(54) Title: TREATING CARDIOVASCULAR DISEASE

(57) Abstract: A method for treating cardiovascular disease comprises applying peripheral blood from a patient or subject to an apheresis column loaded with a solid support comprising one or more binding reagents capable of specifically binding to a chemokine receptor, optionally the chemokine receptor CCR2, CCR1 and/or CCR7 immobilized directly or indirectly on the support thus removing chemokine receptor, optionally CCR2, CCR1 and/or CCR7 expressing cells from the peripheral blood of the patient or subject. Various companion diagnostic methods and useful binding reagents are also described.
TREATING CARDIOVASCULAR DISEASE

FIELD OF THE INVENTION
The various embodiments of the present invention relates to products for and methods of treating inflammatory conditions, such as cardiovascular disease, including arteriosclerosis and in particular atherosclerosis. Companion diagnostics are also described.

BACKGROUND OF THE INVENTION
Heart or cardiovascular diseases are the class of diseases that involve the heart or blood vessels. Thus disease may relate to the arteries and/or veins.

Obesity and diabetes mellitus are often linked to cardiovascular disease. C-reactive protein (CRP) is a common inflammatory marker that has been found to be present in increased levels in patients at risk for cardiovascular disease. Also osteoprotegerin, which is involved with regulation of a key inflammatory transcription factor called NF-κB has been found to be a risk factor of cardiovascular disease and mortality.

Atherosclerosis is a condition in which fatty material collects along the walls of arteries. This fatty material thickens, hardens to form a plaque, and may eventually block the arteries resulting in myocardial infarction. Atherosclerosis is a type of arteriosclerosis (which refers to any stiffening of the arteries).

Pieces of plaque can break off and move through the affected artery to smaller blood vessels, blocking them and causing tissue damage or death (embolization). This is a common cause of heart attack and stroke. Blood clots can also form around a tear (fissure) in the plaque leading to blocked blood flow. If the clot moves into an artery in the heart, lungs, or brain, it can cause a stroke, heart attack, or pulmonary embolism. In some cases, the atherosclerotic plaque is associated with a weakening of the wall of an artery leading to an aneurysm.

Apheresis is a treatment used for depletion of blood components, such as antibodies, low-density lipoproteins (LDL) and blood cells. Leukapheresis is the apheresis treatment used for removal of white blood cells, leukocytes. The patient is connected to an extracorporeal blood circulating system; the blood is drawn from a vein in one arm, passed through a column device and returned into the other arm of the patient. WO2010/029317 describes apheresis columns useful for treating inflammatory conditions including a chemokine immobilised on a solid support.

SUMMARY OF THE INVENTION
Chemokines are a class of cytokine molecules involved in cell recruitment and activation in inflammation. Chemokines cause chemotaxis and activation of various subpopulations of cells in the immune system. The activity of chemokines is mediated primarily through tight binding to their receptors on the surface of leukocytes. In certain embodiments the present
invention is based on the realisation that the interaction between chemokines and cells expressing their receptors may be exploited for the treatment of specific inflammatory cardiovascular disease. In particular, various cardiovascular diseases, such as arteriosclerosis, in particular atherosclerosis, include an inflammatory component. The inventors have determined that targeting increased recruitment of specific chemokine receptor-expressing cells to the site of inflammation presents a new therapeutic approach to treat such conditions. Moreover, in such conditions, chemokine receptor expression on each cell may be increased again providing a therapeutic approach to treat such conditions. It is shown herein that subjects suffering from atherosclerosis exhibit increased frequency of chemokine receptor expressing cells in the peripheral blood, in particular CCR1 expressing monocytes, compared to healthy controls. It is also shown herein that the CCR1 cells can be removed using a suitable binding reagent, in particular RANTES (in biotinylated form) immobilized on a suitable matrix. Similarly, it is shown herein that CCR2-expressing cells can be depleted in atherosclerosis patients using a suitable binding reagent, in particular MCP-1, in biotinylated form, immobilized on a suitable matrix.

Thus, in certain embodiments the invention serves to reduce the recruitment of inflammatory leukocytes, which express characteristic chemokine receptors, and possibly express characteristic chemokine receptors at increased levels, to sites of inflammation linked to cardiovascular diseases, such as arteriosclerosis, in particular atherosclerosis. This is achieved using specific binding reagents to capture specific chemokine receptor-expressing inflammatory leukocytes from the patient. Accordingly, in certain embodiments the invention provides in a first aspect a method for treating cardiovascular disease comprising applying peripheral blood from a patient to an apheresis column loaded with a solid support comprising one or more binding reagents capable of specifically binding to a chemokine receptor, in particular a chemokine receptor selected from CCR1, CCR2 and CCR7, immobilized directly or indirectly on the support thus removing chemokine receptor, in particular CCR1, CCR2 and CCR7, expressing cells from the peripheral blood of the patient. The peripheral blood from which the chemokine receptor expressing cells have been removed may then be returned to the patient in order to complete the treatment. The invention may thus rely on a continuous extracorporeal circuit in some embodiments. Alternatively, the invention may comprise steps of obtaining peripheral blood from the patient, applying the peripheral blood to the column and subsequently returning the peripheral blood from which the chemokine receptor expressing cells have been removed to the patient.

As shown herein, suitable binding reagents can be immobilized onto a solid support, either directly or indirectly, to generate an apheresis column suitable for capturing relevant chemokine receptor-expressing cells. Where increased levels of chemokine receptor expression are observed, such cells may be preferably removed from the peripheral blood using the columns of the various embodiments of the invention in some embodiments. Thus, the methods of the various embodiments of the invention may preferably target CCR1\textsuperscript{hi}, CCR2\textsuperscript{hi} and/or CCR7\textsuperscript{hi} cells as defined herein for removal from the peripheral blood. "High"
expression may be determined according to standard flow cytometry techniques. The level is measured relative to levels of expression of the chemokine receptor in cells taken from a healthy subject. The attached Figure 12 provides an example of a gating strategy.

Herein, reference to CCR1, CCR2 and/or CCR7 is intended to encompass selection of any one or more, up to all, of the chemokine receptors listed. In addition, the combination of CCR2 and/or CCR7 is explicitly contemplated as a separate grouping, to include any one or more of CCR2 and CCR7.

In other embodiments the invention further provides one or more binding reagents capable of specifically binding to a chemokine receptor, in particular to a chemokine receptor/chemokine receptor selected from CCR1, CCR2 and CCR7, for use in the treatment of cardiovascular disease, wherein the binding reagent is immobilized, directly or indirectly, on a solid support contained within an apheresis column, to which is applied peripheral blood from a patient thus removing chemokine receptor/CCR1, CCR2 and/or CCR7 expressing cells from the peripheral blood of the patient. In certain embodiments the invention also provides for use of a binding reagent capable of specifically binding to a chemokine receptor/the chemokine receptor CCR1, CCR2 and/or CCR7 for use in the manufacture of an apheresis column for treatment of cardiovascular disease, wherein the binding reagent is immobilized on a solid support contained within the apheresis column, to which is applied peripheral blood from a patient thus removing chemokine receptor/CCR1, CCR2 and/or CCR7 expressing cells from the peripheral blood of the patient.

All embodiments described in respect of the methods of treatment of the various embodiments of the invention apply to these aspects *mutatis mutandis* and are not repeated for reasons of conciseness. Thus, the following discussion made with reference to the methods of treatment is also applicable to the medical use aspects of the various embodiments of the invention.

In certain embodiments the invention aims to treat cardiovascular disease. MCP-1 is secreted from endothelial cells and smooth muscle cells and plays a critical role in the development of cardiovascular diseases. It has been shown (Niu and Kolattakudy. Clinical Science (2009) 117, 95–109) that MCP-1, by its chemotactic activity, causes diapedesis of monocytes from the lumen to the subendothelial space where they become foam cells, initiating fatty streak formation that leads to atherosclerotic plaque formation. Inflammatory macrophages probably play a role in plaque rupture and the resulting ischaemic episode as well as restenosis after angioplasty. There is strong evidence that MCP-1 plays a major role in myocarditis, ischaemia/reperfusion injury in the heart and in transplant rejection. MCP-1 also plays a role in cardiac repair and manifests protective effects under certain conditions.

Such protective effects may be due to the induction of protective ER (endoplasmic reticulum) stress chaperones by MCP-1. Under sustained ER stress caused by chronic exposure to MCP-1, the protection would break down resulting in the development of heart failure. MCP-1 is also involved in ischaemic angiogenesis. In mice, blocking of MCP-1 or CCR2 show
beneficial effects against cardiovascular disease. In particular, MCP-1 knockout (KO) mice or CCR2 KO mice have reduced atherosclerosis, whereas MCP-1 overexpression increases foam cell formation and atherosclerosis. Any relevant condition including an inflammatory component may be treated according to the methods of the invention. Specific conditions including an inflammatory component are thus cardiovascular disease, including arteriosclerosis and in particular atherosclerosis.

By “treatment” is meant a reduction in the specific chemokine receptor expressing cells in the peripheral blood of the patient. The reduction may comprise a reduction in cells that express chemokine receptors, in particular CCR1, CCR2 and/or CCR7, at increased levels in diseased patients. The patient is typically a human patient but the term patient may include both human and non-human animal subjects in some embodiments. In the context of the various embodiments of the present invention, this typically involves a reduction in CCR1, CCR2 and/or CCR7 expressing cells, such as “CCR1, CCR2 and/or CCR7” expressing cells, in the peripheral blood of the patient. The CCR1, CCR2 and/or CCR7 expressing cells comprise, consist essentially of or consist of monocytes, including differentiated forms thereof such as macrophages and foam cells and/or lymphocytes, in particular T-lymphocytes, and/or dendritic cells, in particular plasma dendritic cells in certain embodiments. In specific embodiments the cells removed in order to treat atherosclerosis comprise monocytes, in particular CCR1 and/or CCR2 expressing monocytes. Monocytes are produced by the bone marrow from haematopoietic stem cell precursors called monoblasts. Monocytes may differentiate into macrophages or dendritic cells. Monocytes and their macrophage and dendritic cell progeny serve a number of functions in the immune system including phagocytosis, antigen presentation and cytokine production. Monocytes may be characterized with reference to expression of the cell surface marker CD14, optionally together with CD16. Classical monocytes may be characterized by high level expression of the CD14 cell surface receptor (CD14++ CD16- monocyte). Non-classical monocytes may be characterized by low level expression of CD14 and with additional co-expression of the CD16 receptor (CD14+CD16++ monocyte). Intermediate monocytes may be characterized by high level expression of CD14 and low level expression of CD16 (CD14++CD16+ monocytes). Macrophages are derived from monocytes and are responsible for protecting tissues from foreign substances. They are cells that possess a large smooth nucleus, a large area of cytoplasm and internal vesicles for processing foreign material. The term “macrophage” may refer to a monocyte-derived cell expressing one or more of the following cell surface markers CD14, CD11b, Lysozyme M, MAC-1/MAC-3 and CD68. The term macrophage includes both activated and un-activated macrophages. Activated macrophages may be characterized by expression of one or more of CD69, ENG, FCER2 and IL2RA, HLADR, CD86. Un-activated macrophages have not yet received activating signals through for example TLR receptors and therefore they express less activation markers on the cell surface which correlates with lesser maturation. M1 macrophages may be characterized by expression of one or more of CD16+CD32+CD64+ and secrete mainly IL-23 and IL-1, TNF, IL-6 and high levels of IL-12 and in addition effector molecules such as iNOS and ROI. M1 macrophages have cytotoxic features as opposed to M2 macrophages. M2 macrophages
may be characterized by expression of one or more of SRA/B*CD163*MR*CD14* and express TGFβ, IL-10 and IL-1Ra. Tumour associated macrophages (TAMs) share many characteristics with the M2 macrophages and are considered as M2 polarised macrophages. The M1/M2 paradigm can also be found in monocyte subsets where CD14*CD16*CX3C3R1<sub>low</sub> monocytes are considered the “inflammatory” subset and the CD14<sup>high</sup>CD16<sup>low</sup>CX3C3R1<sup>high</sup> are “resident” monocytes. A critical event in atherogenesis is the focal accumulation of lipid-laden foam cells (FC) derived from macrophages (MF), smooth muscle cells, and other vascular cells with subsequent fatty streak formation. Macrophages engulf oxidized low-density lipoproteins (LDLs) by endocytosis. The oxidized LDL accumulates in the phagocytes, forming a foam cell. Characteristic markers of foam cells include CD68 and alphaVbeta3 integrin.

The three major types of lymphocyte are T cells, B cells and natural killer (NK) cells. The term “T-lymphocyte” includes CD4<sup>+</sup> T cells such as Th1 cells and Th2 cells, and CD8<sup>+</sup> T cells such as cytotoxic T cells. Th1 cells may be characterized by expression of CCR5 and/or production of IFN-γ. Th2 cells may be characterized by expression of CCR3 and/or production of IL-4.

Dendritic cells (DCs) are the most important class of “antigen presenting cells” and as such, play an central role in the activation of the immune response. Immature DCs reside in tissues throughout the body and may become activated in response to a variety of stimuli indicating the presence of antigen. Once activated, DCs can release a plethora of cytokines that activate cells of the innate immune system including eosinophils, macrophages and NK cells. Activated DCs also take up and process antigen and, as a result, actively transport antigen to secondary lymphoid organs. At these sites, antigen is presented on the surface of mature DCs, in the context of MHC class I and class II complexes, to immature T and B cells leading to both cellular and humoral immune responses.

DCs represent a heterogeneous class of cells of which there are two main subtypes: myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). These subtypes can be further divided into different subsets which may be classified according to the differential expression of a variety of cell surface markers. Importantly, the different subsets of DCs appear to drive different immune effector responses; for example, CD14<sup>+</sup> mDCs appear to specialize in the generation of humoral immunity whereas BDCA3<sup>+</sup> mDCs elicit CD8<sup>+</sup> T cell responses.

Furthermore, pDCs can be distinguished by the surface expression of CD2, and are typically involved in the generation of anti-viral immune responses as a result of rapid type I interferon production.

CCR1, CCR2 and CCR7 expressed on these aforementioned cells binds to chemokines such as monocyte chemoattractant protein-1 (MCP-1). MCP-1 is a major chemoattractant for monocytes and memory T cells by means of their binding to its specific cell-surface receptor, CC-chemokine receptor-2 (CCR2). CCR2 is the gene symbol approved by the HUGO Gene Nomenclature Committee for chemokine (C-C motif) receptor 2. The HGNC ID for this gene
is 1603. The gene is located at chromosome position 3p21. The previous symbol and name for the gene is CMKBR2. Synonyms for this gene include CC-CKR-2, CD192, CKR2, FLJ78302, MCP-1-R. The NCBI Reference Sequence is NM_001123041.2 as available on 13 June 2011, which is incorporated herein by reference in its entirety.

CCR7 is the gene symbol approved by the HUGO Gene Nomenclature Committee for chemokine (C-C motif) receptor 7. The HGNC ID for this gene is 1608. The gene is located at chromosome position 17q12-q21.2. The previous symbol and name for the gene is CMKBR7, EBi1. Synonyms for this gene include BLR2, CD197 and CDw197. The RefSeq reference sequence for CCR7 is NM_001838.3 as available on 13 June 2011, which is incorporated herein by reference in its entirety.

CCR1 is the gene symbol approved by the HUGO Gene Nomenclature Committee for chemokine (C-C motif) receptor 1. The HGNC ID for this gene is 1602. The gene is located at chromosome position 3p21. The previous symbol and name CMKBR1, SCYAR1. Synonyms for this gene include CD191, CRK-1, MIP1αR. The RefSeq reference sequence for CCR1 is NM_001295.2 as available on 13 June 2011, which is incorporated herein by reference in its entirety.

The various embodiments of the methods of the invention may involve specific binding interactions with any one or more of these further cell-surface markers in addition to the removal based upon binding to CCR1, CCR2 and/or CCR7. Suitable binding reagents can be prepared to specifically bind to these cell-surface markers. The discussion of CCR1, CCR2 and/or CCR7 specific binding reagents thus applies *mutatis mutandis*.

Treatment according to the various embodiments of the invention may result in alleviation or amelioration of symptoms, prevention of progression, regression of the condition, or complete recovery. Measurable parameters of successful treatment include one or more, up to all, of ECG, angiography, MRI, Technicium sestamibi scintigraphy (SPECT). In specific embodiments, a single treatment is sufficient to cause a depletion of around 10%, 20%, 30%, 40%, 50%, 60% or 70%, or higher up to 80%, 90%, 95% or more, or any range of values between and including these amounts, of the specific chemokine receptor, in particular CCR1, CCR2 and/or CCR7, expressing cells from the peripheral blood of the patient. In specific embodiments, at least around 50% depletion is achieved in a single treatment. Thus, successful treatment may be defined with reference to depletion of CCR1, CCR2 and/or CCR7 expressing cells. Treatment may lead to depletion of between approximately 100 and 500 million CCR1, CCR2 and/or CCR7 expressing cells, such as monocytes, in certain embodiments and more particularly to about 100, 150, 200, 250, 300, 350, 400, 450, or 500 million CCR1, CCR2 and/or CCR7 expressing cells.

By binding to the column through the binding reagent-chemokine receptor interaction, chemokine receptor expressing cells are immobilized. These immobilized cells express further unoccupied chemokine receptors, which may be of the same or different type to those
used for capture. These additional chemokine receptors may permit circulating chemokines which contribute to the inflammatory condition to be captured from the peripheral blood. Thus, a reduction in circulating (specific) chemokine levels may provide a measure of successful treatment.

The duration of treatment may be readily determined by one skilled in the art and will depend upon factors such as the flow rate of the peripheral blood. Duration of treatment may be tied into monitoring of the treatment itself, with the treatment considered complete once a measurable parameter of treatment has reached a defined threshold. Any suitable parameter may be employed as discussed herein. Thus, for example, treatment may be considered complete when a reduction in CCR1, CCR2 and/or CCR7 expressing cells, such as a 50% reduction in CCR1, CCR2 and/or CCR7 expressing cells, has been achieved. The apheresis system may be operated at a flow rate of around 10-80 mL/min, or more specifically between around 20-70 mL/min, or between around 30-60 mL/min. In specific embodiments, the treatment is performed for a period of around 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120 etc., or any range of values between and including these amounts, minutes. The treatment is typically not aimed to remove all of the cells expressing the chemokine receptor in the peripheral blood, as a basal level of those cells is required in healthy subjects. However, it has been found that only low blood volumes need to be applied to the columns of the various embodiments of the invention in order to achieve effective levels of depletion of the chemokine receptor-expressing cells. Thus, in certain embodiments, around 10-80% or more specifically around 20, 30, 40 or 50%, or any range of values between and including these amounts, of the patient’s blood is applied to the column in a single treatment. The volume of blood circulated through the apheresis column or system may be in the region of around 1000-3000ml, such as around 1000, 1200, 1400, 1600, 1800 or 2000ml or any range of values between and including these amounts. The treatment may be considered complete once this volume of blood has been circulated. The patient may be administered anticoagulants prior to each treatment session. A suitable solution, such as a sterile saline solution, optionally including an anticoagulant such as Heparin, may be used for priming the apheresis (extracorporeal) system. An additional volume of anticoagulant may be added to the circuit at the start of each treatment session, for example as a bolus injection.

