Supplemental Methods and Results

Methods

Human Cardiovascular Tissue Samples

Human tissues were obtained with informed consent from the Papworth Hospital Research Tissue Bank (REC reference 08/H0304/56) and experimental work carried out with local ethical approval (REC 05/Q0104/142). Saphenous vein (SV), mammary artery (IMA) and radial artery (RA) were from 98 patients receiving coronary artery bypass grafts (mean age 66±1 years, 86 male, 12 female, 1 unknown). Other cardiovascular tissues including coronary artery (CA), left ventricle (LV), atria, pulmonary artery (PA), aorta, failed saphenous vein graft (SVG) and dispersed cardiomyocytes were from 75 patients undergoing cardiac or lung transplantation (mean age of knowns 47±1.4 years; 49 males, 11 female, details for 15 patients unavailable) for conditions including cardiomyopathies, ischaemic heart disease and lung disease. Pre-operative drugs included positive inotropes, anti-arrhythmics, anti-platelet/anti-coagulants, β-blockers, ACE inhibitors/AT1 receptor antagonists, Ca²⁺ channel blockers, nitrates, diuretics and statins.

Human in vitro Pharmacology

Experiments were carried out as previously described.¹ Human saphenous vein and coronary artery were cleaned of connective tissue, cut into 4 mm rings and their luminal surface rubbed gently with a metal seeker to remove the endothelium. Vessels were set up for isometric force recordings in 5 ml organ baths containing oxygenated Krebs solution (37°C) and allowed to equilibrate for one hour. Contractile responses were then recorded to 100 mmol/L KCl at incrementally increasing levels of basal tension until no further increase in KCl response was obtained. This determined the optimum resting tension for each preparation and
was followed by a further 30 minutes equilibration period. Responses were obtained to 1μmol/L phenylephrine in the absence and then addition of 1μmol/L ACh to confirm absence of a functional endothelium. We compared the ability of CCR5 ligands to contract human blood vessels to phenylephrine that activates the α₁ receptor that mediates adrenergic tone and to the potent vasoconstrictor peptides angiotensin-II and endothelin-1 (ET-1). Cumulative concentration response curves were then constructed to CCL4 or CCL5 (10pmol/L-110nmol/L), angiotensin-II (10pmol/L-100nmol/L), ET-1 (0.1nmol/L-300nmol/L) and phenylephrine (1nmol/L-100μmol/L) and experiments were terminated by addition of 100 mmol/L KCl to determine the maximum possible response for each preparation. Additional experiments were carried out in saphenous vein for CCL4 in the absence and presence of 300 nmol/L maraviroc or 10 and 100 nmol/L PF-232796, with antagonists or vehicle (DMSO) added 30 minutes earlier. Agonist responses were normalised to the last KCl response of the normalisation procedure. Data were analysed using the iterative curve fitting programmes Fig P (Biosoft, Cambridge, UK) or GraphPad Prism5 (GraphPad Inc, La Jolla, USA) to give values of pD₂ ((negative log10 of the EC₅₀ (the molar concentration of agonist producing 50% of maximum response)) and E₅₀ (maximum agonist response as a % KCl). All data were expressed as mean±SEM.

For dilator studies rings of endothelium-denuded saphenous vein were preconstricted with 10 nmol/L ET-1 and once a stable response was established cumulative concentration-response curves were constructed to CCL4 (10pmol/L–110nmol/L) to identify any direct smooth muscle dilator action and compared to concentration-response curves to the nitric oxide donor S-nitroso-N-acetyl-DL-penicillamine (SNAP). Experiments with CCL4 were terminated by the addition of
30 μmol/L SNAP (0.1nmol/L–30μmol/L) to confirm that any lack of response to CCL4 was not a result of the inability of any particular tissue to relax. Adjacent rings of vein were set up and contracted with ET-1 and used as time matched controls. Data were analysed as above to give values of pD2 ((negative log10 of the EC50 (the molar concentration of agonist producing 50% inhibition of the ET-1 constriction) and EMAX (maximum agonist response as a % reversal of ET-1). Modified Krebs solution had the following composition (mmol/L): NaCl, 90; KCl, 5; MgSO4.7H2O, 0.5; Na2HP04, 1; NaHCO3, 45; CaCl2, 2.25; glucose, 10; Na pyruvate, 5; fumaric acid, 5; L-glutamic acid, 5.

Mouse in vitro Pharmacology

Animals were housed in an Home Office accredited facility and handled according to guidelines complying with the European Commission guidelines for the Care and Use of Laboratory Animals. Experiments were performed with local ethical approval. Mice (C57/Bl6, male or female 12-20 weeks old) were killed by CO2 inhalation and the aortae removed and cleaned of fat and connective tissue. For each animal four consecutive rings (1-2 mm long) were cut from the thoracic portion of the aorta and mounted for the measurement of isometric tension in a wire myograph, maintained at 37°C containing oxygenated Krebs’ solution. Aortic rings were automatically normalised and set to 90% of the internal circumference they would have if fully relaxed and under a transmural pressure of 100 mmHg. Vessels were then challenged three times with high K+ Krebs’ solution (95mmol/L K+) at 15 min intervals and allowed to re-equilibrate for 60 min. Cumulative concentration-response curves were constructed to mouse CCL4 (1x10^{-11}-1.1x10^{-7}mol/L), phenylephrine (1x10^{-9} – 1x10^{-8}mol/L) and U-46619 (10^{-10}-3x10^{-6}mol/L). Experiments were terminated by the addition of 95 mmol/L K+ and agonist responses were expressed as a percentage of
the maximum response (%KCl). Data were analysed as described above using
GraphPad Prism5.

**Human Saphenous Vein Organ Culture**

Saphenous vein organ culture is an in vitro system in which to study the initial events in the development of saphenous vein intimal thickening and mimics many aspects of vein graft stenosis. An advantage over cell culture models is the maintenance of vessel wall structure and interaction between cellular components. Saphenous vein organ culture was performed as described previously. Saphenous veins were collected after surgery in sterile transfer medium comprising RPMI-1640 (20mmol/L HEPES) (Sigma, UK) with 100 units/mL penicillin and streptomycin (Invitrogen, UK), 2 mmol/L L-glutamine (Invitrogen, UK) and 2.5 μg/mL amphotericin B (Sigma, UK).

