

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
6 May 2010 (06.05.2010)

(10) International Publication Number
WO 2010/049152 A1

PCT

(51) International Patent Classification:
C07D 513/12 (2006.01) *C12N 5/0784* (2010.01)
A61K 31/54 (2006.01)

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(21) International Application Number:
PCT/EP2009/007746

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(22) International Filing Date:
29 October 2009 (29.10.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/109,381 29 October 2008 (29.10.2008) US

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(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))

(54) Title: NOVEL COMPOSITION FOR THE PREPARATION OF MATURE DENDRITIC CELLS

(57) Abstract: The present invention relates to a method for *in vitro* maturation of at least one immature dendritic cell, comprising stimulating said immature dendritic cell with TNF α , IL-1 β , IFN γ , a TLR7/8 agonist and prostaglandin E2 (PGE2), wherein said TLR7/8 agonist is a thiazoloquinolone derivative type immune response modifying compound. The invention further relates to a mature dendritic cell or a population of mature dendritic cells, obtainable by the method of the invention, as well as to a pharmaceutical composition comprising said mature dendritic cell or cells. Moreover, one aspect of the invention is the use of a TLR7/8 agonist for the preparation of at least one mature dendritic cell, wherein the TLR7/8 agonist is a thiazoloquinolone derivative type immune response modifying compound according to the invention, in particular wherein said TLR7/8 agonist is used in combination with TNF α , IL-1 β , IFN γ , prostaglandin E2 (PGE2), and, optionally, with a TLR3 agonist, preferably poly (I:C). A further aspect of the invention is a composition comprising TNF α , IL-1 β , IFN γ , a TLR7/8 agonist as defined in the present invention, prostaglandin E2 (PGE2), and optionally a TLR3 agonist, preferably poly (I:C), in particular wherein said TLR7/8 agonist is 2-propylthiazolo [4,5-c]quinolin-4-amine (CL075).



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Novel Composition for the Preparation of Mature Dendritic Cells

- 5 The invention relates to novel compositions for the preparation of mature dendritic cells as well as to methods for *in vitro* maturation of immature dendritic cells and to therapeutic uses of the dendritic cells obtainable by the method of the invention.

Background of the Invention

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Dendritic cells (DCs) have a high potential as adjuvants in the induction of tumor-specific killer and helper cells in the patient (Schuler et al., 2003, Review; Banchereau et al., 2005, Review). For this purpose, mature dendritic cells which have been matured *in vitro* from immature dendritic cells are loaded with tumor-specific antigens and reinjected into the
15 body, preferably next to or in the lymph nodes. Within lymph nodes dendritic cells interact with naive T cells resulting in active signal transduction during the so called immunological synapse and subsequent proliferation of effector T cells, which, in turn, mediate anti-tumor responses like cytotoxicity (cytotoxic T lymphocytes = CTLs), activation of macrophages and delayed type hypersensitivity reactions. DCs regulate CD4
20 positive T helper (h) cell polarizations. Th1 cells, for example, support CTLs by secretion of certain cytokine patterns [e.g. Interferon gamma (IFN- γ) and IL-2, TNF-beta (TNFSF1)]. On the other hand, Th2 cells induce antibodies as well as eosinophiles and degranulation of mast cells by IL-4, IL-5, IL-10 and IL-13 (Langenkamp et al., 2000; O’Gara A., 1998).

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For the therapy with dendritic cells, it is essential that a sufficient number of mature DCs are available. Since, in the individual, only 0.2 % of the white blood cells are dendritic cells, it is necessary to have an efficient method for the *in vitro* production of mature dendritic cells.

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In the art, various methods have been proposed for the preparation of mature dendritic cells starting from peripheral blood mononuclear cells, monocytes, or other myeloid progenitor

cells (Jonuleit et al., 1997; Mailliard et al., 2004; Napolitani et al., 2005). The most common protocol for maturation of dendritic cells (DC) from monocytes present among peripheral blood mononuclear cells (PBMC) is made over a period of seven days (7-day DC) using the cocktail as described by Jonuleit et al. (1997). An alternative protocol has been developed that allows dendritic cells to be produced in fewer days (3-day DC), these are also referred to as fast DC (Dauer et al., 2003). The monocytes can be isolated either from PBMC through a plastic adherence step, by isolation of CD14⁺ cells with monoclonal antibodies, or through leukapheresis and elutriation, as performed on day 0 (see patent application WO 07/110240 and Zobywalski et al., 2007). The monocytes are cultured in DC medium, which can be of various sources, the sources of which are known to a person skilled in the art, and can include various sources of serum or plasma (e.g. autologous human sources, pools prepared from human donors, or fetal calf serum). Alternatively, serum-free medium of various types that contain substitutes for serum can be used.

It is accepted that the cultivation of peripheral blood mononuclear cells, monocytes or other myeloid progenitor cells with GM-CSF and either IL-4 or IL-13 results in the production of immature dendritic cells *in vitro* (Ahn and Agrawal, 2005). However, to date, there is no generally accepted method available for the maturation of the immature dendritic cells. Jonuleit et al. (1997) describe such a maturation process using a composition comprising TNF- α , IL-1 β , IL-6 and prostaglandin E2 (PGE2), namely the so-called "Jonuleit cocktail". Dendritic cells produced by incubation of immature dendritic cells with this composition show the surface markers for mature dendritic cells and can be well harvested. However, these cells fail to produce biological active IL-12(p70), which is the most important factor for the induction of Th1 cells in the lymph nodes.

Mailliard et al. describe a composition comprising TNF- α , IL-1 β , interferon- α , interferon- γ and poly (I:C) (polyinosinic:polycytidylic acid, Mailliard et al., 2004). In contrast to the above mentioned Jonuleit cocktail, incubation of immature dendritic cells with this so-called "Kalinski cocktail" results in mature dendritic cells (as demonstrated by the respective surface markers), which produce IL-12(p70). However, these cells are very adherent to the bottom of the culture flasks and are, therefore, nearly impossible to harvest (Zobywalski et al., 2007). It is, therefore, very difficult, if not impossible, to obtain sufficient mature dendritic cells for the vaccination therapy with this method.

WO 00/47719 describes the compound resiquimod (R848) which is proposed for the preparation of mature dendritic cells. In the experiments described therein, immature dendritic cells are stimulated with R848 only. However, R848 as a single maturation substance is not sufficient to provide a maturation process resulting in appropriate dendritic cells suitable for clinical purposes. All experiments have been carried out with FCS (fetal calf serum) and are, therefore, not applicable under GMP (good manufacturing process) conditions because fetal calf serum-free conditions are crucial for a GMP process.

Peng et al. discloses the generation and maturation of dendritic cells for clinical application under serum-free conditions using various Toll-like receptor (TLR) ligands (Peng et al., 2005).

The mammalian Toll-like receptors (TLRs) belong to an evolutionarily ancient recognition and signalling system which was initially discovered in the fruitfly *Drosophila melanogaster*, and which plays an important role in innate immunity in most, if not all, multicellular organisms. TLRs recognize elements of most pathogenic microorganisms and microbes, and thus play a key role in the response to infections. Some mammalian TLRs act as cell-surface receptors by directly interacting with molecules on the pathogen surface, whereas others are located in the membranes of endosomes and function intracellularly by recognizing microbial components, such as DNA, that are accessible only after the microbe has been broken down. In humans, there are at least 10 functional *TLR* genes. Toll-like receptors are expressed by dendritic cells and macrophages. Monocyte-derived dendritic cells express all known TLRs except for TLR9. TLR9, however, is expressed by plasmacytoid dendritic cells along with TLR1 and TLR7. Immature dendritic cells, originating from bone marrow progenitors, can be converted into mature dendritic cells by TLR signalling and signals received from chemokines. TLR signalling results in a significant alteration of the chemokine receptor profile expressed by dendritic cells, involving induction of the chemokine receptor CCR7, which in turn facilitates their entry into peripheral lymphoid tissues.

Several Toll-like receptors (TLR) ligands have been described in the art to trigger immunostimulatory activity. Natural agonists for TLR2, TLR3, TLR4, TLR5, TLR2/6, and TLR9 include peptidylglycan, dsRNA [poly (I:C)], LPS, flagelin, macrophage-activating lipopeptide 2, and bacterial DNA-containing CpG motifs, respectively. Natural agonists

for TLR7 and TLR8 include guanosine- and uridine-rich ssRNA. Small molecular mass (< 400 Da) synthetic imidazoquinoline-like molecules including e.g. imiquimod (R837), resiquimod (R848), S-27609, CL097, CL075, adenine derivatives such as CL087, or guanosin analogues such as loxoribone function as synthetic ligands in activating NF- κ B and preferentially triggering the production of various cytokine and chemokines such as TNF- α , INF- γ , IL-6, IL-10, IL-12, and MIP-1 α (Gorden et al., 2005; Philbin and Levy, 2007).

WO 07/110240 discloses a method for *in vitro* maturation of immature dendritic cells in which said dendritic cells are stimulated with a cytokine cocktail comprising TNF- α , IL-1 β , IFN γ , a TLR7/8 agonist and prostaglandin E2 (PGE2).

Dendritic cells can take up, process, and present a wide variety of pathogens and antigens and are considered to be the most important activators of naive T cells during T cell mediated immunity.

There is always a need for improved methods for the preparation of mature dendritic cells out of immature dendritic cells.

Summary of the Invention

The present invention is based on the finding that a TLR7/8 agonist, which is a thiazoloquinolone derivative type immune response modifying compound, is especially suitable for promoting the *in vitro* maturation of dendritic cells. Especially, as demonstrated by the Example, the mature dendritic cells obtained by using said compound surprisingly (i) secrete significantly higher amounts of IL-12(p70), (ii) express significantly increased amounts of CCR7 chemokine receptors on their surfaces which increases their migratory activity, and (iii) have an improved capacity to retain viable CD8⁺ T cells, and thus to activate antigen-specific T cells as compared to the dendritic cells prepared by the methods known in the art. Accordingly, the present invention enables the production of optimized and superior mature dendritic cells as an important tool for clinical purposes for the reasons as follows: IL-12(p70) is the most important factor for the

induction of Th1 cells in the lymph nodes, and a strong expression and secretion of IL-12(p70) over, e.g., IL-10 allows for a polarization of T cell responses in favour of Th1 cells, since IL-12(p70) induces CD4 T cell differentiation into Th1-like cells. Moreover, expression of the chemokine receptor CCR7 directs their migration to lymph nodes where they encounter lymphocytes for activation, and augments the expression of co-stimulatory molecules, e.g. B7, and MHC molecules, since activated dendritic cells are rendered sensitive to chemokines like CCL19 and CCL21 by the expression of CCR7. In addition, the functional capacity of mature dendritic cell populations to activate antigen-specific T cells is a crucial aspect of efficient T cell mediated immunity.

The present invention thus allows for obtaining mature dendritic cells with improved and optimized immunostimulatory activity, as demonstrated in the Examples.

In a first aspect, the present invention provides a method for *in vitro* maturation of at least one immature dendritic cell, comprising stimulating said immature dendritic cell with TNF- α (also known as TNFSF2), IL-1 β , IFN γ , a TLR7/8 agonist and prostaglandin E2 (PGE2), wherein said TLR7/8 agonist is a thiazoloquinolone derivative type immune response modifying compound.

The invention further relates to a mature dendritic cell or population of mature dendritic cells, obtainable by the method of the invention.

In a further aspect, the present invention also relates to a pharmaceutical composition comprising the mature dendritic cell or the mature dendritic cells according to the present invention.

Furthermore, the invention also relates to the mature dendritic cell or to the population of mature dendritic cells of the invention for use in a method of treating a disease selected from the group consisting of tumorigenic diseases and infectious diseases (e.g. provoked by viruses, bacteria, intracellular bacteria or fungi). Additionally, the present invention relates to a method for treating a patient with a tumorigenic disease or an infectious disease, wherein an effective amount of the mature dendritic cell of the invention is administered to said patient.

In another aspect, the invention relates to the use of a TLR7/8 agonist for the preparation of at least one mature dendritic cell, wherein the TLR7/8 agonist is a thiazoloquinolone derivative type immune response modifying compound according to the present invention.

- 5 Another aspect of the invention relates to a composition comprising TNF- α , IL-1 β , IFN γ , a TLR7/8 agonist, prostaglandin E2 (PGE2) and, optionally, a TLR3 agonist, wherein said TLR7/8 agonist is a thiazoloquinolone derivative type immune response modifying compound according to the present invention.

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Detailed Description

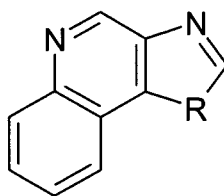
In a first aspect, the present invention provides a method for *in vitro* maturation of at least one immature dendritic cell, comprising stimulating said immature dendritic cell with
15 TNF- α (also known as TNFSF2), IL-1 β , IFN γ , a TLR7/8 agonist and prostaglandin E2 (PGE2), wherein said TLR7/8 agonist is a thiazoloquinolone derivative type immune response modifying compound.

The term “agonist” as used herein generally refers to any kind of substance that interacts
20 and/or binds to a specific receptor, resulting in the activation of said receptor and in subsequent triggering of an intracellular response. This response includes, but is not limited to, activation of gene expression. The activation of gene expression might be mediated by receptor-mediated signalling cascades, and can be measured by means of standard procedures including, but not limited to, mRNA and/or protein expression
25 profiling. These methods are well known to a person skilled in the art and described, e.g., by Gordon et al. (2005). An agonist might be a natural or synthetic ligand and mimic the action of an endogenous ligand that binds to the same receptor.

