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(71) Applicant *(for all designated States except US)*: **CELL MED RESEARCH GMBH** [AT/AT]; Magnesitstr. 1, A-3502 Krems-Lerchenfeld (AT).

(72) Inventor; and
(71) Applicant : **MOLZER, Sylvia** [AT/AT]; Paracelsusg. 36, A-3003 Gablitz (AT).

(74) Agents: **GASSNER, Birgitta** et al.; REDL Life Science Patent Attorneys, Donau-City-Str. 1, A-1220 Vienna (AT).

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(54) Title: NATIVE DENDRITIC CELLS

(57) Abstract: The present invention relates to a pharmaceutical composition comprising native dendritic cells expressing/presenting at least one universal and/or embryonic tumor associated antigen.

NATIVE DENDRITIC CELLS

The present invention relates to native dendritic cells (DCs) produced according to a new and inventive protocol including cryopreservation in the absence of serum and any plasma protein, and their use.

DCs are bone-marrow-derived antigen-presenting cells (APCs) that play a pivotal role in both induction and regulation of the immune response (7). It has been described that the *in vitro* generation and manipulation of human DCs can be particularly effective to stimulate the immune system against cancer, being a new powerful tool in the fight against tumor (7). Indeed, it has been shown that in patients suffering from different types of cancer such as breast, ovarian, head and neck, colorectal and renal tumor, hepatocellular carcinoma and malignant melanoma, the incapability of the host's immune system to fight efficiently the cancer establishment strictly correlates with diminished DC function. These changes have been mainly detected in blood, in cells infiltrating cancers and in lymph nodes (LNs). In addition, it has been documented that most chemotherapies deeply impair DC function, whereas the capability of patients' T-cell to drive an effective immune response is comparable with that observed in healthy people. This observation implies that the openness to tumor establishment displayed by cancer patients' immune system is mostly due to the presence of functional defects in the DCs such as maintenance of an immature phenotype and/or incorrect balance between IL-12 and IL-10 secretion. Accordingly, it has been shown that the serum levels of IL-10 levels are significantly higher in patients suffering from cancer than in healthy age- and sex-matched controls. IL-10 has been shown to have a significant inhibitory effect on several aspects of DC function, such as the expression of costimulatory molecules and the ability to secrete IL-12. Importantly, IL-10 treated DCs become tolerogenic. In addition, it has been reported that in patients with progressively tumor growing, the DCs are both immature and switched off. Since the antitumor effect of DCs depends on their level of activation and maturation, it is likely that the failure of induction of a tumor-specific T-cell driven cytotoxic response depends on the wrong balance between IL-10 and IL-12 as well as on the absence of maturity markers, required to elicit the expected T-cell activation. Indeed the immune suppressive function of IL-10 can be modulated by a simultaneous production of IL-12. For example, it has been shown that monocyte-derived mature DCs commonly secrete both IL-12 and IL-10 upon stimulation with CD40L. In this case, the coupled secretion of IL-10 and IL-12 induces a more effective immune response against cancer than that

observed upon secretion of IL-12 alone. In the presence of mild-high levels of IL-12 production, IL-10 secretion promotes the activation of the T-helper cells ($CD4^+$ T-cells) and therefore triggers the adaptive arm of the immune system.

One of the main goals of the current immune therapy strategies is to activate efficiently the host's immune system against cancer. Among them, the use of *in vitro* cultured and modified DCs and T-cells are the most frequently applied methods. The most common approach to use DCs for vaccines is to prepare large numbers of autologous mature myeloid DC (MDCs) *ex vivo*, load them with cancer-specific antigen(s), and inject them back into the subject. Since the rate of transdifferentiation from monocytes to mature DCs is low, it is important to gain an amount of DC precursors compatible with large-scale production of mature DCs. This goal is usually achieved alternatively by (1) differentiating DCs from leukapheresis / elutriation-derived monocytes with Granolulocyte-Macrophage Colony Stimulating Factor (GM-CSF) and IL-4 or with GM-CSF and IL-13 or (2) directly isolating DCs from leukapheresis products by density gradient centrifugation or with commercially available closed systems, based on the use of immunomagnetic beads.

Once transdifferentiated from monocytes, the immature DCs (iDCs) could be loaded and matured *in vitro*. While the loading step can be performed according to several methods with a single physiological or modified protein antigen, mRNA, cDNA, tumor lysate, etc., the maturation is usually performed by means of addition of one of the following cocktails: (a) IL-lβ, IL-6, TNF-α, and prostaglandin E2 (PGE2); (b) lipopolysaccharides (LPS) and interferon-γ (IFN-γ); (c) Ribomunyl and IFN-γ. Although in the art it is observed that DCs matured with a cocktail including PGE2 still express CCR7 and induce ThI as well as $CD8^+$ T-cell responses, they fail to secrete detectable bioactive IL-12p70, in contrast to DCs matured with LPS/Ribomunyl and IFN-γ.

Schmitz et al. discloses the specific tomour cell killing activity of DCs. The cells were isolated from blood of healthy donors and had been differentiated into DCs in the presence of IL-4, GM-CSF and 10 % human serum, followed by stimulation by growth in the presence of IFN-gamma for 6 h (8).

WO 2004/050909 describes the storage of frozen native differentiated DCs in 4 % human serum albumin and 10 % DMSO. These immature DCs were thawed, loaded with the peptide antigen (the M158-66 peptide derived from M1 protein of the influenza virus, Melan-A/MART1 peptide, the peptide derived the influenza matrix protein or PSA1 peptide) and matured in the presence of Ribomunyl and IFN-gamma.

Osada et al. described clinical trials involving DC-based vaccines against cancer, for which DCs were generated in serum-free media (9).

In US 2005/0003533 the use of DCs matured by growth in IFN-gamma containing medium for the treatment of cancer is described.

Onaitis et al. summarized the clinical experiments, in which DC vaccination for tumor treatment were used (10).

The inventors have discovered methods which allow the preparation of a pharmaceutical composition of native, unloaded dendritic cells useful as vaccine, which exhibit beneficial immunologic properties. This allows the manufacture of a pharmaceutical composition comprising native DCs which express tumor associated antigens (TAAs) on their surface without the additional step of loading the DCs with exogenous antigens due to the new and inventive protocol. That means there is neither need for the production or purchasing as well as selection of tumor associated antigens nor the establishment of challenging loading procedures.

These native DCs should preferably show (a) reduced phagocyting capability in comparison with iDCs; (b) capability to migrate in response to stimuli (such as but not only CCL19 and/or CD40L; (c) a mature phenotype i.e. high surface expression of CD80, CD86, CD40, CD83, HLA-ABC and HLA-DR; (d) mild to high levels of IL-12 secretion with or without IL-10; (e) surface expression of TAAs.

The activation and establishment of both the short-term immune response by $CD8^+$ T-cells (cytotoxic) and of the long-term immune response by $CD4^+$ T-cells (adaptive) is a further goal to be achieved by these native DCs.

In another embodiment the present invention relates to native DCs which express at least one TAA protein or fragment thereof.

The native, endogenous available antigen will be processed by the DCs and exposed on their cell membrane complexed to MHC-I. In this way, it triggers the cytotoxic response, due to the fact that MHC-I exposes antigens exclusively to $CD8^+$.

