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(54) Title: METHODS FOR GENERATING CELL MICROPARTICLES

(57) Abstract: The present invention relates to a method for generating cell microparticles comprising a step of consisting of contacting a cell with an agonist of the CD47/IAP receptor for a time sufficient to induce microparticles shedding by said cell.



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METHODS FOR GENERATING CELL MICROPARTICLES

FIELD OF THE INVENTION:

5 The present relates to methods for generating cell microparticles.

BACKGROUND OF THE INVENTION:

10 Microparticles (MP) are cellular fragments of a diameter below 1 μm , released during cell activation or cell death. MP comprise a lipid membrane similar to and derived from the parent cell membrane, and may also contain other components from the parent cell, such as cytosol. It is generally accepted that MP carry a broad spectrum of the parent cell proteins and other cell surface determinants, such as CD molecules and cell surface receptors. MP might thus theoretically fulfill functions comparable to those of the parent cell. At present, cell MP have mainly been envisaged for pronostic or diagnostic applications. However, recent
15 publications report that MP trigger a great variety of signals in target cells. This suggests that MP will soon be envisaged for innovative therapeutic applications, but methods for generating MP are needed.

SUMMARY OF THE INVENTION:

20 The present invention relates to a method for generating cell microparticles comprising a step of consisting of contacting a cell with an agonist of the CD47/IAP receptor for a time sufficient to induce microparticles shedding by said cell.

DETAILED DESCRIPTION OF THE INVENTION:

25 The invention is a novel technique to generate cell MP from a variety of parent cells. The inventors indeed demonstrate that the induced MP can function as information vectors from a parent cell to a target cell. They demonstrate that MP preparations generated from different parent cell types do not always bear the same characteristics, nor carry the same activation potential for target cells. They also show that MP shed from a selected cell type
30 using the novel technique have specific characteristics that other MP generated by different means do not always share. Hence, MP generated by said technique are unique and present specific advantages that may prove useful for therapy. In particular, the inventors show how MP generated by the technique can be used to modulate the homing and engraftment of transplanted cells.

Accordingly the present invention relates to a method for generating cell microparticles comprising a step of consisting of contacting a cell with an agonist of the CD47/IAP receptor for a time sufficient to induce microparticles shedding by said cell.

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As used herein, the term "cell microparticle" denotes a plasma membrane vesicle shed from an apoptotic or activated cell (Boulanger and Dignat-George, 2011, *Arterioscler Thromb Vasc Biol.* 2011;31:2-3). The size of cell microparticle ranges from 0,1 μm to 1 μm in diameter. Typically, said cell microparticle expresses different cell surface markers that are the same as the parent cells. For example, an endothelial cell microparticle expresses a surface marker selected from the group consisting of CD31, CD144, VE-Cadherin, and CD146. As endothelial cell, endothelial microparticles do not express specific surface markers such as CD41, CD4; CD14; CD235a; and CD11a. Therefore a typical endothelial microparticle is as CD31+CD41- microparticle.

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As used herein, the term "cell" refers to any eukaryotic cell. Eukaryotic cells include without limitation ovary cells, epithelial cells, circulating immune cells, hematopoietic cells, bone marrow cells, circulating vascular progenitor cells, cardiac cells, chondrocytes, bone cells, beta cells, hepatocytes, and neurons... Moreover the term includes pluripotent stem cells. As intended herein, the expression "pluripotent stem cells" relates to division-competent cells which are liable to differentiate in one or more cell types. Preferably, the pluripotent stem cells are not differentiated. Pluripotent stem cells encompass stem cells, in particular adult stem cells (e.g. mesenchymal stem cells (MSC)) and embryonic stem cells. The term also encompasses induced pluripotent stem cells (IPS). Accordingly the term includes purified primary cells and immortalized cell lines. The term also refers to cells in suspension (e.g. circulating leukocytes (PBMC)), or adherent cells (e.g. endothelial cells). According to the invention said cells express the CD47/IAP receptor.

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As intended herein the expression "express a CD47/IAP receptor" means that the cells comprise an mRNA which encodes the CD47/IAP receptor, or an RNA precursor thereof, and/or a protein consisting of the CD47/IAP receptor. The detection of the mRNA, or of its precursors, can be carried out by various techniques well-known to one of skill in the art, such as RT-PCR for instance. The detection of the protein can also be carried out by various

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techniques well-known to one of skill in the art, such as immunodetection using anti-CD47/IAP antibodies for instance.

In a particular embodiment, said cell is genetically transformed with a vector encoding for the CD47/IAP receptor, in the case where the cells do not express the CD47/IAP in a sufficient amount for inducing microparticle shedding.

As used herein, the terms "vector", "cloning vector" and "expression vector" mean the vehicle by which a DNA or RNA sequence (e.g. a foreign gene) can be introduced into a host cell, so as to transform the host and promote expression (e.g. transcription and translation) of the introduced sequence. Any expression vector for animal cell can be used. Examples of suitable vectors include pAGE107 (Miyaji H et al. 1990), pAGE103 (Mizukami T et al. 1987), pHSG274 (Brady G et al. 1984), pKCR (O'Hare K et al. 1981), pSG1 beta d2-4- (Miyaji H et al. 1990) and the like.

Other examples of plasmids include replicating plasmids comprising an origin of replication, or integrative plasmids, such as for instance pUC, pcDNA, pBR, and the like.

Other examples of viral vectors include adenoviral, retroviral, herpes virus and AAV vectors. Such recombinant viruses may be produced by techniques known in the art, such as by transfecting packaging cells or by transient transfection with helper plasmids or viruses. Typical examples of virus packaging cells include PA317 cells, PsiCRIP cells, GPenv+ cells, 293 cells, etc. Detailed protocols for producing such replication-defective recombinant viruses may be found for instance in WO 95/14785, WO 96/22378, US 5,882,877, US 6,013,516, US 4,861,719, US 5,278,056 and WO 94/19478.

Examples of promoters and enhancers used in the expression vector for animal cell include early promoter and enhancer of SV40 (Mizukami T. et al. 1987), LTR promoter and enhancer of Moloney mouse leukemia virus (Kuwana Y et al. 1987), promoter (Mason JO et al. 1985) and enhancer (Gillies SD et al. 1983) of immunoglobulin H chain and the like.

As intended herein, the expression "agonist of the CD47/IAP receptor" relates to any molecule which is liable to induce a response of the CD47/IAP receptor similar in nature to the response which is induced by binding of the TSP1 protein or the RFYVVMWK peptide (SEQ ID NO: 1). By way of example, agonists of CD47/IAP are liable to promote the cytoskeletal remodeling as described in the EXAMPLE 1.

For instance, the agonist of the CD47/IAP receptor can be an anti-CD47 agonist antibody, such as the B6H12 antibody described by Gresham et al. (1989) J. Cell. Biol. 108:1935-1943; Wang and Frazier (1998) Mol Cell Biol 9:865-874; Ticchioni et al. FASEB J

(2001) 15:341-350; and Barazi et al. (2002) J Biol Chem 277:42859-42866, the CIK α 1 antibody, as described by Wilson et al. (1999) J Immunol. 163:3621-3628, or the 1F7 antibody, as described by Wang and Frazier (1998) Mol Cell Biol 9:865-874.

The agonist can also be the SIRP α 1 protein as described by Jiang et al. (1999) J Biol Chem 274:559-562; Babic et al. (2000) J Immunol 164:3652-3658; Seiffert et al. (2001) Blood 97:2741-2749; and Liu et al. (2006) J Mol Biol 365:680-693.

However, it is preferred that the agonist is a polypeptide which comprises the amino acid sequence VVM. Such agonists are well-known to the man skilled in the art. In particular, they are selected from CD47/IAP receptor-binding peptides, such as those described in:

Voit et al. (2003) FEBS Letters 544:240-245; Barazi et al. (2002) J Biol Chem 277:42859-42866 (peptide 4N1-1 of sequence RFYVVMWK SEQ ID NO:1);

Gao et al. (1996) J Cell Biol 135:533-544; Wang et al. (1999) J Cell Biol 147:389-399; Kanda et al. (1999) Exp Cell Res 252:262-272; Ticchioni et al. (2001) FASEB J 15:341-350; Barazi et al. (2002) J Biol Chem 277:42859-42866; and Li et al. (2005) J Immunol 174:654-661 (peptide 4N1K of sequence KRFYVVMWKK, SEQ ID NO: 4);

Wilson et al. (1999) J Immunol 163:3621-3628; Barazi et al. (2002) J Biol Chem 277:42859-42866; and Isenberg et al (2006) J Biol Chem 281:26069-26080 (peptide 7N3 of sequence FIRYVVMYEGKK (SEQ ID NO: 5)); and

U.S. Pat. No. 6,469,138 (RFYVVMWKQVTQS (SEQ ID NO: 6); and FIRVVMYEGKK (SEQ ID NO: 4)).

