

METHODS FOR THE STIMULATION OF DENDRITIC CELL (DC) PRECURSOR POPULATION “PRE-DC” AND THEIR USES THEREOF

TECHNICAL FIELD

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[0001] The present invention generally relates to methods for stimulating pre-DC to increase the immune response for treating or preventing certain diseases in a subject in need thereof. The present invention also relates to molecules that are capable of effectively stimulating pre-DC to increase a subject's immune response, and molecules that are capable of being effective indicators of pre-DC stimulation and activation. The present invention further relates to an immunogenic composition for treating or preventing diseases or improving immunization by targeting pre-DC for an increased immune response.

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BACKGROUND

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[0002] Dendritic cells (DC) are professional pathogen-sensing and antigen-presenting cells that are central to the initiation and regulation of immune responses. The DC population is classified into two lineages: plasmacytoid DC (pDC), and conventional DC (cDC), the latter comprising cDC1 and cDC2 sub-populations.

[0003] Both pDC and cDC arise from DC restricted bone-marrow (BM) progenitors known as common DC progenitors (CDP). Along the differentiation pathway of CDP giving rise to cDC, from BM to peripheral blood, it is believed that there is an intermediate population of cells called the precursor of cDC (pre-DC). The pre-DC compartment contains distinct lineage committed sub-populations including one early uncommitted CD123^{high} pre-DC subset and two CD45RA⁺CD123^{low} lineage-committed subsets called pre-cDC1 and pre-cDC2, which exhibit functional differences. Pre-cDC1 and pre-cDC2 eventually differentiate into cDC1 and cDC2, respectively.

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[0004] The heterogeneous DC population is capable of processing and presenting antigens to naïve T cells to initiate antigen-specific immune responses. In many cases, increasing immune response to combat certain diseases is necessary to achieve desirable therapeutic effects. The conventional way of manipulating DC to increase immune responses in a subject includes stimulating various receptors expressed on the surface of DC. However,

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conventionally-defined pDC population is heterogeneous, incorporating an independent pre-DC sub-population. This makes it difficult to target specific populations of cells within the heterogeneous population to treat specific diseases. In addition, there is limited understanding of the pre-DC sub-population functions, especially the role of pre-DC in eliciting and increasing immune responses. Also, there has been no development of pre-DC specific therapeutic interventions, for example, in vaccines or treatment of diseases.

[0005] There is a need to provide means for stimulating pre-DC to increase the immune response for treating or preventing certain diseases in a subject in need thereof, that overcomes, or at least ameliorates, one or more of the disadvantages described above.

10 [0006] There is also a need to provide molecules which are capable of effectively stimulating pre-DC to activate or increase a subject's immune response, and molecules which are capable of being effective indicators of pre-DC stimulation and activation.

[0007] There is further a need to provide an immunogenic composition for treating or preventing diseases or improving immunization by targeting pre-DC for an increased immune response.

SUMMARY

[0008] According to a first aspect, there is provided a method of treating or preventing an infection, a neoplastic disease or an immune-related disease in a subject in need thereof, the method comprising contacting a therapeutically effective or immuno-effective amount of an TLR9 agonist with a precursor dendritic cell (pre-DC), wherein the TLR9 agonist stimulates the pre-DC to secrete one or more cytokines, to thereby activate or increase the subject's immune response for treating or preventing the infection, the neoplastic disease or the immune-related disease.

[0009] According to a second aspect, there is provided use of one or more TLR9 agonists in the manufacture of a medicament for treating or preventing an infection, a neoplastic disease or an immune-related disease in a subject in need thereof, wherein the TLR9 agonist stimulates pre-DC to secrete one or more cytokines to thereby activate or increase the subject's immune response for treating or preventing the infection, the neoplastic disease or the immune-related disease.

[0010] According to a third aspect, there is provided an immunogenic composition comprising one or more TLR9 agonists capable of stimulating pre-DC to secrete one or more cytokines.

5 [0011] According to a fourth aspect, there is provided an adjuvant composition comprising a TLR9 agonist that is capable of stimulating pre-DC to secrete one or more cytokines for increasing a subject's immune response to treat or prevent an infection, a neoplastic disease or an immune-related disease.

[0012] According to a fifth aspect, there is provided a method of diagnosing a deficient immune system in a subject, said method comprising:

- 10 (a) obtaining a sample comprising pre-DC from the subject;
(b) contacting the sample with one or more TLR9 agonists;
(c) detecting the presence or absence of one or more cytokines in the sample; and
(d) diagnosing the subject as one having a deficient immune system when the one or more cytokines in the sample is absent (or not detected) or is present in a lower level when
15 compared to a control sample.

[0013] According to a sixth aspect, there is provided a method of eliciting an immune response against an infection, a neoplastic disease or an immune-related disease in a subject in need thereof, the method comprising contacting an immuno-effective amount of an TLR9 agonist with pre-DC, wherein the TLR9 agonist stimulates the pre-DC to secrete one or more
20 cytokines, to thereby elicit an immune response against the infection, the neoplastic disease or the immune-related disease.

[0014] According to a seventh aspect, there is provided a kit for diagnosing a deficient immune system in a subject according to the method as described herein.

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DEFINITION OF TERMS

[0015] The following words and terms used herein shall have the meaning indicated:

[0016] The term "marker" refers to any biological compound, such as a protein and a fragment thereof, a peptide, a polypeptide, or other biological material whose presence,
30 absence, level or activity is correlative of or predictive of a characteristic such as a cell type. Such specific markers may be detectable by using methods known in the art, such as but are not limited to, flow cytometry, fluorescent microscopy, immunoblotting, RNA sequencing,

gene arrays, mass spectrometry, mass cytometry (Cy TOF) and PCR methods. A marker may be recognized, for example, by an antibody (or an antigen-binding fragment thereof) or other specific binding protein(s). Reference to a marker may also include its isoforms, preforms, mature forms, variants, degraded forms thereof (such as fragments thereof), and metabolites thereof.

[0017] The term “treatment” and variations of that term includes any and all uses which remedy a disease state or symptoms, prevent the establishment of disease, or otherwise prevent, hinder, retard, or reverse the progression of disease or other undesirable symptoms in any way whatsoever. Hence, “treatment” includes prophylactic and therapeutic treatment.

[0018] The term “preventing” a disease refers to inhibiting completely or in part the development or progression of a disease (such as an immune-related disease) or an infection (such as an infection by a virus or bacteria). Vaccination is a common medical approach to prevent diseases where upon vaccination, immunization is initiated such that the body’s own immune system is stimulated to protect the subject from infection or disease, or from subsequent infection or disease. Immunization may, for example, enable a continuing high level of antibody and/or cellular response in which T-lymphocytes can kill or suppress the pathogen in the immunized subject. The pathogen may be one which the subject has been previously exposed to.

[0019] The term “subject” refers to patients of human or other mammals, and includes any individual it is desired to be treated using the immunogenic compositions and methods of the disclosure. However, it will be understood that “subject” does not imply that symptoms are present. Suitable mammals that fall within the scope of the disclosure include, but are not restricted to, primates, livestock animals (e.g. sheep, cows, horses, donkeys, pigs), laboratory test animals (e.g. rabbits, mice, rats, guinea pigs, hamsters), companion animals (e.g. cats, dogs) and captive wild animals (e.g. foxes, deer, dingoes).

[0020] The term “contacting” and variations of that term including “contact”, refers to incubating or otherwise exposing a compound or composition of the disclosure to cells (such as the pre-DC cells) of an organism (such as a subject as described herein) . The contacting may occur in vitro, in vivo or ex vivo. The term “contacting” may also refer to administration of a compound or composition of the disclosure to an organism (such as a subject as described herein) by any appropriate means as described below.

[0021] The term “in vitro” as used herein refers to conducting a process or procedure outside a living organism, such as in a test tube, a culture vessel or a plate, or elsewhere outside the living organism.

5 [0022] The term “in vivo” as used herein refers to a process or procedure which is being performed in a subject.

[0023] The term “ex vivo” as used herein refers to a process or procedure conducted on live isolated cells outside a subject, and then returned to the living subject. For example, pre-DC may be extracted from a subject, contacted with a TLR9 agonist (for example, in a test tube, a culture vessel or a plate), and then returned to the subject to induce an immune response.

[0024] The term “administering” and variations of that term including “administer” and “administration”, includes contacting, applying, delivering or providing a compound or composition of the disclosure to an organism (such as a subject as described herein), or a surface by any appropriate means.

15 [0025] The term “immunogenic composition” as used herein refers to a composition which is capable of stimulating the immune system of a subject to produce an immune response. An immunogenic composition may comprise, for example, a specific type of antigen against which an immune response is desired to be elicited.

[0026] “Immune response” refers to conditions associated with, or caused by, inflammation, trauma, immune disorders, or infectious or genetic disease, and can be characterized by expression of various factors, e.g., cytokines, chemokines, and other signaling molecules, which may affect cellular and systemic defence systems.

20 [0027] The term “agonist”, when used in reference to TLR9, refers to a molecule which intensifies or mimics the biological activity of TLR9. Agonists may include proteins, nucleic acids, carbohydrates, small molecules, or any other compounds or compositions which modulate the activity of TLR9, either by directly interacting with TLR9 or by acting as components of the biological pathways in which TLR9 participates.

25 [0028] The term “antigen” refers to a molecule or a portion (such as a fragment) of a molecule capable of being recognized by antigen-binding molecules of the immune system, and inducing an immune response in the subject. Sources of antigen may be, but are not limited to, toxins, pollen, bacteria (or parts thereof), viruses (or parts thereof) or other microorganisms (or parts thereof). Parts of bacteria, viruses or other microorganisms which

may act as antigens may be, but are not limited to, coats, capsules, cell walls, flagella, and fimbriae. If an antigen causes a specific disease (such as a disease caused by the host bacteria, virus or other microorganism which is the source of the antigen), then the antigen may be said to be associated with the disease.

5 [0029] Unless specified otherwise, the terms “comprising” and “comprise”, and grammatical variants thereof, are intended to represent “open” or “inclusive” language such that they include recited elements but also permit inclusion of additional, unrecited elements.

[0030] Throughout this disclosure, certain examples may be disclosed in a range format. It should be understood that the description in range format is merely for convenience and
10 brevity and should not be construed as an inflexible limitation on the scope of the disclosed ranges. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4,
15 from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

[0031] Certain examples may also be described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the disclosure. This includes the generic description of the examples with a proviso or
20 negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

DETAILED DISCLOSURE OF THE EMBODIMENT

25 [0032] According to a first aspect, there is provided a method of treating or preventing an infection, a neoplastic disease or an immune-related disease in a subject in need thereof, the method comprising contacting a therapeutically effective or immuno-effective amount of an TLR9 agonist with a precursor dendritic cell (pre-DC), wherein the TLR9 agonist stimulates the pre-DC to secrete one or more cytokines, to thereby activate or increase the subject's
30 immune response for treating or preventing the infection, the neoplastic disease or the immune-related disease. In one example, the pre-DC presents an antigen (or a fragment thereof) associated with the infection, the neoplastic disease or the immune-related disease in

the subject. In another example, the pre-DC does not present any antigen. In one example, pre-DC were found to produce significantly more of the cytokines TNF- α and IL-12p40 when exposed to CpG ODN 2216 (also referred to as CpG, a TLR9 agonist), than either LPS (a TLR4 agonist) or polyI:C (TLR3 agonist)(see Fig. 5C). Cytokines such as TNF- α are known to exert a variety of effects on the immune response of a host such as in controlling infection and to modulate macrophage activity to control disease pathology. TNF- α has also been previously shown to exert a variety of effects in controlling infection. IL-12p40, another cytokine, is known to have protective function during infections. . Thus, the contacting of TLR9 agonist with pre-DC enables a subject's immune response to be stimulated through the release of TNF- α and IL-12p40 cytokines to a therapeutically effective or immune-effective level for treating and preventing infections, neoplastic diseases or immune-related diseases.

[0033] Dendritic cells, such as pre-DC, are involved in the initiation of immune response to bacterial and viral infections. Upon infection by a pathogenic bacteria or virus, dendritic cells, such as pre-DC, will take up the bacterial or viral antigens in the peripheral tissues, process the antigens into proteolytic peptides, and load these peptides onto major histocompatibility complex (MHC) class I and II molecules. The dendritic cells, such as pre-DC, then become competent to present antigens to T lymphocytes, thus initiating antigen-specific immune responses. During this immune response, the TLR-9 agonist functions to specifically stimulate pre-DC to release cytokines to activate and/or enhance the immune response against the antigens.

[0034] Exemplary diseases in which the method as disclosed herein may be useful include but are not limited to bacterial infections, and viral infections, or the like. Examples of viruses which may cause viral infections are DNA viruses, and RNA viruses. Examples of DNA viruses are herpes simplex virus (HSV-1), cytomegalovirus (CMV), adenovirus, poxvirus, hepatitis B virus (HBV), or the like. Examples of RNA viruses are human immunodeficiency virus (HIV), hepatitis A virus (HAV), hepatitis C virus (HCV), respiratory syncytial virus (RSV), influenza, Zika virus, or the like.

[0035] In one example, the immune-related disease is an inflammatory disease. In another example, the immune-related disease is an autoimmune disease. Immune-related diseases may be caused by dysfunction or abnormality in the immune response. The dysfunction or abnormality in the immune response may be caused by genetic mutations, reaction to a drug, radiation therapy, or other chronic and/or serious disorders (such as cancer or diabetes).

[0036] In one example, the autoimmune disease is selected from the group consisting of systemic lupus erythematosus (SLE) and Sjögren's syndrome.

[0037] Exemplary TLR9 agonists which may be useful for stimulating the pre-DC cells include but is not limited to an oligodeoxynucleotides (ODN), or a biological or functional
5 variant thereof.

[0038] Exemplary CpG oligodeoxynucleotides include CpG ODN Class A, CpG ODN Class B and CpG ODN Class C. In one example, the CpG oligodeoxynucleotide is CpG ODN 2216, or a biological or a functional variant thereof.

[0039] The biological variant of a CpG ODN is expected to display substantially the same
10 biological activity as the CpG ODN 2216 of which it is a variant. For example, the biological variant of CpG ODN 2216 is expected to display substantially the same biological activity as CpG ODN 2216 as an agonist of TLR9. Alternatively, the TLR9 agonist may be a functional variant of a CpG ODN. A functional variant typically has substantial or significant sequence identity or similarity to the CpG ODN of which it is a variant, such as at least 80% (e.g. 80%,
15 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%) identity to the CpG ODN sequence of which it is a variant, and retains the same activity as the CpG ODN.

[0040] The TLR9 agonist (or a composition thereof) may be contacted with a pre-DC or administered in a therapeutically effective amount or an immune-effective amount. A therapeutically effective amount includes a sufficient but non-toxic amount of a TLR9
20 agonist (or a composition thereof) to provide the desired therapeutic effect. An immune-effective amount includes a sufficient but non-toxic amount of a TLR9 agonist (or a composition thereof) to provide the desired immunoprotective effect. The exact amount required will vary from subject to subject depending on factors such as the species being treated, the age and general condition of the subject, the severity of the condition being
25 treated, the particular agent or composition being contacted or administered, the mode of contact or administration, and so forth. Thus, it is not possible to specify an exact "effective amount". However, for any given case, an appropriate "effective amount" may be determined by one of ordinary skill in the art using only routine experimentation. For example, an effective amount to result in therapeutic or immunoprotective amount may be an
30 amount sufficient to result in the improvement of the pathological symptoms of a target disease or an amount sufficient to result in protection against a target infectious disease. Generally, an effective dosage may be in the range of about 100 ng/kg to about 100 mg/kg,

about 100 ng/kg to about 90 mg/kg, about 100 ng/kg to about 80 mg/kg, about 100 ng/kg to about 70 mg/kg, about 100 ng/kg to about 60 mg/kg, about 100 ng/kg to about 50 mg/kg, about 100 ng/kg to about 40 mg/kg, about 100 ng/kg to about 30 mg/kg, about 100 ng/kg to about 20 mg/kg, about 100 ng/kg to about 10 mg/kg, about 90 ng/kg to about 100 mg/kg, about 80 ng/kg to about 100 mg/kg, about 70 ng/kg to about 100 mg/kg, about 60 ng/kg to about 100 mg/kg, about 50 ng/kg to about 100 mg/kg, about 40 ng/kg to about 100 mg/kg, about 30 ng/kg to about 100 mg/kg, or about 20 ng/kg to about 100 mg/kg, and includes any subranges therein, as well as individual numbers within the ranges and subranges.

[0041] Exemplary cytokines which may be produced by pre-DC upon stimulation with a TLR9 agonist include but are not limited to tumor necrosis factors, interleukins, interferons, and chemokines, or the like.

[0042] In one example, the tumor necrosis factor that is produced by pre-DC upon stimulation with a TLR9 agonist is TNF- α . In one example, CpG ODN 2216 was shown to stimulate pre-DC to produce high levels of cytokine, specifically TNF- α (see Fig. 5C).

[0043] In another example, the interleukin that is produced by pre-DC upon stimulation with a TLR9 agonist is IL-12p40. In one example, IL-12p40 was shown to be readily secreted by pre-DC when stimulated with TLR9 agonists (see Fig. 2G).

[0044] In yet another example, the interferon that is produced by pre-DC upon stimulation with a TLR9 agonist is IFN- α .

[0045] Pre-DC is a subset of CD33⁺CD45RA⁺CD123⁺ cell which gives rise to cDC subsets (**Fig. 2A**, and **Fig. 10A**). Pre-DC cells also express CX3CR1, CD2, CD303 and CD304, with low CD11c expression (**Fig. 2, A and B**, and **Fig. 10, B and C**). The pre-DC may be identified based on the expression of pre-DC-specific marker genes such as those listed in **Fig. 27** and **Fig. 28**. For example, the pre-DC may be isolated based on the specific marker genes through conventional gating strategy such as, but not limited to those, described in **Fig. 10A, 11, 12A-C, 14, 15, 18** and **19**.

[0046] In another example, the pre-DC comprises one or more markers selected from the group consisting of CD123, CD303, CD304, CD327, CD45RA, CD85j, CD5 and BTLA. The expression of the markers may be determined based on the gene expression or protein expression levels using methods known in the art, such as but are not limited to, flow cytometry, fluorescent microscopy, immunoblotting, RNA sequencing, gene arrays, mass spectrometry, mass cytometry (Cy TOF) and PCR methods.

[0047] Early pre-DC can differentiate to both cDC subsets, and committed pre-DCs such as pre-conventional dendritic cells 1 (pre-cDC1) and pre-conventional dendritic cells 2 (pre-cDC2) differentiate exclusively into cDC1 and cDC2 subsets, respectively (**Fig. 3H**, **Fig. 18D**, and **Fig. 19**).

