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(54) Title: METHODS FOR OBTAINING DENDRITIC CELLS

(57) Abstract: The invention relates to methods for obtaining DNNGR-1⁺ dendritic cells, believed to be the human equivalents of murine CD8a⁺ dendritic cells, by differentiation in vitro from hematopoietic stem cells (HSCs) using specific cocktails of differentiation factors. The invention also extends to generation of such dendritic cells in reconstituted mice, as well as their use in immunotherapeutic methods including antigen pulsing and adoptive transfer approaches.



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Methods for obtaining dendritic cellsField of the Invention

The invention relates to methods for obtaining a human
5 equivalent of murine CD8 α ⁺ dendritic cells (DC) and the
exploitation of such cells in immunotherapeutic approaches.

Background to the Invention

10 Mouse CD8 α ⁺ DC have attracted much attention and have been
studied extensively. These cells possess several functional
properties that distinguish them from other DC subtypes. These
include a superior capacity to cross-present exogenous
antigens on MHC class I, to ingest material from dead or dying
cells and to produce IL-12 in response to innate and T cell
15 derived stimuli (Heath and Carbone, 2009; Naik, 2008;
Villadangos and Schnorrer, 2007). Because of these properties,
CD8 α ⁺ DC have emerged as an attractive cellular target for
vaccination strategies, in particular those aimed at eliciting
cytotoxic T cell (CTL) responses against tumour or virus-
20 infected cells. In addition, CD8 α ⁺ DC can also induce
conversion of antigen specific T cells into regulatory T cells
(Treg), suggesting that antigen delivery to CD8 α ⁺ DC could be
used to dampen immune reactivity (Yamazaki et al., 2008).

25 Unfortunately, the translation of mouse experiments into human
studies and clinical protocols has been hampered by the fact
that CD8 α ⁺ DC have not been identified in humans.

30 DNNGR-1 is a C-type lectin which has recently been identified
as a novel marker for mouse CD8 α ⁺ DC (Caminschi et al., 2008;
Sancho et al., 2008; WO2009/013484) and also of an apparently
equivalent subset of dendritic cells in humans. Jongbloed et
al. (J. Exp. Med. 2020, 207(6): 1247-60), published after the
priority date of this application, has recently confirmed that
35 human DNNGR-1⁺ DCs are functionally similar to murine CD8 α ⁺ DC.

There remains a need for reliable and reproducible methods for isolation and/or generation of such cells in order to facilitate their further study and use.

5 **Summary of the Invention**

The present inventors have identified DC which express DNGR-1 (DNGR-1 DC) in human secondary lymphoid tissues, specifically in primary human spleen. However, only a low yield of such cells could be obtained, due to restricted access to tissue, paucity of DC in samples, and difficulty in recovering live
10 cells from frozen samples.

Consequently, the inventors have developed protocols for obtaining DNGR-1⁺ DC from hematopoietic stem cells (HSCs). The
15 ability to generate these cells in reconstituted mice, or to grow them *in vitro*, offers tremendous opportunities for studying their properties and for using them in immunotherapeutic methods including antigen pulsing and adoptive transfer approaches.

20 Thus, in a first aspect, the invention provides a method of obtaining a dendritic cell population comprising one or more DNGR-1⁺ DC, the method comprising the step of contacting a HSC with a differentiation cocktail *ex vivo* or *in vitro*, wherein
25 the differentiation cocktail contains a) GM-CSF (granulocyte-macrophage colony stimulating factor), IL-4 (Interleukin-4), and Flt3L (Fms-related tyrosine kinase 3 ligand); or b) GM-CSF, IL-4 and TGF- β (Transforming growth factor β).

30 It may also be desirable to contact the HSC with one or more stem cell viability factors. Stem cell viability factors are useful to maintain stem cell viability. For example HSCs may be co-cultured with stromal elements supportive of HSC survival or cultured in media conditioned by being previously
35 inoculated onto stromal feeder layers which secrete factors and proteins conducive to the maintenance and survival of HSCs. A specific example of such a factor is stem cell factor

(SCF) although others may be contemplated such as Thrombopoietin (TPO), Pleiotrophin, HoxB4, HoxA9, Wnt3a, Notch1 and Jagged-1. Factors such as Thrombopoietin, Pleiotrophin, HoxB4, HoxA9, Wnt3a, Notch1 and Jagged-1 may be used alone, or in combination. These factors may be used alone or in combination with one another. For example, any one or more of TPO, Pleiotrophin, HoxB4, HoxA9, Wnt3a, Notch1 and Jagged-1 may be used in combination with SCF.

It may be desirable to replenish or renew the differentiation cocktail periodically. Thus the differentiation cocktail may be replenished during the contacting step. The differentiation cocktail will typically be replenished once, but may be replenished twice, three times, four times or more if desired e.g. every 5, 6, 7 or 8 days. The HSCs may be contacted with the differentiation cocktail for as long as required for emergence of DNGR-1⁺ DC. Typically they will be contacted with the differentiation cocktail for at least 6 days, e.g. at least 7, at least 8, at least 9, at least 10 days, or at least 21 days; e.g. up to 10 days, up to 11 days, up to 12 days, up to 13 days, up to 14 days, up to 15 days, up to 16 days, up to 17 days, up to 18 days, up to 19 days, or up to 21 days.

HSCs for use in the claimed method may be obtained from any suitable source. Sources include but are not limited to embryonic yolk sac, the embryonic aorta-gonad-mesonephrous region, fetal liver or spleen, adult spleen, adult bone marrow, fetal bone marrow, peripheral blood and umbilical cord blood. Thus the method may comprise the preliminary step of providing a sample containing HSCs. The HSCs may be from any desired mammalian species, e.g. human, other primate, or domestic, laboratory or livestock animals, such as a rodent (e.g. mouse, rat or guinea pig), lagomorph (e.g. rabbit), cat, dog, pig, cow, horse, sheep or goat. In certain embodiments, the HSCs are human. In certain embodiments, the HSCs are derived from a human donor.

The sample may be contacted directly with the differentiation cocktail. However, it may be desirable to enrich the sample for HSCs and/or expand the HSCs in the sample before such a contacting step. This may involve a positive selection for cells expressing markers associated with HSCs. Additionally or alternatively enrichment for HSCs may involve negative selection to remove cells which are not HSCs. HSCs typically do not display lineage specific markers. Thus it may be desirable to deplete the sample of cells expressing lineage specific markers (so-called "lineage positive cells"). Such enrichment or depletion may be performed in addition to, or as an alternative to, an expansion step as described below.

It may be desirable to induce proliferation of a HSC, or population of HSCs, prior to contacting the HSCs with the differentiation cocktail. This allows the number of precursor cells to be increased before differentiation begins, thus increasing the final yield of DNCR-1⁺ DC. Thus the method may additionally comprise providing a first cell population comprising one or more HSCs, and expanding the number of HSCs in the first cell population, prior to contacting the cell population with the differentiation cocktail. The expansion step may comprise, for example, contacting said first cell population with one, more than one, or all of SCF, Flt3L, IL-3 (Interleukin-3) and IL-6 (Interleukin-6). Other factors such as Thrombopoietin, Pleiotrophin, HoxB4, HoxA9, Wnt3a, Notch1 and Jagged-1 may be used alone or in combination, particularly in combination with SCF.

In certain embodiments it may be desirable to deplete the sample of cells expressing lineage specific markers in order to obtain an enriched HSC population, and subsequently to expand the number of HSCs in the sample.

It will be understood that the dendritic cell population obtained by contacting HSCs with the differentiation cocktail may additionally include other cell types, including dendritic

cells that do not express DNGR-1, such as cells which express one or more of BDCA2, IRF4, TLR7 and TLR9. Such cells may be plasmacytoid dendritic cells (pDC).

5 The invention also relates to methods of obtaining DNGR-1⁺ DC from rodents having xenogeneic immune cells (i.e. immune cells from another species). The rodent is typically immunocompromised, i.e. it does not have a functional immune system of its own and thus is not capable of mounting an
10 immune response against a graft of immunologically different (allogeneic or xenogeneic) cells or tissue. Thus the invention provides a method of obtaining a dendritic cell population comprising one or more DNGR-1⁺ DC, the method comprising a) obtaining cells from the lymphoid tissue of a rodent
15 reconstituted with xenogeneic immune cells; and b) isolating DNGR-1⁺ DC. The method may further comprise the step of reconstituting the rodent. Thus the method of the invention may further comprise a) providing an immunocompromised rodent; and b) introducing xenogeneic immune cells into said rodent.
20 The rodent may thus be reconstituted with xenogeneic immune cells which may be obtained from HSCs. The step of isolating DNGR-1⁺ DC may additionally comprise positive selection for cells expressing BDCA3 and/or HLA-DR.
25 The rodent may be a mouse, for example, a NOD/SCID mouse, in particular a NOD/SCID/ β_2m null mouse or a NOD/SCID/ γc null mouse.

The xenogeneic HSCs may be derived from any desired mammalian
30 species, e.g. human, other primate, or domestic, laboratory or livestock animals, such as another species of rodent (e.g. mouse, rat or guinea pig), lagomorph (e.g. rabbit), cat, dog, pig, cow, horse, sheep or goat. In certain embodiments, the HSCs are human HSCs.

35 The lymphoid tissue may be taken from any suitable lymphoid organ but will often be derived from the spleen.

Whichever of the above-described methods is used to provide the DC population, a DC population is obtained which comprises one or more DNGR-1⁺ DC.

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In a further aspect it may be desirable to further isolate or purify one or more DNGR-1⁺ DC. Therefore the method may additionally comprise the step of isolating a DNGR-1⁺ DC, or a population thereof.

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A DNGR-1⁺ DC, or population thereof, may be isolated by contacting the cells with a binding agent capable of binding to DNGR-1. Antibodies directed against (e.g. specific for) DNGR-1, and functional fragments of such antibodies having an antibody binding site, are particularly suitable for use as

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If desired, the binding agent or antibody may be provided on a solid support (such as a particle or bead, e.g. a magnetic bead) to facilitate isolation of the desired cells.

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Alternatively, the step of isolating DNGR-1⁺ DC may be performed using cell sorting techniques such as flow cytometry, e.g. FACS.

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It is believed that most or all human DNGR-1⁺ DC also express BDCA3 and HLA-DR. Thus the method may additionally comprise positive selection for cells expressing BDCA3 (BDCA3⁺ DC) and/or HLA-DR, e.g. using a binding agent (such as an antibody or antibody fragment) having the ability to bind to BDCA3 or HLA-DR.

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The invention provides a dendritic cell population comprising one or more DNGR-1⁺ DC as obtainable by any one of the methods of the invention as described above.

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Having been isolated to the desired level of purity, the DNGR-1⁺ DC isolated by the methods described above may find application in various ways.

- 5 For example, the cells may be administered directly to a subject, or formulated for administration to a subject directly. Thus the invention provides a dendritic cell population as obtained or obtainable by any of the methods of the invention as described above, for use in a method of
- 10 medical treatment. The invention further provides a dendritic cell population as obtained or obtainable by any of the methods of the invention, for use in the treatment of any suitable condition as described in more detail below. The invention further provides the use of a dendritic cell
- 15 population as obtained or obtainable by any of the methods of the invention, in the preparation of a medicament for the treatment of any suitable condition as described in more detail below.
- 20 It may be desirable to expose the DNGR-1⁺ DC to an antigen in order to enable that antigen to be presented to T cells. This approach is sometimes described as "antigen pulsing". It may be desirable that the antigen is thus cross-presented to T cells via MHC class I molecules.
- 25 Thus the invention further provides a method comprising contacting the DC population obtained or obtainable by the methods described above *in vitro* or *ex vivo* with an antigen.
- 30 The DC population may also be contacted with an adjuvant. Contacting may take place *in vitro*, for example at or approximately at the same time as contacting with the antigen, or at or after administration to the recipient subject. The nature of the adjuvant may be selected depending on the nature
- 35 of the desired immune response.

The antigen-pulsed DC population may then be administered to a subject, or formulated for administration to a subject. The subject may be the same individual from whom the HSCs were originally derived.

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Thus the invention provides an antigen-pulsed dendritic cell population as obtained or obtainable by any of the methods of the invention as described above, for use in a method of medical treatment. The invention further provides an antigen-pulsed dendritic cell population as obtained or obtainable by any of the methods of the invention, for use in the treatment of any suitable condition as described in more detail below. The invention further provides the use of an antigen-pulsed dendritic cell population as obtained or obtainable by any of the methods of the invention, in the preparation of a medicament for the treatment of any suitable condition as described in more detail below.

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The antigen-pulsed DC population may further be contacted with T cells *in vitro* or *ex vivo* in order to generate or stimulate T cells (particularly CTLs, but also helper T cells or Tregs) specific for the antigen. The T cells in the population may be allowed to expand in culture in order to increase the number or proportion of T cells in the population which are specific for the antigen. The T cells may then be administered to a subject or formulated for such administration. Optionally the T cells are separated from other cells in the population before administration. As in other aspects of the invention, the dendritic cells and T cells may also be contacted with an adjuvant. The nature of the adjuvant may be selected depending on the nature of the desired immune response.

35

Preferably, the T cells and dendritic cells are autologous, i.e. they are derived from the same subject, or from genetically identical subjects.

The T cells may be re-administered to the subject from whom they (or their progenitors) were derived.

Again, an adjuvant may be administered with the T cells.

Thus the invention provides a population of T cells as obtained or obtainable by the above methods, for use in a method of medical treatment. The invention further provides such a population of T cells, for use in the treatment of any suitable condition as described in more detail below. The invention further provides the use of such a population of T cells in the preparation of a medicament for the treatment of any suitable condition as described in more detail below.

Description of the Drawings

Figure 1. DNCR-1⁺ BDCA3⁺ DC are found in spleen of humans

(A) Flow cytometry analysis of human spleen cell suspensions from cadaveric donors. Live HLA-DR⁺ Lin⁻ cells were gated as shown (upper left panel) and analyzed for expression of BDCA3 versus DNCR-1 (upper right panel). Three populations were defined (gate I: BDCA3^{hi}, DNCR-1⁺; gate II: BDCA3^{int}, DNCR-1⁻; gate III: BDCA3⁻, DNCR-1⁻) and analyzed for the expression of CD11c versus BDCA1, BDCA2 or CD11b (lower panels). Numbers indicate percentage of cells in each of the indicated gates or quadrants. Arrows show gating strategy. (B) Normalized expression of DNCR-1, Necl2 and IRF8 mRNA on sorted HLA-DR⁺ Lin⁻ BDCA2⁻ BDCA1⁻ DCs, from human spleen, expressing high (BDCA3^{hi}), intermediate (BDCA3^{int}), or low levels of BDCA3 (BDCA3⁻). ND, not detectable. Data in A and B are representative of at least 3 cadaveric donors. Additional data analysis is shown in Fig. 9.

Figure 2. DNCR-1⁺ BDCA3^{hi} DC are found in the spleens of humanized mice

HLA-DR⁺ Lin⁻ live spleen cells from humanized mice were analyzed as in Figure 1 for the expression of BDCA3 versus BDCA2 (upper right), BDCA3 versus DNNGR-1 (middle left), and BDCA2 versus DNNGR-1 (middle right). The scatter profile of BDCA3⁺ and BDCA2⁺ cells is also shown (lower panels). Numbers indicate percentage of cells in each of the indicated gates or quadrants. Data are representative of multiple mice from 3 independent cohorts of mice engrafted with human HSCs.

