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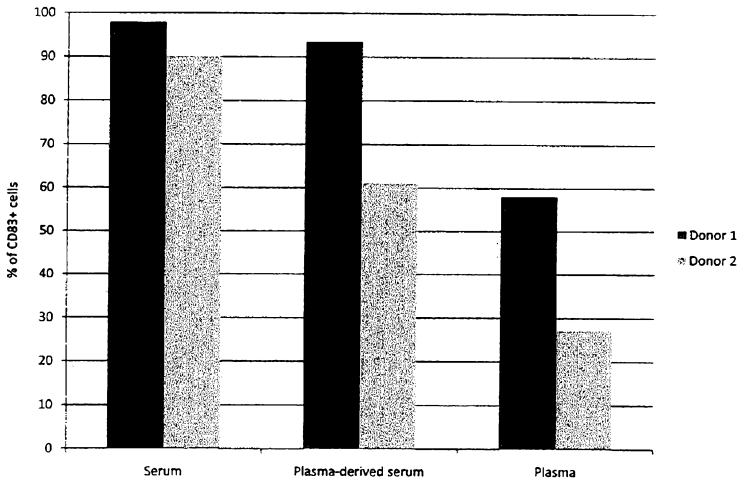
(54) **Title**: COMPOSITIONS AND METHODS FOR PRODUCING DENDRITIC CELLS

Figure 1



(57) **Abstract**: The present invention relates to compositions and methods for producing dendritic cells and particularly to compositions and methods for producing immature dendritic cells that are immunocompetent. We describe a method of producing dendritic cells by cultivation of monocytes, characterised by at least one of: pre-treatment of a tissue culture surface with at least one of: a substantially plasma-free and serum-free pre-treatment medium, a pre-treatment medium comprising heparin, and a pre-treatment medium comprising a protein solution; adsorption of monocytes using at least one of: a substantially plasma-free and serum-free adsorption medium; cultivation of monocytes using a substantially plasma-free and serum-free cultivation medium. We also describe compositions including the dentritic cells and uses of the dentritic cells.

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Compositions and methods for producing dendritic cells

The present invention relates to compositions and methods for producing dendritic cells and particularly to compositions and methods for producing immature dendritic cells that are immunocompetent.

Tumour cells, similar to many infectious agents, express specific protein antigens that are absent in normal cells. Potentially, the immune system is able to recognize these tumour cells as foreign and eliminate them. The major effector cell population able to mediate tumour cell recognition and destruction is cytotoxic T lymphocytes (CTL). In order to induce a CTL response, the antigens should be presented to CTL precursors by antigen-presenting cells (APC). During the last 15 to 20 years, immunotherapy research has focussed on the use of dendritic cells as the most efficient antigen-presenting cells (References 1 and 2).

The most convenient source for preparation of dendritic cells is blood monocytes. Peters described the ability of monocytes to transform, in culture, into dendritic cell-like cells, both spontaneously (3) and in the presence of two cytokines (Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) and Interleukin-4 (IL-4)) (4). Following research by Romani et al., (1994) (5) and Sallusto and Lanzavecchia (1994) (6), monocytes cultured in the presence of GM-CSF and IL-4 became the most widely-used source for dendritic cell preparation. Preparation of dendritic cells from monocytes is traditionally carried out in the presence of fetal calf serum (FCS). FCS is currently not recommended for use in the preparation of compositions for immunotherapy since the patient risks development of type 1 hypersensitivity to bovine serum albumin (BSA) (10).

There are two types of dendritic cells - mature and immature. Mature dendritic cells can be characterised by the presence of CD83, which is a well known marker of mature dendritic cells (18). Immature dendritic cells can be identified by expression of CD1a and CD4, which are characteristic markers for immature dendritic cells. The immature state of dendritic cells is their natural state in an organism. After appearance of an infection or diseased cells, dendritic cells will localize in the

effected organs or tissue and phagocytize the infected agents or diseased cells. They will then migrate to regional lymph nodes to present the processed antigens to antigen-specific T lymphocytes. During the process of migration, and probably during initial interaction with antigen-specific T cells, dendritic cell maturation is initiated, leading to increased T-cell stimulatory activity. The common conception is that, to be effective antigen-presenting cells, dendritic cells should be mature, since mature dendritic cells (DC) express high levels of co-stimulatory molecules, such as CD80 and CD86, and are better able to migrate than immature DC.

There are, however, some studies demonstrating that immunization with immature dendritic cells might have a clinical effect. A recent review of the use of dendritic cells for treatment of melanoma patients (11) illustrates examples where immature dendritic cells effectively elicit an immune response, indicating their potential immunocompetence. In fact, the clinical effects demonstrated in one of the first studies of dendritic cell-based immunotherapy of melanoma patients were obtained using immature dendritic cells (9).

Use of immature DC as antigen-presenting cells has potential advantages, compared with use of mature DC. Mature dendritic cells stimulate lymphocytes in an antigen-nonspecific way (see, for example, WO 2008/081035) - possibly as a result of high levels of expression of co-stimulatory molecules by fully mature DC. It has also been demonstrated that, whereas mature dendritic cells induce a strong CTL response, immature dendritic cells induce a strong central memory T cell response (12), i.e., immature DC preferentially stimulate a memory-type immune response. Induction of a memory-type immune response is important in inducing effective anti-tumour immunity because memory cells have the ability to re-circulate (due to the presence of adhesion molecules such as CD62L (13)), to be activated after initial contact with tumour cells, and to proliferate after destroying tumour cells (14, 15).

The present invention seeks to provide a method of generating immunocompetent immature DC, which show similar properties to the immature DC found *in vivo*. A particular problem the present invention seeks to address is to reduce or eliminate foreign body reactions during the preparation of immature DC. The present invention also aims to provide a method of generating clinically-useful and pharmaceutically-

relevant immature DC. In this regard, the present invention seeks to generate dendritic cells that have the same properties as DC produced using FCS, but without using FCS in the preparation procedure.

In its broadest aspect, the present invention provides a method of producing immature dendritic cells, particularly immunocompetent immature dendritic cells, which are able to stimulate a memory-type anti-tumour T cell response. In another aspect, the present invention provides a method of producing dendritic cells that express IL-15.

According to the present invention, there is provided a method of producing dendritic cells by cultivation of monocytes, characterised by at least one of: pre-treatment of a tissue culture surface with at least one of: a substantially plasma-free pre-treatment medium, a pre-treatment medium comprising heparin, and a pre-treatment medium comprising a protein solution; adsorption of monocytes using at least one of: a substantially plasma-free adsorption medium, and a substantially serum-free adsorption medium; and cultivation of monocytes using a substantially plasma-free cultivation medium.

Preferably, the protein solution comprises human serum. Suitably, the human serum has a concentration of between 2 and 10 %.

In one embodiment, the pre-treatment medium has a heparin concentration of between 10 and 200 U/ml. Ideally, the pre-treatment medium has a heparin concentration of between 25 and 100 U/ml.

In one aspect, there is provided a composition comprising dendritic cells produced in accordance with the methods described above. In one embodiment, the composition is injectable directly into a tumour.

In another aspect, the present invention provides use of immature dendritic cells produced in accordance with the methods described above for preparation of a pharmaceutical composition for treating cancer by immunotherapy.

A method of treating or preventing cancer by administering a vaccine comprising immature dendritic cells produced in accordance with the methods described above forms another aspect of the present invention. In one embodiment, the method comprises injecting the vaccine directly into a tumour.

One aspect of the present invention provides a composition comprising immature dendritic cells produced in accordance with the methods described above for treatment of cancer by immunotherapy.

A further aspect provides an antigen-presenting composition produced from immature dendritic cells produced in accordance with the methods described above.

Further aspects of the present invention include:

a method of preparing a cytotoxic composition, comprising using an antigen-presenting composition as described above to activate T cell lymphocytes, thereby obtaining a cytotoxic composition containing tumour-specific CD8+ cytotoxic T lymphocytes;

a method of treating cancer by stimulating an immune response against tumours, comprising administering an antigen-presenting composition as described above to a cancer patient to stimulate an immune response to the tumour antigens;

a method of treating cancer by adoptive T cell therapy, comprising preparing a cytotoxic composition in accordance with the method described above, and administering the cytotoxic composition to a cancer patient;

use of a dendritic cell composition as described above as antigen presenting cells for activation of cytotoxic T lymphocytes; and

use of tumour-specific CD8+ cytotoxic T lymphocytes obtained by the method described above for preparation of a pharmaceutical composition for adoptive T cell immunotherapy.

The above and other aspects of the present invention will now be described in further detail, by way of example only, with reference to the accompanying figures and examples, in which:

Figure 1 illustrates the effect of inclusion of plasma in the plastic pre-treatment medium on phenotype of mature dendritic cells generated;

Figure 2 illustrates the effect of inclusion of heparin in the pre-treatment medium on the proportion of lymphocytes in the final product;

Figure 3 illustrates the effect of inclusion of heparin in the pre-treatment medium on the phenotype of the mature dendritic cells generated;

Figure 4 illustrates the effect of inclusion of heparin in the pre-treatment medium on the production of IL-12p70 by the mature dendritic cells generated;

Figure 5 illustrates the effect of inclusion of human serum in the adhesion medium on the phenotype of the mature dendritic cells generated;

Figure 6 illustrates the effect of inclusion of human serum in the adhesion medium on the production of IL-12p70 by the mature dendritic cells generated;

Figure 7 illustrates the phenotype of immature dendritic cells;

Figure 8 illustrates expression of IL-15 in the immature dendritic cells;

Figure 9 illustrates detection of IL-15 protein in the lysates of immature and mature dendritic cells;

Figure 10 illustrates uptake of labelled lysate;

Figure 11 illustrates the phenotype of lymphocytes stimulated two times with dendritic cells loaded with lysate of breast cancer cell line MDA-MB-231;

Figure 12 illustrates the cytotoxic activity of the generated immune lymphocytes of HLA-A2-positive donor on HLA-A2-positive tumour cell lines MCF-7 and MDA-MB-231; and

Figure 13 illustrates the cytotoxic activity of the generated immune lymphocytes of HLA-A2-positive donor on HLA-A2-positive tumour cell line LNCaP and HLA-A2-negative cell line T47D.