In certain embodiments the invention relies upon a binding reagent which is capable of specifically binding to a chemokine receptor. This specific binding reaction permits cells expressing the chemokine receptor to be removed from the peripheral blood of the patient when the blood is passed over the solid support upon or within which the binding reagent is immobilized. Specific chemokine receptors of interest include CCR1, CCR2 and CCR7. The binding reagent can be any binding reagent capable of specifically binding to the receptor in question. By “specific binding” is meant that the binding reagent displays sufficient specificity of binding and appropriate binding affinity/kinetics to permit removal of cells expressing CCR1, CCR2 and/or CCR7 from the peripheral blood. Whilst it is not precluded that the binding reagent is capable of binding to other molecules, such as other chemokine receptors,
the binding reagent will preferentially bind to cells expressing CCR1, CCR2 and/or CCR7 and in particular to cells expressing increased levels of CCR1, CCR2 and/or CCR7 (as defined further herein). The binding reagent capable of specifically binding to CCR1, CCR2 and/or CCR7 may be either an agonist or an antagonist of CCR1, CCR2 and/or CCR7. As the disease condition relies upon up-regulation of expression of or signaling via CCR1, CCR2 and/or CCR7, in certain embodiments the binding reagent capable of specifically binding to CCR1, CCR2 and/or CCR7 is an antagonist of CCR1, CCR2 and/or CCR7. Chemokines are typically, although not necessarily exclusively (particularly in the case of truncated or modified forms) agonists of their cognate receptor and serve to activate the cells expressing the relevant receptor, as would be appreciated by one skilled in the art. Antibodies against the relevant chemokine receptor are generally considered to be antagonists, as would be appreciated by one skilled in the art. Specific examples of binding reagents include proteins or polypeptides, such as antibodies and receptor ligands, in particular chemokines. The binding reagent may be a nucleic acid molecule in certain embodiments. In some embodiments, the nucleic acid is an aptamer. Nucleic acid aptamers are polynucleotides of approximately 15-40 nucleotides long. Nucleic acid aptamers can be made using the SELEX process (systemic evolution of ligands by exponential enrichment) or any other process known to those of skill in the art.

In other embodiments, the binding reagent may be a peptide, and in certain instances, a peptide aptamer. Peptide aptamers are artificial recognition molecules that consist of a variable peptide sequence inserted into a constant scaffold protein (Baines IC, Colas P. Peptide aptamers as guides for small molecule drug discovery. Drug Discov Today. 2006;11:334–341, incorporated herein by reference). A number of methodologies, such as phage display, ribosome display and yeast two-hybrid screening systems are available for screening a library of potential peptide-based binding agents. Similarly protein scaffolds based on domains such as fibronectins, ankyrin repeats, protein A, SH3 domains, lipocalins, and ubiquitin can be used as the binding agent. Again a number of technologies such as phage display and ribosome display are available for screening a library of protein – based binding agents. Similarly, libraries of candidate chemical compounds can be screened for specific binding to the relevant chemokine receptor using suitable screening techniques known in the art, which may be high throughput screens in certain embodiments. The candidate binding agent may be immobilized on a solid support and the ability of the agent to specifically retain cells expressing the chemokine receptor of interest or labelled chemokine receptors determined. A range of cell types may be applied to the solid supports to confirm specificity of binding, or alternatively a mixed sample (such as peripheral blood) may be applied to the solid support. Retention of the cell type of interest (expressing the appropriate chemokine receptor) can be confirmed to identify suitable binding agents. A range of small-molecule antagonists of CCR-2 are discussed by Xia M and Sui Z in Expert Opin Ther Pat. 2009 Mar;19(3):295-303 - Recent developments in CCR2 antagonists, and incorporated herein by reference.
In the context of the various embodiments of the present invention the term “chemokine” also comprises biotinylated or otherwise labelled chemokines. The term “chemokine” also comprises modified and truncated versions of the chemokine and chemokine fragments with the proviso that the modified or truncated form retains its ability to bind to its cognate receptor (and thus remains functional in the context of the various embodiments of the invention). The chemokine does not necessarily need to retain biological activity as it is specific binding affinity for CCR1, CCR2 and/or CCR7 that is required. In certain embodiments, the chemokine lacks biological activity, for example in terms of activation of the (CCR1, CCR2 and/or CCR7) receptor. Modifications may be made to improve protein synthesis, for example uniformity of product and yield. As known to those skilled in the art, exemplary modifications may comprise amino acid additions, substitutions, deletions or other modifications to one or more amino acids in the chemokine. Modifications may comprise substitution of the wild type amino acid with non-natural amino acids such as norleucine (NLeu) and derivatized amino acids such as pyroglutamic acid (pyrGlu). Such modifications may be made to minimize side-product formation during storage and use of the columns of the various embodiments of the invention. Modifications may be made to improve labelling, for example inclusion of a polyethylene glycol (PEG) spacer to facilitate biotinylation. The biotinylation and/or conjugation with fluorochromes or other labelling groups of the chemokine is performed in a manner which does not substantially affect the receptor binding capacity. Site specific biotinylation or other labelling is preferred as non-selective labelling of chemokines may compromise receptor binding activity. Bioinylation or other labelling is generally preferred at or towards the C-terminus of the protein as the inventors have found that modifications in this area are generally well tolerated (in terms of a minimal effect on receptor binding capability). Biotinylation may be carried out site-specifically at any suitable amino acid. Examples of suitable amino acids include lysine, diaminopropionic acid and ornithine. Truncations may involve deletion of either N or C terminal amino acids as appropriate, or both. Typically, the truncated version will retain the residues required for the chemokine to fold correctly, for example to retain a chemokine fold structure, consistent with the requirement that a truncated version must retain the ability to bind to the relevant receptor (expressed by (on the surface of) a leukocyte). Chemokine molecules typically include disulphide bonds between the 1st and 3rd and 2nd and 4th cysteine residues respectively, as would be understood by one skilled in the art. Where sequences are presented herein, it is assumed that these disulphide bonds will form in the folded protein (unless otherwise stated). Truncated versions may comprise anywhere between 1 and 100 less amino acids, such as 1, 2, 3, 4, 5 etc amino acids, than the wild type amino acid sequence in certain embodiments. Of course, truncated versions may comprise further modification as detailed herein. The modified or truncated version may have 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more overall amino acid sequence identity with the full length wild type chemokine (where a deletion is counted as a difference in amino acid sequence) in certain embodiments. Over the common sequence between the molecules (i.e the amino acids that have not been deleted), there may be 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% amino acid sequence identity in certain embodiments. Sequence identity may be determined using known algorithms, such as BLAST or GAP analysis (GCG...
Program), (applying default settings), which are freely available. Chemokines may lack the N-terminal signal peptide which is cleaved off during synthesis in vivo. Biotinylation may be carried out site-specifically at any suitable amino acid. Examples of suitable amino acids include lysine, diaminopropionic acid and ornithine. Generally, reference may be made to Natarajan S et al, Int. J. Pept. Protein Res., 1992, 40, 567-74; Baumeister B, Int. J. Peptide Res. And Therapeutics, 2005, 11, 139-141; Bioconjugate techniques 2nd edition, Greg T. Hermanson, incorporated by reference herein in its entirety.

Specific chemokines useful in the present various embodiments of the invention include MCP-1, MCP-2, MCP-3, MCP-4 and MCP-5. Both MCP-1 and MCP-5 bind solely to the chemokine receptor CCR2 and so these chemokines may be preferred in some embodiments. Each chemokine is able to bind to a chemokine receptor implicated in a cardiovascular disease associated disorder or condition. More specifically, each of MCP-1, MCP-2, MCP-3, MCP-4 and MCP-5 are useful for removing CCR2 expressing cells from the blood of a patient. CCL19 (MIP-3beta) and CCL21 (SLC) each bind to the chemokine receptor CCR7 and may be useful in the present invention. CCL5 (RANTES) binds to CCR1 and may be useful in the various embodiments of the present invention.

The modified and truncated chemokines described in greater detail herein (with reference to the relevant amino acid sequences, as set forth in the SEQ ID NOs and accompanying experimental examples) may each be applied according to the present invention. Such modified forms may instruct the skilled person regarding additional modified forms of the same and other chemokines which may be suitable for use in the invention.

Chemokines show variable sequence homology varying from less than 20% to over 90% but all share very similar tertiary structures consisting of a disordered N-terminus, followed by a long loop (the N-loop) that ends in a βα helix, a 3-stranded β-sheet and a C-terminal helix. The overall topology is stabilised by disulphide bonds. This common tertiary structure is a common feature of the chemokine protein family (Fernandez EJ and Lolis E., Annu. Rev. Pharmacol. Toxicol., 2002, 42, 469-99; Allen SJ et al, Annu. Rev. Immunol., 2007, 25, 787-820, incorporated herein by reference).


Truncations at the C-terminus of the chemokine can also be made and maintain receptor binding activity (Treating Inflammatory Disorders, Ola Winqvist and Graham Cotton, WO2010/029317, incorporated herein by reference in its entirety).

In other embodiments, fragments and variants of chemokines are used in the devices and methods as disclosed herein. More particularly, such fragments and variants retain the ability
to specifically bind to their cognate chemokine receptor. Chemokines are known by those skilled in the art to share specific receptor binding domains, including a similar monomeric fold, characterized, for example, by a disordered amino-terminal domain, followed by a conserved core region, consisting of the so called “N-loop,” three anti-parallel β-strands, and a carboxyl-terminal α-helix. While not being bound by theory, it is believed that the chemokine-chemokine receptor interaction is a two-step mechanism, in which the core of the chemokine interacts first with a binding site formed by the extracellular domains of the receptor, while another interaction is formed between the chemokine N terminus and a second binding site on the receptor in order to trigger receptor activation. Thus, a “fragment,” such as a functional fragment of a chemokine is intended to mean a portion of the amino acid sequence of the protein that retains binding for its cognate receptor. The fragment may include, for example, the monomeric fold region, or portions thereof such as the amino-terminal domain, the conserved core region and/or the “N-loop,” the anti-parallel β-strands, and/or the carboxyl-terminal α-helix or combinations and portions thereof.

Further, it is recognized that a polypeptide can be considerably mutated without materially altering one or more of the polypeptide’s functions, for example, without altering specific binding and/or the folding of the protein. The genetic code is well known to be degenerate, and thus different codons encode the same amino acids. Even where an amino acid substitution is introduced, the mutation can be conservative and have no material impact on the essential functions of a protein (see for example, Stryer, Biochemistry 4th Ed., W. Freeman & Co., New York, NY, 1995). This includes, for example, the ability of the protein to bind and interact with other proteins, such as a truncated chemokine binding to its cognate receptor.

In some examples, part of a polypeptide chain can be deleted without impairing or eliminating all of its functions. For example, the deletion of between about 1 and about 20 amino acids on the C- and/or N-terminus, such as deletions of about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acids at the C- and/or N-terminus, can result in a chemokine that retains function, such as specific binding of its cognate receptor. Such truncations can retain the full function of an entire protein, and/or can allow for retained functions such as protein-protein interactions as in the case of ligand-receptor interactions. Chemokines having deletions of a small number of amino acids, for example, less than about 20% (such as less than about 18%, less than about 15%, less than about 10%, less than about 8%, less than about 5%, less than about 2%, or less than about 1%) of the total number of amino acids in the wild type chemokine can also be used in the methods and devices disclosed herein. Moreover, insertions or additions can be made in the polypeptide chain for example, adding epitope tags, without impairing or eliminating its functions (Ausubel et al., Current Protocols in Molecular Biology, Greene Publ. Assoc. and Wiley-Intersciences, 1998). Other modifications that can be made without materially impairing one or more functions of a polypeptide include, for example, in vivo or in vitro chemical and biochemical modifications or the incorporation of unusual amino acids. In some examples, a functional fragment of a chemokine may consist of about 10 or more, about 25 or more, about 50 or
more, about 75 or more, about 100 or more, about 125 or more, about 150, about 175 or more, or about more or 200 or more amino acid residues of a chemokine amino acid sequence.

In some examples, the chemokine or a functional fragment thereof has an amino acid that has at least about 60% or 65% sequence identity, about 70% or 75% sequence identity, about 80% or 85% sequence identity, about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity over its full length as compared to a reference sequence, such as those detailed herein, for example using the NCBI Blast 2.0 gapped BLAST set to default parameters. Alignment may also be performed manually by inspection. One or more conservative amino acid modifications can also be made in the chemokine amino acid sequence, whether an addition, deletion or modification, that does not substantially alter the 3-dimensional structure of the polypeptide or its ability to bind to the cognate receptor. For example, a conservative amino acid substitution does not affect the ability of the chemokine to specifically bind its cognate receptor. Conservative substitution tables providing functionally similar amino acids are well known in the art. The following six groups each contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Serine (S), Threonine (T); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

Peptides, such as chemokines and fragments thereof, can be modified by a variety of chemical techniques to produce derivatives having essentially the same activity or function—such as binding to a cognate receptor—as the unmodified peptides, and optionally having other desirable properties. For example, carboxylic acid groups of the protein, whether carboxyl-terminal or side chain, may be provided in the form of a salt of a pharmaceutically-acceptable cation or esterified to form a C1-C16 ester, or converted to an amide of formula NR1R2 wherein R1 and R2 are each independently H or C1-C16 alkyl, or combined to form a heterocyclic ring, such as a 5- or 6- membered ring. Amine groups of the peptide, whether amino-terminal or side chain, may be in the form of a pharmaceutically-acceptable acid addition salt, such as the HCl, HBr, acetic, benzoic, toluene sulfonic, maleic, tartaric and other organic salts, or may be modified to C1-C16 alkyl or dialkyl amino or further converted to an amide.

Hydroxyl groups of the peptide side chains can be converted to C1-C16 alkoxy or to a C1-C16 ester using well-recognized techniques. Phenyl and phenolic rings of the peptide side chains can be substituted with one or more halogen atoms, such as F, Cl, Br or I, or with C1-C16 alkyl, C1-C16 alkoxy, carboxylic acids and esters thereof, or amides of such carboxylic acids. Methylene groups of the peptide side chains can be extended to homologous C2-C4 alkenes. Thioles can be protected with any one of a number of well-recognized protecting groups, such as acetal groups. Those skilled in the art will also recognize methods for introducing cyclic structures into the peptides of this disclosure to select and provide conformational constraints to the structure that result in enhanced stability. For example, a
C- or N-terminal cysteine can be added to the peptide, so that when oxidized the peptide will contain a disulfide bond, generating a cyclic peptide. Other peptide cyclizing methods include the formation of thioethers and carboxyl- and amino-terminal amides and esters.

Peptidomimetic and organomimetic embodiments are also within the scope of the present disclosure, whereby the three-dimensional arrangement of the chemical constituents of such peptido- and organomimetics mimic the three-dimensional arrangement of the peptide backbone and component amino acid side chains, resulting in such peptido- and organomimetics of the proteins of this disclosure. For computer modelling applications, a pharmacophore is an idealized, three-dimensional definition of the structural requirements for biological activity. Peptido- and organomimetics can be designed to fit each pharmacophore with current computer modeling software (using computer assisted drug design or CADD). See Walters, "Computer-Assisted Modeling of Drugs", in Klegerman & Groves, eds., 1993, Pharmaceutical Biotechnology, Interpharm Press: Buffalo Grove, IL, pp. 165 174 and Principles of Pharmacology Munson (ed.) 1995, Ch. 102, for descriptions of techniques used in CADD. Also included within the scope of the disclosure are mimetics prepared using such techniques.


CCL2 is the gene symbol approved by the HUGO Gene Nomenclature Committee for chemokine (C-C motif) ligand 2, also known as MCP-1. The HGNC ID for this gene is 10618. The gene is located at chromosome position 17q11.2-q21.1. The previous symbol and name for the gene is SCYA2 "small inducible cytokine A2 (monocyte chemotactic protein 1, homologus to mouse Sig-je)". Synonyms for this gene include GDCF-2, HG11, MCP1, MGC9434, SMC-CF, "monocyte chemoattractant protein-1", "monocyte chemotactic and activating factor", "monocyte chemotactc protein 1, homologous to mouse Sig-je", "monocyte secretory protein JE", "small inducible cytokine subfamily A (Cys-Cys), member 2". The Genbank reference sequence for CCL2 is BC009716.1 as available on 13 June 2011, which is incorporated herein by reference in its entirety.
CCL8 is the gene symbol approved by the HUGO Gene Nomenclature Committee for chemokine (C-C motif) ligand 8, also known as MCP-2. The HGNC ID for this gene is 10635. The gene is located at chromosome position 17q11.2. The previous symbol and name for the gene is SCYA8, "small inducible cytokine subfamily A (Cys-Cys), member 8 (monocyte chemotactic protein 2)". Another synonym for this gene is HC14. The Genbank reference sequence for CCL8 is X99886.1 as available on 13 June 2011, which is incorporated herein by reference in its entirety.

CCL7 is the gene symbol approved by the HUGO Gene Nomenclature Committee for chemokine (C-C motif) ligand 7, also known as MCP-3. The HGNC ID for this gene is 10634. The gene is located at chromosome position 17q11.2-q12. The previous symbol and name for the gene is SCYA6, SCYA7, "small inducible cytokine A7 (monocyte chemotactic protein 3)". Synonyms for this gene include FIC, MARC, MCP-3, MCP3, NC28, "monocyte chemoattractant protein 3", "monocyte chemotactic protein 3". The Genbank reference sequence for CCL7 is AF043338 as available on 13 June 2011, which is incorporated herein by reference in its entirety.