In another embodiment of the present invention, the native DCs preferably express embryonic TAAs (eTAAs). eTAAs includes universal TAAs (or uTAAs, which are present in more than 2, preferably more than 5, more preferably more than 10, even more preferably more than 20, in particular more than 30, different types of tumors). That means this method can be applied to a combined use of the two most common uTAAs: survivin and Telomerase Reverse Transcriptase (TERT). Indeed the expression of these two antigens is restricted to tumor cells, while being not expressed

at all or to very negligible levels by healthy cells/tissues (3, 5). Survivin rescues cells from apoptosis (programmed cell death) and TERT promotes cell immortalization, two features, which are typical for de-regulated rather than healthy cells. Accordingly, survivin is almost exclusively expressed during embryonic and fetal development but it becomes undetectable in terminally differentiated normal adult tissue, while it is re-expressed in tumor cell lines and several human cancer cells at a frequency of 34-100%. Also TERT protein, unlikely from its RNA subunit, is expressed almost only by tumor cells, even at very early stages of tumorgenesis. As a prognostic factor, survivin and TERT expression is significantly associated with poor clinical outcome in cancers, such as breast cancer, ovarian cancer, neuroblastoma, colorectal cancer, lung cancer and esophageal cancer. As survivin and TERT are preferentially expressed in tumor versus normal tissue, adverse effects on normal, differentiated cells are unlikely. Indeed, recent studies demonstrated that patient treated with DCs loaded with survivin or TERT protein, peptides or nucleic acids do not develop any adverse events related to immune therapy, other than a local reaction at the vaccination site, despite induction of impressive immune reactivity against survivin or TERT as measured by IFN-γ ELISPOT. Moreover, the strong immunological response against survivin and TERT at the time of tumor response underscores the tumor regression, following vaccination.

In yet another embodiment, the native DCs of the present invention can be used to treat and/or prevent specific tumors.

Definitions:

The term “native dendritic cell” as used herein refers to naïve dendritic cells which have not been exposed to an exogenous antigen, e.g. nucleic acid, protein, peptide or fragment of it.

“Mature” dendritic cells are those cells which have fully differentiated into cells which exhibit the characteristic morphology and function of DCs, for example an array of protrusions (dendrites) and the ability to be potent stimulators of allogenic T cells (6).

Herein, the term "serum- free" refers to a formulation which does not include any form of whole serum or plasma or and any plasma proteins (f.e. albumin), neither animal nor human.

The term “autologous” as used herein indicates the origin of a cell. Thus, a cell being administered to an individual (the “recipient”) is autologous if the cell was derived from that individual (the “donor”).

An “autologous vaccine” therefore is manufactured from precursor cells obtained from a patient cultured and differentiated and administered to that patient.

The term “isolated” means separated from constituents, cellular and otherwise, in which the cells are physiologically associated.

The term “cryoprocessed” as used herein refers to a method of freezing and storing a composition, preferably a pharmaceutical preparation.

As used herein, the term "fragment" relates to a part or stretch of universal tumor associated antigen, which retains at least one epitop of the wild-type protein.

Consequently, a "fragment" of universal tumor associated antigen binds to at least one T-cell receptor, which promotes an immune response highly specific against cells expressing such universal associated antigen. The "fragment" relates as well to a part or stretch of universal tumor associated antigen.

As used herein the term "universal tumor associated antigen" refers to tumor associated antigens which comprise or consist of proteinaceous structures like polypeptides, proteins and peptides. Fragments of tumor associated antigen protein of the present invention have at least 10, preferably at least 20, more preferably at least 30, even more preferably at least 50, contiguous amino acid residues of said tumor associated antigens. Universal tumor associated antigens are for example TERT, survivin, CEA, CYP1B, MUC16, MDM2, and cyclin (D1).

The term “pharmaceutical formulation” as used herein refers to a ready to use formulation in the absence of serum and any plasma protein. The formulation may comprise additives, selected from the group consisting of suspending fluids (e.g. sterile water, saline or fluids containing protein), preservatives and stabilizers (albumin, phenols, antibiotics and glycine, MSG, 2-phenoxy-ethanol), cryoprotectants (DMSO or glycerol) and optionally adjuvants or enhancers (aluminum gels or salt cryoprotectants). Other cryoprotectants, such as methoxylated compounds, are also used and are often considered less toxic and more penetrating. Still further examples of cryoprotectants include ethanol, ethylene glycol, 2-methoxy ethanol, 1,2-dimethoxyethane, propylene glycol, 1- methoxyl-2-propanol, and glycerol derivatives, such as 3-methoxy-1,2-propanediol or 1,3-dimethoxy-2-propanol.

“Conditions simulating physiological processes observed after application of DCs to the patient by use of specific media as well as temperature, humidity and carbon dioxide concentration” refer to re-cultivation conditions of frozen and thereafter thawed native DCs in CellGro medium supplemented with CD40L (mimicing the

presence of lymphocytes and stimulating DCs to produce IL-10 and IL-12), Ribomunyl and Imukin (activation of DCs by mimicking the inflammatory stimuli) for 2-20 h at 37 ˚C.

The native dendritic cells of the present invention have several advantages over loaded DCs known in the art: (a) There is no need for the production respectively purchasing of highly cost intensive GMP conform antigen for loading DCs. (b) There is no need for the establishment of proper loading strategies for the different biological variants of exogenous antigens to be loaded; e.g. protein, peptide, nucleic acid. (c) Skipping any loading step during the generation of DCs results in less stress to the culture thereby achieving better DC yields. (d) There is no more need for selecting tumor specific or associated antigen for each type and stage of cancer since universal and/or embryonic tumor associated antigens are expressed in almost every cancer as well as metastatic tumors. (e) The use of native, unloaded DCs, which are produced in a way, that they are expressing their endogenous available universal and/or embryonic TAA on their surface may not only be used for the treatment of cancer patients but might also be useful for the prevention of cancer of healthy people, especially in any events of immunodeficiency. (f) The use of autologous DCs with no exogenous antigen drastically reduces the risk of any severe adverse effects. (g) Since native DCs are expressing their endogenous available TAA in MHC I as well as MHC II, they can stimulate a short term, cytotoxic, transient as well as a long term, adaptive, memory response. (h) The use of native, unloaded DCs expressing universal and/or embryonic TAA on their surface abolish HLA-type restriction for the patients, what is a limitation in case of cellular therapies based on DCs loaded with peptides.

The native DCs of the present invention are able to express at least one uTAA and to decrease the capability to phagocyte. This is important to ensure the specific activation of the T-cells and remain capable to migrate in response to stimuli, such as CCL19 and/or CD40L. This feature allows the native DCs to move from the injection site towards the lymphonodes and/or where there is a high concentration of T-cells. The native dendritic cells show a mature phenotype i.e. high surface expression of CD80, CD86, CD40, CD83, HLA-ABC and HLA-DR. This feature is important to dock and activate the right population of T-cells. The native dendritic cells secrete mild to high levels of IL-12 and mild to none IL-10. This feature guarantees that the native DCs sustain the cytotoxic response (IL-12) and potentially increase the activation of the T-helper cells (IL-10). Further, the native dendritic cells show the surface

expression of the antigen(s) upon freezing, thawing and reculturing. This feature is important to determine the specificity of the DC stimulation of the T-cells. Further, upon harvesting and re-culturing after a cycle of freezing/thawing, the native dendritic cells show the typical dendritic morphology characterized by adherence and presence of several fingers, elongating from the small, round cell body as well as by the presence of cell clusters. This feature constitutes a hallmark of DC functionality as APCs, which can be stored between -80 and -180° C without loosing their active phenotype and/or functionality.

The present invention relates to native mature DCs which have been formulated as a ready for use pharmaceutical preparation and frozen, wherein the pharmaceutical preparation is free of serum and any plasma protein.