The procedure of the present invention for generation of dendritic cells provides significant improvements to a well-known method of generating DC from monocytes. This well-known method comprises isolation of monocytes from peripheral blood,

followed by culture of the isolated monocytes in the presence of GM-CSF and IL-4 for 5-7 days. The improvements of the present invention generate advantageous properties in the dendritic cells, such as low levels of co-stimulatory molecules and high endocytic activity. The properties of immunocompetent immature dendritic cells made according to the preparation procedures of the present invention are similar to the properties of immature dendritic cells *in vivo*. The key properties of immature DC *in vivo* are: high endocytic activity, the ability to produce Interleukin-15 (IL-15), the ability to differentiate into fully mature non-exhausted dendritic cells upon addition of an appropriate maturation agent, and the ability to induce, *in vitro*, a memory-type antigen-specific CTL response.

To isolate monocytes from a population of mononuclear cells, obtained by centrifugation of peripheral blood, a plastic surface is commonly used to adsorb the monocytes. Mononuclear cells include monocytes, lymphocytes and a variable proportion of platelets, which are particularly difficult to remove by centrifugation of peripheral blood. Isolation by adsorption is based on the high adhesive properties of monocytes. It is expected that only monocytes will adhere to the surface, while other cellular elements will not. Therefore, monocytes should be easy to isolate by removing the non-adherent cells and washing the monolayer of adherent monocytes. In practice, however, a significant proportion of other cellular elements will also adhere to the surface (see Figure 1 in ref. (22)).

The presence of other cellular elements during transformation of monocytes into dendritic cells can compromise the properties of the dendritic cells. Several previous studies have reported that immature dendritic cells generated *in vitro* lack immunocompetence (7, 8). Differences between immature DC produced *in vitro* and those found *in vivo* could arise from compromised differentiation of monocytes into DC *in vitro*. Differentiation could be compromised as a result of foreign body reactions that occur when monocytes and other cells present in a population of mononuclear cells contact plastic tissue culture surfaces (16, 17). Foreign body reactions can cause activation of mononuclear cells leading to production of factors that inhibit the ability of the monocytes to differentiate into fully competent immature dendritic cells.

Production of dendritic cells typically comprises plastic pre-treatment, adsorption and cultivation steps. In the method of the present invention, the process of adsorption of monocytes has been improved by decreasing adhesion of non-monocyte mononuclear cellular elements while retaining high adhesion of monocytes.

Non-specific adsorption of lymphocytes during the adsorption step of the preparation of dendritic cells is a common problem (22). In the present invention, pre-treatment of tissue culture plastic with a medium containing human serum (2-10 %) is employed to coat plastic surfaces and decrease non-specific adsorption of lymphocytes. This effect of human serum is associated with the presence of fibronectin, since monocytes have receptors to fibronectin and are able to adhere to the surface-bound fibtonectin (28, 29). Use of human serum rather than fibronectin derived from another source is advantageous in the preparation of compositions for administration to immunotherapy patients.

A further decrease in non-specific adsorption and lymphocyte expansion is achieved in the present invention by inclusion of heparin in the plastic pre-treatment medium. Heparin is often used to decrease adsorption and activation of human blood cells during various types of extracorporeal blood processing (26). In these systems, covalent attachment of heparin to all surfaces used in extracorporeal devices and directly contacting with blood is employed. In generation of dendritic cells, heparin has been used as additive to the cultivation medium to prepare cells with high levels of expression of CD1a (27). In one aspect of the method of the present invention, however, heparin is used during the plastic pre-treatment step, particularly by inclusion of heparin in a tissue culture plastic pre-treatment medium.

The inventors have also found that adsorption and activation of platelets can be prevented if plasma is excluded from the sample medium during plastic pre-treatment, adsorption or cultivation. Addition of plasma to the culture medium is often used for preparation of dendritic cells (22, 23). Plasma comprises fibrinogen, which can mediate adsorption and activation of platelets (24), which are often present in a preparation of mononuclear cells.

In one aspect of the present invention, non-specific activation of monocytes is decreased during the adsorption step. Non-specific activation of monocytes usually promotes their differentiation into macrophages, compromising their differentiation into dendritic cells. In the present invention, a decrease in non-specific activation of monocytes is achieved by exclusion of adult human serum from the adsorption steps.

The methods described herein for generation of immature dendritic cells generate cells that are suitable for use as a dendritic cell vaccine. For example, immature dendritic cells produced according to the present invention can be injected directly into a tumour as part of an immunotherapy cancer treatment. The methods of the present invention also generate dendritic cells with an ability to induce strong antigen-specific CTL responses. When these immature dendritic cells are used as antigen presenting cells in *in vitro* immunization experiments, the responses generated have characteristics of memory-type responses, such as, expression by CTL of CD62L, specific recognition and killing of target cells and intensive proliferation after initial contact with target cells.

Example 1 – Preparation of dendritic cells

Dendritic cells were generated from buffy coats. 60 ml of buffy coat was diluted with 60 ml of Ca/Mg-free phosphate buffered saline (DPBS) (Cambrex), and applied on Lymphoprep (four 50-ml tubes each with 14 ml of the Lymphoprep). In order to minimize contamination of peripheral blood mononuclear cells (PBMCs) with platelets, the Lymphoprep centrifugation (200 g, 20°C) was interrupted after 20 min according to procedure described by Romani et al., 1996 (25). The top 15-20 ml, containing most of the platelets, was transferred into 50-ml tubes and centrifugation was resumed (460 g, 20 min, 20°C).

Coating of plastic tissue culture flasks was initiated by adding RPMI pre-treatment medium containing 5 % of human AB serum to the flasks (T25). The pre-treatment medium was later removed from the T25 flasks and the flasks rinsed with 5 ml of RPMI 1640.

After termination of Lymphoprep centrifugation, mononuclear cells were harvested from the interface, diluted twice with cold EDTA-containing DPBS (Cambrex) and

washed by 3 centrifugations, the first at 250 g, the second at 200 g and the last at 150 g. Centrifugation was carried out at 4°C for 12 min. After the final centrifugation, cells were re-suspended in 30 ml of cold Ca/Mg-free DPBS and counted using Coulter Counter. The quantity of monocytes was estimated as the number of cells in a peak with average size around 9 µm.

For the generation of dendritic cells, cell suspensions containing 4-8 Mio of monocytes per T25 polystyrene flask were transferred into centrifuge tubes and centrifuged at 250 g for 12 min at 4°C. 4 ml of AIM-V adsorption medium (with or without the addition of plasma and/or serum) was then added to each flask. After further centrifugation, the obtained pellet was re-suspended in the adsorption medium at a concentration of 4-8 x 10^6 monocytes/ml, and 1 ml of cell suspension was added to each T25 flask. After 30 min of adsorption at 37°C, non-adherent cells were removed, adherent cells were rinsed twice with warm RPMI 1640 medium and 5 ml of cultivation medium (AIM-V medium) was added to each flask. The flasks were placed into a CO_2-incubator at 37°C. Cytokines (GM-CSF, final concentration 100 ng/ml, and IL-4, final concentration 25 ng/ml) were added the next day and on day 3.

At day 4, the ability to maturate was determined by addition the maturation cocktail consisting of: 10 ng/ml of TNF-alpha, 1000 U/ml of IL-6, 10 ng/ml of IL-1 and 0.1 µg/ml of prostaglandin E2. At day 6, the cells were harvested, their phenotype was determined by FACS analysis, and production of IL-12p70 in the supernatant was determined by ELISA analysis. Cells were stained with directly conjugated antibodies to CD83 (phycoerythrin (PE)) and CD86 (PE), all from Pharmingen, Becton Dickinson, Broendby, Denmark. Appropriate isotype controls were used. Samples were analysed using FC500 Flow Cytometer (Beckman Coulter).

The CD83 level of DC was used to indicate full maturation. To indicate absence of exhaustion, production of cytokine IL-12p70 - that is considered to be important for Th1 polarization of immune response (19) - was measured. Absence of IL-12p70 production is considered to be a marker of exhausted mature dendritic cells (20, 21).

Example 2 - Effect of plasma in the adsorption medium.

Dendritic cell preparation as described in Example 1 was repeated with adsorption media comprising either: serum, plasma-derived serum or plasma, in addition to AIM-V medium.

The results of one of the experiments with cells from two donors are shown in Figure 1. Plasma had a negative effect on the degree of maturation, while plasma-derived serum has smaller inhibitory effect. The negative effect of plasma-derived serum could be associated with incomplete removal of fibrinogen from plasma during clotting induced by addition of $CaCl_2$. In summary, plasma had a negative effect on the ability of the dendritic cells to reach full maturation.

Example 3 - Effect of heparin in the plastic pre-treatment medium.

Dendritic cells were prepared as described in Example 1. In the experiments of Example 3, heparin was added to the plastic pre-treatment medium so that plastic cell culture surfaces were coated with heparin during the pre-treatment step.

The effect of heparin coating on the proportion of lymphocytes in the final product is demonstrated in Figure 2. For the majority of donors, the proportion of lymphocytes decreased two-fold as a result of inclusion of heparin in the pre-treatment medium, while final yield of dendritic cells was either unchanged, or decreased insignificantly. Flow cytometry of the dendritic cells generated did not show changes in the levels of maturation of dendritic cells (Figure 3).

For these dendritic cells the levels of IL-12p70 during maturation was also measured. Production of IL-12p70 was measured by sandwich ELISA that included capture Abs, standard or sample, biotinylated detection Abs, and HRP-streptavidin. Kit "DuoSet ELISA development System" for IL-12 p70 (R&D Systems) was used essentially according to the manufacturer's recommendations with some modifications. After overnight binding of capture antibodies to the Nunc maxisorp 96-well plates and washing, the blocking step was extended to at least 3 hrs at room temperature (r.t.). A standard curve was generated by seven serial dilutions of the standard, starting at 500 pg/ml. Standards and samples were incubated at r.t. for 2 hrs followed by incubation at 4°C overnight. The subsequent steps were performed according to the

manufacturer's protocol. Hydrogen peroxide-tetramethylbenzidine mixture was used as a substrate solution for HRP, and after terminating the enzymatic reaction, optical density was measured with wavelength correction as the difference between readings at 490 and 620 nm.