Preferably, the agonist is a polypeptide which comprises or consists in RFYVVMWK (4N1-1, SEQ ID NO: 1), such as KRFYVVMWKK (4N1K, SEQ ID NO: 4), or a derivative of RFYVVMWK.

4N1-1 notably represents amino acids 1034-1041 of human TSP1.

As intended herein, a derivative of RFYVVMWK, relates to any polypeptide derived from RFYVVMWK by insertion, deletion or insertion of at least one amino-acid and/or by chemical treatment, provided that the derivative presents essentially the same agonist properties than RFYVVMWK vis-à-vis the CD47/IAP receptor.

Preferably, the agonist is a fragment of the TSP1 protein. It is preferred that the TSP1 protein as intended herein is a human TSP1 protein, notably represented by GenBank reference NP-003237, a mouse TSP1 protein, notably represented by GenBank reference AAA50611, or a rat TSP1 protein, notably represented by GenBank reference NP-0001013080. Most preferably, the TSP1 protein is a human TSP1. In particular, as will be

apparent to the man skilled in the art, any fragment of the TSP1 protein which comprises RFYVVMWK can be considered as an agonist of the CD47/IAP receptor as intended herein.

As intended herein an "analog" of the RFYVVMWK peptide relates to any molecule which is similar in shape, charge repartition and hydrophilicity/hydrophobicity repartition to peptide RFYVVMWK.

In a particular embodiment, said cells are contacting with said agonist of the CD47/IAP receptor for a very short time, preferably for less than 15 min, more preferably for less than 10 min. The inventors indeed demonstrate that contacting said cell with said agonist during 5 min is sufficient to induce massive microparticles shedding by said cells.

In a particular embodiment, cells are maintained in a cell culture medium. Any Cell culture medium known in the art may be suitable provided that said culture medium comprises an amount of calcium (Ca^{2+}). Typically the concentration of calcium corresponds to the in vivo physiological concentration of extracellular calcium.

In a particular embodiment, the cells may be in suspension, and accordingly the agonist is provided directly in said suspension. In a particular embodiment, a shear stress may be applied to said cell suspension.

Alternatively the cells may consist in adherent cells. For example, said cells may be coated in a cell culture surface. The term "cell culture surface" or "cell culture matrix" refers to every type of surface or matrix suitable for cell culture. The term "cell culture surface" includes but is not limited to tissue culture plate, dish, well or bottle. In a particular embodiment, the culture surface is plastic surface of the culture plate, dish, well or bottle. The cell culture surface is to be compatible with the coating of cells. In those cases, the agonist is provided in the cell culture medium that surrounds the adherent cells.

In a particular embodiment, the method further comprises a step consisting of concentrating said microparticles. Microparticles may be concentrated by ultracentrifugation. Moreover ultracentrifugation may be useful for i) differential separation of microparticles from parent cells and for ii) differential separation of microparticles from the agonist of the CD47/IAP receptor. The agonist can be entirely removed from the microparticle preparation.

In a particular embodiment, the method further comprises a step consisting of isolating the microparticles of interest from the supernatant of the cells.

Standard methods for isolating microparticles are well known in the art. For example the methods may consist in collecting the population of microparticles present in the supernatant of the cells and using differential binding partners directed against the specific surface markers of the microparticles of interest, wherein microparticles are bound by said binding partners to said surface markers.

In a particular embodiment, the methods of the invention comprise contacting the supernatant with a set of binding partners capable of selectively interacting with microparticles present in said supernatant. The binding partner may be an antibody that may be polyclonal or monoclonal, preferably monoclonal, directed against the specific surface marker of the microparticles. In another embodiment, the binding partners may be a set of aptamers.

Polyclonal antibodies of the invention or a fragment thereof can be raised according to known methods by administering the appropriate antigen or epitope to a host animal selected, e.g., from pigs, cows, horses, rabbits, goats, sheep, and mice, among others. Various adjuvants known in the art can be used to enhance antibody production. Although antibodies useful in practicing the invention can be polyclonal, monoclonal antibodies are preferred.

Monoclonal antibodies of the invention or a fragment thereof can be prepared and isolated using any technique that provides for the production of antibody molecules by continuous cell lines in culture. Techniques for production and isolation include but are not limited to the hybridoma technique originally described by Kohler and Milstein (1975); the human B-cell hybridoma technique (Cote et al., 1983); and the EBV-hybridoma technique (Cole et al. 1985).

In another embodiment, the binding partner may be an aptamer. Aptamers are a class of molecule that represents an alternative to antibodies in term of molecular recognition. Aptamers are oligonucleotide or oligopeptide sequences with the capacity to recognize virtually any class of target molecules with high affinity and specificity. Such ligands may be isolated through Systematic Evolution of Ligands by EXponential enrichment (SELEX) of a random sequence library. The random sequence library is obtainable by combinatorial chemical synthesis of DNA. In this library, each member is a linear oligomer, eventually chemically modified, of a unique sequence. Possible modifications, uses and advantages of this class of molecules have been reviewed in Jayasena S.D., 1999. Peptide aptamers consist of conformationally constrained antibody variable regions displayed by a platform protein,

such as E. coli Thioredoxin A, that are selected from combinatorial libraries by two hybrid methods.

The binding partners of the invention such as antibodies or aptamers, may be labelled with a detectable molecule or substance, such as a fluorescent molecule, a radioactive molecule or any others labels known in the art. Labels are known in the art that generally provide (either directly or indirectly) a signal.

As used herein, the term "labelled", with regard to the antibody or aptamer, is intended to encompass direct labelling of the antibody or aptamer by coupling (i.e., physically linking) a detectable substance, such as a radioactive agent or a fluorophore (e.g. fluorescein isothiocyanate (FITC) or phycoerythrin (PE) or Indocyanine (Cy5)) to the antibody or aptamer, as well as indirect labelling of the probe or antibody by reactivity with a detectable substance. An antibody or aptamer of the invention may be labelled with a radioactive molecule by any method known in the art. For example radioactive molecules include but are not limited radioactive atom for scintigraphic studies such as I123, I124, In111, Re186, Re188.

Preferably, the antibodies against the surface markers are already conjugated to a fluorophore (e.g. FITC-conjugated and/or PE-conjugated). Examples include monoclonal anti-human CD62E-FITC, CDC105-FITC, CD51-FITC, CD106-PE, CD31-PE, and CD54-PE, available through Ancell Co. (Bayport, Minn.).

The aforementioned assays may involve the binding of the binding partners (ie. Antibodies or aptamers) to a solid support. Solid supports which can be used in the practice of the invention include substrates such as nitrocellulose (e. g., in membrane or microtiter well form); polyvinylchloride (e. g., sheets or microtiter wells); polystyrene latex (e.g., beads or microtiter plates); polyvinylidene fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, and the like. The solid surfaces are preferably beads. Since microparticles have a diameter of roughly 0,1-1 μm , the beads for use in the present invention should have a diameter larger than 1 μm . Beads may be made of different materials, including but not limited to glass, plastic, polystyrene, and acrylic. In addition, the beads are preferably fluorescently labelled. In a preferred embodiment, fluorescent beads are those contained in TruCount(TM) tubes, available from Becton Dickinson Biosciences, (San Jose, California).

According to the invention, methods of flow cytometry are preferred methods for determining the concentration of microparticles in biological fluid samples obtained from the patient. Biological fluid samples may preferably include blood and plasma samples. For

example, fluorescence activated cell sorting (FACS) may be therefore used to separate in the supernatant the desired microparticles. In another embodiment, magnetic beads may be used to isolate microparticles (MACS).

For instance, beads labelled with monoclonal specific antibodies may be used for the positive selection of microparticles. Other methods can include the isolation of microparticles by depletion the microparticles that are not of interest (negative selection).

The microparticle isolation methods may also consist in collecting the population of microparticles present in the supernatant of the cells and using size exclusion columns or filters to purify microparticles of specific sizes out of the cell supernatants.

A further aspect of the invention relates to an isolated cell microparticle obtainable by the method of the invention.

More particularly, the present invention relates to a population of cell microparticles obtainable by the method of the invention.

Said population is typically a substantially pure homogenous population of microparticles obtainable by the method of the invention. The term "substantially pure homogenous population", as used herein, refers to a population of cell microparticles wherein the majority (e.g., at least about 80%, preferably at least about 90%, more preferably at least about 95%) of the total number of said cell microparticles have the specified characteristics of the microparticles of interest.

The population of cell microparticles according to the invention may be easily conserved in appropriate medium and therefore may be stored so as to form bank of cell microparticles.

The present invention also provides pharmaceutical compositions comprising a population of the cell microparticles according to the invention. Such compositions comprise a therapeutically effective amount of said population, and a pharmaceutically acceptable carrier. In a specific embodiment, As used herein, the term "pharmaceutically acceptable carrier or excipient" refers to a carrier medium which does not interfere with the effectiveness of the biological activity of the cell microparticles of the invention, and which is not excessively toxic to the host at the concentrations at which it is administered. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the cell microparticles

of the invention are administered. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E. W. Martin. Such compositions will contain a therapeutically effective amount of the population of said microparticles, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer.