CCL13 is the gene symbol approved by the HUGO Gene Nomenclature Committee for chemokine (C-C motif) ligand 13, also known as MCP-4. The HGNC ID for this gene is 10634. The gene is located at chromosome position 17q11.2-q12. The previous symbol and name for the gene is SCYA6, SCYA7, "small inducible cytokine A7 (monocyte chemotactic protein 3)". Synonyms for this gene include FIC, MARC, MCP-3, MCP3, NC28, "monocyte chemoattractant protein 3", "monocyte chemotactic protein 3". The Genbank reference sequence for CCL13 is AJ001634 as available on 13 June 2011, which is incorporated herein by reference in its entirety.

MCP-5 is a mouse chemokine in the CC chemokine family. It is also known as Chemokine (C-C motif) ligand 12 (CCL12) and, due to its similarity with the human chemokine MCP-1, sometimes it is called MCP-1-related chemokine. The gene for MCP-5 is found in a cluster of CC chemokines on mouse chromosome 11. The NCBI reference sequence for CCL12 is NC_000077.5 as available on 13 June 2011, which is incorporated herein by reference in its entirety.

CCL19 is the gene symbol approved by the HUGO Gene Nomenclature Committee for chemokine (C-C motif) ligand 19, also known as MIP-3b. The HGNC ID for this gene is 10617. The gene is located at chromosome position 9p13. The previous symbol and name for the gene is SCYA19, "small inducible cytokine subfamily A (Cys-Cys), member 19". Synonyms for this gene include "beta chemokine exodus-3", "CC chemokine ligand 19", "CK beta-11", CKb11, "EB1-ligand chemokine", ELC, exodus-3, "macrophage inflammatory protein 3-beta", MIP-3b. The Genbank reference sequence for CCL19 is AB000887.1 as available on 13 June 2011, which is incorporated herein by reference in its entirety.
CCL5 is the gene symbol approved by the HUGO Gene Nomenclature Committee for chemokine (C-C motif) ligand 5, also known as RANTES. The HGNC ID for this gene is 10632. The gene is located at chromosome position 17q11.2-q12. The previous symbol and name for the gene is D17S136E, SCYA5, "small inducible cytokine A5 (RANTES)". Synonyms for this gene include "beta-chemokine RANTES", MGC17164, RANTES, "regulated upon activation, normally T-expressed, and presumably secreted", "SIS-delta", SISd, "small inducible cytokine subfamily A (Cys-Cys), member 5", "T-cell specific protein p288", "T-cell specific RANTES protein", TCP228. The Genbank reference sequence for CCL5 is AF043341.1 as available on 13 June 2011, which is incorporated herein by reference in its entirety.

CCL21 is the gene symbol approved by the HUGO Gene Nomenclature Committee for chemokine (C-C motif) ligand 21. The HGNC ID for this gene is 10620. The gene is located at chromosome position 9p13. The previous symbol and name for the gene is SCYA21, "small inducible cytokine subfamily A (Cys-Cys), member 21". Synonyms for this gene include 6CKine, "beta chemokine exodus-2", CKb9, ECL, "Efficient Chemoattractant for Lymphocytes", exodus-2, "secondary lymphoid tissue chemokine", SLC, TCA4. The Genbank reference sequence for CCL21 is AB002409.1 as available on 13 June 2011, which is incorporated herein by reference in its entirety.

Examples of suitable modified chemokines of the various embodiments of the invention containing modifications and/or truncations and specifically adapted for use in the invention are described in detail herein. MCP-1 has been produced with residue 75, which may be a lysine, as the site of biotinylation on the chemokine (numbering based upon the mature protein having the amino acid sequence of SEQ ID NO: 2). Biotinylation permits immobilization of MCP-1 on a solid support (via a biotin-avidin interaction). The basic amino acid sequence of MCP-1, including a 23 amino acid leader sequence is set forth as SEQ ID NO: 1. The amino acid sequence of the mature protein is set forth as SEQ ID NO: 2. The inventors have determined that chemokines may display improved binding properties where the chemokine is biotinylated via a spacer group. The spacer may prevent the biotin group from impacting on the binding affinity of the chemokine. Any suitable spacer group may be employed. Further modifications may provide the molecule with advantageous properties. The invention also relates to derivatives of truncated MCP-1 chemokines. The amino acid sequence of the truncated version is set forth as SEQ ID NO:3.

Accordingly, the invention also provides a modified MCP-1 chemokine comprising, consisting essentially of or consisting of the amino acid sequence set forth as SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3 in which one or more of the following modifications have been made: a) the glutamine residue 1 of SEQ ID NO: 2 has been replaced with pyroglutamine
b) the C terminus is produced as an amide derivative (this may be achieved by synthesis on an amide linker)
c) the (C terminal region) residue at position 98 of SEQ ID NO: 1 or position 75 of SEQ ID NO:2 or position 67 of SEQ ID NO: 3, which may be a lysine or ornithine residue, is
biotinylated, optionally via a spacer group, in order to permit immobilization of the chemokine on a solid support; and/or
d) the methionine residue at position 87 of SEQ ID NO: 1 or position 64 of SEQ ID NO: 2 or position 56 of SEQ ID NO: 3 has been replaced with norleucine.

The (C terminal region) amino acid, which may be a lysine residue or a functional equivalent, at position 98 of SEQ ID NO: 1 or position 75 of SEQ ID NO: 2 or position 67 of SEQ ID NO: 3 may be biotinylated via a suitable spacer group, such as a polyethylene glycol (PEG) spacer group, in order to permit immobilization of the chemokine on a solid support. In specific embodiments, the PEG spacer is 3,6-dioxo aminooctanoic acid. The sequence and biotinylation of the modified MCP-1 chemokines of the invention are shown in figures 7 to 9 respectively. The modified MCP-1 chemokines may be agonists or antagonists of CCR2 activity. They can be tested for activity in a suitable assay, such as cell-based assays. In particular, agonist and antagonist properties may be determined in functional cell-based assay on human CCR2 receptor.

MCP-5 only binds CCR2 and should be selective in its removal of CCR2 expressing cells. The full length amino acid sequence, including the signal peptide, is set forth as SEQ ID NO: 4. The amino acid sequence of N-terminal processed MCP-5 chemokine is 82 amino acids long and is set forth as SEQ ID NO: 5. An amino acid sequence alignment suggests that MCP-5 has a C-terminal extension when compared to the amino acid sequence of MCP-1. The results of this alignment are shown in figure 10. C-terminal truncated versions of MCP-5 can thus be synthesised. This truncated version will comprise, consist essentially of or consist of MCP-5 residues 1-76, set forth as SEQ ID NO: 6.

Accordingly, the invention also provides a modified MCP-5 chemokine comprising the amino acid sequence set forth as SEQ ID NO: 4, SEQ ID NO: 5 or SEQ ID NO: 6 in which the isoleucine residue at position 97 of SEQ ID NO: 4 or at position 75 of SEQ ID NO: 5 or SEQ ID NO: 6 has been replaced with lysine. In certain embodiments, the modified MCP-5 chemokine comprises, consists essentially of or consists of the amino acid sequence of SEQ ID NO: 7. The modified MCP-5 chemokine may be biotinylated at the lysine (or a functional equivalent) residue at position 97 of SEQ ID NO: 4 or at position 75 of SEQ ID NO: 5 or SEQ ID NO: 6. Biotinylation may be via a suitable spacer group. Specific examples of the spacer group include a PEG spacer, optionally 3,6-dioxo aminooctanoic acid. In some embodiments, the C terminus is produced as an amide derivative. This may be achieved by synthesis on an amide linker. In certain embodiments, the modified MCP-5 chemokine comprises, consists essentially of or consists of the sequence and biotinylation shown in figure 11. The modified MCP-5 chemokine may be an agonist or an antagonist of CCR2 activity. Chemokines and modified chemokines can be tested for activity in a suitable assay, such as cell-based assays. In particular, agonist and antagonist properties may be determined in a functional cell-based assay on human CCR2 receptor.
An example of a chemokine of the various embodiments of the invention containing modifications and specifically adapted for use in the invention is described in detail herein (see Example 3 below). The modified CCL8 (MCP-2) corresponds to residues 1 to 76 of the full length mature protein (and lacks the N-terminal signal peptide of 23 amino acids, which is cleaved off) and thus retains the chemokine fold. The Gln at the N-terminus of the protein is subject to pyroGlu formation under physiological conditions. Thus Gln1 of the sequence may thus be substituted with pyroglutamine to prevent mixed species of N-terminal Gln and pyroGlu being generated (SEQ ID NO: 14). This improves the yield of synthesis and ensures a homogeneous chemokine preparation through column manufacture and use.

FmocLys(ivDde)-OH is incorporated as residue 75 to facilitate site-specific labelling at this position of the protein (SEQ ID NO: 15). The naturally occurring lysine at position 75 is modified through biotinylation. A PEG spacer may be incorporated between the ε-amino functionality and the biotin (SEQ ID NO: 16).

Thus, in certain embodiments the invention also relates to a modified chemokine comprising, consisting essentially of or consisting of the amino acid sequence of SEQ ID NO: 14:

\[
\text{XPDSVSIPICTCFVINRKLPIQRLESYTRITNQCPKEAVIFKTKRKGKEVCDPKE RWVRSMKHDQIFQNLXP}
\]

X1 = pyroGlu (but may remain as Gln in some embodiments)

X75 = an amino acid residue that can be biotinylated, such as lysine, ornithine or dLaminopropionic acid and optionally is biotinylated, optionally via a spacer molecule such as PEG, e.g. K(PEG-Biotin).

Or SEQ ID NO: 16

\[
\text{XPDSVSIPICTCFVINRKLPIQRLESYTRITNQCPKEAVIFKTKRKGKEVCDPKE RWVRSMKHDQIFQNLXP}
\]

X1 = pyroGlu (but may remain as Gln in some embodiments)

X75 = K(PEG-Biotin).

A further example of a chemokine of the various embodiments of the invention containing modifications and specifically adapted for use in the invention is described in detail herein (see Example 4 below). The modified CCL5 (RANTES) corresponds to residues 1 to 68 of the full length mature protein (and lacks the N-terminal signal peptide of 23 amino acids, which is cleaved off) and thus retains the chemokine fold. The single methionine (Met67) within the sequence is mutated to lysine, to mitigate against oxidation of this residue during the chain assembly (SEQ ID NO: 11). This Met to Lys substitution provides a lysine at position 67 which can be modified through biotinylation. FmocLys(ivDde)-OH is incorporated as residue 67 to facilitate site-specific labelling at this position of the protein (SEQ ID NO: 12). The biotinylated version comprises, consists essentially of or consists of the amino acid sequence of SEQ ID NO: 13.

Thus, in other embodiments the invention also relates to a modified chemokine comprising, consisting essentially of or consisting of the amino acid sequence of SEQ ID NO: 13:
SPYSSDTPCCCFAYARPLPRAHIKEYFYTSGKCSNPAVVFVTXKRKNRQVC
ANPEKKWVREYINLEX

X is an amino acid residue that can be biotinylated, such as lysine, ornithine or
diaminopropionic acid and optionally is biotinylated, optionally via a spacer molecule such as
PEG (e.g. K(Biotin))

A further example of a chemokine of the various embodiments of the invention containing
modifications and specifically adapted for use in the invention is described in detail herein
(see Example below). The modified CCL2 (MCP-1) corresponds to residues 1 to 76 of the
full length mature protein (and lacks the N-terminal signal peptide of 23 amino acids, which is
cleaved off) and thus retains the chemokine fold. The Gln at the N-terminus of the protein
(Gln1) is substituted with pyroglutamine to prevent mixed species of N-terminal Gln and
pyroGlu being generated (SEQ ID NO: 8). This improves the yield of synthesis and ensures a
homogeneous chemokine preparation through column manufacture and use.
FmocLys(ivDde)-OH is incorporated as residue 75 to facilitate site-specific labelling at this
position of the protein (SEQ ID NO: 9). A suitable spacer, such as a PEG spacer, may be
incorporated between the ε-amino functionality and the biotin. The biotinylated version
comprises, consists essentially of or consists of the amino acid sequence of SEQ ID NO: 10.

Thus, in other embodiments the invention also relates to a modified chemokine comprising,
consisting essentially of or consisting of the amino acid sequence of:
SEQ ID NO:8:
ADPKQKWVDHMHLKDQTPKT
X = pyroGlu or Gln
And/or SEQ ID NO: 10:
ADPKQKWVDHMHLKDQTPKT
X1 = pyroGlu or Gln
X75 is an amino acid residue that can be biotinylated, such as lysine, ornithine or
diaminopropionic acid and optionally is biotinylated, optionally via a spacer molecule such as
PEG, optionally K(PEG-Biotin)

A further example of a chemokine of the various embodiments of the invention containing
modifications and specifically adapted for use in the invention is described in detail herein
(see Example 9 below). The modified CCL19 (MIP-3β) corresponds to residues 1 to 77 of
the full length mature protein (and lacks the N-terminal signal peptide of 21 amino acids,
which is cleaved off) and thus retains the chemokine fold. An additional lysine is inserted at
the C-terminus, at position 78. The chemokine may thus comprise, consist essentially of or
consist of the amino acid sequence of SEQ ID NO: 17. FmocLys(ivDde)-OH is incorporated
as residue 78 to facilitate site-specific labelling at this position of the protein (SEQ ID NO:
18). The ε-amino side chain functionality of Lys(78) is modified through biotinylation. The
final protein may thus comprise, consist essentially of or consist of the amino acid sequence of SEQ ID NO: 19.

Thus, in other embodiments the invention also relates to a modified chemokine comprising, consisting essentially of or consisting of the amino acid sequence of SEQ ID NO: 17 or 19:

SEQ ID NO: 17
GTNDAEDCCLSVTQKPPIPQYIVRNFHLYLKDGRVPAVVFTTLRGRQLCA
PPDQPWVERIIQLRQRTSAKMKRRSSX

X is an amino acid residue that can be biotinylated, such as lysine, ornithine or diaminopropionic acid and optionally is biotinylated (e.g. K-biotin), optionally via a spacer molecule such as PEG, in particular K(PEG-Biotin)

SEQ ID NO: 19
GTNDAEDCCLSVTQKPPIPQYIVRNFHLYLKDGRVPAVVFTTLRGRQLCA
PPDQPWVERIIQLRQRTSAKMKRRSSX
X is K(Biotin)

Chemokines useful in the various embodiments of the invention may be synthesised through any suitable means known in the art. Preferably, the chemokines are chemically synthesised as this facilitates modification and labelling etc. However, recombinant DNA based approaches may also be employed in combination with appropriate labelling and modification technologies as required. Thus, in certain embodiments the invention also provides a nucleic acid molecule encoding the chemokines of the various embodiments of the invention. In certain embodiments the invention also relates to a vector containing such a nucleic acid molecule and a host cell containing the vector. The vector may additionally comprise a suitable promoter operably linked to the nucleic acid molecule, to facilitate transcription of the corresponding mRNA molecule. The host cell may be capable of expressing the protein by transcription and translation of the nucleic acid molecule encoding a chemokine of the various embodiments of the invention.

The chemokines useful in the various embodiments of the invention can be biotinylated by methods known in the art such as described in WO 00/50088 A2, which is incorporated herein by reference in its entirety. As indicated above, site-specific labelling of the chemokines of the various embodiments of the invention is preferable, although any labelling technique which does not significantly affect the receptor-binding capacity of the chemokine may be employed. Various site-specifically biotinylated chemokines and native chemokines are available commercially, for instance from Almac, Craigavon, UK. In specific embodiments the one or more chemokines are biotinylated via a spacer group. The spacer may be employed to prevent the biotin group from impacting on the activity of the chemokine, in particular binding of the chemokine to its cognate receptor. Any suitable spacer that facilitates retention of receptor binding properties of the chemokine may be employed in the various embodiments of the invention. Thus, in the specific embodiments described above,
spacers other than PEG spacers may be employed as appropriate. In specific embodiments, the spacer is a polyethylene glycol (PEG) spacer. PEG has been shown to be an effective spacer permitting attachment of biotin to the chemokine (which can then be immobilized on the solid support through interaction with streptavidin) without compromising receptor binding capability.

In the context of the various embodiments of the present invention the term “antibody” includes all immunoglobulins or immunoglobulin-like molecules with specific binding affinity for the relevant chemokine receptor (including by way of example and without limitation, IgA, IgD, IgE, IgG and IgM, combinations thereof, and similar molecules produced during an immune response in any vertebrate, for example, in mammals such as humans, goats, rabbits and mice). Specific immunoglobulins useful in the various embodiments of the invention include IgG isotypes. The antibodies useful in the various embodiments of the invention may be monoclonal or polyclonal in origin, but are typically monoclonal antibodies. Antibodies may be human antibodies, non-human antibodies, or humanized versions of non-human antibodies, or chimeric antibodies. Various techniques for antibody humanization are well established and any suitable technique may be employed. The term “antibody” also refers to a polypeptide ligand comprising at least a light chain or heavy chain immunoglobulin variable region which specifically recognizes and binds an epitope of an antigen, and it extends to all antibody derivatives and fragments that retain the ability to specifically bind to the relevant chemokine receptor. These derivative and fragments may include Fab fragments, F(ab')2 fragments, Fv fragments, single chain antibodies, single domain antibodies, Fc fragments etc. The term antibody encompasses antibodies comprised of both heavy and light chains, but also heavy chain (only) antibodies. In specific embodiments, the antibodies may be engineered so as to be specific for more than one chemokine receptor, for example bi-specific to permit binding to two different chemokine receptors. Suitable commercially available antibodies which bind to the chemokine receptors of interest are listed in table 1 below. They may or may not be labelled. General reference may be made to “Antibodies a laboratory manual: By E Harlow and D Lane, pp 726. Cold Spring Harbor Laboratory. 1988”, which reference is incorporated herein in its entirety.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorophore</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR1</td>
<td>Alexa Fluor 647</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CCR2</td>
<td>PerCP Cy5.5</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CCR7</td>
<td>PerCP Cy5.5</td>
<td>Biolegend</td>
</tr>
</tbody>
</table>

Table 1. Commercially available fluorophore labelled antibodies against specific chemokine receptors

Anti-CCR2 antibodies are described for example in WO 2010/021697, incorporated herein by reference. Further examples of potentially useful antibodies include MLN-1202, an anti-CCR2 monoclonal antibody currently undergoing clinical trials (Millennium Pharmaceuticals).
The chemokine receptor expressing cells may thus be targeted using alternative binding agents, such as antibodies or other chemical compounds, as defined herein, rather than the natural chemokine binding partner. This approach is a new approach to treating inflammatory conditions.