Under sterile conditions, veins were flushed with transfer medium and an undamaged section of vein lacking ties or staples was chosen, cleaned of adventitia and opened up longitudinally to expose the endothelium and consecutive segments approximately 1cm long were cut with a sterile scalpel blade (Swann-Morton No. 22, Thermo Fisher Scientific, UK) and placed into culture. One segment was designated a pre-culture ("Day 0") control, washed in PBS and immediately fixed in 10% NBF for 24 hours at 4°C. Segments to be cultured were placed, luminal surface uppermost, onto a small piece of mesh and pinned to approximately in situ length using 0.2 mm diameter pins in a glass petri dish (60 mm diameter, VWR International Ltd. UK) filled with a base of silicone elastomer (Sylgard® 184 Elastomer Kit General Purpose Encapsulant, VWR International Ltd. UK). Segments were then covered with 6mL of sterile RPMI-1640 culture medium (sodium bicarbonate 2 g/L) (Sigma, UK) with 100 units/mL penicillin and streptomycin (Invitrogen, UK), 0.8 mmol/L L-glutamine (Invitrogen, UK) and 5 μg/mL amphotericin B (Sigma, UK) supplemented with 30%
foetal bovine serum (PAA Laboratories Ltd, UK). CCR5-selective antagonists maraviroc and PF-232796 (supplementary table 2 and supplementary table 3), in dimethyl sulphoxide (DMSO), were used at 1µM, with the same volume of DMSO (final concentration 0.1%) added to control segments. A high concentration of drug was used as plasma protein binding has been reported to be 75% for maraviroc. In cell culture studies maraviroc up to 10µM was non-toxic with respect to cell proliferation and DMSO has no detrimental effects up to 2%. Vein segments were incubated in a humidified chamber at 37°C, 5% CO₂ with culture medium changed every 2-3 days. Conditioned culture medium from each medium change was frozen at -70°C until use. After 14 days culture, segments were fixed in 10% NBF for 24 hours at 4°C and paraffin embedded. Transverse sections were stained with a combination of Alcian blue, Miller’s elastin and van Gieson histological stains, to determine neointimal hyperplasia. Sections rehydrated and heat-mediated antigen retrieval performed as above and then slides were incubated in Alcian blue solution (VWR International, Ltd. UK) for 30 mins at room temperature. Sections were then washed in distilled water then 70% ethanol and stained with Miller’s elastin (BDH Prolabo, VWR International, Ltd. UK) for 2 hours at room temperature and differentiated in 70% ethanol for 5 mins. After washing in distilled water sections were incubated for 20 mins with van Gieson’s solution (0.1% acid fuchsin in saturated picric acid). Finally sections were washed in 70% ethanol, dehydrated through increasing concentrations of ethanol, cleared in xylene for 1 hour and mounted in DePeX medium Gurr (BDH Prolabo, VWR International, Ltd. UK). To determine neointimal thickening slides were viewed under standard bright field microscope (Olympus, UK), images were captured using a U-TV1-X digital camera (Olympus UK) and Cell software (Olympus, UK) was used to make area
measurements. To allow comparisons of neointimal area between veins, neointimal area was expressed as a percentage of total intimal and medial area. Haemotoxylin and eosin staining was performed using an automated multistainer (Leica Microsystems, Germany). Slides were dewaxed in xylene, rehydrated in 100% industrial methylated spirits, stained with haematoxylin, immersed in acid-alcohol, stained with eosin, dehydrated and cleared before viewing under a standard bright field microscope (Olympus, UK). Haemotoxylin and eosin stained sections were used to perform nuclei counting using image analysis software (Definiens XD1.2, Munich, Germany) and cell density expressed as number of nuclei per mm² of vein. Masson’s Trichrome staining was performed using kit HT-15 (Sigma Aldrich, UK) following manufacturer’s instructions. Slides were mounted and viewed as before. Additional immunocytochemical studies were performed as for frozen tissue samples as described below (See Immunohistochemistry).

**Chemokine Multiplex Immunoassay**

A custom 4-plex Human Cell Culture Chemokine Assay for CCL2, CCL3, CCL4 and CCL5 (Mesoscale Discovery, MA, USA) was used to determine chemokine concentrations in vein culture supernatant. Vein culture supernatant was concentrated prior to assaying with Vivaspin centrifugal ultrafiltration units (Sartorius Stedim Biotech, France) with a molecular weight cut-off of 5kDa. An equal volume of blank media or media spiked with CCL3, CCL4 or CCL5 (all 600pg/mL) were concentrated as controls. The custom 4-plex immunoassay for CCL3, CCL5, CCL5 and CCL2 was used according to manufacturer’s instructions (Mesoscale Discovery, US). Chemokine concentrations were corrected for the concentration procedure. The sensitivities of detection (defined as the amount of standard chemokine required to increase the signal three standard deviations above that produced by the blank
(0pg/mL) sample) were 6.5pg/mL, 10.9pg/mL, 12.6pg/mL and 7.9pg/mL for CCL3, CCL4, CCL5 and CCL2 respectively. Recoveries were 77.7%, 33.3% and 62.5% for CCL3, CCL4 and CCL5 respectively. Intra-assay coefficients of variation (CoV) were 11.8%, 10.1%, 5.6% and 6.2% (9 determinations), whilst inter-assay CoV were 18.8%, 21.3%, 7.3% and 8.2% (6 determinations) for CCL3, CCL4, CCL5 and CCL2 respectively. Combined calibration curves of the four chemokines were prepared ranging from 10 000 pg/mL to 2.4 pg/mL. Standards, samples or blanks (all 25μL) were added to wells in duplicate, plates were sealed (FisherBrand, UK) and incubated at room temperature with vigorous chaking (600 rpm). Detection antibody was added at 1μg/mL, plates resealed and incubated for a further 2 hours with shaking. Plates were then washed with 3 x 300μL PBS/T per well using an automated plate washer (Denley Instruments, UK), read buffer added and plates read on a Sector Imager 6000 (Mesoscale Discovery, US). Serial dilution curves for culture supernatant were parallel to the standard curve for each chemokine, confirming suitability of the assay for detection of CCL3, CCL4, CCL5 and CCL2 in tissue culture medium.

**Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Assays**

Human vessels were cleaned of endothelium and adventitia by forceful strokes of a razor blade to leave only the smooth muscle layer of the vessel. Total RNA was extracted from 50-100mg human tissues (saphenous vein, aorta, pulmonary artery, coronary artery, saphenous vein graft medial smooth muscle and dispersed cardiomyocytes) using TRIzol reagent (Invitrogen, UK) and a Fast Prep instrument (QBioGene, CA, USA) and Lysing Matrix D tubes (AnaChem, UK). Tissue was spun at 6.5 m/s for 45 s up to six times (with a cooling period between spins) until homogenised. Following a short incubation at room temperature samples were
shaken with 0.2mL chloroform and centrifuged at 4ºC, 12000 x g, 15 min. The top
phase was recovered and RNA precipitated with 0.5mL isopropanol. The samples
were incubated at room temperature for 10 min, centrifuged at 4ºC, 12000 x g, 10
min and the supernatant removed. The RNA pellet was washed with 1mL ethanol
(75%), centrifuged at 4ºC, 7500 x g, 5 min and then air dried and resuspended in
diethylpyrocarbonate (DPEC)-treated RNase-free water with a short incubation at
55ºC to ensure completely dissolved. Extracted RNA was DNase treated with RQ1
RNase-Free DNase (Promega, USA.) and reverse transcribed using Superscript III
First-Strand Synthesis System for RT-PCR with Oligo(dT)20 primers kit as per
manufacturer’s instructions (Invitrogen, UK). The resulting cDNA was stored at -
70ºC until use. Control reactions used HeLa RNA (10ng/mL); positive controls were
treated as described for tissue samples while Superscript III RT enzyme was omitted
from negative RT reactions. Control reactions were included in every RT
experiments and were checked by PCR.
PCR reactions were carried out with gene specific primers for CCR52, CCL33, CCL43
and CCL54 checked for specificity using NCBI BLAST tool and custom synthesised
by Invitrogen, UK. Were possible, primers flanked intron sites to allow any
contaminating gDNA to be distinguished from cDNA Gsα primers spanning an
intronic region of this housekeeping gene served as a control for sample integrity
and lack of gDNA contamination. The PCR mix contained 10X PCR buffer
(200mmol/L Tris-HCl pH 8.4, 500mmol/ KCl) diluted to 1X, 1.5mmol/L MgCl2,
0.2mmol/L each dNTP, 0.2mmol/L each primer and 1 unit Platinum Taq Polymerase
(all Invitrogen, UK). Primer specific conditions are given in Supplementary Table 1
with a final extension step of 10 min at 72 ºC. Reactions were carried out in a MJ
Research MiniCycler and PCR products were separated by electrophoresis on a 1.5
% agarose gel stained with ethidium bromide and compared with a 25 base pair (Promega, US) or 100 base pair (Invitrogen, UK) DNA molecular weight standard. All cDNA samples used were checked for integrity and gDNA contamination using primers spanning an intronic region of the housekeeping gene $G_{\alpha}$ and negative controls consisting of omission of cDNA were run with each reaction. CCR5 primers were designed to flank the CCR5 delta 32 deletion region.

**Quantitative RT-PCR**

Total RNA was extracted as above and additionally from human left ventricle. Ventricular tissue was homogenised using a Polytron PT.K homogeniser fitted with a PTA 10S probe (Philip Harris Scientific, UK). RNA purity and yield determined by spectrophotometric analysis using the Nanodrop 1000 (Thermo Fisher Scientific, UK). Reverse transcription was carried out as described above using 1µg RNA diluted in DPEC-treated water. Negative RT reactions consisted of omission of SuperSriipt III RT as before. Expression of CCR5, CCL3, CCL4 and CCL5 and the endogenous control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined using cDNA-specific TaqMan Gene Expression Inventoried Assays (assay ID: CCR5, Hs00152917_m1; CCL3, Hs00234142_m1; CCL4, Hs00237011_m1; CCL5, Hs00174575_m1; GAPDH, Hs99999905_m1; Applied Biosystems, UK). Primer sets were intron spanning to eliminate false signals from gDNA contamination. Results were normalised using GAPDH as the endogenous control or active reference gene to control for differences in cDNA between wells. Assays comprised a primer set and probe, with FAM (6-carboxyfluorescein) as the reporter dye, TAMRA (6-carboxy-4,7,2,7'-tetramethylrhodamine) as the quencher dye and ROX (carboxy-X-rhodamine) as a passive reference dye to normalise reporter dye fluorescence for
volume and concentration changes in each well. TaqMan Gene Expression assays had an amplification efficiency of 100%.

Reactions (20μL, run in triplicate in 96 well plates with ultra clear cap strips (Thermo Fisher Scientific, UK)) comprised 10μL X2 TaqMan Universal PCR Master Mix (Applied Biosystems, UK), 1μL gene expression assay and 95 ng cDNA in 9μL RNase free water and were carried out on an ABI PRISM 7700 sequence detection system (Applied Biosystems, UK) with cycle conditions 2 min at 50°C; 15 min at 95°C; 40 cycles of 15s at 95°C and 1 min at 60°C. No template control reactions consisted of negative RT reactions. Gene expression was quantified using the comparative (ΔΔCt) method and presented as relative levels between tissues and genes. Comparisons were not carried out between tissues with statistically significant differences in housekeeping gene expression.