5 [0048] Therefore, in one example, the pre-DC is selected from the group consisting of early pre-DC, pre-conventional dendritic cells 1 (pre-cDC1), and pre-conventional dendritic cells 2 (pre-cDC2).

[0049] In one example, the subject is a human. The subject may be one suffering from any of the diseases disclosed herein and is in need of treatment. The subject may also be a human
10 at risk of any of the bacterial or viral infections disclosed herein, such as subjects living in (or in close proximity to areas) with a bacterial or viral outbreak who may require vaccination against these infections. The human subjects can be either adults or children. In another example, the subject is a human suffering from any of the immune-related disease disclosed herein. In yet another example, the subject is a human with a deficient immune system. The
15 methods of the disclosure can also be used on other subjects at risk of any of the bacterial or viral infections disclosed herein or suffering from any of the diseases disclosed herein such as, but not limited to, non-human primates, livestock animals (eg. sheep, cows, horses, donkeys, pigs), laboratory test animals (eg. rabbits, mice, rats, guinea pigs, hamsters), companion animals (eg. cats, dogs) and captive wild animals (eg. foxes, deer, dingoes).

20 [0050] The TLR9 agonist may be administered to the subject by any route suitable for administration of such compounds, such as, intramuscular, intradermal, subcutaneous, intravenous, oral, and intranasal administration. Thus, the TLR9 agonist of the disclosure may be in a formulation suitable for parenteral administration (that is, subcutaneous, intramuscular or intravenous injection), in the form of a formulation suitable for oral
25 ingestion (such as capsules, tablets, caplets, elixirs, for example), or in an aerosol form suitable for administration by inhalation (such as by intranasal inhalation or oral inhalation).

[0051] For administration as an injectable solution or suspension, non-toxic parenterally acceptable diluents or carriers can include Ringer's solution, isotonic saline, phosphate buffered saline, ethanol and 1,2 propylene glycol.

30 [0052] For oral administration, suitable carriers, diluents, excipients and adjuvants include peanut oil, liquid paraffin, sodium carboxymethylcellulose, methylcellulose, sodium alginate, gum acacia, gum tragacanth, dextrose, sucrose, sorbitol, mannitol, gelatine and lecithin. In

addition these oral formulations may contain suitable flavouring and colourings agents. When used in capsule form the capsules may be coated with compounds such as glyceryl monostearate or glyceryl distearate which delay disintegration.

- [0053] Solid forms for oral administration may contain binders acceptable in human and veterinary pharmaceutical practice, sweeteners, disintegrating agents, diluents, flavourings, coating agents, preservatives, lubricants and/or time delay agents. Suitable binders include gum acacia, gelatine, corn starch, gum tragacanth, sodium alginate, carboxymethylcellulose or polyethylene glycol. Suitable sweeteners include sucrose, lactose, glucose, aspartame or saccharine. Suitable disintegrating agents include corn starch, methylcellulose, polyvinylpyrrolidone, guar gum, xanthan gum, bentonite, alginic acid or agar. Suitable diluents include lactose, sorbitol, mannitol, dextrose, kaolin, cellulose, calcium carbonate, calcium silicate or dicalcium phosphate. Suitable flavouring agents include peppermint oil, oil of wintergreen, cherry, orange or raspberry flavouring. Suitable coating agents include polymers or copolymers of acrylic acid and/or methacrylic acid and/or their esters, waxes, fatty alcohols, zein, shellac or gluten. Suitable preservatives include sodium benzoate, vitamin E, alpha-tocopherol, ascorbic acid, methyl paraben, propyl paraben or sodium bisulphite. Suitable lubricants include magnesium stearate, stearic acid, sodium oleate, sodium chloride or talc. Suitable time delay agents include glyceryl monostearate or glyceryl distearate.
- [0054] Liquid forms for oral administration may contain, in addition to the above agents, a liquid carrier. Suitable liquid carriers include water, oils such as olive oil, peanut oil, sesame oil, sunflower oil, safflower oil, arachis oil, coconut oil, liquid paraffin, ethylene glycol, propylene glycol, polyethylene glycol, ethanol, propanol, isopropanol, glycerol, fatty alcohols, triglycerides or mixtures thereof.
- [0055] Suspensions for oral administration may further comprise dispersing agents and/or suspending agents. Suitable suspending agents include sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethyl-cellulose, poly-vinyl-pyrrolidone, sodium alginate or acetyl alcohol. Suitable dispersing agents include lecithin, polyoxyethylene esters of fatty acids such as stearic acid, polyoxyethylene sorbitol mono- or di-oleate, -stearate or -laurate, polyoxyethylene sorbitan mono- or di-oleate, -stearate or -laurate and the like.

[0056] The emulsions for oral administration may further comprise one or more emulsifying agents. Suitable emulsifying agents include dispersing agents as exemplified above or natural gums such as guar gum, gum acacia or gum tragacanth.

[0057] Drops for oral administration according to the present disclosure may comprise
5 sterile aqueous or oily solutions or suspensions. These may be prepared by dissolving the immunogenic agent in an aqueous solution of a bactericidal and/or fungicidal agent and/or any other suitable preservative, and optionally including a surface active agent. The resulting solution may then be clarified by filtration, transferred to a suitable container and sterilised. Sterilisation may be achieved by: autoclaving or maintaining at 90°C-100°C for half an hour,
10 or by filtration, followed by transfer to a container by an aseptic technique. Examples of bactericidal and fungicidal agents suitable for inclusion in the drops are phenylmercuric nitrate or acetate (0.002%), benzalkonium chloride (0.01%) and chlorhexidine acetate (0.01%). Suitable solvents for the preparation of an oily solution include glycerol, diluted alcohol and propylene glycol.

[0058] Upon contact with the TLR9 agonist (or a composition comprising the TLR9 agonists described above) with pre-DC, the subject's immune response may be stimulated through the release of cytokines (such as, but not limited to, TNF- α and IL-12p40) to a therapeutically effective or immune-effective level for treating and preventing infections, neoplastic diseases or immune-related diseases.

[0059] According to a second aspect, there is provided use of one or more TLR9 agonists in the manufacture of a medicament for treating or preventing an infection, a neoplastic disease or an immune-related disease in a subject in need thereof, wherein the TLR9 agonist stimulates pre-DC that present an antigen (or a fragment thereof) associated with the infection or immune-related disease in the subject to secrete one or more cytokines to thereby
25 increase the subject's immune response for treating or preventing the infection, the neoplastic disease or the immune-related disease.

[0060] In one example, the medicament is a vaccine for preventing an infection, a neoplastic disease or an immune-related disease in a subject in need thereof.

[0061] According to a third aspect, there is provided an immunogenic composition
30 comprising: (a) an antigen (or a fragment thereof) associated with an infection, a neoplastic disease or an immune-related disease, and (b) one or more TLR9 agonists capable of

stimulating pre-DC that present the antigen (or a fragment thereof) to secrete one or more cytokines.

[0062] As described herein, an immunogenic composition is a composition which is capable of stimulating the immune system of a subject to produce an immune response
5 against an antigen. Sources of antigen may be, but are not limited to, toxins, pollen, bacteria (or parts thereof), viruses (or parts thereof) or other microorganisms (or parts thereof). Parts of bacteria, viruses or other microorganisms which may act as antigens may be, but are not limited to, coats, capsules, cell walls, flagella, and fimbriae.

[0063] In one example, the immunogenic composition is a vaccine.

10 [0064] In general, suitable immunogenic compositions may be prepared according to methods which are known to those of ordinary skill in the art and accordingly may include a pharmaceutically acceptable carrier, diluent and/or adjuvant. The carriers, diluents and adjuvants must be "acceptable" in terms of being compatible with the other ingredients of the composition, and not deleterious to the recipient thereof.

15 [0065] One skilled in the art would be able, by routine experimentation, to determine an effective and safe amount of the immunogenic composition for contact or administration to achieve the desired immunogenic response.

[0066] Generally, an effective dosage to achieve the desired immunogenic response is expected to be in the range of about 0.0001mg to about 1000mg per kg body weight per 24
20 hours; typically, about 0.001mg to about 750mg per kg body weight per 24 hours; about 0.01mg to about 500mg per kg body weight per 24 hours; about 0.1mg to about 500mg per kg body weight per 24 hours; about 0.1mg to about 250mg per kg body weight per 24 hours; about 1.0mg to about 250mg per kg body weight per 24 hours. More typically, an effective dose range is expected to be in the range about 1.0mg to about 200mg per kg body weight per
25 24 hours; about 1.0mg to about 100mg per kg body weight per 24 hours; about 1.0mg to about 50mg per kg body weight per 24 hours; about 1.0mg to about 25mg per kg body weight per 24 hours; about 5.0mg to about 50mg per kg body weight per 24 hours; about 5.0mg to about 20mg per kg body weight per 24 hours; about 5.0mg to about 15mg per kg body weight per 24 hours.

30 [0067] Alternatively, an effective dosage to achieve the desired immunogenic response may be up to about 500mg/m². Generally, an effective dosage is expected to be in the range of about 25 to about 500mg/m², preferably about 25 to about 350mg/m², more preferably

about 25 to about 300mg/m², still more preferably about 25 to about 250mg/m², even more preferably about 50 to about 250mg/m², and still even more preferably about 75 to about 150mg/m².

[0068] According to a fourth aspect, there is provided an adjuvant composition comprising a TLR9 agonist that is capable of stimulating pre-DC that present an antigen (or a fragment thereof) associated with an infection, a neoplastic disease or an immune-related disease in a subject to secrete one or more cytokines for increasing the subject's immune response to treat or prevent the infection, the neoplastic disease or the immune-related disease.

10 [0069] As an adjuvant composition, the adjuvant composition comprising a TLR9 agonist is capable of increasing the effectiveness of a composition for stimulating immune response, for example through stimulation of cytokines release from pre-DC.

[0070] As described herein, in one example, the subject who may benefit from the methods or compositions of the disclosure is one who has a deficient immune system. A subject with deficient immune system may be one who is unable to activate the immune response, or one whose immune system is partially activated (for example, activated to only a certain extent, such as in the range of about 10% to about 90%, about 10% to about 80%, about 10% to about 70%, about 10% to about 60%, about 10% to about 50%, about 10% to about 40%, about 10% to about 30%, about 10% to about 20%, and includes any subranges therein, as well as individual numbers within the ranges and subranges, compared to a subject without a deficient immune system). Such a condition may be due to abnormal pre-DC cells which are unable to produce cytokines, resulting in a deficient level of cytokines required for activation of the immune response. For example, while a normal pre-DC is able to secrete cytokines, such as TNF- α and IL-12p40, when stimulated, a subject with abnormal pre-DC may secrete lower levels of cytokines (or no cytokines) compared to a healthy subject.

25 [0071] Therefore, according to a fifth aspect, there is provided a method of diagnosing a deficient immune system in a subject, said method comprising:

- (a) obtaining a sample comprising pre-DC from the subject;
- (b) contacting the sample with one or more TLR9 agonists;
- 30 (c) detecting the presence or absence of one or more cytokines in the sample; and

(d) diagnosing the subject as one having a deficient immune system when the one or more cytokines in the sample is absent (or not detected) or is present in a lower level when compared to a control sample.

[0072] Samples suitable for use in the methods described herein include tissue culture, blood, apheresis residue, tissue (from various organs, such as spleen, kidney, etc.), peripheral blood mononuclear cells or bone marrow. The samples may be obtained by methods, such as but not limited to, surgery, aspiration or phlebotomy. The samples may be untreated, treated, diluted or concentrated from the subject.

[0073] The contacting of the samples with one or more TLR9 may be conducted in vitro, in vivo or ex vivo.

[0074] The cytokines may be detected using methods known in the art, such as but are not limited to, labelling with cytokine-specific antibodies followed by flow cytometry analysis, ELISA, or other commercially available cytokine detection assay kits (such as the Luminex assay kits).

[0075] In the context of detecting cytokine, such as a TNF- α and IL-12p40, the term “absence” (or grammatical variants thereof) can refer to when cytokine cannot be detected using a particular detection methodology. For example, cytokine may be considered to be absent in a sample if the sample is free of cytokine, such as, 95% free, 96% free, 97% free, 98% free, 99% free, 99.9% free, or 100% free of cytokine, or is undetectable as measured by the detection methodology used. Alternatively, if the level of cytokine (such as TNF- α and IL-12p40) is below a previously determined cut-off level, the cytokine may also be considered to be “absent” from the sample.

[0076] In the context of detecting cytokine, such as a TNF- α and IL-12p40, the term “presence” can refer to when a cytokine can be detected using a particular detection methodology. For example, if the level of cytokine (such as TNF- α and IL-12p40) is above a previously determined threshold level, the cytokine may be considered to be “present” in the sample.

[0077] A control sample that may be used in the methods disclosed herein includes, but is not limited to, a sample which is not contacted with one or more TLR9 agonist or a sample from a healthy subject (for example, a subject whose immune system is not deficient) which has been contacted with one or more TLR9 agonist.

[0078] In one example, the method further comprises treating the subject diagnosed with a deficient immune system by administering a composition described herein, to thereby increase the subject's immune response.

[0079] According to a sixth aspect, there is provided a method of eliciting an immune response against an infection, a neoplastic disease or an immune-related disease in a subject in need thereof, the method comprising contacting an immuno-effective amount of an TLR9 agonist with a pre-DC, wherein the TLR9 agonist stimulates precursor dendritic cells (pre-DC) that present an antigen (or a fragment thereof) associated with the infection, the neoplastic disease or the immune-related disease in the subject to secrete one or more cytokines, to thereby elicit an immune response against the infection, the neoplastic disease or the immune-related disease.

[0080] The immune response may be considered "elicited" when the humoral and/or cell-mediated immune responses are triggered, resulting in protection of the subject from subsequent infections, removal of pathogenic bacteria, virus or microorganisms, and/or inhibition of the development or progression of a disease or infection by a virus or bacteria.

[0081] According to a seventh aspect, there is provided a kit for diagnosing a deficient immune system in a subject according to the method as described herein. Other components of a kit may include, but are not limited to, one or more of the TLR9 agonist described above, one or more cytokine-specific antibodies, one or more buffers, and one or more diluents.

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BRIEF DESCRIPTION OF THE DRAWINGS

[0082] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings, in which:

[0083] **Fig. 1. MARS-seq and CyTOF identify rare CD123⁺CD33⁺ putative DC precursors (pre-DC).** (A-E) Lin(CD3/CD14/CD16/CD20/CD34)⁻HLA-DR⁺CD135⁺ sorted PBMC were subjected to MARS-seq. (A) shows a t-stochastic neighbor embedding (tSNE) plot of 710 cells fulfilling all quality criteria, displayed by clusters identified by tSNE plus Seurat clustering, or by the relative signature score for pDC, cDC1 and cDC2. (B) illustrates a connectivity MAP (cMAP) analysis showing the degree of enrichment for pDC or cDC signature genes in the tSNE/Seurat clusters. (C) shows Mpath analysis applied to the

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tSNE/Seurat clusters defining their developmental relationship. Representations of the 710 cells by (D) Monocle, (E) Principal component analysis (PCA) and (F) Diffusion Map, highlighting the tSNE/Seurat clusters identified in (A). (G) shows violin plots of tSNE/Seurat pDC clusters, cluster #4 and cDC clusters showing the expression of pDC and cDC signature genes with differential expression between cluster #4 and pDC clusters. Adjusted P-values were calculated by Kruskal-Wallis test followed by Dunn's multiple comparisons procedure. (H, I) provide tSNE plots of CyTOF data from CD45⁺Lin(CD7/CD14/CD15/CD16/CD19/CD34)⁻HLA-DR⁺ PBMC, showing in (H) gates defining the CD123⁺CD33⁺ cells and DC subsets, and in (I) relative expression of selected markers. In (J), subsets defined in (H) were overlaid onto 2D-contour plots for phenotypic comparison. The gating strategy prior to MARS-seq is shown in Fig. 7A.

[0084] **Fig. 2. Characterization of human pre-DC.** (A) shows flow cytometric identification of pre-DC and pDC within PBMC and spleen cell suspensions. (B) shows expression of CD303/CD304/CD123/CD11c by blood pre-DC and DC subsets. (C) shows % pre-DC within spleen (n=3) and PBMC (n=6) CD45⁺ populations. (D) shows Wright-Giemsa staining of sorted blood pre-DC and DC subsets. (E) shows electron micrographs of pre-DC and pDC (RER (arrowheads), centriole (C) and microtubules (small arrows), near RER cisterna are indicated). (F) shows DC subsets or pre-DC co-cultured for 5 days with MS-5 feeder-cells, FLT3L, GM-CSF and SCF. Their capacity to differentiate into cDC1 or cDC2 was measured by flow cytometry. (n=3) (G) shows intracellular detection of cytokines in DC subsets and pre-DC post-TLR stimulation. IFN- α and IL-12p40 production by pDC and pre-DC, alongside mean % cytokine-positive pre-DC and DC subsets exposed to LPS, LPS+IFN γ (L+I), polyI:C (pI:C), CL097 (CL) or CpG-ODN2216 (CpG) (n=4) are shown. (H) shows the proliferation of naïve CD4⁺ T cells cultured for 6 days with allogeneic pDC, total CD123⁺HLA-DR⁺ cells or pre-DC (n=2). (I) shows frequency of pDC and pre-DC from control subjects (Ctrl, n=11) and Pitt-Hopkins Syndrome (PHS) patients (n=4). P-values were calculated by Mann-Whitney test. Error bars represent mean +/- SEM.

[0085] **Fig. 3. Identification of committed human pre-DC subsets.** (A-B) shows single-cell mRNA sequencing (scmRNAseq) of 92 Lin(CD3/14/16/19/20)⁻HLA-DR⁺CD33⁺CD123⁺ cells (sort gating strategy shown in Fig. 14A). (A) shows the connectivity MAP (cMAP) enrichment score of cells (cDC1- vs cDC2-specific signatures). (B) shows the Mpath analysis showing the developmental relationship between "unprimed", cDC1-primed or cDC2-primed

cells defined in (A). (C) shows Lin⁻HLA-DR⁺CD33⁺ PBMC analyzed by flow cytometry and visualized as 3D-PCA of three cell clusters (pre-DC, cDC1 and cDC2) and the relative expression of CADM1, CD1c and CD123. (D) shows relative expression of CD45RA, BTLA, CD327, CD141 and CD5 in the same 3D-PCA plot. The dashed black circles indicate the intermediate CD45RA⁺ population. (E) shows CD45RA/CD123 dot plots showing overlaid cell subsets defined in the 3D-PCA plot (left panel) with the relative expression of BTLA, CD327, CD141 and CD5. (F) shows overlay of the Wanderlust dimension (progression from early (dark) to late (clear) events is shown) onto the 3D-PCA and CD45RA/CD123 dot plots. (G) illustrates the gating strategy starting from live CD45⁺Lin(CD3/14/16/19/20)⁻CD34⁻HLA-DR⁺ PBMC to define pre-DC subsets among CD33⁺CD45RA⁺ cDC. (H) shows pre-DC subsets co-cultured for 5 days with MS-5 feeder-cells, FLT3L, GM-CSF and SCF (n=3). Their capacity to differentiate into Clec9A⁺CADM1⁺ cDC1 (middle panel), or CD1c⁺CD11c⁺ cDC2 (right panel) was analyzed by flow cytometry. (I) shows scanning electron microscopy of pre-DC and DC subsets (scale bar: 1μm).

