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(54) **METHODS OF DONOR SPECIFIC CROSSMATCHING**

VERFAHREN FÜR DONORSPEZIFISCHES CROSSMATCHING

METHODES D'ESSAI DE COMPATIBILITE CROISEE SPECIFIQUE DU DONNEUR

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(73) Proprietor: **Absorber AB**  
**103 95 Stockholm (SE)**

(72) Inventor: **SUMITRAN-HOLGERSSON, Suchitra**  
**S-141 33 Huddinge (SE)**

(74) Representative: **Crump, Julian Richard John et al**  
**Mintz, Levin, Cohn, Ferris, Glovsky and Popeo IP, LLP**  
**Alder Castle**  
**10 Noble Street**  
**London EC2V 7JX (GB)**

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**Description****FIELD OF THE INVENTION**

5 **[0001]** The invention relates to a method for the direct isolation of endothelial cells from whole blood for routine donor-specific crossmatching to detect anti-endothelial cell antibodies prior to organ transplantation.

**BACKGROUND OF THE INTENTION**

10 **[0002]** The presence of donor lymphocyte-reactive Human Leukocyte Antigen (HLA)-specific antibodies either before and/or after renal allograft transplantation has been associated with hyperacute rejections, early acute rejections, and poor graft survival. However, rejections may occur in the absence of detectable lymphocytotoxic antibodies, suggesting that non-HLA antigenic systems may also play a role in renal allograft hyperacute and acute rejections. Antibodies reactive with endothelial cells and monocytes (also called the EM-antigenic system), or only with endothelial cells, have  
15 been described and reported to have a deleterious effect in several organ transplantations.

**[0003]** Recently, the major histocompatibility class I-related chain A antigen (MICA) expressed on endothelial cells was identified as one of the target antigens of humoral immunity associated with irreversible rejections of kidney allografts. Studies of HLA-identical living-related donor allografts showed that the presence of endothelial cell/monocyte reactive antibodies correlated with rejection, graft loss, and poor allograft function. It was reported that this reactivity could be  
20 responsible for up to 80% of irreversible rejections in this group of patients. However, the routinely used lymphocyte cross-match (LXM) does not permit detection of the clinically relevant HLA class-I, class-II, endothelial/monocyte-reactive and endothelial cell-specific antibodies. Although the presence of circulating endothelial cells in whole blood has been a subject of debate for many years, the existence of circulating precursor endothelial cells in adult humans has recently  
25 been reported by some investigators. However, there is currently no suitable method available to perform a routine donor-specific endothelial cell-crossmatch (ECXM).

**[0004]** Therefore, there is a need to efficiently perform routine donor-specific endothelial cell cross-matching to aid in the identification of better donor-recipient combinations, which will thereby have a greater impact on transplant survival than the current method of lymphocyte cross-match.

30 F. GEORGE ET AL, THROMBOSIS AND HAEMOSTASIS, vol. 67, no. 1, 1992, pages 147-153 relates to the rapid isolation of human endothelial cells from whole blood using s-endo1 monoclonal antibody coupled to immuno-magnetic beads and endothelial injury after angioplasty.

ROSELLA SBARBATI ET AL: BLOOD, vol. 77, no. 4, 15 February 1991 (1991-02-15), pages 764-769 relates to the immunologic detection of endothelial cells in human whole blood.

35 M.K. JONES ET AL: JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY, vol. 51, no. 4, 2000, pages 813-820 relates to the isolation and characterization of rat gastric microvascular endothelial cells as a model for studying gastric angiogenesis in vitro.

MARIO PEICHEV ET AL: BLOOD, vol. 95, no. 3, 1 February 2000 (2000-02-01), pages 952-958 relates to the identification of a population of functional endothelial precursors through the expression of VEGFR-2 and AC133.

**SUMMARY OF THE INVENTION**

40 **[0005]** The invention pertains to a method of isolating precursor endothelial cells which are useful in donor specific crossmatching prior to transplantation. The isolated precursor endothelial cells are also useful in diagnosing various vascular and autoimmune related disorders.

45 **[0006]** The invention includes a method of isolating a precursor endothelial cell from a biological sample by providing a sample known to contain, or suspected of containing, a precursor endothelial cell. The precursor endothelial cell is Tie-2 positive. The sample is contacted with a detection reagent to form a detection reagent-endothelial cell complex. The precursor endothelial cell is isolated by separating the complex from the sample. Separation is accomplished, for example, by flow cytometry or use of a magnetic field.

50 **[0007]** The detection reagent is an antibody specific for Tie-2. The detection reagent can be attached to a solid support, such as a non-magnetic, magnetic, or paramagnetic bead.

**[0008]** The biological sample is whole blood, sera, peripheral blood mononuclear cells (PBMCs) or leucapherisate.

55 **[0009]** The invention further provides a method of cross-matching a donor and a recipient by providing a biological sample from a donor that is known to contain a precursor endothelial cell, contacting the donor sample with detection reagent to isolate a precursor endothelial cell. A recipient biological sample is screened for reactivity with the donor isolated precursor endothelial cell. No reactivity between the recipient sample and the isolated precursor endothelial cell indicates compatibility between the donor and recipient and a higher probability that the organ transplant will be successful.

**[0010]** In a further aspect, the invention includes a method of diagnosing an autoimmune related or vascular disorder

in a subject by providing a first sample from a subject known to contain, or suspected of containing, a precursor endothelial cell. The first sample is contacted with a second sample. The second sample is from a subject known to contain, or suspected of containing, an auto antibody. Alternatively the second sample contains reagents (*e.g.* antibodies) that recognize endothelial cell surface markers associated with the particular disorder. Complex formation between the precursor endothelial cell and the second sample is determined. The presence of the complex indicates the disorder in the subject.

**[0011]** In yet another aspect, the invention includes a method of determining the efficacy of treatment or prognosis of an autoimmune related or vascular disorder in a subject by providing a first sample from a subject known to contain, or suspected of containing, a precursor endothelial cell, contacting the precursor endothelial cell with a second sample from a subject known to contain, or suspected of containing, an autoantibody, measuring any autoantibody-endothelial cell precursor complex present to yield a subject profile. The subject profile is compared with a reference profile, wherein a similarity between the subject profile and reference profile indicates that the treatment is efficacious or a favorable prognosis.

**[0012]** Autoimmune related or vascular disorders include vasculitis, atherosclerosis, bleeding disorders, angiogenesis, thrombosis, defective wound healing, and transplantation.

**[0013]** Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

**[0014]** Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

## BRIEF DESCRIPTION OF THE DRAWINGS

### [0015]

Figure 1 shows dot plots representing forward and side scatter profile as well as fluorescence of peripheral blood mononuclear cells (PBMCs) stained with anti-Tie-2+ monoclonal antibodies (Mabs). Tie-2+ cells (grey dots) appeared in the lymphocyte gate. Histograms show the percentage of Tie-2+ cells (app.  $2 \pm 3\%$ ) in PBMCs stained with negative control abs and Tie-2 Mabs.

Figures 2a-c are photographs of the morphology of Tie-2+ cells under light microscope at various time points after isolation from peripheral blood. Initially, these cells appeared either as single cells or as clusters of round cells (a), which after few days of culture converted into adherent cells with extended cytoplasm, (the dark objects are beads) (b). After 7-12 days in culture the cells gradually developed a spindle shape (c).

**Figure 3a** is a series of photographs showing Tie-2+ cells stained positive for the endothelial associated markers, acetylated low density lipoprotein (Ao-LDL), von Willebrand factor (vWF), vascular endothelial growth factor receptor-1 (VEGFR-1) and the vascular cell adhesion molecule (VCAM)(on activated Tie-2+ cells). Tie-2+ cells also constitutively expressed the human leukocyte antigen class I (HLA class I) and the monocyte/macrophage marker CD68.

**Figure 3b** shows that quiescent Tie-2+ cells expressed small amounts of the clinically important MICA antigens (dark grey), and HLA class II (light grey), control antibodies (black).

**Figure 4a** is a dot plot that represents the forward and side scatter of rosettes of Tie-2+ cells and paramagnetic beads (gate R1) and paramagnetic beads only (gate R2).