As illustrated in Figure 4, the production of IL-12p70 by maturing dendritic cells was increased in several, but not all cultures.

In summary, inclusion of heparin in the plastic pre-treatment medium significantly decreases contamination of the final dendritic cells with lymphocytes without compromising the properties of the dendritic cells.

Example 4 - Effect of human serum in the adsorption medium.

The method described in Example 1 was replicated with and without use of human serum in the adsorption medium and the properties of the resulting DC were investigated. Figures 5 and 6 illustrate the results obtained with cells from two donors. The presence of human serum during the adsorption step decreases the ability of dendritic cells to mature upon addition of the standard maturation cocktail, which is illustrated by the decrease in the proportion of CD83+ cells (Figure 5) as well as by a decrease in the production of IL-12p70 (Figure 6). This effect may be the result of presence of immunoglobulins in the serum that induce activation of monocytes (16).

Example 5 - Final characterization of dendritic cells.

Final characterization of immature dendritic cells was done using the following methods: FACS analysis for expression of the markers characteristic for immature dendritic cells present *in vivo* (CD1a and CD4), expression of IL-15, and uptake of labelled lysate.

The results from FACS analysis of immature dendritic cells are presented in Figure 7. Cells were positive for CD1a and CD4. CD4 is a receptor for IL-16 produced by lymphocytes and for MHC class II. Interaction of CD4 on the surface of dendritic cells with ligands (MHC class II or IL-16) may induce activation of dendritic cells that can further increase their co-stimulatory function.

IL-15 was determined by two methods: mRNA expression by RT-PCR (conventional and real-time), and protein expression by sandwich ELISA.

Expression of mRNA for IL-15

RNA was isolated from the samples, stored in RNAlater (Ambion), DNase treated, reverse transcribed, and used in PCR. Preparations without reverse transcription were used as negative controls, in which the absence of PCR products indicated a complete lack of contaminating genomic DNA. Primers for human IL-15 are given in reference (30): TAAAACAGAAGCCAACTG (sense) and CAAGAAGTGTTGATGAACAT (antisense). After initial denaturation at 95°C for 5 min, samples were amplified in a DNA thermocycler for 35-38 cycles, each consisting of: denaturing for 60 seconds at 95°C, annealing for 60 seconds at 63°C (cycle 1-3), 59°C (cycle 4-6), and 56°C (cycle 7-38) and extension for 45 seconds at 72°C, followed by a final extension at 72°C for 10 min. Aliquots of PCR products were then electrophoresed on 2% agarose gel and visualized by ethidium bromide staining.

Real-time RT–PCR was performed using a Lightcycler Fastart DNA Master Plus SYBR green I and a Lightcycler 2.0 instrument (Roche Applied Science), primers and conditions were as in reference (31): sense - 5'-GCCCTGGATATCTGTTCCAA-3', antisense - 5'-GCTCGACACATTTCGTCTCA-3', resulting in a PCR product size 177 bp. Annealing was carried out at 61 °C. Genes encoding ß-actin and GAPDH were used as house-keeping gene controls.

Determination of IL-15 by ELISA

For IL-15 determination, 0.35 ml of each dendritic cell lysate was collected and kept frozen (-20°C) until analysis. Concentrations of IL-15 were determined using "Ready-Set-Go" ELISA kit (eBioscience, San Diego, Ca, USA) combined with ELIST amplification system (PerkinElmer LAS, Inc.). The "Ready-Set-Go" kit included capture Abs, standard, biotinylated detection Abs, and HRP-streptavidin. The procedure was performed essentially according to the manufacturer's recommendations with the following modifications: 1) After overnight binding of capture antibodies to the Nunc Maxisorp 96-well plates and washing the plates, the blocking step was extended to at least 3 hrs at r.t.; 2) The standard curve was generated by seven serial dilutions of the standard, starting with 500 pg/ml of IL-15,

and the standards and samples in triplicates were incubated at r.t. for 2 hrs followed by incubation at 4° C overnight.

After binding of biotinylated detection Abs followed by HRP-streptavidin, amplification by ELIST was performed essentially according to the PerkinElmer's protocol. Then the enzymatic reaction of HRP was measured using tetramethylbenzidine (TMB) as a substrate. After terminating the reaction by sulfuric acid, optical density was measured with wavelength correction as the difference between readings at 450 and 550 nm.

The results of determination of IL-15 are presented in Figure 8 (RT-PCR analysis) and Figure 9 (ELISA analysis). The results show that immature dendritic cells produced according to the present invention express mRNA for IL-15 and express the protein in the cells. It is of interest to note that upon maturation, the amount of IL-15 in the cells decreased in some experiments. Data from real-time PCR confirm the expression of mRNA for IL-15 (not shown). IL-15-expressing DC have been shown to be superior in induction of a CTL response compared to non-IL-15-producing cells (32, 33, 34). IL-15 is also able to drive generation and maintenance of memory CD8+ cells (35, 36). Therefore, IL-15 expression may reflect the high competence of the dendritic cells generated by the present method for inducing memory type CTL responses.

To demonstrate the ability of the generated dendritic cells to take up lysate from tumour cells, the lysate - made as described in the Example 6 - was labeled using the following procedure. The tumour cell lysate (2-4 mg protein/ml) was prepared in DPBS with Ca/Mg, 1M NaOH was added to raise pH to 9.0. 3 mg of N-Hydroxysuccinimide–Fluorescein (NHS-Fluorescein, Pierce) was dissolved in 50µl dry DMSO, and added to the lysate. The mixture was incubated at 37^0 C for 1.5 hr in the dark. The modified lysate was dialyzed, with 1.5 l DPBS (without Ca/Mg) being replaced at 12 hr intervals, until no fluorescence is detected in the dialysate under UV, 254 nm. 1.5 µl of the final lysate and NHS-Fluorescein solution were spotted on a TLC plate with a fluorescent indicator (Kieselgel 60 F_{254}, Merck) and developed in a chromatographic system acetonitryl:H_20/ 4:1. The plate was viewed under UV 254

nm in order to ensure that all label in the lysate was covalently attached to the protein and that the lysate did not contain free label.

Figure 10 demonstrates microscopy of dendritic cells incubated for 60 minutes with the labelled lysate at 37°C (a – light microscopy; b – fluorescent microscopy). It can clearly be seen that all the dendritic cells demonstrate intensive uptake of the labelled lysate.

In summary, the dendritic cells generated by the described method, are highly homogeneous, they express the required set of surface molecules characteristic for dendritic cells present *in vivo*, they express IL-15 and they have high ability to uptake exogenously added lysate of tumour cells.

Example 6 - Immature dendritic cells loaded with lysate from a tumour cell line stimulate *in vitro* generation of a tumour-specific CTL response with the characteristics of a memory-type response.

The ability of dendritic cells, prepared by the method of the present invention and loaded with tumour antigens, to stimulate the generation of an anti-tumour CTL response was tested *in vitro* using lysate of breast cancer cell line MDA-MB-231 as a source of tumour antigens. This cell line expresses broad spectrum cancer/testis antigens (see WO 2008/081035).

Tumour cells were cultured in RPMI 1640 medium supplemented with L-glutamine and 10 % of fetal calf serum. For preparation of lysate, cells were harvested by trypsinization, washed, counted, suspended in RPMI 1640 medium at a concentration of 10^7/ml and subjected to 5 cycles of freezing (liquid nitrogen) and thawing (water bath, 37°C). The resultant lysate was clarified by centrifugation (3000 g, 60 min, 4°C) and stored in aliquots at -80°C.

Dendritic cells were prepared as described in Example 1. At day 4 or 5, 10% of tumour lysate was added to the culture of the dendritic cells for loading the dendritic cells with tumour lysate.

After overnight incubation, cells were harvested, washed, re-suspended in lymphocyte cultivation medium (AIM-V with addition of 5% of autologous serum). For *in vitro* generation of a tumour-specific CTL responses the loaded dendritic cells were mixed with autologous non-adherent lymphocytes at 1:40 ratio and placed into 24-well plates, 2 Mio of the lymphocytes per well. At days 2, 5 and 8, half of medium was removed and 1 ml of fresh medium containing 50 U/ml of IL-2 was added into each well. At day 10 or 11, the cells were harvested, and the concentration of large activated lymphocytes was determined using Coulter Counter. The cells were then washed, mixed with thawed lysate-loaded dendritic cells at a ratio of 10:1 and placed into the wells of 24-well plates ($0.5x10^6$ lymphocytes per well), in 2 ml of lymphocyte culture medium.

At days 2 and 5, half of the medium was removed and 1 ml of fresh medium containing 50 U/ml of IL-2 was added into each well. At day 7, cells were harvested, counted, and used for FACS analysis and for testing for cytotoxicity against a panel of tumour cell lines.

Phenotypic analysis of lymphocytes generated in one of several experiments is demonstrated in Figure 11. As can be seen, a significant portion of the cells are CD8+ T lymphocytes and the majority of them express high levels of CD62L.

Cytotoxic activity of the activated lymphocytes was determined morphologically for HLA-A2-positive donors, using a panel of tumour cell lines: breast cancer cell lines MCF-7 (HLA-A2+), MDA-MB-231 (HLA-A2+) and T47D (HLA-A2-), prostate cancer cell line LNCaP (HLA-A2+). $5x10^4$ tumour cells were seeded in 24-well plates in 1 ml of RPMI-1640 medium with addition of 10% of FCS and incubated for 2 days before testing. 1 ml of the suspension of the isolated lymphocytes containing $1x10^6$ cells was added to the tumour cells.