[0086] **Fig. 4. DC and pre-DC subset gene expression analysis.** (A) shows microarray data from sorted DC and pre-DC subsets (shown in Fig. 3) were analyzed by 3D PCA using differentially-expressed genes (DEG). For each PCA dimension (principal component, PC), the variance explained by each component is indicated. (B-D) show heat maps of DEG between (B) early pre-DC/pDC, (C) early pre-DC/pre-cDC1/cDC1 and (D) early pre-DC/pre-cDC2/cDC2. (E) shows expression profiles of 62 common genes identified from DEG analysis comparisons along the lineage progression from early pre-DC to mature cDC, for cDC1 and cDC2 respectively. The profiles were plotted with the log2 fold-change values (versus early pre-DC). (F) shows expression level of CD327 (SIGLEC6), CD22 and AXL proteins by DC and pre-DC subsets evaluated by flow cytometry. The mean fluorescence intensities are indicated. (G) shows expression profiles of selected transcription factors.

[0087] **Fig. 5. Functional analysis of DC and pre-DC subsets.** (A) shows frequency of cytokine production by pre-DC and DC subsets upon TLR stimulation measured by intracellular flow cytometry. Dot plots (left panel) show IFNα, IL-12p40 and TNF-α production by pDC, early pre-DC, pre-cDC2, cDC2, pre-cDC1 and cDC1. Bar charts (right panel) show the mean relative numbers of pre-DC and DC subset cells producing IFN-α, IL-12p40 or TNF-α in response to LPS, LPS+IFNγ (L+I), pI:C, CL097 (CL) or CpG ODN2216 (CpG) (n=4). (B) shows expression level (represented as mean fluorescence intensity (MFI))

of costimulatory molecules (CD40, CD80, CD83, CD86) by blood pre-DC and DC subsets (n=4). (C) shows proliferation of naïve CD4⁺ T cells after 6 days of culture with allogenic pre-DC and DC subsets (n=3). P-values were calculated by Mann-Whitney test. Error bars represent mean +/- SEM.

- 5 [0088] **Fig. 6. Unsupervised mapping of DC ontogeny using CyTOF.** CyTOF data from bone marrow (BM) and PBMC were analyzed using isoMAP dimensionality reduction to compare overall phenotypic relatedness of cell populations, and were automatically subdivided into clusters using the phenograph algorithm. (A, B) show IsoMAP1-2 plots showing the expression level of common DC progenitor (CDP), pDC, pre-DC and cDC
10 specific markers within (A) BM and (B) blood Lin(CD3/CD7/CD14/CD15/CD19/CD34)⁻ HLA-DR⁺CD123⁺ cells. (C) shows phenotypic association between Lin-HLA-DR⁺CD123^{hi} BM and CD123⁺ PBMC, showing progression from CDP towards pDC or pre-DC in the BM, and the clear separation of pDC and pre-DC in the blood. Cells within the pre-DC phenograph clusters (clusters #1 and #2 in the BM, and #6 in the blood) and cells within the
15 pDC phenograph clusters (clusters #3 and #4 in the BM, and #7 in the blood) were further analyzed by isoMAP to define pre-DC subsets (left panels, and **Fig. 26, C and D**) and heterogeneity among pDC (right panels, and **Fig. 26, D and E**).

- [0089] **Fig. 7.** (A) shows gating strategy for FACS of single cells from total Lin⁻HLA-DR⁺CD135⁺ cells. (B) shows the workflow of the MARS-seq single cell data analysis. (C)
20 shows the association between molecule counts and cells. Cell IDs were sorted from highest to lowest number of unique molecular identifier (UMI) or molecule counts. The data are presented on a log₁₀ axis. The three horizontal lines correspond to molecule counts of 650 (bottom), 1,050 (middle) and 1,700 (top) per cell. The shaded area indicates the range of molecule counts from 400 to 1,200 UMIs per cell. Cells with <1,050 molecules were
25 removed from the analysis (n=1,786 cells). A total of 710 high-quality cells were used for further downstream analyses. (D) shows a density plot (top panel) representing the distribution of cells with a certain number of molecules, and the first (middle panel) and second derivative (bottom panel) of the density function. The three lines correspond to molecule counts of 650 (left), 1,050 (middle) and 1,700 (right) per cell. (E) shows principal
30 component analysis (PCA) after simulation at different normalization thresholds. Points were colored according to the different runs. (F) shows a correlation plot of average expression of genes in run2 (y-axis) versus average expression of genes in run1 (x-axis). The data are

presented on a $\log_{10}$ axis. The Pearson correlation coefficient was 0.99. **(G)** shows t-distributed stochastic neighbor embedding (tSNE) analysis of the 710 single cells, colored by run association (run 1: dark, run 2: light), showed an even distribution of the cells within the tSNE plot. Lines represent a linear fit of the points. The distributions of the points along the tSNE component 1 and component 2 were represented as density plots on the top or right panel, respectively. **(H)** shows frequency of cells in the five determined clusters for run1 and run2. **(I)** shows that the mean-variability plot showed average expression and dispersion for each gene. This analysis was used to determine highly variable gene expression (labeled by gene symbol). The 36 highly variable genes were used to perform a dimensionality reduction of the single-cell data by PCA. In **(J)**, the highest gene loadings in the first and second principal component (PC1 and PC2) from the PCA of 710 high quality cells are shown.

[0090] **Fig. 8.** **(A)** shows the relative expression of signature genes of pDC (TCF4), cDC1 (CADM1) and cDC2 (CD1D) in Mpath clusters defined in **Fig. 1C**. **(B)** shows the weighted neighborhood network of the Mpath analysis shown in **Fig. 1C**. **(C)** shows the analysis of MARS-seq data using the Wishbone algorithm. In the 2D-t-distributed stochastic neighbor embedding (tSNE) plot (upper panels) and in the 3D-Diffusion Map (lower panels) (See **Fig. 1, A and F**, respectively), cells were displayed according to the values of the Wishbone trajectory (left panels) or the values of the Wishbone branches (right panels). Line chart (top right panel) shows the expression of signature genes along Wishbone trajectory. X-axis represents pseudo-time of Wishbone trajectory. Solid line represents backbone trajectory, dotted lines represent separate trajectories along the two branches. Heat maps (bottom right panels) show the expression of signature genes along Wishbone trajectory on the two branches.

[0091] **Fig. 9.** **(A)** shows the gating strategy of CD45⁺Lin(CD7/CD14/CD15/CD16/CD19/CD34)⁻HLA-DR⁺ blood mononuclear cells from CyTOF analysis for downstream t-distributed stochastic neighbor embedding (tSNE) as shown in **Fig. 1, E to G**. The name of the excluded population(s) is indicated in each corresponding 2D-plot. **(B)** shows tSNE plots of the CyTOF data from **Fig. 1, H to J** showing the expression level of cDC2-, cDC1- and pDC- specific markers. **(C)** shows that unsupervised phenograph clustering identified 10 clusters that were overlaid onto the tSNE1/2 plot of the CyTOF data from **Fig. 1, H and I**.

[0092] **Fig. 10.** (A) shows the gating of flow cytometry data to identify the Lin⁻HLA-DR⁺ cell population displayed in **Fig. 2A** (blood data displayed). (B) shows classical contour plots of CyTOF data from **Fig. 1** showing the same gating strategy as applied in the flow cytometry analyses shown in **Fig. 2A**. (C) shows flow cytometry data of the relative expression of CD33, CX3CR1, CD2, CD141, CD11c, CD135, CD1c and CADM1 by pre-DC, pDC, cDC1 and cDC2 defined in **Fig. 2A** in the blood (upper panels) and spleen (lower panels). (D) shows a ring graphical representation of the proportion of pre-DC, cDC1 and cDC2 among total Lin⁻CD34⁻HLA-DR⁺CD33⁺ cDC defined in **Fig. 2A** in the spleen (left) and blood (right). (E) shows representative electron micrographs showing morphological characteristics of a pre-DC. (F) shows histograms of the mean relative numbers of CD123⁺CD172a⁻ cells, Clec9A⁺CADM1⁺ cDC1 or CD172a⁺CD1c⁺ cDC2 from the *in vitro* differentiation assays as described in **Fig. 2F** (n=4). Error bars represent mean ± SEM.

[0093] **Fig. 11.** Gating strategy for the fluorescence-activated cell sorting of DC subsets and pre-DC used in the *in vitro* differentiation assays (**Fig. 2F**). (A) shows pre-sorted data and (B-E) show post-sorted re-analysis of (B) pre-DC, (C) cDC1, (D) cDC2, and (E) pDC.

[0094] **Fig. 12.** (A)-(C) show comparison of (A) the gating strategy from Breton *et al.* (32) (Pre-DC are shown in the two plots on the top right.) and (B) the gating strategy used in **Fig. 2A** and **Fig. 10A** (pre-DC displayed in purple) to define pre-DC. The relative numbers of pre-DC defined using the two gating strategies among live CD45⁺ peripheral blood mononuclear cells are indicated in the dot plots. (C) shows graphical representation of the median relative numbers of pre-DC defined using the two gating strategies among live CD45⁺ blood mononuclear cells (n=4). The median percentages of CD45⁺ values are indicated. P-values were calculated using the Mann-Whitney test. (D) illustrates a histogram showing the expression of CD117 by DC subsets and pre-DC determined by flow cytometry. (E)-(F) show identification of pre-DC, cDC1 and cDC2 among Lin⁻HLA-DR⁺ (E) ILT3⁺ILT1⁻ cells (33) or ILT3⁺ILT1⁺ (cDC), and (F) CD4⁺CD11c⁻ cells (34) or CD4^{int}CD11c⁺ cDC.

[0095] **Fig. 13.** shows pDC, pre-DC, cDC1 and cDC2 isolated by fluorescence-activated cell sorting were stimulated *in vitro* with LPS, LPS+IFN γ (L+I), Flagellin (Flag), polyI:C (pI:C), CL097 (CL) or CpG ODN2216 (CpG), and the soluble mediators (as indicated above each histogram) in the culture supernatants were quantified by Luminex Multiplex Assay (n=2).

[0096] **Fig. 14.** shows identification of CD33⁺CX3CR1⁺ pre-DC among Lin⁺HLA-DR⁺CD303⁺CD2⁺ cells (36).

[0097] **Fig. 15.** shows the gating strategy for the fluorescence-activated cell sorting analysis of peripheral blood mononuclear cells from control subjects (Ctrl, n=11) and patients with Pitt-Hopkins Syndrome (PHS; n=4). pDC (circled in blue) and pre-DC (circled in purple) were defined among Lin⁺HLA-DR⁺CD45RA⁺CD123⁺ cells.

[0098] **Fig. 16.** (A) shows the gating strategy for FACS of Lin⁺HLA-DR⁺CD33⁺CD45RA⁺CD1c^{lo/-}CD2⁺CADM1^{lo/-}CD123⁺ pre-DC analyzed by C1 single cell mRNA sequencing (scmRNAseq). (B) shows quality control (removing low-quality cells and minimally-expressed genes below the limits of accurate detection; low-quality cells that were identified using SINGuLAR toolbox; minimally-expressed genes with transcripts per million (TPM) values ≥ 1 in $<95\%$ of the cells) and (C) shows the work flow of the C1 scmRNAseq analyses shown in **Fig. 3A-B**. Error bars represent the maximum, third quartile, median, first quartile and minimum.

[0099] **Fig. 17.** shows the relative expression levels of signature genes of cDC1 (BTLA, THBD and, LY75) and cDC2 (CD2, SIRPA and ITGAX) in Mpath clusters defined in **Fig. 3B**.

[00100] **Fig. 18.** (A) shows the expression level of markers in the 3D-Principal Component Analysis (PCA) plots from **Fig. 3, C and D**. (B) shows the sequential gating strategy of flow cytometry data starting from Live CD45⁺Lin⁺(CD3/14/16/19/20)⁻CD34⁻HLA-DR⁺ peripheral blood mononuclear cells defining CD33⁻CD123⁺CD303⁺ pDC, CD33⁺CD45RA⁻ differentiated cDC (CADM1⁺ cDC1, CD1c⁺ cDC2), and CD33⁺CD45RA⁺ cells (comprising CD123⁺CD45RA⁺ pre-DC and CD123^{lo}CD45RA⁺ intermediate cells). (C) shows the proportion of CD45⁺ mononuclear cells in spleen (n=3) (left) and peripheral blood (n=6) (right) of the above-mentioned pre-DC subsets. (D) shows histograms of the mean proportion of CD303⁺CD172a⁻ cells, Clec9A⁺CADM1⁺ cDC1 or CD1c⁺CD11c⁺ cDC2 obtained in the *in vitro* differentiation assays as described in **Fig. 3H** (n=3). Error bars represent mean $\pm$ SEM.

[00101] **Fig. 19.** shows the gating strategy for sorting of pre-DC subsets used in the *in vitro* differentiation assays (**Fig. 3G**). (A) shows pre-sorted data and (B-D) show the post-sorted re-analysis of (B) early pre-DC, (C) pre-cDC1, and (D) pre-cDC2.

[00102] **Fig. 20.** (A) shows expression level in terms of mean fluorescence intensity (MFI) of the side scatter area (SSC-A) indicating cellular granularity of blood pre-DC and DC subsets from five individual human donors (n=5). Error bars represent mean $\pm$ SEM. (B-C) show the flow cytometry data of the relative expression of (B) CD45RA, CD169, CD11c, CD123, CD33, Fc ϵ RI, CD2, Clec9A, CD319, CD141, BTLA, CD327, CD26, CD1c, CD304 or of (C) IRF4 and IRF8 by pDC, early pre-DC, pre-cDC2, cDC2, pre-cDC1 and cDC1 defined in **Fig. 3G** and in **Fig. 18B**.

[00103] **Fig. 21.** shows 2D-plots showing combinations of Principal Component Analysis components 1, 2 or 3 (PC1-3) using differentially-expressed genes from the microarray analysis of **Fig. 4**.

[00104] **Fig. 22.** shows heat maps of relative expression levels of all differentially-expressed genes, with magnifications of the specific genes in early pre-DC (region within the first magnified box, middle panel) and pre-cDC1 (region within the second magnified box, middle panel) from the microarray analysis of **Fig. 4**.

[00105] **Fig. 23.** shows a Venn diagram showing genes common between the lists of cDC1 DEGs (the union of DEGs from comparing pre-cDC1 vs early pre-DC and cDC1 vs pre-cDC1) and cDC2 DEGs (the union of DEGs from comparing pre-cDC2 vs early pre-DC and cDC2 vs pre-cDC2). These 62 genes were then plotted in **Fig. 4E** with the log₂ fold-change values (versus early pre-DC).

[00106] **Fig. 24.** (A-B) show the ingenuity Pathway analysis (IPA) based on genes that were differentially-expressed between (A) cDC and early pre-DC or (B) pDC and early pre-DC. Only the DC biology-related pathways were shown, and all displayed pathways were significantly enriched ($P < 0.05$, right-tailed Fischer's Exact Test). The heights of the bars correspond to the activation z-scores of the pathways. Enriched pathways predicted to be more activated in early pre-DC pathways and enriched pathways predicted to be more activated in cDC or pDC are shown. IPA predicts pathway activation/inhibition based on the correlation between what is known about the pathways in the literature (the Ingenuity Knowledge Base) and the directional expression observed in the user's data. IPA Upstream Regulator Analysis Whitepaper (56) and IPA Downstream Effectors Analysis Whitepaper (57) provide full description of the activation z-score calculation. (C) shows gene Ontology (GO) enrichment analysis of differentially-expressed genes (DEGs) in early pre-DC and pDC indicating biological processes that were significantly enriched (Benjamini-Hochberg

adjusted p value < 0.05) with genes expressed more abundantly in early pre-DC as compared to pDC. No biological process was significantly enriched with genes expressed more abundantly in pDC as compared to early pre-DC.

[00107] **Fig. 25.** (A) shows normalized abundance of all TLR mRNA in DC and pre-DC subsets obtained from the microarray analysis of **Fig. 4.** (B) shows polarization of naïve CD4⁺ T cells into IFN γ ⁺IL-17A⁻ Th1 cells, IL-4⁺ Th2 cells, IL17A⁺IFN γ ⁻ Th17 cells and IL-22⁺IFN γ ⁻IL-17A⁻ Th22 cells after 6 days of culture in a mixed lymphocyte reaction with allogenic pre-DC and DC subsets (n=3). Error bars represent SEM.

[00108] **Fig. 26.** (A) shows the isoMAP1-2 plot of bone marrow (BM) Lin(CD3/CD7/CD14/CD15/CD19/CD34)⁻CD123^{hi} cells (upper panel) and graphics of the binned median expression of defining markers along the phenotypic progression of cells defined by the isoMAP1 dimension (lower panels). (B) shows the expression level of selected markers in the isoMAP1-2 3D-plots (**Fig. 6C**, lower left panel) corresponding to cells within the pre-DC phenograph clusters (#1 and #2) of the blood Lin⁻CD123⁺ cells isoMAP analysis. (C) shows the expression level of selected markers in the isoMAP1-2 plots (**Fig. 6C**, upper left panel) corresponding to cells within the pre-DC phenograph clusters (#3 and #4) of the BM Lin⁻CD123^{hi} cells isoMAP analysis. (D) shows pDC defined in BM Lin⁻CD123^{hi} (phenograph clusters #3 and #4) or blood Lin⁻CD123⁺ (phenograph cluster #7) cells of **Fig. 6A** and **6B**, respectively, which were exported and analyzed using the isoMAP method and subdivided into clusters using the phenograph algorithm. BM and blood concatenated and overlaid BM and blood isoMAP1/3 plots are shown (left panels). Expression level of CD2 in BM (left) and blood (right) pDC is shown in the isoMAP1/3 plot. (E) Expression level of selected markers is shown in the BM and blood concatenated isoMAP1/3 plot of **Fig. 6C** (right panels).

[00109] **Fig. 27.** is a schematic representation of the expression of major pre-DC, cDC1 and cDC2 markers as pre-DC differentiate towards cDC.