**Figures 4b-g** are histograms showing that pro-transplant sera from two kidney-transplanted patients with hyperacute rejections reacted strongly with their respective donor-specific Tie-2+ cells (b & e), while their Tie-2- fractions, which included lymphocytes did not (d & g). Histograms c & f show that the paramagnetic beads alone did not react with the sera non-specifically. The grey lines represent control or negative sera, and the black lines represent reactivity with patient's sera.

## DETAILED DESCRIPTION OF THE INVENTION

**[0016]** The invention is based in part on the discovery that targeting a single specific cell population allows for the detection of clinically relevant donor-specific human leukocyte antigens(HLA) class I, class II, endothelial-monocytes,

or endothelial cell-specific antibodies prior to transplantation of organs. Routine use of the endothelial cell cross matching will aid in identifying better donor-recipient combinations and thus have a greater impact on transplant survival as compared to traditional lymphocyte cross matching.

**[0017]** The clinical importance of endothelial cell (EC) antibodies in allo-transplantation has been reported. However, lack of a suitable method for isolation of donor-specific ECs has prevented routine detection of these antibodies prior to transplantation. The invention provides a quick and simple method for the direct isolation of precursor ECs from whole blood, for routine cross-matching to detect anti-EC antibodies. The presence of endothelial cell- reactive antibodies has previously been detected using human umbilical vein endothelial cell (HUVEC) lines, keratinocyte cell lines, or monocytes as targets, or by immunohistochemistry. However, these methods are cumbersome because endothelial cell culturing is tedious and a generally large panel of donors of HUVEC lines/keratinocytes is required to represent all the known polymorphic alleles for screening of endothelial cell- reactive antibodies. Moreover, these methods do not permit the detection of donor-specific endothelial cell antibodies. Thus, use of an endothelial cell cross-match is advantageous over use of lymphocyte cross-match because lymphocyte cross-match does not allow for such detection or isolation of donor-specific endothelial cell-reactive antibodies.

**[0018]** ECs were isolated using magnetic beads coated with antibodies against the angiopoietin receptor, Tie-2 that is expressed on EC precursors. Tie genes play an important role in renal vascular development, and based on transplantation experiments these precursors have been shown to contribute to the generation of glomerular maturation. A retrospective analysis of 50 previously well characterized crossmatch sera taken immediately prior to transplantation from patients with end-stage kidney disease were tested. Tie-2+ cells expressed HLA class I, class II and other endothelial cell markers. Sera known to contain only EC specific or EC and monocyte (EM) reactive antibodies reacted positively with Tie-2+ cells, but not with Tie-2-cells from the same individual. In addition, the Tie-2+ cells reacted with sera containing only HLA class I or class II abs. In all, 3/25 sera from patients with stable graft outcome and no rejections reacted with Tie-2+ cells. This antigen-antibody interaction is relevant to the pathogenesis of rejection, since in many studies these antibodies are not detected in the serum of patients with good graft function or in non-transplantation patients.

#### Methods of Isolating Precursor Endothelial Cells

**[0019]** The invention includes methods of isolating a precursor endothelial cell from a mixture of cells by contacting the mixture of cells with a detection reagent to form a precursor endothelial cell- detection reagent complex. The complex is formed via a specific affinity interaction between the detection reagent and the cell. The complex is separated from the mixture to isolate the precursor endothelial cell. The complex is separated from the mixture using techniques known in the art, such as by, *e.g.*, liquid chromatography (*e.g.*, HPLC or FPLC), High Performance Membrane Chromatography (HPMC), flow cytometry or use of a magnetic field. Alternatively, the complex is separated from the mixture by attaching the detection reagent to a solid support. A washing step may be employed by re-suspending the complex in a biologically compatible solution. The complex can be re-suspended, *i.e.*, washed as many times as desired. Typically the particles are washed three times. A biologically compatible solution include biological buffers known in the art such as phosphate buffer saline (PBS).

**[0020]** The detection reagent is an antibody specific for Tie-2. The antibody is a monoclonal antibody or a polyclonal antibody. The term antibody encompasses not only an intact antibody, but also an immunologically-active antibody fragment, *e.g.*, a Fab or (Fab)<sub>2</sub> fragment; an engineered single chain Fv molecule; or a chimeric molecule, *e.g.*, an antibody which contains the binding specificity of one antibody, *e.g.*, of murine origin, and the remaining portions of another antibody, *e.g.*, of human origin. For example the detection reagent is monoclonal antibody Tie-2.

**[0021]** The detection reagent can be attached to a solid support. The solid support is a particle, a polymer (*e.g.*, polystyrene, polyethylene), a vessel, a chamber, a dipstick, beads, particles, membranes (*e.g.*, nylon, nitrocellulose or polyvinylidenedifluoride (PVDF)), or other forms known in the art.

**[0022]** The solid support may carry functional groups such as hydroxyl, carboxyl, aldehyde or amino groups. The solid support may be positively charged, negatively charged or hydrophobic. Functionalized coated supports for use in the present invention may be prepared by modification of the support. For example, uncoated support is treated with a polymer carrying one or such functional groups, such as polyurethane together with a polyglycol to provide hydroxyl groups, or a cellulose derivative to provide hydroxyl groups, a polymer or copolymer of acrylic acid or methacrylic acid to provide carboxyl groups, or an aminoalkylated polymer to provide amino groups. US Pat No. 4,654, 267 describes the introduction of many surface coatings.

**[0023]** The particle is made of metal compounds, silica, latex, polymeric material, or a silica, latex or polymer nuclei coated with a metal or metal compound. Preferably, the particle is made of a metal compound, such as iron, gadolinium, zinc, indium, gold, silver, cobalt, copper, or magnesium. Most preferably, the particle is magnetizable or magnetic. By "magnetizable or magnetic" is meant that the particle is capable of having a magnetic moment imparted to it when it is placed in a magnetic field.

**[0024]** The detection reagent is labeled with a detectable marker. For example the detection reagent to labeled with

a radioactive isotopes (e.g.,  $^{125}\text{I}$ , and  $^{131}\text{I}$ ), enzymes (e.g., peroxidase, beta-galactosidase, alkaline phosphatase) or fluorescent substances (e.g., fluorescein isothiocyanate (FITC)). The labels are quantified by the conventional methods well-known in the art, thereby the formed immune complex is quantified. The mixture of cells is any sample known to or suspected of containing an endothelial cell. For example the mixture is a biological sample such as whole blood, sera,

leucapherisate, bone marrow, peripheral blood mononuclear cells or a tissue homogenate.  
**[0025]** An endothelial cell is any cell derived from any part of the vascular tree. For example the endothelial cell is from large and small veins and arteries capillaries, the umbilical vein of newborns, blood vessels in the brain or from vascularized solid tumors. The endothelial cell is an endothelial cell precursor which is Tie-2 positive.

#### Methods of Donor Specific Crossmatching

**[0026]** Cross-matching detects those antigenic differences to which the recipient is already sensitized. A donor is crossmatched to a recipient by contacting a donor sample with a detection reagent to isolate a precursor endothelial cell. The recipient sample is contacted with the isolated precursor endothelial cell and reactivity of the recipient sample with the isolated precursor endothelial cell is determined. By "reactivity" it is meant that that a complex is formed via a specific affinity interaction between the recipient sample and the cell. No reactivity of the recipient sample with the isolated precursor endothelial cell indicates compatibility between the donor and recipient sample. In contrast, reactivity of the of the recipient sample with the isolated precursor endothelial cell indicates non-compatibility between the donor and recipient sample. Compatibility is measured by no or low hyperacute rejection of the donor transplant by the recipient.

**[0027]** The donor and the recipient are, e.g., any mammal, e.g., a human, a pig, a cow or a horse. The donor and the recipient are the same species. Alternatively, the donor and the recipient are of different species.

**[0028]** The donor and recipient sample is, for example, whole blood, sera, leucapherisate, bone marrow, peripheral blood mononuclear cells or a tissue homogenate. Optionally, the samples are subjected to a pre-purification step prior to crossmatching.