A further aspect of the invention relates to the use of native mature DCs as medicament.

The present invention further relates also to native mature DCs which have been formulated as a ready for use pharmaceutical preparation, frozen, and thawed before use.

The present invention further relates also to native mature DCs which have been formulated as a ready for use pharmaceutical preparation, frozen and optionally stored. The frozen pharmaceutical preparation is thawed and recultivated under biological conditions. Upon recultivation, at least 20 % of the dendritic cells present a universal TAA in the reculturing test.

A further aspect of the invention relates to the use of native mature DCs in the treatment of patients, suffering from different solid and/or blood cancers.

Another embodiment of the invention relates to the use of native mature DCs, capable of expressing at least survivin and/or TERT, preferably capable to migrate with decreased phagocytic activity, in the treatment of patients, suffering from different solid and/or blood cancers.

The present invention, especially its preferred embodiments, offers advantages over the prior art, such as the isolation of high quantity of monocytes, transformation rate of peripheral monocytes (pMos) into mature DCs between 40-80 %, GMP- and *re-vivo* use conform protocols, standard production in GMP conform lab, the possibility of a serum-free process, a therapy virtually applicable to any type of solid and/or blood tumor, availability of high quantity of phenotypic and functional mature DCs, a ready-to-use product that can be stored for years, with cytotoxic response, as well as

adaptive response, the inhibition of the T-reg response, a technique with minimal cell manipulation, no requirement for tumor tissue/cells as well as exogenous antigen, the possibility to expose universal tumor antigens with MHC-I coupled to simultaneous activation of the MHC-II , universal expressed TAAs, a high cell viability after a cycle of freezing/thawing, and the total preservation of cell functionality and phenotype after a cycle of freezing/thawing.

The inventors have discovered that native dendritic cells and native matured dendritic cells can be formulated in the absence of serum and any plasma protein, and frozen. After thawing, the dendritic cells are capable of expressing eTAA, optionally tested in a reculturing assay. These results are surprising, as it was commonly believed that specific immunoactive DCs need to be loaded respectively transfected with exogenous antigens for achieving specific T-cell response. There was no idea about the circumstance that DCs could just simply use their own pool of TAAs by their activation and expression.

In general TAAs comprise autologous cellular antigens which are expressed at high levels by tumor cells, but can be also presented at lower levels by normal healthy cells. As mentioned, survivin, which is one of the best known TAA, is found to be expressed at high levels by hematopoietic progenitor and stem cells in contrast to blood mononuclear cells, indicating its down-regulation during hematopoietic cell differentiation. Likewise, expression of TERT, other well known TAA, is high in embryonic stem cells and repressed in most adult somatic cells. However we detected survivin and TERT by immunofluorescence microscopy in native, unloaded DCs differentiated from Peripheral Blood Mononuclear Cells (PBMCs) on their surface. Interestingly, it was shown by other groups that survivin was expressed by immature and mature DCs differentiated *in vitro* from PBMCs (1, 2). However they never checked a surface expression of survivin or distinguished a specific immunoactive surface expression from an intracellular one. Therefore, survivin protein was simply detected by Western Blot and ELISA and survivin mRNA was detected by real time reverse transcriptase PCR (1). Similarly, TERT mRNA was detected by reverse transcriptase PCR in PBMCs from healthy donors, but at levels much lower than in case of cells from patients with cancer (4). One of the explanations for the presence of survivin and TERT in native, unloaded DCs can be that during differentiation or maturation processes growth factors induced re-expression of them in DCs. For example, it was shown that GM-CSF, which is used for differentiation of monocytes

into DCs, increased survivin expression in mature neutrophils, which normally do not express survivin.

DCs, frozen in a serum- and any plasma protein-free pharmaceutical formulation, decrease capability to phagocyte after thawing but retain the capability to migrate in response to stimuli; display high IL-12 production with or without mild IL-10 secretion; enhance T-cell cytotoxic response and induce T-mem response, since the endogenous antigen(s) expressed by DCs are complexed with MHC-I and MHC-II and do not activate the T-reg.

The dendritic cell production of the present invention may express any kind of embryonic TAA and/or a universal TAA selected from the group consisting of survivin and TERT.

According to an embodiment of the present invention the dendritic cells are of human or animal origin.

According to a preferred embodiment of the present invention the dendritic cells are of human blood, especially of peripheral or cord blood origin.

The dendritic cells of the present invention are preferably based on the expansion of autologous DCs from a human individual's peripheral blood. PBMCs are collected through leukapheresis followed by elutriation or gradient centrifugation (i.e. Ficoll gradient centrifugation), in order to increase the monocyte (Mo) fraction, which constitute the selected DC precursors. This way to obtain Mos from individuals ensures both high purity and large amounts of DCs precursors that can be therefore cultured immediately, without the need of intermediate steps, such enrichment through the use of beads, which are on the one hand, not GMP-conformed and on the other hand, may be a source of contamination. The Mos are thereafter differentiated into DCs in a GMP-conform laboratory, with a culture medium, not only GMP-, but as well serum-free and *re-vivo* conformed.

The DCs can be matured by means of adding one of the currently available standard cocktails, but preferentially by the use of Ribomunyl and INF-γ.

The mature DCs are preferably injected into the patients only if at least 20%, preferably at least 40%, more preferably at least 60% of them express the desired universal TAA(s).

Another aspect of the present invention relates to a method for manufacturing native dendritic cells according to the present invention, comprising the steps of:

• providing native dendritic cells, preferably isolated from human blood,

• formulating a pharmaceutical composition in the absence of serum and any plasma protein (f.e. albumin), and

• freezing said pharmaceutical composition.

According to one aspect of the invention the human blood is obtained from healthy subjects.

According to a preferred embodiment of the present invention the native dendritic cells source consists of their autologous precursors, i.e. PBMC-derived monocytes. The monocytes are transdifferentiated into immature DCs upon 5-6 days culture in CellGro, added up with GM-CSF and IL-4.

The dendritic cells may be alternatively directly isolated from whole blood. Protocols for direct isolation of dendritic cells and transdifferentiation of monocytes into dendritic cells are known in the art. The present protocol ensures that 40-80 % of the initial Mos become DCs, whereas current protocols guarantee about 10-20 %.

The native DCs are finally matured *in vitro* in order to increase the levels of the costimulatory molecules and to trigger a strong and durable tumor-specific T-cell cytotoxic response. The maturation-cocktail according to the present invention is completely GMP-conform and ensures a prolonged IL-12 production, with or without mild IL-10 secretion in comparison with the currently employed methods. Moreover, these native DCs are able to produce high levels of IL-12 and mild amounts of IL-10 upon stimulation with CD40L and remain viable, phenotypic, morphological and functional active upon a freezing/thawing cycle even after one year of deep-frozen preservation in a cryoprotectant, preferably in DMSO 10 % or Glycerol 10 % (-150° C) . When the method described in this patent is used to manufacture a GMP-conformed final product, its releasing is conditional to the successful pass of a strict End Product Control (EPC), designed to determine soundly the viability, the phenotypic, the morphological and the functional activity, the stability upon deep-frozen preservation and a freezing/thawing cycle, the purity and the sterility of the DCs, which must be applied to the patient. The final product is stored in a pharmaceutical formulation which is also the freezing media which preferentially contains glycerol (10 %) or DMSO (10 %). The freezing media make the product ready-to-use by preserving its quality for a long period, that are GMP-conformed and that do not trigger side effects at the used concentrations. These freezing media grant as well the long-lasting activity (IL-12 secretion, T-cells stimulation and migration) of the product. Native matured DCs decrease their capability to phagocyte but retain their capability to migrate in response to

stimuli and display high IL-12 production with or without mild IL-10 secretion; and enhance T-cell cytotoxic and T-mem response, without triggering the activation of the T-reg.