Thus, in certain embodiments the invention also provides an apheresis column loaded with a solid support comprising a binding reagent capable of specifically binding to a chemokine receptor immobilized directly or indirectly on the support to permit removal of a cell expressing the chemokine receptor from the peripheral blood of a patient/subject, wherein the binding reagent is not a chemokine. The binding reagent capable of specifically binding to the chemokine receptor may be an agonist or an antagonist of the chemokine receptor. In specific embodiments, the binding reagent capable of specifically binding to the chemokine receptor is selected from an antibody and a chemical compound.

In other embodiments the invention thus also provides a method for treating an inflammatory condition comprising applying peripheral blood from a patient/subject to an apheresis column as defined above (an apheresis column loaded with a solid support comprising a binding reagent capable of specifically binding to a chemokine receptor immobilized directly or indirectly on the support to permit removal of a cell expressing the chemokine receptor from the peripheral blood of a patient, wherein the binding reagent is not a chemokine) thus removing chemokine receptor expressing cells from the peripheral blood of the patient/subject. The method may comprise returning the blood depleted of the chemokine receptor expressing cells to the patient/subject.

Similarly, in other embodiments the invention provides a binding reagent capable of specifically binding to a chemokine receptor for use in the treatment of an inflammatory condition, wherein the binding reagent is immobilized on a solid support contained within an apheresis column as defined above (an apheresis column loaded with a solid support comprising a binding reagent capable of specifically binding to a chemokine receptor immobilized directly or indirectly on the support to permit removal of a cell expressing the chemokine receptor from the peripheral blood of a patient/subject, wherein the binding reagent is not a chemokine), to which is applied peripheral blood from a patient thus removing chemokine receptor expressing cells from the peripheral blood of the patient.

These aspects of the various embodiments of the invention may be integrated into the more focussed therapeutic aspects of the various embodiments of the invention (i.e. treating cardiovascular disease and various aspects thereof) and thus, the remainder of the disclosure, including all specific embodiments applies mutatis mutandis.

Solid support materials for immobilizing the binding reagents of the various embodiments of the invention are known in the art. “Solid support” refers to, for example, materials having a rigid or semi-rigid surface or surfaces, and may take the form of beads, resins, gels,
microspheres, or other geometric configurations. A useful support material is one that does not activate blood cells so as to make them coagulate or adhere to the support as peripheral whole blood is applied to the device. In certain embodiments, a support treated with an agent to provide it with anti-coagulation properties, in particular a heparinized support is employed. Alternatively, the blood of the patient may be treated with an anti-coagulant such as heparin prior to application to the support. Useful support materials comprise high molecular weight carbohydrates, in particular carbohydrates having a molecular weight of 100 kDa or more, such as agarose, in particulate form, optionally cross-linked, and cellulose. Other preferred support materials are polymers, such as carboxylated polystyrene, and glass. The support of the various embodiments of the invention may be provided in the form of particles or fibres. The support particles may have regular form, such as spheres or beads, or irregular form. They may be porous or non-porous. A preferred average particle size of the support is from 50 μm to 2 mm. In certain embodiments Sepharose™ , a cross linked, beaded form of agarose, is used as column matrix. It is chosen for its optimal distribution capacity and can provide a large available area for affinity binding. Solid supports may be provided in the form of magnetic beads, with the specific binding reagent immobilized on the magnetic beads. Following capture of the (CCR1, CCR2 and/or CCR7) chemokine receptor expressing cells from the blood, the beads can be removed from the blood with the aid of an appropriate magnetic separator.

Methods for immobilizing binding reagents on a solid support are known in the art. A binding reagent, such as a chemokine, antibody, peptide, nucleic acid or chemical compound, can be immobilized on the support in a direct or indirect manner. Immobilization can be by means of a suitable linker in some embodiments. A preferred method of indirect immobilization of a binding reagent, such as a chemokine, relies upon the interaction between biotin and avidin molecules. “Avidin” or “avidin molecule” refers to any type of protein that specifically binds biotin to the substantial exclusion of other (small) molecules that might be present in a biological sample. Examples of avidin include avidins that are naturally present in egg white, oilseed protein (e.g., soybean meal), and grain (e.g., corn/maize), and streptavidin, which is a protein of bacterial origin. Thus, biotinylation of the binding reagent and use of an avidin molecule such as streptavidin immobilized on the solid support allows reliable attachment of the binding reagent to the solid support according to methods known in the art. Specifically, such a method may comprise providing the binding reagent in biotinylated form, providing a solid support having streptavidin immobilized on its surface, contacting the support with an aqueous solution of the biotinylated binding reagent, and rinsing the support with an aqueous solvent. In addition, binding pair interactions, such as antibody - antigen interactions, may also be utilised for indirect immobilisation of binding reagent onto a support. In such embodiments the support may be derivatised with one member of a binding pair, such as an antibody or fragment or derivative thereof, as defined herein, which has known affinity for a particular peptide sequence or small molecule hapten. Incorporating the other member of the binding pair, such as an antigen, peptide sequence or the hapten onto or into the binding reagent facilitates immobilisation onto a solid support coated with the corresponding antibody or fragment or derivative thereof. Thus, the binding
reagent may be modified to include the peptide sequence or hapten into the linear molecule or may be added as a side chain or label. Any suitable antibody-antigen pair may be employed. The antibody fragment or derivative may be any fragment or derivative that retains specific binding affinity for the appropriate antigen. Examples include Fab, F(ab')2 fragments, scFv, VH domains, single domain antibodies (such as nanobodies), heavy chain antibodies and humanized version of non-human antibodies etc. Other high affinity interactions can be utilised for immobilisation of binding reagents, as long as the binding reagent can be synthesised or derivatised with one of the interacting partners and the solid support synthesised or derivatised with the other interacting partner without loss of binding activity (i.e. binding of the binding reagent to the appropriate chemokine receptor).

Immobilization may occur via essentially the same interaction in reverse in some embodiments. Thus, the binding reagent which may be a chemokine for example, may be attached to an antibody as defined herein, and the solid support derivatised with the antigen. The chemokine may be produced as a fusion protein with the antibody.

Alternatively binding reagents, such as chemokines and antibodies, can be immobilised directly onto a solid support using bioconjugation techniques established in the field. For example direct immobilisation of proteins onto cyanogen bromide activated solid supports via amino functionalities within the primary sequence of the protein. Alternatively, sulphhydryl functionalities within proteins can be used to directly immobilise the protein to alkyl halide derivatised supports or supports containing free thiol functionalities. In further embodiments, proteins containing α-thioester functionalities can be directly immobilised on supports containing 1,2 amino thiol moieties (eg N-terminal cysteine) using the native chemical ligation reaction. Alternatively proteins modified with ketones and aldehydes can be immobilised on solid supports derivatised with hydrazinyl, hydrazide and aminooxy functionalities using hydrazone / oxime bond forming ligation reactions (and vice versa). Alternatively ‘Click’ chemistry can be used to immobilise proteins onto solid supports, whereby the protein and the support are derivatised with the appropriate mutually reactive chemical functionalities (azides and alkynes). In other embodiments Staudinger ligation chemistry can be used to immobilise appropriately derivatised proteins onto the appropriately derivatised solid supports.

The solid support is contained within or carried by the apheresis column. Thus, by “loaded” is meant that the column carries or contains the solid support in a manner such that (peripheral) blood can flow through the column in contact with the solid support. Thus, the solid support provides a matrix within the column through which blood flows, in continuous fashion in certain embodiments. This permits cells expressing the specific chemokine receptor to be removed from the blood passing through the column, such that blood exiting the column is depleted of the specific chemokine receptor-expressing cells. In specific embodiments, the apheresis column is loaded with a support comprising streptavidin immobilized on the support and one or more biotinylated binding reagents, such as chemokines, bound to the streptavidin on the support. The solid support may be comprised of a high-molecular weight carbohydrate, optionally cross-linked, such as agarose.
As discussed above, the binding reagent is coupled to the solid support. The relative amounts of binding reagent may be controlled to ensure that coupling between the solid support and the binding reagent will be immediate, minimising the risk of binding reagent decoupling from the solid support. Thus, it may be ensured that there is a relative excess of immobilization sites for the binding reagent on the solid support. Alternatively, or additionally, following immobilization of the binding reagent on the solid support, the solid support may be washed to remove any unbound binding reagent.

The apheresis column utilised in the various embodiments of the present invention acts as a leukapheresis treatment for cardiovascular disease. The column acts to specifically remove CCR1, CCR2 and/or CCR7-expressing monocytes by exploiting the interaction between CCR1, CCR2 and/or CCR7 expressed on the cell surface and a specific binding reagent immobilized on a solid support contained within or carried by the column. The overall column typically comprises, consists of, or consists essentially of three combined components: 1) a housing which contains or carries 2) the solid support and 3) one or more binding reagents (immobilized thereon) which specifically bind one or more chemokine receptors. The housing may be manufactured from any suitable material for clinical use. In certain embodiments the housing is composed of a plastic material. The housing includes an in flow site for entry of blood and an out flow site for blood (depleted of target cells) to exit the column. The housing may be designed to maintain a continuous blood flow through the solid support matrix. The housing (as shown for example in Figure 3) may include a top portion which comprises a distribution plate (2) at the inflow site (1) to spread the blood evenly over the entire matrix area. The distribution plate may act as a first safety barrier preventing larger particles flowing through the column and into the patient. However, the distribution plate is not essential and may be removed in some embodiments to decrease the overall resistance in the system. The column may contain one or more safety filter units (3 and 4) placed at the inflow (1) and/or outflow (5) sites of the plastic housing. Such filter units may act to prevent particles larger than blood cells passing in and/or out of the column. The safety filter units may contain a plurality of filters, such as two, three or four filters designed to be a robust barrier and stop all particles larger than blood cells passing through the column. Inclusion of safety filters (3 and 4) at both ends of the column serves to minimize the risk of leakage of particles into the patient, including in the event that the device is incorrectly connected resulting in blood flow in the opposite direction to that intended. The safety filters may comprise of any suitable pore size to prevent particles larger than blood cells from passing through the column, as would be readily understood by one skilled in the art. Suitable filters are commercially available. In specific embodiments, the pore size of the filter(s) is between approximately 60µm and 100µm, more specifically approximately 80 µm. The solid support and binding reagent components are discussed in further detail herein.

The volume of the housing may be varied depending upon the blood volumes intended to pass through the column. Typically, the volume of the housing is between approximately 40ml and 200ml, more specifically 50ml to 150ml or 60ml to 120ml.
The column is generally applied in the form of an apheresis circuit. In this context, the overall system includes the apheresis column, tubing and an appropriate pump to pump the blood around the circuit. The system is illustrated in figure 4. The patient (1) is connected to the extracorporeal circuit via sterile needles to veins in the right and the left arms. A saline bag (3) is also connected and the saline solution is pumped with a suitable pump (2). Blood is drawn from one arm of the patient through the sterile tubing system by the blood pump (4) and passed through the column (6) and back to the patient. The tubing system may be connected to the column via any suitable coupling, such as standard dialysis luer-lock couplings. The couplings on the column may be colour-coded for correct assembly. For example, red tubing for inflow to the red column top and blue tubing for outflow back to the patient. An air detector (8) may be present in the circuit. Inlet pressure (5) and/or Pven sensors (7) may additionally be employed to monitor the pressure in the circuit.

An apheresis pump, such as the 4008 ADS pump manufactured by Fresenius Medical Care or the Adamonitor pump, may monitor the patient's inflow and outflow. The pump may also monitor the pressure in the extracorporeal circulation. The pump may be able to discriminate air by a bubble catcher and air detector. A clot catcher filter may be positioned inside the bubble catcher. The pump may also incorporate an optical detector to distinguish between light, e.g. saline solution or air present in the tubing system and dark e.g. blood present in the tubing system.

A schematic diagram of a suitable pump, showing the air detector and optical filter is shown in figure 5. If the pump system detects air bubbles and optical fluctuations or if extracorporeal pressure values are out of the set range, then the pump may stop immediately. Alternatively or additionally a visual/audible alarm may be emitted.

The treatment methods of the various embodiments of the invention may thus rely upon an extracorporeal circuit. The methods may be considered as *ex vivo* or *in vitro* methods and be defined solely with reference to steps performed outside of the patient. In some embodiments, however, the method further comprises, prior to application of the blood to the column, collecting peripheral blood from the patient. In a further embodiment, the method further comprises, following the application of the blood to the column, infusing the blood depleted of (CCR1, CCR2 and/or CCR7) chemokine receptor expressing cells to the patient. This is then a complete leukapheresis treatment method. Thus, a leukapheresis method, for treating a cardiovascular disease, comprises collecting peripheral blood from the patient; applying the peripheral blood to an apheresis column loaded with a solid support comprising a binding reagent capable of specifically binding to a chemokine receptor, in particular the chemokine receptor CCR1, CCR2 and/or CCR7, immobilized directly or indirectly on the support thus removing CCR1, CCR2 and/or CCR7 expressing cells from the peripheral blood of the patient; and infusing the depleted blood (of chemokine receptor expressing cells) to the patient.
The peripheral blood may be continuously collected from the patient. Similarly, the depleted blood may be continuously infused to the patient, through use of an appropriate circuit as described herein. Thus, the support may be disposed in a column through which the blood is made to flow. This may be achieved using a suitable pump for example, as also described herein. Blood flow through the column enables the binding reagent(s) immobilized on the solid support to capture the cells expressing the chemokine receptor, thus depleting them from the blood and preventing their contribution to the inflammatory cardiovascular disease.

The methods of the various embodiments of the invention and binding reagents for use in the methods of the various embodiments of the invention may require that the patient has been selected for treatment on the basis of detecting an increase in the level of chemokine receptor, in particular, CCR1, CCR2 and/or CCR7 expressing cells in a sample obtained from the patient. Such companion diagnostic methods are described in greater detail herein and are based, for example, on the observation that CCR1, CCR2 and/or CCR7 expression on specific cells may be elevated in patients with cardiovascular disease. More specifically, it is shown herein that levels of CCR1 expressing leukocytes, in particular monocytes, are increased in atherosclerosis patients (compared with healthy controls).

Thus, (in this context) in certain embodiments the invention also provides a method of diagnosing, monitoring progression of, or monitoring treatment of cardiovascular disease comprising determining:

a) the levels of the chemokine receptor CCR1, CCR2 and/or CCR7 expressing cells
b) levels of expression of CCR1, CCR2 and/or CCR7; and/or
c) levels of cells with high expression of CCR1, CCR2 and/or CCR7
in a sample obtained from a subject, wherein high levels of CCR1, CCR2 and/or CCR7 expressing cells, high levels of expression of CCR1, CCR2 and/or CCR7 or high levels of cells with high expression of CCR1, CCR2 and/or CCR7 or increased levels of CCR1, CCR2 and/or CCR7 expressing cells compared to control, increased levels of expression of CCR1, CCR2 and/or CCR7 compared to a control or increased levels of cells with high expression of CCR1, CCR2 and/or CCR7 compared to a control indicate the presence or progression of cardiovascular disease. Levels of chemokine receptor expression, as opposed to cell numbers, may also be investigated as increased levels of chemokine receptor expression per cell may also be diagnostically relevant. The cells may be monocytes such as CCR1 expressing, and possibly also CCR2 expressing, monocytes.

"Diagnosing" is defined herein to include screening for a disease/condition or pre-indication of a disease/condition, identifying a disease/condition or pre-indication of a disease/condition and checking for recurrence of disease/condition following treatment. The methods of the various embodiments of the invention may also have prognostic value, and this is included within the definition of the term "diagnosis". The prognostic value of the methods of the various embodiments of the invention may be used as a marker of potential susceptibility to cardiovascular disease by identifying levels of CCR1, CCR2 and/or CCR7 expression linked to conditions associated with that syndrome. Thus patients at risk may be identified before
the disease has a chance to manifest itself in terms of symptoms identifiable in the patient. In certain embodiments, diagnosis may be made in conjunction with other objective indicators of cardiovascular disease. Thus, in specific embodiments, diagnosis is made in conjunction with one or more of the following indicators:

Clinical indicators may be employed, as would be understood by one skilled in the art. The diagnosis of cardiovascular disease is made on patients history with exercise induced chest pain, shortness of breath. Physical examination including ECG, exercise ECG (stress test) and cardiac catheterization. Lab test include cardiac enzymes. These findings can be combined with the determination of circulating proinflammatory cells expressing coronary vessel homing cells.

"Monitoring progression of" includes performing the methods to monitor the stage and/or the state and progression of the cardiovascular disease. Monitoring progression may involve performing the diagnostic methods multiple times on the same patient to determine whether the levels of CCR1, CCR2 and/or CCR7 expressing cells are increasing, decreasing or remaining stable over a certain time period. This may be in the context of a treatment regime.

"Monitoring the success of a particular treatment" is defined to include determining the levels of CCR1, CCR2 and/or CCR7 expressing cells before and after a treatment. The treatment is generally one aimed at treating cardiovascular disease and may be a treatment according to one of the methods of the various embodiments of the invention as defined herein. Successful treatment may be determined with reference to a decrease in CCR1, CCR2 and/or CCR7 expressing cells as a result of, or following, the treatment. Thus, in such methods a level of CCR1, CCR2 and/or CCR7 expressing cells is determined prior to treatment. This level is recorded and a further assessment made at a predetermined time following the treatment. The comparison of levels of CCR1, CCR2 and/or CCR7 expressing cells permits the success of the treatment to be monitored. In specific embodiments, a single treatment is sufficient to cause a depletion of around 10%, 20%, 30%, 40%, 50%, 60% or 70%, or higher, up to 80%, 90%, 95% or more, or any range of values between and including these amounts, of the specific chemokine receptor, in particular CCR1, CCR2 and/or CCR7, expressing cells from the peripheral blood of the patient. In specific embodiments, at least around 50% depletion is achieved in a single treatment. Thus, successful treatment may be defined with reference to depletion of CCR1, CCR2 and/or CCR7 expressing cells. Treatment may lead to depletion of between approximately 100 and 500 million CCR1, CCR2 and/or CCR7 expressing cells, such as monocytes, in certain embodiments. Additional factors may be included to determine successful treatment. For example, a lack of increase in CCR1, CCR2 and/or CCR7 expressing cells following treatment may indicate successful treatment in terms of preventing further progression of the condition, optionally combined with an improvement in other markers or staging of the cardiovascular disease.