In the context of the present invention, the term “TLR7/8 agonist” more specifically refers
30 to an activator of Toll-like receptor 7 (TLR7) and/or Toll-like receptor 8 (TLR8). Activation of said TLRs might result in downstream activation of NK- κ B and other transcription factors, and in transcriptional activation of numerous genes, including cytokines, chemokines, and co-stimulatory markers. TLR signalling might result in a significant alteration in the chemokine receptors expressed by dendritic cells which directs

their migration to and facilitates their entry into lymphoid tissues. Many different signalling proteins can be induced by TLR activation, including, but not limited to, various MAP kinases and PI 3-kinase. In addition, TLR activation might result in the upregulation of expression of MHC class I and MHC class II molecules which enables dendritic cells to stably present peptides and/or antigens already taken up. Moreover, TLR activation in the context of the present invention might induce the expression of high levels of co-stimulatory molecules including, but not limited to, B7 which, in turn, provide co-stimulatory signals for T cell activation by interacting with receptors on the surface of naïve T cells. Methods for analysing transcriptional gene activation by TLR agonists include all kinds of standard procedures including, but not limited to, quantitative RT-PCR methods and Western Blots, and are described, e.g., by Gordon et al. (2005).

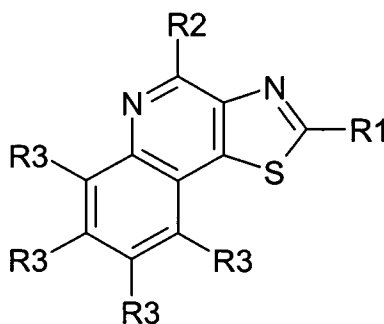
In the context of the present invention, a thiazoloquinolone derivative is a compound of the general formula (I) or a pharmaceutically acceptable salt thereof



(I)

wherein the residue R is S or S = O, preferably S. A thiazoloquinolone derivative in the context of the present invention can be substituted or unsubstituted in any position of the ring structure.

Preferably, the thiazoloquinolone derivative of the present invention is a compound of general formula (II) or a pharmaceutically acceptable salt thereof



(II)

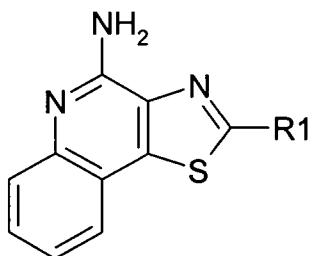
wherein

R1 represents a substituted or unsubstituted alkyl or alkoxy residue, preferably an unsubstituted alkyl or alkoxy residue or an alkyl or alkoxy residue substituted with
5 halogen, preferably fluorine, more preferably a C1 to C12 alkyl or alkoxy residue, in particular a C2 to C6 alkyl or alkoxy residue, for example an ethyl, propyl, butyl residue;

R2 represents NH₂, NHR₄, N(R₄)₂, SH or OH, wherein R₄ is a C1 to C6 alkyl group, preferably methyl or ethyl, preferably R2 represents NH₂;

each R3 independently represents hydrogen or halogen, preferably hydrogen or fluorine, more preferably hydrogen.

More preferably, the thiazoloquinolone derivative according to the present invention is a
15 compound of general formula (III) or a pharmaceutically acceptable salt thereof

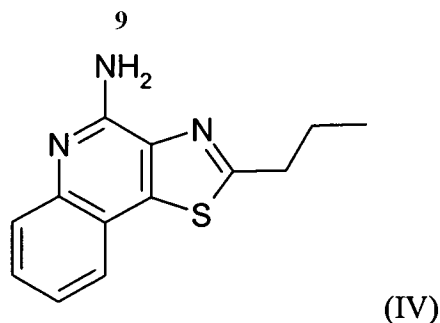


(III)

wherein

R1 represents a substituted or unsubstituted alkyl or alkoxy residue, preferably an unsubstituted alkyl or alkoxy residue or an alkyl or alkoxy residue substituted with
20 halogen, preferably fluorine, more preferably a C1 to C12 alkyl or alkoxy residue, in particular a C2 to C6 alkyl or alkoxy residue, for example an ethyl, propyl, butyl residue.

In a particularly preferred embodiment, the thiazoloquinolone derivative is a compound of
25 formula (IV) named 2-propylthiazolo[4,5-c]quinolin-4-amine, also known in the art as CL075 or 3M-002 (Gordon et al., 2005; Philbin and Levy, 2007; molecular weight 243; commercially available from InvivoGen™, San Diego, CA, USA):



CL075 is a small molecule imidazoquinoline that preferentially activates NF- κ B through TLR8 (Gorden et al., 2005).

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Individual techniques for the preparation of mature dendritic cells, e.g. starting from human peripheral blood mononuclear cells, monocytes or other myeloid progenitor cells, and from immature DCs themselves, which have been directly isolated from the blood, are known in the art (Berger et al., 2005; Campbell et al., 2005). Therefore, the basic techniques such as incubation periods, media used, etc., for producing mature dendritic cells out of immature dendritic cells, are known in the art.

The present invention relates to the novel use of a specific group of TLR7/8 agonists in the context of these prior art techniques. The method of the present invention can, therefore, be easily practiced by the person skilled in the art, simply by performing prior art methods, but using the above identified combination of factors during the incubation of immature dendritic cells in order to obtain mature dendritic cells.

Furthermore, since each of the individual components with the exception of the thiazoloquinolone derivative type TLR7/8 agonist has already been individually used in the art, the person skilled in the art can easily determine in which concentration each factor should be used. Additionally, the skilled person would be able to adapt the individual concentration of a given factor depending on compositions of the cell culture medium especially growth factors and serum components.

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As a general guidance, TNF- α and IL-1 β might be used at concentrations from 1 ng/ml to 50 ng/ml, more preferably from 5 ng/ml to 40 ng/ml, and even more preferably at 10 ng/ml. PGE2 might be used at concentrations from 50 ng/ml to 5000 ng/ml, preferably from 50 ng/ml to 1000 ng/ml, even more preferably from 50 ng/ml to 500 ng/ml or at 100

ng/ml or 250 ng/ml. IFN γ might be used at a concentration between 500 U/ml and 10000 U/ml, preferably between 1000 and 5000 U/ml, and more preferably either at 1000 or 5000 U/ml. Finally, the thiazoloquinolone derivative type TLR7/8 agonist, preferably CL075, might be used at a concentration between 0.2 and 5 μ g/ml, preferably 0.5 μ g/ml to 2 μ g/ml, more preferably 1 μ g/ml.

According to the invention, immature dendritic cells are cultivated with the above combination of factors. This can be performed by adding the factors to the culture medium. Alternatively, the culture medium in which the immature dendritic cells have been grown is replaced by a medium already containing the factors. In a further preferred embodiment, the substances mentioned above are part of a composition added to the culture medium of said immature dendritic cell.

Said culture medium may be of any suitable kind, i.e. it may contain human serum or not, may be supplemented with or without any other animal supplements, like proteins, amino acids, or antibiotics. In a preferred embodiment, the medium is produced and used under GMP conditions.

After the maturation period is completed, DCs may be harvested by up and down pipetting, shaking (by hand or mechanically) and rinsing with salt solution, medium components (e.g. RPMI) or complete medium without cytokines. Cells may be collected, centrifuged and cytokines may be washed out by at least one more resuspension of pelleted DCs.

The immature dendritic cells may further be treated with a TLR3 agonist, preferably poly (I:C), e.g. at a concentration of between 10 and 50 ng/ml, preferably 20 ng/ml. The TLR3 agonist may be added separately to the cells or may be part of the composition comprising also the other factors.

As shown in the Examples, the further administration of said TLR3 agonist results in an increase of the ratio of IL12(p70) and IL-10 produced by the mature dendritic cell, which facilitates a selective Th1 stimulation.

In the context of the present invention, a TLR3 agonist means an activator of Toll-like receptor 3 (TLR3). In innate immune response, TLR3 is activated upon recognition of

double-stranded RNA. The activation of TLR3 by, e.g., virus-derived or synthetic double-stranded RNA might result in the production of an antiviral cytokine and/or interferon.

5 In another preferred embodiment, the cells are not stimulated with IL-6. Stimulation of cells with IL-6 might result in an inhibition of IL-12(p70) synthesis.

10 In a further preferred embodiment, the immature dendritic cell used as the starting material of the method of the invention is a monocyte derived immature dendritic cell. Preferably, a monocytic progenitor obtained from peripheral blood or leukapheresis and enriched by density gradient centrifugation, elutriation or simply plastic adherence techniques is used.

15 Alternatively, it is also possible to obtain a monocytic progenitor cell from CD34 positive progenitor cells by *in vitro* differentiation to CD14 positive cells, e.g. with FLT3L, SCF, TPO, IL-3 and/or IL-6.

20 Preferably, said immature dendritic cell is obtained by incubating human peripheral blood mononuclear cells, monocytes or other myeloid progenitor cells with GM-CSF and IL-4 or IL-13. As already discussed above, corresponding methods are known in the art. Furthermore, such methods are described in the Examples.

25 Any medium suitable for physiological conditioning of mammalian cells e.g. containing standard amino acids, growth factors, carbon source, buffer system, or certain salts may be used. Cell culture may be performed at 37°C according to medium composition at certain CO₂ concentrations.

Furthermore, the immature DC may be obtained directly from peripheral blood, e.g. via leukapheresis.

30 In an especially preferred embodiment, the immature dendritic cell is of human origin, although situations, e.g. scientific research or veterinary medicine applications, may be feasible where immature dendritic cells of mammalian origin may be used.

Consequently, in a further preferred embodiment, the method of the invention comprises the following steps:

- a) preparing mononuclear cells from peripheral blood,
- b) incubating the mononuclear cells of step a) with GM-CSF and IL-4 or IL-13,
- c) incubating the cells obtained in step b) with a cocktail comprising TNF α (also known as TNFSF2), IL-1 β , IFN γ , a TLR7/8 agonist, wherein said TLR7/8 agonist is a thiazoloquinolone derivative type immune response modifying compound according to the present invention, prostaglandin E2 (PGE2), and, optionally, a TLR3 agonist, preferably poly (I:C), and
- d) harvesting the mature dendritic cell or cells.

10 In step a), the mononuclear cells may be obtained by leukapheresis from peripheral blood or fresh blood, e.g. buffy coat. Furthermore, mononuclear cells may be isolated by magnetic or FACS sorting, elutriation or plastic adherence or density gradient centrifugation (e.g. metricamide).

15 In a preferred embodiment, the mononuclear cells of step a), which are subsequently used for incubation in step b), are CD14 positive (CD14⁺) monocytes.

The term “CD14 positive monocytes” as used herein refers to mononuclear cells which express CD14 (i.e. cluster of differentiation 14) cell surface receptors specific for bacterial lipopolysaccharide (LPS).

In the context of the present invention, it has been found that stimulation of immature dendritic cells according to the present invention under shortened incubation times, e.g. within 3-days, results in the generation of mature dendritic cells with equivalent immunostimulatory activity as compared to mDCs prepared by the 7-day protocol.

Throughout the invention, the term “immunostimulatory activity” refers to the capability of a mature dendritic cell or of a mature dendritic cell population to produce and/or to secrete sufficient amounts of specific cytokines which mediate the stimulation and the maturation of effector T cells.

Accordingly, in a preferred embodiment, the incubation in step b) takes 1 to 9, preferably 1 to 7, more preferably 1 to 3 days. However, it is also feasible to spare steps a) or b) if using

freshly isolated immature DCs from peripheral blood/leukapheresis. Furthermore, it is possible that step b) lasts only hours and may be performed in combination with step c).

The incubation in step c) may take preferably 24 hours to 72 hours.

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As already discussed above, the skilled person will be able to adapt these incubation periods, if necessary.

The incubation of the immature dendritic cells and the harvesting have already been described above.

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In a further preferred embodiment of the invention, the immature or mature dendritic cell or cells is /are further loaded *in vitro* with one or more antigens.

15 Loading of immature or mature dendritic cells with respective antigens could be carried out by competitive displacement of peptides within solutions from the MHC binding groove, or for more complex antigens, like proteins and original tumor lysates or lysates of tumor cell lines, through phagocytosis of immature DCs and proper processing. Such techniques are known in the art (Dieckmann et al., 2005; Grunebach et al., 2005; Kyte et al., 2005; Su et al., 2005).

20

Preferably, said antigen or antigens are supposed to trigger the effector T cell maturation within secondary lymphatic organs. Said secondary lymphatic organs include, but are not limited to, e.g. the lymph nodes.

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The term "triggering effector T cell maturation and/or function" as used herein refers to any kind of biological process in which T cells are exposed to mature dendritic cells within secondary lymphatic organs (e.g. lymph nodes), resulting in priming of naïve T cells, reactivation of effector cell function and stimulation of memory T cells. The naïve T cell can be a naïve CD4 T cell or a naïve CD8 T cell, respectively. Naïve CD4 T cells can differentiate upon activation into various subpopulations (e.g. Th1, Th2 or Th17 cells). Naïve CD8 T cells can differentiate into cytotoxic effector cells or cytokine (e.g. IFN- γ) secreting T cells upon activation. To mediate interactions with T cells, the mature dendritic cells may express high levels of co-stimulatory molecules, such as B7 molecules. The

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generation of effector cells from T cells may take several days. At the end of this period, the effector T cells might leave the lymph nodes and re-enter the bloodstream to migrate to sites of infection.

