according to the manufacturer’s instructions. The CD11b selected monocyte suspension was spun and the pellet re-suspended in fresh media, counted and treated according to the experimental plan. To differentiate blood-derived monocytes into macrophages, monocytes were cultured for 6-7 days with RPMI/FCS plus 20ng/µl recombinant mouse M-CSF or GM-CSF (Miltenyi Biotec, Surrey, UK) at 37 °C.

**RNA Extraction and Reverse Transcription-PCR (RT-PCR)**

The Qiagen miRNeasy kit was used for micro RNA extraction (Qiagen Ltd, Crawley West Sussex, UK) according with manufacturer protocol. Recover-All kit (Ambion) was used for RNA isolation from paraffin-embedded sections according with the product data sheet. RNA samples were quantified with a NanoDrop ND-1000 spectrophotometer (LabTech International Ringmer, East Sussex, UK). miScript Reverse Transcription Kit (Qiagen Ltd, Crawley West Sussex, UK) was used to obtain equal amounts of cDNA from RNA samples including microRNA, sample preparation and reaction mix was performed in accordance with the manufacturer's instructions. Samples were incubated first at 42°C for 30 minutes then at 95°C for 3 minutes. The cDNA obtained was stored at -80°C.

**Quantitative PCR (Q-PCR)**

QuantiTect SYBR Green PCR Kit (for mRNA detection) or miScript SYBR Green PCR Kit (for microRNA detection) (Qiagen Ltd, Crawley West Sussex, UK) were used to carry out quantitative PCR using a Roche LightCycler 1.5 (Roche). 8ng or 1ng of cDNA sample was used to amplify messenger RNA or microRNA respectively in accordance with the manufacturer’s instructions. All primers (except microRNA primers) were home designed and ordered from Sigma (see Table I). GAPDH, 36B4 and β-actin were used as house-keeping genes. MicroRNA detection by Q-PCR was performed using commercial primers.
from the Qiagen miScript range (Qiagen Ltd, Crawley West Sussex, UK). Scarna17 was used as a house-keeping miR.

**Western Blotting**

Adherent cells were washed with PBS on ice, and then lysed with ice-cold 1% SDS lysis buffer. The Bicinchoninic acid (BCA) kit (Pierce) was used to estimate protein concentration (all in duplicate) of cell lysates according to kit protocol. Protein concentration was read at 560 nm on a Multiskan Ascent (Thermo Electron Corporation) plate reader.

NuPAGE Novex Bis-Tris Mini Gel system (Invitrogen, Life Technologies, Paisley, UK) was used for western blot experiments. 10µg of total protein was loaded on 4-12% Bis-Tris-HCl buffered (pH 6.4) polyacrylamide precast gels (Invitrogen, Life Tecnologies, Paisley, UK) and run in MES-SDS Running Buffer 1x (Invitrogen, Life Tecnologies, Paisley, UK) according to manufacturer instructions. After electrophoresis, gels were transferred to a 0.45µm nitrocellulose membrane in NOVEX NuPAGE Transfer Buffer 1x plus 5% Methanol (v/v). Following transfer, the nitrocellulose membrane was blocked in 5% Milk Solution in Tris buffered solution containing Tween (TBS-T) (200 mM Tris, 2% Tween 20, pH 7.6). The membrane was then incubated over night at 4°C with rabbit polyclonal MMP-14 primary antibody (Chemicon) diluted in 2 ml of SignalBoost™ Solution 1 (Merck Millipore, Watford, UK). The following day the membrane was incubated with 5% Milk solution in TBS-T and then with the anti-rabbit Horseradish Peroxidase (HPR)-labelled secondary antibody (Dako, Dorset, UK) diluted 1:1000 in SignalBoost™ Solution 2 (Calbiochem-Novabiochem Ltd, Nottingham, UK). After incubation the membrane was washed in TBS-T. To detect the peroxidase labelled proteins, Luminata™ Forte Western HRP substrate Chemiluminescence detection system (Merck Millipore, Watford, UK) was used. The membrane was incubated with HRP substrate and exposed to X-ray film for the desired length of time. B-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as loading controls.
Detected bands were quantified using a Bio-Rad GS-690 scanning densitometer (Bio-Rad, Hemel Hempstead, UK) and were normalised by GAPDH and/or β-actin values.