In specific embodiments, the cardiovascular disease is atherosclerosis.
The sample in which CCR1, CCR2 and/or CCR7 expressing cell levels, levels of expression of CCR1, CCR2 and/or CCR7 and/or levels of cells with high expression of CCR1, CCR2 and/or CCR7 (defined as CCR1^hi, CCR2^hi and/or CCR7^hi) are determined may comprise any suitable tissue sample or body fluid sample. Generally, the test sample is obtained from a human subject. Typically, the sample is a blood sample, in particular a peripheral blood sample. The sample may comprise an adipose tissue biopsy in certain embodiments. The methods may involve determining levels of CCR1, CCR2 and/or CCR7 expressing monocytes, macrophages, dendritic cells or lymphocytes in certain embodiments.

Levels of CCR1, CCR2 and/or CCR7 expressing cells, levels of expression of CCR1, CCR2 and/or CCR7 and/or levels of cells with high expression of CCR1, CCR2 and/or CCR7 (defined as CCR1^hi, CCR2^hi and/or CCR7^hi) may be determined according to any suitable method. For example, flow cytometry may be employed in order to determine the number of cells expressing CCR1, CCR2 and/or CCR7 in the sample, to determine levels of CCR1, CCR2 and/or CCR7 expression and/or to identify levels of CCR1^hi, CCR2^hi and/or CCR7^hi cells. Flow cytometric techniques are described herein and examples of commercially available antibodies suitably labelled for use in flow cytometry are set out in Table 1 for example. Alternatively, the method may involve steps of collecting and fixing the cells in the sample, followed by incubation with a suitable binding reagent that binds specifically to the CCR1, CCR2 and/or CCR7 chemokine receptor expressing cells in the sample. Any suitable binding reagent, as defined herein, may be employed. For example, a CCR-2 specific antibody may be employed. A wash step may be adopted following an incubation period to remove any unbound reagent. Suitable wash steps and incubation conditions would be well known to one skilled in the art. The binding reagent may be directly labeled in order to permit antibody binding to be directly determined. Alternatively a secondary binding reagent, such as an antibody, may be employed which binds to the first binding reagent and carries a label. Again, suitable incubation conditions and wash steps would be apparent to one skilled in the art. The primary and secondary binding reagents may form two halves of a binding pair. The binding interaction should not prevent the primary binding reagent binding to the CCR1, CCR2 and/or CCR7 receptor expressing cells, unless a competition assay is being employed. The two halves of a binding pair may comprise an antigen-antibody, antibody-antibody, receptor-ligand, biotin-streptavidin pair etc. in certain embodiments. Other techniques used to quantify chemokine (CCR1, CCR2 and/or CCR7) receptor expressing cell levels, to quantify levels of CCR1, CCR2 and/or CCR7 expression and/or to quantify levels of CCR1^hi, CCR2^hi and/or CCR7^hi cells include PCR-based techniques such as QT-PCR and protein based methods such as western blot. Quantitation may be achieved with reference to fixed cell lines carrying known numbers of various receptor expressing cells and/or known levels of receptor expression per cell. Such fixed cell lines are available commercially (for example ChemiScreen™ cell lines from Millipore). Methods analogous to the treatment methods of the invention may also be employed, with binding of CCR1, CCR2 and/or CCR7 expressing cells to the solid support being determined following peripheral blood being passed through the column.
The levels of CCR1, CCR2 and/or CCR7 expressing cells, levels of expression of CCR1, CCR2 and/or CCR7 and/or levels of cells with high expression of CCR1, CCR2 and/or CCR7 (defined as CCR1^{hi}, CCR2^{hi} and/or CCR7^{hi}) may be determined relative to a suitable control.

When diagnosing a cardiovascular disease, a threshold level of cells, level of expression of CCR1, CCR2 and/or CCR7 and/or level of cells with high expression of CCR1, CCR2 and/or CCR7 (defined as CCR1^{hi}, CCR2^{hi} and/or CCR7^{hi}) may be set at or over which a positive diagnosis is made. This threshold may be determined based upon measuring levels of CCR1, CCR2 and/or CCR7 expressing cells, levels of expression of CCR1, CCR2 and/or CCR7 and/or levels of cells with high expression of CCR1, CCR2 and/or CCR7 (defined as CCR1^{hi}, CCR2^{hi} and/or CCR7^{hi}) in samples obtained from diseased patients and comparing these levels with levels of CCR1, CCR2 and/or CCR7 expressing cells, levels of expression of CCR1, CCR2 and/or CCR7 and/or levels of cells with high expression of CCR1, CCR2 and/or CCR7 (defined as CCR1^{hi}, CCR2^{hi} and/or CCR7^{hi}) in samples obtained from healthy subjects.

In certain embodiments, a cardiovascular disease such as atherosclerosis is diagnosed on the basis of levels of chemokine receptor expressing cells, such as CCR1, CCR2 and/or CCR7 expressing cells. A positive diagnosis may be made in subjects based upon the presence of greater than about 10%, greater than about 20%, greater than about 30%, greater than about 40%, greater than about 50%, greater than about 55%, greater than about 60%, greater than about 65%, greater than about 70%, greater than about 75%, greater than about 80%, greater than about 85%, greater than about 90%, greater than about 95%, or more chemokine receptor expressing cells in the sample, as a percentage of total cells in the sample. In other embodiments, a cardiovascular disease such as atherosclerosis is diagnosed on the basis of the presence of a about a 1.2 fold or greater increase, such as about a 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 10, 20, 50 or 100 or greater fold increase in chemokine receptor expressing cells, relative to healthy controls.

In specific embodiments, a cardiovascular disease such as atherosclerosis is diagnosed on the basis of levels of CCR1 expressing cells, in particular monocytes. A positive diagnosis may be made in subjects based upon the presence of greater than about 70%, greater than about 75%, greater than about 80%, greater than about 85% or greater than about 90% CCR1 expressing monocytes in the sample, as a percentage of total cells in the sample. Cardiovascular disease such as atherosclerosis may also be diagnosed on the basis of the presence of about a 1.2 fold or greater increase, such as about a 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 10, 20, 50 or 100 or greater fold increase in CCR1 expressing cells, in particular monocytes, relative to healthy controls.

In certain embodiments, progression of cardiovascular disease such as atherosclerosis, which may be in the context of a treatment regime, is monitored on the basis of levels of chemokine receptor expressing cells at different time points, such as CCR1, CCR2 and/or CCR7 expressing cells. Progression of cardiovascular disease such as atherosclerosis may
be indicated in subjects based upon an increase of greater than about 10%, such as an increase of greater than about 15%, greater than about 20%, greater than about 25%, greater than about 30%, greater than about 35%, greater than about 40%, greater than about 45%, greater than about 50%, greater than about 55%, greater than about 60%, greater than about 65%, greater than about 70%, greater than about 75% or more chemokine receptor expressing cells in the sample, as a percentage of total cells in the sample, compared to a sample taken from the same subject at an earlier time point. In other embodiments, progression of cardiovascular disease such as atherosclerosis is confirmed on the basis of the presence of a about a 1.2 fold or greater increase, such as about a 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 10, 20, 50 or 100 or greater fold increase in chemokine receptor expressing cells, relative to a sample taken from the same subject at an earlier time point.

In specific embodiments, cardiovascular disease such as atherosclerosis is monitored on the basis of levels of CCR1 expressing cells, in particular monocytes. Progression of the disease, which may be in the context of a treatment regime, may be indicated in subjects based upon the presence of an increase of greater than about 10%, such as greater than about 15%, greater than about 20%, greater than about 25%, greater than about 30%, greater than about 35%, greater than about 40%, greater than about 45%, greater than about 50%, greater than about 55%, greater than about 60%, greater than about 65%, greater than about 70%, greater than about 75% or more chemokine receptor expressing cells in the sample, as a percentage of total cells in the sample, compared to a sample taken from the same subject at an earlier time point. In other embodiments, progression of cardiovascular disease such as atherosclerosis is confirmed on the basis of the presence of a about a 1.2 fold or greater increase, such as about a 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 10, 20, 50 or 100 or greater fold increase in CCR1 expressing cells, in particular monocytes, relative to a sample taken from the same subject at an earlier time point.

Regression or successful treatment may be monitored based upon similar decreases over various time points. For example, regression or successful treatment may be indicated in subjects based upon a decrease of about 10%, such as a decrease of about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75% or more chemokine receptor expressing cells in the sample, as a percentage of total cells in the sample, compared to a sample taken from the same subject at an earlier time point. In other embodiments, regression of cardiovascular disease such as atherosclerosis is confirmed on the basis of the presence of a about a 1.2 fold or greater decrease, such as about a 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 10, 20, 50 or 100 or greater fold decrease in chemokine receptor expressing cells, relative to a sample taken from the same subject at an earlier time point.

In specific embodiments, cardiovascular disease such as atherosclerosis is monitored on the basis of levels of CCR1 expressing cells, in particular monocytes. Regression or successful treatment of the disease may be made in subjects based upon a decrease of about 10%, such as such as a decrease of about 15%, about 20%, about 25%, about 30%, about 35%,
about 40%, about 45%, about 50%, about 55% or more CCR1 expressing cells, in particular monocytes in the sample, as a percentage of total cells in the sample or by a decrease of about 10%, such as a decrease of about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75% or more CCR1 expressing cells, in particular monocytes in the sample, as a percentage of total cells in the sample, compared to a sample taken from the same subject at an earlier time point. In other embodiments, regression of cardiovascular disease such as atherosclerosis is confirmed on the basis of the presence of a about a 1.2 fold or greater decrease, such as about a 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 10, 20, 50 or 100 or greater fold decrease in CCR1 expressing cells, in particular monocytes, relative to a sample taken from the same subject at an earlier time point.

Suitable software is freely available (such as the R project for statistical computing) to perform the necessary statistical analysis of the data obtained to calculate a useful threshold. The threshold may be set to maximize sensitivity and/or specificity of the test. Performance of the test in these respects may be measured by plotting a receiver operating characteristics (ROC) curve (sensitivity versus specificity). The area under the curve provides an indication of the overall performance of the test. Thus, once thresholds have been set for diagnosing the condition, a separate control experiment does not necessarily have to be run each time a sample is tested. Rather reference can simply be made to the pre-existing thresholds to determine the diagnosis. However, in certain embodiments, the sample is tested together with a control sample taken from a healthy subject to provide a comparator based upon essentially identical experimental conditions. The test sample is generally tested in parallel with the control sample. The test sample level of CCR1, CCR2 and/or CCR7 expressing cells, levels of expression of CCR1, CCR2 and/or CCR7 and/or levels of cells with high expression of CCR1, CCR2 and/or CCR7 (defined as CCR1^{hi}, CCR2^{hi} and/or CCR7^{hi}) can then be compared with that of the control sample to make the diagnosis. A control sample from a disease patient may also be tested in certain embodiments. Reference to controls permits relative levels ("high", "low" etc.) of CCR1, CCR2 and/or CCR7 expressing cells in the test sample to be readily identified and the significance thereof interpreted. Reference to controls also permits relative levels of CCR1, CCR2 and/or CCR7 expression ("high", "low" etc.) within the cell population to be determined and the significance thereof interpreted. Such determination may, for example, indicate the average levels of CCR1, CCR2 and/or CCR7 expression per cell in the test sample.

Thus, in specific embodiments, high or higher levels of CCR1, CCR2 and/or CCR7 expressing cells or high or higher levels of CCR1, CCR2 and/or CCR7 expression, for example average CCR1, CCR2 and/or CCR7 expression per cell, or high or higher levels of CCR1^{hi}, CCR2^{hi} and/or CCR7^{hi} cells correlate with active disease or more active disease associated with cardiovascular disease. Similarly, lower or low levels of CCR1, CCR2 and/or CCR7 expressing cells, or low or lower levels of CCR1, CCR2 and/or CCR7 expression, for example average CCR1, CCR2 and/or CCR7 expression per cell, or low or lower levels of CCR1^{hi}, CCR2^{hi} and/or CCR7^{hi} cells may correlate with a lack of active inflammation or
disease associated with cardiovascular disease. This may be defined as "less active
disease". It can readily be envisaged that control samples may be assessed and levels of
CCR1, CCR2 and/or CCR7 expressing cells, levels of expression of CCR1, CCR2 and/or
CCR7 and/or levels of cells with high expression of CCR1, CCR2 and/or CCR7 (defined as
CCR1\(^{hi}\), CCR2\(^{hi}\) and/or CCR7\(^{hi}\)) determined across the range of severities of cardiovascular
disease. This may assist in correlating the levels of CCR1, CCR2 and/or CCR7 expressing
cells, levels of expression of CCR1, CCR2 and/or CCR7 and/or levels of cells with high
expression of CCR1, CCR2 and/or CCR7 (defined as CCR1\(^{hi}\), CCR2\(^{hi}\) and/or CCR7\(^{hi}\)) in the
test sample with the relative severity of the condition.

When monitoring progression of, or monitoring treatment of a cardiovascular disease, the
control samples may be taken from the subject at an earlier time point. They may, however,
be based upon known reference values as discussed above. Thus, relative levels of CCR1,
CCR2 and/or CCR7 expressing cells, relative levels of CCR1, CCR2 and/or CCR7
expression including relative levels of average CCR1, CCR2 and/or CCR7 expression per
cell or relative levels of CCR1\(^{hi}\), CCR2\(^{hi}\) and/or CCR7\(^{hi}\) cells may be with reference to
samples taken from the same subject at a different point in time. In certain embodiments,
decreased levels of CCR1, CCR2 and/or CCR7 expressing cells decreased relative levels of
CCR1, CCR2 and/or CCR7 expression including decreased relative levels of average CCR1,
CCR2 and/or CCR7 expression per cell or decreased relative levels of CCR1\(^{hi}\), CCR2\(^{hi}\)
and/or CCR7\(^{hi}\) cells correlate with successful treatment. The treatment may be any suitable
treatment, but in specific embodiments is a treatment according to the various embodiments
of the invention. When monitoring progression of a cardiovascular disease, increased levels
of CCR1, CCR2 and/or CCR7 expressing cells increased relative levels of CCR1, CCR2
and/or CCR7 expression including increased relative levels of average CCR1, CCR2 and/or
CCR7 expression per cell or increased relative levels of CCR1\(^{hi}\), CCR2\(^{hi}\) and/or CCR7\(^{hi}\) cells
may indicate the progression of condition or disease. Thus, if levels of CCR1, CCR2 and/or
CCR7 expressing cells, levels of expression of CCR1, CCR2 and/or CCR7 and/or levels of
cells with high expression of CCR1, CCR2 and/or CCR7 (defined as CCR1\(^{hi}\), CCR2\(^{hi}\) and/or
CCR7\(^{hi}\)) are increased in a sample taken later than a sample from the same patient this may
indicate progression of the condition.

Since the levels of CCR1, CCR2 and/or CCR7 expressing cells, levels of CCR1, CCR2
and/or CCR7 expression or levels of CCR1\(^{hi}\), CCR2\(^{hi}\) and/or CCR7\(^{hi}\) cells are diagnostically
relevant, determining such levels in a sample obtained from a subject may influence
treatment selection for that subject. Accordingly, in certain embodiments the invention
provides a method of selecting a suitable treatment for cardiovascular disease comprising
determining:

a) the levels of the chemokine receptor CCR1, CCR2 and/or CCR7 expressing cells

b) levels of expression of CCR1, CCR2 and/or CCR7; and/or

c) levels of cells with high expression of CCR1, CCR2 and/or CCR7

in a sample obtained from a subject, wherein high levels of CCR1, CCR2 and/or CCR7
expressing cells, high levels of expression of CCR1, CCR2 and/or CCR7 or high levels of
cells with high expression of CCR1, CCR2 and/or CCR7 or increased levels of CCR1, CCR2 and/or CCR7 expressing cells compared to control, increased levels of expression of CCR1, CCR2 and/or CCR7 compared to a control or increased levels of cells with high expression of CCR1, CCR2 and/or CCR7 compared to a control, result in selection of a treatment as defined herein for treatment of the cardiovascular disease. In certain embodiments, the chemokine receptor expressing cells are high chemokine receptor expressing cells, in particular, high CCR1, CCR2 and/or CCR7 expressing cells. The cells may be monocytes such as CCR1 expressing, and possibly also CCR2 expressing, monocytes.

In specific embodiments, cardiovascular disease such as atherosclerosis is treated on the basis of measuring levels of chemokine receptor expressing cells, such as CCR1, CCR2 and/or CCR7 expressing cells. Thus, a treatment according to the various embodiments of the invention may be applied based upon the presence of greater than about 10%, greater than about 20%, greater than about 30%, greater than about 40%, greater than about 50%, greater than about 55%, greater than about 60%, greater than about 65%, greater than about 70%, greater than about 75%, greater than about 80%, greater than about 85%, greater than about 90%, greater than about 95%, or more chemokine receptor expressing cells in the sample, as a percentage of total cells in the sample. In other embodiments, cardiovascular disease such as atherosclerosis is treated according to the various embodiments of the invention on the basis of the presence of a about a 1.5 fold or greater increase, such as about a 2, 2.5, 3, 3.5, 4, 4.5, 5, 10, 20, 50 or 100 or greater fold increase in chemokine receptor expressing cells, relative to healthy controls.

In specific embodiments, cardiovascular disease such as atherosclerosis is treated on the basis of measuring levels of CCR1 expressing cells, in particular monocytes. Thus, a treatment according to the various embodiments of the invention may be applied based upon the presence of greater than about 70%, greater than about 75%, greater than about 80%, greater than about 85% or greater than about 90% CCR1 expressing monocytes in the sample, as a percentage of total cells in the sample or on the basis of the presence of a about a 1.5 fold or greater increase, such as about a 2, 2.5, 3, 3.5, 4, 4.5, 5, 10, 20, 50 or 100 or greater fold increase in CCR1 expressing cells, in particular monocytes, relative to healthy controls.

For the avoidance of doubt, all embodiments described in respect of the methods of the various embodiments of the invention apply to these aspects *mutatis mutandis* and are not repeated for reasons of conciseness. Specifically, cardiovascular disease may be indicated in conjunction with one or more of the following indicators: Clinical indicators may be employed, as would be understood by one skilled in the art. The diagnosis of cardiovascular disease is made on patients history with exercise induced chest pain, shortness of breath. Physical examination including ECG, exercise ECG (stress test) and cardiac catheterization. Lab test include cardiac enzymes. These findings can be combined with the determination of circulating proinflammatory cells expressing coronary vessel homing cells.
The cardiovascular disease may be atherosclerosis. In specific embodiments, the sample is a peripheral blood sample.