Where necessary, the composition may also include a solubilising agent and a local anaesthetic such as lignocaine to ease pain at the site of the injection.

The amount of the population of cell microparticles of the invention which will be effective in the body can be determined by standard clinical techniques. In addition, in vitro and in vivo assays may optionally be employed to help identify optimal dosage ranges. However, suitable dosage ranges for intravenous administration are generally about 20-500 micrograms of active component per kilogram body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 pg/kg body weight to 1 mg/kg body weight. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems. A typical dose for a human might be 10 pg to 10 mg, preferably 20 mg to 5 mg, preferably 40 pg to 2 mg, preferably 100 pg to 1 mg, preferably about 0.5 mg (calculated either per kg body weight or as total dose per subject).

Pharmaceutical preparations for topical and local use may consist in lotions and creams which comprise a liquid or semi-solid oil-in-water or water-in-oil emulsion, and ointments (which preferably comprise a preservative). Suitable for the treatment of the eyes are eye drops which comprise the active ingredient in aqueous or oily solution, and eye ointments which are preferably manufactured in sterile form. Suitable for the treatment of the nose are aerosols and sprays (similar to those used in the treatment of the respiratory tract), coarse powders which are administered by rapid inhalation through the nostrils, and especially nose drops which comprise the active ingredient in aqueous or oily solution; suitable for local treatment of the buccal cavity are lozenges which comprise the active ingredient in a mass generally formed of sugar and gum arabic or tragacanth, to which

flavourings may be added, and pastilles which comprise the active ingredient in an inert mass, for example of gelatine and glycerine or sugar and gum arabic. Pharmaceutical preparations suitable for administration in the form of aerosols or sprays comprise, for example, suitable pharmaceutically acceptable solvent, such as, especially, ethanol and water, or a mixture of such solvents. They may, as necessary, comprise other pharmaceutical adjuncts, such as non-ionic or anionic surface-active agents, emulsifiers and stabilisers, and also active ingredients of other kinds, and especially advantageously they can be mixed with a propellant gas, such as an inert gas under elevated pressure or especially with a readily volatile liquid, preferably a liquid that boils under normal atmospheric pressure below customary room temperature (for example from approximately -30 to +10° C), such as an at least partially fluorinated polyhalogenated lower alkane, or a mixture of such liquids. Such pharmaceutical preparations, which are used predominantly as intermediates or stock mixtures for the preparation of the corresponding medicaments in finished form, comprise the active ingredient customarily in a concentration of from approximately 0.1 to approximately 10% by weight, especially from approximately 0.3 to approximately 3% by weight. For the preparation of medicaments in finished form, such a pharmaceutical preparation is introduced into suitable containers, such as flacons and pressurised bottles, which are provided with a spray device or valve suitable for such purposes. The valve is preferably constructed in the form of a metering valve which on operation releases a predetermined amount of liquid, corresponding to a predetermined dose of the active ingredient. In the preparation of the finished medicament form, it is also possible for corresponding amounts of the pharmaceutical preparation in stock solution form and of the propellant to be introduced separately into the containers and to be mixed with one another only at that stage.

Delivery of the cell microparticles of the invention to a subject to be treated may be achieved by providing the product locally, such as to the appropriate tissue or organ. For example, the administration of the product may be intravenous, rectal, oral, auricular, intraosseous, intra-arterial, intramuscular, subcutaneous, cutaneous, intradermal, intracranial, intrathecal, intraperitoneal, topical, intrapleural, intra-orbital, intra-cerebrospinal fluid, intranodal, intralesional, transdermal, intranasal (or other mucosal), pulmonary, or by inhalation to a site of interest. The cell microparticles may, for example, be provided by local injection. The cell microparticles may be provided by injection into a blood vessel or other vessel that leads to the desired target site. The product may be administered by local injection to the desired tissue. The cell microparticles may be administered by any of the routes

mentioned herein such as intra-muscular injection or by ballistic delivery. In preferred embodiments the cell microparticles may be administered via direct organ injection, vascular access, or via intra-muscular, intra-peritoneal, or sub-cutaneous routes.

5 The present invention also relates to an *in vitro* method for preparing cells for engraftment in a target tissue of a subject, wherein the cells to be engrafted are contacted with a population of cell microparticles according to the invention prior to engraftment.

10 As intended herein the expression “engraftment” relates to the delivery and the fixation of cells within a target tissue, or the prolonged physical interaction with the target tissue.

15 As intended herein the expression “target tissue” relates to any group of cells, which cells have similar or different phenotypes, which exhibits one or several characteristics, as a whole, which makes it distinguishable from its environment. Target tissues as intended herein can be found in a multicellular organism, preferably an animal organism, more preferably a mammal organism, and most preferably a human organism. Preferably, the tissue is selected from the group constituted of cardiac and skeletal muscle, brain, pancreas, skin, kidney, blood vessels and other vascular structures.

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 In an advantageous embodiment of the above-defined method, the prepared cells exhibit an improved adhesion to the target tissue with respect to similar cells which have not been prepared according to said method.

25 In another advantageous embodiment of the above-defined method, the prepared cells are liable to differentiate into mature cells in the target tissue and differentiation of the prepared cells is accelerated with respect to similar cells which have not been prepared according to said method.

30 In a further embodiment of the above-defined method, the cells are preferably contacted with the population of cell microparticles according to the invention for 10 seconds to 2 hours.

In another embodiment, the above method comprises a step of further selecting adherent cells among prepared cells, for instance after adhesion to extracellular matrices such as gels of gelatine/vitronectin or fibrin.

Indeed cells may respond differently to the contacting phase, thus separating cells into
5 adherent and less-adherent fractions, and using the fraction with enhanced adhesion capacity is advantageous to improve the rate of therapeutic cell engraftment.

The present invention also relates to the cells prepared by the *in vitro* method as defined above, for use in a method for enhancing cell engraftment in a subject in need thereof
10 wherein a therapeutically effective amount of cells prepared by the *in vitro* method as defined above is administered to said subject.

The present invention further relates to the above-defined agonist of the CD47/IAP receptor for use in a method for enhancing cell engraftment in a subject in need thereof. For
15 example, said method may be useful for cell therapy, wherein a therapeutically effective amount of at least one of the above-defined agonist of the CD47/IAP receptor, preferably in association with cells to be engrafted, is administered to said subject.

The present invention further relates to a population of cell microparticles according to
20 the invention for use in a method for enhancing cell engraftment in a subject in need thereof. For example, said method may be useful for cell therapy, wherein a therapeutically effective amount of a population of cell microparticles according to the invention, preferably in association with cells to be engrafted, is administered to said subject.

25 As intended herein the subject preferably relate to an animal, more preferably to a mammal, and most preferably to a human. Typically, the subject may suffer from an insufficiency of the vascular system. The subject may be in need of blood vessel reconstruction or neoformation. The subject may suffer from a pathology selected from the group constituted of atherosclerosis, diabetes, obesity, coronaropathy, diabetic retinopathy,
30 nephroangiosclerosis, cerebral ischemia, thrombosis, endothelial dysfunction, pulmonary hypertension, traumatic cutaneous wounds, ulcers, and burns.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1. TSP1 triggers MP shedding via CD47

We investigated MP shedding in response to TSP1. First, we injected TSP1 to wild type and CD47^{-/-} transgenic mice (n>15) by intravenous injection (1,3 mg/kg). We then collected their plasma after 5 minutes. (A) We quantified circulating PS⁺ MP by FACS after annexin-V labeling. (*) indicate $p < 0.05$ vs wild type (wt). (C). Next, we treated BMC with recombinant TSP1 (10 $\mu\text{g/ml}$) in static conditions or under shear rate applied in a Lorca cone-and-plate ektacytometer (1500 s^{-1}), for 5 minutes. (D-E) We quantified PS⁺ MP in the supernatant by FACS after annexin-V labelling. (E) To assess the rôle of the CD47 receptor, we treated wild type or CD47^{-/-} BMC with TSP1 (10 $\mu\text{g/ml}$) as above, or its carboxyterminal, CD47 agonist peptide 4N1-1 (25 μM), and quantified PS⁺ MP in the supernatant. (*) indicate $p < 0.05$ vs none, (\$) indicate $p < 0.05$ vs static.

Figure 2. 4N1-1 triggers rapid and Ca²⁺-dependent MP shedding

We treated wild type BMC in static conditions, with (A) 4N1-1, truncated peptide 4N1-2, mutated peptide 4NGG (all 25 μM) for 10 minutes, or with stroma-derived factor 1 (SDF1) or recombinant Fc fragment-coupled ephrine-B2 (EphB2-Fc) for 2 hours, or deprived the cells from serum for 72 hours (starvation). We quantified PS⁺ MP in the supernatants. (B) Alternatively, we treated BMC with 4N1-1 for 10 minutes and determined MP shedding in the presence of inhibitors of G(α)i proteins (Pertussis toxin, PTX; 1 $\mu\text{g/ml}$), phospholipase C (U73122; 10 μM), IP₃-receptor (Xestospongin, 3 μM), or with an intracellular Ca²⁺ chelator BAPTA-AM (10 μM) or in the absence of extracellular Ca²⁺ (PBS no Ca²⁺). We also tested the impact of ROCK inhibitor HA1977 (10 μM). (*) indicate $p < 0.05$ vs 4N1-1 alone.