Figure 3. Phenotype of in vitro generated DNNGR1⁺ BDCA3⁺ DCs

CBDCs were generated as described in the methods. Live cells were analyzed for the expression of BDCA3 versus DNNGR-1 (A), CD1a versus DNNGR-1 (B, left panel) and CD1a versus HLA-DR (B, middle panel). Gated CD1a⁺ HLA-DR⁺ cells were analyzed for the expression of BDCA3 versus DNNGR-1 (B, right panel). Numbers indicate percentage of cells in each of the indicated gates or quadrants. (C) CBDCs were treated with or without poly I:C (10 µg/ml) overnight and subsequently sorted into live HLA-DR⁺ Lin⁻ DNNGR-1⁺ cells. Cytospins were prepared and morphology assessed by hematoxylin-eosin staining. Bars: 5 µm. (D) Live DNNGR-1⁺ DC were analyzed for the expression (red) of CD11c, BDCA1, CD8α, BDCA2, CD83, CD86, CD40, CD103, CD45RO, CD123, CCR7, EpCam and CD11b. Isotype-matched control mAb staining is shown in the blue histograms. Data in A-D are representative of multiple CBDC cultures with 2 independent pools of cord blood derived HSCs.

Figure 4. DNNGR-1⁺ BDCA3⁺ DCs display a gene expression profile characteristic of mouse CD8α⁺ DCs

Normalized expression of DNNGR-1, Necl2, CD207, BATF3, IRF8, IRF4, TLR7, TLR9, DAP12, TLR8 and TLR3 mRNA in BDCA3⁺ DNNGR-1⁺ DCs (DNNGR-1⁺) purified either from CBDCs or from pooled spleens of 2 to 5 humanized mice (hu mice). Expression was compared to that of BDCA2⁺ pDCs purified from the same humanized mouse spleens. Data are representative of 2 independent experiments. ND, not detectable.

Figure 5. DNNGR-1⁺ BDCA3⁺ DCs respond to TLR3 and TLR8 but not TLR7 agonists

(A) Purified BDCA3⁺ DNNGR-1⁺ DCs from CBDCs (DNNGR-1⁺ CBDCs) or from humanized mice (DNNGR-1⁺ hu mice), or BDCA2⁺ pDCs from the same humanized mice (BDCA2⁺ hu mice), were cultured with poly I:C (10µg/ml), imiquimod (10µg/ml) or medium alone. After overnight incubation, culture supernatant was tested for TNF-α and IL-6 content.

(B) DNNGR-1⁺ CBDCs as in (A) were cultured with resiquimod (10µg/ml), poly U (10µg/ml) or medium alone. Supernatant was tested for IL-6 after overnight culture. (C) 5x10³ DNNGR-1⁺ BDCA3⁺ DCs purified from CBDCs (DNNGR-1⁺ CBDCs) or human spleen (DNNGR-1⁺ hu spleen) were cultured with a mix of TLR agonists and cytokines with or without T cells and antigen, as described in the methods. Supernatant was tested for IL-12p70 after overnight culture. Data are representative of at least 2 independent experiments with independent sources of cells. ND, not detectable.

Figure 6. DNNGR-1⁺ BDCA3⁺ DCs efficiently capture dead-cells and cross-present exogenous antigens

(A) CBDCs or Mo-DCs were incubated with labelled dead melanoma cells at the indicated ratios, as described in the methods. Uptake of dead cell material by DNNGR-1⁺ DCs or Mo-DC was quantified by flow cytometry (left) and confirmed by confocal microscopy (right; blue shows dead cell material; bar: 5 µm). Data are mean ± SEM of three biological replicates from one experiment and are representative of two independent experiments.

(B) 10⁴ purified BDCA3⁺ DNNGR-1⁺ CBDCs or Mo-DC, as indicated, were pulsed for 2-3 h with 1 µM MelanA/MART-1 long peptide as antigen source for cross-presentation or the same concentration of short peptide as a processing-independent control, in the presence or absence of poly I:C. Cells were subsequently washed twice and co-cultured with a MelanA-specific CD8⁺ T cell clone at a 5:1 cell ratio. IFN-γ accumulation in culture supernatants was assessed after 40 h.

Data are the mean value of duplicate wells $\pm$ range and are representative of 2 independent experiments.

Figure 7. Determination of the appropriate differentiation cocktails

A number of factors were tested in different combinations for the ability to generate DNDR-1⁺ DC from HSCs. Twenty combinations were tested and 2 were found suitable as indicated (V). DNDR-1⁺ BDCA3⁺ DC could be obtained after culture with SCF, Flt3L, GM-CSF and IL-4. DNDR-1⁺ BDCA3⁺ DC could also be obtained by substituting Flt3L for TGF- β . Cultures containing TNF α did not produce DNDR-1⁺ DC.

Figure 8. In vitro generation of DNDR-1⁺ DC with the GM-CSF, IL-4, TGF- β differentiation cocktail

CBDCs were generated as described in the methods. Live cells were analyzed for the expression of BDCA3 versus DNDR-1 at day 6 of culture. Numbers indicate percentage of cells in each of the specified gates. Data shown are representative of multiple CBDC cultures with 2 independent pools of cord blood derived HSCs.

Figure 9. Flow cytometry analysis of human spleen cell suspensions from cadaveric donors

Live HLA-DR⁺ Lin⁻ cells (same as in Fig. 1) were analyzed for expression of CD11c versus DNDR-1 (upper panel). Three populations were identified (gate I: CD11c⁺, DNDR-1⁺; gate II: CD11c⁺, DNDR-1⁻; gate III: CD11c⁻, DNDR-1⁻) and analyzed subsequently for the expression of BDCA3 versus BDCA1, BDCA2, or CD11b (lower panels). Numbers indicate percentage of cells in each of the indicated gates or quadrants. Data are representative of 3 cadaveric donors.

Figure 10. DNDR-1⁺ BDCA3⁺ CBDCs do not express CD14 and do not develop in the absence of SCF, GM-CSF, Flt3L or IL-4

(A) The requirement for each growth factor in the CBDC differentiation cocktail was assessed by culturing amplified

cord blood HSCs with differentiation cocktails lacking SCF (GM-CSF, Flt3L, and IL-4 (no SCF)); GM-CSF (SCF, Flt3L, and IL-4 (no GM-CSF)); IL-4 (SCF, GM-CSF, and Flt3L (no IL-4)); or Flt3L (SCF, GM-CSF, IL-4 (no Flt3L)). After 13 days, live cells were analyzed for the expression of BDCA3 versus DNNGR-1. Numbers indicate percentage of cells in each of the indicated gates or quadrants.

(B) Live HLA-DR⁺ Lin⁻ (CD3/CD16/CD19/CD20/CD56) DNNGR-1⁺ BDCA3⁺ DC (DNNGR-1⁺) (left) were compared to DNNGR-1⁻ BDCA3⁻ (DNNGR-1⁻) (right) in the same CBDC culture for the expression of CD14 (red histograms). Isotype-matched control staining is shown by the blue histograms. Data in A and B are representative of multiple CBDC cultures with 2 independent pools of cord blood derived HSCs.

Figure 11. Poly I:C stimulates production of cytokines by CBDCs and upregulation of HLA-DR in DNNGR-1⁺ BDCA3⁺

Bulk CBDCs were cultured with poly I:C (10µg/ml), imiquimod (10µg/ml) or medium alone. After overnight incubation, culture supernatant was tested for TNF-α content (left) or the cells were recovered and live DNNGR-1⁺ BDCA3⁺ DC tested for expression of HLA-DR (right; mean fluorescence intensity of HLA-DR staining). Data are representative of 2 independent experiments.

Figure 12. CBDCs efficiently cross-present exogenous antigens

10⁴ bulk CBDCs or Mo-DCs were pulsed for 2-3 h with 10µM NY-ESO-1 protein (left) or 1µM pre-processed peptide (right) in the presence or absence of poly I:C before co-culture with an NY-ESO-11₁₅₇₋₁₆₅-specific T cell clone at a 5:1 T:DC ratio. Fixed DC were used as a control to exclude extracellular antigen processing. IFN-γ accumulation in culture supernatants was assessed after 40 h of T/DC co-culture. Data are representative of 2 independent experiments. ND, not detectable.

Detailed Description of the Invention**DNGR-1⁺ dendritic cells**

Dendritic cell, NK lectin group receptor 1 (DNGR-1) (also known as Clec9a - see for example WO2009/013484) is a C-type lectin expressed on certain dendritic cells.

These cells are capable of cross-presenting extracellular antigen via MHC class I molecules. By "extracellular" is meant that the antigen has been taken up by the cell from its extracellular environment, typically by endocytosis or phagocytosis.

As used in this specification, the term DNGR-1 is intended to embrace the human protein, the murine protein, their homologues (especially orthologues) in other species, and variants and derivatives thereof which retain DNGR-1 activity. Such variants and derivatives preferably have at least about 30% sequence identity, more preferably at least about 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95% sequence identity to the human protein sequence shown below, or at least about 35% identity, more preferably at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95% identity to the extracellular domain (CTLD) of the human protein sequence shown below.

Human DNGR-1 cDNA sequence:

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ATGCACGAGGAAGAAATATACACCTCTCTTCAGTGGGATAGCCCAGCACCAGACACTTACCA
GAAATGTCTGTCTTCCAACAAATGTTTCAGGAGCATGCTGTCTTGTGATGGTGATTTTCATGTG
TTTTCTGCATGGGATTATTAACAGCATCCATTTTCTTGGGCGTCAAGTTGTTGCAGGTGTCC
ACCATTGCGATGCAGCAGCAAGAAAACTCATCCAACAAGAGAGGGCACTGCTAAACTTTAC
AGAATGGAAGAGAAGCTGTGCCCTTCAGATGAAATATTGCCAAGCCTTCATGCAAACTCAT
TAAGTTCAGCCCATAACAGCAGTCCTTGTCCAAACAATTGGATTTCAGAACAGAGAAAAGTTGT
TACTATGTCTCTGAAATTTGGAGCATTTGGCACACCCAGTCAAGAGAATTGTTTAAAGGAAGG
TTCCACGCTGCTACAAATAGAGAGCAAAGAAGAAATGGATTTTATCACTGGCAGCTTGAGGA
AGATTAAAGGAAGCTATGATTACTGGGTGGGGTTGTCTCAGGATGGACACAGCGGACGCTGG
CTTTGGCAAGATGGCTCCTCTCCTTCTCCTGGCCTGTTGCCAGCAGAGAGATCCCAGTCAGC

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TAACCAAGTCTGTGGATACGTGAAAAGCAATTCCCTTCTTTCTGCTAACTGCAGCACGTGGA
AGTATTTTATCTGTGAGAAGTATGCGTTGAGATCCTCTGTCTGA

Human DNGR-1 protein sequence:

5 MHEEEIYTSLQWDSAPADTYQKCLSSNKCSGACCLVMVISCVFCMGLLTASIFLGVKLLQVS
TIAMQQQEKLIQQERALLNFTEWKRSALQMKYCQAFMQNSLSSAHNSSPCPNWIONRESC
YYVSEIWSIWHTSQENCLKEGSTLLQIESKEEMDFITGSLRKIKGSYDYWVGLSQDGHSGRW
LWQDGSSPSPGLLPAERSQSANQVCYVKSNSLLSSNCSTWKYFICEKYALRSSV
(The transmembrane portion is shown in italics and the CTLD is
10 underlined.)

Murine DNGR-1 cDNA sequence:

ATGCATGCGGAAGAAATATATACCTCTCTTCAGTGGGACATTCCTACCTCAGAGGCCTCTCA
GAAGTGCCAATCCCCTAGCAAATGTTCAGGAGCATGGTGTGTTGTGACGATGATTTCTGTG
15 TGGTCTGTATGGGCTTGTTAGCAACGTCCATTTTCTTGGGCATCAAGTTCTTCCAGGTATCC
TCTCTTGTCTTGGAGCAGCAGGAAAGACTCATCCAACAGGACACAGCATTGGTGAACCTTAC
ACAGTGGCAGAGGAAATACACACTGGAATACTGCCAAGCCTTACTGCAGAGATCTCTCCATT
CAGGCACAGATGCTTCTACTGGACCAGTTCTTCTGACCTCTCCACAGATGGTTCACAGACC
CTGGACAGCAAGGAAACAGGTAGTGAAGTGCAGCCCTTGTCCACACAACTGGATTGAGATGG
20 AAAAAGTTGTTACTATGTCTTTGAACGCTGGGAAATGTGGAACATCAGTAAGAAGAGCTGTT
TAAAAGAGGGCGCTAGTCTCTTTCAAATAGACAGCAAAGAAGAAATGGAGTTCATCAGCAGT
ATAGGGAACTCAAAGGAGGAAATAAATATTGGGTGGGAGTGTTCAGATGGAATCAGTGG
ATCTTGGTTCTGGGAAGATGGCTCTTCTCCTCTCTCTGACTTGTTGCCTGCAGAAAGACAGC
GATCAGCCGGCCAGATCTGTGGATACCTCAAAGATTCTACTCTCATCTCAGATAAGTGCGAT
25 AGCTGGAAATATTTTATCTGTGAGAAGAAGGCAGTTTGGATCCTGCATCTGA

Murine DNGR-1 protein sequence:

MHAEEIYTSLQWDIPTSEASQKQSPSKCSGAWCVVTMISCVVCMGLLTASIFLGKFFQVS
SLVLEQQERLIQQDTALVNLTQWQRKYTLEYCQALLQRLHSGTDASTGPVLLTSPQMVPQT
30 LDSKETGSDCSPCPHNWIONGKSCYYVFERWEMWNISKKSCLKEGASLFQIDSKEEMEFISS
IGKLKGGNKYWVGVFQDGISGSWFWEWGSSPLSDLLPAERQRSAGQICGYLKDSTLISDKCD
SWKYFICEKKAFGSCI

(The transmembrane portion is shown in italics and the CTLD is
underlined.)

35

DNGR-1 is highly restricted to BDCA3⁺ DC among peripheral blood
mononuclear cells (Caminschi et al., 2008; Huysamen et al.,

2008; Sancho et al., 2008). BDCA3 is one of a group of markers known as Blood DC Antigens (BDCA) which enable distinct DC subsets to be phenotypically identified in human blood (Dzionek et al., 2000). BDCA3 marks a small subset of DC that appear myeloid in origin (Dzionek et al., 2000; MacDonald et al., 2002). By contrast, BDCA2 appears to be a marker for circulating human pDC and is not believed to be expressed on DNNGR-1⁺ DC.

DNNGR-1⁺ DC also express HLA-DR, which is a general marker for antigen presenting cells, and it is believed that DNNGR-1⁺ BDCA-3⁺ HLA-DR⁺ cells are a distinct phenotypic population of cells in humans. Furthermore, DNNGR-1⁺ DC express Nect2 and do not express CD14 or CD11b distinguishing these cells from other DC subsets.

Murine CD8 α ⁺ DC express TLR9, CD103 and CD8 α but it appears that human DNNGR-1 cells do not express TLR9, CD103 or CD8 α , or express them only at very low levels.

The dendritic cell population obtained by the methods of the invention may additionally include dendritic cells that do not express DNNGR-1, such as cells which express one or more of BDCA2, IRF4, TLR7 and TLR9. Such cells may be plasmacytoid dendritic cells (pDC). BDCA2⁺ pDC respond to imiquimod but not poly I:C, while DNNGR-1⁺ DC respond to poly I:C but not imiquimod. Thus, DNNGR-1⁺ BDCA3⁺ DC and pDC have reciprocal patterns of response to TLR7 vs. TLR3 agonists, which could be exploited in vaccination strategies designed to mobilize one and/or the other cell type in the DC population.