[00110] **Fig. 28.** is a schematic representation of the expression of major pre-DC, cDC1 and cDC2 markers as pre-DC differentiate towards cDC.

[00111] **TABLES**

[00112] **Table 1.** Number of detected genes per cell in the total DC MARS-seq experiment.

Cell	Count	Cell	Count	Cell	Count	Cell	Count	Cell	Count
SCB_105	787	SCB_247	729	SCB_102	711	SCB_549	787	SCB_339	770

SCB_106	785	SCB_248	722	SCB_103	753	SCB_550	771	SCB_340	730
SCB_107	744	SCB_249	734	SCB_104	781	SCB_551	750	SCB_341	767
SCB_108	774	SCB_250	722	SCB_360	761	SCB_552	746	SCB_342	786
SCB_109	779	SCB_251	729	SCB_361	754	SCB_553	742	SCB_343	709
SCB_110	755	SCB_252	725	SCB_362	775	SCB_554	767	SCB_344	767
SCB_111	770	SCB_253	763	SCB_363	762	SCB_555	743	SCB_345	778
SCB_112	740	SCB_254	778	SCB_364	779	SCB_556	750	SCB_346	745
SCB_113	766	SCB_255	768	SCB_365	782	SCB_557	744	SCB_347	778
SCB_114	751	SCB_256	748	SCB_366	763	SCB_558	756	SCB_348	759
SCB_115	749	SCB_257	787	SCB_367	779	SCB_559	765	SCB_349	755
SCB_116	780	SCB_258	736	SCB_368	786	SCB_560	759	SCB_350	733
SCB_117	764	SCB_259	730	SCB_369	748	SCB_561	741	SCB_351	759
SCB_118	734	SCB_260	782	SCB_370	779	SCB_562	730	SCB_352	708
SCB_119	742	SCB_261	753	SCB_371	764	SCB_563	762	SCB_353	721
SCB_120	787	SCB_262	758	SCB_372	745	SCB_564	737	SCB_354	792
SCB_121	766	SCB_263	690	SCB_373	754	SCB_565	770	SCB_355	761
SCB_122	766	SCB_264	735	SCB_374	778	SCB_566	774	SCB_356	686
SCB_123	755	SCB_265	735	SCB_375	802	SCB_567	720	SCB_357	733
SCB_124	758	SCB_266	739	SCB_376	788	SCB_568	763	SCB_358	765
SCB_125	762	SCB_267	682	SCB_377	732	SCB_569	725	SCB_359	756
SCB_126	767	SCB_268	788	SCB_378	718	SCB_570	735	SCB_628	763
SCB_127	758	SCB_269	729	SCB_379	698	SCB_571	713	SCB_629	715
SCB_128	756	SCB_270	729	SCB_380	761	SCB_572	747	SCB_630	719
SCB_129	783	SCB_271	764	SCB_381	747	SCB_573	750	SCB_631	774
SCB_130	744	SCB_272	746	SCB_382	812	SCB_574	763	SCB_632	691
SCB_131	766	SCB_273	774	SCB_383	784	SCB_575	768	SCB_633	691
SCB_132	729	SCB_274	759	SCB_384	781	SCB_576	800	SCB_634	687
SCB_133	717	SCB_275	749	SCB_385	715	SCB_577	788	SCB_635	706
SCB_134	781	SCB_276	773	SCB_386	717	SCB_578	726	SCB_636	708
SCB_135	794	SCB_277	777	SCB_387	773	SCB_579	761	SCB_637	702
SCB_136	775	SCB_278	755	SCB_388	699	SCB_580	764	SCB_638	743
SCB_137	745	SCB_279	748	SCB_389	703	SCB_581	735	SCB_639	752
SCB_138	784	SCB_280	755	SCB_390	768	SCB_582	729	SCB_640	772
SCB_139	745	SCB_281	752	SCB_391	712	SCB_583	812	SCB_641	739
SCB_140	748	SCB_282	762	SCB_392	759	SCB_584	718	SCB_642	733
SCB_141	771	SCB_283	723	SCB_393	747	SCB_585	745	SCB_643	767
SCB_142	767	SCB_284	742	SCB_394	747	SCB_586	742	SCB_644	735
SCB_143	768	SCB_285	776	SCB_395	776	SCB_587	728	SCB_645	756

SCB_144	670	SCB_286	726	SCB_396	794	SCB_588	752	SCB_646	775
SCB_145	701	SCB_287	786	SCB_397	788	SCB_589	758	SCB_647	728
SCB_146	752	SCB_1	721	SCB_398	770	SCB_590	769	SCB_648	750
SCB_147	746	SCB_2	768	SCB_399	734	SCB_591	742	SCB_649	768
SCB_148	726	SCB_3	746	SCB_400	719	SCB_592	752	SCB_661	723
SCB_149	750	SCB_4	791	SCB_401	752	SCB_593	777	SCB_662	804
SCB_150	781	SCB_5	734	SCB_402	774	SCB_594	718	SCB_663	713
SCB_151	738	SCB_6	754	SCB_403	768	SCB_595	777	SCB_664	699
SCB_152	775	SCB_7	760	SCB_404	754	SCB_596	776	SCB_665	766
SCB_153	750	SCB_8	757	SCB_405	764	SCB_597	706	SCB_666	768
SCB_154	788	SCB_9	763	SCB_406	729	SCB_598	750	SCB_667	759
SCB_155	781	SCB_10	706	SCB_407	750	SCB_599	777	SCB_668	765
SCB_156	773	SCB_11	713	SCB_408	731	SCB_600	761	SCB_669	784
SCB_157	770	SCB_12	776	SCB_409	784	SCB_601	731	SCB_670	702
SCB_158	762	SCB_13	749	SCB_410	785	SCB_602	729	SCB_671	703
SCB_159	766	SCB_14	765	SCB_411	738	SCB_603	776	SCB_672	775
SCB_160	768	SCB_15	762	SCB_412	775	SCB_604	717	SCB_673	753
SCB_161	752	SCB_16	772	SCB_413	722	SCB_605	747	SCB_674	764
SCB_162	767	SCB_17	767	SCB_414	803	SCB_606	757	SCB_675	680
SCB_163	719	SCB_18	705	SCB_415	782	SCB_607	737	SCB_676	768
SCB_164	748	SCB_19	721	SCB_416	778	SCB_608	760	SCB_677	709
SCB_165	774	SCB_20	740	SCB_417	768	SCB_609	804	SCB_678	761
SCB_166	769	SCB_21	765	SCB_418	749	SCB_610	758	SCB_679	777
SCB_167	792	SCB_22	774	SCB_419	770	SCB_611	771	SCB_680	719
SCB_168	772	SCB_23	766	SCB_420	731	SCB_612	767	SCB_681	761
SCB_169	721	SCB_24	765	SCB_421	785	SCB_613	762	SCB_682	784
SCB_170	752	SCB_25	682	SCB_422	747	SCB_614	747	SCB_683	718
SCB_171	745	SCB_26	772	SCB_423	733	SCB_615	764	SCB_684	771
SCB_172	749	SCB_27	730	SCB_424	732	SCB_616	761	SCB_685	766
SCB_173	774	SCB_28	763	SCB_425	732	SCB_617	746	SCB_686	733
SCB_174	745	SCB_29	735	SCB_426	759	SCB_618	782	SCB_687	767
SCB_175	780	SCB_30	754	SCB_427	740	SCB_619	777	SCB_688	793
SCB_176	763	SCB_31	737	SCB_428	741	SCB_620	700	SCB_689	758
SCB_177	770	SCB_32	787	SCB_429	769	SCB_621	757	SCB_690	768
SCB_178	777	SCB_33	758	SCB_430	713	SCB_622	747	SCB_691	764
SCB_179	755	SCB_34	768	SCB_431	720	SCB_623	770	SCB_692	811
SCB_180	719	SCB_35	713	SCB_432	773	SCB_624	772	SCB_693	779
SCB_181	756	SCB_36	722	SCB_433	753	SCB_625	792	SCB_694	691

SCB_182	759	SCB_37	765	SCB_434	742	SCB_626	733	SCB_495	694
SCB_183	720	SCB_38	741	SCB_435	721	SCB_627	776	SCB_496	766
SCB_184	730	SCB_39	757	SCB_436	798	SCB_699	769	SCB_497	756
SCB_185	741	SCB_40	759	SCB_437	756	SCB_700	805	SCB_498	780
SCB_186	741	SCB_41	750	SCB_438	767	SCB_701	799	SCB_499	770
SCB_187	760	SCB_42	776	SCB_439	790	SCB_702	712	SCB_500	757
SCB_188	783	SCB_43	713	SCB_440	768	SCB_703	672	SCB_501	776
SCB_189	760	SCB_44	675	SCB_441	771	SCB_704	788	SCB_502	806
SCB_190	757	SCB_45	775	SCB_442	738	SCB_705	672	SCB_503	737
SCB_191	786	SCB_46	757	SCB_443	760	SCB_706	755	SCB_504	769
SCB_192	753	SCB_47	760	SCB_444	765	SCB_707	708	SCB_505	754
SCB_193	786	SCB_48	764	SCB_445	770	SCB_708	709	SCB_506	736
SCB_194	761	SCB_49	730	SCB_446	752	SCB_709	752	SCB_507	773
SCB_195	749	SCB_50	755	SCB_447	799	SCB_710	718	SCB_508	726
SCB_196	737	SCB_51	751	SCB_448	749	SCB_288	716	SCB_509	773
SCB_197	720	SCB_52	774	SCB_449	712	SCB_289	767	SCB_510	756
SCB_198	781	SCB_53	743	SCB_450	777	SCB_290	770	SCB_677	690
SCB_199	749	SCB_54	714	SCB_451	700	SCB_291	720	SCB_678	728
SCB_200	780	SCB_55	739	SCB_452	748	SCB_292	704	SCB_679	725
SCB_201	793	SCB_56	750	SCB_453	795	SCB_293	787	SCB_680	749
SCB_202	747	SCB_57	758	SCB_454	738	SCB_294	732	SCB_681	759
SCB_203	771	SCB_58	755	SCB_455	782	SCB_295	728	SCB_682	746
SCB_204	719	SCB_59	776	SCB_456	742	SCB_296	746	SCB_683	740
SCB_205	754	SCB_60	759	SCB_457	763	SCB_297	782	SCB_684	689
SCB_206	779	SCB_61	697	SCB_458	762	SCB_298	682	SCB_685	698
SCB_207	742	SCB_62	721	SCB_459	665	SCB_299	760	SCB_686	737
SCB_208	750	SCB_63	741	SCB_460	707	SCB_300	687	SCB_687	741
SCB_209	751	SCB_64	682	SCB_511	787	SCB_301	745	SCB_688	729
SCB_210	756	SCB_65	756	SCB_512	779	SCB_302	777	SCB_689	808
SCB_211	732	SCB_66	766	SCB_513	753	SCB_303	701	SCB_690	701
SCB_212	760	SCB_67	725	SCB_514	766	SCB_304	773	SCB_691	789
SCB_213	734	SCB_68	774	SCB_515	775	SCB_305	748	SCB_692	775
SCB_214	740	SCB_69	733	SCB_516	771	SCB_306	772	SCB_693	811
SCB_215	714	SCB_70	710	SCB_517	777	SCB_307	795	SCB_694	727
SCB_216	727	SCB_71	758	SCB_518	774	SCB_308	753	SCB_695	778
SCB_217	748	SCB_72	743	SCB_519	757	SCB_309	753	SCB_696	718
SCB_218	772	SCB_73	758	SCB_520	756	SCB_310	714	SCB_697	724
SCB_219	772	SCB_74	740	SCB_521	750	SCB_311	758	SCB_698	690

SCB_220	743	SCB_75	725	SCB_522	758	SCB_312	695	SCB_650	797
SCB_221	686	SCB_76	713	SCB_523	719	SCB_313	748	SCB_651	736
SCB_222	758	SCB_77	735	SCB_524	731	SCB_314	747	SCB_652	773
SCB_223	771	SCB_78	768	SCB_525	736	SCB_315	750	SCB_653	703
SCB_224	766	SCB_79	715	SCB_526	744	SCB_316	746	SCB_654	772
SCB_225	755	SCB_80	713	SCB_527	765	SCB_317	774	SCB_655	769
SCB_226	709	SCB_81	751	SCB_528	755	SCB_318	723	SCB_656	797
SCB_227	733	SCB_82	745	SCB_529	737	SCB_319	753	SCB_657	765
SCB_228	758	SCB_83	742	SCB_530	768	SCB_320	741	SCB_658	764
SCB_229	756	SCB_84	782	SCB_531	769	SCB_321	718	SCB_659	741
SCB_230	709	SCB_85	783	SCB_532	796	SCB_322	744	SCB_660	732
SCB_231	756	SCB_86	753	SCB_533	757	SCB_323	750	SCB_661	768
SCB_232	748	SCB_87	744	SCB_534	726	SCB_324	711	SCB_662	758
SCB_233	782	SCB_88	743	SCB_535	741	SCB_325	731	SCB_663	773
SCB_234	688	SCB_89	741	SCB_536	731	SCB_326	764	SCB_664	753
SCB_235	626	SCB_90	736	SCB_537	802	SCB_327	699	SCB_665	745
SCB_236	730	SCB_91	691	SCB_538	731	SCB_328	755	SCB_666	709
SCB_237	757	SCB_92	772	SCB_539	715	SCB_329	716	SCB_667	705
SCB_238	726	SCB_93	764	SCB_540	785	SCB_330	783	SCB_668	662
SCB_239	734	SCB_94	748	SCB_541	758	SCB_331	739	SCB_669	729
SCB_240	757	SCB_95	770	SCB_542	779	SCB_332	747	SCB_670	784
SCB_241	773	SCB_96	744	SCB_543	800	SCB_333	752	SCB_671	726
SCB_242	745	SCB_97	732	SCB_544	741	SCB_334	766	SCB_672	691
SCB_243	750	SCB_98	749	SCB_545	779	SCB_335	715	SCB_673	782
SCB_244	725	SCB_99	763	SCB_546	729	SCB_336	765	SCB_674	651
SCB_245	725	SCB_100	718	SCB_547	737	SCB_337	745	SCB_675	760
SCB_246	711	SCB_101	781	SCB_548	773	SCB_338	698	SCB_676	705

[00113] **Table 2.** DC subsets signature genes derived from Gene Expression Omnibus data series GSE35457 and used for MARS-seq and C1 data analyses.

pDC signature genes		cDC1 signature genes		cDC2 signature genes
ABCA7	MTMR2	ABCB4	STX11	ABCG1
ABCB6	MUPCDH	ABI3	STX6	ACP5
ABHD15	MX1	ABR	SVIL	ACP6
ABTB2	MYB	ACER3	SWAP70	ACSL1
ACACB	MYBPH	ACOT11	SYN1	ACSL5
ACN9	MYH3	ACPP	SYT11	ACSS2
ACSBG1	MYL6B	ACTA2	SYTL3	ACTB

ACSM3	N4BP2L1	ACVRL1	TBL1X	ACTR3
ADA	N6AMT1	ADAM15	TBXAS1	ADAD2
ADAM19	NADK	ADAM8	TESC	ADAM28
ADARB1	NAT8L	ADAMTSL4	TICAM2	ADORA2B
ADAT3	NCF1C	ADAP1	TIMP1	ADORA3
ADC	NCLN	AGTPBP1	TIPARP	AGPAT1
ADI1	NCRNA00153	ALDH3B1	TKT	AGPS
AEBP1	NDST2	ALOX5	TLE4	AIG1
AHI1	NEK8	AMICA1	TLR2	AIM2
AJAP1	NFATC2IP	AMOT	TLR5	ALDH1A1
AKRIC3	NFX1	ANG	TLR8	ALDH3A2
ALDH5A1	NGLY1	ANXA1	TM6SF1	AMY1A
ALOX5AP	NHEDC1	ANXA2	TMC6	ANPEP
ANKRD33	NIN	ANXA2P1	TMEM154	ANXA6
APOBEC3D	NIPA1	ANXA5	TMEM173	AP3M2
APP	NLRP2	AOAH	TMEM2	APOL1
ARHGAP25	NLRP7	APAF1	TMEM71	APOL2
ARHGAP27	NOP56	APLP2	TNFAIP2	APOL3
ARHGAP9	NOTCH3	ARAP3	TNFRSF10D	ASAP1
ARHGEF10	NOTCH4	ARHGAP10	TNFRSF1A	ASB2
ARHGEF4	NPAL3	ARL4A	TNFRSF1B	ATG3
ARID3A	NPC1	ARRDC2	TNFSF10	ATL1
ARMC5	NPC2	ASCL2	TNFSF12	ATP1A1
ARMET	NR5A1	ASGR1	TNFSF13B	AZI1
ARRDC5	NRP1	ASGR2	TOB1	B4GALT5
ASIP	NTAN1	ATP1B1	TPPP3	BAG3
ATP10A	NUCB2	ATP6V1B2	TREM1	BATF3
ATP13A2	NUMA1	BACH2	TRIB1	BCAR3
ATP2A3	OAS1	BATF	TRIB2	BCL6
ATP8B2	ODC1	BLVRA	TSC22D3	BEND5
AUTS2	OFD1	BTBD11	TSPAN32	BIK
AVEN	OGT	C10orf11	TSPAN4	BIVM
B4GALT1	OPN3	C10orf54	TSPO	BTLA
BAIAP2L1	OPTN	C15orf39	TTYH3	C10orf105
BCAS4	OR3A3	C16orf7	UBAC1	C10orf64
BCL11A	P2RX1	C17orf44	UPP1	C13orf15
BEND6	P4HB	C2CD2	USP3	C13orf31
BLK	PACAP	C3orf59	VCAN	C15orf38