Figure 3. MP shedding, membrane and cytoskeletal remodeling

We investigated the remodeling of BMC that leads to MP shedding in response to 4N1-1 or control truncated 4N1-2 (25 μM). (A) We treated BMC for 10 minutes with the

peptides, fixed them for electron microscopy, and monitored cell surface blebbing (red arrows) and fragments (blue arrows). (C) We revealed by a decrease in both forward (FSc) and side (SSc) light scatter indices. (E) We quantified PS⁺ and total MP in the supernatants of 4N1-1-treated BMC after single labeling, or double labeling with annexin-V (PS⁺) and PKH26 membrane dye (Total).

EXAMPLE 1: GENERATION OF CELL MICROPARTICLES FROM BONE MARROW MONONUCLEAR CELLS (BMC).

Material & Methods

Reagents and antibodies

Human recombinant TSP1 was from EMP-Genetech (Germany). Synthetic peptides ¹ 4N1-1 (RFYVVMWK) (SEQ ID NO:1) and 4N1-2 (RFYVVM) (SEQ ID NO:2) were from Bachem, 4NGG (RFYGGMWK) (SEQ ID NO:3), were from Genecust (France).

Animals, ischemia and cell therapy

To describe the technique, we used mouse cells as parent cells. Mice for blood and bone marrow collection were 10-14 week old C57Bl/6 males (Charles River, France). We obtained CD47-deficient mice as a kind gift from Dr Frazier (University of Washington in Missouri, St Louis, MO, USA). Mice for the vascular injury model and intravital microscopy were 4-8 week old C57Bl/6 males. In some experiments, recombinant human TSP1 (1,3 mg/kg) or mouse BMC ($2 \cdot 10^6$ cells/mouse) were administered intravenously by retro-orbital injection under light sedation, following the ethical rules and guidelines of Inserm.

Cell culture

All media were passed through a 0,2 μ m mesh filter to remove any material of similar size to cell MP. BMC were collected as previously described ^{2, 3} and kept in endothelial cell basal medium from Promocell (EBM). Mouse SVEC4-10 endothelial cells from ATCC (#CRL-2181) were grown at 37°C in a culture chamber at 5% CO₂ and 98 % humidity, in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% NCS. Mouse primary peripheral blood mononuclear cells (PBMC) were isolated from wild type mice. Mouse blood was obtained via intracardiac puncture under terminal anesthesia, anti-coagulated with heparin and separated on Ficoll Hypaque-1088 gradient (Sigma) by centrifugation at 600 g for

45 minutes². For apoptotic MP induction, cells were left in culture in the absence of serum for up to 72 hours and the supernatants were collected.

Plasma, platelets and platelet degranulation products

5 For platelet-free plasma (PFP) and platelets, mouse blood was obtained via intracardiac puncture under terminal anesthesia, anti-coagulated with ACD-C buffer and incubated with prostaglandin E1 (PGE1 10 nM). After low speed centrifugation to eliminate red blood cells, platelet-rich plasma was collected and centrifuged to pellet the platelets, before re-suspension in reaction buffer and numeration. The supernatant was kept as PFP.
10 Platelet suspensions (400 µl, 600,000 platelets/µl) were brought to 37°C and aggregation was initiated upon addition of calcium and Thrombin Receptor Activating Peptide (TRAP, 100 µM), or 4N1-1 peptide (25 µM). After 5 min, platelets were pelleted and eliminated. Supernatants contained platelet MP.

MP characterization

15 MP were isolated from mouse cell culture supernatants after a first low speed centrifugation (600 g, 5 minutes) to remove large cell fragments. The supernatants were then ultracentrifuged at 20,800 g for 4 hours at 4°C to pellet MP. The MP were then resuspended in 200 µl of filtered medium and quantified. For MP quantification, MP were reacted with
20 fluorescein-coupled Annexin-V (Annexin-V Fluos kit, Roche Diagnostics according to the manufacturer's instructions, and analyzed by FACS by comparison against size-calibrated fluorescent microbeads (0.5, 0.9 and 3 µm diameter, Megamix, Biocytex). For MP typing, MP were immunoreacted with phycoerythrin-coupled anti-mouse CD31 (clone 390, Biolegend), APC-coupled anti-mouse CD41 (clone MWReg30, eBiosciences), Alexa700-
25 coupled anti-mouse CD45 (clone 30-F11, Biolegend), Phycoerythrin-Cy7-coupled anti-mouse Ter119 (clone Ter-119, Biolegend) antibodies, or matched-coupled non immune IgG as controls (Biolegend and eBiosciences).

Real time monitoring of BMC recruitment in experimental thrombosis

30 We adapted our experimental model of mouse mesenteric thrombosis induced by superfusion of iron chloride (FeCl₃)⁴. Mice were laparotomized after terminal anesthesia and local cutaneous application of Xylocaine. BMC were labeled with fluorescent 'Cell Tracker-Orange' dye (5 µM; Molecular Probes), and primed with synthetic peptide 4N1-1 or 4N1-2

(50 μ M), or control BSA (1%). BMC were administered through the retro-orbital sinus ($2 \cdot 10^6$ cells/animal). Mesenteric vessels were exposed for intra-vital microscopy and placed on the stage of an inverted fluorescence microscope. BMC interactions with the intact wall of a selected venule were recorded (VHS) for 2 min, at rest. A 2 mm³ piece of 1% agar gel containing 500 mM FeCl₃ was applied locally to trigger progressive thrombosis. BMC interactions with the vessel were recorded for a further 15 min. We counted the number of novel BMC/vascular wall interactions as a rate (number per minute, every minute). Firm adhesions (immobilization for over 2 minutes) were accumulated over the whole observation period, after induction of injury. Average BMC rolling speeds were calculated for subject cells ($n > 20$) with calibrated screens.

Statistical analysis

All experiments were repeated at least three times. Data represent mean $\pm$ sem. For in vitro experiments, statistical analysis was performed with the Student T-test. In vivo data were analyzed with the ANOVA test, and inter-group comparisons (5-10 animals/group) were performed with the Mann-Whitney test. Significance was achieved when $p < 0.05$.

Results

We formed the hypothesis that TSP1 triggers cytoskeletal remodeling and MP shedding from circulating cells, and that these MP activate other circulating cells to promote their adhesion to injured vessels. We used bone marrow mononuclear cells as a model of PBMC and addressed successively the mechanisms leading to MP shedding, and the effects of MP on cell adhesion during thrombosis.

TSP1 triggers MP shedding via CD47

We first asked the question whether TSP1 stimulated MP shedding in BMC. Cellular MP are generally identified by a combined evaluation of size and PS externalization versus calibrated microbeads. TSP1 is thought to occur in the plasma at concentrations thought to reach up to 20 μ g/ml during thrombosis. We administered TSP1 to wild type mice by intravenous injection, collected their plasma and measured MP by FACS after annexin-V labeling. We found that TSP1 triggered a 5 to 6 fold increase in total circulating MP levels. However, we found no such stimulation when we performed the same experiment in CD47^{-/-} BMC. Next we double-labeled the plasma MP with annexin-V and anti-mouse CD31, CD41,

CD45 and Ter119 antibodies to characterize the cellular origin of the circulating MP. We found that CD41⁺ platelet MP represented about 80% of circulating MP, CD31⁺ CD41⁻ endothelial MP about 20%, CD45⁺ leukocyte and Ter119⁺ erythrocyte MP about 10% each in mice at rest. However, CD45⁺ leukocyte MP rose up to 40% after TSP1 injection, and
5 CD31⁺ CD41⁻ endothelial MP went up to 25%, whereas platelet and erythrocyte MP dropped.

We then used purified BMC as an in vitro model for leukocytes. We found that BMC treated with 10 µg/ml TSP1 did not shed MP in static suspensions. However, TSP1 is thought to exert pro-thrombotic and other effects under shear rate. We resorted to a cone and plate
10 Lorca ektacytometer to apply shear rate (1500 s⁻¹) on BMC suspensions for 10 minutes. We found that TSP1 combined with shear rate triggered a doubling in PS⁺ MP shedding versus shear alone. Among TSP1 cell surface receptors, CD47 had previously been linked to cytoskeletal remodeling and adhesion in multiple cell types, and CD47 activation can be recapitulated by synthetic peptides comprising the VVM motives of TSP1. To investigate a
15 possible connection with MP shedding, we evaluated wild type and CD47^{-/-} BMC in response to shear rate combined with TSP1, or the 6 amino-acid peptide 4N1-1 that repeats the CD47-specific sequence of the TSP1 carboxyterminus, TSP1 and 4N1-1 both triggered MP shedding in wild type BMC, but their effects were greatly reduced in CD47^{-/-} BMC.