Western Blot

Human saphenous vein was homogenised using a Polytron PT.K homogeniser fitted with a PTA 10S probe (Philip Harris Scientific, UK) in Tris lysis buffer (mmol/L: Tris 50, MgCl2 2.5, EDTA 5, EGTA 1, pH 7.5) with protease inhibitor cocktail (μmol/L: 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) 104, pepstatin A 1.5, E-64 1.4, bestatin 4, leupeptin 2, aprotinin 0.08). The suspension was centrifuged at 30 000 g for 30 min at 4 °C, re-suspended in Tris lysis buffer and re-centrifuged. The resultant pellet was re-suspended in HEPES buffer (mmol/L: HEPES 50, KCl 13.4, pH 8) and protein content determined by Bio-Rad DC protein assay (Bio-Rad Laboratories, UK). Samples were diluted in HEPES buffer and stored at –70 °C. For SDS-PAGE samples were diluted with an equal volume of reducing sample buffer (5 parts 2X Laemmli sample buffer (mL: 10% sodium dodecyl sulphate (SDS), 4; glycerol, 2; 1 mol/L Tris pH 6.8, 1.2; H2O, 2.8; 1% bromophenol blue 0.1) 4 parts de-ionised water
and 1 part Dithiothreitol) and denatured at 95 °C for 10 min. SDS-PAGE was carried out on a 10 % polyacrylamide gel with molecular weight markers (Dual Colour Precision Plus Protein Standards, Bio-Rad Laboratories, UK) run alongside. Proteins (~20μg) were transferred to an activated polyvinylidene difluoride membrane (Amersham Biosciences, GE Healthcare, UK) by a semi-dry blotting method (transfer buffer: Tris 48 mmol/L, glycine 39 mmol/L, 0.0375% SDS, 20% methanol, pH 9.2). Ponceau S red staining was used to evaluate equal protein transfer. Membranes were agitated for 24 hours at 4°C in a solution of 5 % non-fat milk in Tris buffered saline (TBS, mmol/L: Tris, 20; NaCl, 137; pH 7.6)) to block non-specific binding sites and probed with rabbit anti-human CCR5 antibody (N-terminus) diluted 1:500 in TBS with 0.1% Tween-20 (TBS/T) with 3 % non-fat dry milk for 24 hours at 4 °C. Following washing in TBS/T, membranes were probed with HRP-conjugated donkey anti-rabbit IgG antibody diluted 1:5000 in TBS/T with 3 % non-fat milk for 1 hour at room temperature. After further washing the chemiluminescent reporter system ECL Plus reagent (Amersham Biosciences, GE Healthcare, UK) was applied to membranes for 5 min at room temperature, excess reagent drained, membranes wrapped in cling film and membranes, wrapped in cling film, were apposed to photographic film (Hyperfilm™ ECL, Amersham Biosciences, GE Healthcare, UK) for varying periods of time. Flims were developed manually. No bands were evident on omission of primary antibody as negative controls.

**Immunohistochemistry**

Immunohistochemistry (IHC) with colourimetric detection and dual-labelling fluorescence IHC were carried out on frozen sections of human normal and diseased cardiovascular tissues and paraffin embedded sections of cultured saphenous vein (Day 0 and Day 14). For frozen sections, tissues were mounted on metal chucks
with cryogenic mounting medium (O.C.T. Mounting Medium, BDH Prolabo, VWR International Ltd, UK). Cryostat (Bright Instrument C. Ltd, UK) cut tissue sections (30\( \mu \)m) were thaw mounted onto gelatine or poly-L-lysine coated slides and were left to dry overnight at room temperature. Sections were stored at -70\(^{\circ}\)C until required. Saphenous vein from organ culture experiments was fixed in 10% neutral buffered formalin (NBF), paraffin embedded and transverse sections of vein cut on a microtome as before.

**Colourimetric IHC**

Immunohistochemistry on sections of normal and diseased coronary artery, saphenous vein, saphenous vein graft and left ventricle was carried out as described previously\(^5\) using rabbit anti-human CCR5 (N-terminus diluted 1:500), CCL3 (1:300), CCL4 (1:300) and CCL5 (1:300), mouse anti-CD3 (1:100) and mouse anti-CD68 (1:2000). Thawed sections were fixed in ice-cold acetone for 10 mins and then incubated with 10% normal donkey serum or rabbit serum (normal serum from species in which secondary antibody was raised) in phosphate buffered saline (PBS) for 2 hours at room temperature to block non-specific protein interaction. Tissues were incubated with primary antisera at the indicated optimum dilution in PBS containing 0.1% Tween-20 (PBS/T) and 3% normal serum overnight (18 hours) at 4\(^{\circ}\)C. Sections were washed 3x 5 mins in PBS/T before incubation with the biotinylated donkey anti-rabbit antiserum (1:500) or biotinylated rabbit anti-mouse antiserum (1:200) in 3% normal serum in PBS/T, for 1 hour at room temperature. After 3 x 5 mins washes in PBS/T sections were incubated with Vectastain Elite reagent (Vector Laboratories, UK) for 1 hour at room temperature. Following a further wash step, specific staining was revealed with 3,3’–diaminobenzidine tetrahydrochloride (DAB) as the chromogenic substrate at a final concentration of 0.6
mg/mL in 0.05mol/L Tris-HCl buffer and 0.03% hydrogen peroxide. The reaction was stopped by immersion in de-ionised water and slides were dehydrated in an alcohol series (increasing concentrations of industrial methylated spirits, absolute ethanol, acidified ethanol, absolute ethanol) cleared in xylene (1 hour, room temperature) and mounted in DePex mounting medium (BDH Prolabo, VWR International Ltd, UK). Sections were examined using a standard bright field microscope (Olympus UK, London, UK). Images were captured using a U-TV1-X digital camera (Olympus UK) and AnalySis software (Soft Imaging System, Münster, Germany). Specificity of staining was confirmed by attenuation of the positive staining by pre-absorption of the primary antisera with the corresponding immunising peptide for rabbit antisera. The diluted primary antibody was incubated with an excess of immunising peptide in PBS/T with 3% of the appropriate normal serum for 24 hours at 4°C and then used as per the protocol above. For mouse anti-CD68 and anti-CD3 antisera, mouse IgG1 (Dako, UK) was used as a negative control in place of the primary antibody. Negative controls were performed by omission of the primary antibody and all controls were performed in adjacent sections.