- 5 More preferably, said loading is performed by incubating the immature or mature dendritic cell or cells with at least one protein or one peptide of said antigen, or by transfecting the dendritic cell or cells with antigen encoding RNA or DNA.

10 In the context of the present invention, transfection methods include, but are not limited to, lipofection, electroporation, viral vector systems, simply incubation of naked nucleic acids or fusion of DCs with infected cells or tumor cells. These standard methods are well known in the art and are feasible and introduce nucleic acids, such as antigen encoding plasmids, RNA of them or DNA, and especially RNA from original tumors or tumor cell lines into the DCs. There might also be other antigenic combinations with original MHC
15 molecules conceivable such as membrane fragments or exosomes to use as antigen sources of any kind.

In a further preferred embodiment, the loading of the immature or mature dendritic cell or cells further comprises introducing a specific cytokine inhibitor into said immature or
20 mature dendritic cell or cells, e.g. an inhibitor of IL-10 or IL-12. This could e.g. facilitate the production of mature dendritic cells which specifically trigger either a Th1 or a Th2 response.

According to the present invention, the term “inhibitor” refers to any kind of biochemical
25 or chemical compound which inhibits or reduces the activity of specific genes or proteins. The inhibition of protein expression can occur via specific inhibition or suppression of the respective gene expression. Inhibition or suppression of gene expression can further occur on a transcriptional or post-transcriptional level including, but not limited to, the use of antisense oligonucleotides.

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Preferably, the inhibitor is either an antisense oligonucleotide, an siRNA, or a ribozyme.

Throughout the invention, the term “antisense oligonucleotide” as used herein refers to any kind of nucleic acid molecule which is capable of specifically inhibiting gene function

within a cell. This nucleic acid molecule can be capable of hybridizing to a sequence-specific portion of an endogenously expressed RNA (preferably mRNA) by virtue of some sequence complementarity. The antisense oligonucleotide may be complementary to a coding and/or noncoding region of said RNA. The antisense oligonucleotide may be encoded by a DNA vector and transcribed within the target cell.

The production and use of siRNAs as tools for RNA interference in the process to down regulate or to switch off gene expression is e.g. described in Elbashir et al. (2001). Preferably, siRNAs exhibit a length of less than 30 nucleotides, wherein the identity stretch of the sense strand of the siRNA is preferably at least 19 nucleotides.

Ribozymes are also suitable tools to inhibit the translation of nucleic acids, because they are able to specifically bind and cut the mRNAs. Such tools are known in the art.

The inhibition of gene expression can be measured by means of standard procedures including, but limited to, Northern and/or Southern Blot analysis, quantitative RT-PCR methods, Western Blot analysis, or mRNA and protein expression profiling experiments, also including, e.g., chip arrays. Furthermore, inhibition of gene expression can occur via inhibition of the activity of the respective protein, e.g. by binding to it.

The invention further relates to a mature dendritic cell or population of mature dendritic cells, obtainable by the method of the invention. As discussed above, the mature dendritic cells obtained by the method of the invention produce significant high amounts of IL-12(p70), express significant levels of CCR7 chemokine receptors, and reveal a superior capacity to activate antigen-specific T cells as compared to the mature dendritic cells known in the art so far.

Consequently, in a further aspect, the present invention also relates to a pharmaceutical composition comprising the mature dendritic cell or the mature dendritic cells according to the present invention. Furthermore, the invention also relates to the mature dendritic cell or to the population of mature dendritic cells of the invention for use in a method of treating a disease selected from the group consisting of tumorigenic diseases and infectious diseases (e.g. provoked by viruses, bacteria, intracellular bacteria or fungi). Furthermore, the present invention relates to a method for treating a patient with a tumorigenic disease or an

infectious disease, wherein an effective amount of the mature dendritic cell of the invention is administered to said patient.

In the context of the present invention, the term “pharmaceutical composition” as used
5 herein refers to any kind of drug suitable for the treatment of the respective diseases.

Moreover, the terms “treatment” and “treating” as used herein generally mean to obtain a desired pharmacologic and/or physiologic effect, and covers any treatment of a disease in a mammal, particularly a human, including:

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(1) preventing the disease or symptom from occurring in a subject which may be predisposed to the disease or symptom, but has not yet been diagnosed as having it;

(2) inhibiting the disease symptom, i.e., arresting its development; or

15

(3) relieving the disease symptom, i.e., causing regression of the disease or symptom.

The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete stabilization or cure for a disease and/or adverse effect attributable to the disease.

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In the context of the present invention, the term “tumorigenic disease” refers to any kind of disease provoked by a tumor (malignant or benign). Preferably, the tumorigenic disease treated by use of the mature dendritic cell or the mature dendritic cells according to the present invention is selected from the group of tumors consisting of astrocytoma,
25 oligodendroglioma, meningioma, neurofibroma, glioblastoma, ependymoma, Schwannoma, neurofibrosarcoma, medulloblastoma, melanoma, malignant melanoma, pancreatic cancer, prostate carcinoma, head and neck cancer, breast cancer, lung cancer, preferably small cell lung cancer, non-small cell lung cancer, colon cancer, preferably adenocarcinoma of the colon, colorectal cancer, gastrointestinal stromal tumor, ovarian
30 cancer, endometrial cancer, renal cancer, neuroblastomas, squamous cell carcinomas, medulloblastomas, hepatoma and mesothelioma, epidermoid carcinoma, clear cell adenocarcinoma and serous adenocarcinoma of the uterine corpus, cervix carcinoma, urinary tract adenocarcinoma, Pheochromocytoma, neuroma, neurilemmoma, and paraganglioma.

The term "infectious disease" as used herein refers to any kind of clinically evident disease resulting from the presence of pathogenic microbial agents, including pathogenic viruses, pathogenic bacteria, fungi, protozoa, or multicellular parasites.

5

The mature dendritic cell or the mature dendritic cells can be formulated, in accordance with routine procedures, as a pharmaceutical composition or a medicament adapted for various administration routes (see below). Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lidocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water-free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water, saline or isotonic aqueous buffers/media. Where the composition is administered by injection, an ampoule of sterile water, saline or isotonic aqueous buffers/media for injection can be provided so that the ingredients may be mixed prior to administration. The pharmaceutical composition of the present invention may further comprise pharmaceutically acceptable salts include those formed with free carboxyl groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., those formed with free amine groups such as those derived from isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc., and those derived from sodium, potassium, ammonium, calcium, and ferric hydroxides, etc.

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Throughout the invention, the patient is preferably a mammal, and more preferably a human patient.

As indicated above, the dendritic cells can be administered directly to the organism to produce T cells active against a selected, e.g. cancerous cell type. Administration of these cells, often with pharmaceutically acceptable carriers, is by any of the routes normally used for introducing a cell into ultimate contact with a mammal's blood or tissue cells.

30

Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, intranodal and subcutaneous routes (preferably intradermal, intranodal or subcutaneous), and carriers include aqueous isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. Intradermal, subcutaneous, intranodal or intravenous administration are preferred methods of administration for dendritic cells of the invention.

The dose of the dendritic cells administered to a patient, in the context of the present invention should be sufficient to effect a beneficial therapeutic response in the patient over time, or to inhibit growth of cancer cells, or to clear infection. Thus, cells are administered to a patient in an amount sufficient to elicit an effective effector cell response to the virus or tumor antigen and/or to alleviate, reduce, cure or at least partially arrest symptoms and/or complications from the disease or infection. An amount adequate to accomplish this is defined as a "therapeutically effective dose." The dose will be determined by the activity of dendritic cell produced and the condition of the patient, as well as the body weight or surface area of the patient to be treated. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular cell in a particular patient. In this context, effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems. In determining the effective amount of the cell to be administered in the treatment or prophylaxis of diseases such as cancer (e.g., metastatic melanoma, prostate cancer, etc.), the physician needs to evaluate circulating plasma levels, T cell function, progression of the disease, and the induction of immune response against any introduced cell type.

Prior to infusion, blood samples are obtained and saved for analysis. Generally about 10^5 to 10^7 cells are infused into a 70 kg patient. Preferably, cell numbers of at least 10^7 /vaccination point are used. The injections may be e.g. 4 times repeated in a 2 weeks interval and should be given preferably near lymph nodes by intradermal or subcutaneous injections or injected directly into the lymph nodes. Booster injections may be performed after a 4 weeks pause. Vital signs and oxygen saturation by pulse oximetry are closely monitored. Blood samples are obtained 5 minutes and 1 hour following infusion and saved

for analysis. Cell reinfusion are repeated roughly every month for a total of 10-12 treatments in a one year period. After the first treatment, infusions can be performed on an outpatient basis at the discretion of the clinician. If the reinfusion is given as an outpatient, the participant is monitored for at least 4 hours following the therapy.

5

For administration, cells of the present invention can be administered at a rate determined by the LD-50 (or other measure of toxicity) of the cell type, and the side-effects of the cell type at various concentrations, as applied to the mass and overall health of the patient. Administration can be accomplished via single or divided doses. The cells of this invention can supplement other treatments for a condition by known conventional therapy, including cytotoxic agents, nucleotide analogues and biologic response modifiers. Similarly, biological response modifiers are optionally added for treatment by the dendritic cells. For example, the cells are optionally administered with an adjuvant, a cytokine such as GM-CSF, IL-12, or IL-2, or with KLH.

10

In the art, the use of a thiozoloquinolone derivative type TLR7/8 agonist according to the present invention has not yet been described in the context of stimulation and/or maturation of immature dendritic cells.

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Therefore, in another aspect, the invention relates to the use of a TLR7/8 agonist for the preparation of at least one mature dendritic cell, wherein the TLR7/8 agonist is a thiozoloquinolone derivative type immune response modifying compound according to the present invention.

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In a preferred embodiment, said TLR7/8 agonist is used in combination with TNF- α (also known as TNFSF2), IL-1 β , IFN γ , prostaglandin E2 (PGE2) and, optionally, with a TLR3 agonist, preferably poly (I:C), for the preparation of at least one mature dendritic cell. More preferably, said TLR7/8 agonist is 2-propylthiazolo[4,5-c]quinolin-4-amine (CL075).

25

In a further preferred embodiment, said TLR7/8 agonist is not used in combination with IL-6.

Furthermore, another aspect of the invention relates to a composition comprising TNF- α (also known as TNFSF2), IL-1 β , IFN γ , a TLR7/8 agonist, prostaglandin E2 (PGE2) and,

optionally, a TLR3 agonist, preferably poly (I:C), wherein said TLR7/8 agonist is a thiazoloquinolone derivative type immune response modifying compound according to the present invention. As indicated above, preferably said TLR7/8 agonist is 2-propylthiazolo[4,5-c]quinolin-4-amine (CL075).

5

In a further preferred embodiment, said composition does not contain IL-6.

The invention will be further described by the following figures, tables and Example, which are not intended to limit the scope of protection as defined in the claims.

10

Short Description of the Figures

Figure 1

15 **Schedule for generation of mature DCs (mDC) from immature DCs (iDC) generated from peripheral blood monocytes using a 3-day and a 7-day protocol**

Immature dendritic cells (iDC) were prepared from monocytes by culture in medium containing GM-CSF and IL-4 added on d0 in the 3-day protocol and on d1 and d3 in the 7-day protocol. Maturation cocktails were added to the immature DC cultures on d2 in the 3-day protocol and on d6 in the 7-day protocol. Mature DCs were harvested 24 hours later.

20

Figure 2

Comparison of DC surface marker expression in DC1 (C1 cocktail) versus DC6 (C6 cocktail) using the 3-day and a 7-day protocol

25 Figure 2a shows typical maturation markers (CD83, CD209 and HLA-DR) to demonstrate optimal maturation using the two different maturation protocols, i.e. the 3-day (3d) vs. the 7-day (7d) protocol. No differences could be detected for expression of CD83, and only small variations in CD209 were noted. Levels of HLA-DR were higher on 3-day DCs.

30 The expression of the co-stimulatory molecules (CD80, CD86 and CD274) is shown in Figure 2b. The changes in expression of these molecules turned out to be more dramatic. Comparing the 7-day (7d) protocol with the 3-day (3d) protocol, a decreased expression level of activating co-stimulators (CD80) was observed on 7-day DCs, whereas the expression of the inhibitory molecule CD274 was increased on 7-day DCs.

Figure 3**Expression of surface markers in differently matured DCs**

All DC populations were matured by the 3-day protocol, immature DCs were harvested on day 2. The DCs were matured with cocktails C1, C4, C5, C6, and C7 yielding the DC populations DC1, DC4, DC5, DC6 and DC7, respectively. The expression of surface markers was measured by flow cytometry using specific monoclonal antibodies. Data are given as percentage positive cells. The DC maturation marker CD83 was up-regulated by every maturation cocktail, whereas the CD14 molecule was always down-regulated. Expression of co-stimulatory molecules (CD80 and CD86) and HLA-DR expression was high. Significantly more cells expressed CCR7 after maturation with C6 and C7. Each maturation cocktail was evaluated using different numbers of independent DC donors: iDC: 2; DC1: 4; DC4: 4; DC5: 4; DC6: 3; DC7: 4.

Figure 4**Cytokine secretion assessed by a signal-3-assay**

All DC populations were matured using the 3-day protocol, immature DCs were harvested on day 2. Independent experiments were performed with a number of $n = 1$ for iDC; $n = 2$ for DC1, DC4, DC5, DC6; $n = 3$ for DC7. CD40-ligand transfected mouse fibroblasts were used as stimulators for DC, which mimics the encounter of matured DC with T cells that express CD40L. Supernatants were collected after 24 h of co-culture. Cytokines were measured by standard ELISA. Shown is the ratio of IL-12(p70) to IL-10 detected in the supernatant of the co-cultures. All ratios are normalized to the ratio of DC1, which was set to 1. The best ratio could be detected in the co-culture of DC matured with cocktail 6.