**[0029]** Reactivity is determined my methods known in the art. For example reactivity is measured by an ELISA assay, flow cytometry (e.g. flow cytometric cross-match), or complement-dependent lymphotoxicity cross-match.

#### Vascular and Immune Disorders

**[0030]** The invention also provides methods of diagnosing, accessing the prognosis or monitoring the course of treatment of vascular and autoimmune disorders.

**[0031]** In these methods a first test sample is provided from a subject The sample is known to or suspected of containing a precursor endothelial cell. Optionally, the precursor endothelial cell is isolated from the first sample.

**[0032]** An autoimmune disorder is diagnosed by contacting the first sample to a second sample from the subject known to contain, or suspected of containing, an auto-antibody and identifying an autoantibody- precursor endothelial cell complex. The presence of an autoantibody-precursor endothelial cell complex indicates the subject is suffering from or predisposed to an autoimmune disorder. In contrast, the absence of an autoantibody-precursor endothelial cell complex indicates the subject is not suffering from or predisposed to an autoimmune disorder.

**[0033]** A vascular disorder is diagnosed by contacting the first sample to a second sample. The second sample is derived from the subject. Alternatively, the second sample comprises antibodies to cell surface markers know to be associated with a particular vascular disorder. The presence of a second sample-precursor endothelial cell complex indicates the subject is suffering from or predisposed to a vascular disorder. In contrast, the absence of a second sample-precursor endothelial cell complex indicates the subject is not suffering from or predisposed to a vascular disorder.

**[0034]** The methods allow the course of treatment of a vascular or autoimmune related disorder to be monitored or the prognosis of the subject to be determined. In this method, a test sample is provided from a subject undergoing treatment for the disorder. If desired, test samples are obtained from the subject at various time points before, during, or after treatment. The presence of a precursor endothelial cell complex is then determined to create a subject profile. The subject profile is compared to a reference profile whose vascular disorder or autoimmune disorder state is known. The reference profile has not been exposed to the treatment. The reference profile is derived from a sample type as similar to test sample. Optionally, the reference profile is derived from a database of molecular information derived from samples for which the assayed parameter or condition is known.

**[0035]** If the reference profile contains no autoantibody-precursor endothelial cell complexes, a similarity in the amount of complexes between the subject profile and the reference profile indicates that the treatment is efficacious (e.g., that one or more symptoms of the immune disorder are alleviated or that the severity of the disorder is reduced), and thus, a favorable prognosis for the subject. However, a shift in the amount of complexes between the subject profile and the reference profile indicates that the treatment is not efficacious, and thus, an unfavorable prognosis for the subject.

**[0036]** When the reference profile contains autoantibody-precursor endothelial cell complexes, e.g., when the reference profile includes complexes taken from the subject at the time of diagnosis but prior to beginning treatment, a similarity

in the expression of complexes pattern between the subject profile and the reference profile indicates the treatment is not efficacious. In contrast, a shift in expression complexes in the subject profile and this reference profile indicates the treatment is efficacious.

**[0037]** By "efficacious" it is meant that the treatment leads to a decrease in any of the symptoms of an autoimmune disorder in a subject previously noted. When treatment is applied prophylactically, "efficacious" means that the treatment retards or prevents an autoimmune related or vascular disorder.

**[0038]** An autoimmune disorder includes disorders mediated by an immune mechanism such as deposition of immune complexes, inflammation, direct attack by circulating antibodies. An autoimmune disorder or an autoimmune related disorder includes those disorders caused by an immune response against the body's own tissues. Autoimmune disorders result in destruction of one or more types of body tissues, abnormal growth of an organ, or changes in organ function. The disorder may affect only one organ or tissue type or may affect multiple organs and tissues. Organs and tissues commonly affected by autoimmune disorders include blood components such as red blood cells, blood vessels, connective tissues, endocrine glands such as the thyroid or pancreas, muscles, joints, and skin. Autoimmune disorders include for example, autoimmune hemolytic anemia, autoimmune hepatitis, Berger's disease, chronic fatigue syndrome, Crohn's disease, Hashimoto's thyroiditis, fibromyalgia, systemic lupus erythematosus, Graves' disease, idiopathic thrombocytopenia purpura, multiple sclerosis, psoriasis, rheumatic fever, rheumatoid arthritis,

**[0039]** Vascular disorders include disease associated with the vascular system. For example vasculitis, atherosclerosis, bleeding disorders, defective wound healing.

**[0040]** Symptoms of an autoimmune disorder depend on the specific disease and the organ or tissue that is affected. For example, systemic lupus erythematosus may cause kidney failure, arthritis, and a skin rash on the face. Autoimmune hemolytic anemia causes anemia, or low red blood cell counts. Generally symptoms of autoimmune disorders may include: low-grade fever, malaise, which is a vague feeling of illness, fatigue, or tiring easily. Autoimmune disorders are diagnosed based on symptoms, a physical exam and the results of blood tests.

**[0041]** Treatments to reduce symptoms may include: nonsteroidal anti-inflammatory drugs (NSAIDs), including aspirin or ibuprofen, to relieve fever, joint pain, and muscle aches corticosteroids, or steroid, help reduce inflammation. These medications are often used on a short-term basis to get a person through a sudden episode or flare-up, medications to suppress the immune system, such as methotrexate, azathioprine, and cyclophosphamide, which help to reduce inflammation and organ damage. In some cases, other treatments may be needed. For example, surgery may be needed for blockage of the bowels, which may occur in Crohn's disease. Blood transfusions may be needed in severe cases of autoimmune hemolytic anemia. Insulin is given to individuals with type 1 diabetes to control blood glucose levels.

**[0042]** The subject is preferably a mammal. The mammal can be, e.g., a human, non-human primate, mouse, rat, dog, cat, horse, or cow.

## GENERAL METHODS

**[0043]** The data described herein was generated using the following reagents and methods.

### **EXAMPLE 1: Coupling of anti-Tie-2 Mabs to magnetic beads**

**[0044]** Mouse anti-human Tie-2 monoclonal abs. (Mabs) (BD Pharmingen, Oxford, UK) were first coupled to pan-mouse Dynabeads with a DNA-linker (cat. no. 115.19)(DYNAL, Oslo, Norway). For this purpose, 10  $\mu$ g of Tie-2 Mabs were added to 500  $\mu$ s of pan-mouse Dynabeads. The bead-Mab suspension was rotated on a rock n' roller for 24 hrs at 4°C. Excess Mab was removed and the Tie-2 Mab coated beads were blocked with 2 ml phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) on a rock n' roller at 4°C for 10 min. The blocking step was repeated six times after which, the beads were resuspended in original volume (500  $\mu$ s) of PBS/0.1% BSA.

### **EXAMPLE 2: Isolation of Tie-2+ cells from peripheral blood mononuclear cells (PBMC)**

**[0045]** Due to the large numbers of cells required to establish the specificity of the Tie-2+ cells, in the initial experiments PBMCs were isolated from leucapherisate of healthy blood donors by gradient centrifugation with lymphoprep (Nycomed-Oslo, Norway). ECs were isolated from PBMCs using anti-Tie-2 Mabs coated magnetic beads. PBMCs were first distributed into several tubes each containing 40 x 10<sup>6</sup> cells. Fifteen  $\mu$ ls of pre-coated Tie-2 magnetic beads were added to each tube and incubated in a volume of 500  $\mu$ ls RPMI medium (GIBCO, Paisley, UK) supplemented with 2 mM L-glutamine and 10% heat inactivated fetal calf serum. Cells+beads were incubated on rock 'n roller at 4°C for 30 min. Tie-2+ cells were separated after extensive washing (5-6 times) with PBS using a magnet. Tie-2+ cells from all tubes were pooled together and rosettes were counted under a light microscope.