According to another preferred embodiment of the present invention the immature DCs are matured. The DCs are preferably matured by adding Ribomunyl and INF-γ.

Another aspect of the present invention relates to native DCs transdifferentiated and/or cultured and matured according to the method described in the present invention.

Yet another aspect of the present invention relates to a pharmaceutical/clinical grade preparation comprising native dendritic cells according to the present invention. The native dendritic cells of the present invention may be provided in a pharmaceutical/clinical grade preparation. Said preparation may be employed in the treatment and/or prevention of virtually any type of solid and blood cancers. The native mature DCs are preferentially inoculated weekly or fortnightly. Since this product consists of an autologous product and therefore may show many individualistic components, the route of administration may vary between weekly to monthly administration, according to the results observed in the patient during the continuous monitoring. The native mature DCs can be administered preferentially intradermally or intranodally. Alternatively they can be administered para-, perinodally, subcutaneously or intravenously, depending on the cancer treated.

The native DCs are preferably administered as close as possible to the tumor. The standard dose comprises preferably approximately 7-13*10^6 DCs, solved in a final volume of 0.5 - 1 mL of the pharmaceutical preparation.

The therapy is made up of a number of injections comprised between 2 and 20. The final product is preferentially but not exclusively stored in 10 % glycerol or 10 % DMSO thawed for about 10 min at RT and immediately administered.

According to a preferred embodiment of the present invention the administration of the product can be accompanied by the administration of immunostimulatory agents and/or adjuvants. Its usage is as well suitable in concomitance with chemotherapy as well as during the pauses between chemotherapeutic cycles but not in concomitance with immune suppressive treatments and/or in presence of severely reduced amounts of T-cells (preferential targets of the DC-driven activation of the immune system against cancer).

A further aspect of the present invention relates to the use of dendritic cells according to the present invention for the manufacture of a vaccine for treating and/or preventing cancer in an individual.

The native dendritic cells of the present invention may be used to treat an individual suffering from cancer or to prevent that an individual develops cancer.

The present invention is further illustrated by the following figures and example, however, without being restricted thereto.

Fig.1 shows the expression of survivin, HLA-DR and nuclei in monocytes and the expression of survivin and HLA-DR in native DCs.

Fig.2 shows the expression of survivin, HLA-DR and nuclei in single slices of monocytes and the expression of survivin and HLA-DR in single slices of native DCs.

Fig.3 shows the expression of TERT, HLA-ABC and nuclei in monocytes and the expression of TERT and HLA-ABC in native DCs.

Fig.4 shows the expression of TERT, HLA-ABC and nuclei in single slices of monocytes and the expression of TERT and HLA-ABC in single slice of native DCs.

Examples:

1. Native dendritic cells

1.1. Cell isolation

Leukaphereses and elutriations are performed with FENWAL and GAMBRO equipment and following the manufacture instruction.

Monocytes are isolated from patients' leukapheresate and monitored by flow cytometry for the following markers with regard to their respective isotype / negative control:

FITC	PE	ECD	PC5	PC7
CD14	CD19	CD3	CD16	CD45
CD41	GPA	n.a.	n.a.	CD45
CD45	n.a.	n.a.	7-AAD	n.a.

n.a. – not applicable

1.2. Ficoll purification

10 mL of blood/apheresate were mixed with 30 mL of PBS + 10% citrate buffer. Thereafter 30 mL of the mix are added to 20 mL of Ficoll and centrifuged 20 min at 2000 rpm at RT. Afterwards the interfaces were collected and centrifuged 10 min at 1200 rpm at RT. Finally, the pellets were resuspended in PBS, washed twice,

centrifuged 10 min at 1200 rpm at RT and resuspended in CellGro followed by a 2h adherence step.

1.3. Cell culture

DCs were cultured in plastic flasks in CellGro added up with GM-CSF (400-1000 U/mL) and IL-4 (100-400 U/mL) 5-6 days and matured upon addition of Ribomunyl (0.1-1 µg/mL) and INF-γ (200-1000 U/mL) for 24h.

Alternatively, PBMCs collected by leukapheresis were ficollized, recounted and re-monitored as described before. At this point, ficollized-cells or cells derived from elutriation were set for the adherence for 2h in CellGro. Afterwards, the non-adherent cells were discarded with the supernatant and the adherent cells are further cultured in CellGro, added up with GM-CSF (1000-2500 U/mL) and IL-4 (400-1000 U/mL), in a concentration of 1-2 Mio/mL for 5-6 days in order to transdifferentiate the monocytes into immature DCs. Alternatively for cells derived from elutrations the adherence step can be skipped. Thereafter, the culture media is changed and added up with Ribomunyl (1-100 µg/mL) and INF-γ (400-1000 U/mL) and let mature for 8-24h. The mature DCs were stored in aliquots of 7-13*10^6 DCs each in Glycerol or DMSO (aqueous solution) 10%.

1.4. Recultivation and the quality control (QC)

One aliquot of cells is thawed and analyzed directly. Second aliquot of cells is re-cultured for 2-20h as described below.

Recultivation:

Recultivation conditions providing conditions simulating physiological processes observed after application of DCs to the patient by use of specific media as well as temperature, humidity and carbon dioxide concentration:

Vials of frozen native DCs were thawed, cells were resuspened in 10 ml PBS, centrifuged at 215 g for 5 min at RT; subsequently resuspended in 1 ml of PBS. Cells were counted with Neubauer-counting chamber and seeded at a concentration of 1-2 x 10^6/well in 6-well plate wells and cultured for 2-20 h in CellGro medium supplemented with 0.25-2.5 µg CD40L/ml; 0.1 - 1000 µg Ribomunyl/ml and 200-1000 U IFBγ/ml at 37 °C. Thereafter cells were harvested, stained with indicated antibodies and analyzed via flow cytometer.

Phenotype:

FITC	PE	PC5	PC7
CD14	CD19	CD3	CD45
CD80	CD40	HLA-DR	CD45
CD83	CD86	HLA-ABC	CD45
TERT	SURVIVIN	HLA-ABC	CD45
CD45	n.a.	7-AAD	n.a.

n.a. – not applicable

Mature native DCs show down-regulation of CD14 and up-regulation of CD83 and high expression of CD40, CD80, CD86, HLA-ABC and HLA-DR and increased surface expression of at least one TAA like TERT and/or survivin after reculturing.

Cell viability is measured by specific staining with 7-AAD.

The contamination by T-cells and B-cells is monitored by detecting via flow cytometry the number of CD3 and CD19 positive cells. The contamination is always kept under 25 %.

Functionality:

The immune activity test is performed through the analysis of the production of IL-12 vs IL-10 via an ISO 9002 validated ELISA assay and induction of lymphocytes proliferation via mixed leukocyte reaction.

The phagocytosis and migration activity of the DCs is monitored by FITC-dextran uptaking and Transwell-assay, respectively.

2. Immunofluorescence and microscopy of native DCs (TERT and survivin staining)

The aim of this study is to investigate the potential presentation of TERT and survivin in the context of MHC class I (MHC I) and MHC class II (MHC II) by native DCs, by immunofluorescence microscopy.

2.1 Materials and Methods

2.1.1. General information

Native DCs prepared as described above were used. Monocytes were obtained from a healthy donor and differentiated into dendritic cells. After maturation the DCs were harvested and frozen.