The methods and medical uses of the various embodiments of the invention thus can be tailored to the need of individual patients or groups of patients on the basis of the various diagnostic methods of the various embodiments of the invention. By removing from the circulation CCR1, CCR2 and/or CCR7 expressing cells, such as monocytes, macrophages and lymphocytes, in particular monocytes, upregulated in various inflammatory cardiovascular disease, an important factor in the inflammatory process of cardiovascular disease can be controlled. The method of the invention may be effective in treating or reversing conditions such as atherosclerosis.

The various embodiments of the invention will now be described in more detail by reference to the following non-limiting embodiments and examples:

DESCRIPTION OF THE FIGURES

FIG. 1a, 1b & 1c - the binding of biotinylated MCP-1 by CD4+, CD8+ T-cells and CD14+ monocytes respectively, obtained from peripheral blood of a healthy donor.

FIG. 2a - binding of MCP-1 to monocytes (dashed line) in peripheral blood taken from IBD patients. The graph represents a summary of four tests. FIG. 2b - binding of CCR2-antibody to monocytes (line) in peripheral blood taken from IBD patients. The graph represents a summary of four tests.

FIG. 3 - The plastic house and top showing the distribution plate (2) and safety filter units (3 and 4).

FIG. 4 - The overall leukapheresis system.

FIG. 5 - The pump with air detector and optical detector (4).

FIG. 6 - Results of in vitro depletion tests performed on the bMCP-1 coupled matrix showing ability to eliminate CCR2-expressing cells from blood from three healthy donors.

FIG. 7 - Sequence and biotinylation, via a spacer group, of mature protein MCP-1 derivative containing Gln to pyroGlu modification.

FIG. 8 - Sequence and biotinylation, via a spacer group, of mature protein MCP-1 derivative containing Gln to pyroGlu modification and Met to Norleu substitution.

FIG. 9 - Sequence and biotinylation, via a spacer group, of truncated MCP-1 derivative containing Met to Norleu substitution.
FIG. 10 - Alignment of MCP-1 and MCP-5 amino acid sequences.

FIG. 11 - Sequence and biotinylation, via a spacer group, of (C-terminal) truncated MCP-5 derivative containing Ile to Lys modification.

FIG. 12 - Example of gating criteria for CCR2 expressing monocytes.

FIG. 13 - Frequency of CCR1 expressing monocytes in 20 healthy controls and one patient with atherosclerosis (AS). The expression of chemokine receptors and specific cell markers were analysed with flow cytometry.

FIG. 14 - Binding of bRANTES to blood monocytes from a patient with atherosclerosis. The binding of chemokine and expression of specific cell markers were analysed with flow cytometry.

FIG. 15 - Depletion of CCR1 expressing monocytes with Sepharose Streptavidin-matrix conjugated with bRANTES. Blood cells from an atherosclerosis patient were incubated with biotinylated chemokine-Sepharose Streptavidin-matrix. Unbound cells were retrieved by washing the matrix. The cells (After Depletion) were then analysed with flow cytometry and compared with cells that had not been incubated with biotinylated-chemokine-matrix (Before Depletion).

FIG. 16 - Expression of CCR2 on monocytes from one patient with atherosclerosis. The expression of chemokine receptors, binding of chemokine and specific cell markers were analysed with flow cytometry.

FIG. 17 - Binding of the chemokine bMCP-1 to monocytes. Bars represent frequency of bMCP-1 binding monocytes and CCR2 expressing monocytes in blood from a patient with atherosclerosis. Blood was incubated with biotinylated chemokine and analysed with flow cytometry.

FIG. 18 - Depletion of CCR2 expressing monocytes with Sepharose Streptavidin-matrix conjugated with bMCP-1. Blood cells from a patient with atherosclerosis were incubated with biotinylated chemokine-Sepharose Streptavidin-matrix. Unbound cells were retrieved by washing the matrix. The cells (After Depletion) were then analysed with flow cytometry and compared with cells that had not been incubated with b-chemokine-matrix (Before Depletion).

DESCRIPTION OF PREFERRED EMBODIMENTS

MCP-1 is secreted from endothelial cells and smooth muscle cells and plays a critical role in the development of cardiovascular diseases. It has been shown (Niu and Kolattakudy, Clinical Science (2009) 117, 95–109) that MCP-1, by its chemotactic activity, causes
diapedesis of monocytes from the lumen to the subendothelial space where they become foam cells, initiating fatty streak formation that leads to atherosclerotic plaque formation. Inflammatory macrophages probably play a role in plaque rupture and the resulting ischaemic episode as well as restenosis after angioplasty. There is strong evidence that MCP-1 plays a major role in myocarditis, ischaemia/reperfusion injury in the heart and in transplant rejection.

MCP-1 also plays a role in cardiac repair and manifests protective effects under certain conditions. Such protective effects may be due to the induction of protective ER (endoplasmic reticulum) stress chaperones by MCP-1. Under sustained ER stress caused by chronic exposure to MCP-1, the protection would break down resulting in the development of heart failure. MCP-1 is also involved in ischaemic angiogenesis. In mice, blocking of MCP-1 or CCR2 show beneficial effects against cardiovascular disease. In particular, MCP-1 knockout (KO) mice or CCR2 KO mice have reduced atherosclerosis, whereas MCP-1 overexpression increases foam cell formation and atherosclerosis. On this basis, the inventors have selected CCR2 expressing cells as a target for treatment of cardiovascular disease using specific binding interactions with CCR2 expressing cells.

It is shown herein that subjects suffering from atherosclerosis exhibit increased frequency of chemokine receptor expressing cells in the peripheral blood, in particular CCR1 expressing monocytes, compared to healthy controls. It is also shown herein that the CCR1 cells can be removed using a suitable binding reagent, in particular RANTES (in biotinylated form) immobilized on or in a suitable matrix. Similarly, it is shown herein that CCR2-expressing cells can be depleted in atherosclerosis patients using a suitable binding reagent, in particular MCP-1, in biotinylated form, immobilized on or in a suitable matrix.

**EXAMPLES 1 and 2**

**Materials and methods**

*Isolation of peripheral blood leukocytes.* Heparinized peripheral blood from healthy blood donors was fixed with 4% paraformaldehyde for 4 minutes, hemolyzed for 15 minutes with a 0.83 % ammonium chloride solution and washed twice in FACS buffer to obtain a suspension of blood leukocytes.

*Chemokines.* The leukocytes were incubated for 30 min in the dark at 4 °C with biotinylated and Alexa647 Fluor® labeled MCP-1 (in concentrations 10 ng/μL and 50 ng/μL). The cells were then washed with FACS-buffer and analyzed by flow cytometry. All chemokines used in the Examples were provided by Almac Sciences Scotland Ltd, Edinburgh, Scotland.

*Flow cytometry assay.* The flow cytometry assay was performed on a two laser FACS Calibur cytometer (BD Immunocytometry systems, San José, Ca, USA). Ten thousand cells were counted and analysed in each sample. For data analyses, Cell Quest Pro software from Becton Dickinson was used.

**EXAMPLE 1** - *Binding of monocytes to MCP-1.* In the experiment with biotinylated MCP-1 it was found that about 90% of the monocytes obtained from peripheral blood of healthy donors
had bound to the cytokine after 30 min of incubation (Fig. 1a), whereas CD4+ and CD8+
lymphocytes had not bound (Fig. 1b and 1c).

**EXAMPLE 2** - Monocytes were investigated for their expression of CCR2 (FIG. 2b) and their
ability to bind MCP-1 (FIG. 2a). CCR2 expression was noted on all monocytes with the
majority of monocytes expressing high levels, using an anti-CCR2 antibody (FIG. 2b). The
MCP-1 binding to monocytes shown in FIG. 2a corresponds to the CCR2\textsuperscript{hi} expressing
population shown in FIG. 2b. Thus, MCP-1 binds favourably to CCR2\textsuperscript{hi} expressing cells.

**EXAMPLE 3** - Tailored leukapheresis

**COLUMN DESIGN AND PROPERTIES**

**Introduction**

Apheresis is an established treatment used for depletion of blood components, such as
antibodies, low-density lipoproteins (LDL) and blood cells. Leukapheresis is the apheresis
treatment used for removal of white blood cells, leukocytes. The patient is connected to an
extracorporeal blood circulating system; the blood is drawn from a vein in one arm, passed
through a column device and returned into the other arm of the patient. Side effects of
leukapheresis treatments are varying from mild events like headache, dizziness,
hypotension, palpitation and flush seen in 0.1 to 5% of treated patients.

**The column**

The column is intended to be used as a leukapheresis treatment for a cardiovascular
disease. It will specifically remove CCR1, CCR2 and/or CCR7-expressing leukocytes, in
particular monocytes, through the use of a binding reagent, more specifically an MCP-1,
MCP-2, MCP-3, MCP-4 and/or MCP-5 containing resin, or a CCL19 containing resin,
exploiting the CCR1, CCR2 and/or CCR7-chemokine interaction. The column consists of
three combined components, the plastic house, the streptavidin (SA) Sepharose\textsuperscript{TM} BigBeads
matrix and the binding reagent bound to the matrix. The treatment is conducted using the
same techniques as a standard apheresis procedure.

**The plastic house (FIG. 3)**

The plastic house, designed to keep a continuous blood flow through the matrix, consists of a
transparent body and red-coloured top. The top has a distribution plate (2) at the inflow site
(1) to spread the blood evenly over the entire matrix area. The plate is the first safety barrier
preventing larger particles flowing through the column and into the patient. Safety filter units
(3 and 4) are placed at the inflow (1) and outflow (5) sites of the plastic housing. The safety
filter unit contains three filters designed to be a robust barrier and stop all particles larger
than blood cells passing through the column. The plastic housing design is shown in Figure
3. The design with safety filters (3 and 4) at both ends of the column device will minimize the
risk of leakage of particles into the patient, including in the event that the device is placed up
side down with the blood flow in the opposite direction to that anticipated.

Streptavidin Sepharose\textsuperscript{TM} BigBeads
The second component in the device is the affinity matrix called streptavidin Sepharose™ BlgBeads (Sepharose™ GE Healthcare, Sweden). Sepharose™ is a cross linked, beaded-form of agarose, which is a polysaccharide extracted from seaweed. Sepharose™ and agarose are commonly used as column matrices in biomedical affinity techniques. It is chosen for its optimal distribution capacity and can provide a large available area for affinity binding.

Binding reagent
Coupled to the matrix is the third component of the device, the binding reagent that binds specifically to CCR1, CCR2 and/or CCR7. Chemokines such as MCP-1, MCP-2, MCP-3, MCP-4, MCP-5, CCL19 and/or CCL5 may be employed. These peptides may be synthetic, engineered versions of the human chemokine, which are truncated and biotinylated, but retain binding activity to the CCR1, CCR2 and/or CCR7 receptor. By biotinylating the engineered chemokine, it is able to bind to the streptavidin molecules in the Sepharose™ matrix. The biotin-streptavidin binding is known be one of the strongest biological interactions with a Kd in the order of 4 x 10^{-14} M. The calculated ratio of streptavidin:biotin binding sites in the column is 10:1. Therefore, the coupling between the matrix and chemokine will be immediate, minimising the risk of chemokine decoupling from the matrix.

The apheresis system
To conduct the leukopheresis the following components are needed; the column, tubing system, and a 4008 ADS pump (Fresenius Medical Care).

The circuit
The system is illustrated in Figure 4. The patient (1) is connected to the extracorporeal circuit via sterile Venflon needles to veins in the right and the left arms. A saline bag (3) is also connected and the saline solution is pumped with an ACD pump (2). Blood is drawn from one arm of the patient through the sterile tubing system by the blood pump (4) and passed through the column (6) and back to the patient. The tubing system is connected to the column via standard dialysis luer-lock couplings. The couplings on the column are colour-coded for correct assembly; red tubing for inflow to the red column top and blue tubing for outflow back to the patient. An air detector (8) is present. Inlet pressure(5) and Pven sensors (7) are employed to monitor the pressure in the circuit.

The 4008 ADS pump
An apheresis pump, from Fresenius Medical Care, monitors the patient's inflow and outflow, the pressure in the extracorporeal circulation and can discriminate air by a bubble catcher and air detector. A clot catcher filter is placed inside the bubble catcher. The pump also has an optical detector to distinguish between light, e.g. saline solution or air present in the tubing system and dark e.g. blood present in the tubing system.

A schematic diagram of the pump, showing the air detector and optical filter is shown in Figure 5. If the pump system detects air bubbles and optical fluctuations or if extracorporeal
pressure values are out of the set range, then the pump stops immediately and a visual/audible alarm are emitted.

Legend for FIG. 5:

1. Monitor
2. Holder for waste bag
3. Modules (left to right – Blood pump, ACD pump, Air detector)
4. Reserve places for further modules
5. Absorber holder
6. Drip detector
7. IV pole

Preparation of the patient
The patient will be administered anticoagulants prior to each treatment session. A sterile saline solution with 5000 IE Heparin will be used for priming the extracorporeal system, thereafter a bolus injection with 4000 IE Heparin will be added into the circuit at the start of each treatment session.

Leukapheresis time and flow rate
The apheresis system should be operated at a flow rate of 30-60 mL/min. A treatment is finalised after 1800mL of blood has been circulated.

Storage conditions
The column devices should be stored between 1 and 25°C avoiding freezing and more elevated temperatures. Stability data > 3 months indicate no difference in functionality over time or by temperature (room temperature and refrigerated). The columns will be kept in refrigerated conditions until use. Mechanical damage as those resulting from violent vibrations and trauma should be avoided. Column stored outside of these recommendations should not be used.

Transport conditions
The column devices will be transported under refrigerated condition, avoiding freezing and more elevated temperatures. Mechanical damage such as those resulting from violent vibrations and trauma should be avoided.

In-vitro depletion of target cell populations
To investigate the ability to eliminate CCR2-expressing cells, in vitro tests have been performed on the bMCP-1 coupled matrix. Blood was collected from blood donors and passed through the column device containing bMCP-1 coupled matrix. Blood samples were taken before and after column passage and analyzed by flow cytometry (FACS) for the depletion of CCR2-expressing cells.

The results demonstrate significant depletion of the target population CCR2-expressing monocytes post matrix perfusion. Depletion tests were performed on blood from three healthy donors. The results are shown in Figure 6.
In conclusion, the in-vitro results demonstrate a specific reduction of up to 80% of the CCR2-expressing cells by the column. Notably, individuals with fewer CCR2 expressing cells initially achieved lower depletion. The remaining levels of monocytes were around 20-30% in each case, irrespective of the starting point. Non-CCR2-expressing cells remained unaffected (data not shown).

**EXAMPLE 4 - MCP1 DERIVATIVES**

MCP-1 has been produced with residue 75 as the site of biotinylation on the chemokine (numbering based upon the mature protein having the amino acid sequence of SEQ ID NO: 2). Biotinylation permits immobilization of MCP-1 on a solid support (via a biotin-avidin interaction). The basic amino acid sequence of MCP-1, including a 23 amino acid leader sequence is set forth as SEQ ID NO: 1,

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MKVSAAALLCLLLIAATFIPOQLAQPDAINAPVTCCYNFTNRKISVQRLASYRRITSSKCPKEAVIFKTIVAIECADPKQKWVQDSMDHLDKQTQTPKT
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The amino acid sequence of the mature protein is set forth as SEQ ID NO: 2,