Lytic activity of lymphocytes was detected morphologically after 24-48 hours of incubation. The results of such experiments are shown in the Figures 12 and 13. As could be seen, significant lysis was seen with all HLA-A2-positive tumour cell lines, while HLA-A2-negative T47D tumour cells were resistant to lysis. Intensive proliferation of lymphocytes is seen in cultures with HLA-A2-positive tumour cells,

while no proliferation is seen in the culture with the resistant HLA-A2-negative T47D cell line.

These findings - high level of expression of CD62L, lysis of only HLA-A2+ tumour cell lines, and intensive proliferation of lymphocytes after contact with, and recognition of, the appropriate tumour antigens - point to generation of specific memory type CTL response in the system of the present invention.

In summary, the improved method of the present invention for generating DC results in fully immunocompetent immature dendritic cells able to stimulate a specific memory-type antitumour T cell response.

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Claims

1. A method of producing immature dendritic cells by cultivation of monocytes, the method comprising:

pre-treatment of a tissue culture surface with a pre-treatment medium, the pre-treatment medium comprising heparin and a protein solution;

adsorption of monocytes using an adsorption medium that is either substantially plasma free, or substantially serum free; and

cultivation of monocytes using a cultivation medium that is either substantially plasma free or substantially serum free.

2. A method as claimed in claim 1 wherein the protein solution comprises human serum.

3. A method as claimed in claim 2 wherein the human serum has a concentration of between 2 and 10 %.

4. A method as claimed in any one of the preceding claims wherein the pre-treatment medium has a heparin concentration of between 10 and 200 U/ml.

5. A method as claimed in claim 4 wherein the pre-treatment medium has a heparin concentration of between 25 and 100 U/ml.

6. A composition comprising immature dendritic cells produced in accordance with the method of any one of claims 1 to 5.

7. A composition as claimed in claim 6 wherein the composition is injectable directly into a tumour.

8. Use of immature dendritic cells produced in accordance with the method of any one of claims 1 to 5 for the preparation of a pharmaceutical composition for treating cancer by immunotherapy.

9. A vaccine comprising immature dendritic cells produced in accordance with the method of any one of claims 1 to 5 when used in treating or preventing cancer.

10. A vaccine as claimed in claim 9 wherein the vaccine is injected directly into a tumour.

11. A composition comprising immature dendritic cells produced in accordance with the method of any one of claims 1 to 5 when used in the treatment of cancer by immunotherapy.

12. An antigen-presenting composition produced from immature dendritic cells produced in accordance with the method of any one of claims 1 to 5.

13. A method of preparing a cytotoxic composition, comprising using an antigen-presenting composition according to claim 12 to activate T cell lymphocytes, thereby obtaining a cytotoxic composition containing tumour-specific CD8+ cytotoxic T lymphocytes.

14. An antigen-presenting composition according to claim 12 when used in treating cancer by stimulating an immune response against tumours or tumour antigens.

15. A cytotoxic composition prepared in accordance with the method of claim 13 when used in treating cancer by adoptive T cell therapy.

16. A composition according to claim 6 when used as antigen presenting cells for activation of cytotoxic T lymphocytes.

17. Use of tumour-specific CD8+ cytotoxic T lymphocytes obtained by the method of claim 13 for preparation of a pharmaceutical composition for adoptive T cell immunotherapy.

Figure 1

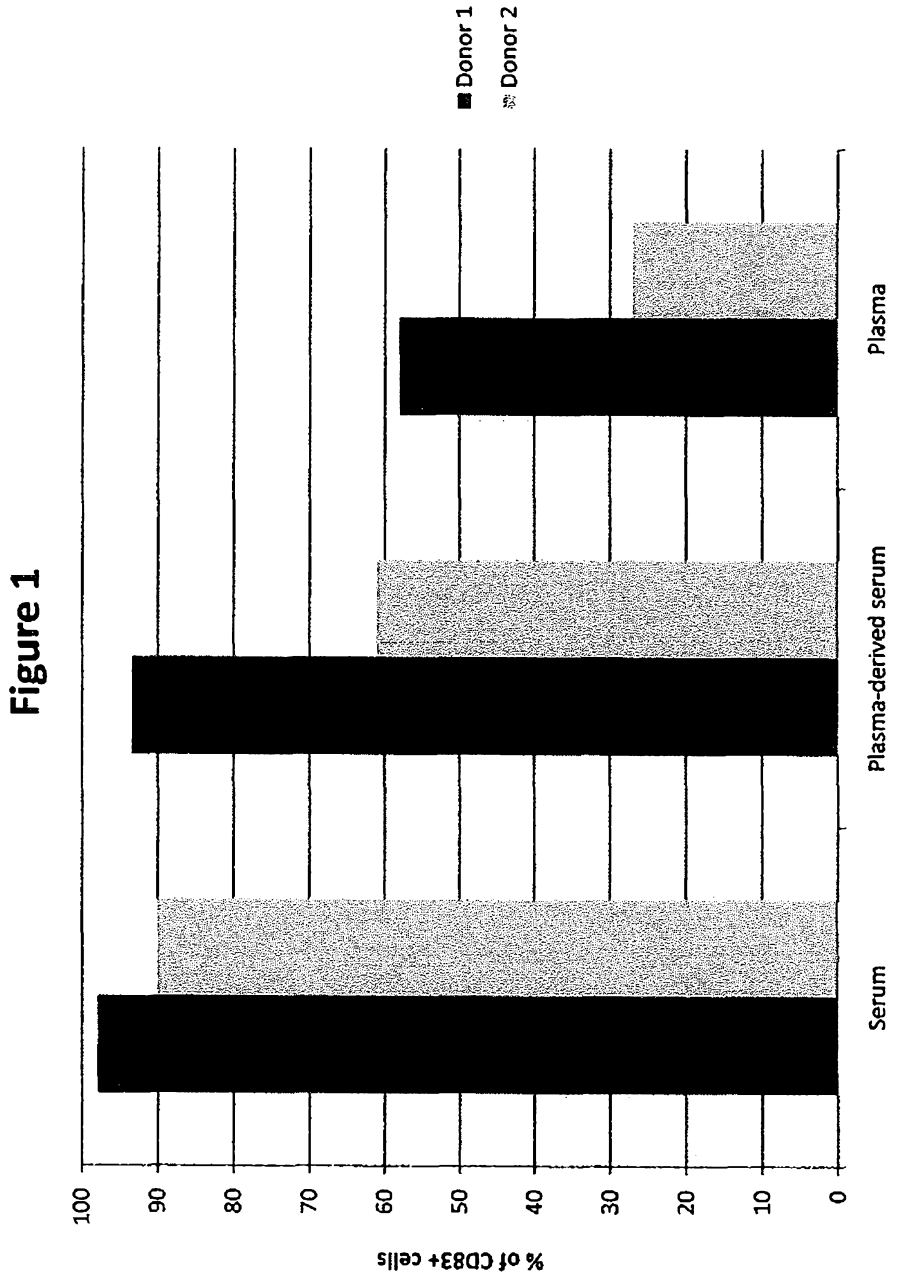

Donor 1
Donor 2
% of CD83+ cells
100
90
80
70
60
50
40
30
20
10
0
Serum
Plasma-derived serum
Plasma

Figure 2

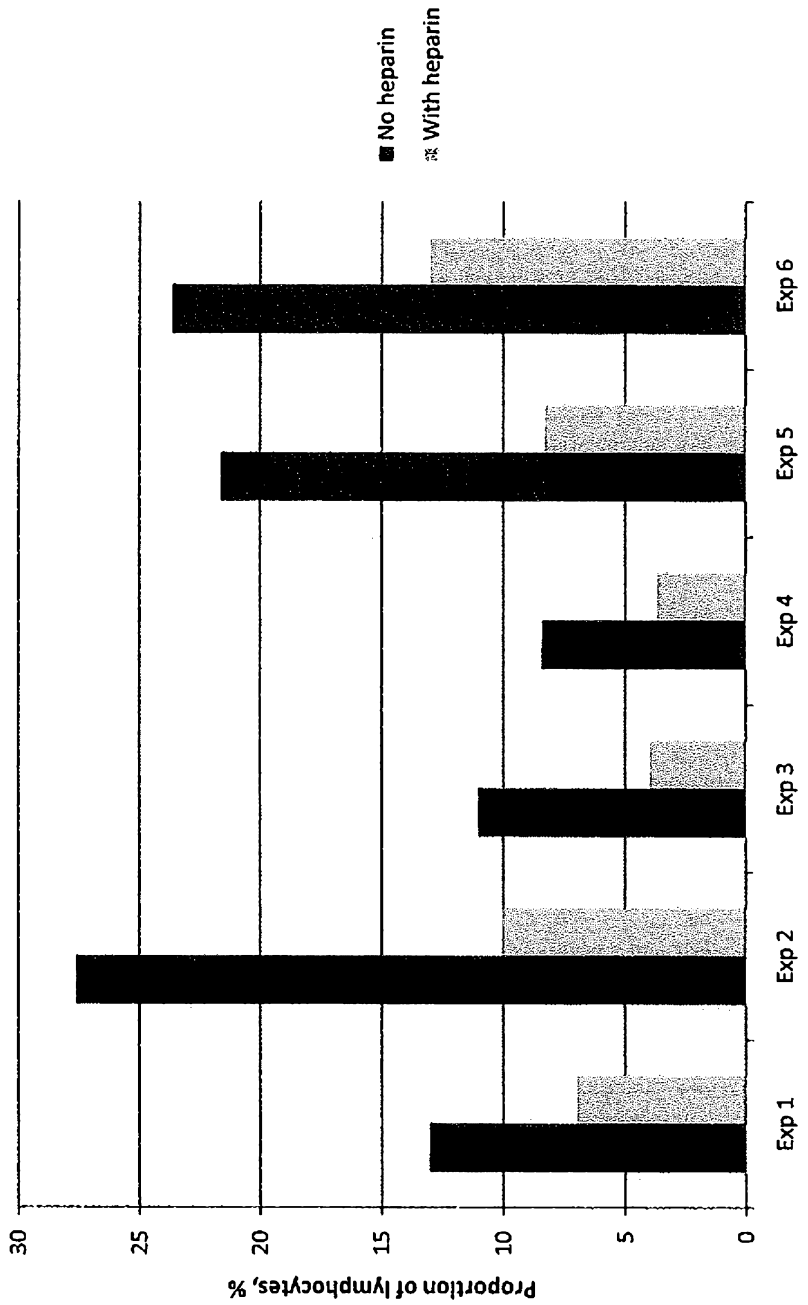

No heparin
With heparin
Proportion of lymphocytes, %
0
5
10
15
20
25
30
Exp 1
Exp 2
Exp 3
Exp 4
Exp 5
Exp 6