Figure 5**Capacity of DC populations to stimulate different T cells subpopulations**

Differently matured DCs generated in a 3-day protocol were pulsed with a CEF-peptide pool. They were co-cultured with autologous donor PBL to induce a memory response. After a 7-day co-culture the different T cell subpopulations were assessed by FACS using CD3, CD4 and CD8 specific antibodies. DC6 as well as DC1 were able to activate CD8⁺ T cells. Furthermore DC6 was able to induce a shift within the T cell populations (CD8⁺ > CD4⁺).

Figure 6**Capacity of DC populations to activate T cells**

Differently matured DCs were pulsed with a CEF-peptide pool and co-cultured with autologous donor PBL to induce a memory response. After a 7 day co-culture the different activated T cell subpopulations were assessed by FACS using CD3, CD4, CD8 and CD137-specific antibodies. DC6 showed a superior capacity to induce activated CD4 and CD8 T cells expressing CD137 (Wölfel et al., 2007).

Figure 7**Migratory capacity to CCR7 ligands.**

Depicted is a trans-well migration assay using CCL19 as a chemo-attractant for DC1, DC5, DC6 and DC7. Pore size of the membrane was 5 μ m; chemokine concentration of CCL19 was 100 ng/ml. Shown is one representative experiment of three with a mean deviation estimated from duplicates.

Figure 8**Activation of NK cells by mDC.**

NK cells were prepared from two unrelated donors and incubated for 24 h with washed DC that were matured in cocktails C1, C5, C6 and C7. a) Secretion of IFN-gamma was quantified by standard ELISA. b) Cocultured cells were stained for CD3, CD56 and CD69 expression. Depicted is the CD56/CD69 stain of the CD3⁻ CD56⁺ population.

Figure 9**Activation of T cells by mDC.**

PBMC from an HLA-A2⁻ donor were stimulated with mDC derived from an HLA-A2⁺ donor for 7 days. After 5 h stimulation with PMA and ionomycin, intracellular IFN- γ and IL-4 was analyzed using flow cytometry. a) Shown is a representative example of CD4⁺ gated cells depicting the intracellular double staining of IFN-gamma and IL-4. b) IFN-gamma positive cells are depicted as percentages of CD4⁺ cells (gray) and CD8⁺ cells

(black), respectively. Solid line (at 65%) represents control stimulation of the CD4⁺ clone 234 and the dotted line (at 20%) represents the control stimulation of the CD8⁺ clone JB4.

5 Short Description of the Tables

Table 1

Composition of cocktails used for DC cultivation and maturation

Cocktail C1 is equivalent to the standard cocktail of Jonuleit which does not include any TLR3 or TLR7/8 ligands, and includes IL-6 as published (Jonuleit et al, 1997). Cocktails C4 and C5 are published in WO 2007/110240 and in Zobywalski et al. (2007). Both use R848 as the TLR7/8 ligand and poly (I:C) as a TLR3 ligand in C5. It is known also to interact with other pattern recognition receptors (Dong et al., 2008). Cocktails C6 and C7 are new cocktails that utilize CL075. They differ in the use of poly (I:C) which is present only in C6. These four cocktails (C4, C5, C6, C7) differ from Jonuleit cocktail (C1) in the inclusion of interferon-gamma (5000 U/ml), the exclusion of IL-6, and the reduction of PGE2 to 250 ng/ml in C4,C5, C6, C7 versus 1000 ng/ml in Jonuleit cocktail. The star indicates an incubation at 55 °C for 5 minutes at room temperature and then diluted 1:10 with endotoxin-free water.

Table 2

Recoveries of DCs after maturation in various cocktails

iDC: immature DCs cultured only in GM-CSF and IL-4; *DC1*: immature DCs matured with Jonuleit cocktail (C1); *DC4*: immature DCs matured with cocktail 4 (C4); *DC5*: immature DCs matured with cocktail 5 (C5); *DC6*: immature DCs matured with cocktail 6 (C6); *DC7*: immature DCs matured with cocktail 7 (C7). For the composition of cocktail C1, C4, C5, C6, and C7, respectively, see Table 1. Mature DCs were prepared from 3 or 4 unrelated donors, and the percentage recoveries were calculated by the quotient of monocytes that were seeded in the cultures on day 0 divided by of the numbers of DCs recovered on day 3. The mean was estimated by the quotient of seeded cells and recovered cells. No statistical differences were found in the percentages of DCs recovered after maturation in the various cocktails. N* indicates the number of independent donors; m+: mean; std#: standard deviation.

Table 3**Expression intensity of typical DC surface marker**

Table 3 summarizes the levels of expression of the surface markers measured by flow cytometry that are depicted as percentages of positive cells in Figure 2. Data represent mean fluorescence intensities (MFI) +/- with standard errors for 3-4 independent donors. The percentages of CCR7-positive cells were higher in DC populations matured with C6 and C7 as compared to C4 and C5 (see Figure 2), but also their levels of CCR7 expression by MFI were significantly higher compared to DC matured in C4 and C5.

Table 4**Levels of IL-10 and IL-12(p70) produced by DCs**

Table 4 shows the production of the cytokines IL-10 and IL-12(p70) from DC populations of two donors that were matured in cocktails C1, C4, C5, C6 and C7, respectively. Cytokines were measured in standard ELISA using supernatants obtained upon harvesting of the DC on day 3, representing the accumulated cytokine over the 3-day culture period. Data represent amounts of cytokines given in pg/ml as measured in a standard ELISA; n.d. non detectable. The levels of IL-12(p70) produced by DC populations matured with C6 and C7 were significantly higher compared to C1, C4 and C5.

Examples**1. Material and Methods****Leukapheresis and elutriation**

To obtain monocytes as a progenitor cell population for generation of human dendritic cells, we used a closed system of elutriation by ELUTRA (Gambro BCT, Lakewood, USA). After informed consent, healthy, unmobilized donors underwent 180 minute leukaphereses with the COBE Spectra cell separator (Gambro BCT, Inc. Lakewood, USA) using a modified MNC program (V6.1): separation factor was set to 700 with a collection rate of 0.8 ml/min and a target hematocrit of only 1-2%. Resulting blood cells were analysed by automatic blood counter ACT Dif (Beckman Coulter, Krefeld, Germany) to set up conditions for ELUTRA system.

Leukapheresis products were processed by ELUTRA (Gambro BCT, Lakewood, USA) according manufacturer's instructions by a method of counter-flow centrifugal elutriation using a fixed rotor speed (2400rpm) and computer controlled stepwise adjustment of media flow rate followed by rotor-off harvesting. Therefore 5000ml of running buffer containing HANKs buffered salt solution (Biochrom, Berlin, Germany) with 1% human serum albumin (Octalbine[®], Octapharma, Langen, Germany) were prepared. ELUTRA process resulted in five fractions, with enriched monocytes in the rotor-off fraction. Cellular composition of fractions were characterised by automatic blood counter ACT Diff (Beckman Coulter, Krefeld, Germany) and FACS analysis.

Generation of mature monocyte-derived dendritic cells from elutriated monocytes

Cells from rotor-off fraction or the subsequently named fraction 5 were frozen in aliquots of 50×10^6 monocytes in a freezing medium (11 % HSA (Octapharm, Langenhagen, Germany), 20 % DMSO (Merck, Darmstadt, Germany) and 10 % Glucose (Braun, Melsungen, Germany)). Monocytes were thawed and washed with endotoxine free PBS (Biochrom, Berlin, Germany) at 1500 rpm for 10 minutes. Cells were resuspended and seeded at $4,5 \times 10^7$ per "nuncleon-surface"-flask (80 cm^2) (NUNC, Wiesbaden, Germany) in 15 ml DC medium containing RPMI 1640 with very low endotoxin (Biochrom, Berlin, Germany) and 1,5% human serum (pool of AB-positive adult males) (Blood Bank, University of Tuebingen, Germany) and cultivated for 50 minutes by 37°C, 5% CO₂ in a humidified atmosphere. Afterwards cells were washed twice with RPMI 1640 very low endotoxin and 15 ml DC medium was added. At day 0 or at day 1 and 3 cell cultures were supplemented with 100 ng/ml GM-CSF (Leukine[®] by Berlex, Richmond, USA) and 20 ng/ml recombinant human IL-4 (R&D Systems, Wiesbaden, Germany) in 3 ml fresh DC medium per flask in the 3-day-protocol or the 7-day-protocol, respectively. Full DC maturation was achieved by addition of the different cocktails (see table 1) on day 2 (3-day-protocol) or on day 6 (7-day-protocol).

Harvesting of dendritic cells

After incubation of DCs with the maturation cocktails for 24 hrs, cells were harvested by washing twice with PBS + 0, 5% human serum with light shaking, cells were counted by Neubauer chamber and prepared for the analyses.

Flow cytometric (FACS) analysis/ DC phenotyping

DCs were labeled with the following fluorescence-conjugated monoclonal mouse antibodies: CD14 (FITC, MΦP9), CD86 (FITC, clone: 2331 FUN-1), CD80 (PE, clone: L307.4) (BD Biosciences, Heidelberg, Germany), CD274 (FITC, clone: MIH1; ebioscience), CD209 (PE, clone: DCN46) (Pharmingen, San Diego, USA), HLA-DR (PE, clone: B8.12.2), and CD83 (PE, clone: HB15a) (Immunotech, Marseille, France).

CCR7 staining was performed with a rat hybridoma BLR-2 (clone 8E8) (E. Kremmer, GSF) in comparison to isotype control for IgG2a of hybridoma EBNA-A2 (clone R3) by incubation of DCs in culture supernatant for 60 minutes and followed by after washing, and detection with secondary mouse antibody against rat IgG conjugated with cyanin 5 (Jackson Immuno, West Grove, USA). To test vitality, DCs were pelleted and resuspended for 20 minutes in 7- Aminoactinomycin D (Sigma-Aldrich, Deisenhofen, Germany) at final concentrations of 10µg/ml in PBS + 2% fetal calf serum. After washing, cells were analyzed in the third channel of the FACS Calibur machine.

Signal 3-Assay

DCs were co-cultured with T cell-mimicking cells as described previously (Mailliard et al., 2004). Briefly, matured, harvested and washed DCs were reseeded in 96 well plates at concentrations of 2×10^4 /well and incubated together with mouse fibroblasts stably transfected with human CD40L at concentrations of 5×10^4 /well. To control cytokine secretion of each cell population alone, DCs with out any additions and CD40L-fibroblasts in standard medium were tested. After 24 hrs, plates were centrifuged and supernatants of 8 replicate wells were pooled for analyses of IL-10 and IL-12(p70) by ELISA.

ELISA (IL-12(p70)/IL-10)

Secretion of IL-12(p70) and IL-10 by DCs during maturation process (primary DCs) and DCs within Signal 3-assay were detected by standard quantitative ELISA. ELISA was performed utilizing pre-tested antibody duo sets for detection of IL-12(p70) and IL-10 (R&D Systems, Wiesbaden, Germany) according to manufacturer's instructions. Colorimetric substrate reaction with tetramethylbenzidine and H_2O_2 was measured after stopping with H_3PO_4 at 450nm and wavelength correction by 620nm and analyzed by software easy fit (SLT, Crailsheim, Germany).

Autologous DC and T cell cultures with CEF peptide pool

To assess the functional capacity of the differently matured DC we used two leukapheresis fractions, which contain mainly monocytes or lymphocytes, donated by a healthy HLA-A2 positive donor. DCs were generated by the 3-day protocol, described above. After harvesting and washing, 10^5 DC were pulsed with 1 μ g of the CEF peptide pool. These peptides are restricted to HLA-A2 and are listed below. After 90 minutes of incubation 10^6 PBL were added to each DC culture and incubated for 7 days. To analyze the different T cell subpopulations and their activation status of those we preformed a FACS analysis (antibodies used are listed below). The gates were set on living cells and afterward on CD3 positive cells. Out of this population $CD4^+$ and $CD8^+$ cells, as well as activated $CD4^+$ $CD137^+$ and $CD8^+$ $CD137^+$ were calculated as percent of total CD3 population.

CEF peptide pool:

CEF peptide pool (HLA-A2-restricted)	PANTecs, Tübingen	amino acid equence
	Influenza A	GILGFVFTL
	Influenza A	FMYSDFHFI
	EBV	CLGGLLTMV
	EBV	GLCTLVAML
	HCMV	NLVPMVATV

Antibodies:

antibody	labeling	clone	company	isotype
CD3	PE	UCHT1	Beckman C.	IgG2a
CD3 PerCP	PerCP	SK7	Becton Dickinson	IgG2a
CD4	FITC	13B8.2	Beckman C.	IgG2a
CD8	APC	SK1	Becton Dickinson	IgG2a
CD137	PE	1AH2	Becton Dickinson	IgG2a
IgG2a	PE	G155-178	Becton Dickinson	
IgG2a	APC	X39	Becton Dickinson	
IgG2a	FITC	X39	Becton Dickinson	
IgG2a	PerCP	X39	Becton Dickinson	

Method of migration assay

After harvesting and washing, mDC were analyzed in a transwell-migration assay. In brief, the lower culture chamber of a 24-trans-well plate (Costar, Corning, USA) was filled with 600 μ l migration medium, consisting of RPMI-VLE, 500 U/ml GM-CSF, 250 U/ml IL-4 and 1% human serum, with or without chemokine CCL19 at 100 ng/ml (R&D Systems).

mDC were seeded in the upper chamber at 2×10^5 cells/well and incubated for 2 h at 37°C in 5% CO₂ in a humidified atmosphere. DC from the upper and lower chambers were collected and counted using a Neubauer-hemocytometer.