2.1.2. Materials

2.2.1. Antibodies

Primary antibodies, working dilution:

- TERT: rabbit monoclonal anti-TERT peptide antibody (Abcam, ab32020); 1:10
- HLA-ABC: rat monoclonal anti-human HLA Class I antibody (Acris, SM2012P), 1:100
- survivin: mouse monoclonal anti-human survivin antibody (R&D Systems, MAB886); 1:10
- HLA-DR: rat monoclonal anti-HLA-DR antibody (Santa Cruz; sc-59257); 1:100

Secondary antibodies, working dilution:

- Cy5-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, 711-175-152); 1:500
- Texas Red-conjugated goat anti-rat IgG (Santa Cruz; sc-2782); 1:200
- Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, A-11029); 1:1000

Nuclei staining:

Hoechst dye; 1:3000

2.1.3. Methods

Each combination of staining is performed for native DCs and monocytes. Staining is performed on two different days.

1.) Control for staining, native DCs, methanol fixation:

2nd antibodies (anti-mouse Alexa fluor 488 and anti-rat Texas Red)

2.) Control for staining, native DCs, methanol fixation:

2nd antibodies (anti-rabbit, Cy5 and anti-rat Texas Red)

3.) Double staining, native cells, methanol fixation:

1st antibodies: anti-HLA-ABC (rat) and anti-TERT peptide (rabbit)

2nd antibodies: anti-rat Texas Red and anti-rabbit Cy5

4.) Double staining, native cells, methanol fixation:

1st antibodies: anti-HLA-ABC (rat) and anti-survivin (mouse)

2nd antibodies: anti-rat Texas Red and anti-mouse Alexa fluor 488

5.) Double staining, native cells, methanol fixation:

1st antibodies: anti-HLA-DR (rat) and anti-TERT peptide (rabbit)

2nd antibodies: anti-rat Texas Red and anti-rabbit Cy5

6.) Double staining, native cells, methanol fixation:

1st antibodies: anti-HLA-DR (rat) and anti-survivin (mouse)

2nd antibodies: anti-rat Texas Red, anti-mouse Alexa fluor 488

7.) Control for staining, native DCs, PFA fixation:

2nd antibodies (anti-mouse Alexa fluor 488 and anti-rat Texas Red)

8.) Control for staining, native DCs, PFA fixation:

2nd antibodies (anti-rabbit, Cy5 and anti-rat Texas Red)

9.) Double staining, native cells, PFA fixation:

1st antibodies: anti-HLA-ABC (rat) and anti-TERT peptide (rabbit)

2nd antibodies: anti-rat Texas Red and anti-rabbit Cy5

10.) Double staining, native cells, PFA fixation:

1st antibodies: anti-HLA-ABC (rat) and anti-survivin (mouse)

2nd antibodies: anti-rat Texas Red and anti-mouse Alexa fluor 488

11.) Double staining, native cells, PFA fixation:

1st antibodies: anti-HLA-DR (rat) and anti-TERT peptide (rabbit)

2nd antibodies: anti-rat Texas Red and anti-rabbit Cy5

12.) Double staining, native cells, PFA fixation:

1st antibodies: anti-HLA-DR (rat) and anti-survivin (mouse)

2nd antibodies: anti-rat Texas Red, anti-mouse Alexa fluor 488

If indicated additional staining of nuclei was performed.

Staining of monocytes was performed on the first day of the production.

Vials of native DCs were thawed, cells resuspended in 10 mL PBS, centrifuged at 215 g for 5 min at RT; subsequently resuspended in 1 mL PBS. Cells were counted with Neubauer-counting chamber. Cells were seeded at a concentration of $0.5x10^6$/well on glass coverslips put in 24-well plate wells and cultured for at least 3.5 h (maximum 6 h) in stimulating medium (with 2.5 µg CD40L/mL; 100 µg Ribomunyl/mL, 1000U Imukin/mL).

Fixation:

a) Samples with methanol (-20ºC)

The medium was removed from the wells and the cells washed 1x with PBS. Afterwards PBS was removed from the wells and cold methanol was applied (-20ºC; approximately 500 µL per well). Then the cells were incubated with methanol for 90 sec at -20ºC. The fixative was discarded immediately and the cells were washed 3x with PBS.

b) Samples with 4 % PFA

4 % PFA was applied with 22 % sucrose to the cells in a 1+1 dilution with the medium (final concentration of PFA 2 %) and the cells were incubated for 30 min at RT. The fixative was discarded immediately and the cells were washed 3x with PBS.

Blocking:

500 µL of blocking solution were apply (2 % BSA; 0.02 % NaN_3 in PBS) to each well and the cells were incubated in the blocking solution for 1 h at RT. Afterwards blocking solution was removed and cells were washed 3x with PBS.

Primary Immunoreaction:

Dilutions of the primary antibodies were prepared in blocking solution. A wet chamber was prepared as follows: a square filterpaper was cut, put in a plastic dish, soaked in PBS and covered with parafilm. Then 50 µL of the primary antibodies solution was applied to the parafilm. The coverslips from the wells were taken and PBS was let to run off the coverslips on a paper towel. Coverslips were placed on the drop of the primary antibodies solution on the parafilm and the cells were incubated with the primary antibodies in the closed chamber for 1 h at RT. Then the antibody solution was removed from the wells and PBS was put into the wells. The coverslips were placed into the wells and the cells washed with PBS 1x10 min and 2x5 min.

Secondary Immunoreaction:

Dilutions of the secondary antibodies were prepared in PBS. A wet chamber was prepared analogicous to the primary antibodies step. 50 µL of the secondary antibodies solution were applied to the parafilm. The coverslips were taken from the wells and PBS was let to run off the coverslips on a paper towel. Coverslips were placed on the drop of the secondary antibodies solution on the parafilm and the cells were incubated with the secondary antibodies in the closed chamber for 1h at RT. The coverslips were placed in the wells and washed with PBS 3x5min.

Staining of nuclei:

Hoechst dye was applied in a 1:3000 dilution to the coverslips in the 24-well plate and incubated for 5 min at RT. The staining solution was removed from the wells and the coverslips washed with distilled H_2O 1x5min.

Mounting of the coverslips on the slides:

Mowiol was thawed and a drop of 10 µL was applied to each coverslip on the slide. The coverslips were taken from the wells and H_2O was let to run off the coverslips on a paper towel. Coverslips were placed on mowiol with cells directed to

the slide. The slides were dried at RT over night in the dark and stored at 4°C in the dark until microscopy was performed.

2.2. Results:

Different methods of cells fixation (cold methanol or 2 % paraformaldehyd (PFA)) were used to analyze the presence of TERT and survivin on the cell surface. Methanol fixes and permealbilizes cells, thus both extra- and intracellular epitops can be stained, while PFA fixes cells and crosslinks proteins, but does not permeabilize the surface, therefore is more suitable for visualization of cell surface associated proteins (complexes). As anti-survivin antibody worked with both fixation methods and the potential complexes survivin/HLA are expected to be presented on the cell surface, further staining for survivin was performed for cells fixed with PFA. Anti-TERT antibody worked only for cells fixed with methanol, resulting in staining of both cytoplasmic and cell surface bound TERT. TERT expressed on the cell surface showed characteristic ring-shape pattern.

Monocytes were used as a negative control for survivin and TERT expression in the context of MHC. These cells were positive for HLA-DR, HLA-ABC, TERT and survivin, but TERT and survivin were expressed only within the cell, while both types of HLA were expressed within the cell and on the cell surface. This conclusion is drawn from the fact that in case of cells fixed with PFA no signal of survivin and TERT was observed, while HLA-ABC and HLA-DR showed a ring-like shape pattern. Moreover, in case of monocytes fixed with methanol survivin and TERT seemed to be expressed only within cytoplasm (no ring-shape pattern).