Figure 3

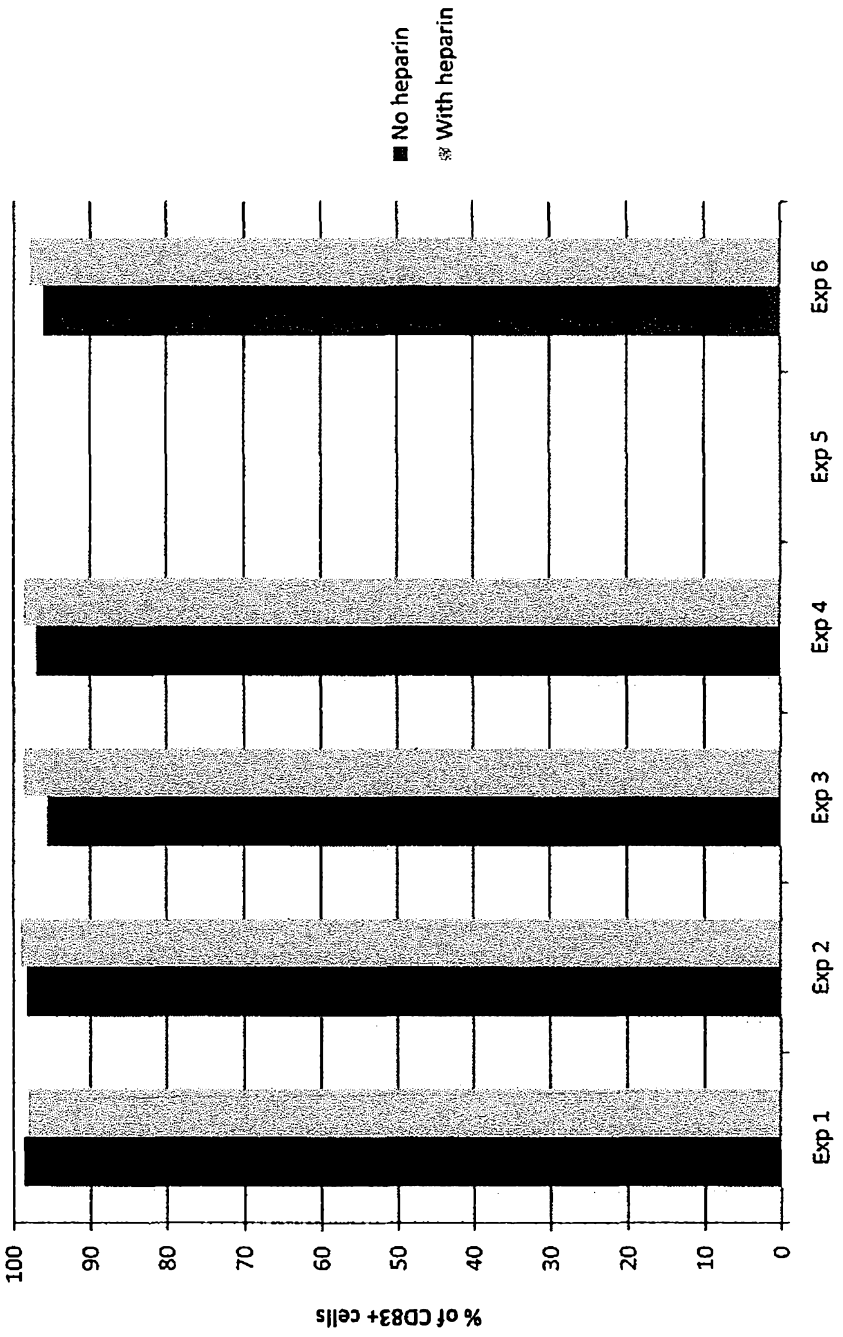

No heparin
With heparin
% of CD83+ cells
100
90
80
70
60
50
40
30
20
10
0
Exp 1
Exp 2
Exp 3
Exp 4
Exp 5
Exp 6

Figure 4

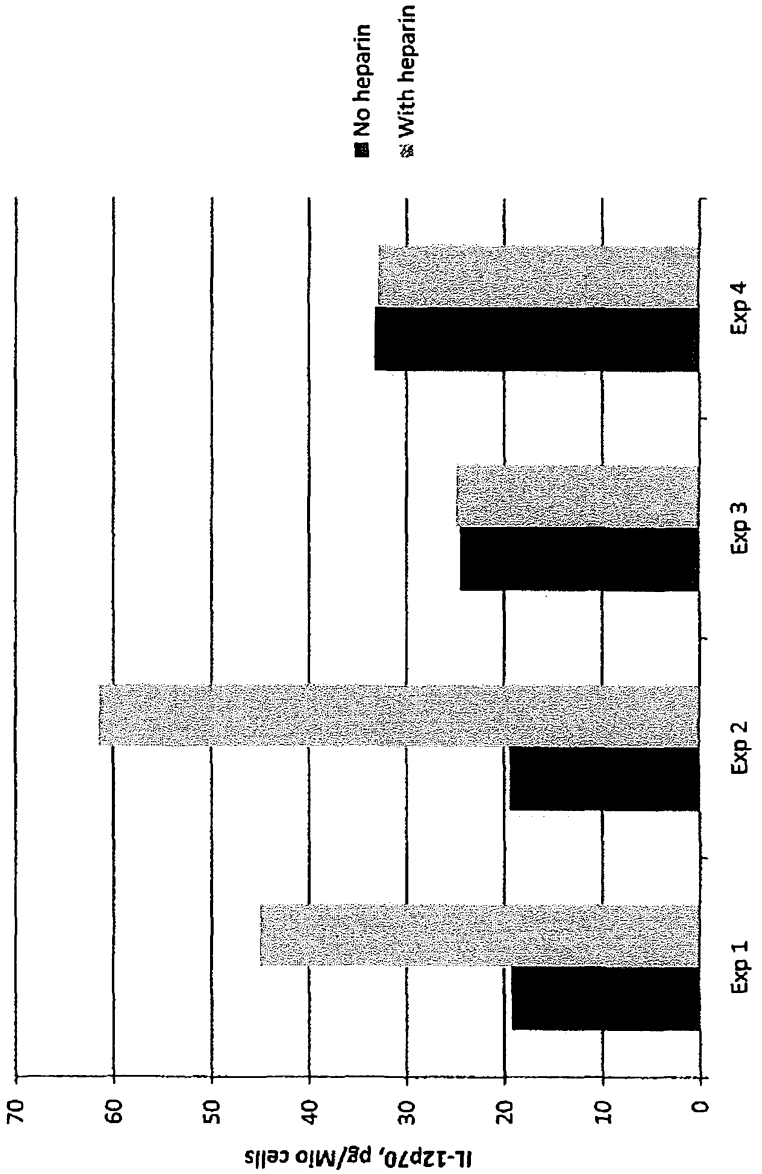

No heparin
With heparin
IL-12p70, pg/Mio cells
0
10
20
30
40
50
60
70
Exp 1
Exp 2
Exp 3
Exp 4