BLNK	PACSIN1	C4orf18	VENTX	C17orf58
BSPRY	PAFAH2	C9orf72	VIPR1	C1orf115
BTAF1	PAG1	CA2	VPS37C	C1orf162
BTG1	PANX2	CACNA2D3	VSIG4	C1orf165
C10orf141	PAPLN	CALHM2	XAF1	C1orf186
C10orf47	PARP10	CAPN2	XYLT1	C1orf21
C10orf58	PARVB	CARD16	YIF1B	C1orf24
C11orf24	PBX3	CARD9	ZAK	C1orf51
C11orf67	PCNT	CASP1	ZBP1	C1orf54
C11orf80	PCNX	CASP4	ZEB2	C20orf27
C12orf23	PCSK4	CAST	ZFAND5	C21orf63
C12orf44	PDCD4	CCL5	ZFP36	C5orf30
C12orf57	PDIA4	CCND2	ZNF562	C8orf47
C13orf18	PDXP	CCR6	ZYG11B	CADM1
C14orf4	PFKFB2	CD14		CAMK2D
C14orf45	PFKP	CD151		CAMP
C16orf33	PGD	CD163		CBL
C16orf58	PGM2L1	CD1A		CCDC6
C16orf93	PHACTR1	CD1B		CCDC62
C18orf25	PHEX	CD1C		CCDC90A
C18orf8	PHF16	CD1D		CCND1
C1orf109	PI4KAP2	CD1E		CCR9
C20orf100	PIK3AP1	CD2		CD226
C20orf103	PIK3CD	CD209		CD38
C20orf132	PIK4CA	CD244		CD48
C21orf2	PLAC8	CD300A		CD59
C2orf55	PLAU	CD300C		CDCA7
C3orf21	PLD6	CD300LF		CDH2
C4BPB	PLEKHG4	CD33		CDK2AP1
C5orf62	PLP2	CD5		CDK6
C6orf170	PLS3	CD52		CHD7
C7orf41	PMEPA1	CD69		CHST2
C7orf54	PNOC	CDC42EP4		CLEC1A
C8orf13	POLB	CDCP1		CLEC9A
C9orf127	POLE	CDH23		CLNK
C9orf128	POMGNT1	CDS1		CLSTN2
C9orf142	POU4F1	CEBPA		CNTLN
C9orf37	PPM1J	CEBPB		CPNE3

C9orf45	PPP1R14A	CEBPD	CREG1
C9orf91	PPP1R14B	CENPN	CSRP1
C9orf95	PPP1R16B	CENTA1	CST3
CA8	PPP2R1B	CENTG3	CTPS2
CADM4	PPP2R5C	CES1	CXCL16
CARD11	PRAGMIN	CFD	CXCL9
CASZ1	PRIM1	CFP	CYB5R3
CBLB	PRKCB	CHD1	CYP2E1
CBX4	PRKCB1	CHST13	DBN1
CBX6	PRKD1	CIDEB	DCLRE1A
CCDC102A	PROC	CLEC10A	DCTPP1
CCDC50	PSCD4	CLEC12A	DFNA5
CCDC69	PSD4	CLEC4A	DHCR24
CCR2	PTCRA	CLEC4F	DHRS3
CCR3	PTGDS	CMTM1	DLG3
CCR7	PTGR1	COL9A2	DOCK7
CCS	PTK7	COQ10A	DPP4
CD164	PTPRCAP	CPNE8	DSE
CD247	PTPRM	CPPED1	DYSF
CD2AP	PVRL1	CREB5	EGLN3
CD320	QDPR	CRTAP	EHD4
CD36	RAB15	CRYL1	ELOVL5
CD4	RAB38	CRYZL1	ENOX1
CD68	RAB40B	CSF1R	ENPP1
CD7	RAB9P1	CSF3R	ENPP3
CD99	RABGAP1L	CST7	ENPP4
CDC14A	RALGPS1	CSTA	ERAP2
CDCA7L	RASD1	CTSH	ERMP1
CDH1	RBM38	CX3CR1	ERO1L
CDK2	RECQL5	CXCR7	EVI2A
CDK5R1	RELN	CYBRD1	EVL
CDKN2D	REXO2	CYFIP1	FAH
CDR2	RGS1	DAGLB	FAM102A
CDYL	RGS7	DDX60L	FAM125B
CENPV	RHBDF2	DEM1	FAM129A
CETP	RIMS3	DENND3	FAM149A
CHST12	RLTPR	DEPDC6	FAM160A2
CHST15	RNASE6	DHRS9	FAM20C

CIB2	RNASEL	DOK2	FAM57A
CIRBP	RNF11	DPEP2	FAR2
CLDN23	RNF121	DPYD	FARS2
CLEC4C	RNF165	DTD1	FBXL20
CLIC3	RPA1	DTNA	FKBP1B
CLN8	RPP25	ECGF1	FLJ10916
CMTM3	RPPH1	EFNB1	FLJ22795
CNTNAP1	RPS6KA2	EMP1	FLT3
COBL	RPS6KA4	EMR2	FMNL2
COBLL1	RRBP1	EMR3	FNBP1
COL24A1	RSPH1	ENHO	FNIP2
CORO1C	RTKN	ENTPD1	FUCA1
CPLX1	RUNX2	EPB41L2	FUT8
CREB3L2	RWDD2A	EPB41L3	GCET2
CRTC3	SAP130	EPSTI1	GFOD1
CRYM	SBDS	ERMAP	GINS2
CTNS	SBF1	ETS2	GLTP
CTSB	SCAMP5	F13A1	GNAZ
CTSC	SCARA5	FAM102B	GPER
CTSL2	SCARB1	FAM104B	GPR126
CUEDC1	SCARB2	FAM109A	GPRIN3
CUTL1	SCN9A	FAM110A	GPSM1
CUX2	SCYL3	FAM111A	GPT2
CXCR3	SDC1	FAM129B	GSTP1
CXorf12	SDK2	FAM38A	GYPC
CXorf57	SEC11C	FAM46A	H2AFY2
CXXC5	SEC61A1	FBLN2	HCP5
CYBASC3	SEC61A2	FBN2	HLA-DOB
CYBB	SEC61B	FCGBP	HLA-DPA1
CYFIP2	SEL1L3	FCGR2A	HLA-DPB1
CYP2J2	SELL	FCGR2B	HLA-DQB1
CYP46A1	SELS	FCN1	HLA-DRB1
CYSLTR1	SEMA4D	FCRLB	HLA-DRB3
CYTH4	SEMA5A	FGD4	HLA-DRB4
CYYR1	SEPHS1	FILIP1L	HMOX1
DAAM1	SERPINF1	FLVCR2	HN1
DAB2	SERPING1	FOSB	HOXA9
DACH1	SETBP1	FOXO1	HPS5

DAPK2	SH2D3C	FPR1	HSD17B8
DBNDD1	SH3D19	FPR3	HSDL2
DCK	SH3PXD2A	FRAT2	HYAL3
DCPS	SHD	FXYD5	ICA1
DDB1	SIDT1	FYB	ICAM3
DDIT4	SIK1	GABBR1	ID2
DEDD2	SIRPB1	GADD45B	IDO1
DERL3	SIVA	GALM	IDO2
DEX1	SIVA1	GAPDH	IFNGR2
DHRS7	SLA2	GBP1	IFT20
DHTKD1	SLAMF6	GBP2	IL15
DIP2A	SLC15A4	GBP3	INADL
DKFZP58611420	SLC20A1	GBP4	INDO
DKFZp761P0423	SLC23A1	GBP5	IRAK2
DNASE2	SLC25A4	GHRL	ITGB7
DPPA4	SLC29A1	GIMAP1	ITPR3
DRD4	SLC2A1	GIMAP2	KATNA1
DSG2	SLC2A6	GIMAP4	KIAA1598
DSN1	SLC2A8	GIMAP6	KIAA1688
DTX2	SLC35A3	GIMAP7	KIF16B
DUSP28	SLC35C2	GIMAP8	KIF20B
DUSP5	SLC35F3	GK	KIT
DYRK4	SLC37A1	GLIPR2	KLHL22
E2F2	SLC39A6	GPBAR1	KLHL5
E2F5	SLC3A2	GPR162	KLRG1
EBI2	SLC43A3	GPR44	LAT
EIF4A3	SLC44A2	GRK5	LFNG
EIF4ENIF1	SLC47A1	HBEGF	LIMA1
ELMO2	SLC7A5	HDAC4	LMNA
EMID2	SLC7A6	HK1	LOC100133583
ENOSF1	SLC9A3R1	HK2	LOC100133866
ENPP2	SLFN11	HK3	LOC150223
EPDR1	SLITRK5	HNMT	LOC25845
EPHB1	SMARCAL1	HSPA1A	LOC439949
ERCC1	SMC6	HSPA6	LOC642073
ERN1	SMPD3	HSPA7	LOC642590
ESR2	SNAP91	ICAM2	LOC645638
ETS1	SNCA	IER5	LOC649143

FAM107B	SNRNP25	IFI30
FAM108C1	SNRPN	IFI6
FAM113B	SORL1	IFIH1
FAM129C	SPCS1	IFIT1
FAM167A	SPHK1	IFIT3
FAM43A	SPIB	IFITM1
FAM65A	SPNS3	IFITM2
FAM81A	SPOCK2	IFITM3
FAM82A2	SRPR	IFT57
FANCD2	SRPX	IGLL1
FBXO18	SSR4	IGSF6
FCHSD2	ST3GAL2	IL13RA1
FCRLA	ST3GAL4	IL17RA
FEZ2	ST6GALNAC4	IL1B
FGFR3	ST6GALNAC6	IL1R1
FHL1	STAG3L2	IL1R2
FLJ21986	STAG3L3	IL1RN
FLJ42627	STAMBPL1	INPP1
FMNL3	STAT4	IRAK3
FYCO1	STK11IP	IRF1
FZD3	STK32B	ISG15
GAL3ST4	STMN1	ITGA5
GARNL4	STOX1	ITGAM
GAS6	STT3A	ITSN1
GFI1	SUGT1	JDP2
GGA2	SUPT3H	JHDM1D
GGH	SUPT5H	JUN
GIN53	SUSD1	JUP
GLCE	SYCP2L	KCNK13
GLDN	SYS1	KCNQ1
GLS	SYTL2	KIAA0922
GLT25D1	TACC1	KIAA1683
GLT8D1	TARBP1	KLF11
GNG7	TATDN3	KLF2
GPM6B	TAX1BP3	KLF4
GPR114	TBC1D14	KLF9
GPR183	TBC1D16	LACTB
GPRC5C	TBC1D4	LAMP3

LOC653344
LOC730101
LONRF1
LPAR5
LPCAT2
LRBA
LRRC1
LRRCC1
LRRK2
LYRM4
MARCKSL1
MATK
MCM4
MESP1
MFNG
MGC4677
MIST
MMP25
MND1
MPP3
MYC
MYLK
MYO1D
NAAA
NAALADL1
NAPIL1
NAV1
NBEAL2
NCALD
NCKAP5
NET1
NETO2
NLRX1
NMNAT3
OSBPL3
OSBPL9
P2RY10
PAM

GPX7	TBX19	LAYN
GRAMD1B	TCF3	LDLR
GRB14	TCF4	LGALS1
GRIN1	TCL1A	LILRA2
GSDMB	TCL1B	LILRA3
GZMB	TEX2	LILRA6
GZMH	TFIP11	LILRB3
HCST	TGFBR2	LIMCH1
HERC5	TLCD1	LIMS1
HERPUD1	TLR7	LMO2
HHAT	TLR9	LOC100129550
HIGD1A	TM7SF2	LOC100130520
HIST1H2BD	TM9SF2	LOC100170939
HIST1H2BK	TMEM109	LOC143941
HOXB2	TMEM141	LOC153561
HPS4	TMEM149	LOC338758
HRASLS3	TMEM170B	LOC391075
HSP90B1	TMEM175	LOC644237
HVCN1	TMEM187	LOC645626
IDH3A	TMEM194A	LOC648984
IFI44	TMEM194B	LOC653778
IFI44L	TMEM44	LOC654103
IFT12	TMEM53	LOC728093
IFNAR1	TMEM63A	LOC728519
IFNAR2	TMEM91	LOC728666
IGF2R	TMEM98	LOC728855
IGFBP3	TNFRSF17	LOC729708
IGJ	TNFRSF21	LOC730994
IL18RAP	TNNI2	LOC731486
IL28RA	TOM1	LOC88523
IL3RA	TOX2	LRRC25
INSM1	TP53I13	LRRC33
INTS12	TPM2	LST1
IRF4	TPRG1L	LYL1
IRF7	TPST2	LYST
ISCU	TRAF3	MAFB
ITCH	TRO	MAP3K6
ITGAE	TRPM2	MARCO

PAPSS1
PARM1
PARP3
PDE8B
PDLIM7
PFKFB3
PIGZ
PIK3CB
PITPNC1
PITPNM1
PKP2
PKP4
PLCD1
PLEKHA5
PLEKHA6
PLEKHO2
PLXNA1
PLXNB1
PMM1
PNLDC1
PNMA1
POLA2
PPA1
PPAP2A
PPM1H
PPM1M
PPT1
PPY
PRKCZ
PSEN2
PSMB9
PTGER2
PTK2
PTPLB
QPRT
RAB11FIP4
RAB30
RAB32

ITM2C	TSEN54	MBOAT7	RAB33A
KANK1	TSPAN13	MEFV	RAB3IP
KATNAL1	TSPAN3	MEGF9	RAB7B
KCNA5	TSPYL2	MLKL	RAB7L1
KCNH8	TUBB6	MMD	RAB8B
KCNK1	TUBG1	MOV10	RALB
KCNK10	TUBG2	MPZL2	RASGRP3
KCNK17	TULP4	MS4A14	RGS10
KCTD5	TXN	MS4A7	RGS12
KIAA0226	TXNDC3	MSLN	RUSC1
KIAA0513	TXNDC5	MSN	RYK
KIAA1147	UBE2E3	MT1A	S100A10
KIAA1274	UBE2J1	MTMR11	Septin 3
KIAA1370	UBQLNL	MYBPC3	SERPINB6
KIAA1545	UGCG	MYO1A	SERPINF2
KIAA1641	ULK1	MYO1F	SH3RF2
KIAA1984	UNC93B1	MYO5C	SHE
KIF13B	USF2	MYPOP	SIGLEC10
KIF26B	USP11	NACC2	SIGLECP3
KLHL13	USP24	NCF2	SLA
KLHL3	USP36	NFE2	SLAMF7
KMO	VASH2	NINJ2	SLAMF8
KRT5	VEGFB	NLRP12	SLC1A3
L3MBTL3	VEZF1	NLRP3	SLC24A4
LAIR1	VIPR2	NOD2	SLC25A25
LAMC1	WDR19	NR1H3	SLC39A8
LAMP1	WDR51A	NR4A2	SLC44A1
LAMP2	WNT10A	OAF	SLC46A3
LAPTM4B	XBPI	OAS3	SLC9A9
LASS6	YPEL1	OLFM1	SLCO3A1
LBH	ZC3H5	OSCAR	SMO
LOC1	ZCCHC11	P2RY13	SNORA57
LEPREL1	ZCWPW1	P2RY2	SNX22
LGMN	ZDHC14	P2RY5	SNX3
LHFPL2	ZDHC17	PAPSS2	SNX30
LILRA4	ZDHC23	PARP14	SP140
LILRB4	ZDHC4	PARP9	SPATS2L
LIME1	ZDHC8	PCCA	SPI1

LMNB2	ZDHH9	PCK2	SPIN3
LOC100128410	ZFYVE26	PCSK5	SPNS1
LOC100129466	ZHX2	PEA15	SPRY2
LOC100129673	ZKSCAN4	PFKFB4	ST3GAL5
LOC100130633	ZMYM6	PHCA	ST5
LOC100131289	ZMYND11	PID1	ST6GALNAC2
LOC100132299	ZNF175	PILRA	ST7
LOC100132740	ZNF185	PION	STK39
LOC100134134	ZNF219	PIP3-E	STOM
LOC100190939	ZNF521	PIP4K2A	STX3
LOC132241	ZNF556	PKIB	SUOX
LOC201175	ZNF589	PLA2G7	SUSD3
LOC221442	ZNF706	PLSCR3	TACSTD2
LOC283874	ZNF767	PLXDC2	TANC2
LOC285296	ZNF789	PNPLA6	TAP1
LOC285359	ZSCAN16	PPEF1	TAP2
LOC347544		PPF1A4	TCEA3
LOC387841		PPFIBP2	TCEAL3
LOC387882		PPM1F	TGM2
LOC389442		PQLC3	THBD
LOC389816		PRAM1	THEM4
LOC399804		PRDM1	TJP2
LOC400027		PRIC285	TLR10
LOC400657		PRKCD	TLR3
LOC442535		PSRC1	TMEM106C
LOC550112		PSTPIP2	TMEM14A
LOC641298		PTAFR	TMEM97
LOC642031		PTGER4	TOX
LOC642299		PTGS1	TPMT
LOC642755		PTGS2	TRAF3IP2
LOC643384		PTK6	TRAF5
LOC644879		PTPN12	TRIB3
LOC646576		PYGL	TSHZ3
LOC647000		RAB24	TSPAN2
LOC647886		RAB27A	TSPAN33
LOC650114		RARA	TSPYL3
LOC651957		RARRES3	TTF2
LOC652128		RASSF4	TUBA4A

LOC653158	RCBTB2	VAC14
LOC728308	RGL1	VAV3
LOC728661	RHOU	VCAM1
LOC728715	RIN2	VPS37D
LOC728743	RIPK5	WARS
LOC729148	RNASE2	WDFY4
LOC729406	RPGRIP1	WDR41
LOC729764	RTN1	WDR91
LOC91431	RXRA	YEATS2
LOXL4	S100A12	ZBTB46
LPXN	S100A4	ZDHHC18
LRP5	S100A8	ZIP36L1
LRP8	S100A9	ZMYND15
LRRC26	SAMD9L	ZNF232
LRRC36	SAP30	ZNF366
LSS	SCO2	ZNF532
LTB	SCPEP1	ZNF627
LTK	SDHALP1	ZNF662
LY9	SERPINA1	ZNF788
MAG	SGK	
MAGED1	SGK1	
MAP1A	SGSH	
MAP4K4	SIDT2	
MAPKAPK2	SIGIRR	
MAST3	SIGLEC14	
MCM6	SIGLEC16	
MCOLN2	SIGLEC9	
MDC1	SIPA1L2	
MEF2D	SIRPA	
MEX3B	SLC11A1	
MGAT4A	SLC16A5	
MGC29506	SLC22A16	
MGC33556	SLC26A11	
MGC39900	SLC27A3	
MGC42367	SLC2A3	
MIB2	SLC31A2	
MIR155HG	SLC40A1	
MKNK1	SLC46A2	

MLL4	SLC7A7
MME	SLITRK4
MMP11	SMAGP
MMP23B	SMAP2
MMRN1	SMARCD3
MNAT1	SNRK
MOXD1	SNTB1
MPEG1	SRBD1
MRPL36	SRGAP3
MS4A4A	ST3GAL6
MSRB3	STEAP3

[00114] **Table 3.** List of anti-human antibodies used for mass cytometry (CyTOF).