For immunohistochemistry on paraffin embedded sections of cultured saphenous vein, sections (5µm) underwent heat-mediated antigen retrieval prior to application of antibodies. Slides were rehydrated and immersed in citrate buffer (Antigen unmasking Solution, Vector LAbso, UK) or Tris/EDTA buffer (mmol/L Tris 10; EDTA 1; pH 9.0) for smooth muscle α-actin staining and then microwaved on full power (2 x 10 min), cooled at room temperature and equilibrated in two changes of PBS. For CCR5 (goat anti-CCR5; 1:500), CCL3 (rabbit anti-CCL3; 1:100), CCL4 (goat anti-CCL4; 1:100), CCL5 (rabbit anti-CCL5; 1:50), detection was as for frozen sections (biotinylated donkey anti-goat or donkey anti-rabbit secondary antibodies used at
(1:500) with counterstaining with Miller’s Elastin (BDH Prolabo, VWR International Ltd, UK) to distinguish the layers of the vessel wall. Sections were incubated with Miller’s Elastin stain (BDH Prolabo, VWR International Ltd, UK) for 15 min at room temperature, with differentiation in 70% ethanol. Slides were then dehydrated through an alcohol series and cleared in xylene for one hour prior to mounting in DePeX mounting medium Gurr (BDH Prolabo, VWR International Ltd, UK). Slides were viewed as before. Negative controls consisted of omission of primary antisera or pre-absorption with immunising peptide where available, on adjacent sections. For smooth muscle α-actin and von Willebrand detection was via the peroxidase anti-peroxidase method. Following incubation with the primary antibodies as before (mouse anti-SMaA, 1:100; mouse antivWF, 1:25) slides were washed in 3 changes of PBS, incubated with polyclonal rabbit anti-mouse antibodies, (1:100 and 1:25 in PBS with 3% normal rabbit serum, respectively), incubated for 1 hour at room temperature and washed. Slides were then incubated for one hour at room temperature with mouse peroxidase anti-peroxidase (1:100 in PBS with 3% normal serum) washed and incubated with DAB solution, as before, and counterstained with Miller’s Elastin (BDH Prolabo, VWR International Ltd, UK). Negative controls consisted of isotype-matched mouse IgG2a (SMαA) or IgG1 (vWF), raised against a non-human antigen (Dako, UK) diluted as the primary antibody.

**Dual Labelled Fluorescent Confocal Microscopy**

Sections of human frozen cardiovascular tissues vein were processed as for avidin-biotin-peroxidase immunohistochemistry with rabbit anti-human CCR5 (N-terminus, 1:500), CCL3 (1:100 – 1:200), CCL4 (1:100 – 1:200) or CCL5 (1:100 – 1:200) diluted in PBS/T with 3% goat serum and one of the following cell markers; mouse anti- von Willebrand factor (1:100) to identify endothelial cells, mouse anti-smooth muscle α-
actin (1:100) to identify vascular smooth muscle cells, mouse anti-CD68 (1:1000-1:2000) to identify macrophages or mouse anti-CD3 (1:10) to detect T-lymphocytes. Secondary antibodies Alexa Fluor® 488-conjugated goat anti-rabbit IgG (1:100) and Alexa Fluor® 568-conjugated goat anti-mouse IgG (1:100) diluted in PBS/T with 3% goat serum were incubated on slides for 1 hour at room temperature in a foil wrapped tray to limit light exposure. Hoechst 33258 dye (Invitrogen, UK) was included at a dilution of 1:100 when staining of cell nuclei was required. Following a wash step, slides were mounted in ProLong Gold reagent (Invitrogen, UK). After a 24 hour cure period at room temperature, slides were viewed with a confocal laser-scanning microscope (Leica Microsystems, Germany) and sequential scanning was performed to avoid bleed-through. Negative controls consisted of omission of the primary antisera and/or pre-absorption with the immunising peptides, if available (as described above) in adjacent tissue sections.

Automated IHC

For immunohistochemical analysis of proliferation and apoptotic markers in paraffin embedded saphenous vein, automated IHC was performed. Cleaved caspase-3, phosphorylated-histone H3 and Ki67 staining was carried out using DABMap detection on the Discovery XT system and NexESTM software (Ventana Medical Systems, Inc., France). All reagents for automated staining were from Ventana Medical Systems, Inc. (France) unless otherwise stated. Slides were dewaxed, underwent heat-mediated antigen retrieval prior to peroxidase blocking, avidin-biotin blocking and serum blocking steps. Following manual addition of appropriate primary antibody (anti-cleaved caspase-3, 1:100, 2 hours at room temperature; anti-phosphorylated histone H3, 1:100, 1 hour at 37°C; anti-Ki67, 1:200, 1 hour at 37°C) slides were incubated as indicated, then briefly fixed for 4 min
using glutaraldehyde and incubated for 30 min at 37 °C with biotinylated secondary antibody, diluted 1:200. Slides were incubated with streptavidin-HRP, followed by addition of DAB with a copper enhancer. A light haematoxylin counterstain was then applied, following which, slides were removed from the machine and washed in warm soapy water. Sections were dehydrated and cleared prior to mounting with DePeX mounting medium Gurr (BDH Prolabo, VWR International Ltd, UK) in a fully automated glass coverslipper (Leica Microsystems, Germany). Slides were viewed on a standard bright field microscope (Leica Microsystems, Germany) or scanned using a NanoZoomer (Hamamatsu Photonics, UK) and viewed using WebSlide Enterprise software (Olympus, UK). Negative controls consisted of omission of primary antisera and isotype control on adjacent sections.

**TUNEL Staining**

Sections were dewaxed, rehydrated and washed for 5 min in 0.85% NaCl followed by PBS. Sections were fixed in 10% NBF for 15 min, washed twice for 5 min in PBS and permeabilised by incubating for 10 min with 20 μg/mL Proteinase K. Slides were washed in PBS for 5 min, briefly refixed in 10% NBF for 5 min and washed twice for 5 min in PBS. Sections were incubated for 10 min with Equilibration Buffer (mM: potassium cacodylate pH 6.6, 200; Tris-HCl pH 6.6, 25; DTT, 0.2; cobalt chloride, 2.5; 0.25 mg/mL BSA), then incubated with rTDT Reaction Mix (98 parts Equilibration Buffer, 1 part biotinylated nucleotide mix, 1 part rTDT enzyme) for one hour in a humidified chamber at 37 °C. The reaction was terminated by immersion of slides in sodium chloride-sodium citrate buffer (mM: NaCl, 300; sodium citrate, 30; pH 7.2) and washing three times in PBS for 5 min. Endogenous peroxidase activity was blocked by 0.3% H₂O₂ (5 min), followed by three washes in PBS. Sections were then incubated with streptavidin-HRP diluted 1:500 in PBS for 30 min and washed
three times in PBS. The detection solution was prepared ($\mu$L: DAB substrate 20X Buffer, 50; deionised water, 950; DAB 20X Chromogen, 50; Hydrogen Peroxide 20X, 50) and added to slides for 7 minutes. Slides were rinsed in three changes of deionised water, lightly counterstained with Mayer’s haemalum for 1 min and blued in running tap water. Sections were then dehydrated and cleared prior to mounting with DePeX mounting medium Gurr (BDH Prolabo, VWR International Ltd, UK) in a fully automated glass coverslipper (Leica Microsystems, Germany). Slides were viewed on a standard bright field microscope (Leica Microsystems, Germany) or scanned using a NanoZoomer (Hamamatsu Photonics, UK) and viewed using WebSlide Enterprise software (Olympus, UK). Positive controls comprised sections from tonsil, whilst negative controls consisted of omission of rTDT enzyme from rTDT reaction mix incubated on sections of tonsil.