**EXAMPLE 3: Isolation of Tie-2+ cells directly from peripheral blood**

[0046] 40 ml-heparinized blood was obtained from normal healthy donors. The blood was washed once as follows: 10 ml blood was diluted with 40 ml PBS/0.1%BSA. The blood was centrifuged at 800 g for 10 min. The supernatant was discarded and the blood cells were resuspended in 10 ml of PBS containing 0.6% sodium citrate. Fifty  $\mu$ ls of Tie-2 Mabs coated magnetic beads were added to each tube and incubated for 30 min at 4°C on a rock 'n roller. Tie-2+ cells were collected by a magnet and washed once with PBS-Na-citrate. After 4-5 washes with PBS, Tie-2+ cells were collected and the concentration of the cells adjusted to approximately  $3-4 \times 10^6$  cells/ml. These cells surrounded by beads were used either in a microcytotoxicity assay or the flow cytometer.

**EXAMPLE 4: Immunocytochemistry**

[0047] The Mabs used for immunocytochemistry and FACs analysis are given in Table 1. Tie-2+ cells obtained from leucapherisate of normal volunteers gave sufficient numbers of cells to perform various immunocytochemical analysis. Tie-2+ cells were grown on fibronectin-coated tissue culture plates. Cells were allowed to attach (24 hrs) prior to staining for various EC-specific markers. Cells were left either untreated or stimulated with TNF- $\alpha$  and IFN- $\gamma$  for 14 hrs. For immunocytochemistry, cells were fixed using 30% acetone in methanol for 1 min. After two washes with PBS, cells were blocked using 1% bovine serum albumin (BSA) in PBS for 1 hr at room temperature. The cells were washed twice with PBS and incubated with the above mentioned primary antibodies diluted 1:100 (in PBS) at 4°C for 2 hrs. The secondary antibody was a goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) diluted 1:500 (in PBS). After incubation for 1 hr at 4°C, the cells were washed twice and analysed under a fluorescence microscope.

**EXAMPLE 5: Flow cytometric assay for detection of anti-endothelial cell antibodies**

[0048] A total of 50 sera from kidney-transplanted patients were studied. Over the past years (1988-2001) the pre-transplant sera from kidney patients has been meticulously characterized, using various methods, for the presence of endothelial cell specific, endothelial-monocyte reactive, and HLA antibodies found to be associated with rejections. During these years 15 pre-transplantation sera, characterized as having either anti-EM or EC specific antibodies from non-alloimmunized patients with rejections, have been collected.

[0049] The general methods described herein are based on these 15 sera. Previous characterization showed that ten sera gave positive reactions with human umbilical vein endothelial cell (HUVECs) lines and monocytes (Table 2, pt. nos. 1-10), and five sera with only ECs (Table 2, pt. nos. 11-15). Since none of the fifteen patients with EM or EC-specific antibodies had been alloimmunized, no detectable HLA alloantibodies were found in these sera. In addition, ten well-characterized sera from alloimmunized patients known to contain only HLA class I or only class II alloantibodies were selected (Table 2). As controls, 25 sera from patients with no graft rejections were also tested.

[0050] Using these sera, it was determined whether the isolated Tie-2+ cells could be suitable targets for detection of clinically relevant antibodies in kidney organ transplantation. A pool of sera from patients who had formed alloantibodies as a result of multiple blood transfusions or organ transplantations was used as a positive control. Sera from healthy non-transfused blood group AB males served as negative controls. The following antibodies, FITC-conjugated F(ab')<sub>2</sub> fragments of goat anti-human IgG (Fc specific), or IgM (Immunotech, USA) were used. For the flow cytometric ECXM 100,000 Tie-2+ cells coupled to beads were used and the procedure carried out as described earlier. The cells were analyzed on a Becton Dickinson flow cytometer (FACSsorter). A shift in the mean fluorescence of 10 channels in the test sample as compared to negative control was considered as positive, determined as described before. This value is arbitrary and should be determined by each transplant laboratory. The Tie-2+ cells were also used in the routinely performed immune-magnetic microcytotoxicity assay as described elsewhere. In addition, Tie-2+ cells immediately after isolation were tested in the flow cytometer for all the endothelial cell surface marker (Table 1).

**EXAMPLE 6: Immunocytochemistry and FACS analysis of endothelial cell markers on isolated Tie-2+ cells**

[0051] Tie-2+ ECs were isolated from human peripheral blood by magnetic bead selection. FACS analysis showed that approximately  $3 \pm 4\%$  of PBMCs were Tie-2+ (Fig. 1). After 24 hours in culture, the majority of Tie-2+ cells attached to fibronectin-coated 24 well plates and became adherent. Initially, these cells appeared either as single cells or as clusters of round cells, which after few days of culture converted into adherent cells with extended cytoplasm. After 4 days in culture the cells gradually developed spindle shape (Fig. 2a-c).

[0052] Figure 3 and Table 1 summarize the results of the immunocytochemical analyses using antibodies to known antigens of endothelial cells. Already at 0 hours, FACS analysis indicated that Tie-2+ cells expressed the acLDL-receptor, vWF, VEGFR-1, and strong expression of HLA class I and HLA class II antigens. Importantly, the cells expressed the clinically important MICA antigen, though weakly. Upon cytokine activation, the cells expressed both the endothelial cell

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specific adhesion molecules CD62 E (E-selectin) and CD 106 (VCAM), as well as increased expression of HLA class II. Thus, both the immunocytochemical and FACS analysis indicated that Tie-2+ cells expressed majority of the endothelial cell markers.

### 5 **EXAMPLE 7: Detection of anti-endothelial cell reactive antibodies**

[0053] On an average, approximately,  $3 \pm 4 \times 10^4$  Tie-2+ cells / $10^6$  PBMCs can be obtained from single donors. Since a large number of sera were analyzed herein Tie-2+ cells from leucapherisate of healthy blood donors were isolated. The results are shown in Table 2. All the 10 sera known to have endothelial-monocyte antibodies and 5 sera with endothelial cell-specific antibodies showed varying patterns of reactivity with the panel of Tie-2+ cells from six different donors. However, the Tie-2- fractions, which included the lymphocytes from the same donors, did not react with any of the sera. The reactivity pattern with the Tie-2+ cell panel of both the endothelial-monocyte and endothelial cell-specific antibodies as seen in Table 2, indicates the existence of polymorphism in these antigenic systems or alternatively presence of antibodies with varied specificities.

15 [0054] Sera known to contain only HIT class I broadly reactive alloantibodies also gave positive reactions in every instance with Tie-2+ cells from single donors. Reactivity of HLA class II alloantibodies (limited specificities) was also observed with unstimulated Tie-2+ cells. 3/25 (12%) sera from control patients with no rejections and with stable graft functions gave positive reactions with four of the Tie-2 panel donors (Table 2). Results obtained using the immunemagnetic microcytotoxicity assay were in agreement with the flow cytometric analysis (Table 3).

20

### **EXAMPLE 8: Donor-specific endothelial cell cross-match**

[0055] Donor-specific endothelial cell crossmatches were retrospectively performed in two cases where frozen donor peripheral blood mononuclear cells were available. The first kidney grafts of both patients were lost in hyperacute rejections in the absence of demonstrable donor-specific HLA antibodies. Cross-match sera from one of these patients had been previously well characterized as having anti-endothelial cell-specific antibodies using HUVECs.

25 [0056] In this instance, Tie-2+ cells from the peripheral blood mononuclear cells of this patient's (point. no.11, Table 2) first donor (father) were isolated and kept frozen in liquid N<sub>2</sub>. A cross-match with the serum of the patient taken immediately prior to the first transplant was retrospectively performed. The results are shown in Figure 4b-d. The results from a second case (point. no. 12, Table 2) are very similar to point no. 11, and are shown in Figure 4e-g. As seen, in Table 2, the sera from both these points gave similar pattern of reactivity with Tie-2+ cell panel. No reactivity with the Tie-2- fractions were observed.