Figure 5

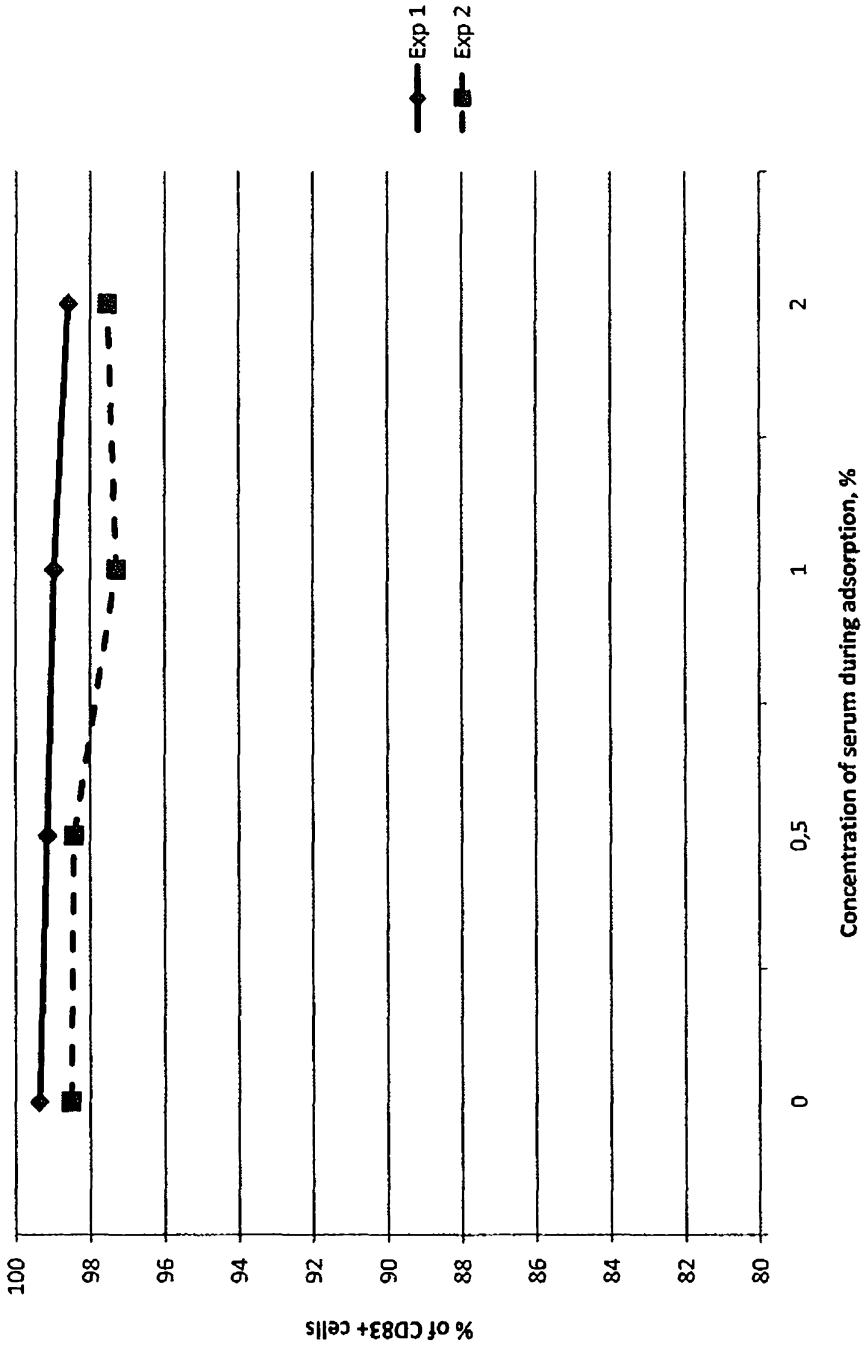

Exp 1
Exp 2
% of CD83+ cells
100
98
96
94
92
90
88
86
84
82
80
0
0,5
1
2
Concentration of serum during adsorption, %

Figure 6

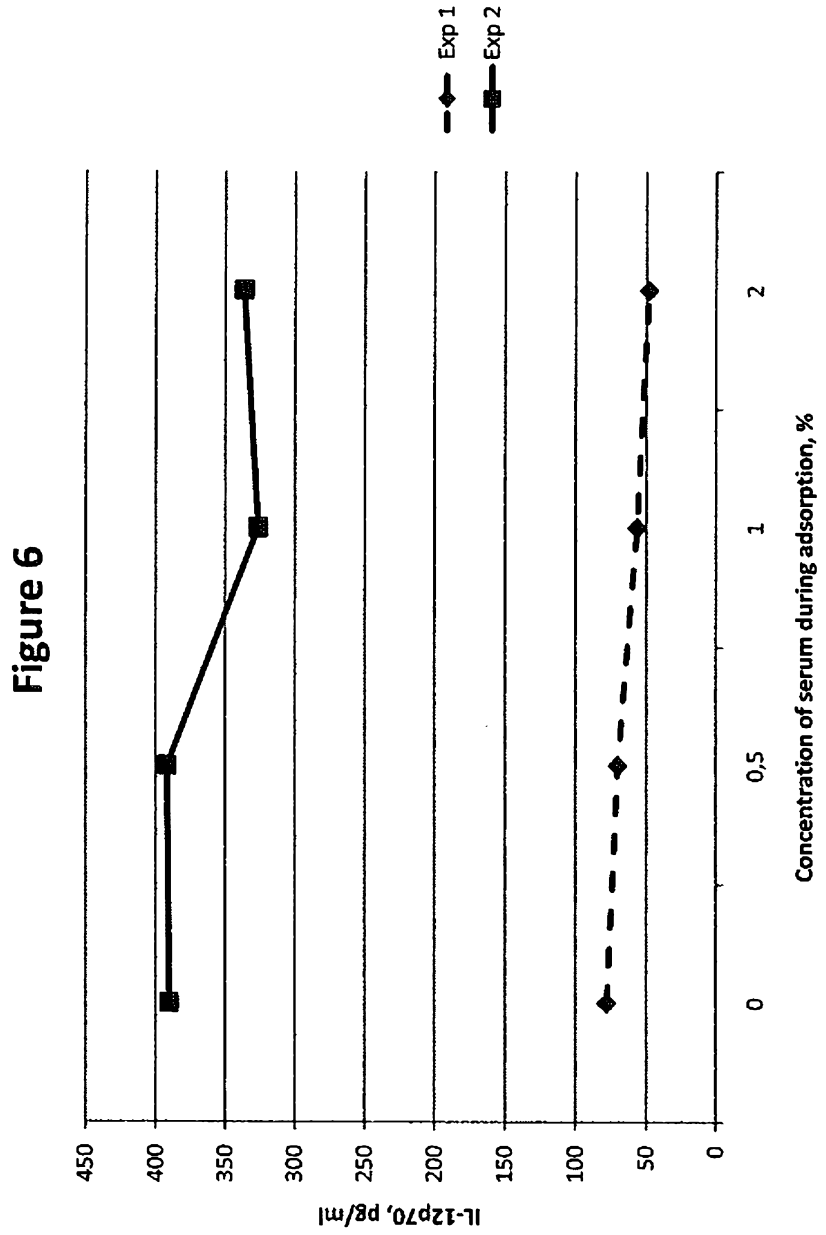

Exp 1
Exp 2
IL-12p70, pg/ml
450
400
350
300
250
200
150
100
50
0
0
0,5
1
2
Concentration of serum during adsorption, %

Figure 7

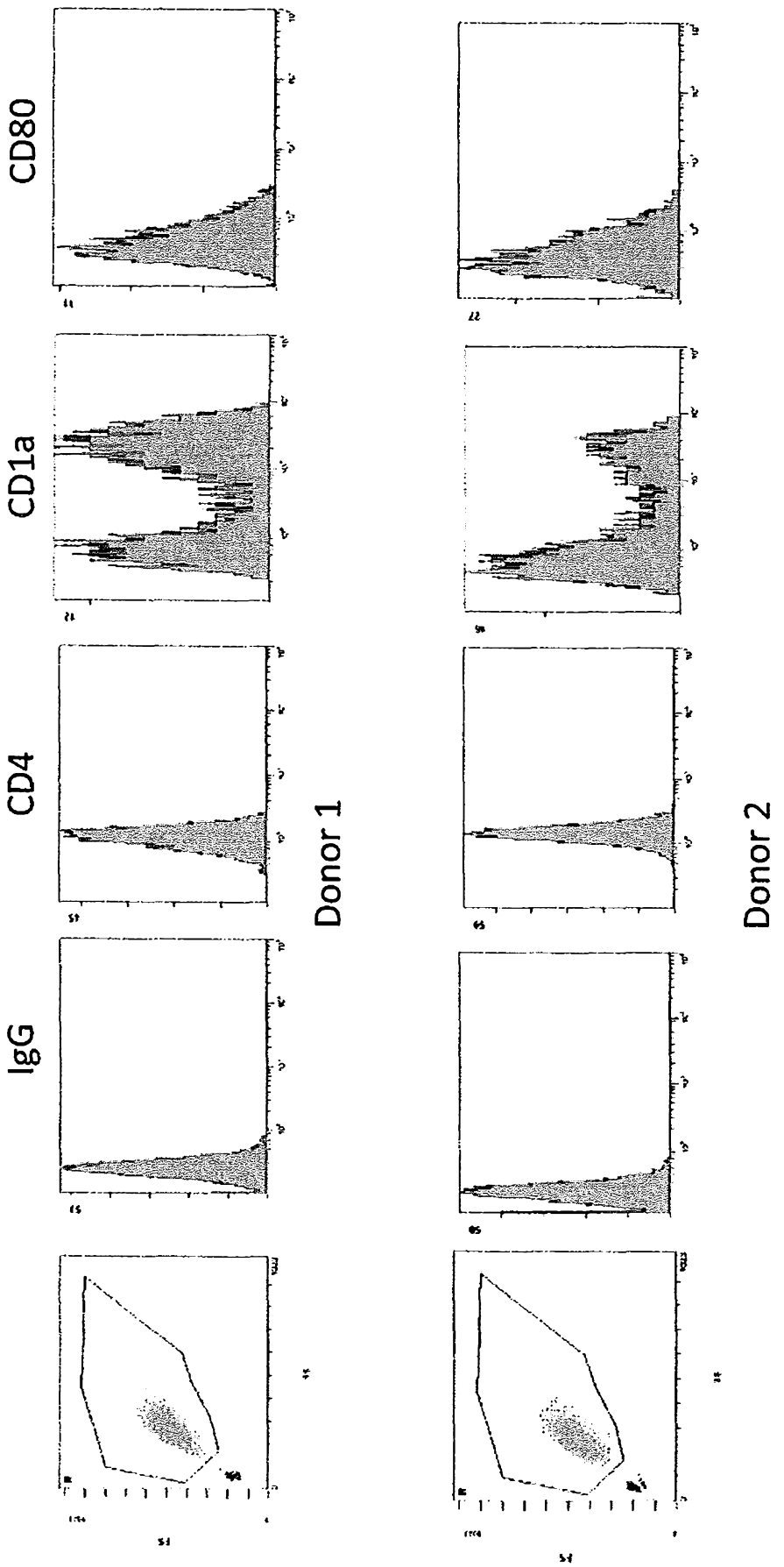
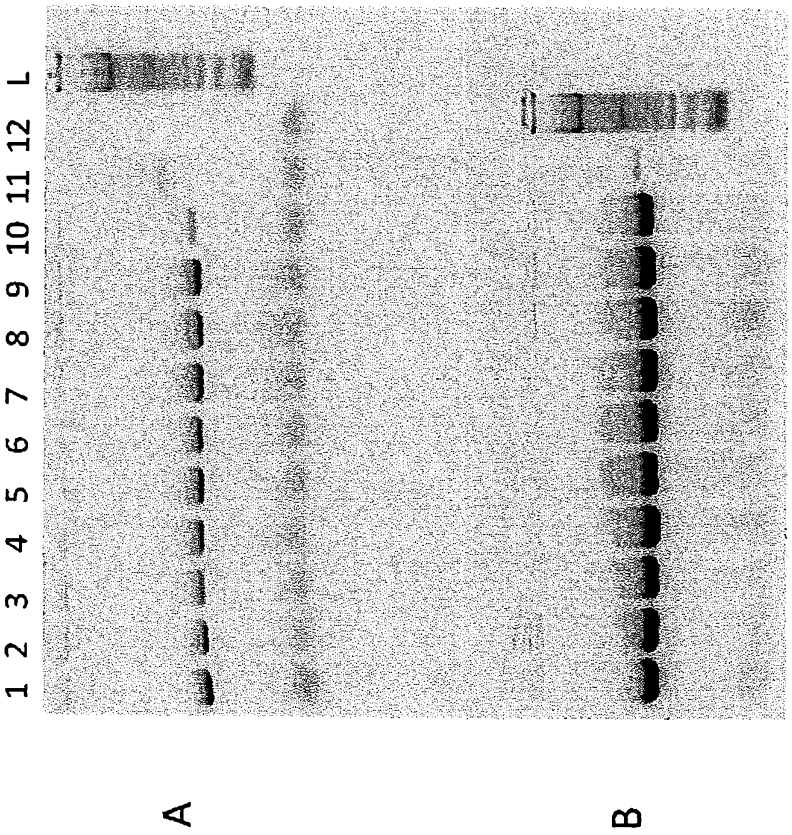


Figure 8



RT-PCR analysis of IL-15(A) and GAPDH (B) expression in DC.
Samples of DC lysates (lanes 1-10), control without RT (lane 11), control in the absence of cDNA (lane 12 A).
The last lane - 100bp ladder.