**Immunocytochemistry (ICC)**

After density gradient separation with Ficoll and subsequent CD11b magnetic-bead isolation (Miltenyi Biotec, Surrey, UK), positively selected monocyte/macrophages were either re-suspended in PBS and adhered to coated slides with Cytospin2 Cytocentrifuge (Shandon), or re-suspended in RPMI/FCS and placed in multi-chamber slides (Millipore, Watford, UK) and cultured as according to the experimental plan. For immunocytochemical analysis, cells were fixed using freshly prepared 3% paraformaldehyde to each slide/well for 10 minutes at room temperature. Cells were then permeabilised in 0.1% PBS/Triton (3x5 minutes) and then incubated in 1% Bovine Serum Albumin (BSA) (75 µl/slide and 100 µl/well) for 30 minutes at room temperature. Serum was then removed and samples incubated over night at 4°C with rabbit polyclonal MMP-14 primary antibody (Abcam) (50 µl/slide and 100 µl/well). A negative control where the primary antibody was replaced with the relevant species IgG at the same dilution was always included. The next day samples were washed in PBS and incubated with the appropriate fluorescent-conjugated secondary antibody for 1 hour at room temperature in the dark. For multi-chamber slides, the chamber and gel surround were removed, and all slides were mounted with large glass coverslips using ProLong® Gold antifade reagent containing DAPI (Invitrogen, Life Technologies, Paisley, UK). Positive cells were counted in 4 x20 magnification fields and expressed as a percentage of total nucleated cells.

**In Vitro Invasion Assay**

Monocyte/macrophage invasion in vitro was assessed using Matrigel™-coated transwell inserts (Merck Millipore, Watford, UK) as described previously [1]. Transwell inserts containing 8µm pore membranes were coated with 25µl/well Basement Membrane Matrix
Lactose-Dehydrogenase-Elevating-Virus (LDEV)-Free (Matrigel) (BD Biosciences, Oxford, UK). 15ng/ml MMP-14 blocking antibody (BAb) (Millipore) or mouse IgG (15ng/ml) was added to the Matrigel of the appropriate inserts. Monocyte-derived macrophages (detached by trypsin) were re-suspended in RPMI/FCS (100μL; 1 x 10^5 cells) and then added to the upper portion of the transwell. RPMI/FCS was supplemented with 100ng/mL M-CSF or GM-CSF, respectively. RPMI/FCS (600 μL) supplemented with 30ng/mL of mouse recombinant monocyte chemoattractant protein-1 (MCP-1) and 30ng/mL of mouse recombinant Fractalkine (CX3CL1) (R&D System, Abingdon, UK) was placed in the lower wells to induce transmigration/invasion. Transwells were incubated for 48 hours and then cells on both the upper and lower surface of the membrane fixed with 3% paraformaldehyde in PBS, stained with haematoxylin and mounted with polyvinyl pyrrolidone (PVP). Cells were counted in 6 x20 magnification fields, and the number of migrated/invaded cells expressed as a percentage of total cells.

**Luciferase Reporter Assay**

Hela cells were cultured in RPMI-1640 10% FCS. The day before transfection cells were detached with trypsin and seeded 1.5x10^5 cells per well in 6 well plates. 18 hours post seeding the media was changed to serum/antibiotic-free RPMI 1640 with L-Glutamine. 4 hours after the cells were transfected with Lipofectamine 2000 (Invitrogen, Life Tecnologies, Paisley, UK) vector. A transfection mix containing Lipofectamine 2000 (1:500 ratio) in serum/antibiotic-free RPMI 1640 with L-Glutamine, 25ng Renilla and 500ng Luciferase reporter plasmids were added to the appropriate samples. Cells were incubated for 4 hours at 37°C and then RPMI/FCS added to all relevant samples. 48 hours after transfection samples were collected in Dual-luciferase Assay Lysis buffer (Promega). Samples were prepared and quantified in accordance with the kit datasheet.