To identify the active region of TSP1, we assessed a panel of carboxyterminal peptides
20 including CD47-specific 4N1-1, truncated 4N1-2, and mutated 4NGG in which the 2 valines of the CD47-binding motif are replaced by glycines. We found that MP shedding was stimulated by 4N1-1 peptide for 10 minutes, with a similar amplitude to serum deprivation for 72 hours, whereas other mediators of BMC adhesion like SDF1 and Fc-coupled Ephrin B2, or the control peptides 4N1-2 and 4NGG had no effects.

25 Next, we assessed the effects of a panel of cell signaling inhibitors on MP shedding in response to 4N1-1 to begin and unravel the intracellular mechanisms that may be at play, including kinase inhibitor HA1977 (ROCK). We also tested the contribution of Ca²⁺ with the intracellular chelator BAPTA-AM, or in the absence of extracellular Ca²⁺. We also tested the impact of G(α)_i proteins with Pertussis toxin (PTX), phospholipase-C with U73122, and
30 IP3-receptor with xestospongin. We found that MP shedding in response to 4N1-1 was entirely blocked by HA1077 and interference with Ca²⁺ mobilization, whereas Pertussis toxin, U73122 and xestospongin had no effect.

MP shedding, membrane and cytoskeletal remodeling

We investigated the remodeling of BMC, likely to accompany MP shedding in response to peptide 4N1-1. We found by electron microscopy that 4N1-1 triggered intense remodeling of the BMC membrane within 5 minutes, reducing microvillousities and promoting blebbing. Fluorescence microscopy after labeling of filamentous (Phalloidin, green) and depolymerized globular (DNase-1, red) actin revealed a re-organization of the cytoskeleton with depolymerization of stress fibers, accumulation of globular actin and relocation of filamentous actin in subcortical foci. We also characterized BMC by FACS and noted progressive PS externalization in response to 4N1-1, in association with a rapid and significant drop in forward (FSc) and side (SSc) light scatter indices. We defined quadrants for large and small BMC, quantified their proportion and characterized an overall reduction in cell size after treatment with 4N1-1. We wondered if the evaluation of PS⁺ MP by FACS after annexin-V labeling allowed the detection of all MP in BMC supernatants. We compared MP labeling with annexin-V (Green), or PKH-26 (red). We found that the numbers of detected MP with either methods were very similar, suggesting that nearly all MP were indeed PS⁺. Quantification of phalloidin⁺ and DNase-1⁺ cells showed no drastic change in overall BMC actin contents, but the shed MP contained both filamentous and globular actin.

CD47-induced MP interact with BMC and enhance their adhesion

We purified MP shed by PKH-26-labeled BMC stimulated with peptide 4N1-1 in vitro for 30 minutes. We incubated fresh naïve BMC with purified PKH-26-labeled MP (MOI 1:50) for 30 minutes, and performed epifluorescence microscopy. We noted that MP bound to the naïve BMC in a heterogeneous manner. To confirm the affinity of MP for BMC, we prepared MP suspensions, incubated BMC in the MP suspension for 30 minutes, and eliminated the BMC by centrifugation. We assessed the MP levels in supernatants by FACS, before and after addition and removal of BMC. As controls, we also quantified MP in the supernatants of BMC alone. We found that incubation with BMC depleted the MP suspensions very significantly. This suggested that the MP are spun out of suspension when the BMC were removed, unveiling strong affinity of MP for BMC.

TSP1 is known to mediate circulating cell adhesion and recruitment to the vascular wall. Hence, our next aim was to find what impact TSP1-induced MP may have on circulating cells. We primed BMC with or without purified MP for 30 minutes, allowing direct contact, or with separation by a 0.2 μ m nylon mesh. The cells were then washed and left to adhere to IL1 β -activated SVEC endothelial monolayers for 30 minutes. We found that MP derived from 4N1-1-treated BMC were pro-adhesive for other naïve BMC, and this was mediated by direct

contact of the MP with the cells. We also found that the addition of integrin-neutralizing cyclic peptide cRGDfv, PS-binding annexin-V, or neutralizing anti-PSGL1 antibody blocked the MP-induced BMC adhesion. Control cRAGfv peptide or non-immune IgG had no effect.

MP shed by BMC, PBMC, platelets and endothelial cells modulate BMC adhesion

We treated mouse endothelial SVEC4-10 cells in culture or fresh PBMC suspensions with 4N1-1, 4N1-2 or 4NGG peptides. We found that only 4N1-1 triggered significant MP shedding in these cells, suggesting a common mechanism of action with BMC. We purified these MP shed by fresh BMC, PBMC, platelets, or cultured SVEC endothelial cells in vitro, in response to 4N1-1 peptide, or after NCS deprivation for 48 h (apoptosis), or after treatment with TRAP peptide. We incubated naïve BMC with or without the different types of MP (MOI 1:50) for 30 minutes. We eliminated excess MP by centrifugation and left the BMC adhere to fibrin gels, or SVEC4-10 endothelial monolayers. We found that MP obtained by 4N1-1 stimulation raised BMC adhesion to fibrin gels and activated endothelium by 50% to 100%, regardless of cellular origin. Platelet MP had the distinct ability to stimulate adhesion to fibrin gels, but not to activated endothelium. On the contrary, apoptotic MP from all cellular origin had reduced or no effect. The results were similar on fibrin and endothelium, although basal levels were slightly higher on fibrin gels.

MP shed by 4N1-1 stimulation enhance BMC affinity for vascular lesions

We purified MP shed by BMC stimulated with 4N1-1 peptide (25 μ M). We incubated Celltracker-orange-labeled BMC with purified MP (MOI 1:50) for 30 minutes, washed the cells from excess MP and injected them intravenously to mice. After 10 minutes, we induced endothelial intravascular thrombosis in mesenteric microvessels by FeCl₃ superfusion (black arrows), and we monitored BMC interacting with the vascular wall by intravital microscopy. We found that only few BMC bounced against the vascular wall at a rate of 3 contacts per minute at rest before injury. There was no overt rolling. After injury, unprimed BMC interacted with the uninjured vascular wall at the increased rate of 5 contacts per minute, with a high rolling speed exceeding 800 μ m/sec. The cells never immobilized. After 5 minutes, the BMC interactions rarified and returned to control 15 minutes after injury. Thrombosis progressed and a thrombus gradually occluded the vessel lumen, and interrupted blood flow within 30 to 45 minutes. Priming BMC with BMC MP did not affect basal recruitment rates without injury. However, there was a slightly accelerated raise in recruitment rates after

vascular injury, suggesting enhanced homing of MP-primed BMC to the lesions. Peak recruitment rates were also slightly enhanced, raising up to 6 contacts per minute, in association with a rolling speed dropping 2 to 3 fold, down to 350 $\mu\text{m}/\text{sec}$. Some cells even began to immobilize in the injury area. Next, we primed BMC with platelet MP. We noted a trend toward slightly delayed recruitment, but the recruitment rates raised gradually to reach over 6 contacts per minute and did not return to baseline within the observation period. Priming with platelet MP reduced BMC rolling speeds down to 400 $\mu\text{m}/\text{sec}$, and favored their immobilization within the lesion.

Based on our data, we propose a novel model of leukocyte recruitment to vascular lesions involving the release and action of MP. In the early phases of thrombosis, activated platelets degranulate and create a local gradient of TSP1, which diffuses and accumulates in the thrombus matrix. The CD47 receptor present at the surface of circulating and vascular cells becomes activated by contacts with TSP1. In response to TSP1, cells passing in the area, including circulating leukocytes and platelets, or nearby vascular cells shed MP. These MP further increase and disseminate the activation signal for circulating cells. Through contact with circulating MP, leukocytes in particular gain a greater adhesive capacity and a greater avidity for substrates present in the vascular injury, such as fibrin and activated endothelium. This enables their specific homing and arrest at sites of vascular injury. Thus, MP shed at sites of vascular injury operate as homing signals.

Our experiments also showed how this novel cell activation pathway can be reproduced experimentally to generate purified MP, using short synthetic peptides encompassing specific 'VVM' motives of the TSP1 carboxyterminus, in a variety of cell types. Our data showed that MP shed under the action of these peptides can be purified and used to modulate the function of selected cells by simple incubation. The cell functions gained by interacting with 4N1-1-stimulated MP include increased ability to adhere, home and engraft into areas of vascular injury. In contrast, MP shed during apoptotic cell death do not always have the same ability to raise circulating cell adhesion and homing.

Hence, 4N1-1 peptide can be envisaged as a novel tool to generate novel therapeutic vectors in the form of MP with specific activity.