5 **Methods of NK cell activation**

NK cells were enriched from cryopreserved PBMC using the Dynabeads® Untouched™ Human NK Cells Kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. NK cells (1×10^6) were seeded in RPMI 1640, supplemented with 200 mM L-glutamine, 100 mM sodium pyruvate, 10^4 U/ml penicillin and streptomycin (all Biochrom) and 10% pooled human serum. NK cells were stimulated with 1×10^5 autologous mDC. After 24 h, supernatant was collected and analyzed using an IFN-gamma ELISA. Cocultured cells were stained afterwards with CD3 (FITC, clone UCHT1; BD), CD56 (APC, clone N901; Immunotech) and CD69 (PE, clone TP1.55.3; Immunotech) antibodies to depict the activated NK cell population. After washing, cells were analyzed by flow cytometry using a LSR II instrument (BD). Post-acquisition data analysis was made with FlowJo 8 software (Tree Star, Inc.).

Methods for activation of allogeneic T lymphocytes

Cryopreserved PBMC isolated from HLA-A2⁻ donors were cocultured with allogeneic mDC prepared from HLA-A2⁺ donors using 1×10^6 PBMC and 1×10^5 mDC in T cell medium (RPMI 1640, 12.5 mM HEPES, 4 mM L-glutamine, 100 U/ml penicillin and streptomycin, supplemented with 10% pooled human serum). Following 7 days of coculture, recovered lymphocytes were analyzed by flow cytometry for intracellular cytokine staining.

Methods for intracellular cytokine staining

PBL activated for 7 days with mDC were harvested, washed and stimulated for 1 h at 37°C with 1 ng/ml phorbol myristate acetate (PMA) and 250 ng/ml ionomycin (Sigma-Aldrich). Afterward, brefeldin A (40 ng/ml) and monensin (0.2 mM) were added and the cells were incubated for an additional 4 h. As positive controls, the CD4⁺ T cell clone 234 and the CD8⁺ T cell clone JB4 were stimulated in a similar manner with PMA/ionomycin. Staining of surface molecules was performed as described above using the following antibodies: CD3 (PerCP, clone SK7; BD); CD4 (PE, clone 13B8.2; Immunotech); CD8 (APC, clone

SK1; BD); CD16 (APC, clone 3G4; Caltag, Buckingham, GB); CD56 (PE, clone N901; Immunotech). Afterwards, cells were fixed with 1% paraformaldehyde, washed twice and permeabilized with 0.1% saponin solution (Sigma-Aldrich). Incubation of cells with IFN-gamma-specific (FITC, clone 25723.11; BD) and IL-4-specific (PE, clone 3010.211; BD) antibodies was done in 0.35% saponin solution in PBS for 20 min at 4°C. After washing, cells were analyzed by flow cytometry as described above. Post-acquisition data analysis was made using FlowJo 8 software (Tree Star, Inc).

2. Results

2.1 Preparation of mature dendritic cells (DC)

Figure 1 provides a schematic representation of the time course for preparation of 3-day versus 7-day DC from monocytes. Monocytes are differentiated into immature DC (iDC) using GM-CSF and IL-4 and then are further incubated with various maturation cocktails to obtain mature DC (mDC). For 3-day DC the immature DC are differentiated over 2 days of culture using GM-CSF (100 ng/ml final conc.) and IL-4 (20 ng/ml final conc.) which are added to the isolated monocytes on day 0 and the maturation cocktails are added on day 2. For 7-day DC the immature DCs are generated from monocytes isolated on day 0 and then the same concentrations of GM-CSF and IL-4 are added on day 1 to initiate differentiation of immature DC. On day 3 the cultures are supplemented with fresh cytokines and DC medium. The maturation cocktails are added on day 6.

Table 2 shows the percentages of DCs recovered after maturation with the different cocktails. Recovery was measured by dividing the seeded monocytes by the number of harvested DCs. In summary, the iDC revealed a lower recovery rate compared to the recovery of mature DCs which was in a similar range for DC1, DC4, DC5, DC6 and DC7, respectively.

Figure 2 illustrates the phenotype of DC prepared according to this scheme using the conventional 7-day protocol and the 3-day protocol. The immature DCs were matured using two different cocktails: the Jonuleit cocktail (C1) yielded the DC1 population of mature DC and cocktail 6 (C6, described below) yielded the DC6 population. DC1 and

DC6 populations were prepared according to the 3-day and 7-day protocols and analyzed for a series of surface molecules that are known to be expressed by mature DC. Surface staining was made using monoclonal antibodies specific for the various markers and detection was done by flow cytometry. CD83 which is a marker of mature DC was found to be expressed by all four DC populations analyzed. HLA-DR molecules are responsible for presentation of MHC class II-restricted peptides to CD4⁺ T cells, which is an important property of DC. All four DC populations expressed HLA-DR and the levels were somewhat higher on 3-day versus 7-day DC, irrespective of the maturation cocktail that was used. CD209 (DC-SIGN) is a C-type lectin receptor on DC and it was detected on all four populations. CD86, CD80 and CD274 are molecules belonging to the B7 family of regulatory molecules that provide co-stimulatory signals to T cells and either enhance or down-regulate T cell function dependent upon the receptors they engage on T cells. They were also present on all four DC populations. These studies demonstrated that both 3-day and 7-day DC, irrespective of maturation with C1 or C6, show highly similar phenotypes for these surface markers, revealing that the phenotypes of mature DC prepared in a 3-day protocol are similar to DC prepared by a 7-day protocol.

2.2 Expression of surface markers

Figure 3 presents the phenotype data of DC prepared from 3-4 donors using cocktails C1, C4, C5, C6 and C7 for DC maturation. All DC were prepared with the 3-day protocol and the phenotype is also presented for immature DC (iDC) for two donors. The iDC still contain substantial numbers of CD14-positive cells, which represent monocytes that have not fully differentiated to immature DC. As expected, the iDC had few cells expressing CD83, which is a marker of mature DC. All mature DC populations had few CD14-positive cells and high percentages of CD83-positive cells. The majority of DC was positive for CD80 and CD86, which are co-stimulatory molecules that provide activation signals to T cells. Somewhat fewer cells were HLA-DR-positive after maturation with C5 and C7, but these differences were not statistically significant. DC matured with cocktail C6 had significantly more HLA-DR-positive cells than DC matured in cocktail C5. CCR7 is a chemokine receptor that can provide intracellular signals to DC and it guides their migration to lymph nodes where they encounter lymphocytes for activation. There were significantly more CCR7-positive DC in populations matured with C6 and C7, containing CL075, in contrast to Jonuleit cocktail C1 and the R848-containing cocktails, C4 and C5.

Table 3 shows the expression levels by MFI (mean fluorescence intensities) of surface markers on DCs matured in different cocktails. Not only the percentages of CCR7-positive cells were higher in DC populations matured with C6 and C7 as compared to C4 and C5 (see Figure 3), but also their levels of CCR7 expression by MFI were significantly higher compared to DC matured in C4 and C5.

2.3 Production of IL-10 and IL-12(p70)

As can be seen from the results presented in Table 4, DC1 prepared from both donors made no or only low levels of IL-10. Higher levels of IL-10 were detected in supernatants from DC matured with C4, C5, C6 and C7. DC1 from donor 1 made low levels of IL-12(p70) but no IL-12(p70) was detected in the culture supernatant of DC1 of donor 2. In contrast, very high levels of IL-12(p70) were found in the supernatants of DC matured with C4, C5, C6 and C7 from both donors. The levels of IL-12(p70) produced by DC matured with C6 and C7 were significantly different and substantially higher from the levels of IL-12(p70) released by DC from donors 1 and 2 following maturation with C4 and C5.

Figure 4 depicts another comparison of IL-10 and IL-12(p70) production by the DC populations matured in various cocktails. Culture medium from the DC cultures was removed and the DC were washed and replated with fibroblasts expressing CD40-ligand (CD40L), in a so-called signal-3 assay, which mimics the encounter of mature DC with T cells that express CD40-ligand. The supernatant media of these cultures of DC and CD40L-fibroblasts were harvested after 24 hours and the content of IL-10 and IL-12(p70) was measured by standard ELISA. The data are presented as the mean quotient of IL-12(p70)/IL-10 for mature DC of 3-4 donors cultured with C1, C4, C5, C6 and C7. Immature DCs (iDCs) from 2 donors were included for comparison. DC matured with C6 showed a clear superiority in the ratio of IL-12(p70) to IL-10. The strong over-expression of IL-12(p70) compared to IL-10 is important for allowing mature DC to polarize T cell responses in a T-helper-1 (Th1) direction. Th1 cells, in turn, are important for development of optimal anti-tumor or anti-viral T cell-mediated immune responses.

2.4 Activation of T cells

To determine the functional capacity of DC populations to activate T cells, peripheral blood lymphocytes (PBL) from an HLA-A2-positive donor were co-cultivated with autologous DC that were pulsed with peptides that were derived from cytomegalovirus, Epstein-Barr virus and influenza virus (CEF peptides) that bind to HLA-A2 molecules. These HLA-A2-CEF peptide complexes have the capacity to reactivate CD8-positive effector memory T cells in healthy HLA-A2-positive donors. DC populations matured in a 3-day protocol using C1, C5 and C6 were harvested, pulsed with CEF peptides, washed and used in co-cultures with autologous peripheral blood lymphocytes (PBL). PBL cultured in the absence of DC and absence of peptides served as controls. After 7 days of co-culture, the PBL were harvested, stained with monoclonal antibodies specific for CD3, CD4, CD8, CD137 which is a marker that is up-regulated on recently activated antigen-specific T cells (Wölfl et al., 2007). Using antibodies that were labelled with different fluorescent dyes and appropriate gating strategies, it was possible to distinguish percentages of CD4 and CD8 T cells among total CD3-positive cells (Figure 5) as well as double-positive antigen-activated CD137-positive T cells (Figure 6).

This analysis showed that DC populations matured in C6 had a superior capacity to retain viable CD8⁺ T cells compared to non-stimulated PBL and to DC matured in C5. The increased numbers of CD8-positive cells account for the decrease in CD4 cells since data are presented as percentages of total CD3-positive T cells (Figure 5). Furthermore, T cells co-cultured with DC1, DC5 and DC6 and characterized for double expression of CD4⁺CD137⁺ and CD8⁺CD137⁺ showed superiority in both cases for the DC population matured with C6, demonstrating that this DC population could also optimally activate antigen-specific T cells (Figure 6).

2.5 Migratory capacities of DC populations matured in different maturation cocktails

DC6 matured in cocktail C6 [CL075 with poly (I:C) as TLR7/8 and TLR 3 agonists, respectively] and DC7 matured in cocktail C7 (CL075 as a single TLR7/8 agonist) show

excellent migration properties that are comparable to DC1 matured in the Jonuleit cocktail (that contains no TLR agonists). Both spontaneous migration in the absence of chemo-attractant signals, as well as CCL19-mediated chemokine attraction are better with DC6 and DC7 cells as compared with DC5 cells that were matured in C5 that uses R848 with poly (I:C) as TLR signals in the DC maturation cocktail (Fig. 7). CCL19 is a chemokine that interacts with the chemokine receptor CCR7.

2.6 Activation of natural killer cells by various DC populations prepared in 3 days using the different maturation cocktails.

It was expected that mDC that secrete bioactive IL-12(p70) would be superior to DC1 populations in the activation of NK cells. This was clearly demonstrated when enriched NK cells prepared from two unrelated donors (A and B) were incubated for 24 h with mDC prepared using cocktails C1, C5, C6 and C7 and analyzed for secretion of IFN-gamma, as one parameter of NK cell activation (Fig. 8, upper panels). Only low levels of IFN-gamma were secreted by NK cells stimulated with DC1, whereas NK cells released substantially more IFN-gamma following contact with DC5, DC6 and DC7. As a second parameter, we measured upregulation of the activation marker CD69 on DC-stimulated NK cells after 24 h. About 45% of the NK cells expressed this marker after 24 h in culture medium alone and this percentage did not change substantially after coculture with DC1 cells, whereas this increased to over 75% of NK cells stimulated with DC6 or DC7. Furthermore, this DC stimulation impacted on CD69 upregulation on both the CD56^{dim} and CD56^{bright} NK cells (Fig. 8, lower panels). Lower percentages (70%) of NK cells were activated by DC5 cells, although these were increased as compared to DC1 stimulation.

2.7 Polarization of CD4 and CD8 T cells to secrete IFN-gamma

To analyze the impact on T cell polarization, we stimulated PBMC, containing mixtures of CD4⁺ and CD8⁺ T cells, with allogeneic DC matured with C1, C5, C6 and C7. After 7 days of coculture, the allo-stimulated lymphocytes were harvested, washed and activated with PMA and ionomycin for 5 h. The lymphocytes were then analyzed by flow cytometry for expression of CD3, CD4 and CD8 surface markers. The fractions of cells producing IFN-

gamma and IL-4 were determined using intracellular cytokine staining. The CD4⁺ T cell clone 234 and the CD8⁺ T cell clone JB4 were used as positive controls and PBMC cultured for 7 days in the absence of DC served as a background control of unstimulated cells.