Immunofluorescence analysis of 3D projection of monocytes and native, unloaded DCs. Monocytes (a) and native, unloaded DCs (b) were grown on glass-coverslips, fixed with 2 % PFA and stained for survivin, HLA-DR and nuclei, as indicated. All immunofluorescence microscopy analysis was carried on a confocal Zeiss Axiovert microscope. All pictures present 3D projection of cells. White arrows point ring-like shape formed by HLA-DR and survivin indicating their cell surface expression (see Fig. 1).

Immunofluorescence analysis of single slices of monocytes and native, unloaded DCs. Monocytes (a) and native, unloaded DCs (b) were grown on glass-coverslips, fixed with 2 % PFA and stained for survivin, HLA-DR and nuclei, as indicated. All immunofluorescence microscopy analysis was carried on a confocal

Zeiss Axiovert microscope. All pictures present single slice from the whole cells. White arrows point potential colocalization areas between HLA-DR and survivin (see Fig. 2).

Immunofluorescence analysis of 3D projections of monocytes and native, unloaded DCs. Monocytes (a) and native, unloaded DCs (b) were grown on glass-coverslips, fixed with methanol and stained for TERT, HLA-ABC and nuclei, as indicated. All immunofluorescence microscopy analysis was carried on a confocal Zeiss Axiovert microscope. All pictures present 3D projection of cells. White arrows point ring-like shape formed by HLA-ABC and TERT indicating their cell surface expression (see Fig.3).

Immunofluorescence analysis of single slices of monocytes and native, unloaded DCs. Monocytes (a) and native, unloaded DCs (b) were grown on glass-coverslips, fixed with methanol and stained for TERT, HLA-ABC and nuclei, as indicated. All immunofluorescence microscopy analysis was carried on a confocal Zeiss Axiovert microscope. All pictures present single slice from whole cells. White arrows point potential colocalization areas between HLA-ABC and TERT (see Fig. 4).

Figures 1-4 contain typical staining profiles of 3D projections and single slices for both for TERT and survivin.

Differences in pattern and intensity of survivin signal were found between native, unloaded DCs and monocytes. An interesting observation is that native DCs show a homogenous distributed survivin signal. In respect to cellular localization, it appears that survivin was presented on the cell surface, since cells showed a staining for survivin after 2 % PFA fixation without permeabilization. Moreover, looking at each slice taken from whole cell, one can observe that survivin is on the whole cell surface at the top of the cell and forms a ring-shape pattern with no signal within the ring when looking at slice from the deeper layers of cell, similarly to MHC. In native, unloaded DCs survivin colocalized with both MHC I and MHC II. In case of monocytes survivin not detected in case of PFA fixation, indicating lack of its presentation by monocytes.

Table 1: Summary of results for survivin (PFA fixation)

	Monocytes	Native DCs
Direction of the loading of survivin	no surface staining	merge with both MHCs
Cellular localization	cytoplasm (detected only after methanol fixation;	cell surface in addition to cytoplasm

	data not shown)	
Intensity of survivin surface signal	no signal	detectable signal, similar intensity to MHCs

Although the TERT signal is weak in comparison to the MHCs, it is localized on the cell surface, in addition to cytoplasm, and appears to be presented in the context with both MHC I and MHC II.

Table 2: Summary of results for TERT (methanol fixation)

	Monocytes	Native DCs
Direction of the loading of TERT	no surface staining	merge with both MHCs
Cellular localization	cytoplasm	cell surface in addition to cytoplasm
Intensity of TERT surface signal	no signal	detectable signal, lower intensity to MHCs

Finally, in case of monocytes and native DCs TERT, survivin, HLA-ABC and HLA-DR do not colocalize with nuclei.

3. Prove of the unique influence of the inventive protocol.

In addition, in order to prove unique influence of the methods of cryopreservation (method of freezing and storing a composition) and recultivation on abilities of native DCs to express/present universal TAAs, following experiments are conducted:

3.1. Analysis of universal TAAs expression by native DCs (survivin, TERT):

- Mature unloaded DCs harvested and not recultivated
- Mature unloaded DCs harvested and re-cultivated for 6 h (CellGro DC media supplemented with Ribomunyl, Imukin and CD40 Ligand)
- Mature unloaded DCs frozen in the abence of serum and any of plasma protein, thawed and re-cultivated for 6 h (CellGro DC media supplemented with Ribomunyl, Imukin and CD40 Ligand)
- Mature unloaded DCs frozen in the abence of serum and not re-cultivated
- Mature unloaded DCs frozen in the presence of serum or any of plasma protein, thawed and re-cultivated for 6 h (CellGro DC media supplemented with Ribomunyl, Imukin and CD40 Ligand)

- Mature unloaded DCs frozen in the presence of serum or any of plasma protein, thawed and not recultivated

3.2. Methods:

a) Production of mature unloaded DCs from healthy donors and freezing as indicated.

b) Flow cytometry analysis of cells for expression of survivin/TERT (optionally other anti-apoptotic proteins, such as XIAP, or heat shock proteins, such as Hsp27) Immunofluorescence Microscopy analysis of potential surface presentation of survivin/TERT at indicated steps.

c) Flow cytometry analysis of maturation markers (CD83, CD80, CD40) at indicated steps to prove functionality/maturity of cells.

d) Optionally ELISA analysis of IL-12 and IL-10 production by DCs at indicated steps to prove functionality of cells.

Additionally, different recultivation conditions are examined in order to analyze and prove an influence of re-cultivation procedure on expression/presentation of universal TAAs (survivin/TERT) by native DCs:

- No re-cultivation (directly after thawing)
- Re-cultivation for 2-20 h in CellGro DC media only
- Re-cultivation for 2-20 h in CellGro DC media supplemented with Ribomunyl, Imukin and CD40 Ligand.

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Claims:

1. A cryoprocessed pharmaceutical composition in the frozen form comprising native dendritic cells in a pharmaceutical formulation which is free of serum.

2. The cryoprocessed pharmaceutical composition according to claim 1, which is thawed and optionally recultivated under conditions simulating physiological processes observed after application of DCs to the patient by use of specific media as well as temperature, humidity and carbon dioxide concentration.

3. The pharmaceutical composition according to claim 1 or 2, wherein the dendritic cells originate from blood, preferably from human peripheral or cord blood.

4. The pharmaceutical composition according to any one of claims 1 - 3, wherein said dendritic cells are mature, interleukin 12 producing dendritic cells.

5. The pharmaceutical composition according to any one of claims 1 - 4, wherein said dendritic cells express at least one universal TAA.

6. The pharmaceutical composition according to claim 5, wherein said universal TAA is selected from TERT, survivin, CEA, CYP1B, MUC16, MDM2, cyclin (D1);

7. The pharmaceutical composition according to any one of claims 1 – 6, wherein at least 20 % of the dendritic cells present a universal TAA in a reculturing test.

8. The pharmaceutical composition according to any one of claims 1 – 7, further comprising pharmaceutically acceptable additives selected from DMSO, glucose, glycerin, salts, as well as adjuvants or stimuli like interferon or Ribomunyl.

9. A method of manufacturing a pharmaceutical composition according to any one of claims 1 – 8, including the steps of:
a) providing native dendritic cells, optionally isolated from human blood,
b) formulating a pharmaceutical composition in the absence of serum, and
c) freezing the composition.