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QPDAINAPVTCCYNFTNRKISVQRLASYRRITSSKCPKEAVIFKTIVAIECADPKQKWVQDSMDHLDKQTQTPKT
```

The inventors have determined that chemokines may display improved binding properties where the chemokine is biotinylated via a spacer group. The spacer may prevent the biotin group from impacting on the binding affinity of the chemokine.

Thus, MCP-1 derivatised at the ε-amino side chain functionality of Lys75 with PEG-Biotin (TFA salt) will be synthesised. The PEG spacer will be 3,6-dioxoaminooctanoic acid. The Gln at the N-terminus of the proteins is subject to pyroGlu formation under physiological conditions. Thus the first glutamine (Gln1) of the sequence will be substituted with pyroglutamate. The molecule will be synthesised as a C-terminal amide (via synthesis on an amide linker). The molecule is shown schematically in Figure 7.

A biotinMCP-1 Met to Nleu analogue will also be synthesised. The single methionine within the sequence will be altered to Norleucine, to mitigate against oxidation of this residue during the chain assembly and improve stability of the final product. This molecule is shown schematically in Figure 8.

Once synthesised, the activity of the various biotinMCP-1 derivatives will be determined in cell-based assays. In particular, agonist and antagonist properties will be determined in aequorin functional cell-based assay on human CCR2 receptor.

**EXAMPLE 5 - SYNTHESIS OF A CCR2 ANTAGONIST BIOTINMCP-1 WHICH BINDS TO THE RECEPTOR WITHOUT ACTIVATION**
Antagonist Activity (J-H Gong and I. Clark-Lewis, J. Exp. Med., 1995, 181, 63) has been shown for an MCP-1 derivative truncated at the N-terminus. In particular, deletion of residues 1-8, results in binding to CCR2 with Kd 8.3 nM. This protein was unable to cause chemotaxis of CCR2 positive cells. (inhibition of chemotaxis IC50 20nM)

The amino acid sequence of the truncated version is set forth as SED ID NO: 3:
VTCCYNFTN RKISVQRLAS YRRITSSKCP KEAVIFKTIV AKEICADPKQ KWVQDSMDHL DKQTQTPKT

A derivative of this truncated version will be synthesised comprising residues 9 to 76 of the mature protein (MCP-1 9-76) with Met64 to Nleu substitution and derivatised at the ε-amino side chain functionality of Lys75 with PEG-Biotin (TFA salt). This molecule is shown schematically in Figure 9. The PEG spacer will be 3,6, - dioxaaminooctanoic acid.

Once synthesised, the activity of the various biotinMCP-1 derivatives will be determined in cell-based assays. In particular, agonist and antagonist properties will be determined in aequorin functional cell-based assay on human CCR2 receptor.

EXAMPLE 6 - DEMONSTRATE REMOVAL OF CCR2 EXPRESSING CELLS USING AN ALTERNATIVE CHEMOKINE LIGAND TO MCP-1

CCR2 also binds chemokines MCP-2, MCP-3, MCP-4, MCP-5, and HCC-4 in addition to MCP-1. MCP-5 only binds CCR2 and should be selective in its removal of CCR2 expressing cells. MCP5 is a mouse chemokine shown to chemotact human CCR2 cells with EC50 < 3 nM.

The full length amino acid sequence, including the signal peptide, is set forth as SEQ ID NO: 4
MKISTLLCLL LIATTISPOQ LAGPDAVSTP VTCCYNVVKQ KIHVRKLKSY RRITSSQCPR EAVIFRTILD KEICADPKEK WVKNSINHLD KTSQTFILEP SCLG

The amino acid sequence of N-terminal processed MCP-5 chemokine is 82 amino acids long and is set forth as SEQ ID NO: 5
GPDAVSTP VTCCYNVVKQ KIHVRKLKSY RRITSSQCPR EAVIFRTILD KEICADPKEK WVKNSINHLD KTSQTFILEP SCLG

An amino acid sequence alignment suggests that MCP-5 has a C-terminal extension when compared to the amino acid sequence of MCP-1. The results of this alignment are shown in figure 10. On this basis a C-terminal truncated version of MCP-5 will be synthesised. This truncated version will comprise MCP-5 residues 1-76, set forth as SEQ ID NO: 6:
GPDAVSTP VTCCYNVVKQ KIHVRKLKSY RRITSSQCPR EAVIFRTILD KEICADPKEK WVKNSINHLD KTSQTFIL
In the truncated version, Ile75 to be substituted with Lys, set forth as SEQ ID NO: 7:
GPDAVSTP VTCCYNVKQ KIHVRKLKSY RRITSSQCPR EAVIFRTILD KEICADPKEK
WVKNSINHLD KTSQTFKIL

Following substitution, the substituted version will be biotinylated at position 75, a lysine or other suitable residue such as ornithine or diaminopropanoic acid via a PEG spacer (3,6-dioxoaminooctanoic acid). The protein will be synthesised on an amide linker to yield a C-terminal amide derivative. This molecule is shown schematically in Figure 11.

Once synthesised, the activity of the various biotinMCP-5 derivatives will be determined in cell-based assays. In particular, agonist and antagonist properties will be determined in (aequorin) functional cell-based assay on human CCR2 receptor.

**EXAMPLES 7 to 10 - Chemokine Synthesis – GENERAL PROTOCOLS**

Assembly:
Chemical synthesis of chemokines was performed using standard Fmoc solid phase peptides synthesis (SPPS) techniques on an ABI 433 peptide synthesiser. DIC (0.5 M in DMF) and OxymaPure (0.5 M in DMF) were used for activation, acetic anhydride (0.5 M in DMF) for capping, and 20% piperidine in DMF for Fmoc deprotection. Rink Amide resin was utilised for the generation of C-terminal amide chemokines and Wang resin for C-terminal acid chemokines. After assembly, the resin was washed with DMF and DCM and then dried *in vacuo*.

Removal of Dde Protection:
The Dde protecting group was removed by treatment of resin with a solution of 2.5% hydrazine in DMF (200ml) over a 2 hour period. The resin was then washed with DMF.

Labelling steps:
1. **Couple Fmoc-8-amino-3,6-dioctanoic acid (PEG)**
   Resin was swollen in DMF and then a solution of Fmoc-8-amino-3,6-dioctanoic acid (0.38g, 1mmol), DIC solution (2ml, 0.5 M in DMF) and OxymaPure solution (2ml, 0.5 M in DMF) was added. The mixture was sonicated for 3 hours and then washed with DMF.
2. **Capping**
The resin was capped with acetic anhydride solution (0.5 M in DMF, 10ml) for 5 minutes and then washed with DMF.
3. **Fmoc deprotection**
   Fmoc deprotection was carried out by treatment with 20% piperidine in DMF solution (2 x 50ml) for 15 minutes each. The resin was washed with DMF.
4. **Couple Biotin-OSu**
A solution of Biotin-OSu (341 mg, 1 mmol) and DIPEA (348 μl) in DMF (10 ml) was added to the resin and the mixture was sonicated for 3 hours. The resin was washed thoroughly with DMF and DCM then dried in vacuo.

5 Cleavage:
Dry resin was treated with TFA (10 ml) containing a scavenger cocktail consisting of TIS (500 μl), thioanisole (500 μl), water (500 μl), DMS (500 μl), EDT (250 μl), NH₄I (500 μg) and phenol (500 μg) and the mixture was stirred at room temperature for 5 hours. The solution was filtered into cold ether and the resin rinsed with TFA. The precipitated peptide was centrifuged, washed with ether, centrifuged and lyophilised.

10 Purification Protocol:
The crude peptide was purified by reverse phase HPLC (RP-HPLC) using a Jupiter C18, 250 x 21 mm column, 9 ml/min, eluting with an optimised gradient [Buffer A: water containing 0.1% TFA, Buffer B: acetonitrile containing 0.1% TFA].

Folding Protocol:
Pure peptide (10 mg) was dissolved into 6 M GSHCl (16 ml) and then rapidly diluted to 2 M GSHCl concentration by the addition of 50 mM TRIS pH 8.5 (84 ml) containing 0.3 mM GSSG and 3 mM GSH. The mixture was stirred at room temperature for 24 hours and then analysed by RP-HPLC (Jupiter C18, 250 x 4.6 mm column, 10-60% B over 30 minutes. Purification by RP-HPLC using an optimised gradient afforded the desired product.

EXAMPLE 7 – biotinMCP-1 (CCL2)
25 Target Molecule: MCP-1 derivatised at the ε-amino side chain functionality of Lys(75) with PEG-Biotin (TFA salt)

Modifications: Human MCP-1 corresponding to residues 1-76, is initially expressed as 99 amino acids comprising the chemokine fold, and a 23 amino acid signal peptide which is cleaved off. The Gin at the N-terminus of the protein is subject to pyroGlu formation under physiological conditions. Thus Gin1 of the sequence was substituted with pyroglutamine to prevent mixed species of N-terminal Gin and pyroGlu being generated. This improves the yield of synthesis and ensures a homogeneous chemokine preparation through column manufacture and use. The naturally occurring lysine at position 75 was modified through biotinylation on the resin. A PEG spacer was incorporated between the ε-amino functionality and the biotin.

35 The linear amino acid sequence (SEQ ID NO: 8) is shown, prior to attachment of the PEG spacer and biotin molecules at amino acid 75 (K):

40 H-XPDAINAPVTCCYNFTNRKISQRLASYRRITSSKCPKEAVIFKTIVAKEIC ADPKQKWQDSMDHLKDQTQTPKT-NH₂
X = pyroGlu or Gin
The engineered MCP-1 sequence was assembled on a solid support (Rink Amide resin), using Fmoc protocols for solid-phase peptide synthesis as described in the general protocols section:

SEQ ID NO: 9
H-XPDAINAPVTCCYNFTNRRKISVQRALASYRRTSSKCPKEAVIFKTIVAKEIC
ADPKQKWVGDSMDHLKDQTPXT-RESIN
X1 = pyroGlu or Gln

X75 = K(ivDde)

FmocLys(ivDde)-OH was incorporated as residue 75 to facilitate site-specific labelling at this position of the protein. Subsequent removal of the ivDde protecting group, followed by coupling of the PEG spacer and Biotin, was carried out as described in the general protocol section. Cleavage, purification and folding protocols were carried out as described to furnish the desired active chemokine.

SEQ ID NO: 10
H-XPDAINAPVTCCYNFTNRRKISVQRALASYRRTSSKCPKEAVIFKTIVAKEIC
ADPKQKWVGDSMDHLKDQTPXT-NH₂
X1 = pyroGlu or Gln

X75 is an amino acid residue that can be biotinylated, such as lysine, ornithine or diaminopropanic acid and optionally is biotinylated, optionally via a spacer molecule such as PEG, optionally K(PEG-Biotin)

Electrospray ionisation with tandem mass spectrometry (ESI-TOF-MS) data of purified folded biotinMCP-1: obtained = 9032.8 Da; expected 9034.4 Da.

Functional Assay Data:

biotinMCP-1 was tested for agonist activity in an Aequorin assay against hCCR2b, (Euroscreen) and an EC₅₀ value of 9.6nM was reported. c.f. EC₅₀ for recombinant native MCP-1 is 3.1nM.

EXAMPLE 8 – biotinRANTES (CCL5)

Target Molecule: RANTES derivatised at the ε-amino side chain functionality of Lys(67) with Biotin (TFA salt)

Modifications: Human RANTES corresponding to residues 1-68, is initially expressed as 91 amino acids comprising the chemokine fold, and a 23 amino acid signal peptide which is cleaved off. The single methionine (Met67) within the sequence was mutated to lysine, to mitigate against oxidation of this residue during the chain assembly, which was observed during the synthesis of the natural sequence derivative. This Met to Lys substitution provided a lysine at position 67 which was modified through biotinylation on the resin.
The linear amino acid sequence (SEQ ID NO: 11) is shown, prior to attachment of the biotin molecule at amino acid 67 (K):

5  H-SPYSSDTTPCCFAYIARPLPRAHKEYFYTSGKCSNPAAVVFVTRKNRQVC
    ANPEKKWVREYINSLEKS-OH

The engineered RANTES sequence was assembled on a solid support (Wang resin), using Fmoc protocols for solid-phase peptide synthesis as described in the general protocols section:

10  H-SPYSSDTTPCCFAYIARPLPRAHKEYFYTSGKCSNPAAVVFVTRKNRQVC
    ANPEKKWVREYINSLEXS-RESIN
    X is K(ivDde)

15  FmocLys(ivDde)-OH was incorporated as residue 67 to facilitate site-specific labelling at this position of the protein (SEQ ID NO: 12). Subsequent removal of the ivDde protecting group, followed by coupling of the Biotin, was carried out as described in the general protocol section. Cleavage, purification and folding protocols were carried out as described to furnish the desired active chemokine (SEQ ID NO: 13).

20  H-SPYSSDTTPCCFAYIARPLPRAHKEYFYTSGKCSNPAAVVFVTRKNRQVC
    ANPEKKWVREYINSLEXS-OH
    X is an amino acid residue that can be biotinylation, such as lysine, ornithine or diaminopropionic acid and optionally is biotinylated, optionally via a spacer molecule such as PEG (e.g. K(Biotin))

Electrospray ionisation with tandem mass spectrometry (ESI-TOF-MS) data of purified folded biotinRANTES: obtained = 8068.9 Da; expected 8070.2 Da.

30  Functional Assay Data:
    BiotinRANTES was tested for agonist activity in an Aequorin assay against hCCR5, (Euroscreen) and an EC50 value of 0.5nM was reported.

35  EXAMPLE 9 – biotinMCP-2 (CCL8)
    Target Molecule: MCP-2 derivatised at the ε-amino side chain functionality of Lys(75) with PEG-Biotin (TFA salt)

    Modifications: Human MCP-2 corresponding to residues 1-76, is initially expressed as 99 amino acids comprising the chemokine fold, and a 23 amino acid signal peptide which is cleaved off. The Gin at the N-terminus of the protein is subject to pyroGlu formation under physiological conditions. Thus Gln1 of the sequence was substituted with pyroglutamine to prevent mixed species of N-terminal Gln and pyroGlu being generated. This improves the
yield of synthesis and ensures a homogeneous chemokine preparation through column manufacture and use. The naturally occurring lysine at position 75 was modified through biotinylation on the resin. A PEG spacer was incorporated between the ε-amino functionality and the biotin.

The linear amino acid sequence (SEQ ID NO: 14) is shown, prior to attachment of the PEG spacer and biotin molecules at amino acid 75 (K):

H-XPDSVSIPIITCCFNVINRKPQRLQYESLYTRITNIQCPKEAVIFKTKRGKEVCA DPKE
RWVRDMKHLDQIFQNLFKP-NH$_2$

X = pyroGlu or Gln

The engineered MCP-2 sequence was assembled on a solid support (Rink Amide resin), using Fmoc protocols for solid-phase peptide synthesis as described in the general protocols section:

H-XPDSVSIPIITCCFNVINRKPQRLQYESLYTRITNIQCPKEAVIFKTKRGKEVCA DPKE
RWVRDMKHLDQIFQNLFKP-NH$_2$

X1 = pyroGlu or Gln

X75 = K(ivDde)

FmocLys(ivDde)-OH was incorporated as residue 75 to facilitate site-specific labelling at this position of the protein (SEQ ID NO: 15). Subsequent removal of the ivDde protecting group, followed by coupling of the PEG spacer and Biotin, was carried out as described in the general protocol section. Cleavage, purification and folding protocols were carried out as described to furnish the desired active chemokine (SEQ ID NO: 16):

H-XPDSVSIPIITCCFNVINRKPQRLQYESLYTRITNIQCPKEAVIFKTKRGKEVCA DPKE
RWVRDMKHLDQIFQNLFKP-NH$_2$

X1 = pyroGlu or Gln

X75 = an amino acid residue that can be biotinylated, such as lysine, ornithine or diaminopropionic acid and optionally is biotinylated, optionally via a spacer molecule such as PEG, e.g. K(PEG-Biotin).

Electrospray ionisation with tandem mass spectrometry (ESI-TOF-MS) data of purified folded biotinMCP-2: obtained = 9263.6 Da; expected 9263.8 Da.

Functional Assay Data:
biotinMCP-2 was tested for activity in an Aequorin assay against hCCR2b, (Euroscreen) and was shown to be a partial agonist with an EC50 value of 50.9nM. c.f. EC50 for recombinant native MCP-2 is 23.5nM (partial agonist).
Example 10 – biotinMIP-3β (CCL19)
Target Molecule: MIP-3β derivatised at the ε-amino side chain functionality of Lys(78) with Biotin (TFA salt)

Modifications: Human MIP-3β corresponding to residues 1-77, is initially expressed as 98 amino acids comprising the chemokine fold, and a 21 amino acid signal peptide which is cleaved off. An additional lysine was inserted at the C-terminus, at position 78, and modified through biotinylation on the resin.

The linear amino acid sequence (SEQ ID NO: 17) is shown, prior to attachment of the biotin molecule at amino acid 78 (K):

H-GTNDAECCLSVTQKIPGIVRNFHILKIDGCRVPAVVFTTLRGRQLCA

PPDQPWWRIQRLQRTSAMKRRSSX-NH₂
X is an amino acid residue that can be biotinylated, such as lysine, ornithine or diaminopropionic acid and optionally is biotinylated (e.g. K-biotin), optionally via a spacer molecule such as PEG, in particular K(PEG-Biotin)

The engineered MIP-3β sequence was assembled on a solid support (Rink Amide resin), using Fmoc protocols for solid-phase peptide synthesis as described in the general protocols section:

H-GTNDAECCLSVTQKIPGIVRNFHILKIDGCRVPAVVFTTLRGRQLCA

PPDQPWWRIQRLQRTSAMKRRSSX-RESIN
X is FmocLys(ivDde)

FmocLys(ivDde)-OH was incorporated as residue 78 to facilitate site-specific labelling at this position of the protein (SEQ ID NO: 18). Subsequent removal of the ivDde protecting group, followed by coupling of the Biotin, was carried out as described in the general protocol section. Cleavage, purification and folding protocols were carried out as described to furnish the desired active chemokine (SEQ ID NO: 19).

H-GTNDAECCLSVTQKIPGIVRNFHILKIDGCRVPAVVFTTLRGRQLCA

PPDQPWWRIQRLQRTSAMKRRSSX-NH₂
X is K(Biotin)

Electrospray ionisation with tandem mass spectrometry (ESI-TOF-MS) data of purified folded biotinMIP-3β: obtained = 9148.8 Da; expected 9149.7 Da.

Functional Assay Data:
biotinMip-3β was tested for agonist activity in an Aequorin assay against hCCR7, (Euroscreen) and an EC50 value of 11.0 nM was reported. c.f. EC50 for recombinant native MIP-3β is 1.6 nM.

5 Example 11 – Diagnosis and treatment of Cardiovascular Disease (P117760US00) using CCR1, CCR2 and biotinylated MCP-1, and RANTES.

Materials and methods

10 1. Flow cytometric analysis of peripheral blood

Peripheral blood from patients and healthy controls was collected in heparin tubes. The red blood cells were lysed using Fix Buffer (Phosphate Buffer Saline (PBS) citrate with 4% paraformaldehyde) for four minutes at 37 °C and Lysing buffer (PBS with 10 mM Tris and 160 mM NH₄Cl, pH 7.5) for 15 min at 37 °C. The cells were washed in PBS with 2% Bovine Growth Serum, incubated with 10% human serum for 15 min at room temperature (RT) and stained with antibodies (Table 2) at 4 °C for 30 min. The cells were analysed by flow cytometry on a FACS Canto flow cytometer with the FACSDiva software (BD Biosciences).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorophore</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR1</td>
<td>Alexa flour 647</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CCR2</td>
<td>PerCPCy5.5</td>
<td>Biolegend</td>
</tr>
<tr>
<td>Streptavidin</td>
<td>APC</td>
<td>BD</td>
</tr>
<tr>
<td>CD14</td>
<td>FITC</td>
<td>Beckman Coulter</td>
</tr>
</tbody>
</table>

20 Table 2. List of antibodies for flow cytometric analysis.

2. Chemokine Binding test

Peripheral blood from patients and healthy controls was collected in heparin tubes. The red blood cells were lysed using Fix Buffer (Phosphate Buffer Saline (PBS) citrate with 4% paraformaldehyde) for four minutes at 37 °C and Lysing buffer (PBS with 10 mM Tris and 160 mM NH₄Cl, pH 7.5) for 15 min at 37 °C. The cells were washed in PBS with 2% Bovine Growth Serum, incubated with 10% human serum 15 min at room temperature (RT) and stained with cell specific antibodies together with biotinylated chemokine (1 μM) or the corresponding chemokine receptor antibody at 4 °C for 30 min (Table 2). The biotinylated chemokine was detected via the interaction between biotin and a fluorophore conjugated Streptavidin. The samples were analysed by flow cytometry on a FACS Canto flow cytometer with the FACSDiva software (BD Biosciences).

3. Cell depletion by matrix conjugated with biotinylated chemokine