5

In a representative example, about 6% of CD4⁺ T cells cultured for 7d in medium alone were positive for IFN-gamma with 1.5% expressing IL-4. This value increased to around 25% of CD4⁺ T cells with IFN-gamma and 6.5% with IL-4 after activation for 7d with DC1 cells. In contrast, 45-50% of CD4⁺ T cells expressed IFN-gamma after 7d coculture with DC5, DC6 or DC7 while CD4⁺ T cells producing IL-4 remained at the background levels of unstimulated PBMC (Fig. 9, upper panels). Similar effects were seen on polarization of CD8⁺ T cells with much higher percentages of cells producing IFN-gamma (Fig. 9, lower panels), without alterations in percentages of IL-4-stained cells, following coculture with DC5, DC6 or DC7 as compared to DC1 (data not shown). The highest percentage of CD8⁺ T cells making IFN-gamma was found after DC6 stimulation and DC7 stimulation yielded percentages of CD8⁺ T cells like those stimulated by DC5, despite the absence of poly (I:C) in C7 and its presence in C5.

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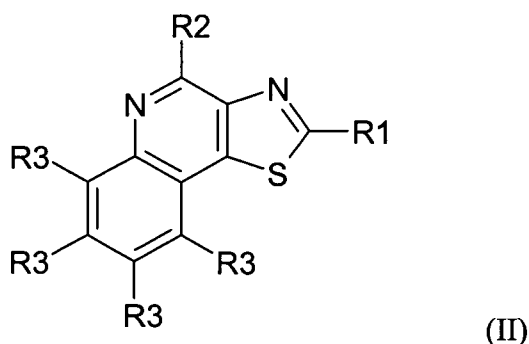
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Claims

- 5 1. A method for *in vitro* maturation of at least one immature dendritic cell, comprising stimulating said immature dendritic cell with $\text{TNF}\alpha$ (TNFSF2), $\text{IL-1}\beta$, $\text{IFN}\gamma$, a TLR7/8 agonist and prostaglandin E2 (PGE2), wherein said TLR7/8 agonist is a thiazoloquinolone derivative type immune response modifying compound.
- 10 2. The method of claim 1, further comprising stimulating said immature dendritic cell with a TLR3 agonist, preferably poly (I:C).
3. The method of any of claims 1 or 2, wherein said cell is not stimulated with IL-6, in particular wherein said substances are part of a composition added to the culture
15 medium of said immature dendritic cell.
4. The method of any of claims 1 to 3, wherein said thiazoloquinolone derivative is a compound of general formula (II) or a pharmaceutically acceptable salt thereof

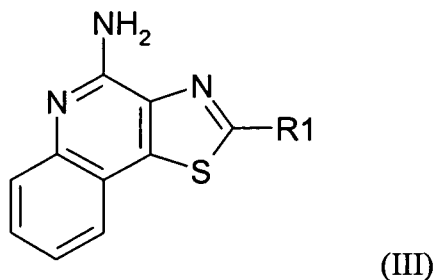


wherein

R1 represents a substituted or unsubstituted alkyl or alkoxy residue, preferably an unsubstituted alkyl or alkoxy residue or an alkyl or alkoxy residue substituted with
25 halogen, preferably fluorine, more preferably a C1 to C12 alkyl or alkoxy residue, in particular a C2 to C6 alkyl or alkoxy residue, for example an ethyl, propyl, butyl residue,

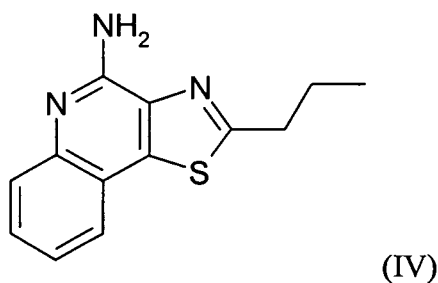
R2 represents NH_2 , NHR_4 , $\text{N(R}_4)_2$, SH or OH, wherein R4 is a C1 to C6 alkyl group, preferably methyl or ethyl, preferably R2 represents NH_2 ,

each R³ independently represents hydrogen or halogen, preferably hydrogen or fluorine, more preferably hydrogen, in particular wherein said thiazoloquinolone derivative is a compound of general formula (III) or a pharmaceutically acceptable salt thereof



wherein

R¹ represents a substituted or unsubstituted alkyl or alkoxy residue, preferably an unsubstituted alkyl or alkoxy residue or an alkyl or alkoxy residue substituted with halogen, preferably fluorine, more preferably a C₁ to C₁₂ alkyl or alkoxy residue, in particular a C₂ to C₆ alkyl or alkoxy residue, for example an ethyl, propyl, butyl residue, more in particular wherein said thiazoloquinolone derivative is a compound of formula (IV) named 2-propylthiazolo[4,5-c]quinolin-4-amine (CL075).



5. The method of any of claims 1 to 4, wherein said immature dendritic cell is a monocyte derived immature dendritic cell, preferably derived from human peripheral blood mononuclear cells, monocytes, other myeloid progenitor cells, or from CD34 positive progenitor cells by *in vitro* differentiation to CD14 positive cells, or wherein said immature dendritic cell is obtained directly from peripheral blood, in particular wherein the immature dendritic cell is obtained by incubating

human peripheral blood mononuclear cells, monocytes or other human myeloid progenitor cells with GM-CSF and IL-4 or IL-13.

6. The method of any of claims 1 to 5, comprising the following steps:

a) preparing mononuclear cells from peripheral blood, in particular wherein the mononuclear cells are obtained by leukapheresis from peripheral blood or fresh blood;

b) incubating the mononuclear cells of step a) with GM-CSF and IL-4 or IL-13, in particular wherein said incubating takes 1 to 9, preferably 1 to 7, more preferably 1 to 3 days, more in particular wherein said mononuclear cells are CD14⁺ monocytes;

c) incubating the cells obtained in step b) with a cocktail comprising TNF α (TNFSF2), IL-1 β , IFN γ , said TLR7/8 agonist, prostaglandin E2 (PGE2), and, optionally, a TLR3 agonist, preferably poly (I:C), in particular wherein said incubation takes 24 hrs to 72 hrs; and

d) harvesting the mature dendritic cell or cells.

7. The method of any of claims 1 to 6, wherein the immature or mature dendritic cell or cells is/are further loaded *in vitro* with one or more antigens, in particular wherein said antigen or antigens are supposed to trigger the effector T cell maturation within secondary lymphatic organs, more in particular wherein said loading is performed by incubating the immature or mature dendritic cell or cells with at least one protein or peptide of said antigen, or by transfecting the dendritic cell or cells with antigen encoding RNA or DNA.

8. The method of claim 7, wherein said loading further comprises introducing a cytokine inhibitor into said immature or mature dendritic cell or cells.

9. A mature dendritic cell or a population of mature dendritic cells, obtainable by the method of any of claims 1 to 8.

10. A pharmaceutical composition comprising the mature dendritic cell or the population of mature dendritic cells of claim 9.

11. The mature dendritic cell or the population of mature dendritic cells of claim 9 for use in a method of treating a disease selected from the group consisting of tumorigenic diseases and infectious diseases.
- 5 12. Use of a TLR7/8 agonist for the preparation of at least one mature dendritic cell, wherein the TLR7/8 agonist is a thiazoloquinolone derivative type immune response modifying compound as defined in any of claims 1, or 4, in particular wherein said TLR7/8 agonist is used in combination with TNF α (TNFSF2), IL-1 β , IFN γ , prostaglandin E2 (PGE2), and, optionally, with a TLR3 agonist, preferably
10 poly (I:C).
13. The use according to claim 12, wherein said TLR7/8 agonist is not used in combination with IL-6, in particular wherein said TLR7/8 agonist is 2-propylthiazolo [4,5-c]quinolin-4-amine (CL075).
15
14. A composition comprising TNF α (TNFSF2), IL-1 β , IFN γ , a TLR7/8 agonist as defined in any of claims 1 or 4, prostaglandin E2 (PGE2), and optionally a TLR3 agonist, preferably poly (I:C), in particular wherein said TLR7/8 agonist is 2-propylthiazolo [4,5-c]quinolin-4-amine (CL075).
20
15. The composition of claim 14, wherein the composition does not contain IL-6.