**Receptor Binding: Autoradiography**

Receptor autoradiography was carried out as previously described using cryostat-cut sections of human tissues and a fixed concentration (0.1nmol/L $^{[125]}$I-CCL4. Non-specific binding (NSB) was defined using 100nmol/L CCL4.

**Receptor Binding: Saturation Analysis**

Saturation biding was carried out as previously described. Saphenous vein, 10$\mu$m sections, were cut onto poly-L-lysine coated microscope slides, air-dried and stored at -70$^\circ$C until required. Following a 30 minute incubation at room temperature in binding buffer (NaH$_2$PO$_4$ 50 mmol/L, NaCl 100 mmol/L, EDTA 5 mmol/L, MgCl$_2$ 5 mmol/L, 0.2% BSA, pH 7.4) sections were incubated for 2 hours, at room temperature (22$^\circ$C), in buffer containing increasing concentrations of $^{[125]}$I-CCL4 (2 pmol/L-2 nmol/L), with non-specific binding determined in adjacent sections using 1 $\mu$mol/L MIP-1$\beta$. To break the equilibrium, sections were rapidly washed in ice-cold
Tris-HCl, pH 7.4 (3x5 minutes), slides were dried an apposed together with standards to radiation-sensitive film for 5 days. The resulting autoradiograms were analysed using computer-assisted densitometry. Protein concentration per tissue section was determined using a DC protein assay kit (Bio-Rad, Herts, U.K.). The iterative, non-linear curve fitting programmes EBDA and LIGAND in the KELL package (Elsevier Biosoft, Cambridge, UK) were used to determine pooled $K_D$, $B_{MAX}$ (normalized to fmol/mg of protein) and Hill slope, expressed as mean±SEM.

**Receptor Binding: Competition Analysis**

Competition binding experiments were carried out essentially as for the saturation experiments but using a fixed concentration of radioligand (0.1nmol/L [$^{125}$I]CCL4) in the absence (Total) and presence of increasing concentrations of unlabelled CCL4 or maraviroc (20pmol/L-1μmol/L). Non-specific binding defined using 1μmol/L CCL4. Data were expressed as % specific binding of 0.1nM [$^{125}$I]CCL4. Curves were analysed using GraphPad Prism5 to determine $K_D$ for CCL4 and $K_B$ (the equilibrium dissociation constant (Molar) of modulator binding) and $\alpha$ (the ternary complex constant) for the allosteric modulator maraviroc.

**Statistical Analysis**

$n$-Values are the number of patients from which tissue was obtained. For in vitro pharmacology data for which there was no evidence of non-normality, $E_{MAX}$ and $pD_2$ values were compared using Student’s two-tailed t-test or by one-way analysis of variance followed by Bonferroni’s multiple comparison tests. Receptor autoradiography by one-way analysis of variance followed by Tukey’s or Bonferroni’s multiple comparison tests. For other data where there was evidence of non-normality non-parametric statistical analysis was carried out with data expressed as median (range). For qRT-PCR, analysis of different genes within the same tissues
was by Friedman test followed by post hoc testing with Bonferroni correction for multiple testing or with Dunn's multiple comparison test. Comparison between different tissues or disease states used Kruskall-Wallis test followed by Mann-Whitney U test with Bonferroni correction applied. For data from saphenous vein culture, comparisons between paired data were by Wilcoxon signed rank test or Friedman test for related samples followed by Dunn's multiple comparison test. Analysis was performed using GraphPad Prism5 (GraphPad Software Inc. La Jolla, USA). \( P \)-values less than 0.05 were considered significant.

**Materials**

Unless otherwise stated all chemicals were from Sigma Aldrich Co. Ltd. (UK). All molecular biology reagents, including primers, were from Invitrogen (UK). Recombinant human CCL4, recombinant human CCL5 and recombinant mouse CCL4 (all purity &gt;97%) were from R&D Systems and were made up as \( 10^{-5} \)M stock solutions in distilled water and stored in 20\( \mu \)L aliquots at -20ºC. \( [^{125}\text{I}] \)-CCL4 (2000 Ci/mmol) was from Perkin Elmer. For Western blotting and immunohistochemistry, rabbit anti-CCR5 antibody, raised against N-terminus amino acids 6-20 (AB 1889), was from Chemicon (Millipore, UK) and rabbit anti-CCR5, raised against CCR5 C-terminus was from Anaspec (ANA54431; Cambridge Bioscience, UK). For immunohistochemistry on paraffin sections, goat anti-CCR5, raised against N-terminus amino acids 3-30, was from Abcam plc, UK (ab 1673). For ligand immunohistochemistry, antibodies raised against human CCL3 (ab 32609), CCL4 (ab 9675) and CCL5 (ab 9679) and corresponding CCL4 and CCL5 antigenic peptides (ab 9676 and ab 9680, respectively), were purchased from Abcam plc (UK). For immunocytochemistry on paraffin sections, goat anti-CCL4 (C-15 C-terminus, sc 1385), goat anti-CCL5 (C-19 C-terminus, sc 1410) and corresponding immunising
peptides (sc 1385 p and sc 1410 p, respectively) were from Santa Cruz Biotechnology, Inc (CA). For dual-labelling fluorescent immunocytochemistry mouse antibodies against human von Willebrand factor (vWF, MO616, clone F8/86), smooth muscle α-actin (SMαA, MO851, clone 1A4), CD3 (MO7254, clone F7.2.38) and CD68 (MO718, clone EBM11) and corresponding negative control antibodies were obtained from Dako (UK). For automated immunohistochemistry, rabbit anti-cleaved caspase-3 (9661, Asp175) and rabbit anti-phosphorylated-Histone H3 (9701, Ser10) were from Cell Signalling Technology, Inc, MA, and rabbit anti-Ki67 (RM-9106-S1, clone SP6) was from Lab Vision, CA. Biotinylated donkey anti-rabbit IgG and donkey anti-goat IgG secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc (USA), and biotinylated rabbit anti-mouse IgG, polyclonal rabbit anti-mouse IgG secondary antibodies, mouse peroxidase anti-peroxidase and mouse IgG1 were from Dako, UK. Vectastain Elite kit, as directions (Vector Laboratories). Fluorescent conjugated secondary antibodies, Alexa Fluor® 488 goat anti-rabbit IgG (A-11008) and Alexa Fluor® were obtained from Molecular Probes (Invitrogen, UK) and ProLong Gold mounting medium was from Invitrogen (UK). Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG antibody, ECL Plus detection reagents, Hyperfilm™ ECL were all obtained from Amersham Biosciences (GE Healthcare, UK). CCR5 antagonists maraviroc (UK-427,857, brand name Celsentri (Europe), Selzentry (US)) and PF-232796 were provided by Pfizer Global Research and Development (Sandwich, UK).
RESULTS