Figure 9

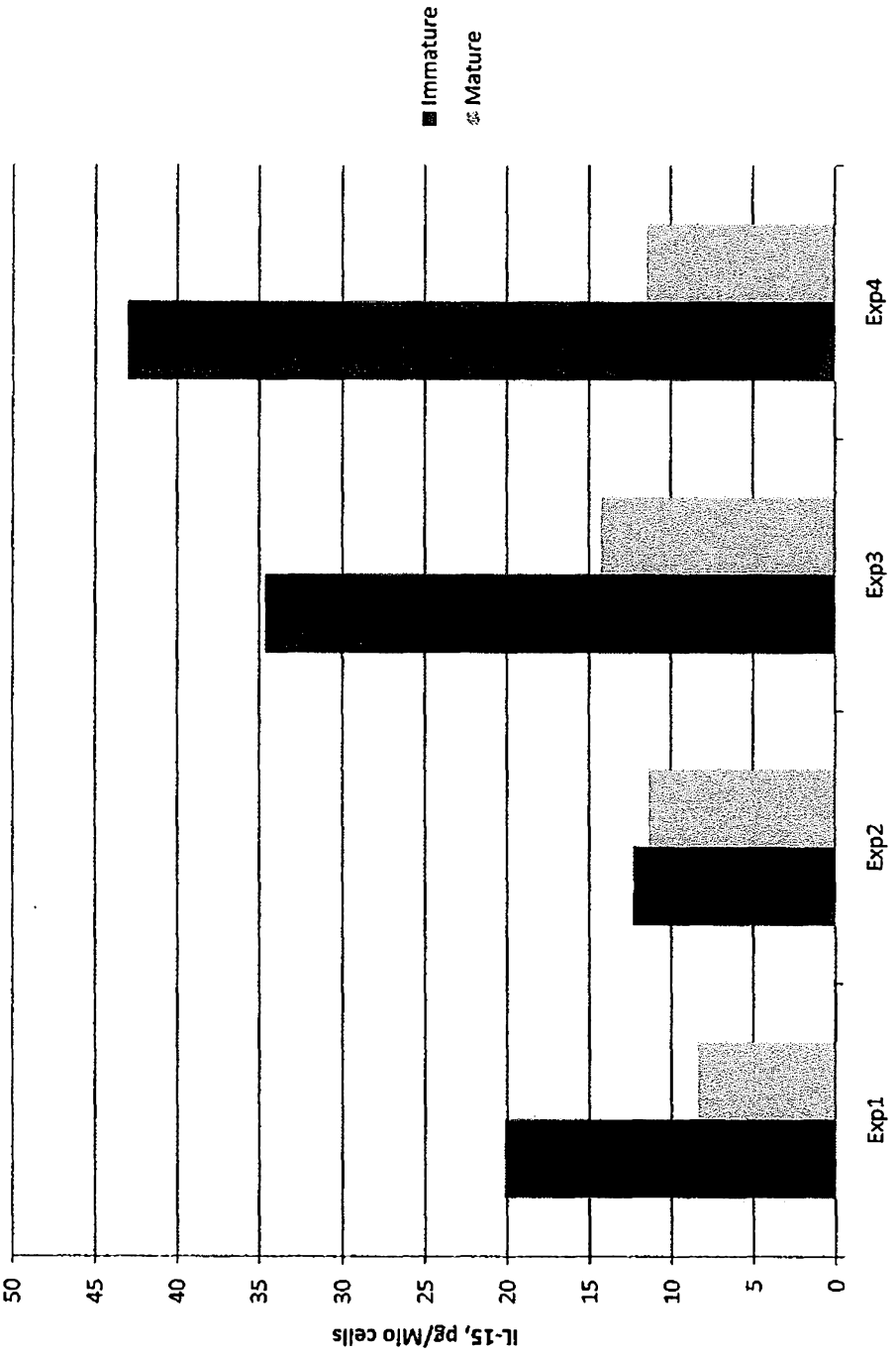

Immature
Mature
IL-15, pg/Mio cells
0
5
10
15
20
25
30
35
40
45
50
Exp1
Exp2
Exp3
Exp4