Hematopoietic stem cells (HSCs)

Hematopoietic stem cells (HSCs) are multipotent stem cells that are capable of giving rise to all blood cell types including myeloid (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, dendritic cells), and lymphoid

lineages (T-cells, B-cells, NK-cells). As stem cells they also have the capacity to self-renew. Cell types referred to as hematopoietic precursor cells (HPCs), hematopoietic progenitor cells (HPCs), or hematopoietic multipotent progenitor cells (MPPs) also have this activity and are included within the term "HSC" as used herein.

HSCs are characterized by the ability to regenerate a multilineage hematopoietic system when introduced into an immunodeficient host. A multilineage hematopoietic system will contain all blood cells and their progenitors. HSCs introduced in such circumstances will be able to sustain multilineage hematopoiesis long term (for example, for the lifespan of the animal model) or transiently (for example, for 4 months or less).

The positive expression or negative expression (expression profile) of certain cell surface proteins may be used to identify HSCs with the potential to differentiate into DN^{GR}-1⁺ DC.

For example, HSCs are lineage negative (Lin⁻) i.e they do not express lineage-specific markers such as CD3, CD14, CD16, CD19, CD20 and CD56. They may also not express CD38, or only at low levels (CD38^{-/10⁺}). They typically express CD34⁺.

The expression profile of other cell surface proteins suitable for identifying HSCs will be known to one skilled in the art.

HSCs obtained from any source are suitable for use in the current method. Sources of HSCs include any tissue which contains any amount of HSCs. These tissues may include but are not limited to embryonic yolk sac, the embryonic aorta-gonad-mesonephrous region, fetal liver or spleen, adult spleen, adult and fetal bone marrow, untreated peripheral blood and umbilical cord blood. HSCs can also be mobilised into the peripheral blood and spleen tissue from the bone marrow by

cytokine treatment, e.g. with G-CSF (granulocyte-macrophage colony stimulating factor) or GM-CSF, and optionally cyclophosphamide. The HSCs may be from any desired mammalian species, e.g. human, other primate, or domestic, laboratory or livestock animals, such as a rodent (e.g. mouse, rat or guinea pig), lagomorph (e.g. rabbit), cat, dog, pig, cow, horse, sheep or goat. In certain embodiments, the HSCs are human. In certain embodiments, the HSCs are derived from human donors.

It may be desirable to enrich a sample for HSCs. HSCs suitable for use in the methods of the invention may be purified from a sample by any suitable technique or device. Positive or negative selection may be employed to enrich for, or deplete of, respectively cells expressing certain surface proteins associated with particular cell lineages or stages of differentiation.

Since HSCs are lineage negative, a sample of cells can be enriched for HSCs by negative selection for cells expressing one or more lineage specific markers (CD3, CD14, CD16, CD19, CD20 and CD56).

Once a population of cells containing HSCs has been obtained, the HSCs in this population may be expanded. The expansion step may occur before or after the cell population is enriched for HSCs. The expansion step may comprise, for example, culturing in hematopoietic bioreactors (see Cabrita et al., 2003), or co-culturing with stromal elements supportive of HSC expansion/survival, or culturing in media conditioned by being previously inoculated onto stromal feeder layers which secrete factors and proteins conducive to the maintenance, survival and expansion of HSCs. The expansion step may comprise contacting said first cell population with one or more of SCF, Flt3L, IL-3 and IL-6, for example, contacting said first cell population with one or more of SCF, Flt3L, IL-3 and IL-6.

Differentiation cocktail

The term "differentiation cocktail" is used in this specification to refer to the combination of cytokines and/or growth factors which are used to differentiate HSCs into DNCR-1⁺ DC. Two such combinations of factors have been identified, namely (a) GM-CSF, IL-4 and Flt3, and (b) GM-CSF, IL-4 and TGF- β .

The differentiation cocktail is contacted with HSCs *in vitro* or *ex vivo*, but typically under cell culture conditions. It will be appreciated that the culture medium may contain additional factors which have roles other than promoting differentiation of HSCs to DNCR-1⁺ DC. These components are not regarded as part of the differentiation cocktail.

For example, the culture medium may contain conventional cell culture components such as antibiotics (e.g. penicillin, streptomycin), nutrients (e.g. glutamine), heat-inactivated fetal calf serum or similar, etc.

The culture medium may also contain components for maintenance of stem cell properties such as viability and/or potency; these stem cell viability factors are described above and can be used individually or combinations as described. For example, SCF may be included in the culture medium but it does not constitute part of the differentiation cocktail. SCF may be provided in a concentration of about 15-25 ng/ml, for example about 20 ng/ml.

However, other cytokines and growth factors besides those present in the relevant differentiation cocktail should not be included, or should not be included at biologically active levels, in the culture medium. For example, G-CSF and TNF- α should not be included at all, TGF- β should not be present in the culture medium when differentiation cocktail (a) is used, and Flt3 should not be present in the culture medium when differentiation cocktail (b) is used.

Of course, this does not prevent the use of certain such factors at later stages of the methods described herein once adequate differentiation of HSCs to DNDR-1⁺ DC has taken place. For example, TNF α can be used as an adjuvant at the antigen pulsing stage or at the stage of antigen presentation by a DNDR-1⁺ DC to T cells, and can influence the resulting T cell response.

The HSCs may remain in contact with the differentiation cocktail for at least 6 days, e.g. at least 7, at least 8, at least 9 or at least 10 days. The contact may be maintained until sufficient differentiation has taken place. Typically contact will be maintained for up to 14 days, e.g. up to 10 days, up to 11 days, up to 12 days or up to 13 days. However it will be understood that longer exposure times may be employed if required, for example, up to 21 days or longer.

Preferably GM-CSF is provided at a concentration of about 15-25 ng/ml, for example at about 20 ng/ml. Preferably IL-4 is provided at a concentration of about 15-25 ng/ml, for example at about 20 ng/ml. Preferably Flt3L is provided at a concentration of about 80-120 ng/ml, for example at about 100 ng/ml. Preferably TGF- β is provided at a concentration of about 8-12 ng/ml, for example at about 10 ng/ml.

In a preferred embodiment the differentiation cocktail comprises about 20 ng/ml GM-CSF, about 20 ng/ml IL-4, and about 100 ng/ml Flt3L. In an alternative, preferred embodiment the differentiation cocktail comprises about 20 ng/ml GM-CSF, about 20 ng/ml IL-4, and about 10 ng/ml TGF- β . The term "about" implies +/- 5% in this context.

Immunocompromised rodent

An immunocompromised rodent suitable for use in the methods of the present invention has a deficient immune system enabling it to accept xenografts. Preferably an immunocompromised rodent will not produce functional B and T lymphocytes or

antibodies. The immunocompromised rodent will preferably show defects in NK cell activity and defects in innate immune effects. In a preferred embodiment the immunocompromised rodent may be reconstituted with tissue from another species without eliciting an immune response in said immunocompromised rodent. In a further embodiment the immunocompromised rodent may be reconstituted with stem cells from another species, without eliciting an immune response in said immunocompromised rodent.

Examples of a suitable immunocompromised rodent include the SCID mouse; SCID beige mouse; NOD/SCID mouse; NOD/SCID/ β_2m null mouse or NOD/SCID/ γc null mouse.

The immunocompromised rodent may be sublethally irradiated. The dose of radiation to be administered will depend on the size of the rodent and its susceptibility to radiation, but should be sufficient to deplete the rodent's immune cells without killing the rodent.

Immunocompromised rodents may then be reconstituted with HSCs. HSCs may be introduced by intraperitoneal or intrahepatic injections. HSCs may be injected directly into stem cell niches, such as bone marrow, or into the uterus. Placing HSCs in suitable haematopoietic microenvironments, such as newborn liver or bone marrow, may help progenitors to survive and engraft. Preferably HSCs are injected intravenously. HSCs may be obtained from the sources mentioned previously.

Immune response

The cell population comprising DNCR-1⁺ DC may play a role in induction of various types of immune response, for example, those involving CD8 T cells or Treg cells. They may be particularly involved in immune responses caused by immunogenic cell death, by taking up cellular debris from the dead or dying cells (or even absorbing the entire cell) and presenting processed fragments to T cells.

In vitro, or following administration to a subject, DNGR-1⁺ DC can induce an immune response to a target antigen with which they have been contacted. The immune response stimulated may be a Th1, Th2, Th17 or Treg response. The immune response may be the proliferation of T cells, which may be CTL or helper T cells. The immune response can be the proliferation of both CD8⁺ T cells and CD4⁺ T cells, and may involve the proliferation of both types of T cell in any given immune response. The cell population comprising DNGR-1⁺ DC may also stimulate and induce proliferation of Treg cells. Treg cells are characterised by the expression of Foxp3. Most Treg cells are CD4⁺ and CD25⁺, and can be regarded as a subset of helper T cells, although a small population may be CD8⁺. However, given that Treg cells may be capable of modulating the response of other cells of the immune system against an antigen in other ways, e.g. inhibiting or suppressing their activity, the effect on the immune system as a whole may be to modulate (e.g. suppress or inhibit) the response against that antigen. Thus an immune response can also comprise modulating the response to an antigen (e.g. inhibiting or suppressing).

It may be particularly desirable to raise a Treg response against an antigen to which a subject exhibits, or is at risk of developing, an undesirable immune response. For example, it may be a self antigen against which an immune response occurs in an autoimmune disease. Examples of autoimmune diseases in which specific antigens have been identified as potentially pathogenically significant include multiple sclerosis (myelin basic protein), insulin-dependent diabetes mellitus (glutamic acid decarboxylase), insulin-resistant diabetes mellitus (insulin receptor), coeliac disease (gliadin), bullous pemphigoid (collagen type XVII), auto-immune haemolytic anaemia (Rh protein), auto-immune thrombocytopenia (GpIIb/IIIa), myasthenia gravis (acetylcholine receptor), Graves' disease (thyroid-stimulating hormone receptor), glomerulonephritis, such as Goodpasture's

disease (alpha3(IV)NC1 collagen), and pernicious anaemia (intrinsic factor). Alternatively the desired immune response may be against an extracellular antigen which stimulates a response which also causes damage to host tissues. For example, acute rheumatic fever is caused by an antibody response to a Streptococcal antigen which cross-reacts with a cardiac muscle cell antigen.

Depletion of Treg cells or impairment of Treg cell function has been shown to result in autoimmune disease in murine models. Disease caused in test animals include arthritis (e.g. rheumatoid arthritis), inflammatory bowel disease, gastritis, pernicious anaemia, thyroiditis, insulinitis, diabetes, sialoadenitis, adrenalitis, autoimmune orchitis/oophoritis, glomerulonephritis, chronic obstructive pulmonary disease and experimental autoimmune encephalitis and multiple sclerosis. Induction of a regulatory T cell type 1 response has also been shown to reduce the development of atherosclerosis in murine models (Mallat Z. et al. Circulation 108:1232-7, 2003). Treg activity has also been shown to be significant in the rate at which allografts are rejected. Depletion of Treg cells or impairment of function accelerates the rate of rejection, while infusion of test animals with syngeneic lymphocytes enriched in Treg cells has been shown to prolong graft survival. The methods of the present invention may therefore find use in the treatment of any of these conditions.

Under certain conditions, it may also be possible to tolerise a subject against a particular antigen. The invention thus provides a method for inducing tolerance in a subject towards an antigen, comprising administering to the subject a composition comprising the antigen and DNGR-1⁺ DC, or antigen pulsed DNGR-1⁺ DC and wherein the antigen and/or DNGR-1⁺ DC is administered or pulsed in the absence of an adjuvant.

DNGR-1⁺ DC are believed to be particularly important in the generation of CTL responses, so the immune response to be

stimulated is preferably a CTL response. The immune response may involve production and/or proliferation of CTLs, which are typically T cells expressing CD8 and are capable of cytotoxic activity against cells displaying their cognate antigen in the context of MHC class I molecules.

In practice, then, the cells produced by the methods of the invention may be used therapeutically or prophylactically to inhibit or suppress an undesirable immune response against a particular antigen, even in a subject with pre-existing immunity or an on-going immune response to that antigen. This may be particularly useful (for example) in the treatment of autoimmune disease.

It will be understood that the cells produced by the methods of the invention may also be used for the prophylaxis and/or treatment of any condition in which it is desirable to induce a CTL response, such as cancer, or infection by an intracellular parasite or pathogen, such as a viral infection.

While DNGR-1⁺ DC may stimulate a Th2 type response it is thought that CD8 α ⁺ DC in mouse are poor Th2 inducers (Maldonado-Lopez et al., 1999). Thus vaccines or immunotherapies employing DNGR-1⁺ DC are unlikely to inadvertently drive a detrimental allergic Th2 immune response.

The magnitude of the immune response may be assessed by any appropriate criteria, such as appearance of inflammation, swelling, cell proliferation (e.g. of Th1, Th2 or Th17 CD4⁺ T cells, or CTLs) or inflammatory cytokine production (e.g. IL-1, IL-4, IL-12, IFN-gamma, TNF-alpha). In certain embodiments, the tolerised individual will display substantially no immune response to that antigen.

Adjuvants

According to the present invention the methods may utilize DNGR-1⁺ DC to stimulate an immune response to a target antigen.

DNGR-1⁺ DC can induce proliferation of both CD8⁺ T cells and CD4⁺ T cells (WO2009/013484), and may stimulate proliferation of both types of T cell in any given immune response.

Therefore, it may be desirable to administer further immunostimulatory agents in order to achieve maximal CTL stimulation and proliferation, and/or stimulation and proliferation of other T cell types.

These may include agents capable of activating dendritic cells and stimulating their ability to promote T cell activation.

Such an agent may be referred to as an adjuvant. The adjuvant may comprise an agonist for CD40 (such as soluble CD40 ligand, or an agonist antibody specific for CD40) or an antagonist of CD40 (such as an anti-CD40 antibody), an agonist of CD28, CD27 or OX40 (e.g. an agonist antibody specific for one of those molecules), a CTLA-4 antagonist (e.g. a blocking antibody specific for CTLA-4), and/or any other agent capable of inducing dendritic cell activation.

A Toll-like receptor (TLR) agonist is a substance which activates a Toll-like receptor. The application demonstrates that human DNGR-1⁺ DC respond to TLR3 and TLR8 agonists. Therefore suitable adjuvants include Poly I:C (polyinosine-polycytidylic acid), which binds TLR3; resiquimod (R-848; 1-[4-amino-2-(ethoxymethyl)imidazo[4,5-c]quinolin-1-yl]-2-methylpropan-2-ol 1-[4-amino-2-(ethoxymethyl)imidazo[4,5-c]quinolin-1-yl]-2-methylpropan-2-ol) or polyU RNA which bind TLR7 in mice and are believed to bind TLR8 in humans. For more details, see Reis e Sousa, Toll-like receptors and dendritic cells. Seminars in Immunology 16:27, 2004. Preferably, the TLR agonist is an activator of TLR3 and/or TLR8.

Adjuvants which may not work via TLRs include 5' triphosphate RNA, β -glucans such as curdlan (β -1,3-glucan) and poly I:C which can function independently of TLRs.

Dectin-1 is also expressed by mouse CD8 α ⁺ DC. Therefore the adjuvant may be a Dectin-1 agonist. Dectin-1 agonists include β -glucans such as curdlan (β -1,3-glucan).