35

**TABLE 1**

<b>Antibodies used in immunocytochemical staining and flow cytometric analysis of Tie-2+ cells isolated from whole blood</b>			
<b>Antibodies</b>	<b>Company</b>	<b>Immuno-cytochemistry</b>	<b>FACS</b>
<b>CD1a</b>	<b>Becton Dickinson (BD)-USA</b>	-	-
<b>CD3</b>	<b>BD</b>	-	-
<b>CD14</b>	<b>BD</b>	(+)/+	(+)/+
<b>CD19</b>	<b>BD</b>	-	-
<b>CD31</b>	<b>BD</b>	-	-
<b>CD34</b>	<b>BD</b>	-	-
<b>CD56+16</b>	<b>BD</b>	-	-
<b>CD68</b>	<b>BD</b>	++	++
<b>CD83</b>	<b>BD</b>	-	-
<b>CD62E (anti-E-selectin)</b>	<b>Biogenesis-UK</b>	+*	+*
<b>CD106 (anti- VCAM)</b>	<b>Biogenesis-UK</b>	++*	++*
<b>CD54 (anti-ICAM)</b>	<b>R&amp;D Systems-UK</b>	+*	+*
<b>VWF</b>	<b>SEROTEC-UK</b>	++	++

55

(continued)

**Antibodies used in immunocytochemical staining and flow cytometric analysis of Tie-2+ cells isolated from whole blood**

Antibodies	Company	Immuno-cytochemistry	FACS
Ac-LDL	Molecular Probes, Inc-USA	+++	+++
VEGF-R1 (Flt-1)	R&D Systems	+++	+++
MHC class I	Serotec-UK	+++	+++
MHC class II	Serotec-UK	+	++
MICA	Dr. Thomas Spies	(+)/+	(+)/+
Anti-Fibroblast	Serotec-UK	-	-
Anti- $\alpha$ -actin	Boehringer Mannheim-Germany	-	-

(+), weak or inconsistent staining; +, moderate staining; ++, strong staining +++, very strong staining vWF, von Willebrand Factor; Ac-LDL, acetylated low-density lipoprotein; MICA, Major histocompatibility complex class I- related chain A.

\* expressed on Tie-2+ cells only after activation with TNF-alpha and IFN-gamma for 12-14 hrs.

**TABLE 2****Flow cytometric analysis of reactivity of anti-endothelial-monocyte, endothelial specific and HLA class I or class II specific antibodies with a panel of Tie-2+ cells**

Pt. No.	Donor 1 Tie-2+/Tie2-	Donor 2 Tie2+/Tie2-	Donor 3 Tie2+/Tie2-	Donor 4 Tie2+/Tie2-	Donor 5 Tie2+/Tie2-	Donor 6 Tie2+/Tie2-
<b>Sera known to contain EM-reactive abs.</b>						
1	+/-	+/-	+/-	+/-	+/-	+/-
2	+/-	+/-	+/-	+/-	+/-	+/-
3	+/-	+/-	+/-	+/-	+/-	+/-
4	+/-	-/-	-/-	+/-	-/-	+/-
5	-/-	+/-	+/-	+/-	+/-	-/-
6	-/-	+/-	+/-	+/-	+/-	-/-
7	-/-	+/-	+/-	+/-	+/-	-/-
8	-/-	-/-	-/-	+/-	-/-	+/-
9	+/-	+/-	-/-	-/-	+/-	-/-
10	+/-	+/-	-/-	-/-	+/-	-/-
<b>Sera known to contain only EC-specific abs.</b>						
11	+/-	+/-	-/-	+/-	-/-	-/-
12	+/-	+/-	-/-	+/-	-/-	-/-
13	-/-	-/-	+/-	-/-	+/-	+/-
14	-/-	-/-	+/-	-/-	+/-	+/-
15	-/-	-/-	+/-	-/-	+/-	+/-

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(continued)

Sera known to contain only HLA class I broadly-reactive abs. (n=5)						
16	+/+	+/+	+/+	+/+	+/+	+/+
Sera known to contain only HLA class II abs. (n=5)						
17	+/+	-/-	-/-	+/+	-/-	+/+
Control sera from pts. with stable graft function (n=25)						
18	-/- *	-/-	-/- *	-/-	-/-	-/- *
*3/25 (12%) sera reacted with some of the Tie-2 cells on the panel						

TABLE 3

Reactivity of anti-endothelial-monocyte, endothelial specific and HLA class I or class II specific antibodies with a panel of Tie-2+ cells using the microcytotoxic assay						
Pt. No.	Donor 1 Tie-2+/Tie2-	Donor 2 Tie2+/Tie2-	Donor 3 Tie2+/Tie2-	Donor 4 Tie2+/Tie2-	Donor 5 Tie2+/Tie2-	Donor 6 Tie2+/Tie2-
Sera known to contain EM-reactive abs.						
1	++/-	+++/-	++/-	++/-	++/-	+++/-
2	++/-	+/-	+++/-	++/-	+++/-	++/-
3	+++/-	++/-	++/-	++/-	++/-	++/-
4	+++/-	-/-	-/-	+++/-	-/-	+++/-
5	-/-	++/-	++/-	++/-	++/-	-/-
6	-/-	++/-	++/-	++/-	++/-	-/-
7	-/-	++/-	++/-	++/	++/-	-/-
8	-/-	-/-	-/-	++/-	-/-	++/-
9	+++/-	++/-	-/-	-/-	++/-	-/-
10	++/-	++/-	-/-	-/-	++/-	-/-
Sera known to contain only EC-specific abs.						
11	+++/-	++/-	-/-	+++/-	-/-	-/-
12	++/-	+++/-	-/-	++/-	-/-	-/-
13	-/-	-/-	++/-	-/-	++/-	++/-
14	-/-	-/-	++/-	-/-	++/-	++/-
15	-/-	-/-	++/-	-/-	++/-	++/-
Sera known to contain only HLA class I broadly-reactive abs. (n=5)						
16	+++/>+++	+++/>+++	++++/>++++	+++/>+++	+++/>+++	++/>++
Sera known to contain only HLA class II abs. (n=5)						
17	++/>++	-/-	-/-	++/>++	-/-	++/>++

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(continued)

Control sera from pts. with stable graft function (n=25)						
5	18	-/- *	-/-	-/- *	-/-	-/-
*2/25 (8%) sera reacted with some of the Tie-2 cells on the panel -, negative; +, 10-25%; ++, 26-50%; +++, 51-75%; +++, 76-100% dead cells.						

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## 35 Claims

1. A method of isolating a precursor endothelial cell from a biological sample comprising:

- 40 (a) contacting a sample containing, or suspected of containing a precursor endothelial cell with a detection reagent to form a detection reagent-precursor endothelial cell complex; and  
(b) separating the detection reagent-precursor endothelial cell complex from the biological sample thereby isolating the precursor endothelial cell,

45 wherein the detection reagent is an antibody specific against Tie-2 and wherein the precursor endothelial cell is CD34-negative.

2. The method of claim 1, wherein the sample is selected from the group consisting of whole blood, sera, peripheral blood mononuclear cells, and leucapherisate.

3. The method of claim 1, wherein the antibody is attached to a solid support.

4. The method of claim 3, wherein the solid support is a non-magnetic, magnetic or a paramagnetic bead.

5. The method of claim 1, wherein the separation is by flow cytometry or a magnetic field.

55 6. A method of cross-matching a donor and a recipient comprising:

- (a) isolating a donor precursor endothelial cell in accordance with the method of any of claims 1 to 5; and  
(b) screening a recipient sample for a reactivity with the isolated precursor endothelial cell;

wherein no reactivity of the recipient sample with the isolated precursor cell indicates compatibility between the donor and recipient

- 5 7. An *ex vivo* method of diagnosing of an autoimmune related or vascular disorder in a subject, comprising:

- (a) isolating a precursor endothelial cell in accordance with the method of claim 1;  
(b) contacting the precursor endothelial cell with a sample containing, or suspected of containing an autoantibody from the subject; and  
10 (c) identifying an autoantibody-precursor endothelial cell complex, if present

wherein the presence of the complex indicates the presence of the disorder in the subject.

- 15 8. An *ex vivo* method of determining the efficacy of treatment, or assessing the prognosis, of an autoimmune related or vascular disorder in a subject, comprising:

- (a) isolating a precursor endothelial cell in accordance with the method of claim 1;  
(b) contacting the precursor endothelial cell with a sample containing, or suspected of containing an autoantibody from the subject;  
20 (c) measuring an autoantibody-precursor endothelial cell complex if present to yield a subject profile; and  
(d) comparing the subject profile with a reference profile,

wherein a similarity between the subject profile and reference profile indicates the treatment is efficacious and a favorable prognosis for the subject.