Moreover, cell therapy with BMC and other cells is currently being developed as a novel approach to help revascularize and repair ischemic or damaged tissues. 4N1-1-stimulated MP may represent a useful tool to enhance the benefits of cell therapy and to

modulate the homing and activity of pro-angiogenic cell preparations such as BMC. In addition, we could characterize the differential activity of MP shed by different cell types, such as platelet versus endothelial cells. In particular, we found that platelet MP had different effects from MP shed by endothelial cells or PBMC. Indeed, platelet MP did not enhance BMC adhesion to endothelium, but favored adhesion to fibrin gels and triggered intense homing to vascular lesions in vivo. Hence, there is an element of parent cell specificity to the activity of the produced MP preparation. Certain cell types may prove more useful than others to generate active MP preparations capable of stimulating cell therapy.

Novelty and technological advantages :

To our knowledge, there is currently not a single report mentioning the shedding of MP by cells stimulated with TSP1 or the 4N1-1 peptide.

In our experiments, we showed **for the first time** that :

- the TSP1-CD47 axis and the 4N1-1 peptide trigger MP shedding in a variety of cell types in vitro and in vivo,

- TSP1 and the 4N1-1 peptide are useful tools to generate cell MP, which can be purified and concentrated by ultracentrifugation,

- Cell MP shed under stimulation with 4N1-1 peptide can activate other cells and carry specific activity not shared by other types of MP,

- The shedding of MP in response to TSP1 may form an intrinsic link between platelet activation and the specific recruitment of CD47-bearing cells towards vascular lesions.

- The generation of MP of multiple cellular origins in response to TSP1 and 4N1-1, in vivo and in vitro, as well as their ability to bind other cell types, suggest that unrelated cells may exchange a significant quantity of material during thrombosis.

- We analyzed circulating cell adhesion and recruitment as one aspect of cell activation by MP. However, many other cell functions may also be modulated by MP, including those modulated by the extracellular matrix and fibrin in particular,

- Specificity of this mode of induction : We demonstrated the ability of TSP1 and TSP1-derived peptides known as CD47 receptor agonists (or Integrin-Associated Protein, IAP) to induce MP shedding. We demonstrated the specific activity of peptide 4N1-1 versus truncated peptide 4N1-2 or mutated peptide 4NGG.

- This MP production technique is extremely rapid, since one to 5 minutes of stimulation suffice to produce massive cell surface vesiculation and MP secretion in most of the tested cell types.

- The active principle, peptide 4N1-1, acts in vitro on cell suspension or on cell cultures, as well as in vivo by intravenous injection.

- The produced MP and the peptide can be separated by pelleting the MP by differential ultracentrifugation. The peptide can be entirely washed off the MP preparation.

- Peptide 4N1-1 is capable of inducing MP shedding from most cell types, including purified primary cells and immortalized cell lines ; cells in suspension like our mouse bone marrow mononuclear cells (BMC), circulating leukocytes (PBMC), and adherents cells such as mouse SVEC4-10 endothelial cells.

- The MP can be stored and frozen for long time conservation, with the possibility to create MP banks.

EXAMPLE 2: GENERATION OF CELL MICROPARTICLES FROM RED BLOOD CELLS (RBC).

We prepared fresh suspensions of purified red blood cells and incubated them for 30 minutes with peptides derived from the thrombospondine-1 carboxyterminus, including the CD47 receptor agonist peptide 4N1-1, or the truncated peptide 4N1-2, or the mutated peptide 4NGG. We collected the supernatants (S/N) and ultracentrifuged them to pellet and discard MP. Then we quantified MP by FACS after labeling with fluorescent annexin-V, compared with calibrated fluorescent microbeads. We found that peptide 4N1-1, derived from TSP1, triggered rapid and intense (about 3 fold) shedding of MP by red blood cells ($p<0.05$), whereas control truncated peptide 4N1-2 and mutated peptide 4NGG failed to do so.

EXAMPLE 3: GENERATION OF CELL MICROPARTICLES FROM MESENCHYMAL STEM CELLS (MSC).

We prepared fresh suspensions of purified mesenchymal stem cells and incubated them with peptides derived from the thrombospondine-1 carboxyterminus, including the CD47 receptor agonist peptide 4N1-1, or the truncated peptide 4N1-2, or the mutated peptide 4NGG. We collected the supernatants and ultracentrifuged them to pellet and discard MP. Then we quantified MP by FACS after labeling with fluorescent annexin-V, compared with

calibrated fluorescent microbeads. We found that peptide 4N1-1, derived from TSP1, triggered rapid and intense (about 3 fold) shedding of MP by mesenchymal stem cells, whereas control truncated peptide 4N1-2 and mutated peptide 4NGG failed to do so.

5

REFERENCES:

Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by
10 reference into the present disclosure.

1. Kosfeld MD, Frazier WA. Identification of a new cell adhesion motif in two homologous peptides from the cooh-terminal cell binding domain of human thrombospondin. *J Biol Chem.* 1993;268:8808-8814

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25

CLAIMS:

- 5 1. A method for generating cell microparticles comprising a step of consisting of contacting a cell with an agonist of the CD47/IAP receptor for a time sufficient to induce microparticles shedding by said cell.
2. The method according to claim 1 wherein said agonist of the CD47/IAP receptor is RFYVVMWK (SEQ ID NO:1).
3. An isolated cell microparticle obtainable by the method according to claim 1 or 2.
- 10 4. A pharmaceutical compositions comprising a population of the cell microparticles according claim 3.
5. An in vitro method for preparing cells for engraftment in a target tissue of a subject, wherein the cells to be engrafted are contacted with a population of cell microparticles according to claim 3.
- 15 6. An agonist of the CD47/IAP receptor for use in a method for enhancing cell engraftment in a subject in need thereof
7. The agonist of the CD47/IAP receptor for use according to claim 6 wherein a therapeutically effective amount of at least one of the above-defined agonist of the CD47/IAP receptor, preferably in association with cells to be engrafted, is
20 administered to said subject.
8. A population of cell microparticles according to claim 3 for use in a method for enhancing cell engraftment in a subject in need thereof.
9. The population of cell microparticles for use according to claim 8 wherein a therapeutically effective amount of a population of cell microparticles according to the
25 claim 3, preferably in association with cells to be engrafted, is administered to said subject.

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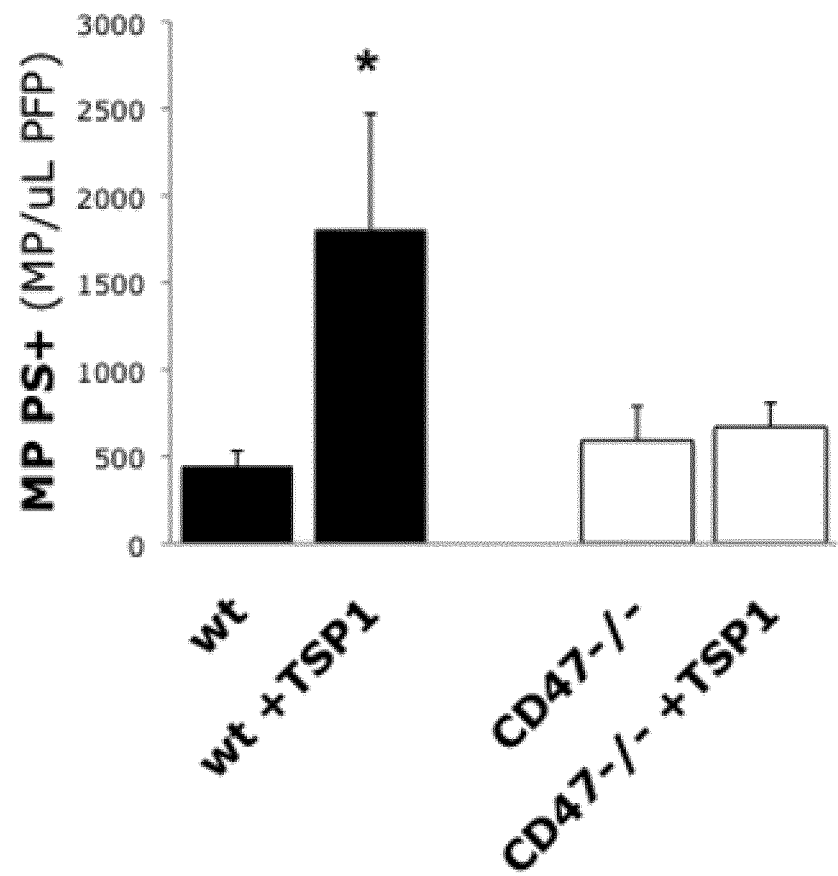