5 It may also be desirable to administer an adjuvant during or after contacting the DC population with T cells; and/or before or after the T cells are administered to a subject. The adjuvant selected at this stage will also influence the immune response generated. The adjuvant may comprise an agonist for
10 CD40 (such as soluble CD40 ligand, or an agonist antibody specific for CD40) or an antagonist of CD40 (such as an anti-CD40 antibody), an agonist of CD28, CD27 or OX40 (e.g. an agonist antibody specific for one of those molecules), a CTLA-4 antagonist (e.g. a blocking antibody specific for CTLA-4). T
15 cells respond to a number of TLR agonists. Suitable TLR agonists include MPL (monophosphoryl lipid A), which binds TLR4; LTA (lipoteichoic acid, which binds TLR2; Poly I:C, which binds TLR3; flagellin, which binds TLR5; resiquimod (R-848; 1-[4-amino-2-(ethoxymethyl)imidazo[4,5-c]quinolin-1-yl]-2-methylpropan-2-ol 1-[4-amino-2-(ethoxymethyl)imidazo[4,5-
20 c]quinolin-1-yl]-2-methylpropan-2-ol) or polyU RNA which bind TLR7 in mice and are believed to bind TLR8 in humans, and CpG (DNA CpG motifs), which binds TLR9; or any other component which binds to and activates a TLR.

25 Adjuvants which may not work via TLRs include 5' triphosphate RNA, poly I:C, and β -glucans such as curdlan (β -1,3-glucan). Pro-inflammatory cytokines such as TNF- α or IL-1 may also be used as adjuvants. TGF- β can act as an adjuvant promoting the
30 conversion of naïve T cells into antigen-specific Tregs in non-inflammatory conditions (Chen et al., 2003; Coombes et al., 2007; Luo et al., 2007; Yamazaki et al., 2008).

35 Certain adjuvants promote stimulation of Treg cells. These include IL-12 and retinoic acid, and in particular all-trans retinoic acid (ATRA), also known as tretinoin. TGF- β can act as an adjuvant promoting the conversion of naïve T cells into

antigen-specific Tregs in non-inflammatory conditions (Chen et al., 2003; Coombes et al., 2007; Luo et al., 2007; Yamazaki et al., 2008).

- 5 Thus, when the immune response to be stimulated is a Treg response it may be appropriate to use a Treg-promoting adjuvant. It may also be possible to stimulate Treg cell stimulation without administration of an adjuvant.
- 10 Without wishing to be bound by theory, it is believed that the nature of the adjuvant used may affect the type of response obtained. DNGR-1⁺ DC can stimulate both CD4⁺ T cells and CD8⁺ T cells, and the nature of the CD4⁺ response in particular may be affected by the adjuvant used. For example, use of poly I:C
- 15 appears to favour generation of a Th1-type CD4⁺ response. Curdlan appears to stimulate a Th17-type CD4⁺ response while the absence of an adjuvant may result in the development of tolerance to the antigen. That is to say, the immune system is induced not to respond to future administrations of the
- 20 same antigen. This may (but need not) involve the generation of Treg cells which are capable of active suppression of the response. Thus further administrations of an antigen to a subject who has been tolerised to that antigen should result in a lesser immune response than in a subject who is naïve for
- 25 that antigen (i.e. whose immune system has not previously been exposed to the antigen).

- The methods of the invention may include contacting or administering a cell, or population of cells with an adjuvant,
- 30 either sequentially or simultaneously, in the same or separate compositions. Thus the methods of the invention may, but need not, comprise contacting or administering with an adjuvant.

Antigen

- 35 The methods of the invention may be used to raise an immune response against a target antigen. The antigen may be any protein or fragment thereof against which it is desirable to

raise an immune response, in particular a CTL response, but also a Th17 response or a Treg response. These may include antigens associated with, expressed by, displayed on, or secreted by cells against which it is desirable to stimulate a CTL response, including cancer cells and cells containing intracellular pathogens or parasites. For example, the antigen may be, or may comprise an epitope peptide from a protein expressed by an intracellular pathogen or parasite (such as a viral protein) or from a protein expressed by a cancer or tumour cell. Thus the antigen may be a tumour-specific antigen. The term "tumour-specific" antigen should not be interpreted as being restricted to antigens from solid tumours, but to encompass antigens expressed specifically by any cancerous, transformed or malignant cell.

It may be particularly desirable to raise a Treg response against an antigen to which the subject exhibits, or is at risk of developing, an undesirable immune response. Thus the Treg response will be to inhibit or suppress an immune response against an antigen. For example, it may be a self antigen against which an immune response occurs in an autoimmune disease. Examples of autoimmune diseases in which specific antigens have been identified as potentially pathogenically significant include multiple sclerosis (myelin basic protein), insulin-dependent diabetes mellitus (glutamic acid decarboxylase), insulin-resistant diabetes mellitus (insulin receptor), coeliac disease (gliadin), bullous pemphigoid (collagen type XVII), auto-immune haemolytic anaemia (Rh protein), auto-immune thrombocytopenia (GpIIb/IIIa), myasthenia gravis (acetylcholine receptor), Graves' disease (thyroid-stimulating hormone receptor), glomerulonephritis, such as Goodpasture's disease (alpha3(IV)NC1 collagen), and pernicious anaemia (intrinsic factor). Alternatively the target antigen may be an exogenous antigen which stimulates a response which also causes damage to host tissues. For example, acute rheumatic fever is caused by an antibody response to a Streptococcal antigen which

cross-reacts with a cardiac muscle cell antigen. Thus these antigens, or particular fragments or epitopes thereof may be suitable antigens for use in the present invention.

5 The antigen is a peptide antigen. The term "peptide" refers to the nature of the antigen, i.e. that it is formed from amino acids linked by peptide bonds, and should not be taken to imply any particular size or length. Typically the peptide antigen will be at least 8 amino acids in length, and may be
10 up to 30 amino acids in length, up to 50 amino acids in length, up to 100 amino acids, up to 200 amino acids, or even longer and may have residues coupled to the amino acids, such as glycon chains. For example, it may be 25 to 35 amino acids in length.

15 Without wishing to be bound by any particular theory, the peptide antigen should be capable of binding to a MHC class II or MHC Class I molecule, or should be capable of being processed within an antigen-presenting cell (such as a
20 dendritic cell) to give rise to one or more peptides capable of binding to a MHC class II molecule or MHC Class I molecule. It has recently been suggested that short epitope peptides of around 8 amino acids in length may induce less sustained CTL reactivity than longer peptides (e.g. around 30 amino acids in
25 length) (Bijker, M.S. et al. *J. Immunol.* 179(8), 5033-5040 (2007)). MHC class I molecules typically bind peptides of 8 or 9 amino acids in length, while MHC class II molecules can bind peptides from 8 amino acids up to 20 amino acids, up to 30 amino acids, or even longer.

30 As described above the therapeutic application of the various cells and medicaments will depend on the identity of the antigen and any adjuvant which is used and the nature of the immune response required.

For example, they may be used for the treatment of cancer, or for the treatment of an infection with a parasite or a pathogen.

5 Alternatively they may be used in the treatment of an inflammatory or autoimmune condition, especially a condition characterised by undesirable CTL activity. The condition to be treated may be selected from:

10 - autoimmune diseases, including rheumatoid arthritis and other types of chronic or acute arthritis or arthropathies with an immune component, systemic lupus erythematosus (which is known to involve particularly high levels of cell death), scleroderma, Sjögren syndrome, autoimmune (particularly Type
15 I) diabetes, thyroiditis, and other organ-specific immune diseases, including psoriasis;

- neurologic diseases, including multiple sclerosis, myasthenia gravis, and other neurologic immune-mediated
20 diseases. Also included are gastrointestinal diseases, including Crohn's disease, colitis, celiac disease and hepatitis;

- cardiovascular diseases, which are now recognised to have a
25 significant immune-mediated component, including atherosclerosis, cardiomyopathy, rheumatic fever, endocarditis, vasculitis, and other immune-mediated cardiovascular diseases;

30 - immune-mediated respiratory diseases, including emphysema, respiratory airways infections, and other immune-mediated respiratory diseases;

- allergic processes and hypersensitivity reactions (type I,
35 II, III, and IV), including asthma, rhinitis, and other immune-mediated hypersensitivity reactions;

- transplant or graft rejection and graft versus host disease, as occurs during or subsequent to, for example, organ transplant, tissue graft, blood transfusion, bone marrow transplant;

5

- immunopathological responses to infectious agents, including septic shock syndromes;

10

- degenerative processes, such as neurodegenerative processes, that implicate immune competent cells such as microglia.

Binding agents

15

Any suitable molecule having a sufficiently high affinity and specificity for the relevant target molecule (e.g. DNGR-1, BDCA3, etc.) may be used as a binding agent. The target molecule may be referred to as its binding partner. The binding agent may be a protein, nucleic acid (e.g. an aptamer), carbohydrate (e.g. oligo- or polysaccharide), small molecule, etc. Particularly preferred binding agents are antibodies and functional fragments thereof.

20

25

The binding agent preferably has a binding affinity (affinity constant) for its binding partner of at least 10^5M^{-1} , at least 10^6M^{-1} , at least 10^7M^{-1} , preferably at least 10^8M^{-1} , more preferably at least 10^9M^{-1} . Where the binding partner is DNGR-1 it is preferred that the binding agent has the relevant degree of affinity for the extracellular domain (ECD). The same is true of other cell surface markers such as BDCA3.

30

The binding agent preferably has an affinity at least 2x, and preferably at least 5x, at least 10x, at least 50x or at least 100x greater than for any other molecule, including other C-type lectins in the case of binding agents for DNGR-1.

35

In certain aspects of the invention, it is desirable to cross-link an antigen (e.g. a protein or peptide antigen) to a binding agent as described, e.g. in order to target or deliver

the antigen to the DNGR-1⁺ DC. The skilled person is well aware of suitable methods and reagents. Where the binding agent is a protein, the antigen may be coupled via a sulphhydryl group of the binding agent. The sulphhydryl group
5 may normally be free, or it may normally be part of a disulphide bond in which case it may be exposed by selective reduction of the binding agent. For example, an antibody can be mildly reduced selectively in the hinge region using the reducing agent mercaptoethanosulfonate. Then, the antigen is
10 activated using sulpho-SMCC, an hetero-bifunctional cross-linking reagent that reacts with the tertiary amines of the protein, generating groups reactive with free sulphhydryls. Then, the antibody and the activated antigen are incubated together resulting in the protein being conjugated to the
15 monovalent antibody. Alternatively, if a suitably immunogenic peptide sequence from the antigen is known, such a peptide containing a cysteine with a free sulphhydryl can be synthesized and coupled to sulpho-SMCC activated antibody, which will remain bivalent and with several peptides bound per
20 molecule of antibody.

Antibodies

It is well-known that fragments of a whole antibody can perform the function of binding antigens. Examples of
25 functional binding fragments are (i) the Fab fragment consisting of VL, VH, CL and CH1 domains; (ii) the Fd fragment consisting of the VH and CH1 domains; (iii) the Fv fragment consisting of the VL and VH domains of a single antibody; (iv) the dAb fragment (Ward, E.S. et al., Nature 341, 544-546
30 (1989)) which consists of a VH domain; (v) isolated CDR regions; (vi) F(ab')₂ fragments, a bivalent fragment comprising two linked Fab fragments (vii) single chain Fv molecules (scFv), wherein a VH domain and a VL domain are linked by a peptide linker which allows the two domains to
35 associate to form an antigen binding site (Bird et al, Science, 242, 423-426, 1988; Huston et al, PNAS USA, 85, 5879-5883, 1988); (viii) bispecific single chain Fv dimers

(PCT/US92/09965) and (ix) "diabodies", multivalent or multispecific fragments constructed by gene fusion (WO94/13804; P. Holliger et al Proc. Natl. Acad. Sci. USA 90 6444-6448, 1993).

5

As antibodies can be modified in a number of ways, the term "antibody" should therefore be construed as covering any specific binding substance having a binding domain with the required specificity. Thus, this term covers the antibody fragments described above, as well as derivatives, functional equivalents and homologues of antibodies, including any polypeptide comprising an immunoglobulin binding domain, whether natural or synthetic. Chimaeric molecules comprising an immunoglobulin binding domain, or equivalent, fused to another polypeptide are therefore included. Cloning and expression of chimaeric antibodies are described in EP-A-0120694 and EP-A-0125023.

It will be appreciated that the binding agents used in the methods described herein are generally required to bind the extracellular domain of DNGR-1 in order to exert the required effect. Reference to a binding agent capable of binding DNGR-1 should be construed accordingly, unless the context allows otherwise.

25

Pharmaceutical compositions

The methods of the invention may be used in the manufacture of medicaments. The medicament may be formulated as pharmaceutical compositions. These medicaments may comprise, in addition to one of the above substances, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraperitoneal routes.

35

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid
5 pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be
10 included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous
15 solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection.
20 Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case
25 may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions
30 on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners.
35 Examples of the techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 20th Edition, 2000, pub. Lippincott, Williams & Wilkins.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

5

Substitutions and sequence identity

A conservative substitution may be defined as a substitution within an amino acid class and/or a substitution that scores positive in the BLOSUM62 matrix.

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According to one classification, the amino acid classes are acidic, basic, uncharged polar and nonpolar, wherein acidic amino acids are Asp and Glu; basic amino acids are Arg, Lys and His; uncharged polar amino acids are Asn, Gln, Ser, Thr and Tyr; and non-polar amino acids are Ala, Gly, Val, Leu, Ile, Pro, Phe, Met, Trp and Cys.

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According to another classification, the amino acid classes are small hydrophilic, acid/acid amide/hydrophilic, basic, small hydrophobic and aromatic, wherein small hydrophilic amino acids are Ser, Thr, Pro, Ala and Gly; acid/acidamide/hydrophilic amino acids are Asn, Asp, Glu and Gln; basic amino acids are His, Arg and Lys; small hydrophobic amino acids are Met, Ile, Leu and Val; and aromatic amino acids are Phe, Tyr and Trp

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Substitutions which score positive in the BLOSUM62 matrix are as follows:

Original Residue	C	S	T	P	A	G	N	D	E	Q	H	R	K	M	I	L	V	F	Y	W
Substitution	-	T A N	S	-	S	-	S D H	N E K	D Q K	E R K	N Y	Q K	E Q R	I L V	M L V	M I V	M I L	Y W	H F W	F Y

30

Percent (%) amino acid sequence identity with respect to a reference sequence is defined as the percentage of amino acid

residues in a candidate sequence that are identical with the amino acid residues in the reference sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity.

% identity values may be determined by WU-BLAST-2 (Altschul et al., Methods in Enzymology, 266:460-480 (1996)). WU-BLAST-2 uses several search parameters, most of which are set to the default values. The adjustable parameters are set with the following values: overlap span = 1, overlap fraction = 0.125, word threshold (T) = 11. A % amino acid sequence identity value is determined by the number of matching identical residues as determined by WU-BLAST-2, divided by the total number of residues of the reference sequence (gaps introduced by WU-BLAST-2 into the reference sequence to maximize the alignment score being ignored), multiplied by 100.