**Macrophage transfection miR-24 inhibitor and mimic**
To facilitate efficient macrophage transfection, we utilised a recently published macrophage-specific RVG-9dR Peptide Transfection Vector [2]. This peptide, a short AchR-binding peptide derived from the rabies virus glycoprotein (RVG), is fused to nona-d-arginine residues (RVG-9dR) to enable siRNA binding. It has been demonstrated to effectively deliver nucleic acids to macrophages through binding to AchR. Accordingly we used the RVG-9dR peptide to deliver siRNA to monocyte/macrophages. M-CSF or GM-CSF differentiated macrophages (7 days in culture) at 40-60% confluence, were transfected with 0.1nM small interfering (si)- micro RNA (miR)-24 (Qiagen Ltd, Crawley West Sussex, UK) or 10nM pre-mir-24 (Ambion, Applied Biosystem, Warrington, UK). Appropriate negative controls were also utilised (0.1 nM All Stars Negative Control siRNA or 10 nM negative control pre-miR; Qiagen and Ambion respectively). 4 hours post-transfection in serum free antibiotic free RPMI, the media was changed and fresh RPMI/FCS was added to each sample. The cells were then cultured for at least 24 hours before collection.

**Sponge Implantation and macrophage/ foam cell macrophage isolation**

In order to isolate in vivo generated mouse macrophages and foam-cell macrophages, we have combined two previously published granuloma models [3, 4]. Mice were anaesthetised by inhalation with isofluorane and 2-6 Matrigel™ (BD Biosciences, Oxford, UK) infused 1cm³ polyurethane sponges (Baxter Scientific, Newbury, Berkshire, UK) placed under the dorsal skin for 11 days, and mice fed normal diet to accumulate monocyte/macrophages or high fat diet to accumulate foam cell macrophages (FCM). After 11 days, mice were terminated and the sponges retrieved and either fixed in 10% formalin for histological analyses, or monocyte/macrophages isolated as described below. Fixed sponges were processed and wax-embedded before eight 3 µm sections were taken and subjected to immunohistochemistry for cell markers as deemed necessary. To isolate monocyte/macrophages, the recovered sponges were gently squeezed over sterile test tubes and the exudates subjected to density gradient separation on Ficoll Paque Plus (ratio 1:1) (GE Healthcare Life Sciences, Buckinghamshire, UK) as described above. One mouse
yielded approximately $1.4 \times 10^6$ monocyte/macrophages. Monocyte/macrophages were then selected by adhesion (60 minutes at 37°C) and MMP-14 positive and negative foam cell macrophages sorted by magnetic-bead separation, as described above.

**In vivo monocyte/macrophage invasion assay**

Ten week old, chow fed, male Apoe KO mice (n=15) were anaesthetised by inhalation with isofluorane and Matrigel™ (BD Biosciences, Oxford, UK) infused sponges placed under the dorsal skin for 11 days to accumulate monocyte/macrophages as previously described [4]. Mice were split into three groups (n=5 per group); control group receiving sponges infused with Matrigel alone; si-miR-24 group receiving sponges infused with Matrigel plus a miR-24 inhibitor (0.1nM); or pre-miR-24 group receiving sponges infused with Matrigel plus an exogenous miR-24 mimetic (10nM). Mice were then returned to a chow diet for a further 11 days, and then terminated and the sponges retrieved and either monocyte/macrophages isolated as described above, or the sponges fixed in 10% formalin for histological analysis. Fixed sponges were then processed and wax-embedded before eight 3µm sections were taken and subjected to immunohistochemistry for macrophages (Mac-3), MMP-14, PCNA to detect proliferative rates and cleaved caspase-3 to determine apoptotic frequencies.