Metal	Name	Clone	Company	Cell expression
89	CD45	HI30	Fluidigm	all leukocytes
112/114	CD14	TUK4	Invitrogen	monocytes
115	CD15	HI98	Biolegend	PMN, monocytes
141	CD7	6B7	Biolegend	T cells, NK cells
142	CD26	BA26	Biolegend	cDC1
143	CD62L	DREG-56	Biolegend	Lymphocytes, monocytes, granulocytes
144	CD48	BL40	Biolegend	Lymphocytes, DCs
145	CD68	KP1	eBioscience	pDC, mono/macro
146	CD5	UCHT2	Biolegend	cDC2
147	CD86	IT2.2	BD Biosciences	DC
148	CD85j	292319	R&D	B cells, DCs, monocytes, NK and T cells
149	HLA-DR	L243	Biolegend	APC
150	CD80	L307.4	BD Biosciences	DC
151	CADM1	3E1	MBL	cDC1
152	CD1c	L161	Biolegend	cDC2
153	FceR1	AER-37	eBioscience	cDC2
154	CD327	767329	R&D	pDC
155	CD123	6H6	BD Biosciences	pDC

156	CD163	GHI	Biolegend	cDC2, mono
157	CXCR3	1C6	BD Biosciences	cDC1
158	CD56	NCAM16.2	BD Biosciences	NK cells, DC subsets
159	CD33	WM53	BD Biosciences	myeloid cells
160	Clec9a	683409	R&D Systems	cDC1
161	CD38	HIT2	Biolegend	HSCs, plasma cells, NK cells, T and B cells
162	CD10	HI10a	Biolegend	B cell precursors, T cell precursors, PMN
163	BTLA	MIH26	Fluidigm	cDC1, cDC2 subset
164	CD141	1A4	BD Biosciences	cDC1
165	CD303	201A	Biolegend	pDC
166	CD16	3G8	Biolegend	mono, NK cells
167	CX3CR1	KO124E1	Biolegend	cDC2, mono
168	CCR2	KO36C2	Biolegend	cDC, mono
169	CD116	4H11	Biolegend	DC
170	CD19	HIB19	Biolegend	B cells
171	CD34	581	Biolegend	HSC
172	CD2	RPA-2.10	Biolegend	cDC2
173	CD13	WM15	Biolegend	cDC1
174	CD45RA	HI100	Biolegend	pDC
175	CD11c	B-Ly6	BD Biosciences	cDC
176	CD11b	ICRF44	Biolegend	cDC2 subset, mono

[00115] **Table 4.** Number of expressed genes detected per cell in the pre-DC C1 scmRNAseq experiment.

Cell ID	Number of detected genes	Cell ID	Number of detected genes
RMS641	4997	RMS687	4667
RMS642	5935	RMS688	5199
RMS643	4873	RMS689	5320
RMS644	5000	RMS690	3683

RMS645	3193	RMS691	3816
RMS646	3255	RMS692	4366
RMS647	2653	RMS693	5400
RMS648	5217	RMS694	5018
RMS649	5191	RMS695	3457
RMS650	5235	RMS696	3660
RMS651	4836	RMS697	4845
RMS652	5715	RMS698	3945
RMS653	5224	RMS699	3801
RMS654	4681	RMS700	5533
RMS655	4014	RMS701	5089
RMS656	4134	RMS702	4365
RMS657	4895	RMS703	4462
RMS658	5094	RMS704	3770
RMS659	5405	RMS705	4897
RMS660	3701	RMS706	5048
RMS661	4432	RMS707	5435
RMS662	3298	RMS708	4930
RMS663	3843	RMS709	5308
RMS664	4417	RMS710	5067
RMS665	5162	RMS711	5536
RMS666	4042	RMS712	3275
RMS667	5172	RMS713	4810
RMS668	5129	RMS714	4878
RMS669	3613	RMS715	5270
RMS670	3571	RMS716	4324
RMS671	5016	RMS717	4130
RMS672	5170	RMS718	3840
RMS673	4996	RMS719	4134
RMS674	5462	RMS720	3592
RMS675	4190	RMS722	4461
RMS676	5206	RMS723	4804

RMS677	5590	RMS724	3950
RMS678	3177	RMS725	4062
RMS679	3938	RMS726	2551
RMS680	1802	RMS727	3749
RMS681	3377	RMS728	3574
RMS682	4166	RMS729	4247
RMS683	3863	RMS730	5363
RMS684	4279	RMS731	5072
RMS685	5128	RMS732	4992
RMS686	4884	RMS733	5301

[00116] **Table 5.** Lists of genes identified from the microarray DEG analysis comparisons along the lineage progression from early pre-DC to mature cDC, for cDC1 and cDC2 respectively, and the list of the 62 common genes.

Profile Genes cDC1	Profile Genes cDC2	62 common elements
ABCA1	ABHD8	ACTN1
ABCB9	ACAD8	ADAM33
ABLM1	ACTN1	ADAMTSL2
ACAA1	ADAM19	ARHGAP22
ACP5	ADAM33	AXL
ACP6	ADAMTSL2	BATF3
ACSS1	AGPAT9	CARD11
ACTN1	AIF1	CCDC50
ACY3	ANXA2P1	CCND3
ADAM33	AOAH	CD22
ADAMTSL2	AP4M1	CD52
ADAP1	APLP2	CLEC4C
AIM1	ARHGAP1	CTSG
ALG5	ARHGAP22	CYP2S1
ALOX5	ARHGAP23	DAB2
ALOX5AP	AXL	EXT1
AMICA1	BACH2	FCN1

ANG	BATF3	GPRC5C
ANPEP	BTBD11	GPX7
ANXA2	C10ORF11	GRINA
APOBEC3H	C10ORF84	HAMP
APOL2	C15ORF48	HRASLS3
APOL3	C16ORF33	HSPA12B
ARHGAP22	C20ORF27	ID2
ARMET	C2ORF89	IL3RA
ASB16	C3ORF60	IRAK3
ASCL2	CARD11	KCNK10
ATN1	CCDC50	LGALS3
ATP2A1	CCL3L1	LILRA4
AXL	CCND3	LIME1
B9D1	CD1C	LIMS2
BAIAP3	CD1D	LOC387841
BATF3	CD1E	LOC387882
BLK	CD207	LOC392382
BTLA	CD22	LOC401720
BUB3	CD52	LTK
C10ORF105	CD81	MARCKS
C11ORF80	CD86	MUPCDH
C15ORF39	CEBPB	MYBPHL
C17ORF61	CHST7	NCLN
C19ORF10	CLEC4C	OSBPL3
C1ORF21	CLIC3	PLAC8
C1ORF54	COQ10A	PLP2
C1RL	CREB5	PPP1R14A
C20ORF100	CSTA	RARRES3
C9ORF91	CTSG	RHOC
CACNA2D3	CXCR3	RPP21
CADM1	CYBASC3	RTN1
CALR	CYP2S1	S100A9

CAMK1G	DAB2	SERPING1
CAPN12	DEF8	SHD
CAPZB	DEK	SIGLEC6
CARD11	DEPDC6	SLC15A2
CASP1	DFFB	SLC20A1
CCDC123	E2F7	SLC44A2
CCDC50	ECE1	STARD7
CCNB2	ELMO1	STMN2
CCND1	ELOVL1	TBC1D19
CCND3	EML4	TCF4
CD22	EXT1	TP53I11
CD27	FAM105A	ZBP1
CD300LB	FAM129B	ZFP36L1
CD300LF	FAM179A	
CD38	FAM26F	
CD5	FBXL6	
CD52	FCGBP	
CD68	FCGR2A	
CD7	FCN1	
CD79A	FCRLA	
CD79B	FLJ22662	
CDC20	GADD45B	
CDC25B	GBP1	
CDC45L	GPRC5C	
CDH1	GPX7	
CDH2	GRINA	
CDKN1A	HAMP	
CDS1	HAPLN3	
CECR1	HK2	
CENPM	HLA-DPB1	
CLEC10A	HLA-DQB1	
CLEC4C	HRASLS3	

CLEC9A	HSPA12B
CLNK	HSPA7
CMTM3	HTR3A
COL18A1	ID2
COMMD4	IL13RA1
CPNE3	IL3RA
CPNE5	IRAK3
CPVL	IRF8
CRKRS	ITGAL
CSF1R	JDP2
CSRP1	KCNK10
CTSG	LAT2
CXCL16	LCNL1
CYP2E1	LGALS3
CYP2S1	LHFPL2
DAB2	LILRA4
DAPK1	LIME1
DBN1	LIMS2
DEXI	LIPT1
DIAPH3	LOC100134361
DUS3L	LOC339352
DUSP3	LOC387841
DYSF	LOC387882
EAF2	LOC389816
EEF1A2	LOC392382
ENO1	LOC401720
ENPP1	LOC440280
EPPB9	LOC642299
EXT1	LOC642367
FAIM3	LOC644879
FAM160A2	LOC728069
FAM30A	LOC729406

FAR2	LOXL3
FBLN2	LRP1
FCER1A	LRP5
FCER1G	LRRC26
FCN1	LTK
FER1L4	MADD
FERMT3	MARCKS
FIS1	MBNL1
FKBP11	MEFV
FKBP1B	MIIP
FLJ40504	MUPCDH
FNDC3B	MYB
GANC	MYBPHL
GAS6	MYL6B
GDPD5	NCKAP1L
GEMIN6	NCLN
GGTL3	NOXA1
GLDC	NRP1
GMPPB	NTAN1
GPER	OGFRL1
GPR162	OLFM1
GPRC5C	OSBPL3
GPRC5D	PACSIN1
GPS2	PAK1
GPX7	PARP10
GRINA	PCBP1
GZMK	PCP4L1
H2AFY2	PCSK4
HAMP	PHYHD1
HCST	PILRA
HEXIM1	PLAC8
HK3	PLOD3

HLA-DOB	PLP2
HN1	POLR2I
HOPX	PPM1J
HRASLS2	PPP1R14A
HRASLS3	PPP1R14B
HSH2D	PROC
HSPA12B	PTGDS
HSPA8	PTGS2
HVCN1	RAB20
ID2	RAB7L1
IDH2	RARRES3
IDO1	RASSF4
IGJ	RHOC
IGLL1	RILPL2
IGLL3	RPP21
IL3RA	RS1
IL7R	RTN1
INDO	S100A8
IRAK2	S100A9
IRAK3	SCMH1
IRF2BP2	SCN9A
IRF4	SERPINA1
ISCU	SERPINF1
ISG20	SERPING1
ITM2C	SGK
ITPR3	SGK1
JARID2	SHANK3
KCNK10	SHD
KCNK12	SIGLEC6
KIAA0101	SLAMF7
KIAA0114	SLC15A2
KIAA1191	SLC20A1

KIAA1545	SLC2A8
KIT	SLC35C2
KLF6	SLC44A2
KRT18P13	SMARCD3
L2HGDH	SOX4
LAMP1	SP140
LGALS3	SPOCK2
LGALS8	SSR1
LILRA2	STARD7
LILRA4	STARD8
LILRB2	STMN2
LILRB4	TBC1D19
LIME1	TCF4
LIMS2	TCL1A
LMNA	TMEM14C
LOC100130171	TMEM2
LOC100130367	TP53H1
LOC100130856	TREM1
LOC100131727	TRIB2
LOC100132444	TSPAN13
LOC144383	TXNIP
LOC286076	USP24
LOC387841	VASN
LOC387882	VCAN
LOC392382	VEGFB
LOC399988	VENTX
LOC401720	VSIG4
LOC642113	ZAK
LOC642755	ZBP1
LOC645381	ZFP36L1
LOC647506	ZNF469
LOC648366	ZNF503

LOC649210

LOC649923

LOC652493

LOC652694

LOC653468

LOC653566

LOC654191

LOC728014

LOC728093

LOC728557

LOC729086

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LST1

LTK

LYN

MARCKS

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MBOAT7

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MED12L

MED27

MEI1

MGC13057

MGC29506

MGC33556

MIF

MIR939

MIST

MLKL

MS4A6A

MUPCDH

MYBPHL

MYO1D

MYO5C

NADK

NAV1

NCF4

NCLN

NDRG1

NDRG2

NFATC2IP

NGFRAP1

NLRC3

NRM

NRSN2

NT5DC2

NUBP1

NUCB2

OSBPL10

OSBPL3

PARM1

PARP3

PCNA

PDE9A

PDIA4

PEPD

PIK3CD

PLAC8

PLCD1

PLD3

PLEKHG2

PLP2

PLXNB2

PMS2L4

POP5

POU2AF1

PPM1H

PPP1R14A

PRDM1

PRDX4

PRKCZ

PRKD2

PRR5

PRSSL1

PSEN2

PSMB8

PSORS1C1

PTGER2

PTTG1

PTTG3P

RAB30

RAB32

RAB43

RARRES3

RASGRP2

RASSF2

RHBDF2

RHOC

RNF130

RNU6-15

RPP21

RPS19BP1

RPS27L

RTN1

RUFY1

S100A4

S100A9

SAMD3

SCPEP1

SDF2L1

SEC11C

SEMA4C

SEPT3

SERPINF2

SERPING1

SH2D3A

SHD

SHE

SHMT2

SIAH1

SIGLEC6

SLC15A2

SLC15A3

SLC20A1

SLC25A4

SLC35A5

SLC41A2

SLC44A1

SLC44A2

SLC9A3R1

SLCO3A1

SMO

SNCA

SNN

SNX22

SNX29

SNX3

SPATS2

SSR4

ST6GALNAC2

STARD5

STARD7

STMN2

SULF2

SUSD3

TACSTD2

TBC1D19

TCF4

TDRD1

TFPI

TGM2

TLR3

TMEM109

TMEM167B

TMEM216

TMEM97

TNFRSF13B

TNFRSF17

TNFRSF21

TNFSF12

TNNI2

TOP2A

TOX2

TP53II1

TP53INP1

TRIB1

TRPM2

TSEN34

TSEN54

TSPAN33

TSPYL1

TUFT1

TXNDC5

TYMS

TYROBP

UBE2C

UBXN11

UGCGL2

UNC119

UNG

VAC14

VISA

VPS37B

VPS37D

WDFY4

WDR34

WFS1

WWC3

XBP1

ZBP1

ZBTB32

ZFP36L1

ZNF662

ZNF821

[00117] **Table 6.** List of anti-human antibodies used for flow cytometry.

Name	Clone	Fluorophore	Source
CADM1	3E1	Purified	MBL
CD116	4H1	Biotin	Biolegend
CD117	104D2	BV421	Biolegend
CD11c	B-ly6	V450	BD Biosciences
CD11c	3.9	BV605	Biolegend

CD123	7G3	BUV395	BD Biosciences
CD123	6H6	PercP/Cy5.5	BD Biosciences
CD135	4G8	PE	BD Pharmigen
CD135	4G8	BV711	BD Biosciences
CD14	RMO52	ECD	Beckman Coulter
CD14	M5E2	BV711	Biolegend
CD14	M5E2	BV650	BD Biosciences
CD141	AD5-14H12	PE/Vio770	Miltenyi Biotec
CD16	3G8	APC/Cy7	Biolegend
CD16	3G8	BV650	BD Biosciences
CD169	7-239	PE	BD Biosciences
CD172 α	SE5a5	PECy7	Biolegend
CD183	1C6/CXCR3	APC	BD Biosciences
CD19	SJ25C1	BV650	BD Biosciences
CD1c	L161	PercP/Cy5.5	Biolegend
CD1c	L161	PE/Cy7	Biolegend
CD1c	L161	APC/Cy7	Biolegend
CD2	RPA-2.10	BV421	BD Biosciences
CD20	2H7	BV650	BD Biosciences
CD22	HIB22	BV421	BD Biosciences
CD26	BA5b	PE/Cy7	Biolegend
CD272	MIH26	PE	Biolegend
CD283	40C1285.6	PE	Abcam
CD289	J15A7	PE	BD Biosciences
CD3	SP34-2	BV650	BD Biosciences
CD303	AC144	Biotin	Miltenyi Biotec
CD319	162.1	PE	Biolegend
CD327	767329	APC	R&D Systems
CD33	WM53	PE-CF594	BD Biosciences
CD33	AC104.3E3	VioBlue	Miltenyi Biotec
CD33	P67.6	PercP/Cy5.5	BD Biosciences
CD335	9E2	PerCP5.5	Biolegend

CD34	581	Alexa Fluor 700	BD Biosciences
CD40	5C3	PercP/Cy5.5	Biolegend
CD45	HL30	V500	BD Biosciences
CD45RA	5H9	FITC	BD Biosciences
CD45RA	L48	PE/Cy7	BD Biosciences
CD5	UCHT2	BB515	BD Biosciences
CD66b	G10F5	PerCP5.5	Biolegend
CD7	124-1D1	PE	eBioscience
CD80	ASL24	PE	Biolegend
CD80	2D10	BV421	Biolegend
CD83	HB15e	PE	Biolegend
CD86	2331 (FUN-1)	Biotin	BD Biosciences
CD88	S5/1	PE/Cy7	Biolegend
Clec9a	8F9	APC	Biolegend
Clec9A	3A4/Clec9A	PE	BD Biosciences
CX3CR1	2A9-1	PE	Biolegend
CX3CR1	K0124E1	PE	Biolegend
CXCR3	G025H7	PE	Biolegend
FcεR1α	AER-37	PerCP	Biolegend
FcεR1α	AER-37	PE	Biolegend
HLA-DR	L243	BV605	Biolegend
HLA-DR	L243	BV785	Biolegend
IFNα	LT27:295	FITC	Miltenyi Biotec
IL-12p40	C8.6	BV421	BD Biosciences
ILT1	REA219	Biotin	Miltenyi Biotec
ILT3	ZM4.1	PE	Biolegend
IRF4	3E4	PE	eBioscience
IRF8	V3GYWCH	PercP/eFluor710	eBioscience
TLR7	A94B10	PE	BD Biosciences
TNFα	Mab11	Alexa Fluor 700	BD Biosciences
secondary reagents:			
Live/Dead blue		equ DAPI	Life Technologies

Streptavidin	BUV737	BD Biosciences
Chicken IgY	Alexa Fluor 647	Jackson ImmunoResearch

DETAILED DESCRIPTION OF THE DRAWINGS

EXAMPLES

[00118] Non-limiting examples of the invention will be further described in greater detail
 5 by reference to specific Examples, which should not be construed as in any way limiting the scope of the invention.

Example 1 – Methods

[00119] **Blood, bone marrow and spleen samples**

[00120] Human samples were obtained in accordance with a favorable ethical opinion from
 10 Singapore SingHealth and National Health Care Group Research Ethics Committees. Written informed consent was obtained from all donors according to the procedures approved by the National University of Singapore Institutional Review Board and SingHealth Centralised Institutional Review Board. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque (GE Healthcare) density gradient centrifugation of apheresis residue samples
 15 obtained from volunteer donors through the Health Sciences Authorities (HSA, Singapore). Blood samples were obtained from 4 patients with molecularly confirmed Pitt-Hopkins syndrome (PHS), who all showed the classical phenotype (1). Spleen tissue was obtained from patients with tumors in the pancreas who underwent distal pancreatectomy (Singapore General Hospital, Singapore). Spleen tissue was processed as previously described (2). Bone
 20 marrow mononuclear cells were purchased from Lonza.