Examples

Example 1: Isolating DNGR-1⁺ BDCA3⁺ DC from human spleen

Mouse CD8 α ⁺ DC have been primarily isolated from spleen and we asked if the latter organ in humans contains DNGR-1⁺ BDCA3⁺ DC. We screened samples from a bank of frozen cell suspensions that had been obtained less than 8h post-mortem by mechanical dissociation of healthy human spleens from cadaveric organ donors. Human DC could be identified in all the samples by flow cytometry as HLA-DR⁺ cells lacking the lineage (Lin) specific markers CD3, CD14, CD16, CD19, CD20 and CD56 (Fig. 1A, top left panel). As in peripheral blood, DNGR-1 was found to be expressed exclusively by BDCA3^{hi} DC (population I in Fig. 1A, top right panel). Like mouse CD8 α ⁺ DC, human spleen DNGR-1⁺ BDCA3^{hi} DC were negative for CD11b and expressed slightly lower levels of CD11c than other DC (Fig. 1A and Fig. 9). Human spleen DNGR-1⁺ BDCA3^{hi} DC also did not express BDCA2, the pDC marker, or BDCA1 (CD1c), a more promiscuous marker also used to define human DC subtypes (Fig. 1A and Fig. 9). The DNGR-1⁺ population included cells lacking BDCA3 (BDCA3⁻, population III

in Fig. 1A, top right panel), as well as cells expressing moderate levels of the marker (BDCA3^{int}; population II in Fig. 1A, top right panel). BDCA3⁻ DC were relatively homogeneous and were mostly negative for CD11c, CD11b, BDCA1 and BDCA2 (Fig. 1A). In contrast, the BDCA3^{int} population was heterogeneous and included CD11c⁻ BDCA2⁺ pDC that lacked CD11b and DNNGR-1. Interestingly, BDCA3^{int} cells also included CD11c⁺ DC that variably expressed BDCA1, BDCA2 and CD11b (Fig. 1A and Fig. 9). We sorted BDCA1⁻ BDCA2⁻ cells into BDCA3^{hi}, BDCA3^{int} and BDCA3⁻ populations. BDCA3^{hi} DC expressed mRNA for DNNGR-1, Necl2 and IRF8 (Fig. 1B), similar to mouse CD8α⁺ DC. BDCA3^{int} and BDCA3⁻ DC expressed low or undetectable levels of DNNGR-1, Necl2 and IRF8 mRNA (Fig. 1B). We conclude that BDCA3 can mark multiple human DC subsets, including pDC, but, at high levels of expression and especially in combination with DNNGR-1, marks a discrete population of putative CD8α⁺ DC equivalents in human spleen.

Example 2: Obtaining DNNGR-1⁺ DC from the lymphoid tissue of mice reconstituted with human stem cells

Two xenotransplantation mouse models were utilized for this purpose: NOD/SCID/β₂m null mice or NOD/SCID/γc null mice reconstituted with purified human CB HSCs. In three independent successful xenotransplantation cohorts, HLA-DR⁺ Lin⁻ human DC were found in the spleens of all mice at 8-12 weeks following reconstitution independently of the host mouse strain (Fig 2 and data not shown). As in human spleen, distinct DC subsets could be defined on the basis of BDCA2, BDCA3 and DNNGR-1 expression. They included DNNGR-1⁻ BDCA3⁻ BDCA2⁻ DC, as well as DNNGR-1⁻ BDCA3^{int} BDCA2⁺ cells that corresponded to pDC. Notably, a separate population of cells that were negative for BDCA2 but bright for BDCA3 and positive for DNNGR-1 could also be identified (Fig 2). These DNNGR-1⁺ BDCA3^{hi} cells were larger than BDCA2⁺ pDC (Fig. 2) and resembled the ones in human spleen (see above). Thus, DNNGR-1⁺ BDCA3^{hi} DC can be found in the spleens of humanized mice, in addition to those of humans.

Example 3: Obtaining DNDR-1⁺ DC from human stem cells in vitro

Cultures were set up as a two step procedure: in the first step, HSCs were amplified by culture in the presence of SCF, Flt3L, IL-3 and IL-6. The expanded cells were then aliquoted and tested in a variety of differentiation conditions for the ability to generate DNDR-1⁺ BDCA3⁺ DC. A number of factors were tested in different combinations (Fig. 7). SCF was included to maintain HSC viability. Notably, we found that DNDR-1⁺ BDCA3⁺ DC could be obtained after culture with SCF, Flt3L, GM-CSF and IL-4 (Fig. 2A, and Fig. 7). DNDR-1⁺ BDCA3⁺ DC could also be obtained by substituting Flt3L for TGF- β (Fig. 7 and Fig. 8). Using HLA-DR and CD1a expression as a phenotypic definition of in vitro HSC-derived DCs (Klechevsky et al., 2008), we found that this culture system generated around 30-50% of Lin⁻ HLA-DR⁺ cells, which included a small percentage of the desired DNDR-1⁺ BDCA3⁺ double positive subset (Fig. 3A). Omission of any one of the four cytokines, SCF, Flt3L, GM-CSF or IL-4, from the differentiation cocktail prevented the emergence of the cells (Fig. 10A) although DNDR-1⁺ BDCA3⁺ cells could still be obtained when substituting TGF- β for Flt3L (data not shown). DNDR-1⁺ BDCA3⁺ cells were not seen at 6 days of the differentiation culture but represented 3-6% of total live cells by 12 days, suggesting that they differentiate or expand as a late event. As for the cells in primary human spleen, cord blood-derived DNDR-1⁺ BDCA3⁺ DC did not express CD14, CD11b or BDCA2 but expressed CD11c (Fig. 3D and Fig. 10B). In contrast to primary human spleen DNDR-1⁺ BDCA3^{hi} DC, those from CBDC expressed BDCA1 (Fig. 3D), perhaps due to GM-CSF driven induction of CD1a-c family members (Porcelli et al., 1992). Cord blood-derived DNDR-1⁺ BDCA3⁺ DC expressed CD86, CD45RO but only low levels of CD123 (Fig. 3D), as reported for blood BDCA3⁺ DC (MacDonald et al., 2002). In contrast to mouse CD8 α ⁺ DCs, DNDR-1⁺ BDCA3⁺ DC did not express CD8 α or CD103. They also did not express high levels of EpCam, indicating that they were not Langerhans cells (Bursch et al., 2007). Finally, cord blood-derived DNDR-1⁺ BDCA3⁺ DC appeared immature in that

they did not express CD40, CD83 or CCR7 (Fig. 3D). Consistent with immaturity, DNNGR-1⁺ BDCA3⁺ DC were not noticeably dendritic until the CBDC cultures were treated with poly I:C (see below) to promote DC maturation (Fig. 3C). Thus, immature DNNGR-1⁺ BDCA3⁺ DC phenotypically similar to primary human spleen DNNGR-1⁺ BDCA3^{hi} DC and to blood BDCA3⁺ DC can be generated in vitro from human HSCs.

Example 4: DNNGR-1⁺ DC derived from HSCs and mouse CD8 α ⁺ DC express similar markers

To establish a possible equivalence between DNNGR-1⁺ BDCA3⁺ human DC and mouse CD8 α ⁺ DC, we analyzed the expression of selected gene products by quantitative PCR. As for the cells isolated from human spleen (Fig. 1B), DNNGR-1⁺ DC purified from bulk CBDC cultures or from the spleens of humanized mice expressed mRNA for DNNGR-1, Necl2 and IRF8 (Fig. 4). IRF8 (and DAP12) was expressed at higher levels by BDCA2⁺ pDC isolated in parallel from humanized mice (Fig. 4), consistent with data in the mouse (Schiavoni et al., 2002). In contrast, the same pDC did not express Necl2 or DNNGR-1 (Fig. 4). Similar to mouse CD8 α ⁺ DC, human DNNGR-1⁺ DC expressed CD207 and BATF3 but did not express mRNA for IRF4 or TLR7, which was restricted to pDC (Fig. 4). Interestingly, TLR9 expression was also not expressed by DNNGR-1⁺ DC but was expressed by pDC (Fig. 4). This is different from the situation with mouse CD8 α ⁺ DC, which express TLR9. Finally, DNNGR-1⁺ DC but not pDC taken from humanized mice expressed TLR3 and TLR8 (Fig. 4). DNNGR-1⁺ cells from CBDC cultures only expressed very low levels of the same TLRs (Fig. 4), possibly due to immaturity. Consistent with the latter, DNNGR-1⁺ CBDC upregulated TLR3 and TLR8 (but not TLR7) upon treatment with type I interferon, a known inducer of nucleic acid-sensing TLRs (data not shown).

Example 5: DNNGR-1⁺ DC respond to adjuvants

Mouse CD8 α ⁺ DCs express TLR3 but not TLR7 mRNA and respond to poly I:C, a TLR3 (and MDA5/RIG-I) agonist, but not to

imiquimod (R837), an agonist for TLR7 (Edwards et al., 2003). We purified DNNGR-1⁺ DC from CBDC cultures or from humanized mouse spleen and stimulated them with poly I:C or imiquimod. Independently of the source, DNNGR-1⁺ DC consistently responded to poly I:C but not imiquimod with production of TNF, IL-6 and other cytokines (Fig. 5A and data not shown). A similar response pattern was seen with total unfractionated CBDC when measuring TNF production or surface HLA-DR upregulation as a marker of DNNGR-1⁺ DC maturation (Fig. 11). In contrast, BDCA2⁺ pDC from humanized mice responded to imiquimod but not poly I:C in the same experiments (Fig. 5A). Notably, when trying various other TLR agonists, we noted that DNNGR-1⁺ DC also responded to resiquimod (R848) and poly U (Fig. 5B). In the mouse, where there is lack of functional TLR8 expression, these compounds have been defined as TLR7 agonists (Diebold et al., 2004). However, in the human, they are reported to additionally stimulate TLR8 (Heil et al., 2004). The potent response of DNNGR-1⁺ BDCA3⁺ DC to resiquimod and poly U but not imiquimod, therefore, indicates that these cells express functional TLR8, consistent with the mRNA analysis above.

Example 6: Human DNNGR-1⁺ DC produce IL-12 in response to innate and T cell-derived stimuli

A hallmark of mouse CD8 α ⁺ DC is their ability to produce high levels of IL-12 in response to TLR agonists, especially in synergy with signals through the CD40, IL-4, GM-CSF or IFN- γ receptors (Shortman and Heath, 2010). DNNGR-1⁺ DC purified from CBDC cultures produced no IL-12 p70 above background in response to stimulation with a cocktail of TLR agonists, even when these were given together with IL-4 and IFN- γ , as well as antigen-specific T cells (Fig. 5C). However, the same cells produced IL-12 p70 when antigen was added to the cultures (Fig. 5C). Notably, this was also the case for primary DNNGR-1⁺ DC isolated directly from human spleen cell suspensions (Fig. 5C). Thus, coordinate delivery of innate and T cell derived stimuli reveals the ability of DNNGR-1⁺ BDCA3⁺ DC to produce bioactive IL-12.

Example 7: DNNGR-1⁺ DC internalize dead cell material and cross-present exogenous proteins to CD8⁺ T cells

Another attribute of mouse CD8 α ⁺ DC is their superior capacity to internalize debris from dead or dying cells (Iyoda et al., 2002 ; Schulz and Reis e Sousa, 2002). DNNGR-1⁺ DC were able to take dead cell material and were superior in this regard to monocyte-derived DC (Mo-DCs) generated with GM-CSF + IL-4 (Sallusto et al., 1995) (Fig. 6A). Internalized dead cell material showed up as multiple small inclusions within a single DNNGR-1⁺ DC by confocal microscopy (Fig. 6A). Finally, we assessed the ability of DNNGR-1⁺ BDCA3⁺ DC to cross present exogenous antigen to CD8⁺ T cells, a functional characteristic of mouse CD8 α ⁺ DC. In the first instance, we used unfractionated CBDC cultures and compared them to Mo-DCs. Unfortunately, the CBDC used for these experiments were only 50% HLA-A2⁺ as they were generated from HSCs purified from pooled CB, whereas Mo-DCs were from a single donor and homogenous for HLA-A2 expression (data not shown). This might be one reason why CBDC were less efficient than Mo-DC at presenting pre-processed antigenic peptides to HLA-A2-restricted T cell clones (Fig. 12). Nevertheless, CBDC were at least as efficient as Mo-DCs at activating NY-ESO-1₁₅₇₋₁₆₅-specific T cells in response to intact recombinant NY-ESO-1 protein, a clinically relevant antigen expressed by a broad range of tumor types (Chen et al., 2005) (Fig. 12). The activation of the NY-ESO-specific T cell clone reflected true NY-ESO-1 protein processing and cross-presentation as it was not seen with DC that were fixed in glutaraldehyde, even though the latter were still able to present the pre-processed determinant (Fig. 12). Interestingly, this cross-presenting ability was only revealed upon treatment of DC with poly I:C (Fig. 12), underscoring the importance of DC maturation in promoting antigen processing and presentation (Delamarre et al., 2003; Inaba et al., 2000). Finally, we tested purified DNNGR-1⁺ HLA-A2⁺ CBDC in a different model of cross-presentation.

We used a long Melan-A derived peptide that requires processing for its (cross)presentation to a specific HLA-A2-restricted T cell line (Faure et al., 2009). As for the NY-ESO-1 protein, presentation of the long Melan-A peptide was only seen upon treatment of the DC with poly I:C (Fig. 6B). DNNGR-1⁺ DC were more efficient at cross-presenting the long peptide than Mo-DC DC, even though the latter were more efficient at presenting the short peptide, representing the pre-processed determinant. We conclude that DNNGR-1⁺ DC have an equal or superior cross presentation ability to that of Mo-DCs.

Methods

Human tissue

Cell suspensions of human cadaveric spleen from healthy victims of traffic accidents (organ donors) were prepared less than 8 hours post mortem by mechanical dissociation followed by Ficoll density centrifugation. Cells were frozen and stored in liquid nitrogen until use. The procedure was approved by the Ethics Committee of Hopital Erasme, Brussels, Belgium. Cord blood was collected from mothers attending the Royal London Hospital, London, UK, after informed consent through a protocol approved by the Local Research Ethics Committee. Mononuclear cells were obtained by Ficoll density centrifugation and ammonium chloride red cell lysis. They were depleted for lineage marker positive cells using the StemSep™ system (Stem Cell Technologies, Canada) to generate Lin⁻ HSCs.

Humanized mice

All animal protocols were carried out under the authority of a project license, in accordance with UK governmental regulations (Animal Scientific Procedures Act 1986). NOD/SCID/ β_2 microglobulin null mice and NOD/Shi-scid/IL-2R γ null (NSG) mice were bred at Charles Rivers Laboratories (Moorgate, UK), housed in micro-isolators and fed sterile food and acidified water. Mice aged 8-12 weeks were sublethally

irradiated (3.75 Gy) up to 24 hours before i.v. injection of
50 000 Lin⁻ human CB cells. Mice were analyzed 8 to 24 weeks
after reconstitution. Spleen cells were prepared by digestion
with liberase and DNase, followed in some cases by an Optiprep
5 gradient to enrich for low density cells (Sigma-Aldrich).

***In vitro* generated DC**

Human Lin⁻ CB cells were prepared as described above and
differentiated into DC using a two-step protocol. In the first
10 step (amplification), Lin⁻ cells were cultured at 5×10^4
cells/ml in StemSpan serum-free medium (StemCell Technologies)
with penicillin, streptomycin, 100 ng/ml SCF, 100 ng/ml Flt3L,
20 ng/ml IL-3 and 20 ng/ml IL-6 (R&D Systems). After 7-11 d of
culture, the expanded cells were frozen until further use or
15 were used directly. In the second step (differentiation),
 6.25×10^4 cells/ml were plated in RPMI 1640 supplemented with
glutamine, penicillin, streptomycin, 2-ME (all from
Invitrogen), 10% heat-inactivated fetal calf serum (Autogen
Bioclear), 20 ng/ml SCF, 20ng/ml GM-CSF, 20 ng/ml IL-4, 100
20 ng/ml Flt3L (R&D Systems). Cultures were kept for 12-14 d and
cytokines were replenished after 6-7 d. Monocyte derived-DCs
were prepared as described (Salio et al., 2001).