**Flow Cytometry**

After appropriate washing and blocking with Fc block TruStain fcX anti-mouse CD16/32 (BioLegend), $1 \times 10^6$ cells were stained for 30 minutes on ice with appropriate antibody. According with experimental plans cells have been stained with: eFluor 450-conjugated anti-mouse CD11b (eBioscence), APC-coniugated anti-mouse F480 (eBioscience), PE-conjugated rabbit polyclonal MMP-14 antibody (Bioss), Alexa Fluor 647-conjugated anti-mouse Ly6G/Ly6C (Gr-1) (BioLegend). Flow-cytometry was performed on a LSRII (BD-Bioscience) and the data analysed using Flowjo software.

**Immunohistochemistry**
Sections were de-waxed in Clearene™ and rehydrated in absolute alcohol. After rinsing in distilled water, slides were subjected to heat-induced antigen retrieval by incubation in citrate buffer (10mM citric acid in 1x PBS, pH 6.0) and microwaved for 10 minutes at full power. Slides were allowed to cool for 30 minutes and then washed with PBS (3 x 2 minutes). 50µl of Image-iT FX Signal Enhancer (Invitrogen, Life Technologies, Paisley, UK) was placed on sections and incubated at room temperature for 30 minutes. The solution was then removed by tapping the slides and 50µl of the appropriate primary antibody (see Table II) added to the samples and incubated either overnight at 4°C or for 1 hour at room temperature. Sections were then washed in PBS (3 x 2 minutes each) and incubated in the dark with either Alexa-Fluor 488 conjugated or Alexa-Fluor 594 conjugated secondary antibody (Invitrogen, Life Technologies, Paisley, UK) for 1 hour at room temperature. Sections were then washed in PBS (3 x 2 minutes) and mounted with ProLong® Gold antifade reagent containing DAPI (Invitrogen, Life Technologies, Paisley, UK) to identify the nuclei. A negative control where the primary antibody was replaced with the relevant species IgG at the same dilution was always included and the cells were counted in 6 x20 magnification fields. Positive cells were counted and expressed as a percentage of total nucleated cells.

Animals
Female mice homozygous null for the apolipoprotein E (ApoE) gene, were derived from a closed outbred colony housed within the Animal Unit of the University of Bristol (originally purchased from Charles River, UK). The strain background of the animals is C57BL/6, as determined by fingerprinting of tail-tip DNA. The housing and care of the animals and all the procedures used in these studies were performed in accordance with the guidelines and regulations of the University of Bristol and the United Kingdom Home Office. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Utilising the previous experiments performed in our laboratory [4-6], we are able to perform power
analyses which demonstrate that group sizes of n=6 will give us 90% power to detect a 40% change in plaque area alongside compositional and cellular markers.

**miR-24 inhibitor tail vein injection**

Apoe KO female mice which had been high-fat fed for 12 weeks to allow the development of intermediate atherosclerotic lesions within the brachiocephalic artery [5], At this stage, 6 female mice were terminated as a satellite group to evaluate atherosclerosis size and composition at the commencement of miR-24 inhibitor infusion (‘Baseline). Remaining animals were subjected to tail vein injection under general anaesthesia (as previously described [6]) with 8mg/Kg dose injection of miR-24 inhibitor or negative control (miRCURY LNA microRNA inhibitors, Exiqon). Injection was repeated after 4 days and mice terminated 3 days after the second injection.

**Termination**

Animals were anaesthetized by intraperitoneal injection of sodium pentobarbitone, before exsanguination by perfusion via the abdominal aorta with PBS at a constant pressure of 100 mmHg, with outflow through the incised jugular veins. This was followed by constant pressure perfusion with 10% formalin.

**Histology**

The brachiocephalic artery each mouse was embedded in paraffin and the proximal aorta frozen with iso-pentane. Sections were cut at 3 µm from the atherosclerosis-prone areas of these vascular beds as previously described [4]. Sections were stained using Miller’s elastin/van Gieson for the detection of elastin, and picro-sirius red for fibrillar collagens.