Human in vitro Pharmacology

In endothelium-denuded saphenous vein preconstricted with ET-1, human CCL4 had no direct dilator actions (n=4) (Supplementary Figure 1A) in contrast to the nitric oxide donor S-nitroso-N-acetyl-DL-penacillamine that produced a significant reversal (P<0.05 compared to time matched control, Student’s two-tailed t-test) of ET-1 contraction, as expected, with pEC$_{50} = 6.31±0.07$ (Supplementary Figure 1B).

Mouse in vitro Pharmacology

Mouse CCL4 contracted mouse aorta with pD$_2 = 9.79±0.23$ and E$_{MAX}$ 9.5±2.5% KCl (n=10) (Supplementary Figure 1C) compared to phenylephrine pD$_2 = 6.42±0.21$, E$_{MAX}$ = 45±9% KCl (n=6) and U-46619 pD$_2 = 7.83±0.13$, E$_{MAX}$ 102±1% KCl (n=10) (Supplementary Figure 1D).

Competition Binding Experiments

In human left ventricle CCL4 competed for 0.1nmol/L $[^{125}\text{I}]$CCL4 binding with K$_D=55.2±11.6$nMol/L (n=3) (Supplementary Figure 1B). Maraviroc is an allosteric modulator of the CCR5 receptor and therefore the "competition" data were analysed using the GraphPad Prism5 allosteric modulator titration function based on the ternary complex model giving K$_D=0.69$nmol/L and $\alpha=0.56$ for maraviroc binding to the CCR5 receptor in human left ventricle (n=3) (Supplementary Figure 1C).

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**Supplementary Table 1** Primer specific conditions for PCR reactions.

| Gene     | Conditions                     | Source                                    |
|----------|--------------------------------|-------------------------------------------|
| Gsα      | 94°C 30 s, 58°C 30 s, 72°C 1 min, 40 cycles | designed by S. Monteith Clinical Pharmacology Unit, University of Cambridge, UK |
|          | Primer Pair (3’ – 5’) F AGATCGACGTGATCAAGCAG R GTCTCAAGATTTCCAGAAGTC |                                          |
| CCR5     | 94°C 30 s, 60°C 30 s, 72°C 1 min, 40 cycles | Reference 2                              |
| CCL3     | 94°C 30 s, 57°C 30 s, 72°C 1 min, 40 cycles | Reference 3                              |
| CCL4     | 94°C 30 s, 55°C 30 s, 72°C 1 min, 40 cycles | Reference 3                              |
| CCL5     | 95°C 30 s, 65°C 30 s, 72°C 1 min, 40 cycles | Reference 4                              |
Supplementary Figure 1. Concentration-response curves to (A) CCL4 (■) and (B) the nitric oxide donor S-nitroso-N-acetyl-DL-penacillamine (■ SNAP) in human endothelium-denuded saphenous vein preconstricted with 10nmol/L ET-1 (n=4). Time matched controls (●) were obtained in adjacent vein rings from the same individual preconstricted with 10nmol/L ET-1 but to which no SNAP or CCL4 was added to indicate any loss of contraction to ET-1 over the time period of the experiment. SNAP but not CCL4 produced a concentration-dependent reversal of the ET-1 contraction (Student’s two-tailed t-test, * P< 0.05 compared to time matched control response). Concentration-response curve to (C) murine CCL4 and (D) phenylephrine and U-46619 in rings of mouse aorta (n=10).

Supplementary Figure 2. Representative photomicrographs show cultured segments at (A) pre-culture (Day 0) and (B) following 14 days in culture (Day 14). Segments have been histologically stained with Alcian Blue, Miller’s Elastin and van Gieson’s stains to identify the vessel wall layers. Arrows identify neointima. Thickened intima in retrieved saphenous vein graft is stained with (C) H&E and (D) Alcian Blue, Miller’s Elastin and van Gieson’s stains for comparison to cultured vein. m denotes medial layer, i intimal layer. (E) Immunoreactivity for the smooth muscle cell marker smooth muscle α-actin (SMαA) was detected in the neointima of cultured vein. (F) Staining was abolished when isotype control antibody was substituted for anti-SMαA. (G) Presence of collagen was identified in the neointima of cultured vein (Day 14) by Masson’s trichrome stain. Endothelial cell marker von Willebrand Factor (vWF) immunoreactivity was observed in (H) uncultured vein (Day 0) and (I) culture vein (Day 14). Scale bars = 50µm. (J) mRNA encoding CCR5 was present at day 0 and levels were not significantly different after Day 14 in culture (P>0.05, Wilcoxon
Signed Rank test, horizontal line indicates median value). (K) Top panel shows CCR5 protein (green fluorescence) and smooth muscle α actin (red fluorescence) co-localised (yellow fluorescence) to the media and neointima of saphenous vein in culture after 14 days (Scale bars = 30μm).