- 25 9. The method of claim 7 or 8, wherein the disorder is selected from the group consisting of vasculitis, atherosclerosis, bleeding disorders, defective wound healing, and transplantation.

### 30 Patentansprüche

1. Verfahren zum Isolieren einer Vorläufer-Endothelzelle aus einer biologischen Probe, aufweisend:

- (a) in Berührung bringen einer Probe, die eine Vorläufer-Endothelzelle enthält, oder von der vermutet wird, dass sie eine Vorläufer-Endothelzelle enthält, mit einem Nachweisreagenz, um einen Nachweisreagenz-Vorläufer-Endothelzelle-Komplex zu bilden; und  
35 (b) Abtrennen des Nachweisreagenz-Vorläufer-Endothelzelle-Komplexes aus der biologischen Probe, wodurch die Vorläufer-Endothelzelle isoliert wird,

wobei das Nachweisreagenz ein gegen Tie-2 spezifischer Antikörper ist, und  
40 wobei die Vorläufer-Endothelzelle CD34-negativ ist.

2. Verfahren nach Anspruch 1, bei dem die Probe ausgewählt wird aus der Gruppe, die aus Vollblut, Seren, einkernigen Peripherblutzellen und Leukapherisat besteht.

- 45 3. Verfahren nach Anspruch 1, bei dem der Antikörper an einen festen Träger gebunden wird.

4. Verfahren nach Anspruch 3, bei dem der feste Träger ein nicht-magnetisches, magnetisches oder ein paramagnetisches Kügelchen ist.

- 50 5. Verfahren nach Anspruch 1, bei dem die Abtrennung mittels Durchfluss-Zytometrie oder mittels eines Magnetfelds durchgeführt wird.

6. Verfahren zur Durchführung einer Kreuzprobe zwischen einem Spender und einem Empfänger, aufweisend:

- 55 (a) Isolieren einer Spender-Vorläufer-Endothelzelle gemäß dem Verfahren nach einem der Ansprüche 1 bis 5; und  
(b) Durchmustern einer Empfängerprobe hinsichtlich einer Reaktivität mit der isolierten Vorläufer-Endothelzelle;

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wobei keine Reaktivität der Empfängerprobe mit der isolierten Vorläuferzelle Verträglichkeit zwischen dem Spender und dem Empfänger anzeigt.

- 5 7. Ex vivo-Verfahren zum Diagnostizieren einer mit Autoimmunität in Beziehung stehenden Erkrankung oder einer Gefäßerkrankung bei einem Individuum, aufweisend:

- 10 (a) Isolieren einer Vorläufer-Endothelzelle gemäß dem Verfahren nach Anspruch 1;  
(b) in Berührung bringen der Vorläufer-Endothelzelle mit einer Probe, die einen Autoantikörper von dem Individuum enthält, oder von der vermutet wird, dass sie einen Autoantikörper von dem Individuum enthält; und  
(c) Identifizieren eines Autoantikörper-Vorläufer-Endothelzelle-Komplexes, falls vorhanden,

wobei das Vorliegen des Komplexes das Vorliegen der Erkrankung bei dem Individuum anzeigt.

- 15 8. Ex vivo-Verfahren zur Bestimmung der Wirksamkeit einer Behandlung, oder zur Ermittlung der Prognose, einer mit Autoimmunität in Beziehung stehenden Erkrankung oder einer Gefäßerkrankung bei einem Individuum, aufweisend:

- 20 (a) Isolieren einer Vorläufer-Endothelzelle gemäß dem Verfahren nach Anspruch 1;  
(b) in Berührung bringen der Vorläufer-Endothelzelle mit einer Probe, die einen Autoantikörper von dem Individuum enthält, oder von der vermutet wird, dass sie einen Autoantikörper von dem Individuum enthält;  
(c) Messen eines Autoantikörper-Vorläufer-Endothelzelle-Komplexes, falls vorhanden, um ein Profil des Individuums zu ergeben;  
(d) Vergleichen des Profils des Individuums mit einem Referenzprofil,

25 wobei eine Ähnlichkeit zwischen dem Profil des Individuums und dem Referenzprofil anzeigt, dass die Behandlung wirksam ist, und eine günstige Prognose für das Individuum anzeigt.

- 30 9. Verfahren nach Anspruch 7 oder 8, bei dem die Erkrankung ausgewählt wird aus der Gruppe, die aus Vasculitis, Atherosklerose, Blutgerinnungsstörungen, unzulänglicher Wundheilung und Transplantation besteht.

### Revendications

- 35 1. Procédé d'isolement d'une cellule endothéliale précurseur d'un échantillon biologique comprenant :

- 40 (a) la mise en contact d'un échantillon contenant, ou susceptible de contenir, une cellule endothéliale précurseur avec un réactif de détection pour former un complexe cellule endothéliale précurseur-réactif de détection ; et  
(b) la séparation du complexe cellule endothéliale précurseur-réactif de détection de l'échantillon biologique, ce qui permet d'isoler la cellule endothéliale précurseur, le réactif de détection étant un anticorps spécifique contre Tie-2 et la cellule endothéliale précurseur étant CD34 négative.

- 45 2. Procédé de la revendication 1, dans lequel l'échantillon est sélectionné dans le groupe constitué de sang entier, de sérum sanguin, de cellules mononucléaires du sang périphérique, et de leucaphérise.

- 50 3. Procédé de la revendication 1, dans lequel l'anticorps est fixé à un support solide.

4. Procédé de la revendication 3, dans lequel le support solide est une bille non magnétique, magnétique ou paramagnétique.

- 55 5. Procédé de la revendication 1, dans lequel la séparation est réalisée par cytométrie de flux ou par un champ magnétique.

6. Procédé de comptabilisation croisée entre un donneur et un receveur comprenant :

- 55 (a) l'isolement d'une cellule endothéliale précurseur de donneur conformément au procédé selon l'une quelconque des revendications 1 à 5; et  
(b) le criblage d'un échantillon receveur à la recherche d'une réactivité avec la cellule endothéliale précurseur isolée ;  
l'absence de réactivité de l'échantillon receveur avec la cellule endothéliale précurseur indiquant une compa-

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tibilité entre le donneur et le receveur.

7. Procédé *ex vivo* de diagnostic d'un trouble vasculaire ou d'un trouble associé à l'auto-immunité chez un sujet, comprenant :

5  
(a) l'isolement d'une cellule endothéliale précurseur conformément au procédé de la revendication 1 ;  
(b) la mise en contact de la cellule endothéliale précurseur avec un échantillon contenant, ou susceptible de contenir, un autoanticorps provenant du sujet ; et  
10 (c) l'identification d'un complexe cellule endothéliale précurseur-autoanticorps, s'il est présent, la présence du complexe indiquant la présence du trouble chez le sujet.

8. Procédé *ex-vivo* de détermination de l'efficacité du traitement, ou d'évaluation du pronostic, d'un trouble vasculaire ou d'un trouble associé à l'auto-immunité, chez un sujet, comprenant :

15 (a) l'isolement d'une cellule endothéliale précurseur conformément au procédé selon la revendication 1 ;  
(b) la mise en contact de la cellule endothéliale précurseur avec un échantillon contenant, ou susceptible de contenir, un autoanticorps provenant du sujet ;  
(c) la mesure d'un complexe cellule endothéliale précurseur-autoanticorps, s'il est présent, pour produire un profil du sujet ; et  
20 (d) la comparaison du profil du sujet avec un profil de référence, une similarité entre le profil du sujet et le profil de référence indiquant un traitement efficace et un pronostic favorable pour le sujet.

9. Procédé de la revendication 7 ou 8, dans lequel le trouble est sélectionné dans le groupe constitué par les vascularites, l'athérosclérose, les troubles de saignement, les troubles de cicatrisation, et les transplantations.