Alternatively, in the second step (differentiation), 6.25×10^4
cells/ml were plated in RPMI 1640 supplemented with glutamine,
25 penicillin, streptomycin, 2-ME (all from Invitrogen), 10%
heat-inactivated fetal calf serum (Autogen Bioclear), 20 ng/ml
SCF, 20ng/ml GM-CSF, 20 ng/ml IL-4, 10 ng/ml TGF- β (R&D
Systems). Cultures were kept for 6d. Particularly high yields
of differentiated DCs were obtained when using culture plates
30 having round-bottomed or "V"-bottomed wells. Monocyte
derived-DCs were prepared as described (Salio et al., 2001).

Antibodies

Anti-HLA-DR (L243), anti-Lineage-1 cocktail (CD3, CD14, CD16,
35 CD19, CD20, CD56), anti-BDCA-3/CD141 (1A4), anti-CD123 (7G3),
anti-CD11c (B-ly6), anti-CD80 (L307.4), anti-CD8 α (RPA-T8),
anti-CD14 (M5E2), anti-CD3 (UCHT1), anti-CD16 (3G8), anti-CD19

(H1B19), and anti-CD56 (B159) were from BD Pharmingen. Anti-BDCA-2/CD303 (AC144), anti-BDCA-1/CD1c (AD5-8E7), anti-CD83 (HB15), anti-EpCam/CD326 (HEA-125) and anti-CCR7/CD197 (FR11-11E8) were from Miltenyi Biotec. Anti-CD1a (201B5.08) was from Dendritics. Anti-CD45RO (UCHL1), anti-CD103 (B-Ly7), anti-CD40 (5C3), anti-CD11b (ICRF44), anti-CD20 (2H7) and isotype-matched control antibodies were from eBioscience. Anti-hDNGR-1 was described previously (Sancho et al., 2008).

Flow cytometry and cell sorting

Cells were pre-incubated on ice with mouse serum (Jackson ImmunoResearch) and purified IgG2a (BD Pharmingen) to block Fc receptors and then stained with appropriate antibody combinations. To avoid cell clumping, primary human spleen cell suspensions were kept in DNase containing buffer during the different procedures. Multiparameter analysis was performed using mainly a LSRII flow cytometer (BD Biosciences), except for primary human spleen cell suspensions, which were analyzed on a FACSaria (BD Biosciences). Dead cells were excluded by a combination of scatter gating and DAPI exclusion. Analysis was performed using FlowJo software (Treestar). For cell sorting (FACSaria), primary human spleen cell suspensions, in vitro generated DC or low density cells from humanized mice were stained as above. Electronic gates were placed on live Lin⁻ HLA-DR⁺ cells and cells were sorted for example into BDCA2⁺ and BDCA3⁺, or into BDCA3⁺/DNGR-1⁻; or into BDCA3^{hi}/BDCA2⁻/BDCA1⁻, BDCA3^{int}/BDCA2⁻/BDCA1⁻ and BDCA3⁻/BDCA2⁻/BDCA1⁻ subsets, as appropriate.

Stimulation with TLR agonists

Sorted DC populations were cultured at 10⁵ cells/ml with selected TLR agonists (Invivogen) used at pre-determined optimal concentrations. Cytokine accumulation in supernatants was measured after 16h using a Becton Dickinson Cytometric Bead Array. Bulk CBDC were stimulated for 16h as above with varying concentrations of TLR3 or TLR7 agonists for assessment

of cytokine secretion or HLA-DR upregulation by flow cytometry. For IL-12p70, sorted HLA-A2-expressing DC populations were cultured at 5×10^4 cells/ml with a mix of different stimuli: TLR 1-9 agonists (human TLR agonist 1-9, Invivogen) used at pre-determined optimal concentrations, IL-4 (10pg/ml), and IFN γ (10pg/ml) (R&D Systems) in the presence or absence of 1 μ M of MelanA/MART-1 short peptide and 5×10^4 MelanA-specific HLA-A2-restricted CD8 $^+$ T cells.

10 RNA isolation and quantitative RT-PCR

RNA from FACS-sorted DC subsets was extracted with an RNeasy Micro Kit and treated with DNase I, according to the manufacturer's protocol (QIAGEN). cDNA was synthesized from total RNA with random hexamer primers and Superscript II RT (Invitrogen). Quantitative PCR was performed with Taqman Universal PCR MasterMix (Applied Biosystems) and preformulated primers and probe mixes (Taqman® Gene Expression Assays; Applied Biosystems). Measurements were performed using a sequence detection system (ABI PRISM 7700; Applied Biosystems). Levels of mRNA for the specific gene being measured were divided by those of GAPDH measured in parallel (normalized expression).

Uptake of dead cells

Human melanoma cells were UV irradiated (2400 J/cm ^{2}), incubated for 8 h at 37°C to allow for apoptosis and secondary necrosis and labeled with CFSE (flow cytometry) or Alexa633-SE (confocal microscopy). Dead cells were added to 5×10^4 CBDC or Mo-DC at different ratios for 2 hours at 4°C or 37°C. For confocal microscopy, cells were subsequently plated on fibronectin coated coverslips for 15 min, fixed in 3.7% paraformaldehyde/PBS for 10 min, permeabilized in 0.1% Triton-X100/PBS for 3 min, blocked with 5% mouse serum and stained for DNGR-1 using Alexa546 coupled antibody. Coverslips were mounted in Fluoromount-G and imaged with a laser scanning confocal microscope Axiovert 100M LSM 510 (Zeiss) with a 63x Plan-Apochromat NA 1.4 oil objective. For flow cytometric

analysis, cells were stained for DNGR-1 and BDCA3 and the percentage of CFSE-positive DNGR-1⁺ BDCA3⁺ cells was calculated by subtracting the frequency of positive events at 4°C (binding) from the frequency at 37°C (binding + uptake).

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Antigen presentation assays

NY-ESO-1₁₅₇₋₁₆₅ peptide (Chen et al., 2000) or NY-ESO-1 full length protein (kindly provided by the Ludwig Institute of Cancer Research) and the MelanA/MART-1 short (ELAGIGILTV) or long (KGHGHSYTTAEAAAGIGILTVILGVL) peptides were used as antigen sources. Antigen presentation assays were carried out as previously described (Faure et al., 2009; Salio et al., 2001) using HLA-A2-restricted NY-ESO-1- or MelanA-specific CD8⁺ T cells.

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Claims:

1. A method of obtaining a dendritic cell (DC) population comprising one or more DNGR-1 expressing dendritic cells (DNGR-1+ DC), the method comprising the step of contacting a hematopoietic stem cell (HSC) with a differentiation cocktail *ex vivo* or *in vitro*, wherein the differentiation cocktail is selected from:
- 5 a) GM-CSF, IL-4 and Flt3L or;
10 b) GM-CSF, IL-4 and TGF- β .
2. The method of claim 1 further comprising contacting the HSC with a stem cell viability factor.
- 15 3. The method of any one of the preceding claims wherein the stem cell viability factor is SCF.
4. The method of any one of the preceding claims wherein the differentiation cocktail is replenished during the contacting step.
- 20 5. The method of any one of the preceding claims wherein the method comprises:
- a) providing a first cell population comprising one or more HSCs, and;
25 b) expanding the number of HSCs in said first cell population to yield a second cell population and;
c) contacting the second cell population with the differentiation cocktail.
- 30 6. The method of claim 5 wherein step (b) comprises contacting the first cell population with one, more than one, or all of SCF, Flt3L, IL-3 and IL-6.
- 35 7. The method of any one of the preceding claims wherein the HSCs are obtained from a sample of embryonic yolk sac, the embryonic aorta-gonad-mesonephrous region, fetal liver or

spleen, adult spleen, adult bone marrow, fetal bone marrow, untreated peripheral blood, mobilized peripheral blood, or umbilical cord blood.

5 8. The method of claim 6 or 7 further comprising the step of depleting the sample for cells expressing lineage positive markers.

10 9. The method of claim 8 further comprising the step of positive selection for cells expressing HSC markers.

15 10. The method of any one of the preceding claims wherein the HSCs are contacted with the differentiation cocktail for at least 6 days.

11. The method of any one of the preceding claims wherein the HSCs are human HSCs.

20 12. The method of any one of the preceding claims further comprising the step of isolating DNGR-1+ DC, or a population thereof.

25 13. The method of claim 12 wherein the step of isolating is performed using a binding agent capable of binding to DNGR-1.

14. The method of claim 13 wherein the binding agent is an antibody, or fragment thereof, against DNGR-1.

30 15. The method of claim 12 further comprising the step of positive selection for cells expressing BDCA3.

16. The method of claims 12 or 15 further comprising the step of positive selection for cells expressing HLA-DR.

35 17. A method of obtaining a DC population comprising one or more DNGR-1+ DC, the method comprising:

- a) obtaining cells from the lymphoid tissue of a rodent reconstituted with xenogeneic HSCs, and;
- b) isolating DNGR-1+ DC.

- 5 18. The method of claim 17 further comprising:
- a) providing an immunocompromised rodent and;
 - b) introducing xenogeneic HSCs into said rodent to obtain a rodent reconstituted with xenogeneic HSCs.
- 10 19. The method of claim 17 wherein step b) further comprises positive selection for cells expressing BDCA3 and/or HLA-DR.
20. The method of any one of claims 17 to 19 wherein the rodent is a NOD/SCID/ β_2m null mouse.
- 15 21. The method of any one of claims 17 to 19 wherein the rodent is a NOD/SCID/ γc null mouse.
- 20 22. The method of claim 17 to 21 wherein the HSCs are human HSCs.
23. The method of any one of claims 17 to 22 wherein the lymphoid tissue is derived from the spleen.
- 25 24. The method of any one of the preceding claims further comprising the step of contacting a DNGR-1+ DC, or population thereof, with an antigen to obtain an antigen-pulsed DC.
- 30 25. A DC population comprising one or more DNGR-1+ DC as obtained or obtainable by the method of any one of claims 1 to 16.
- 35 26. A DC population according to claim 25 further comprising one or more plasmacytoid dendritic cells (pDC).

27. A method comprising the step of contacting the DC population of claims 25 or 26 with an antigen to obtain an antigen-pulsed DC.

5 28. The method of claim 24 or claim 27 wherein the DC, or population thereof, and the antigen are contacted *ex vivo*, or *in vitro*.

10 29. The method of claim 28 further comprising the step of contacting the DC, or population thereof, with an adjuvant.

15 30. The method of claim 29 wherein the DC, or population thereof, is contacted with the adjuvant and the antigen at substantially the same time.

31. The method of any one of claims 28 to 30 further comprising the step of administering the DC cell, or population thereof, to a subject.

20 32. The method of claim 24 or claim 27 wherein the antigen is, or comprises, a peptide from a protein expressed by a pathogen or parasite, or a protein from a cancer cell.

25 33. The method of claim 24 or claim 27 wherein the antigen is a viral protein or a tumour-specific antigen.

34. The method of claim 24 or claim 27 wherein the antigen is an antigen to which a subject exhibits, or is at risk of developing, an undesirable immune response.

30 35. The method of claim 24 or claim 27 further comprising the step of contacting the antigen-pulsed DC, or population thereof, with one or more T cells.

35 36. The method of claim 35 further comprising the step of expanding the number of T cells.

37. The method of claim 35 further comprising the step of administering the T cells to a subject.

38. The method of claim 35 wherein the DC, and the T cells are autologous.

39. The method of claim 35 wherein the T cells and the DC are derived from the same subject.

40. The method of claim 37 wherein the T cells are administered to the subject from whom they are derived.

41. The method of any one of claims 35 to 40 further comprising the step of administering an adjuvant.

42. A DC population as obtained or obtainable by the method of any one of claims 1 to 23, for use in a method of treatment.

43. A DC population as obtained or obtainable by the method of any one of claims 1 to 23, for use in the treatment of any of the following: cancer; infection with a parasite or a pathogen; an inflammatory condition; or an autoimmune condition.

44. An antigen-pulsed DC population as obtained or obtainable by the method of any one of claims 24 and 27 to 34, for use in a method of treatment.

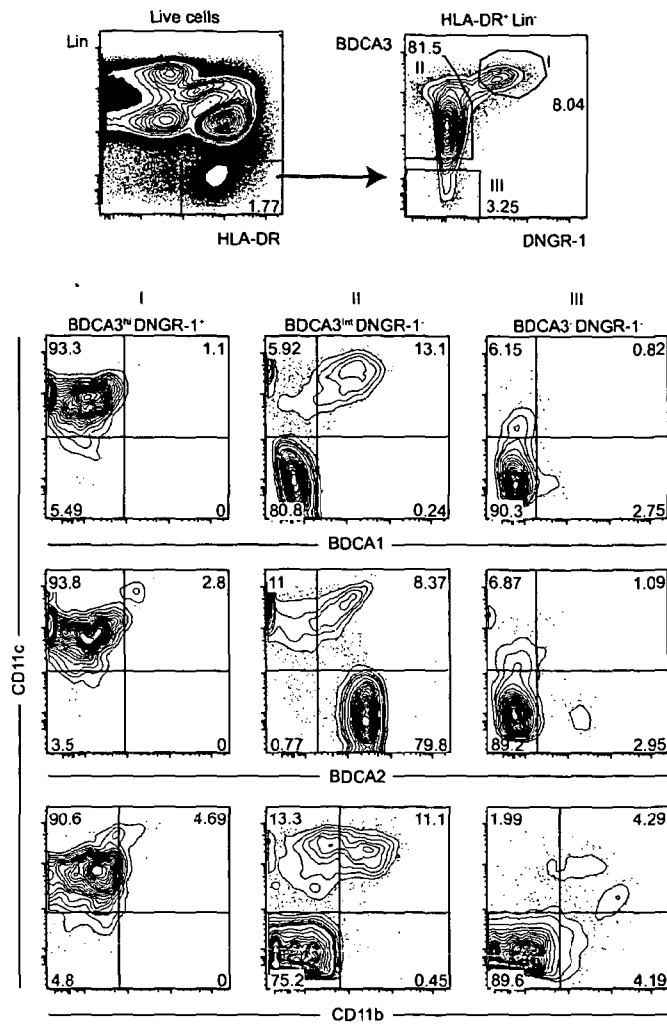
45. An antigen-pulsed DC population as obtained or obtainable by any of the method of claims 24 and 27 to 34, for use in the treatment of any of the following: cancer; infection with a parasite or a pathogen; an inflammatory condition; or an autoimmune condition.

46. A population of T cells as obtained or obtainable by the method of any one of claims 35 to 41 for use in a method of treatment.

- 5 47. A population of T cells as obtained or obtainable by the method of any one of claims 35 to 41, for use in the treatment of any of the following: cancer; infection with a parasite or a pathogen; an inflammatory condition; or an autoimmune condition.

10

A.



B.