**Smooth Muscle Cell and Macrophage Density**

Immunohistochemistry for smooth muscle cells (α-smooth muscle actin) and macrophages (Mac-3) were each performed on four 3 µm paraffin sections respectively from proximal
brachiocephalic plaques of control and miR-24 inhibitor-treated mice (n=6 per group). Cells stained positive with the cell-specific markers were counted and density expressed as the percentage of total nucleated cells stained positive per plaque.

**Plaque Morphometry**

Up to five vessel cross-sections were quantified per mouse. Analysis was performed using a computerised image analysis program (Image Pro Plus, DataCell, Maidenhead, UK). The lengths of the internal and external elastic lamellae were recorded by image analysis. These were used to derive the total vessel area and the (lumen + plaque) area, by assuming them to be the circumferences of perfect circles. Plaque area was measured directly, and was subtracted from the area enclosed by the internal elastica to derive the lumen area.

**In situ zymography**

Gelatinolytic activity was localised in brachiocephalic arteries removed from *Apoe* knockout mice that had received high-fat diet for 13 weeks treated with a miR-24 inhibitor or negative control for the last 7 days, as previously described [4]. In brief, frozen 8 μm cryostat sections were incubated overnight at room temperature in a humidified dark chamber with 20 μg/mL DQ™ Gelatin (Life Technologies Ltd, Paisley, UK) dissolved in developing buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM CaCl$_2$, 0.2 mM sodium azide). Cleavage of the substrate by proteinases results in unblocking of quenched fluorescence and in an increase in fluorescence intensity. Sections were incubated for 24 hours in developing buffer alone, or in the presence of a previously validated MMP-14 blocking antibody (15µg/ml; MAB3328; MerckMillipore, Feltham, UK). Sections were washed in PBS, fixed with 4% paraformaldehyde, and mounted with ProLong® Gold antifade reagent with DAPI (Life Technologies Ltd, Paisley, UK). Using fluorescence microscopy, gelatinolytic activity was identified as green fluorescence.

**Cholesterol Assay**
Cholesterol levels from mouse plasma were quantified as previously described using BioVison Kit. Briefly, HDL and LDL/vLDL present in plasma were separated in precipitation buffer by microcentrifuge and then quantified with colorimetric assay according to manufacturer’s instructions.

**Human Coronary Artery Studies**

Coronary artery segments were collected from cadaveric heart donors to the Bristol Coronary Artery Biobank under National Research Ethics Service approval (08/H0107/48). Paraffin sections from, human coronary artery plaques histologically classified as stable or unstable (n=40 per group) were stained with a CD68 antibody to detect macrophages and a MMP-14 antibody, and the percentage of MMP-14 positive macrophages quantified. Serial sections (n=10 per group) were subsequently subjected to in situ hybridisation using a 5’, 3’ double digoxigenin-labeled locked nucleic acid (LNA)-modified miRCURY miR-24 detection probe or scrambled LNA oligonucleotide to serve as a negative control (Exiqon, Denmark), and expressed as number of miR-24 positive foam cell macrophages as assessed by CD68 IHC on serial sections. Q-CPR for miR-24 was conducted (n=10 per group) and data analysed after normalization to Scarna17 expression and CD68 mRNA expression.

**Statistical Analysis**

Values are expressed as mean ± standard error of the mean (SEM). Group values were compared using the computer program InStat (GraphPad Software, San Diego, California, USA). For the comparison of group means, a check was first made for similar variances: if this was passed then an unpaired two sample two-tailed Student’s t-test was carried out. If the variances were significantly different, then an unpaired two sample two-tailed t-test with Welch’s correction was used. Statistical differences between monocyte/macrophages from the same preparation were analysed by Students paired t-test. For the comparison of multiple groups, an analysis of variance (ANOVA) test was used. In all cases, statistical significance was concluded where the two-tailed probability was less than 0.05.
References