Figure 1 A

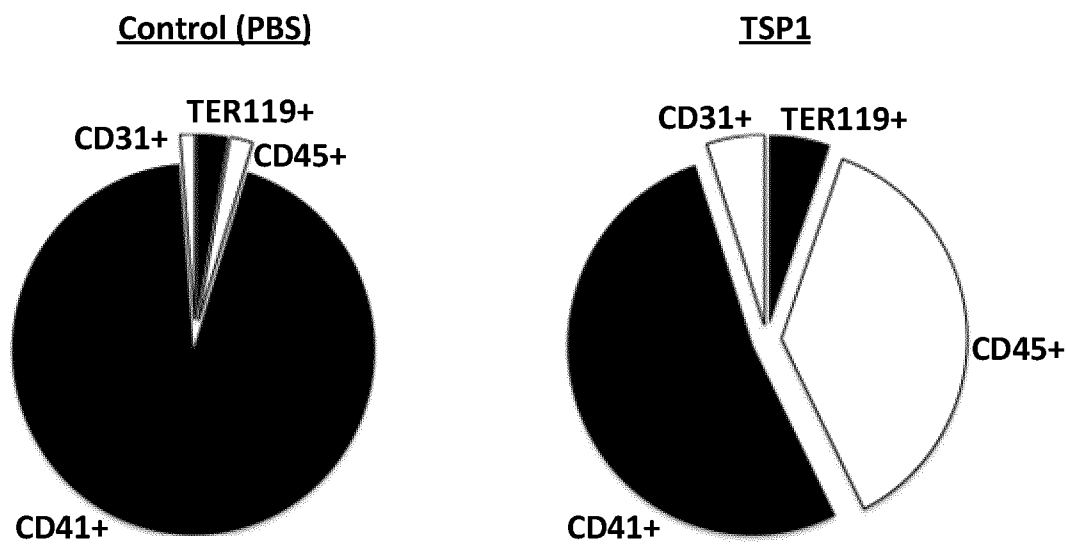


Figure 1C

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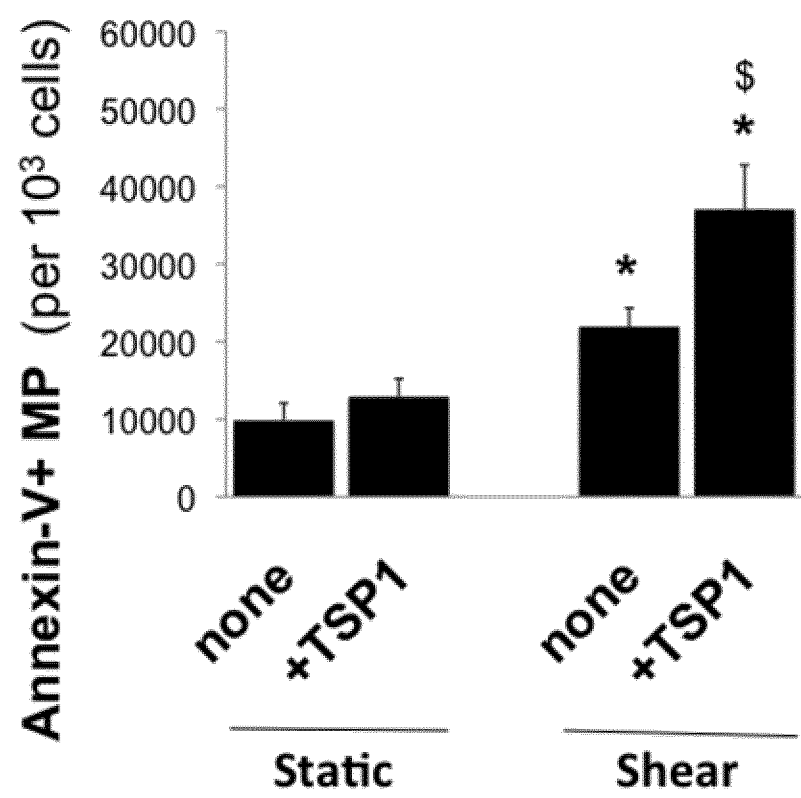