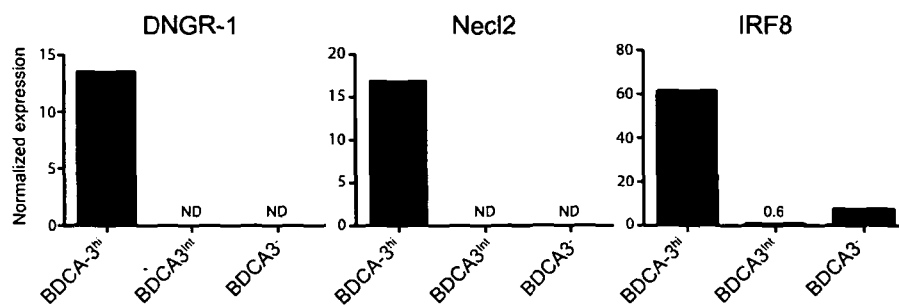


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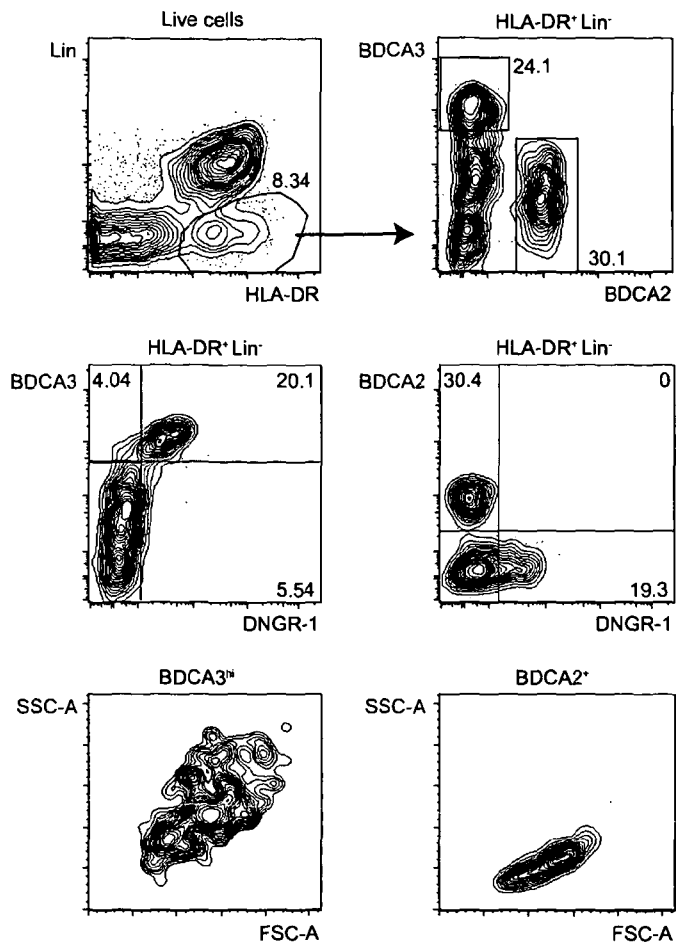


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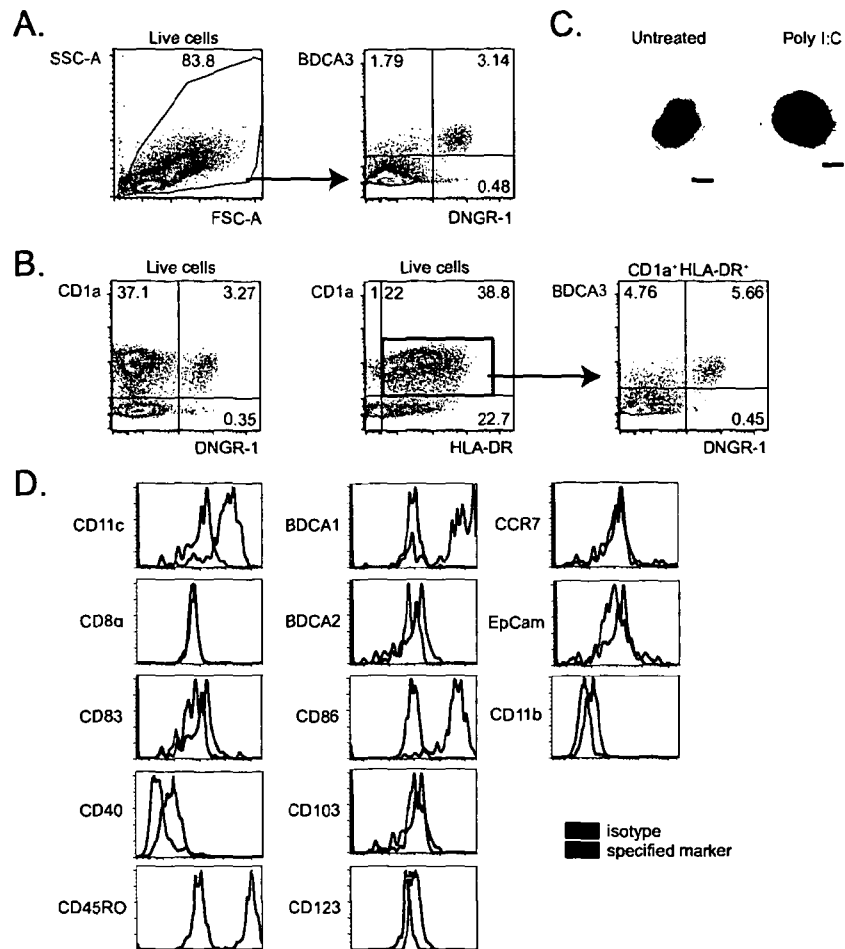


Figure 3

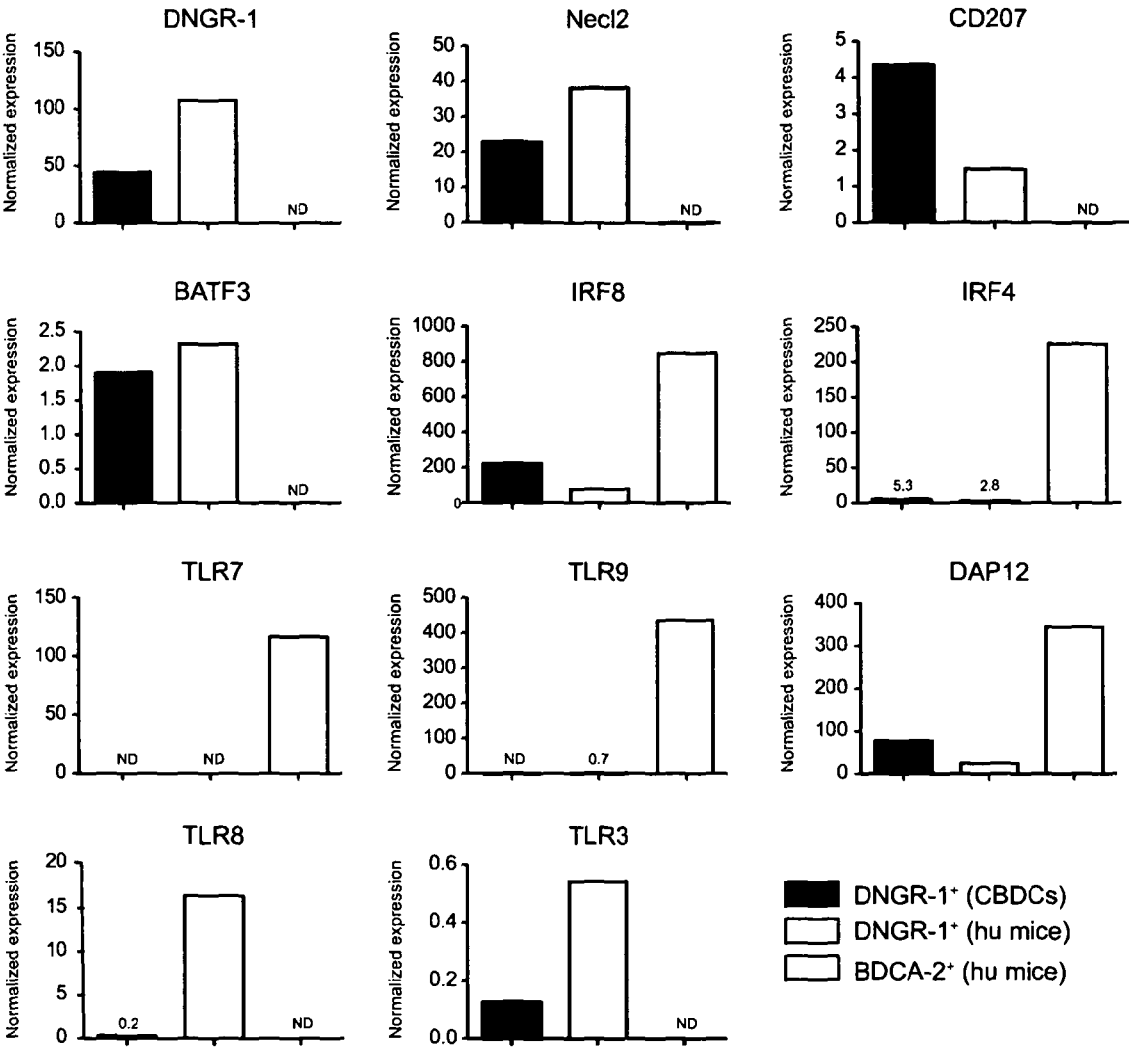
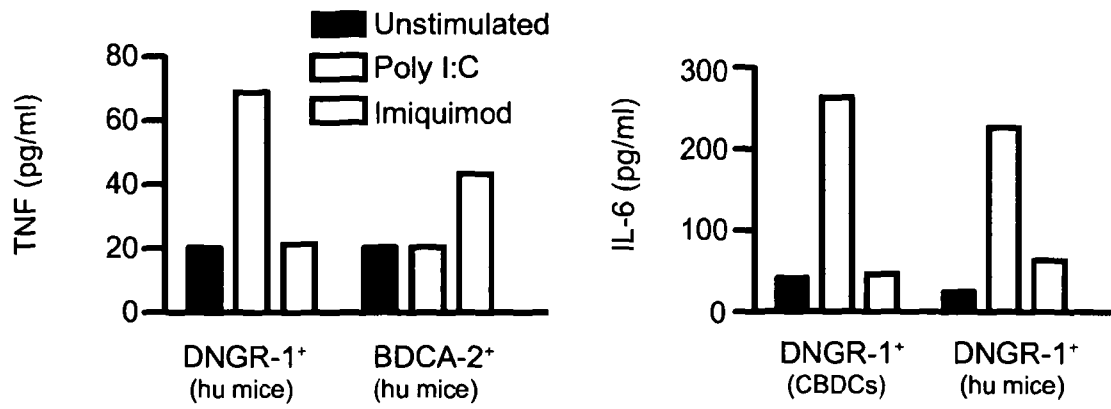
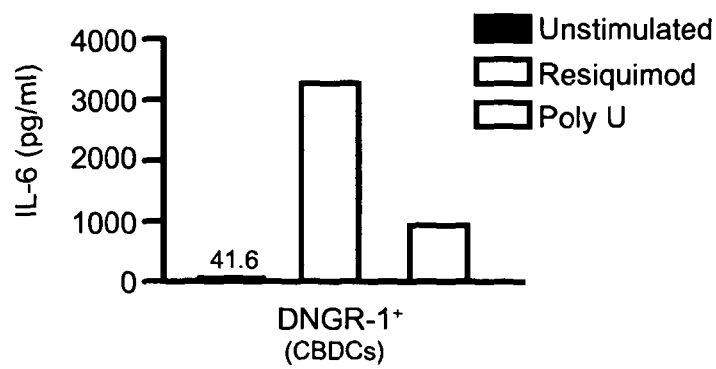


Figure 4
4/12

A.



B.



C.

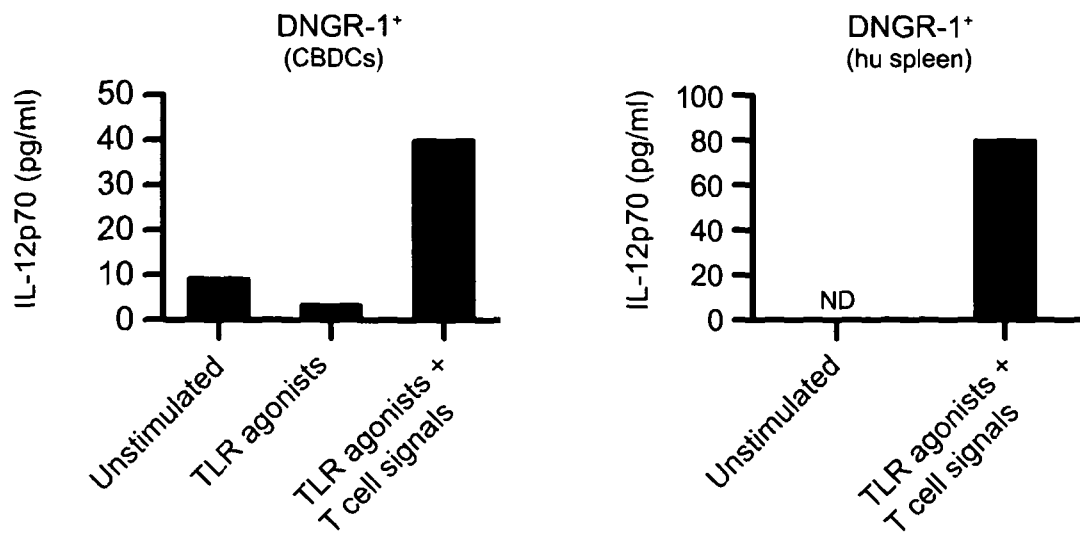


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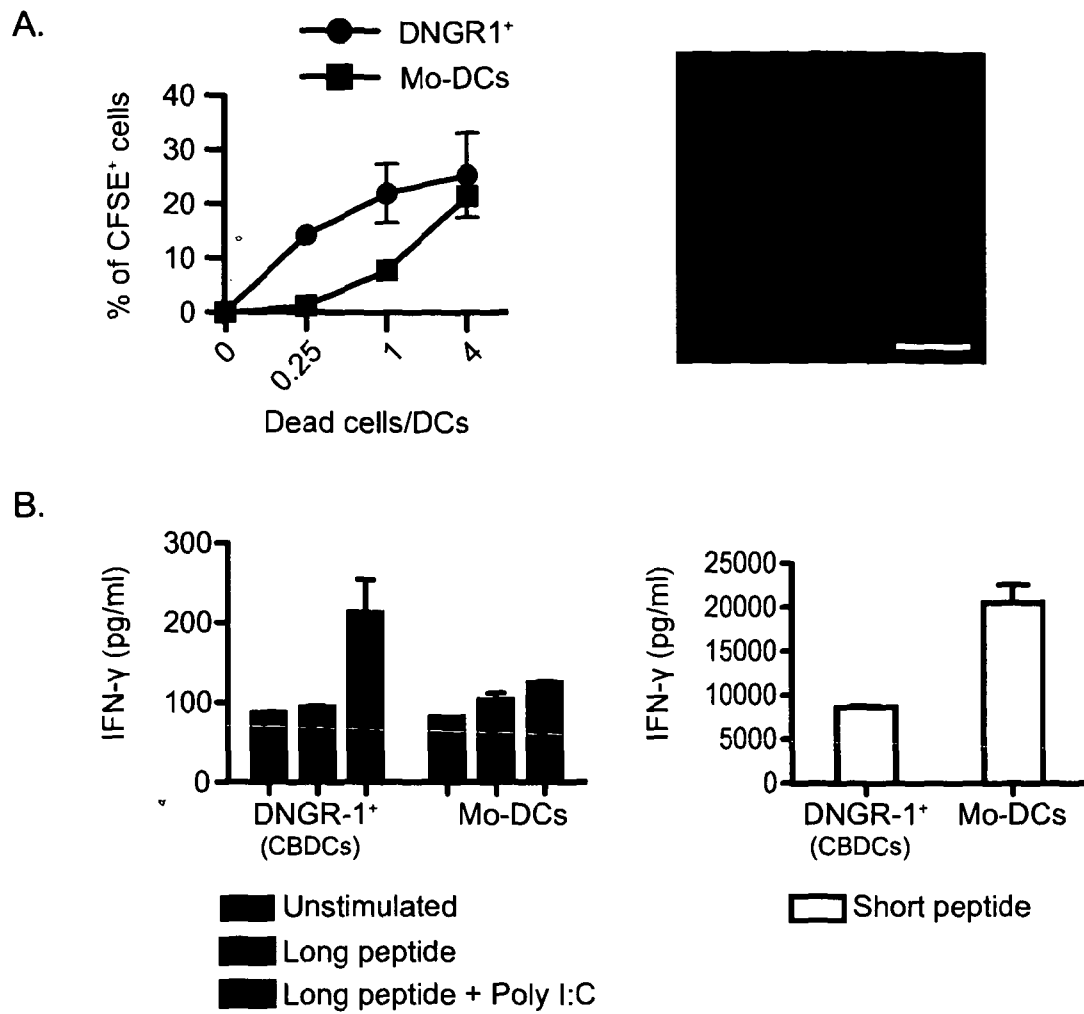