**Supplementary Figure 3.** Representative photomicrographs showing localisation of CCL3 (A), CCL4 (B) and CCL5 (C) protein to the media and neointima of saphenous vein after 14 days in culture. Staining was attenuated by pre-absorption of anti-CCL4 and anti-CCL5 antisera with the appropriate immunising peptides and no signal obtained on omission of primary antibody. (D) Comparison of cumulative release of chemokines CCL3, CCL4, CCL5 and CCL2 (n=9) over 14 days of vein culture with vehicle. *P<0.05, **P<0.01, ***P<0.001, Friedman test followed by Dunn’s multiple comparison test.

**Supplementary Figure 4.** Representative photomicrographs showing co-localisation (orange/yellow fluorescence in overlay) of (A) CCL4, (C) CCL4 and (E) CCL5 (green fluorescence) with the smooth muscle marker smooth muscle α-actin (SMαA, red fluorescence) in the neointima of saphenous vein after 14 days in culture (n=3). No signal was obtained on omission of the primary antibodies for each ligand (B, D, G). Signal was attenuated following pre-absorption of antisera with the immunizing peptide for CCL5 (F). L denotes the luminal surface of the vessel (n=3). Scale bars = 30μm.

**Supplementary Figure 5.** Representative photomicrographs showing discrete positive staining for the apoptosis marker cleaved caspase-3 in (A) the germinal
centres of tonsil that was abolished by (B) omission of the primary antibody. No staining for caspase-3 was apparent in saphenous vein sections at (C) pre-culture (Day 0) (n=8) or (D) following 14 days of culture with vehicle (n=8), (E) maraviroc (MVC) (n=6) or (F) PF-232796 (n=5). L denotes luminal surface of vessel. Scale bars 100μm. Cell density (nuclei mm⁻²) was not different at Day 14 in saphenous vein segments cultured with vehicle or CCR5 antagonists (G) maraviroc (n=6) and (H) PF-232796 (n=5).

**Supplementary Figure 6.** Total release of chemokines during saphenous vein culture in the absence (vehicle ●) and presence of maraviroc (■) or PF-232796 (▲) (n=7). Horizontal bars indicate median values. Significantly different from vehicle ★ P<0.05 Wilcoxon Signed Rank test.

**Supplementary Figure 7.** Representative photomicrographs showing TUNEL staining in human tonsil and cultured saphenous vein. Staining was observed in (A) the germinal centres of tonsil and was abolished (B) by omission of rTDT enzyme (negative control). High background was seen in (C) the positive control outside of the germinal centres; this was not attenuated by (D) omission of rDT enzyme. High background with little or no specific staining was observed in saphenous vein sections at (E) pre-culture Day 0, following 14 days of culture with (F) vehicle, (G) maraviroc or (H) PF-232796. L denotes luminal surface of vessel. Scale bars 100μm.

**Supplementary Figure 8.** Representative photomicrograph showing phosphorylated histone H3-positive nuclei in (A) sections of human ileum with no
staining obtained in (B) isotype control antibody or (C) diluent only. Little or no staining was observed in saphenous vein sections at (D) pre-culture (Day 0) and following 14 days of culture with (E) vehicle, (F) maraviroc or (G) PF-232796. L-denotes luminal surface of vessel. Scale bars = 100μm.

**Supplementary Figure 9.** Representative photomicrograph showing Ki67-positive staining in germinal centres of human tonsil at (A) low power and (B) high power view. Omission of primary antibody abolished staining viewed at (C) low and (D) high power. Little or no staining was observed in saphenous vein sections at (E) pre-culture (Day 0) and following 14 days in culture with (F) vehicle, (G) maraviroc or (H) PF-232796. L denotes luminal surface of vessel. Scale bars = 100μm.

**Supplementary Figure 10:** Representative photomicrographs showing in human saphenous vein (A) attenuation of signal on pre-absorption of CCR5 antisera with immunising peptide. (B, C) Omission of primary and secondary antibodies abolished signal. All n=3. Scale 50μm.

**Supplementary Figure 11.** (A) Representative autoradiograms showing total and non-specific binding (NSB) of $[^{125}I]CCL-4$ to adjacent sections of human cardiovascular tissues. Pulmonary artery (PA), media (m), intima (i). Scale bars are 2mm. Competition binding curves against 0.1nmol/L $[^{125}I]CCL4$ in human left ventricle for (B) CCL4 and (C) maraviroc.

**Supplementary Figure 12.** Representative photomicrographs showing co-localisation of (A) CCL-4 and (B) CCL-5 with the endothelial cell marker von
Willebrand Factor (vWF) and smooth muscle cell marker smooth muscle α-actin (SMαA) in histologically normal coronary artery and saphenous vein and left ventricle containing cardiomyocytes and small intramyocardial vessel. Omission of the primary antibody or primary and secondary antibodies abolished signal. (All n=3).
A

CCL3

No primary

Preabsorption

B

CCL4

Preabsorption

No primary

C

CCL5

Preabsorption

No primary

D

Cumulative chemokine pg/mL

CCL3  CCL4  CCL5  CCL2

**  ***  **  ***
Supplementary Figure 8

A, B, C, D, E, F, G: Images showing tissue samples from different days and treatments.

- **Panel A** and **Panel B**: Images of tissue samples labeled as Day 0.
- **Panel C**: Image with a label indicating an unspecified day.
- **Panel D** and **Panel E**: Images labeled as Day 14 vehicle.
- **Panel F**: Image labeled as Day 14 MVC.
- **Panel G**: Image labeled as Day 14 PF-232796.
Supplementary Figure 10

[Saphenous Vein Normal Coronary Artery Plaque]

A  CCR5  Preabsorption
    Saphenous vein

B  No primary  No primary  Overlay

C  No secondary  No secondary  Overlay

D  CCR5  Preabsorption
    Normal CA

E  CCR5  Preabsorption
    CA Plaque

F  CCR5  vWF  Overlay
    Normal Coronary Vein

G  CCR5  smuA  Overlay

H  CCR5  vWF  Overlay

I  CCR5  smuA  Overlay
Supplementary Figure 11

**Panel B:**
- % Specific Binding
- $0.1\text{mM} \left[{^{125}}\text{I}\right] \text{CCL4}$
- **KD = 55.3 ± 11.6 nM**

**Panel C:**
- % Specific Binding
- $0.1\text{mM} \left[{^{125}}\text{I}\right] \text{CCL4}$
- **KD = 0.69 nM**
- $\alpha = 0.56$