Figure 1D

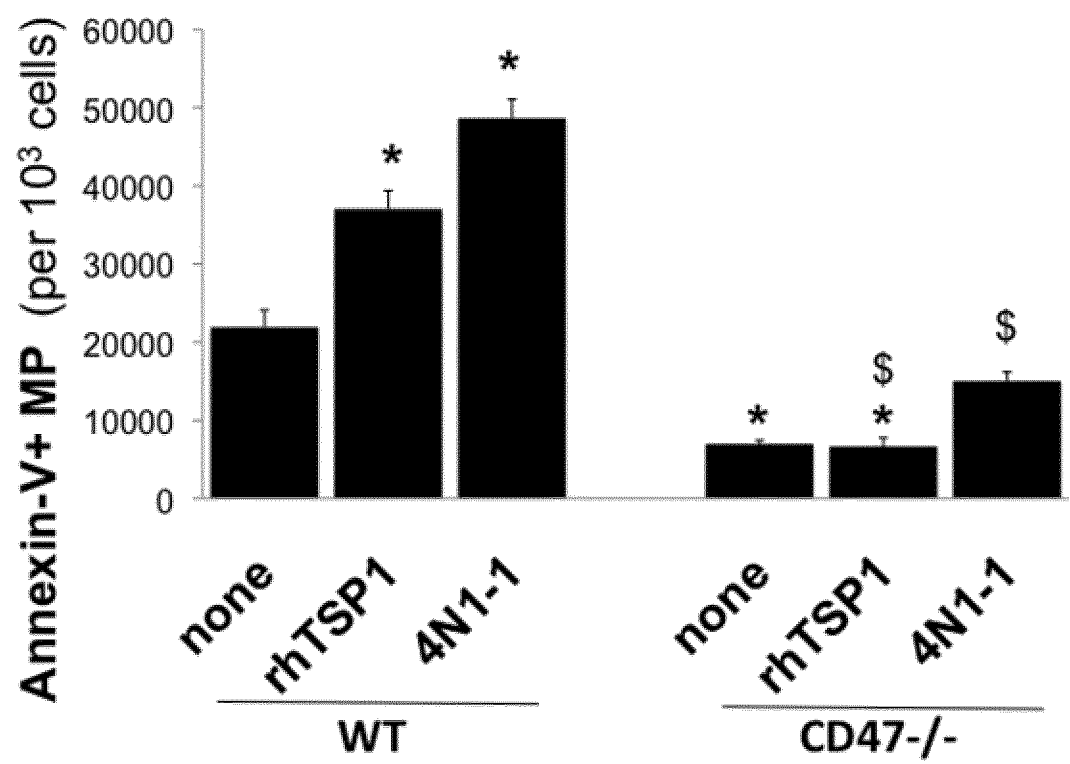


Figure 1E

3/5

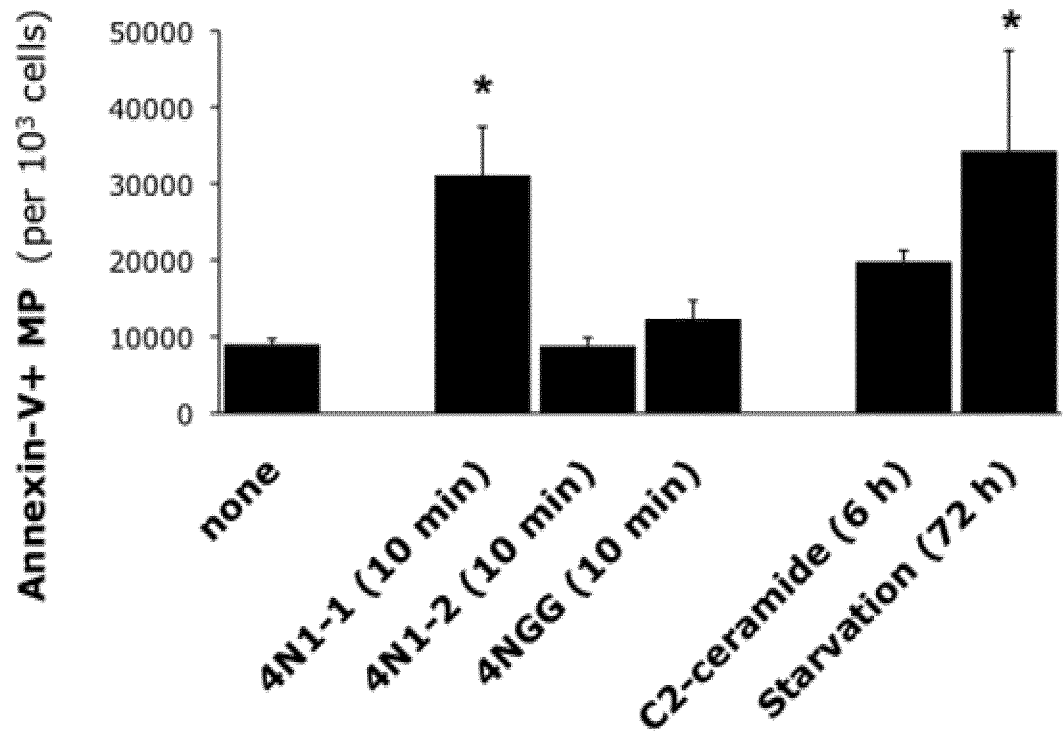


Figure 2A

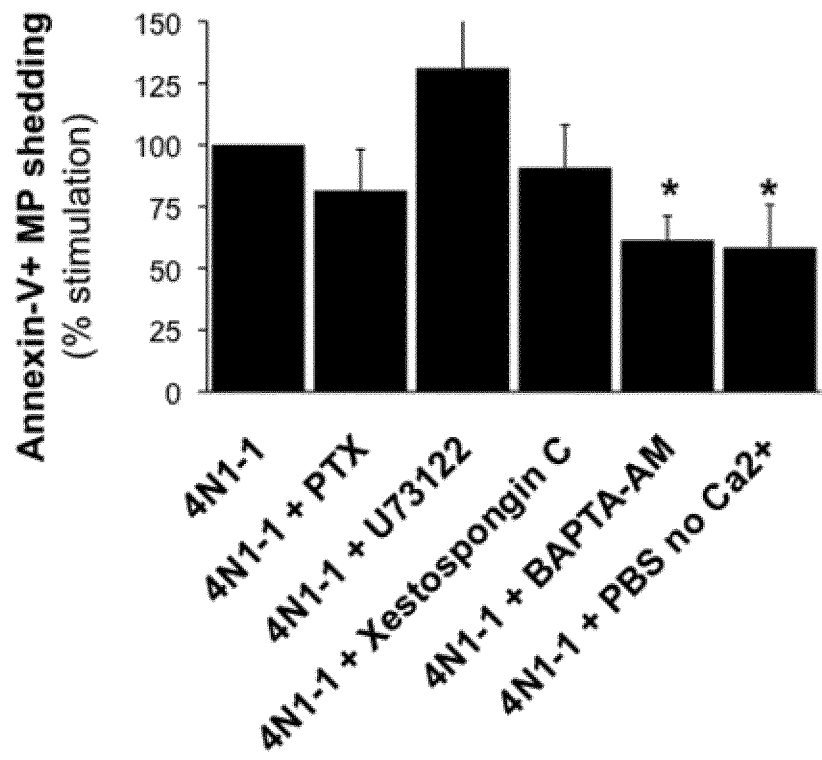


Figure 2B

4/5

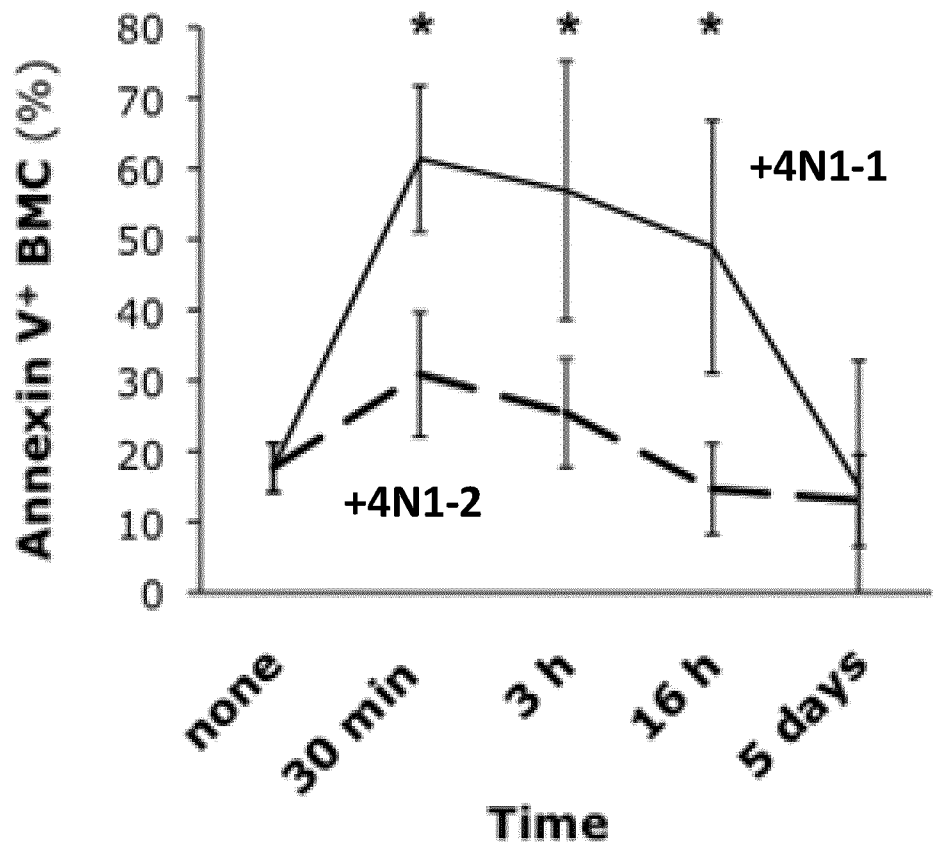


Figure 3C

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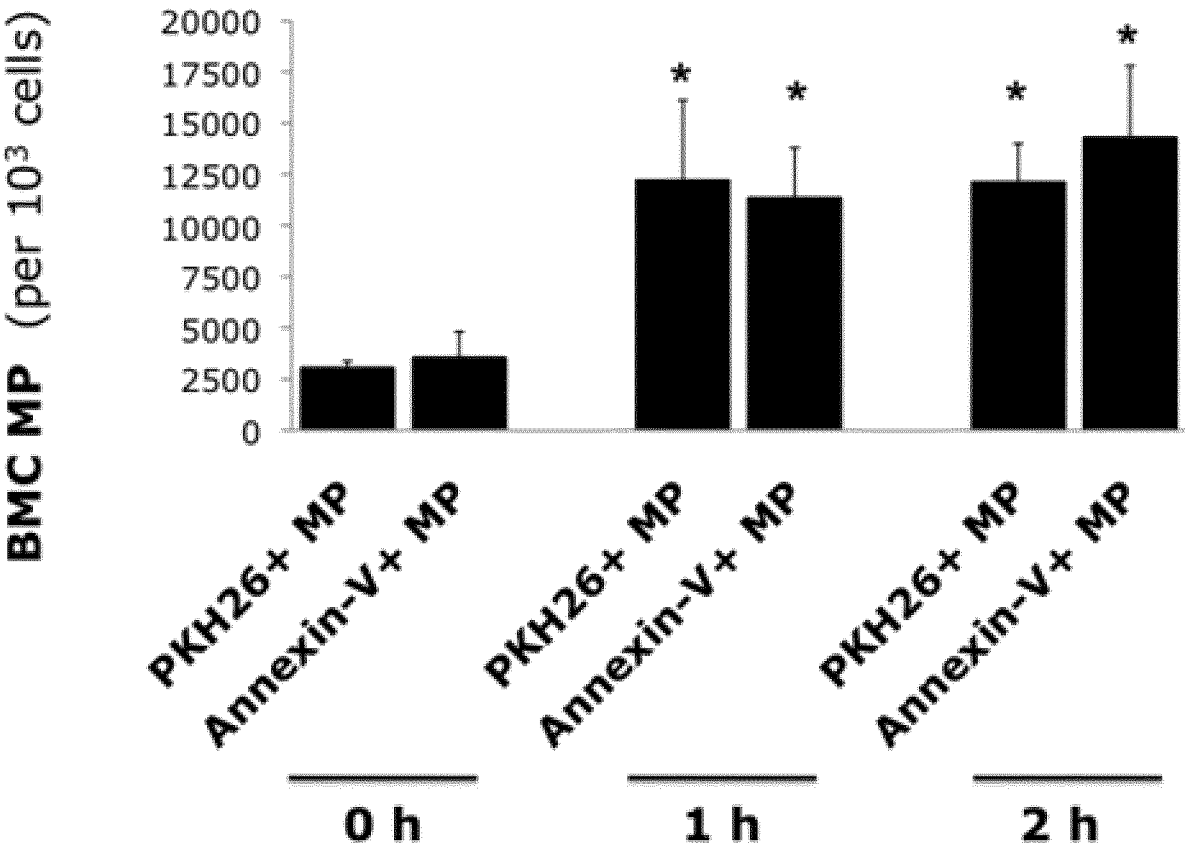


Figure 3E

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2012/054154

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
 - a. (means)

☐

 on paper
 - ☒

 in electronic form
 - b. (time)

☒

 in the international application as filed
 - ☐

 together with the international application in electronic form
 - ☐

 subsequently to this Authority for the purpose of search
2.

☐

 In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/054154

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/39 C07K14/705
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MOREL OLIVIER ET AL: "Cellular mechanisms underlying the formation of circulating microparticles.", ARTERIOSCLEROSIS, THROMBOSIS, AND VASCULAR BIOLOGY JAN 2011 LNKD- PUBMED:21160064, vol. 31, no. 1, January 2011 (2011-01), pages 15-26, XP009151473, ISSN: 1524-4636 page 22, column 2, paragraph 2 - page 23; figure 1 ----- -/--	1-9



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

7 May 2012

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2012/054154

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