[00121] **Generation of single cell transcriptomes using MARS-seq**

[00122] MARS-Seq using the Biomek FXP system (Beckman Coulter) as previously described (3) was performed for scRNAseq of the DC compartment of the human peripheral blood. In brief, Lineage marker (Lin)(CD3/14/16/19/20/34)⁻CD45⁺CD135⁺HLA-
 25 DR⁺CD123⁺CD33⁺ single cells were sorted into individual wells of 384-well plates filled with 2 µl lysis buffer (Triton 0.2 % (Sigma Aldrich) in molecular biology grade H₂O (Sigma Aldrich), supplemented with 0.4 U/µl protein-based RNase inhibitor (Takara Bio Inc.), and barcoded using 400 nM IDT. Details regarding the barcoding procedure with poly-T primers were previously described (3). Samples were pre-incubated for 3 min at 80 °C and reverse
 30 transcriptase mix consisting of 10 mM DTT (Invitrogen), 4 mM dNTPs (NEB), 2.5 U/µl

SuperScript III Reverse Transcriptase (Invitrogen) in 50 mM Tris-HCl (pH 8.3; Sigma), 75 mM KCl (Sigma), 3 mM MgCl₂ (Sigma), ERCC RNA Spike-In mix (Life Technologies), at a dilution of 1:80*10⁷ per cell was added to each well. The mRNA was reverse-transcribed to cDNA with one cycle of 2 min at 42 °C, 50 min at 50 °C, and 5 min at 85 °C. Excess primers were digested with ExoI (NEB) at 37 °C for 30 min then 10 min at 80 °C, followed by cleanup using SPRIselect beads at a 1.2x ratio (Beckman Coulter). Samples were pooled and second strands were synthesized using a Second Strand Synthesis kit (NEB) for 2.5 h at 16 °C, followed by a cleanup using SPRIselect beads at a 1.4x ratio (Beckman Coulter). Samples were linearly amplified by T7-promoter guided *in vitro* transcription using the T7 High Yield RNA polymerase IVT kit (NEB) at 37 °C for 12 h. DNA templates were digested with Turbo DNase I (Ambion) for 15 min at 37 °C, followed by a cleanup with SPRIselect beads at a 1.2x ratio (Beckman Coulter). The RNA was then fragmented in Zn²⁺ RNA Fragmentation Solution (Ambion) for 1.5 min at 70 °C, followed by cleanup with SPRIselect beads at a 2.0 ratio (Beckman Coulter). Barcoded ssDNA adapters (IDT; details of barcode see (3)) were then ligated to the fragmented RNAs in 9.5 % DMSO (Sigma Aldrich), 1 mM ATP, 20% PEG8000 and 1 U/μl T4 RNA ligase I (NEB) solution in 50 mM Tris HCl pH7.5 (Sigma Aldrich), 10 mM MgCl₂ and 1mM DTT for 2 h at 22 °C. A second reverse transcription reaction was then performed using Affinity Script Reverse Transcription buffer, 10 mM DTT, 4 mM dNTP, 2.5 U/μl Affinity Script Reverse Transcriptase (Agilent) for one cycle of 2 min at 42 °C, 45 min at 50 °C, and 5 min at 85 °C, followed by a cleanup on SPRIselect beads at a 1.5x ratio (Beckman Coulter). The final libraries were generated by subsequent nested PCR reactions using 0.5 μM of each Illumina primer (IDT; details of primers see (3)) and KAPA HiFi HotStart Ready Mix (Kapa Biosystems) for 15 cycles according to manufacturer's protocol, followed by a final cleanup with SPRIselect beads at a 0.7x ratio (Beckman Coulter). The quality and quantity of the resulting libraries was assessed using an Agilent 2200 TapeStation instrument (Agilent), and libraries were subjected to next generation sequencing using an Illumina HiSeq1500 instrument (PE no index; read1: 61 reads (3 reads random nucleotides, 4 reads pool barcode, 53 reads sequence), read2: 13 reads (6 reads cell barcode, 6 reads unique molecular identifier)).

[00123] **Pre-processing, quality assessment and control of MARS-seq single cell transcriptome data**

[00124] Cell specific tags and Unique Molecular Identifiers (UMIs) were extracted (2,496 cells sequenced) from sequenced data-pool barcodes. Sequencing reads with ambiguous plate and/or cell-specific tags, UMI sequences of low quality (Phred <27), or reads that mapped to *E. coli* were eliminated using Bowtie1 sequence analysis software (4), with parameters “-M 1
5 -t --best --chunkmbs 64 --strata”. Fastq files were demultiplexed using the fastx_barcode_splitter from fastx_toolkit, and R1 reads (with trimming of pooled barcode sequences) were mapped to the human hg38 + ERCC pseudo genome assembly using Bowtie “-m 1 -t --best --chunkmbs 64 --strata”. Valid reads were then counted using UMIs if they mapped to the exon-based gene model derived from the BiomaRt HG38 data mining tool
10 provided by Ensembl (46). A gene expression matrix was then generated containing the number of UMIs for every cell and gene. Additionally, UMIs and cell barcode errors were corrected and filtered as previously described (3).

[00125] **Normalization and filtering of MARS-seq single cell transcriptome data**

[00126] In order to account for differences in total molecule counts per cell, a down-sampling normalization was performed as suggested by several studies (3, 5). Here, every cell
15 was randomly down-sampled to a molecule count of 1,050 unique molecules per cell (threshold details discussed below). Cells with molecule counts <1,050 were excluded from the analysis (**Table 1**: number of detected genes per cell). Additionally, cells with a ratio of mitochondrial versus endogenous genes exceeding 0.2, and cells with <90 unique genes, were
20 removed from the analysis. Prior to Seurat analysis (4), expression tables were filtered to exclude mitochondrial and ribosomal genes to remove noise.

[00127] **Analysis of MARS-seq single cell transcriptome data**

[00128] Analysis of the normalized and filtered single-cell gene expression data (8,657 genes across 710 single cell transcriptomes used in the final expression table) was achieved
25 using Mpath (6), PCA, tSNE, connectivity MAP (cMAP) (7) and several functions of the Seurat single cell analysis package. cMAP analysis was performed using DEGs between pDC and cDC derived from the gene expression omnibu data series GSE35457 (2). For individual cells, cMAP generated enrichment scores that quantified the degree of enrichment (or “closeness”) to the given gene signatures. The enrichment scores were scaled and assigned
30 positive or negative values to indicate enrichment for pDC or cDC signature genes, respectively. A permutation test (n=1,000) between gene signatures was performed on each enrichment score to determine statistical significance. For the tSNE/Seurat analysis, a Seurat

filter was used to include genes that were detected in at least one cell (molecule count = 1), and excluded cells with <90 unique genes. To infer the structure of the single-cell gene expression data, a PCA was performed on the highly variable genes determined as genes exceeding the dispersion threshold of 0.75. The first two principle components were used to perform a tSNE that was combined with a DBSCAN clustering algorithm (8) to identify cells with similar expression profiles. DBSCAN was performed by setting 10 as the minimum number of reachable points and 4.1 as the reachable epsilon neighbourhood parameter; the latter was determined using a KNN plot integrated in the DBSCAN R package (9) (<https://cran.r-project.org/web/packages/dbscan/>). The clustering did not change when using the default minimal number of reachable points.

[00129] To annotate the clusters, the gene signatures of blood pDC, cDC1 and cDC2 were derived from the Gene Expression Omnibus data series GSE35457 (2) (**Table 2:** lists of signature genes, data processing described below) to calculate the signature gene expression scores of cell type-specific gene signatures, and then these signature scores were overlaid onto the tSNE plots. Raw expression data of CD141⁺ (cDC1), CD1c⁺ (cDC2) DCs and pDC samples from blood of up to four donors (I, II, V and VI) was imported into Partek® Genomics Suite® software, version 6.6 Copyright©; 2017 (PGS), where they were further processed. Data were quantile-normalized and log2-transformed, and a batch-correction was performed for the donor using PGS. Differential probe expression was calculated from the normalized data (ANOVA, Fold-Change ≥ 2 and FDR-adj. p-value < 0.05) for the three comparisons of every cell type against the remaining cell types. The three lists of differentially-expressed (DE) probes were intersected and only exclusively-expressed probes were used for the cell-type specific gene signatures. The probes were then reduced to single genes, by keeping the probe for a corresponding gene with the highest mean expression across the dataset. Resulting gene signatures for blood pDCs, CD1c⁺ and CD141⁺ DCs contained 725, 457 and 368 genes, respectively. The signature gene expression score was calculated as the mean expression of all signature genes in a cluster. In order to avoid bias due to outliers, the trimmed mean (trim = 0.08) was calculated.

[00130] Monocle analysis was performed using the latest pre-published version of Monocle v.2.1.0 (10). The data were loaded into a monocle object and then log-transformed. Ordering of the genes was determined by dispersion analysis if they had an average expression of ≥ 0.5 and at least a dispersion of two times the dispersion fit. The dimensionality reduction was

performed using the reduceDimension command with parameters max_components=2, reduction_method = "DDRTree" and norm_method = "log". The trajectory was then built using the plot_cell_trajectory command with standard parameters.

[00131] Wishbone analysis (11) was performed using the Python toolkit downloaded from
 5 <https://github.com/ManuSetty/wishbone>. MARS-seq data were loaded using the wishbone.wb.SCDData.from_csv function with the parameters data_type='sc-seq' and normalize=True. Wishbone was then performed using wb.run_wishbone function with parameter start_cell= "run1_CATG_AAGACA", components_list=[1, 2, 3, 4], num_waypoints=150, branch = True. Start_cell was randomly selected from the central
 10 cluster #4. Diffusion map analysis was performed using the scdata.run_diffusion_map function with default parameters (12). Wishbone revealed three trajectories giving rise to pDC, cDC1 and cDC2 respectively. Along each trajectory, the respective signature gene shows increasing expression (**Fig. 8C**). Although Wishbone results might be interpreted to suggest that cDC2 are early cells and differentiate into pDC and cDC1 on two separate
 15 branches, this is simply because Wishbone allows a maximum of two branches and assumes all cells fall on continuous trajectories. Nevertheless, it is able to delineate the three trajectories that are in concordance with Mpath, monocle, and diffusion map analysis.

[00132] **C1 Single cell mRNA sequencing**

[00133] Lin(CD3/14/16/19/20)⁻HLA-DR⁺CD33⁺CD123⁺ cells at 300 cells/μl were loaded
 20 onto two 5–10 μm C1 Single-Cell Auto Prep integrated fluidic circuits (Fluidigm) and cell capture was performed according to the manufacturer's instructions. Individual capture sites were inspected under a light microscope to confirm the presence of single, live cells. Empty capture wells and wells containing multiple cells or cell debris were discarded for quality control. A SMARTer Ultra Low RNA kit (Clontech) and Advantage 2 PCR Kit (Clontech)
 25 was used for cDNA generation. An ArrayControl[™] RNA Spots and Spikes kit (with spike numbers 1, 4 and 7) (Ambion) was used to monitor technical variability, and the dilutions used were as recommended by the manufacturer. The concentration of cDNA for each single cell was determined by Quant-iT[™] PicoGreen® dsDNA Reagent, and the correct size and profile was confirmed using DNA High Sensitivity Reagent Kit and DNA Extended Range
 30 LabChip (Perkin Elmer). Multiplex sequencing libraries were generated using the Nextera XT DNA Library Preparation Kit and the Nextera XT Index Kit (Illumina). Libraries were

pooled and subjected to an indexed PE sequencing run of 2x51 cycles on an Illumina HiSeq 2000 (Illumina) at an average depth of 2.5-million raw reads/cell.

[00134] **C1 Single cell analysis**

[00135] Raw reads were aligned to the human reference genome GRCh38 from
5 GENCODE (13) using RSEM program version 1.2.19 with default parameters (14). Gene
expression values in transcripts per million were calculated using the RSEM program and the
human GENCODE annotation version 22. Quality control and outlier cell detection was
performed using the SINGuLAR (Fluidigm) analysis toolset. cMAP analysis was performed
using cDC1 versus cDC2 DEGs identified from Gene Expression Omnibus data series
10 GSE35457 (2), and the enrichment scores were obtained. Similar to the gene set enrichment
analyses, cMAP was used to identify associations of transcriptomic profiles with cell-type
characteristic gene signatures.

[00136] **Mpath analysis of MARS- or C1 single cell mRNA sequencing data**

[00137] Developmental trajectories were defined using the Mpath algorithm (6), which
15 constructs multi-branching cell lineages and re-orders individual cells along the branches. In
the analysis of the MARS-seq single cell transcriptomic data, the Seurat R package was first
used to identify five clusters: for each cluster, Mpath calculated the centroid and used it as a
landmark to represent a canonical cellular state; subsequently, for each single cell, Mpath
calculated its Euclidean distance to all the landmarks, and identified the two nearest
20 landmarks. Each individual cell was thus assigned to the neighborhood of its two nearest
landmarks. For every pair of landmarks, Mpath then counted the number of cells that were
assigned to the neighborhood, and used the determined cell counts to estimate the possibility
of the transition between landmarks to be true. A high cell count implied a high possibility
that the transition was valid. Mpath then constructed a weighted neighborhood network
25 whereby nodes represented landmarks, edges represented a putative transition between
landmarks, and numbers allocated to the edges represented the cell-count support for the
transition. Given that single cell transcriptomic data tend to be noisy, edges with low cell-
count support were considered likely artifacts. Mpath therefore removed the edges with a low
cell support by using (0-n) (n-n represents cell count) to quantify the distance between nodes
30 followed by applying a minimum spanning tree algorithm to find the shortest path that could
connect all nodes with the minimum sum of distance. Consequently, the resulting trimmed
network is the one that connects all landmarks with the minimum number of edges and the

maximum total number of cells on the edges. Mpath was then used to project the individual cells onto the edge connecting its two nearest landmarks, and assigned a pseudo-time ordering to the cells according to the location of their projection points on the edge. In the analysis of the C1 single cell transcriptome data, the cMAP analysis was first used to identify cDC1-primed, un-primed, and cDC2-primed clusters, and then Mpath was used to construct the lineage between these three clusters. The Mpath analysis was carried out in an unsupervised manner without prior knowledge of the starting cells or number of branches. This method can be used for situations of non-branching networks, bifurcations, and multi-branching networks with three or more branches.

10 [00138] **Mass cytometry staining, barcoding, acquisition and data analysis**

[00139] For mass cytometry, pre-conjugated or purified antibodies were obtained from Invitrogen, Fluidigm (pre-conjugated antibodies), Biolegend, eBioscience, Becton Dickinson or R&D Systems as listed in **Table 3**. For some markers, fluorophore- or biotin- conjugated antibodies were used as primary antibodies, followed by secondary labeling with anti-fluorophore metal-conjugated antibodies (such as the anti-FITC clone FIT-22) or metal-conjugated streptavidin, produced as previously described (15). Briefly, 3×10^6 cells/well in a U-bottom 96 well plate (BD Falcon, Cat# 3077) were washed once with 200 μ L FACS buffer (4 % FBS, 2 mM EDTA, 0.05 % Azide in 1X PBS), then stained with 100 μ L 200 μ M cisplatin (Sigma-Aldrich, Cat# 479306-1G) for 5 min on ice to exclude dead cells. Cells were then incubated with anti-CADM1-biotin and anti-CD19-FITC primary antibodies in a 50 μ L reaction for 30 min on ice. Cells were washed twice with FACS buffer and incubated with 50 μ L heavy-metal isotope-conjugated secondary mAb cocktail for 30 min on ice. Cells were then washed twice with FACS buffer and once with PBS before fixation with 200 μ L 2 % paraformaldehyde (PFA; Electron Microscopy Sciences, Cat# 15710) in PBS overnight or longer. Following fixation, the cells were pelleted and resuspended in 200 μ L 1X permeabilization buffer (Biolegend, Cat# 421002) for 5 mins at room temperature to enable intracellular labeling. Cells were then incubated with metal-conjugated anti-CD68 in a 50 μ L reaction for 30 min on ice. Finally, the cells were washed once with permeabilization buffer and then with PBS before barcoding.

30 [00140] Bromoacetamidobenzyl-EDTA (BABE)-linked metal barcodes were prepared by dissolving BABE (Dojindo, Cat# B437) in 100mM HEPES buffer (Gibco, Cat# 15630) to a final concentration of 2 mM. Isotopically-purified PdCl₂ (Trace Sciences Inc.) was then

added to the 2 mM BABE solution to a final concentration of 0.5 mM. Similarly, DOTA-maleimide (DM)-linked metal barcodes were prepared by dissolving DM (Macrocyclics, Cat# B-272) in L buffer (MAXPAR, Cat# PN00008) to a final concentration of 1 mM. RhCl₃ (Sigma) and isotopically-purified LnCl₃ was then added to the DM solution at 0.5 mM final concentration. Six metal barcodes were used: BABE-Pd-102, BABE-Pd-104, BABE-Pd-106, BABE-Pd-108, BABE-Pd-110 and DM-Ln-113.

[00141] All BABE and DM-metal solution mixtures were immediately snap-frozen in liquid nitrogen and stored at -80 °C. A unique dual combination of barcodes was chosen to stain each tissue sample. Barcode Pd-102 was used at 1:4000 dilution, Pd-104 at 1:2000, Pd-106 and Pd-108 at 1:1000, Pd-110 and Ln-113 at 1:500. Cells were incubated with 100 µL barcode in PBS for 30 min on ice, washed in permeabilization buffer and then incubated in FACS buffer for 10 min on ice. Cells were then pelleted and resuspended in 100 µL nucleic acid Ir-Intercalator (MAXPAR, Cat# 201192B) in 2 % PFA/PBS (1:2000), at room temperature. After 20 min, cells were washed twice with FACS buffer and twice with water before a final resuspension in water. In each set, the cells were pooled from all tissue types, counted, and diluted to 0.5x10⁶ cells/mL. EQ Four Element Calibration Beads (DVS Science, Fluidigm) were added at a 1 % concentration prior to acquisition. Cell data were acquired and analyzed using a CyTOF Mass cytometer (Fluidigm).

[00142] The CyTOF data were exported in a conventional flow-cytometry file (.fcs) format and normalized using previously-described software (16). Events with zero values were randomly assigned a value between 0 and -1 using a custom R script employed in a previous version of mass cytometry software (17). Cells for each barcode were deconvolved using the Boolean gating algorithm within FlowJo. The CD45⁺Lin⁻ (CD7/CD14/CD15/CD16/CD19/CD34)⁻HLA-DR⁺ population of PBMC were gated using FlowJo and exported as a .fcs file. Marker expression values were transformed using the logicle transformation function (18). Random sub-sampling without replacement was performed to select 20,000 cell events. The transformed values of sub-sampled cell events were then subjected to t-distributed Stochastic Neighbor Embedding (tSNE) dimension reduction (19) using the markers listed in **Table 3**, and the Rtsne function in the Rtsne R package with default parameters. Similarly, isometric feature mapping (isoMAP) (20) dimension reduction was performed using vegdist, spantree and isomap functions in the vegan R package (21).