Figure 10

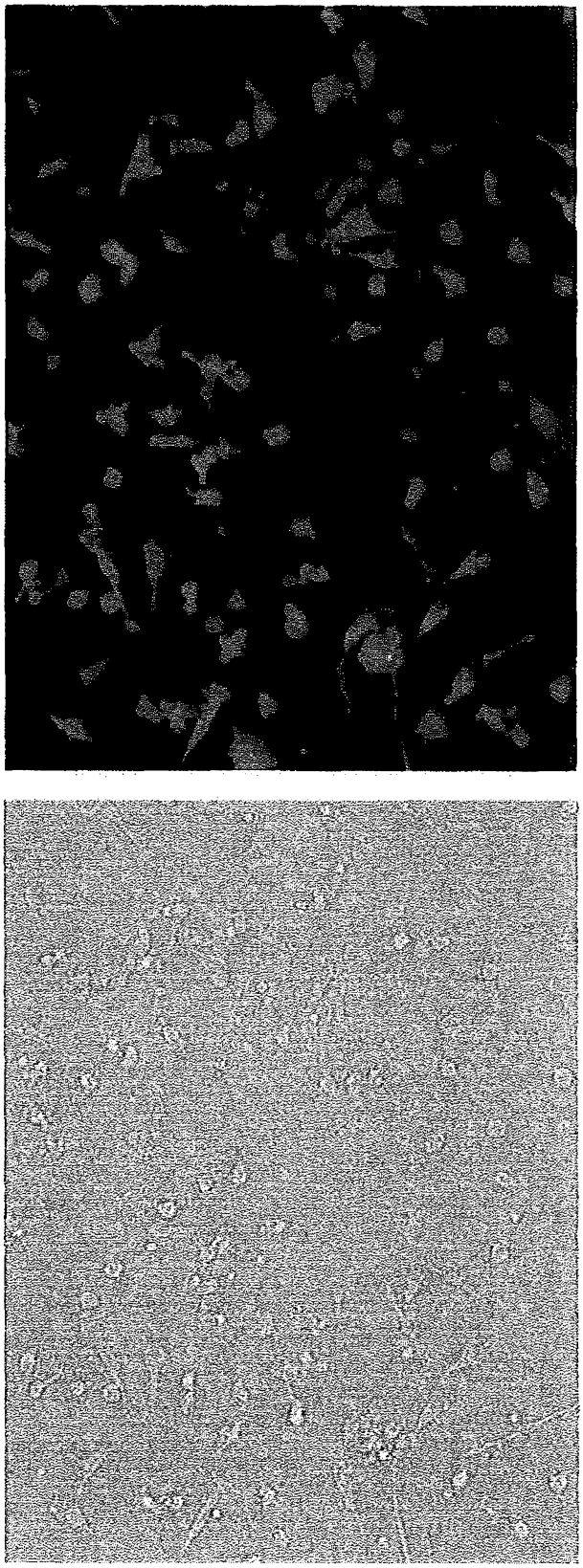


Figure 11

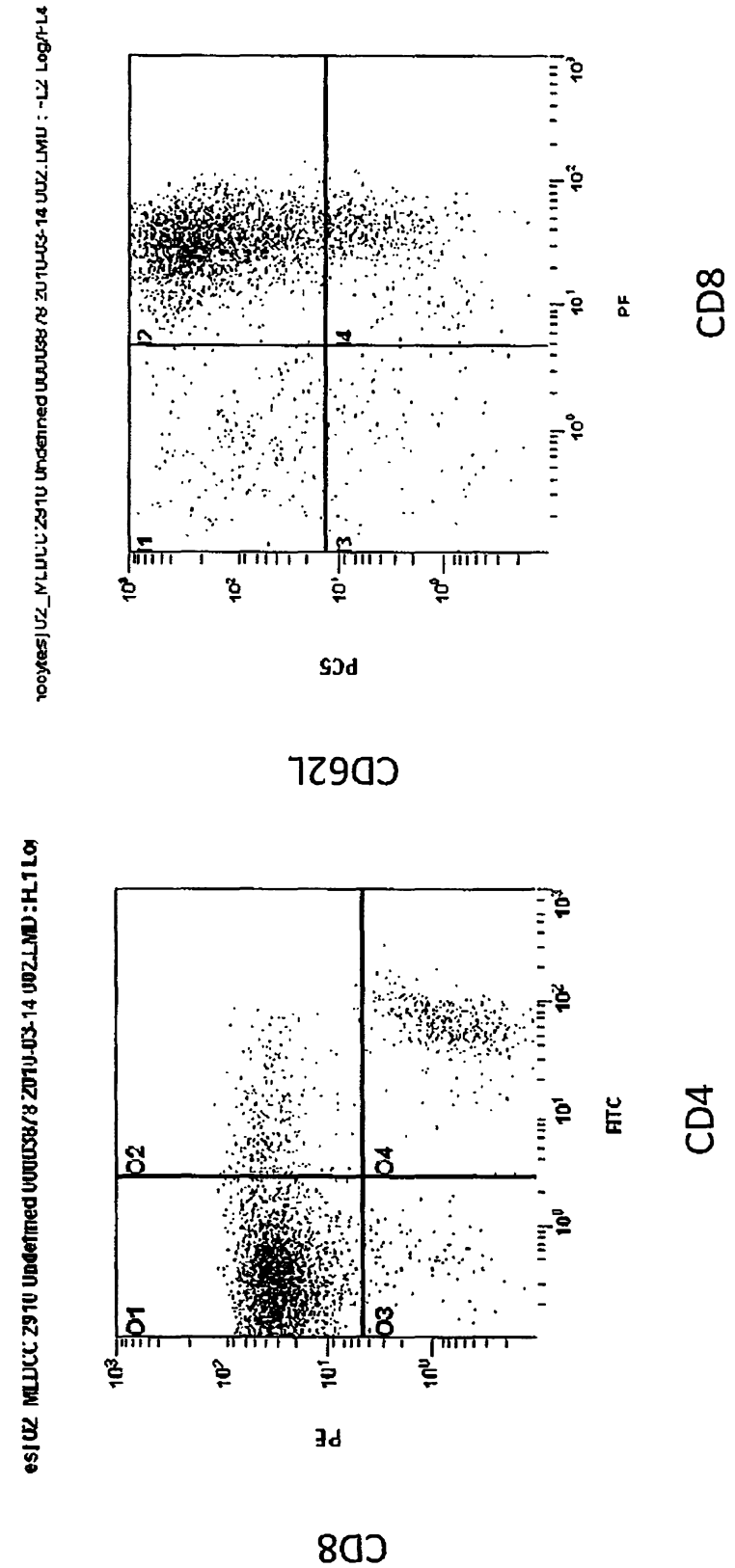


Figure 12

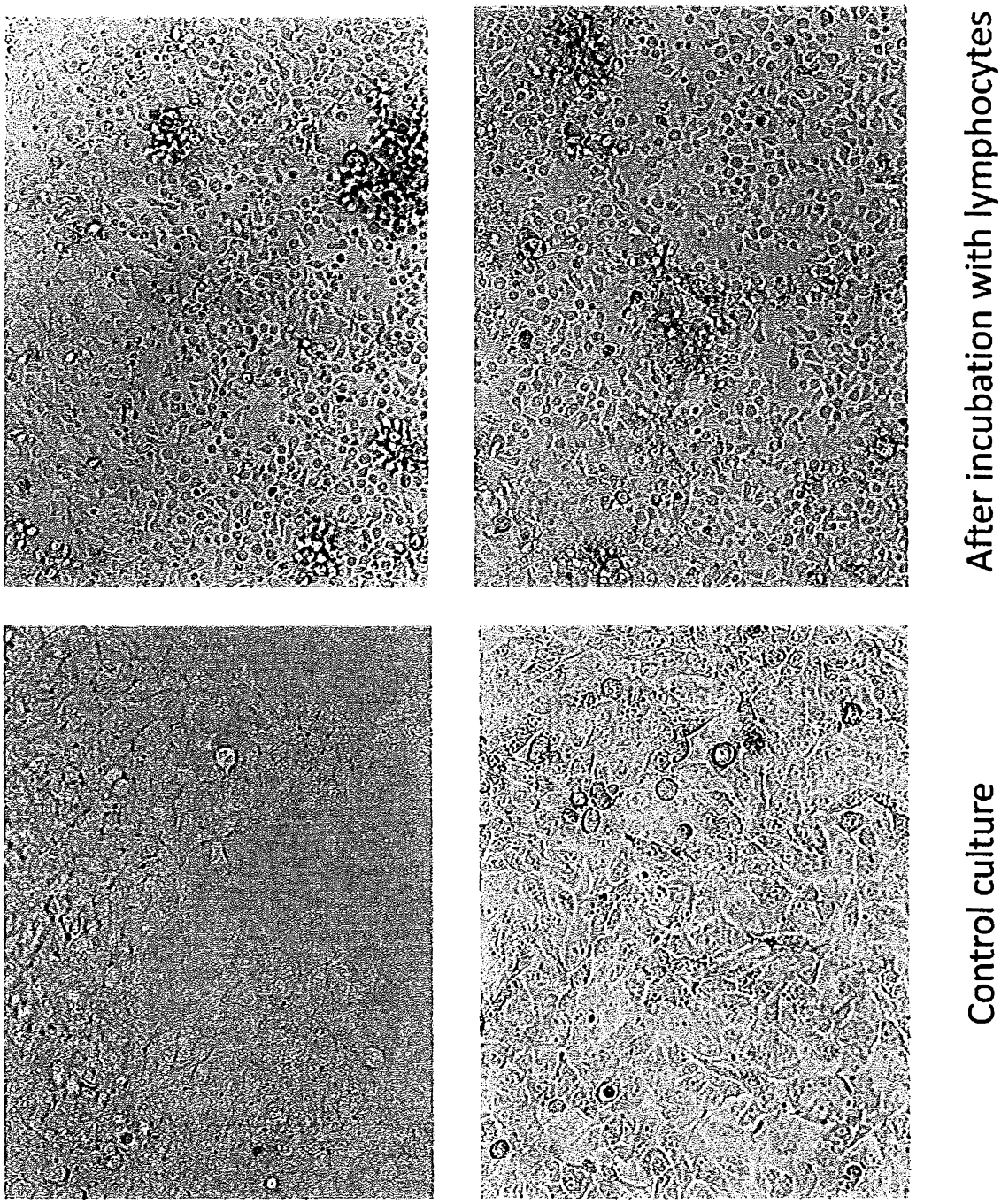
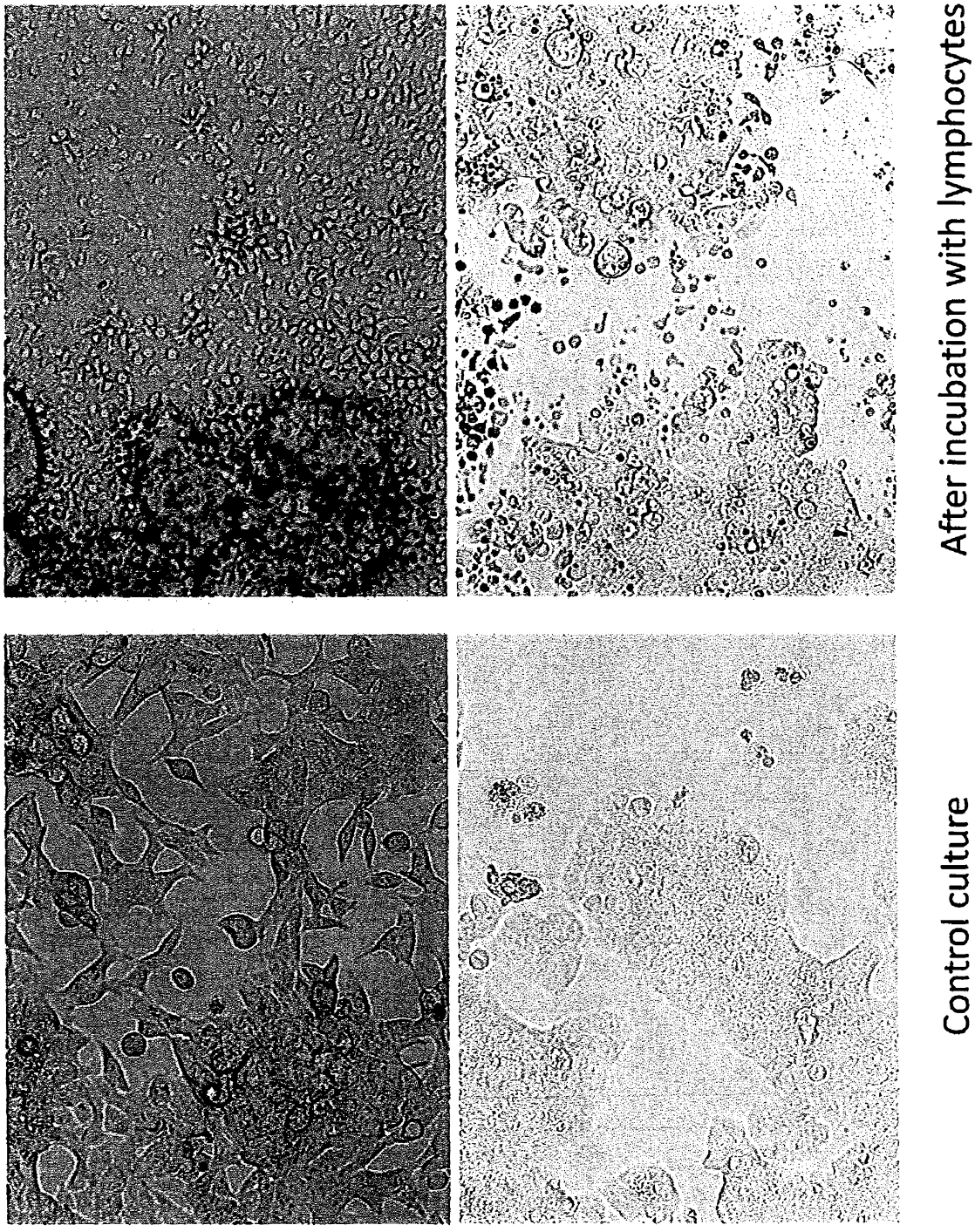

After incubation with lymphocytes
Control culture
MDA-MB-231
MCF-7

Figure 13


After incubation with lymphocytes
Control culture
LNCaP
T47D