10. The method according to claim 9, wherein the peripheral blood is obtained from healthy subjects or tumor patients.

11. The method according to claim 9 or 10, which further comprises thawing the composition ready to use for treating a patient.

12. The method according to any one of claims 9 - 11, which further comprises reculturing the thawed composition under conditions simulating physiological processes observed after application of DCs to the patient by use of specific media as well as temperature, humidity and carbon dioxide concentration.

13. The pharmaceutical composition according to any one of claims 1 – 8 as medicament.

14. The pharmaceutical composition according to any one of claims 1 - 8 for use in the treatment and/or prevention of cancer.

15. A method of determining universal TAA in a pharmaceutical preparation according to any one of claims 1 – 8, comprising the steps of

a) providing native dendritic cells;

b) recultivating the dendritic cells under conditions simulating physiological processes observed after application of DCs to the patient by use of specific media as well as temperature, humidity and carbon dioxide concentration; and

c) determining the distribution of universal, tumor associated antigen positive cells.

Figure 1:

a) Monocytes b) Native DCs

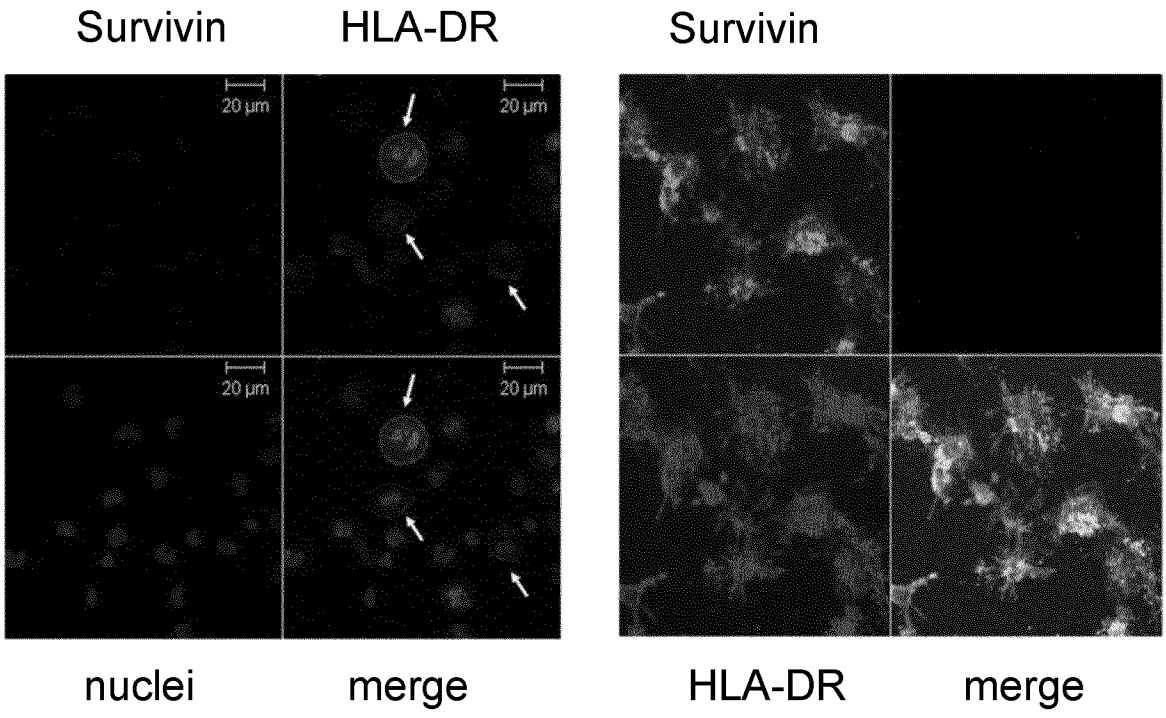


Figure 2

a) Monocytes b) Native DCs

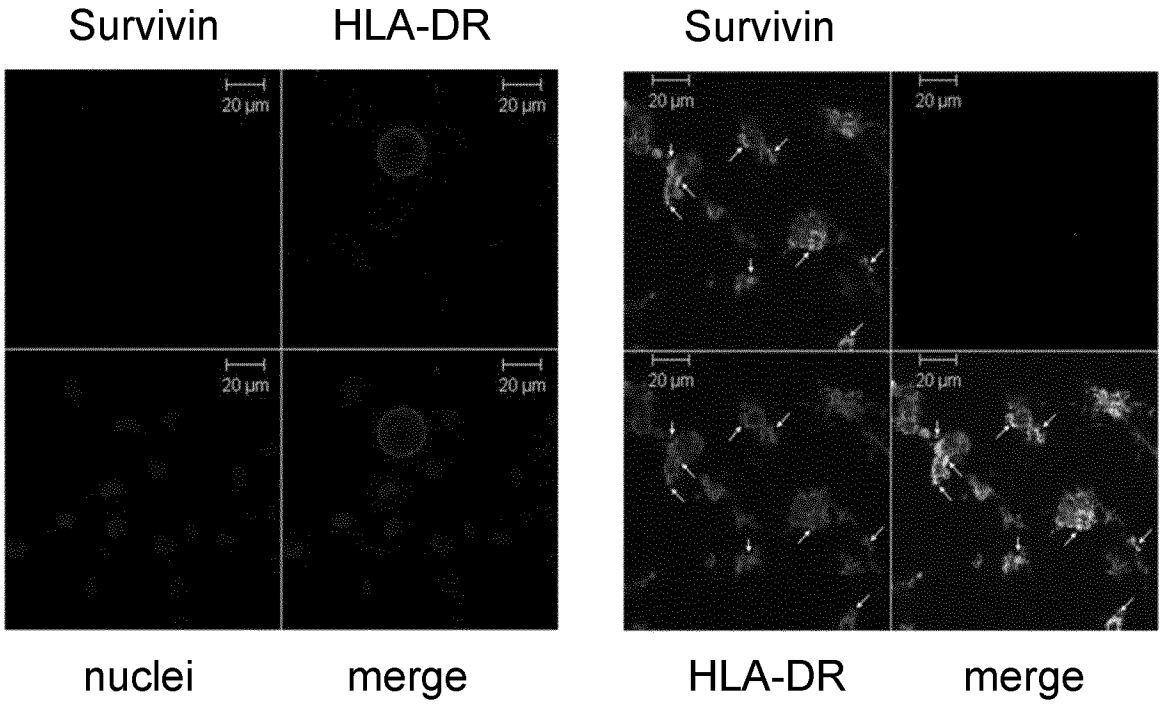


Figure 3:

a) Monocytes b) Native DCs

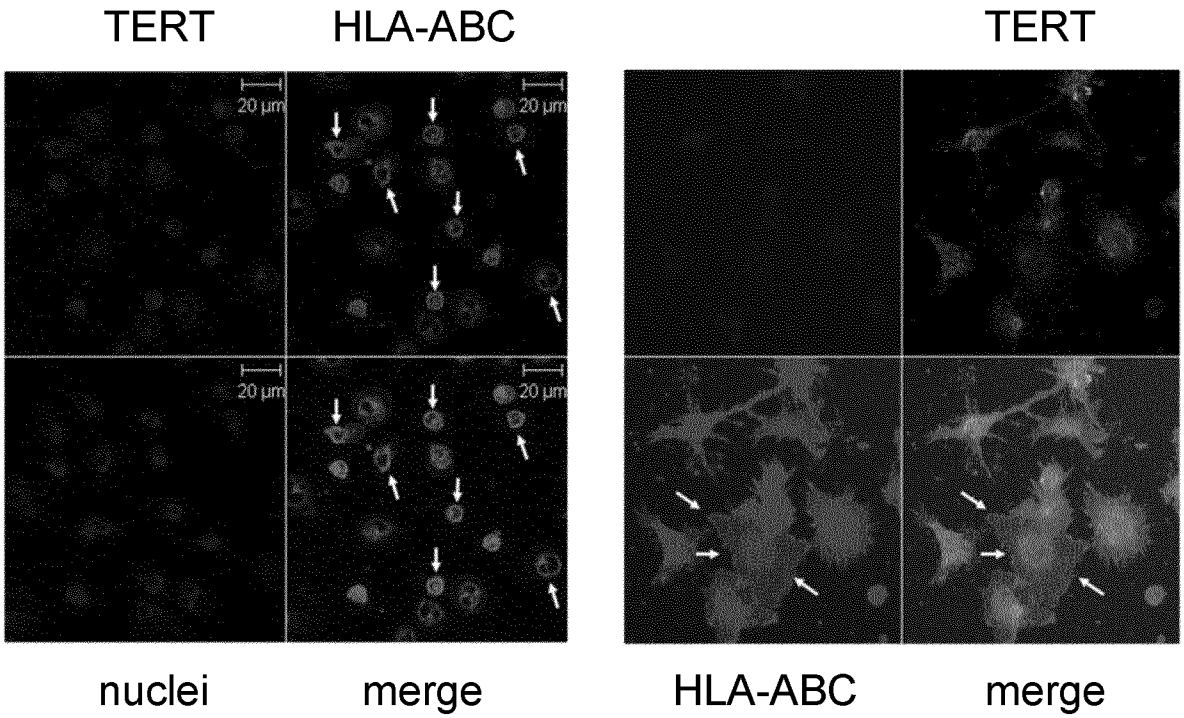
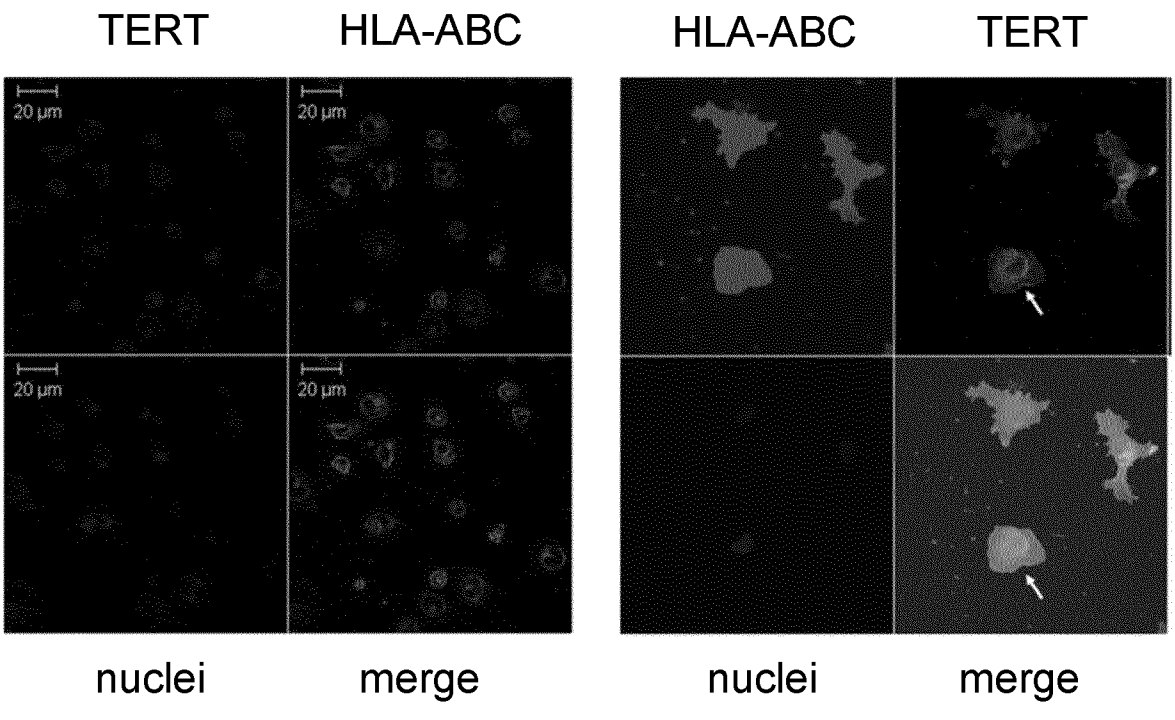


Figure 4:

a) Monocytes b) Native DCs



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/051023

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K35/14 A61K35/16 A61P35/00
ADD.

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

B. FIELDS SEARCHED

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

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCHMITZ MARC ET AL: "Tumoricidal potential of native blood dendritic cells: Direct tumor cell killing and activation of NK cell-mediated cytotoxicity", JOURNAL OF IMMUNOLOGY, vol. 174, no. 7, April 2005 (2005-04), pages 4127-4134, XP002629625, ISSN: 0022-1767 abstract, page 4128, right column, page 4129, left column, paragraph 2 and last para, page 4133, right column. para 2	1-15

X	WO 2004/050909 A2 (DARTMOUTH COLLEGE [US]; IDM IMMUNO DESIGNED MOLECULES [FR]; ABASTADO J) 17 June 2004 (2004-06-17)	1-4,8,9, 12-14
Y	page 54, last para - page 55, para 1, page 52, last para	1-15

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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Date of the actual completion of the international search	Date of mailing of the international search report
10 May 2012	18/05/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Ludwig, Gerald

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	OSADA T ET AL: "Dendritic cell-based immunotherapy", INTERNATIONAL REVIEWS OF IMMUNOLOGY, HARWOOD ACADEMIC PUBLISHERS, LONDON, GB, vol. 25, no. 5-6, 1 December 2006 (2006-12-01), pages 377-413, XP009092541, ISSN: 0883-0185, DOI: DOI:10.1080/08830180600992456 page 382, para 2 - page 383, para 1, abstract	1-15
A	US 2005/003533 A1 (KALINSKI PAWEL [US]) 6 January 2005 (2005-01-06) para 19, lines 1-5; paras 53-54, 57, 59	1-15
A	ONAITIS MARK ET AL: "Dendritic cell gene therapy", SURGICAL ONCOLOGY CLINICS OF NORTH AMERICA, SAUNDERS, PHILADELPHIA, US, vol. 11, no. 3, 1 July 2002 (2002-07-01), pages 645-660, XP009092540, ISSN: 1055-3207, DOI: DOI:10.1016/S1055-3207(02)00027-3 page 651, para 2 - page 652, para 2, page 656, para 2, lines 1-6	1-15
A	CIESIELSKI MICHAEL J ET AL: "Antitumor effects of a xenogeneic survivin bone marrow derived dendritic cell vaccine against murine GL261 gliomas", CANCER IMMUNOLOGY AND IMMUNOTHERAPY, SPRINGER-VERLAG, BERLIN, DE, vol. 55, no. 12, 1 December 2006 (2006-12-01), pages 1491-1503, XP002459738, ISSN: 0340-7004, DOI: DOI:10.1007/S00262-006-0138-6 abstract	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2012/051023

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004050909 A2	17-06-2004	AU 2003288212 A1	23-06-2004
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		EP 1570265 A2	07-09-2005
		WO 2004050909 A2	17-06-2004
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		US 2012009681 A1	12-01-2012