Figure 6

GM-CSF	IL4	TGFb	TNFa	G-CSF	Flt3L	IL3	Result
x	x						v
x	x	x					
x	x	x	x	x	x	x	
x	x	x			x		
x			x				v
x		x	x				
x		x	x		x		
x			x		x		
x	x				x		
		x	x		x		
			x		x	x	
		x	x		x	x	
x	x	x	x	x	x		
x	x	x	x	x			
x	x	x	x				
		x			x		
				x	x		
		x		x	x		
	x	x		x	x		

Figure 7

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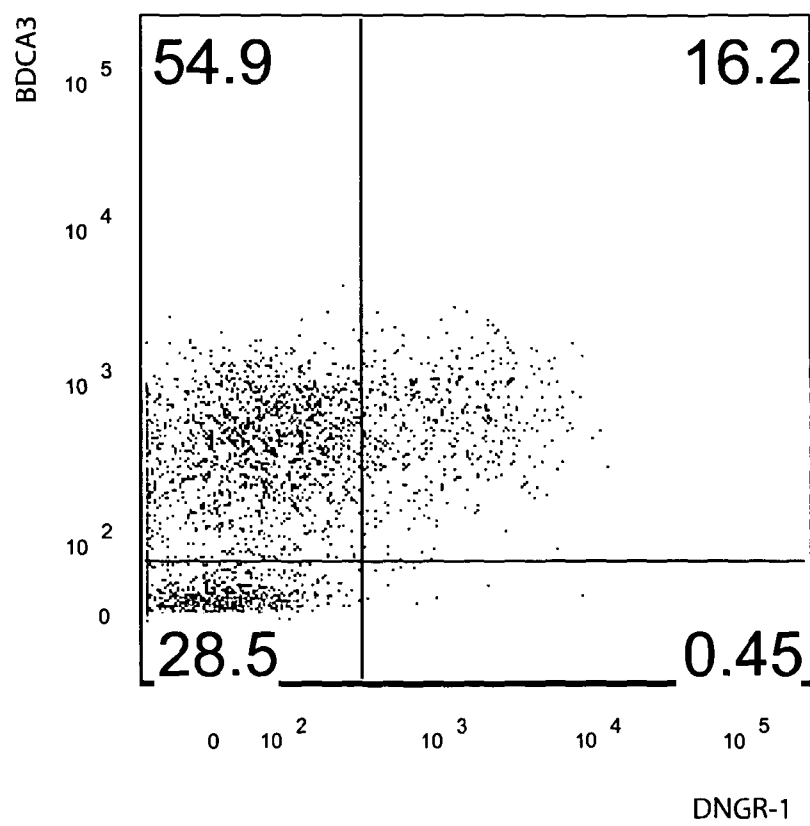


Figure 8

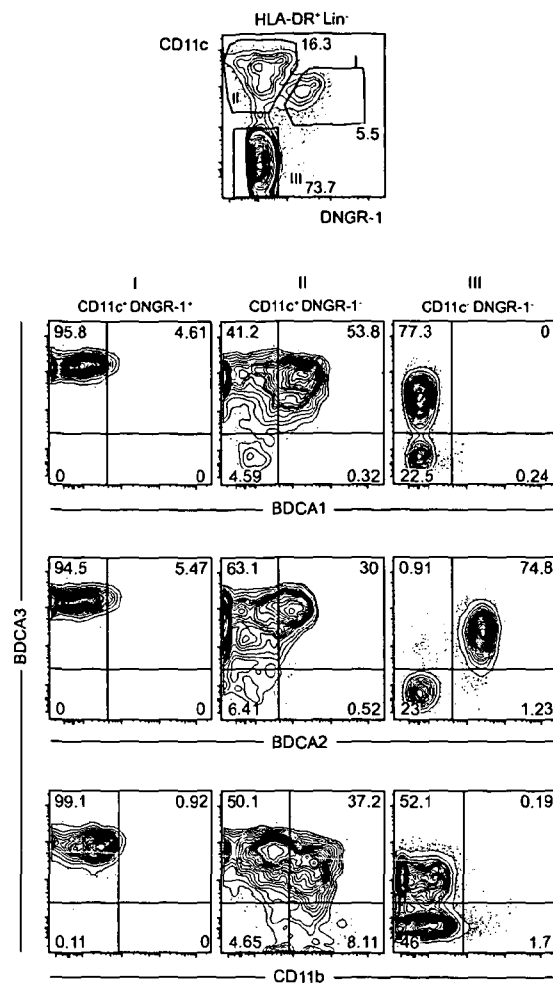


Figure 9

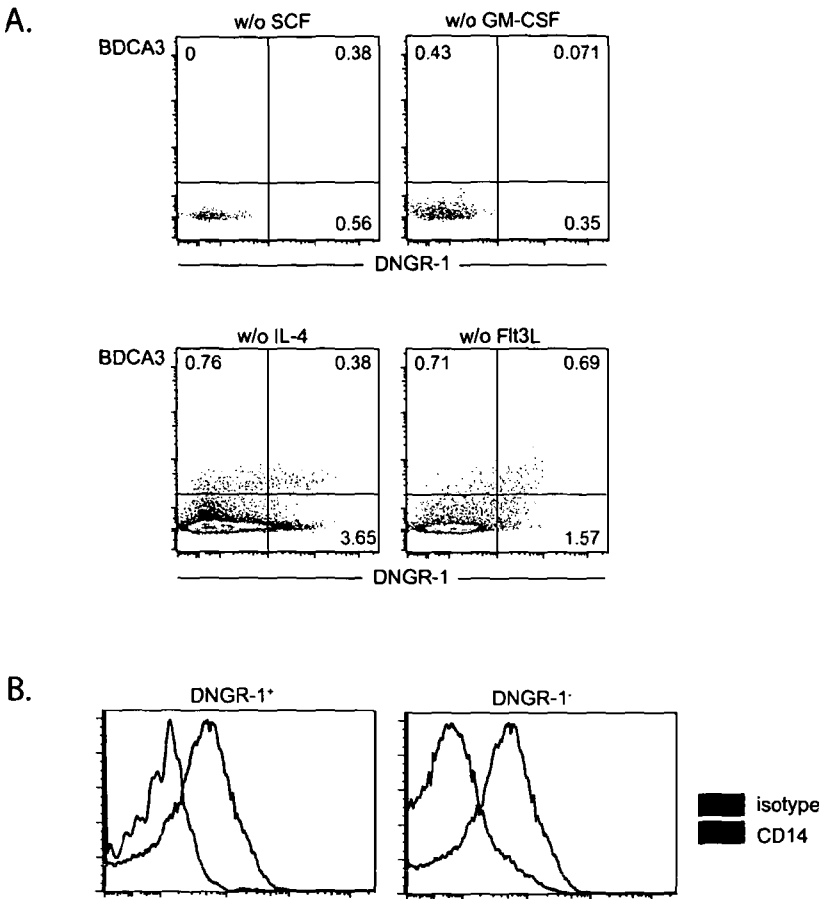


Figure 10
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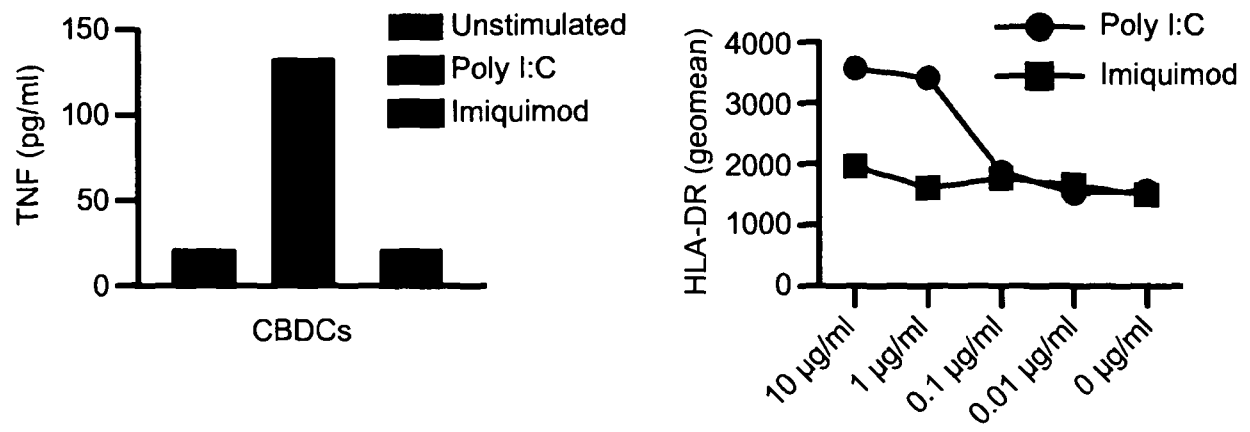


Figure 11

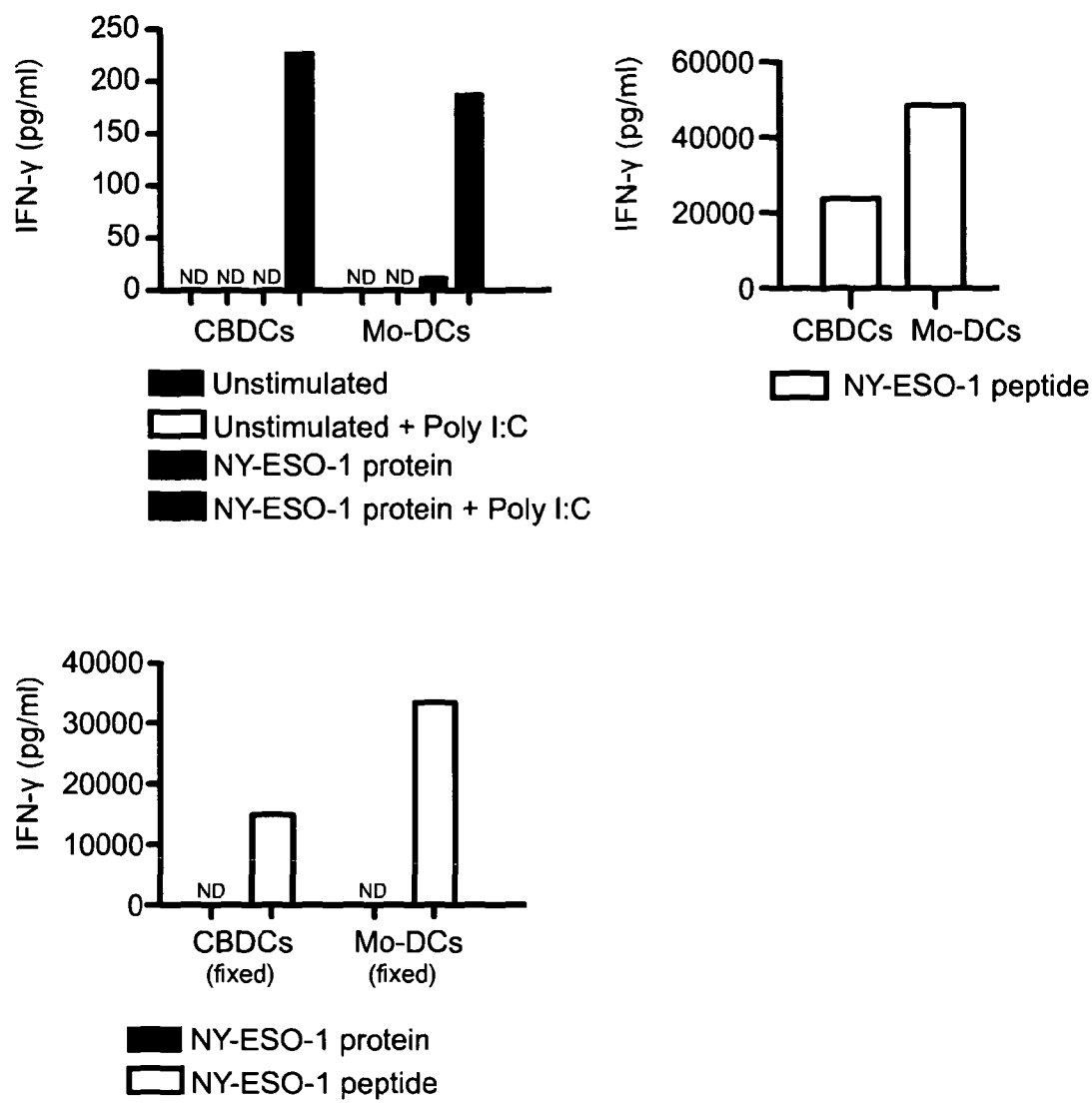


Figure 12
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INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2011/000623

A. CLASSIFICATION OF SUBJECT MATTER
 INV. C12N5/0784
 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)
 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	SANCHO DAVID ET AL: "Tumor therapy in mice via antigen targeting to a novel, DC-restricted C-type lectin.", THE JOURNAL OF CLINICAL INVESTIGATION JUN 2008 LNKD- PUBMED:18497879, vol. 118, no. 6, June 2008 (2008-06), pages 2098-2110, XP55004997, ISSN: 0021-9738 the whole document ----- -/--	1,2, 4-16, 24-27, 29,30, 32-45



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

19 August 2011

Date of mailing of the international search report

06/09/2011

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+31-70) 340-2040,
 Fax: (+31-70) 340-3016

Authorized officer

Manu, Dominique

INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB2011/000623

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

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

☒

 on paper

☒

 in electronic form
 - b. (time)

☐

 in the international application as filed

☐

 together with the international application in electronic form

☒

 subsequently to this Authority for the purpose of search
2.

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

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2011/000623

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CAMINSCHI IRINA ET AL: "The dendritic cell subtype-restricted C-type lectin Clec9A is a target for vaccine enhancement.", BLOOD 15 OCT 2008 LNKD- PUBMED:18669894, vol. 112, no. 8, 15 October 2008 (2008-10-15), pages 3264-3273, XP2503156, ISSN: 1528-0020 the whole document	1,2, 4-16, 24-27, 29,30, 32-45
X	ALINE FLEUR ET AL: "Generation of specific Th1 and CD8+ T-cell responses by immunization with mouse CD8+ dendritic cells loaded with HIV-1 viral lysate or envelope glycoproteins.", MICROBES AND INFECTION / INSTITUT PASTEUR APR 2007 LNKD- PUBMED:17350307, vol. 9, no. 4, April 2007 (2007-04), pages 536-543, XP5934801, ISSN: 1286-4579 the whole document	25,27, 28, 31-34, 42-45
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Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	VILLADANGOS J A ET AL: "Found in translation: The human equivalent of mouse CD8<+> dendritic cells", JOURNAL OF EXPERIMENTAL MEDICINE 20100607 ROCKEFELLER UNIVERSITY PRESS USA LNKD- DOI:10.1084/JEM.20100985, vol. 207, no. 6, 7 June 2010 (2010-06-07), pages 1131-1134, XP55005033, ISSN: 0022-1007 the whole document -----	1-47