Figure 1

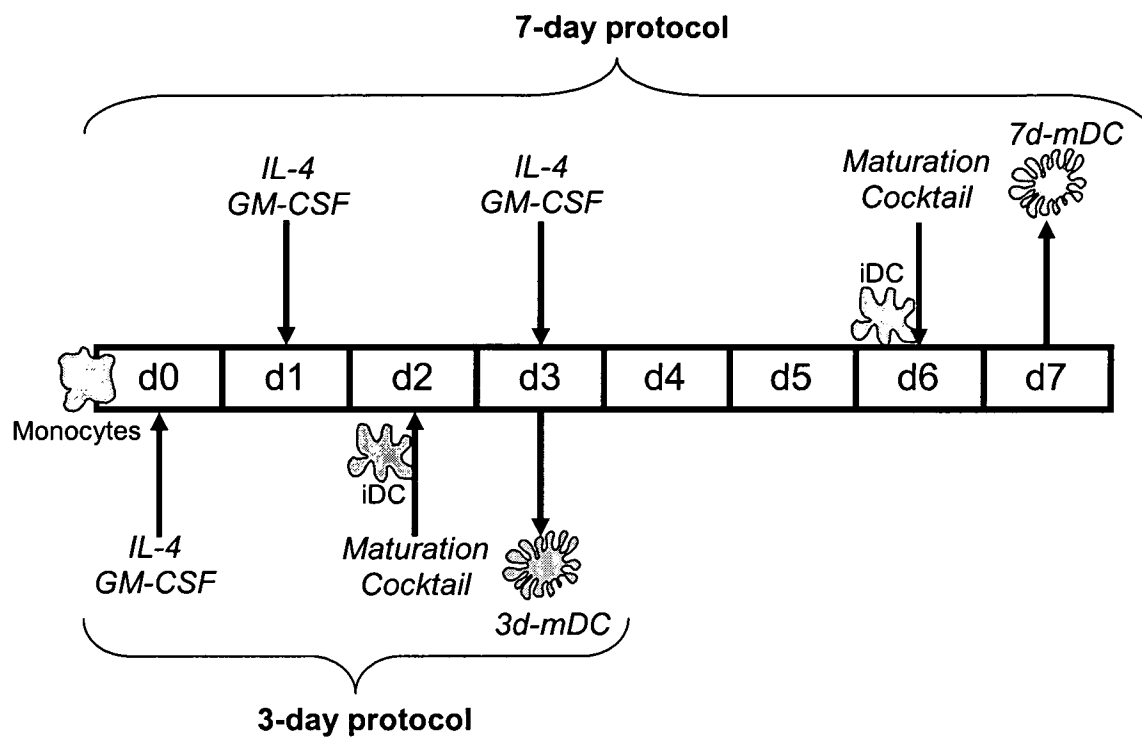


Figure 2a

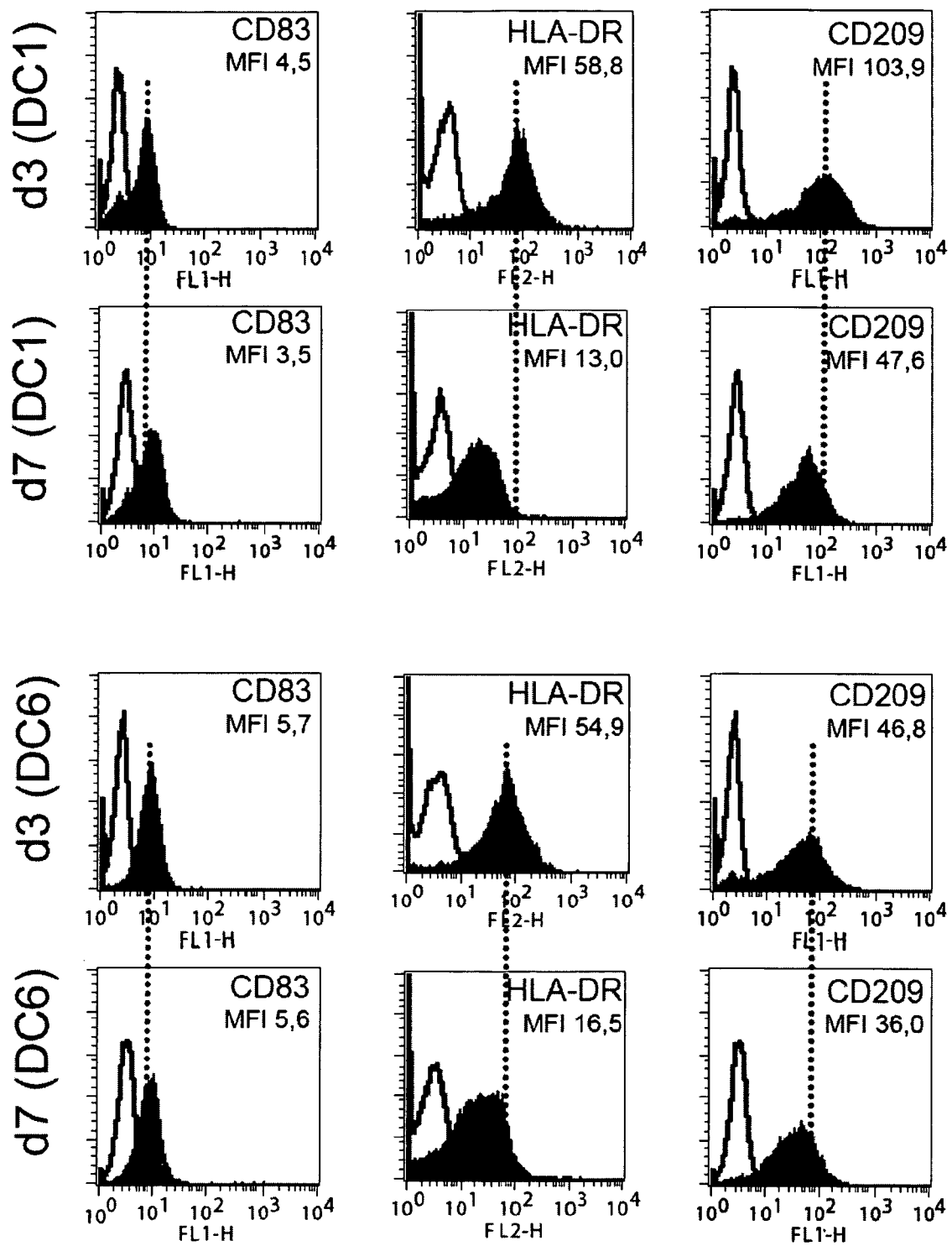


Figure 2b

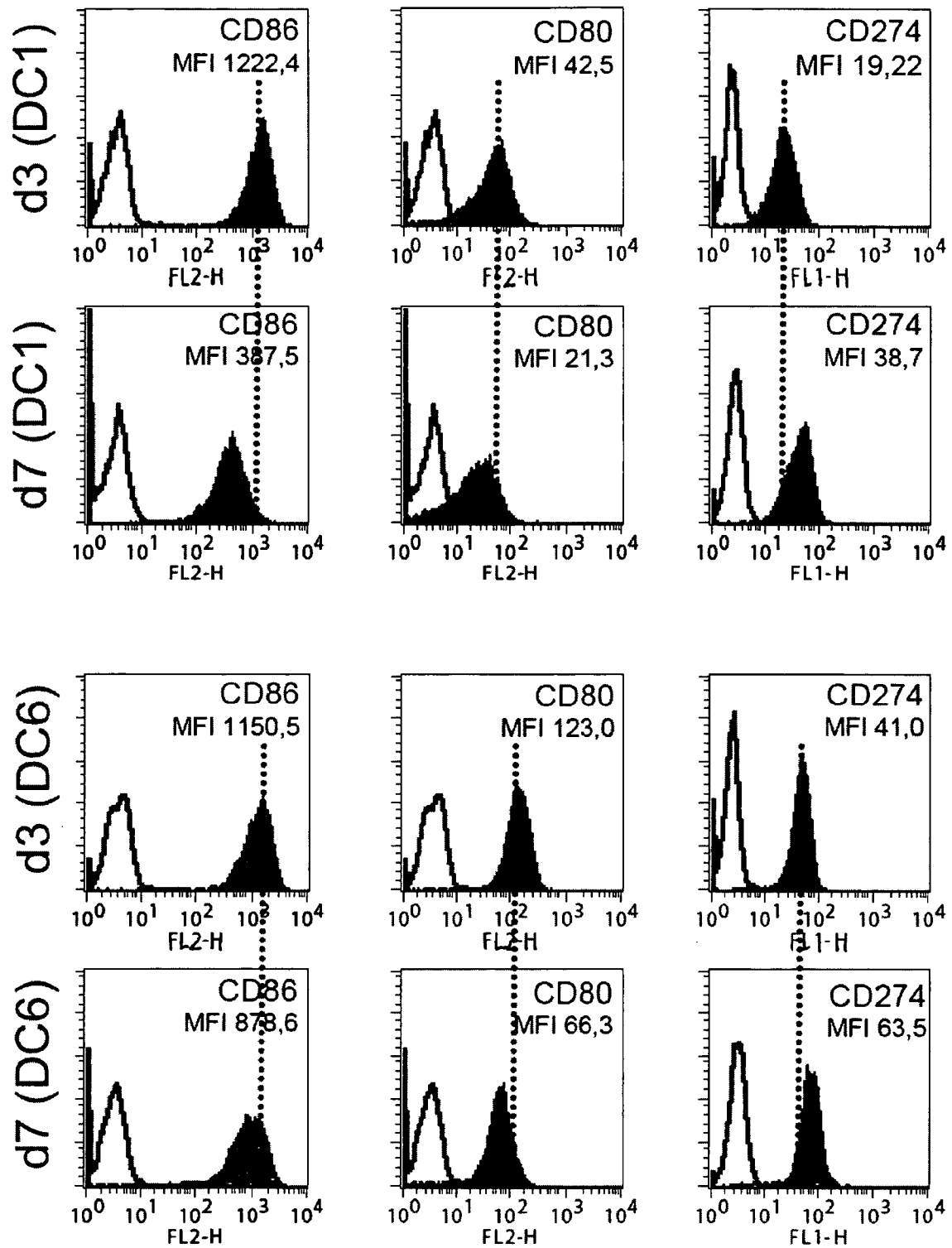


Figure 3

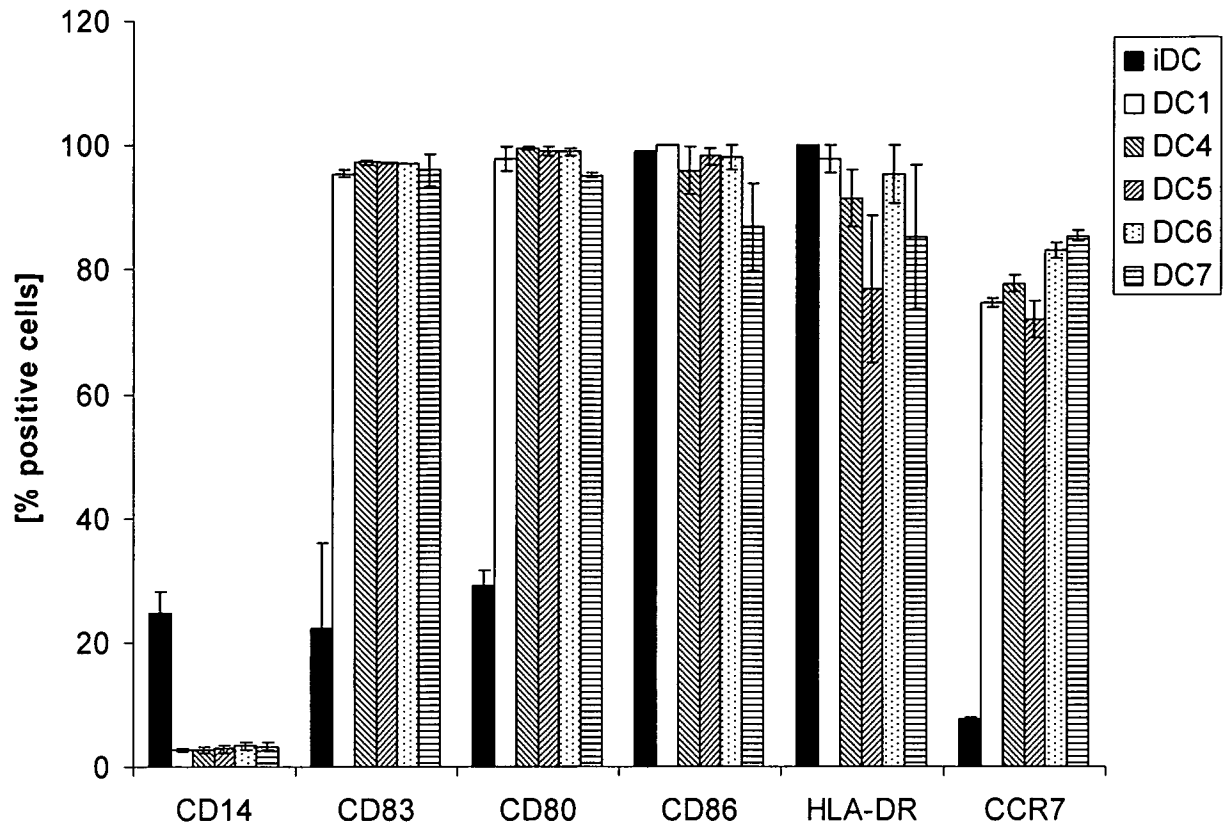


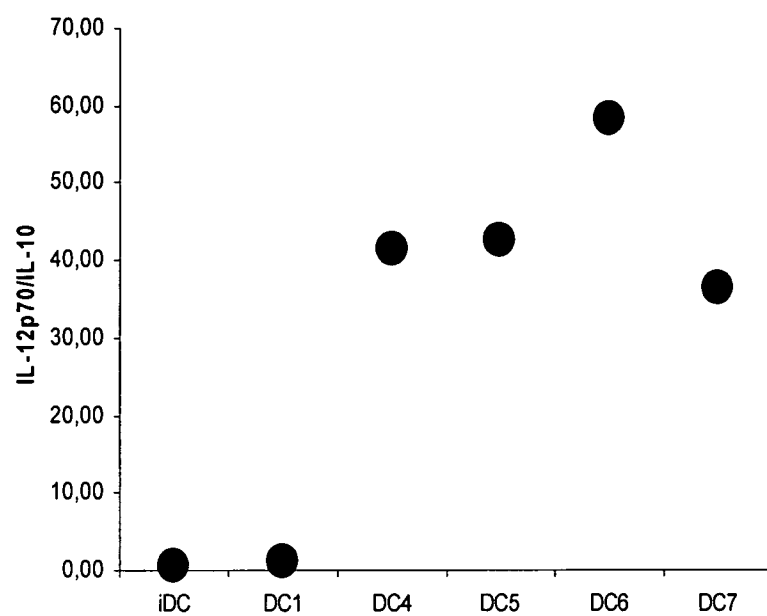
Figure 4

Figure 5

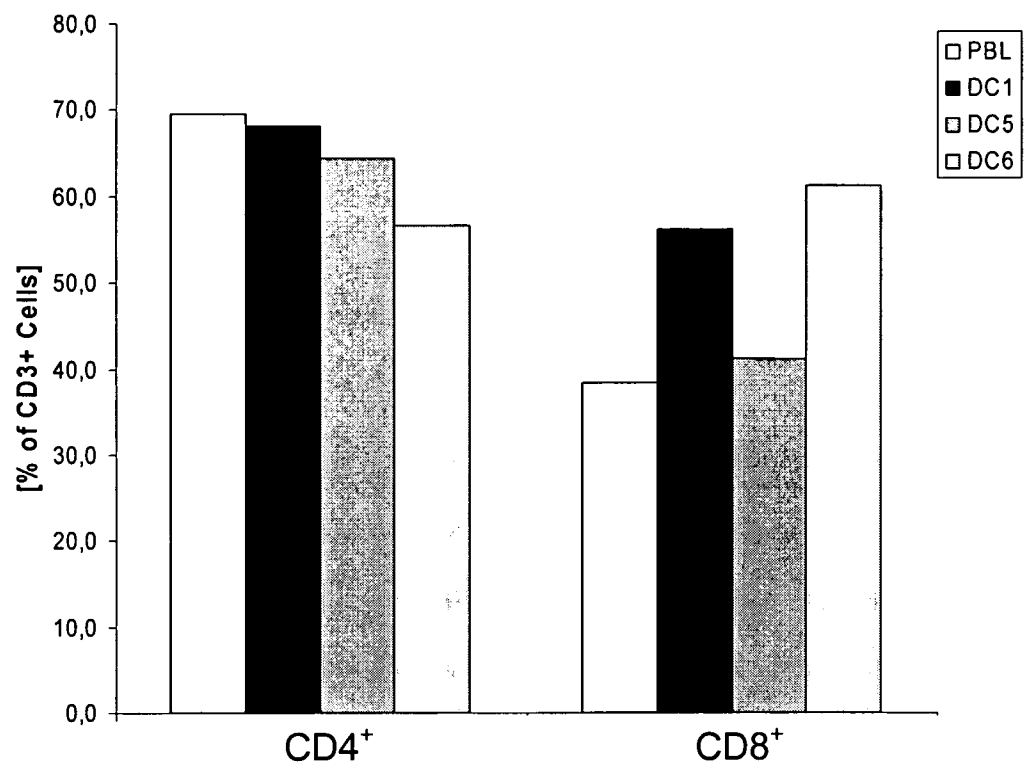


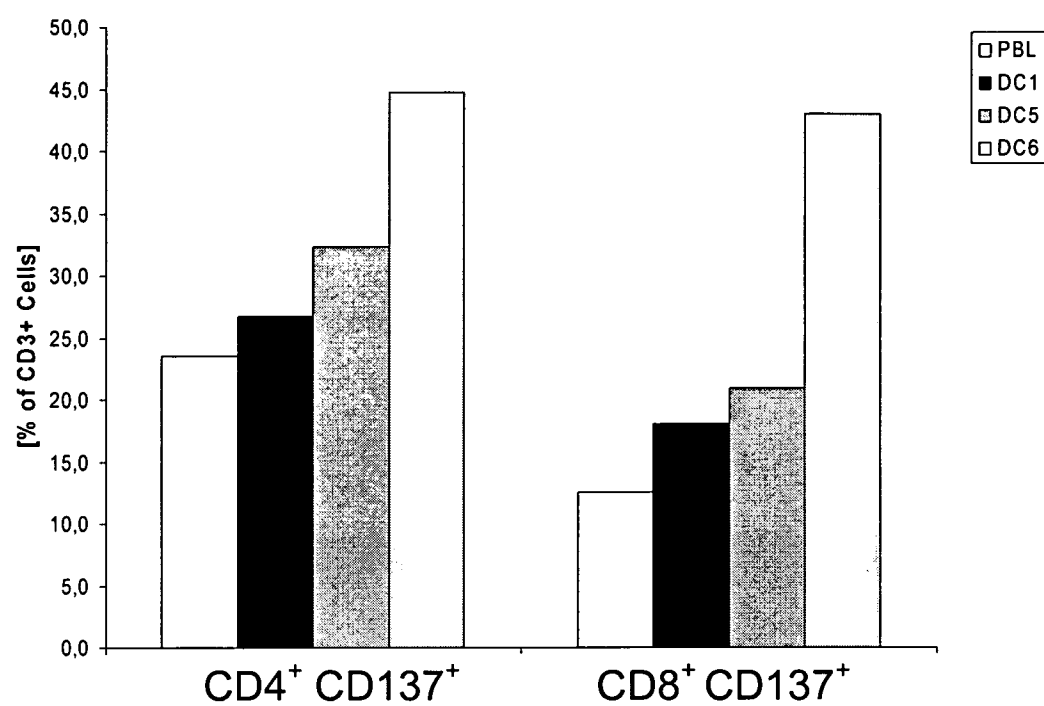
Figure 6

Figure 7

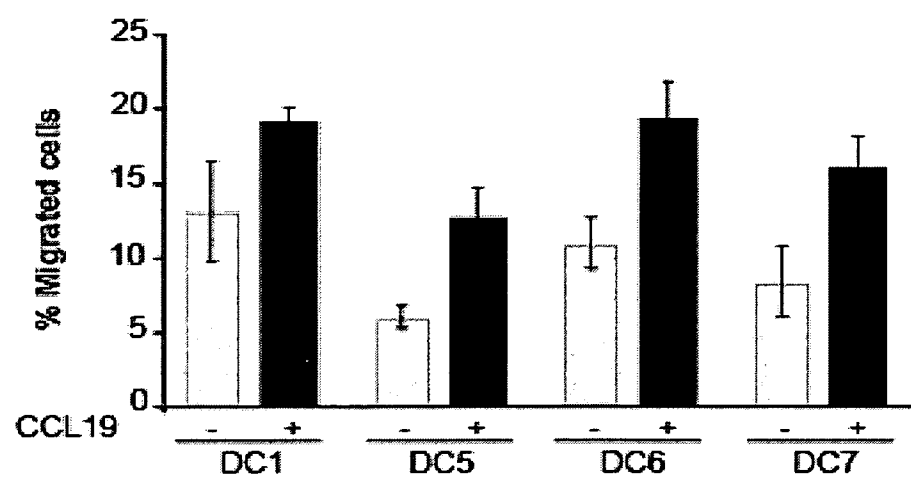


Figure 8

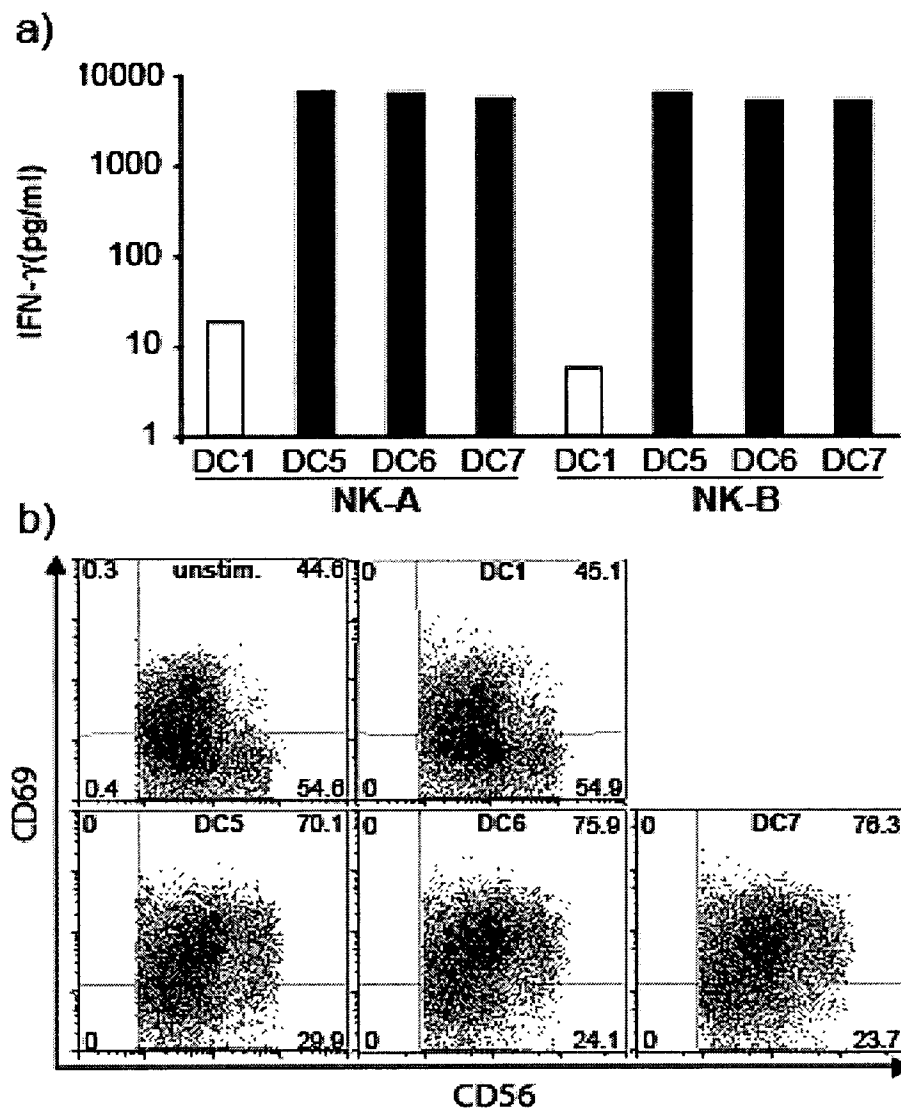


Figure 9

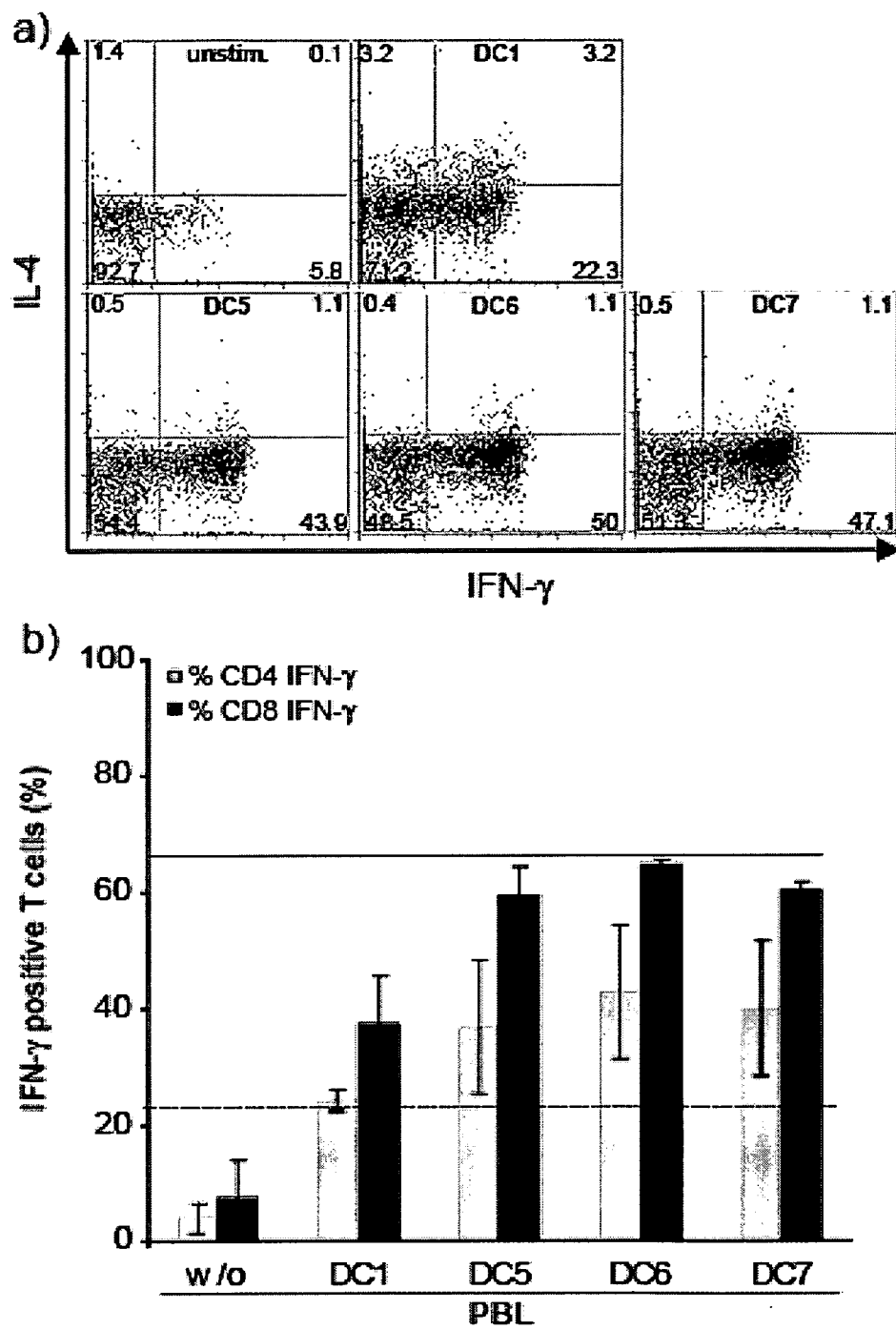


Table 1

	<i>C1 (Jon)</i>	<i>C4</i>	<i>C5</i>	<i>C6</i>	<i>C7</i>
	Final conc.	final conc	final conc.	Final conc	final conc
GM-CSF	100 ng/ml	100 ng/ml	100 ng/ml	100 ng/ml	100 ng/ml
IL-4	20 ng/ml	20 ng/ml	20 ng/ml	20 ng/ml	20 ng/ml
IL-1 β	10 ng/ml	10 ng/ml	10 ng/ml	10 ng/ml	10 ng/ml
TNF- α	10 ng/ml	10 ng/ml	10 ng/ml	10 ng/ml	10 ng/ml
IL-6	15 ng/ml	\	\	\	\
PGE ₂	1000 ng/ml	250 ng/ml	250 ng/ml	250 ng/ml	250 ng/ml
IFN- γ	\	5000 U/ml	5000 U/ml	5000 U/ml	5000 U/ml
R848	\	1 μ g/ml	1 μ g/ml	\	\
CL075	\	\	\	1 μ g/ml	1 μ g/ml
Poly I:C*	\	\	20 ng/ml	20 ng/ml	\

* Incubation at 55 °C for 5 minutes at room temperature and then diluted 1:10 with endotoxin-free water.

Table 2

		iDC	DC1	DC4	DC5	DC6	DC7
Recovery (% seeded cells)	N*	2	4	4	4	3	4
	m+	5,2	7,9	10,2	8,8	9,0	9,0
	std#	0,8	1,7	1,8	2,1	1,9	0,6

Table 3

	CD14	CD83	CD80	CD86	HLA-DR	CCR7
iDC	19 ± 0	14 ± 1	16 ± 2	144 ± 22	497 ± 88	25 ± 5
DC1	20 ± 3	86 ± 14	76 ± 6	760 ± 54	732 ± 101	56 ± 4
DC4	16 ± 3	62 ± 11	146 ± 19	951 ± 111	541 ± 73	51 ± 3
DC5	14 ± 2	61 ± 10	143 ± 17	946 ± 143	542 ± 75	48 ± 5
DC6	12 ± 1	48 ± 3	144 ± 14	842 ± 213	496 ± 91	58 ± 3
DC7	13 ± 2	50 ± 9	136 ± 20	765 ± 145	538 ± 76	63 ± 5

±Data represent mean fluorescence intensities (MFI) ± standard error for 3-4 independent donors

Table 4