Cells were prepared from peripheral blood (section 1). 1 mL Sepharose BigBeads matrix conjugated with 0.4 mg/mL Streptavidin (GE Healthcare) was washed in 50 mL PBS and
added to a 5 mL polystyrene tube (BD Falcon™). Biotinylated chemokine was added to the tube and incubated for 20 min at RT to enable immobilization of the chemokine on the matrix via the biotin-streptavidin interaction. Next, the cells were added to the chemokine-matrix and incubated for 20 min at RT. The cells that did not bind to the matrix were removed by washing the matrix with PBS in a sterile 40um nylon filter (BD Falcon™ Cell Strainer). The flow through cells were stained with antibodies (Table 2), analysed by flow cytometry and compared with cells from peripheral blood that had not been incubated with the chemokine-matrix.

Results and Discussion

1. Flow cytometric analysis of peripheral blood
   White blood cells from one patient with atherosclerosis were analysed for the expression of chemokine receptors with flow cytometry. The patients exhibited increased frequency of monocytes that expressed the receptor CCR1 based upon flow cytometry data and binding of an anti-CCR1 antibody (Figure 13).

   In addition to CCR1, the monocytes express the chemokine receptor CCR2 (Figure 16), based upon flow cytometry data and binding of an anti-CCR2 antibody.

2. Chemokine Binding test
   The ligand for CCR1 is the chemokine RANTES. RANTES is involved in the recruitment of monocytes to atherosclerotic lesions. The monocytes from a patient with atherosclerosis bound biotinylated RANTES (bRANTES) to the same extent as the chemokine receptor expression (Figure 14).

   The ligand for CCR2 is MCP-1 that is increased in atherosclerotic inflammation. In accordance with the CCR2 expression, biotinylated MCP-1 (bMCP-1) could bind to blood monocytes from an atherosclerosis patient (Figure 17).

3. Cell depletion by matrix conjugated with biotinylated chemokine
   The CCR1 expressing monocytes could be efficiently depleted with bRANTES-conjugated Sepharose Streptavidin Matrix (Figure 15).

   The CCR2 expressing monocytes could be depleted with bMCP1-conjugated Sepharose Streptavidin Matrix (Figure 18).

We conclude that the frequency of CCR1 expressing monocytes is enhanced in atherosclerosis. The CCR2 receptor is expressed on monocytes from atherosclerosis patients to the same extent as in the healthy controls, but the CCR2 expressing cells could differ in their pro-inflammatory profile in the patients compared to healthy controls. The CCR1 and CCR2 expressing monocytes bind the chemokines that corresponded with the
chemokine receptor expression, and could be efficiently depleted with the biotinylated chemokine-Sepharose Streptavidin-matrix.

The various embodiments of the present invention are not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the various embodiments of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims. Moreover, all embodiments described herein are considered to be broadly applicable and combinable with any and all other consistent embodiments, as appropriate.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.
CLAIMS
1. A method for treating cardiovascular disease comprising applying peripheral blood from a patient or subject to an apheresis column loaded with a solid support comprising one or more binding reagents capable of specifically binding to a chemokine receptor, optionally the chemokine receptor CCR2, CCR1 and/or CCR7 immobilized directly or indirectly on the support thus removing chemokine receptor, optionally CCR2, CCR1 and/or CCR7 expressing cells from the peripheral blood of the patient or subject.

2. The method of claim 1 wherein the cardiovascular disease is atherosclerosis.

3. The method of claim 1 or 2 wherein the binding reagent capable of specifically binding to CCR2, CCR1 and/or CCR7 is an agonist or an antagonist of CCR2, CCR1 and/or CCR7.

4. The method of any preceding claim wherein the binding reagent capable of specifically binding to CCR2, CCR1 and/or CCR7 is selected from an antibody and a chemokine.

5. The method of claim 4 wherein the chemokine is selected from MCP-1, MCP-2, MCP-3, MCP-4, MCP-5, CCL19 and/or CCL5.

6. The method of claim 5 wherein the chemokine is MCP-1 or MCP-5.

7. The method of any preceding claim wherein the CCR2, CCR1 and/or CCR7 expressing cells are monocytes, macrophages, dendritic cells or foam cells, optionally CCR1 or CCR2 expressing monocytes.

8. The method of any preceding claim wherein the patient or subject has been selected for treatment on the basis of detecting an increase in the level of CCR2, CCR1 and/or CCR7 expressing cells, levels of expression of CCR2, CCR1 and/or CCR7 and/or levels of cells with high expression of CCR2, CCR1 and/or CCR7 in a sample obtained from the patient or subject.

9. The method of any preceding claim wherein around 20-50% of the patient's blood is applied to the column in a single treatment.

10. A binding reagent capable of specifically binding to a chemokine receptor, optionally the chemokine receptor CCR2, CCR1 and/or CCR7 for use in the treatment of a cardiovascular disease, wherein the binding reagent is immobilized on a solid support contained within an apheresis column, to which is applied peripheral blood from a patient thus removing chemokine receptor, optionally CCR2, CCR1 and/or CCR7 expressing cells from the peripheral blood of the patient.
11. The binding reagent of claim 10 wherein the cardiovascular disease is atherosclerosis.

12. The binding reagent of claim 10 or 11 wherein the binding reagent capable of specifically binding to CCR2, CCR1 and/or CCR7 is an antagonist of CCR2, CCR1 and/or CCR7.

13. The binding reagent of any of claims 10 to 12 wherein the binding reagent capable of specifically binding to CCR2, CCR1 and/or CCR7 is selected from an antibody and a chemokine.

14. The binding reagent of claim 13 wherein the chemokine is selected from MCP-1, MCP-2, MCP-3, MCP-4, MCP-5, CCL19 and/or CCL5.

15. The binding reagent of claim 14 wherein the chemokine is MCP-1 or MCP-5.

16. The binding reagent of any one of claims 10 to 15 wherein the CCR2, CCR1 and/or CCR7 expressing cells are monocytes, macrophages, dendritic cells or foam cells, optionally CCR1 or CCR2 expressing monocytes.

17. The binding reagent of any one of claims 10 to 16 wherein the patient has been selected for treatment on the basis of detecting an increase in the level of CCR2, CCR1 and/or CCR7 expressing cells, levels of expression of CCR2, CCR1 and/or CCR7 and/or levels of cells with high expression of CCR2, CCR1 and/or CCR7 in a sample obtained from the patient.

18. The binding reagent of any one of claims 10 to 17 wherein 20-50% of the patient's blood is applied to the column in a single treatment.

19. A method of diagnosing, monitoring progression of, or monitoring treatment of cardiovascular disease comprising determining
   a) the levels of the chemokine receptor CCR2, CCR1 and/or CCR7 expressing cells
   b) levels of expression of CCR2, CCR1 and/or CCR7; and/or
   c) levels of cells with high expression of CCR2, CCR1 and/or CCR7

in a sample obtained from a subject, wherein high levels of CCR2, CCR1 and/or CCR7 expressing cells, high levels of expression of CCR2, CCR1 and/or CCR7 or high levels of cells with high expression of CCR2, CCR1 and/or CCR7 or increased levels of CCR2, CCR1 and/or CCR7 expressing cells compared to control, increased levels of expression of CCR2, CCR1 and/or CCR7 compared to a control or increased levels of cells with high expression of CCR2, CCR1 and/or CCR7 compared to a control indicate the presence or progression of a cardiovascular disease.
20. The method of claim 19 wherein the cardiovascular disease is selected from arteriosclerosis and atherosclerosis.

21. The method of any of claims 19 or 20 wherein the sample is a peripheral blood sample optionally wherein the cells comprise monocytes, optionally CCR1 expressing monocytes.

22. The method of any of claims 19 to 21 wherein higher levels of CCR2, CCR1 and/or CCR7 expressing cells, levels of expression of CCR2, CCR1 and/or CCR7 and/or levels of cells with high expression of CCR2, CCR1 and/or CCR7 correlate with more active disease.

23. The method of any of claims 19 to 22 wherein low levels of CCR2, CCR1 and/or CCR7 expressing cells, levels of expression of CCR2, CCR1 and/or CCR7 and/or levels of cells with high expression of CCR2, CCR1 and/or CCR7 correlate with a lack of active disease or less active disease.

24. The method of any of claims 19 to 23 wherein decreased levels of CCR2, CCR1 and/or CCR7 expressing cells, levels of expression of CCR2, CCR1 and/or CCR7 and/or levels of cells with high expression of CCR2, CCR1 and/or CCR7 correlate with successful treatment.

25. The method of claim 24 wherein the treatment is as claimed in any one of claims 1 to 9.

26. The method of any of claims 19 to 25 wherein increased levels of CCR2, CCR1 and/or CCR7 expressing cells, levels of expression of CCR2, CCR1 and/or CCR7 and/or levels of cells with high expression of CCR2, CCR1 and/or CCR7 indicate the progression of disease.

27. A method of selecting a suitable treatment for cardiovascular disease comprising determining
   a) the levels of the chemokine receptor CCR2, CCR1 and/or CCR7 expressing cells
   b) levels of expression of CCR2, CCR1 and/or CCR7; and/or
   c) levels of cells with high expression of CCR2, CCR1 and/or CCR7
   in a sample obtained from a subject, wherein high levels of CCR2, CCR1 and/or CCR7 expressing cells, high levels of expression of CCR2, CCR1 and/or CCR7 or high levels of cells with high expression of CCR2, CCR1 and/or CCR7 or increased levels of CCR2, CCR1 and/or CCR7 expressing cells compared to control, increased levels of expression of CCR2, CCR1 and/or CCR7 compared to a control or increased levels of cells with high expression of CCR2, CCR1 and/or CCR7 compared to a control, result in selection of a treatment as defined in any one of claims 1 to 9 for treatment of the cardiovascular disease.

28. The method of claim 27 wherein the cardiovascular disease is atherosclerosis.
29. The method of claim 27 or 28 wherein the sample is a peripheral blood sample, optionally wherein the cells comprise monocytes, optionally CCR1 expressing monocytes.

30. A modified MCP-1 chemokine comprising the amino acid sequence set forth as SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3 in which one or more of the following modifications have been made:
a) the glutamine residue at position 1 of SEQ ID NO: 2 has been replaced with pyroglutamine
b) the C terminus is produced as an amide derivative
c) the lysine residue at position 98 of SEQ ID NO: 1 or position 75 of SEQ ID NO: 2 or position 67 of SEQ ID NO: 3 is biotinylated, optionally via a spacer group, in order to permit immobilization of the chemokine on a solid support
c) the lysine residue at position 98 of SEQ ID NO: 1 or position 75 of SEQ ID NO: 2 or position 67 of SEQ ID NO: 3, which may be replaced by a suitable alternative amino acid such as diaminopropionic acid or ornithine, is biotinylated, optionally via a spacer group, in order to permit immobilization of the chemokine on a solid support
d) the methionine residue at position 87 of SEQ ID NO: 1 or position 64 of SEQ ID NO: 2 or position 56 of SEQ ID NO: 3 has been replaced with norleucine

31. The modified MCP-1 chemokine of claim 30 wherein the residue at position 98 of SEQ ID NO: 1 or position 75 of SEQ ID NO: 2 or position 67 of SEQ ID NO: 3 is biotinylated via a polyethylene glycol (PEG) spacer group, in order to permit immobilization of the chemokine on a solid support.

32. The modified MCP-1 chemokine of claim 31 wherein the PEG spacer is 3,6-dioxo aminooctanoic acid.

33. The modified MCP-1 chemokine of any one of claims 30 to 32 having the structure shown in any one of figures 7 to 9.

34. The modified MCP-1 chemokine of any one of claims 30 to 33 which is an agonist or an antagonist of CCR2 activity.

35. A modified MCP-5 chemokine comprising the amino acid sequence set forth as SEQ ID NO: 4, SEQ ID NO: 5 or SEQ ID NO: 6 in which the isoleucine residue at position 97 of SEQ ID NO: 4 or at position 75 of SEQ ID NO: 5 or SEQ ID NO: 6 has been replaced with lysine.

36. The modified MCP-5 chemokine of claim 35 which comprises the amino acid sequence of SEQ ID NO: 7.

37. The modified MCP-5 chemokine of claim 35 or 36 which is biotinylated at the lysine residue at position 97 of SEQ ID NO: 4 or at position 75 of SEQ ID NO: 5 or SEQ ID NO: 6.
38. The modified MCP-5 chemokine of any one of claims 35 to 37 wherein biotinylation is via a spacer group.

39. The modified MCP-5 chemokine of claim 38 wherein the spacer group is a PEG spacer, optionally 3,6-dioxo aminooctanoic acid.

40. The modified MCP-5 chemokine of any one of claims 35 to 39 wherein the C terminus is produced as an amide derivative.

41. The modified MCP-5 chemokine of any one of claims 35 to 40 having the structure shown in figure 11.

42. The modified MCP-5 chemokine of any one of claims 35 to 41 which is an agonist or an antagonist of CCR2 activity.

43. An apheresis column loaded with a solid support comprising a binding reagent capable of specifically binding to a chemokine receptor immobilized directly or indirectly on the support to permit removal of a cell expressing the chemokine receptor from the peripheral blood of a patient, wherein the binding reagent is not a chemokine.

44. The column of claim 1 wherein the binding reagent capable of specifically binding to the chemokine receptor is an agonist or an antagonist of the chemokine receptor.

45. The column of any preceding claim wherein the binding reagent capable of specifically binding to the chemokine receptor is selected from an antibody and a chemical compound.

46. The column of any preceding claim as further defined by any one or more of the features of claims 1 to 42.

47. A method for treating an inflammatory condition comprising applying peripheral blood from a patient to an apheresis column as defined in any one of claims 43 to 46 thus removing chemokine receptor expressing cells from the peripheral blood of the patient.

48. The method of claim 6 as further defined by any one or more of the features of claims 1 to 42.

49. A binding reagent capable of specifically binding to a chemokine receptor for use in the treatment of an inflammatory condition, wherein the binding reagent is immobilized on a solid support contained within an apheresis column as claimed in any one of claims 43 to 46, to which is applied peripheral blood from a patient thus removing chemokine receptor expressing cells from the peripheral blood of the patient.
50. The binding reagent of claim 49 as further defined by any one or more of the features of claims 1 to 42.

51. A modified CCL8 (MCP-2) chemokine comprising the amino acid sequence set forth as SEQ ID NO: 14.

52. The modified CCL8 chemokine of claim 51 wherein the residue at position 75 is biotinylated, optionally via a polyethylene glycol (PEG) spacer group, in order to permit immobilization of the chemokine on a solid support.

53. The modified CCL8 chemokine of claim 52 wherein the PEG spacer is 3,6-dioxo aminooctanoic acid.

54. A modified CCL8 chemokine comprising the amino acid sequence of SEQ ID NO: 16.

55. The modified CCL8 chemokine of any one of claims 51 to 54 which is an agonist or an antagonist of CCR2 activity.

56. A modified CCL5 chemokine comprising the amino acid sequence set forth as SEQ ID NO: 11 or 13.

57. The modified CCL5 chemokine of claim 56 wherein the residue at position 67 is biotinylated optionally via a polyethylene glycol (PEG) spacer group, in order to permit immobilization of the chemokine on a solid support.

58. The modified CCL5 chemokine of claim 56 or 57 which comprises the amino acid sequence of SEQ ID NO: 13.

59. The modified CCL5 chemokine of any one of claims 56 to 58 which is an agonist or an antagonist of CCR1 activity.

60. A modified CCL2 chemokine comprising the amino acid sequence of SEQ ID NO: 10 or SEQ ID NO: 8.

61. The modified CCL2 chemokine of claim 60 wherein the residue at position 75 is biotinylated, optionally via a spacer group, in order to permit immobilization of the chemokine on a solid support.

62. The modified CCL2 chemokine of claim 61 wherein the residue at position 75 is biotinylated via a polyethylene glycol (PEG) spacer group, in order to permit immobilization of the chemokine on a solid support.
63. The modified CCL2 chemokine of claim 62 wherein the PEG spacer is 3,6-dioxo aminooctanoic acid.

64. The modified CCL2 chemokine of any one of claims 60 to 63 which is an agonist or an antagonist of CCR2 activity.

65. A modified CCL19 chemokine comprising the amino acid sequence of SEQ ID NO: 17.

66. The modified CCL19 chemokine of claim 65 wherein the amino acid residue at position 78 of SEQ ID NO: 17 is biotinylated, optionally via a spacer group, in order to permit immobilization of the chemokine on a solid support.

67. The modified CCL19 chemokine of claim 66 wherein the residue at position 78 of SEQ ID NO: 17 is biotinylated via a polyethylene glycol (PEG) spacer group, in order to permit immobilization of the chemokine on a solid support.

68. The modified CCL19 chemokine of claim 67 wherein the PEG spacer is 3,6-dioxo aminooctanoic acid.

69. The modified CCL19 chemokine of any one of claims 65 to 68 comprising the amino acid sequence of SEQ ID NO: 19.

70. The modified CCL19 chemokine of any one of claims 65 to 69 which is an agonist or an antagonist of CCR7 activity.
FIG. 3
FIG. 6
FIG. 7

H-pyroGluPDAINAPVTCCYNFTNRKISVQRLASYRRITSS
KCPKEAVIFKTIVAEEKADPKQKWVQDSMHDLDKQTQTPKT-CONH₂

FIG. 8

H-pyroGluPDAINAPVTCCYNFTNRKISVQRLASYRRITSS
KCPKEAVIFKTIVAEEKADPKQKWVQDSNleuDHDLDKQTQTPKT-CONH₂

FIG. 9

H-VTCCYNFTNRKISVQRLASYRRITSS
KCPKEAVIFKTIVAEEKADPKQKWVQDSNleuDHDLDKQTQTPKT-CONH₂
ulimit -t 30; /usr/molbio/bin/lalign -f -14 -g -4 -K 3 ./wwwtmp/.11134.1.seq
./wwwtmp/.11134.2.seq > ./wwwtmp/.11134.out LALIGN finds the best local
alignments between two sequences version 2.1u09 December 2006 Please cite: X.

Comparison of:

(A) ./wwwtmp/.11134.1.seq MCP1 (human) 76 bp
- 76 aa

(B) ./wwwtmp/.11134.2.seq MCP-5 (mouse) 82 bp
- 82 aa

using matrix file: BL50 (15/-5), gap-open/ext: -14/-4 E(limit)

0.05

68.1% identity in 72 aa overlap (2-73:2-73); score: 370 E(10000):
1.6e-31

10 20 30 40 50 60
MCP1 PDAINAPVTCCYNFTNRKISVQRASYRRITSSSKCPKEAVIFKTVAKEICADPKKQKVQ
.................. ....: :... :..: ...: .................: ...............
MCP-5 PDAVSTPVTCYCNVVKQKHVRDKYSYRRITSSQCPEAVIFRTILDEICADPKKEKWK

70

MCP1 DSDMHLDFQTQTPKT

.:.:..: :::

MCP-5 NSIINHLDKTSQTFILEPSCLG

70

**FIG. 10**

[Diagram of molecular structure]

**FIG. 11**

H-GPDATPVTCCYNVVKQKHVRKLKSYRRITSSQCPEAVIFRTILDEICADPKKEKWK/KNSIINHLDKTSQTFLK-LCONH₂
% CCR1 expressing monocytes

Before depletion

After depletion

FIG. 15

% CCR2 expressing monocytes

FIG. 16