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Figure 1

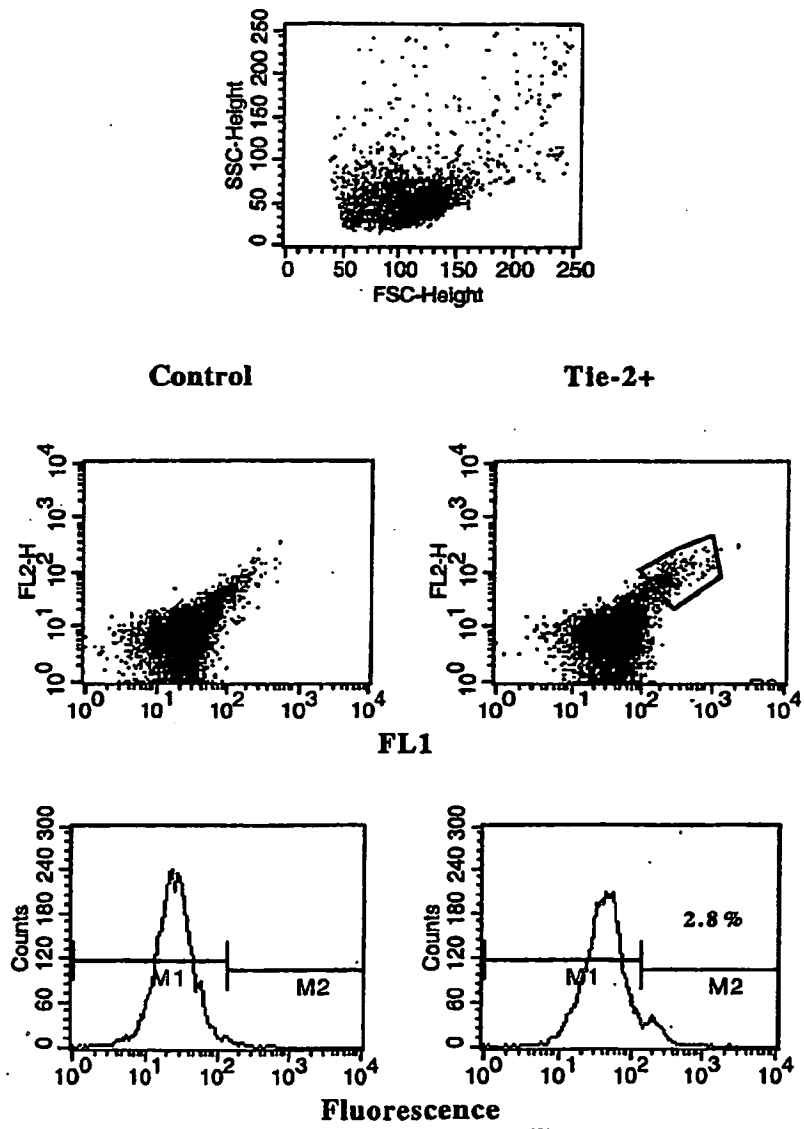


Figure 2

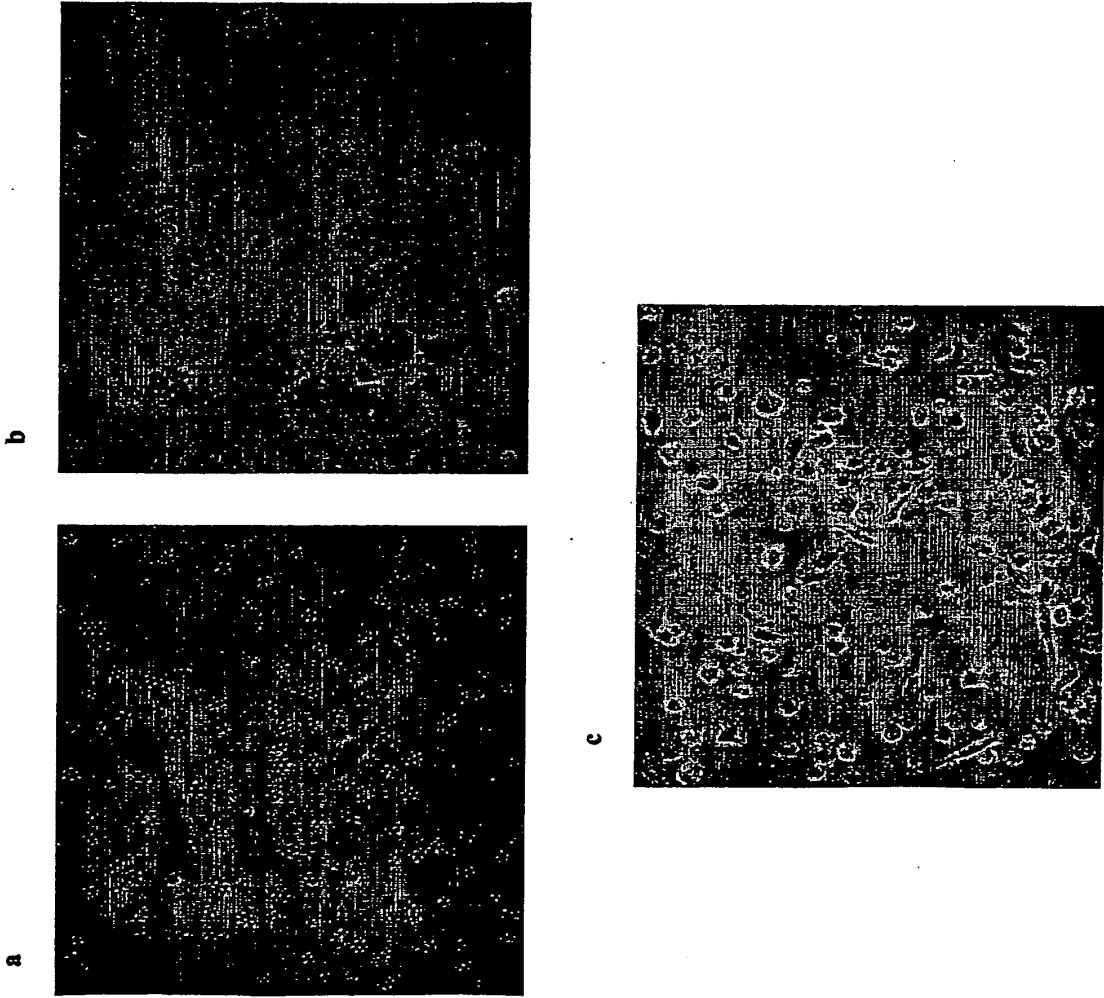


Figure 3

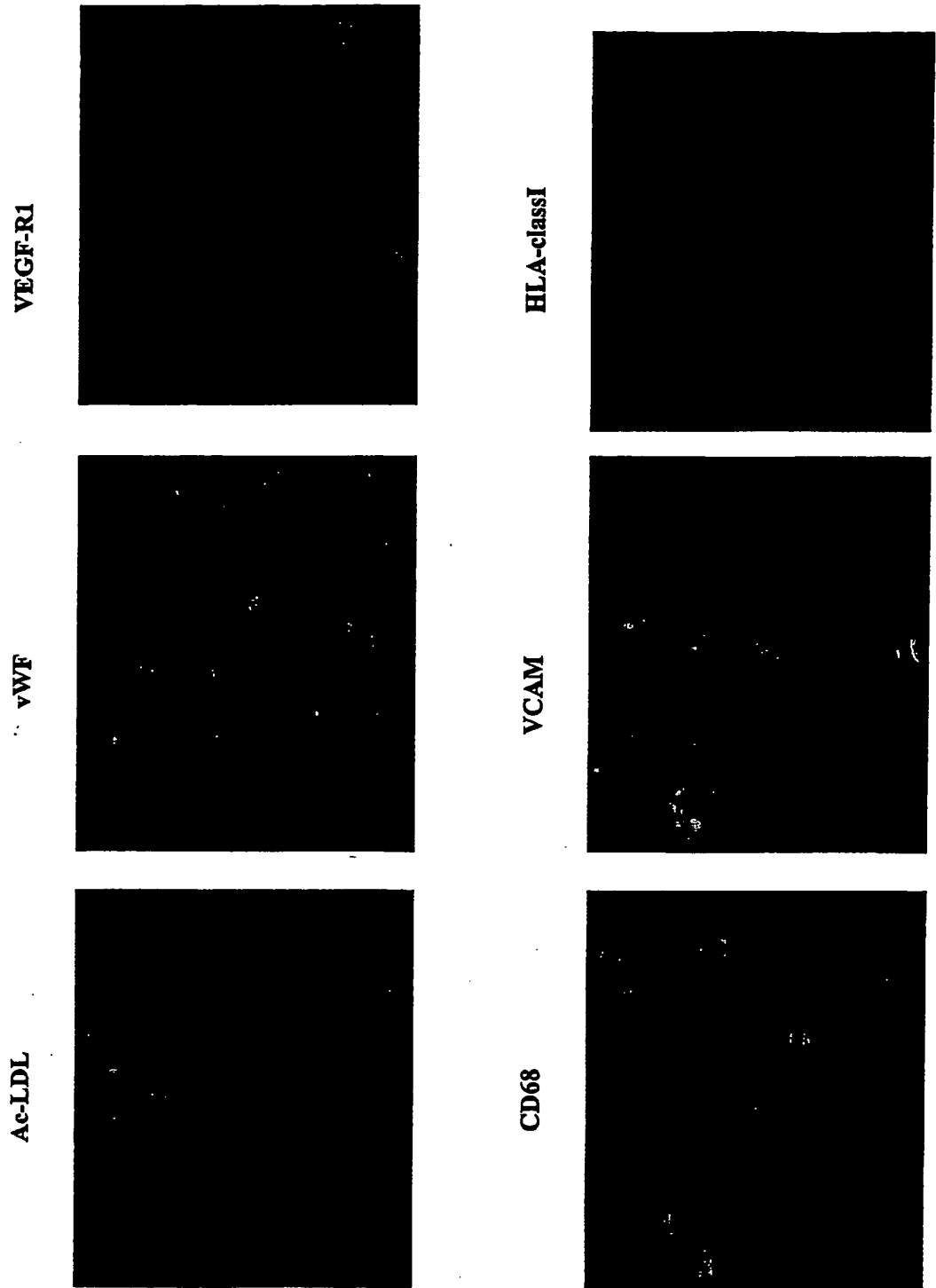


Figure 3b

**MICA and HLA  
class II expression**

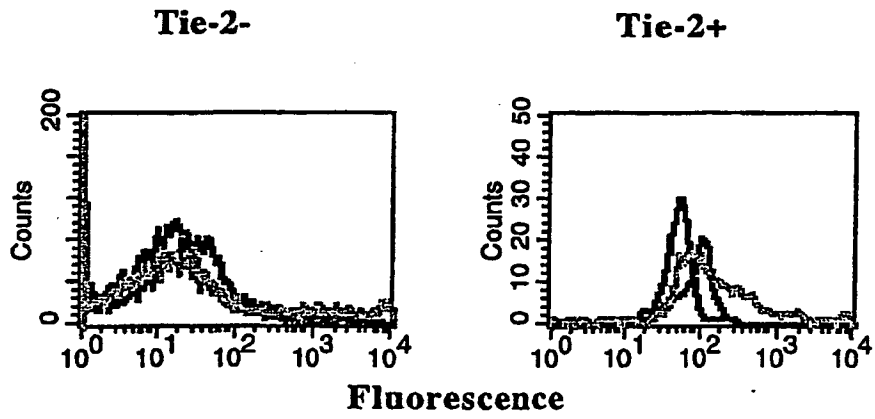
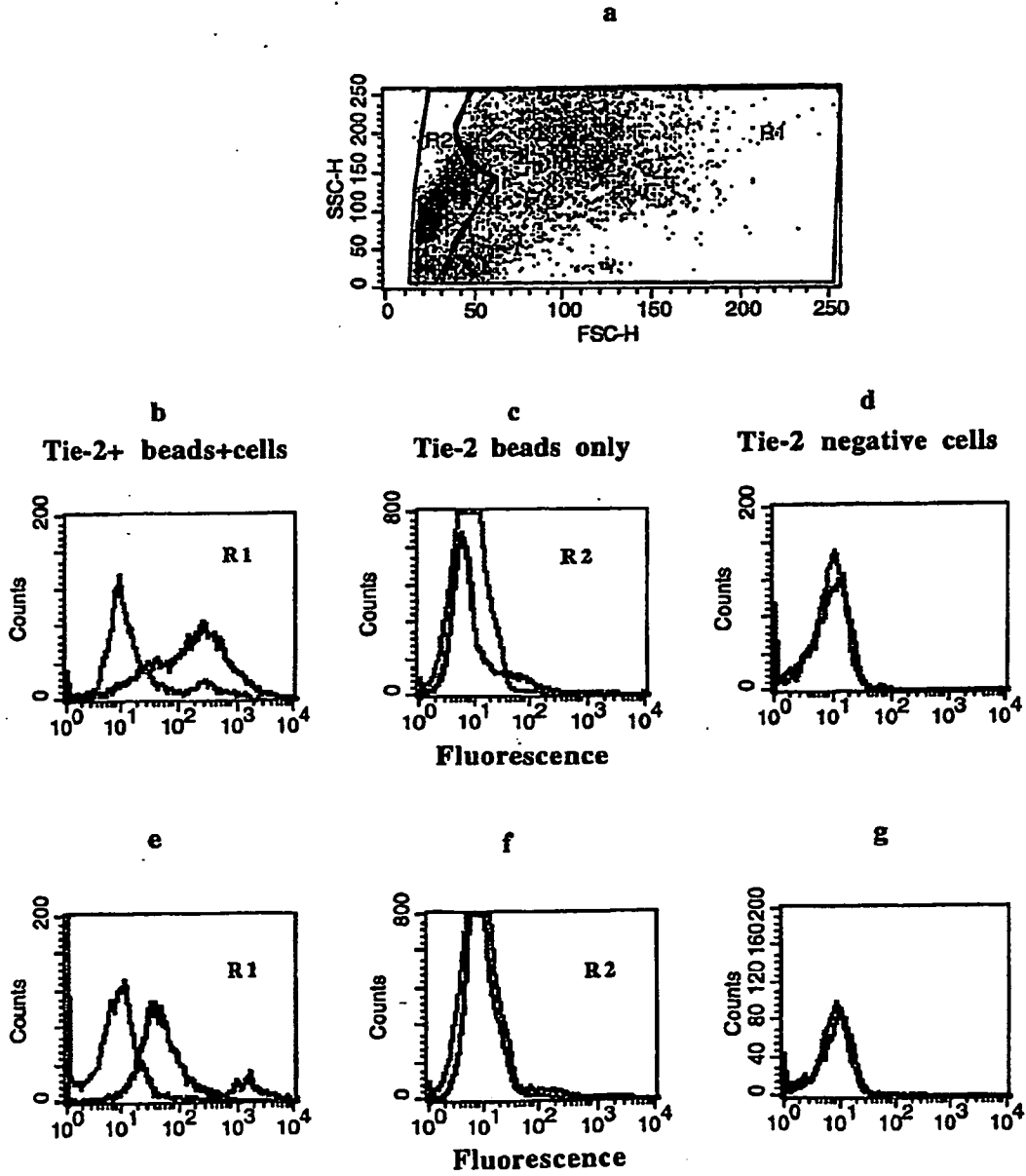


Figure 4

**Donor-specific Tie-2+ (endothelial cells)  
crossmatch**



## REFERENCES CITED IN THE DESCRIPTION

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专利名称(译)	供体特异性交叉配型的方法		
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申请号	EP2003730427	申请日	2003-05-16
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外部链接	<a href="#">Espacenet</a>		

摘要(译)

本发明涉及一种直接从全血中分离内皮细胞的方法，用于常规供体特异性交叉配型，以在器官移植前检测抗内皮细胞抗体。

Figure 1