	Donor 1		Donor 2	
	IL-10	IL-12	IL-10	IL-12
DC1	5 ± 1	673 ± 446	5 ± 0	n.d.
DC4	59 ± 0	16346 ± 111	80 ± 1	30348 ± 1111
DC5	80 ± 6	15952 ± 0	112 ± 6	62450 ± 566
DC6	83 ± 2	47070 ± 252	695 ± 14	205478 ± 19271
DC7	102 ± 5	39604 ± 122	719 ± 33	211411 ± 3380

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/007746

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07D513/12 A61K31/54 C12N5/0784

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)

C07D A61K C12N

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 practical, search terms used)

EPO-Internal, BIOSIS, 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.
X	ZOBYWALSKI ANKE ET AL: "Generation of clinical grade dendritic cells with capacity to produce biologically active IL-12p70" JOURNAL OF TRANSLATIONAL MEDICINE, BIOMED CENTRAL, LONDON, GB, vol. 5, no. 1, 12 April 2007 (2007-04-12), page 18, XP021024929 ISSN: 1479-5876 cited in the application	1-3,5-15
Y	figures 2, 3; table I	4
X	WO 2007/110240 A1 (GSF FORSCHUNGSZENTRUM UMWELT [DE]; SCHENDEL DOLORES J [DE]; BIGALKE IR) 4 October 2007 (2007-10-04)	1-3,5-15
Y	the whole document	4
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☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

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

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

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

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Date of the actual completion of the international search

1 March 2010

Date of mailing of the international search report

23/03/2010

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2009/007746

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Information on patent family members

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