[00143] The `vegdist` function was run with `method="euclidean"`. The `spantree` function was run with default parameters. The `isoMAP` function was run with `ndim` equal to the number of original dimensions of input data, and `k=5`. Phenograph clustering (22) was performed using the markers listed in **Table 3** before dimension reduction, and with the number of nearest neighbors equal to 30. The results obtained from the tSNE, isoMAP and Phenograph analyses were incorporated as additional parameters in the .fcs files, which were then loaded into FlowJo to generate heat plots of marker expression on the reduced dimensions. The above analyses were performed using the `cytokit` R package which provides a wrapper of existing state-of-the-art methods for cytometry data analysis (23).

10 [00144] **Human cell flow cytometry: Labeling, staining, analysis and cell sorting**

[00145] All antibodies used for fluorescence-activated cell sorting (FACS) and flow cytometry were mouse anti-human monoclonal antibodies (mAbs), except for chicken anti-human CADM1 IgY primary mAb. The mAbs and secondary reagents used for flow cytometry are listed in **Table 6**. Briefly, 5×10^6 cells/tube were washed and incubated with Live/Dead blue dye (Invitrogen) for 30 min at 4 °C in phosphate buffered saline (PBS) and then incubated in 5% heat-inactivated fetal calf serum (FCS) for 15 min at 4 °C (Sigma Aldrich). The appropriate antibodies diluted in PBS with 2% fetal calf serum (FCS) and 2 mM EDTA were added to the cells and incubated for 30 min at 4 °C, and then washed and detected with the secondary reagents. For intra-cytoplasmic or intra-nuclear labeling or staining, cells were fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences) or with eBioscience FoxP3/Transcription Factor Staining Buffer Set (eBioscience/Affimetrix), respectively according to the manufacturer's instructions. Flow cytometry was performed using a BD LSRII or a BD FACSFortessa (BD Biosciences) and the data analyzed using BD FACSDiva 6.0 (BD Biosciences) or FlowJo v.10 (Tree Star Inc.). For isolation of precursor dendritic cells (pre-DC), PBMC were first depleted of T cells, monocytes and B cells with anti-CD3, anti-CD14 and anti-CD20 microbeads (Miltenyi Biotec) using an AutoMACS Pro Separator (Miltenyi Biotec) according to the manufacturer's instructions. FACS was performed using a BD FACSAriaII or BD FACSAriaIII (BD Biosciences). Wanderlust analysis (33) of flow cytometry data was performed using the CYT tool downloaded from <https://www.c2b2.columbia.edu/danapeerlab/html/cyt-download.html>. As Wanderlust requires users to specify a starting cell, one cell was selected at random from the CD45RA⁺ CD123⁺ population.

[00146] **Cytospin and Scanning Electron Microscopy**

[00147] Cytospins were prepared from purified cells and stained with the Hema 3 system according to the manufacturer's protocol (Fisher Diagnostics). Images were analyzed at 100X magnification with an Olympus BX43 upright microscope (Olympus). Scanning electron
5 microscopy was performed as previously described (2).

[00148] **Dendritic cell (DC) differentiation co-culture assay on MS-5 stromal cells**

[00149] MS-5 stromal cells were maintained and passaged as previously described (24). MS-5 cells were seeded in 96-well round-bottom plates (Corning) at 3,000 cells per well in complete alpha-Minimum Essential Media (α -MEM) (Life Technologies) supplemented with
10 10 % fetal bovine serum (FBS) (Serana) and 1 % penicillin/streptomycin (Nacalai Tesque). A total of 5,000 sorted purified cells were added 18-24 h later, in medium containing 200 ng/mL Flt3L (Miltenyi Biotec), 20 ng/mL SCF (Miltenyi Biotec), and 20 ng/mL GM-CSF (Miltenyi Biotec), and cultured for up to 5 days. The cells were then resuspended in their wells by physical dissociation and filtered through a cell strainer into a polystyrene FACS
15 tube.

[00150] **Intracellular cytokine detection following stimulation with TLR ligands**

[00151] A total of 5×10^6 PBMC were cultured in Roswell Park Memorial Institute (RPMI)-1640 Glutmax media (Life Technologies) supplemented with 10 % FBS, 1 % penicillin/streptomycin and stimulated with either lipopolysaccharide (LPS, 100 ng/mL; InvivoGen), LPS (100 ng/mL) + interferon gamma ($\text{IFN}\gamma$, 1,000U/mL; R&D Systems),
20 Flagellin (100 ng/mL, Invivogen), polyI:C (10 $\mu\text{g/mL}$; InvivoGen), Imidazoquinoline (CL097; Invivogen) or CpG oligodeoxynucleotides 2216 (ODN, 5 μM ; InvivoGen) for 2 h, after which 10 $\mu\text{g/mL}$ Brefeldin A solution (eBioscience) was added and the cells were again stimulated for an additional 4 h. After the 6 h stimulation, the cells were labeled with
25 cytokine-specific antibodies and analyzed by flow cytometry, as described above.

[00152] **Mixed lymphocyte reaction**

[00153] Naïve T cells were isolated from PBMC using Naïve Pan T-Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions, and labeled with 0.2 μM carboxyfluorescein succinimidyl ester (CFSE) (Life Technologies) for 5 min at 37 °C. A total
30 of 5,000 cells from sorted DC subsets were co-cultured with 100,000 CFSE-labeled naïve T cells for 7 days in Iscove's Modified Dulbecco's Medium (IMDM; Life Technologies) supplemented with 10 % KnockOut™ Serum Replacement (Life Technologies). On day 7,

the T cells were stimulated with 10 µg/ml phorbol myristate acetate (InvivoGen) and 500 µg/ml ionomycin (Sigma Aldrich) for 1 h at 37 °C. 10 µg/ml Brefeldin A solution was added for 4 h, after which the cells were labeled with cytokine-specific antibodies and analyzed by flow cytometry, as described above.

5 [00154] **Electron microscopy**

[00155] Sorted cells were seeded on poly-lysine-coated coverslips for 1 h at 37 °C. The cells were then fixed in 2 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for 1 h, post fixed for 1 h with 2% buffered osmium tetroxide, then dehydrated in a graded series of ethanol solutions, before embedding in epoxy resin. Images were acquired with a Quemesa
10 (SIS) digital camera mounted on a Tecnai 12 transmission electron microscope (FEI Company) operated at 80 kV.

[00156] **Microarray analysis**

[00157] Total RNA was isolated from FACS-sorted blood pre-DC and DC subsets using a RNeasy® Micro kit (Qiagen). Total RNA integrity was assessed using an Agilent
15 Bioanalyzer (Agilent) and the RNA Integrity Number (RIN) was calculated. All RNA samples had a RIN ≥7.1. Biotinylated cRNA was prepared using an Epicentre TargetAmp™ 2-Round Biotin-aRNA Amplification Kit 3.0 according to the manufacturer's instructions, using 500 pg of total RNA starting material. Hybridization of the cRNA was performed on an Illumina Human-HT12 Version 4 chip set (Illumina). Microarray data were exported from
20 GenomeStudio (Illumina) without background subtraction. Probes with detection P-values > 0.05 were considered as not being detected in the sample, and were filtered out. Expression values for the remaining probes were log₂ transformed and quantile normalized. For differentially-expressed gene (DEG) analysis, comparison of one cell subset with another was carried out using the limma R software package (25) with samples paired by donor
25 identifiers. DEGs were selected with Benjamini-Hochberg multiple testing (26) corrected P-value <0.05. In this way, limma was used to select up and down-regulated signature genes for each of the cell subsets in the pre-DC data by comparing one subset with all other subsets pooled as a group. Expression profiles shown in **Fig. 4E** were from 62 common genes identified from the union of DEGs from comparing pre-cDC1 versus early pre-DC and cDC1
30 versus pre-cDC1, and the union of DEGs from comparing pre-cDC2 versus early pre-DC and cDC2 versus pre-cDC2 (see **Table 5** for the lists of DEGs for cDC1 lineage and cDC2

lineage, and the lists of the 62 common genes; **Fig. 23** for Venn diagram comparison of the two lists of DEGs and identification of the 62 common genes).

[00158] Luminex® Drop Array™ assay on sorted and stimulated pre-DC and DC populations

5 [00159] A total of 2,000 cells/well of sorted pre-DC and DC subsets were seeded in V-bottom 96 well plates and then incubated for 18 h in 50 μ L complete RPMI-1640 Glutmax media (Life Technologies) supplemented with 10 % FBS and 1 % penicillin/streptomycin, and stimulated with either LPS, LPS + IFN γ , Flagellin, polyI:C, Imidazoquinoline or CpG oligodeoxynucleotides (ODN) 2216. Cells were then pelleted and 30 μ L supernatant was
10 collected. A Luminex® Drop Array™ was performed using 5 μ L of the supernatant. Human G-CSF, GM-CSF, IFN- α 2, IL-10, IL-12p40, IL-12p70, IL-15, IL-1RA, IL-1a, IL-1b, IL-6, IL-7, IL-8, MIP-1b, TNF- α , TNF- β were tested by multiplexing (EMD Millipore) with DropArray-bead plates (Curiox) according to the manufacturer's instructions. Acquisition was performed using xPONENT 4.0 (Luminex) acquisition software, and data analysis was
15 performed using Bio-Plex Manager 6.1.1 (Bio-Rad).

[00160] Statistical analyses

[00161] The Mann-Whitney test was used to compare data derived from patients with Pitt-Hopkins Syndrome and controls and the intracellular detection of IL-12p40 and TNF- α in pre-DC stimulated with LPS or poly I:C versus CpG ODN 2216. The Kruskal-Wallis test,
20 followed by the Dunn's multiple comparison test, was used to compare the expression level of individual genes in single cells in the MARS-seq single cell RNAseq dataset. Differences were defined as statistically significant when adjusted $P < 0.05$. All statistical tests were performed using GraphPad Prism 6.00 for Windows (GraphPad Software). Correlation coefficients were calculated as Pearson's correlation coefficient.

25

Example 2 – Results

[00162] Unbiased identification of DC precursors by unsupervised single-cell RNAseq and CyTOF

[00163] Using PBMC isolated from human blood, massively-parallel single-cell mRNA
30 sequencing (MARS-seq) (3) was performed to assess the transcriptional profile of 710 individual cells within the lineage marker (Lin)(CD3/CD14/CD16/CD20/CD34) $^-$, HLA-DR $^+$ CD135 $^+$ population (**Fig. 1, A to G**, and **Fig. 7A**: sorting strategy, **Fig. 7, B to J**:

workflow and quality control, **Table 1**: number of detected genes). The MARS-seq data were processed using non-linear dimensionality reduction via t-stochastic neighbor embedding (tSNE), which enables unbiased visualization of high-dimensional similarities between cells in the form of a two-dimensional map (15, 27, 19). Density-based spatial clustering of applications with noise (DBSCAN) (8) on the tSNE dimensions identified five distinct clusters of transcriptionally-related cells within the selected PBMC population (**Fig. 1A**, and **Fig. 7G**). To define the nature of these clusters, gene signature scores were calculated for pDC, cDC1 and cDC2 (as described in (2), **Table 2**: lists of signature genes), and the expression of the signatures attributed to each cell was overlaid onto the tSNE visualization.

Clusters #1 and #2 (containing 308 and 72 cells, respectively) were identified as pDC, cluster #3 (containing 160 cells) was identified as cDC1, and cluster #5 (containing 120 cells) was identified as cDC2. Cluster #4 (containing 50 cells) laid in between the cDC1 (#3) and cDC2 (#5) clusters and possessed a weak, mixed pDC/cDC signature (**Fig. 1A**). A connectivity MAP (cMAP) analysis (7) was employed to calculate the degree of enrichment of pDC or cDC signature gene transcripts in each individual cell. This approach confirmed the signatures of pDC (#1 and #2) and cDC (#3 and #5) clusters, and showed that most cells in cluster #4 expressed a cDC signature (**Fig. 1B**).

[00164] The Mpath algorithm (6) was then applied to the five clusters to identify hypothetical developmental relationships based on these transcriptional similarities between cells (**Fig. 1C**, and **Fig. 8, A and B**). Mpath revealed that the five clusters were grouped into three distinct branches with one central cluster (cluster #4) at the intersection of the three branches (**Fig. 1C**, and **Fig. 8A**). The Mpath edges connecting cluster #4 to cDC1 cluster #3 and cDC2 cluster #5 have a high cell count (159 and 137 cells, respectively), suggesting that the transition from cluster #4 to clusters #3 and #5 is likely valid, and indicates that cluster #4 could contain putative cDC precursors (**Fig. 1C**). In contrast, the edge connecting cluster #4 and pDC cluster #2 has a cell count of only 7 (**Fig. 1C**, and **Fig. 8B**), which suggests that this connection is very weak. The edge connecting cluster #4 and #2 was retained when Mpath trimmed the weighted neighborhood network (**Fig. 8B**), simply due to the feature of the Mpath algorithm that requires all clusters to be connected (6). Monocle (10), principal component analyses (PCA), Wishbone (11) and Diffusion Map algorithms (12) were used to confirm these findings. Monocle and PCA resolved the cells into the same three branches as the original Mpath analysis, with the cells from the tSNE cluster #4 again falling at the

intersection (**Fig. 1, D and E**). Diffusion Map and Wishbone analyses indicated that there was a continuum between clusters #3 (cDC1), #4 and #5 (cDC2): cells from cluster #4 were predominantly found in the DiffMap_dim2^{low} region, and cells from clusters #3 and #5 were progressively drifting away from the DiffMap_dim2^{low} region towards the left and right, respectively. The pDC clusters (#1 and #2) were clearly separated from all other clusters (**Fig. 1F**, and **Fig. 8C**). In support of this observation, cells from these pDC clusters had a higher expression of pDC-specific markers and transcription factors (TF) than the cDC clusters (#3 and #5) and central cluster #4. Conversely, cells in cluster #4 expressed higher levels of markers and TF associated with all cDC lineage than the pDC clusters (**Fig. 1G**). This points to the possibility that cluster #4 represented a population of putative uncommitted cDC precursors.

[00165] Next, CyTOF, which simultaneously measures the intensity of expression of up to 38 different molecules at the single cell level, was employed to further understand the composition of the delineated sub-populations. A panel of 38 labeled antibodies were designed to recognize DC lineage and/or progenitor-associated surface molecules (**Table 3, Fig. 1, H to J**, and **Fig. 9**), and the molecules identified in cluster #4 by MARS-seq, such as CD2, CX3CR1, CD11c and HLA-DR (**Fig. 1I**). Using the tSNE algorithm, the CD45⁺Lin⁻(CD7/CD14/CD15/CD16/CD19/CD34)⁻HLA-DR⁺ PBMC fraction (**Fig. 9A**) resolved into three distinct clusters representing cDC1, cDC2 and pDC (**Fig. 1H**). An intermediate cluster at the intersection of the cDC and pDC clusters that expressed both cDC-associated markers (CD11c/CX3CR1/CD2/CD33/CD141/BTLA) and pDC-associated markers (CD45RA/CD123/CD303) (**Fig. 1, I to J**, and **Fig. 9B**) corresponded to the MARS-seq cluster #4. The delineation of these clusters was confirmed when applying the phenograph unsupervised clustering algorithm (22) (**Fig. 9C**). The position of the intermediate CD123⁺CD33⁺ cell cluster was distinct, and the cells exhibited high expression of CD5, CD327, CD85j, together with high levels of HLA-DR and the cDC-associated molecule CD86 (**Fig. 1, I to J**). Taken together, these characteristics raise the question of whether CD123⁺CD33⁺ cells might represent circulating human pre-DC.

[00166] **Pre-DC exist within the pDC fraction and give rise to cDC**

[00167] The CD123⁺CD33⁺ cell cluster within the Lin⁻HLA-DR⁺ fraction of the PBMC was analyzed by flow cytometry. Here, CD123⁺CD33⁻ pDC, CD45RA⁺/CD123⁻ cDC1 and cDC2, and CD33⁺CD45RA⁺CD123⁺ putative pre-DC were identified (**Fig. 2A**, and **Fig.**

10A). The putative pre-DC expressed CX3CR1, CD2, CD303 and CD304, with low CD11c expression, whereas CD123⁺CD33⁻ pDC exhibited variable CD2 expression (**Fig. 2, A and B**, and **Fig. 10, B and C**).

[00168] The analysis was extended to immune cells from the spleen and a similar putative
5 pre-DC population was identified, which was more abundant than in blood and expressed higher levels of CD11c (**Fig. 2, A and C**, and **Fig. 10D**).

[00169] Both putative pre-DC populations in the blood and spleen expressed CD135 and intermediate levels of CD141 (**Fig. 10C**). Wright-Giemsa staining of putative pre-DC sorted from the blood revealed an indented nuclear pattern reminiscent of classical cDC, a region of
10 perinuclear clearing, and a basophilic cytoplasm reminiscent of pDC (**Fig. 2D**).

[00170] At the ultra-structural level, putative pre-DC and pDC exhibited distinct features, despite their morphological similarities (**Fig. 2E**, and **Fig. 10E**): putative pre-DC possessed a thinner cytoplasm, homogeneously-distributed mitochondria (m), less rough endoplasmic reticulum (RER), an indented nuclear pattern, a large nucleus and limited cytosol, compared
15 to pDC; pDC contained a smaller nucleus, abundant cytosol, packed mitochondria, well-developed and polarized cortical RER organized in parallel cisterna alongside numerous stacks of rough ER membranes, suggesting a developed secretory apparatus, in agreement with previously-published data (28).

[00171] The differentiation capacity of pre-DC to that of cDC and pDC, through stromal
20 culture in the presence of FLT3L, GM-CSF and SCF was compared, as previously described (24). After 5 days, the pDC, cDC1 and cDC2 populations remained predominantly in their initial states, whereas the putative pre-DC population had differentiated into cDC1 and cDC2 in the known proportions found *in vivo* (29, 2, 30, 31) (**Fig. 2F**, **Fig. 10F**, and **Fig. 11**). Altogether, these data suggest that CD123⁺CD33⁺CD45RA⁺CX3CR1⁺CD2⁺ cells are
25 circulating pre-DC with cDC differentiation potential.

[00172] Breton and colleagues (32) recently reported a minor population of human pre-DC (highlighted in **Fig. 12A**), which shares a similar phenotype with the Lin⁻ CD123⁺CD33⁺CD45RA⁺ pre-DC defined here (**Fig. 12, A and B**). The present results reveal that the pre-DC population in blood and spleen is markedly larger than the one identified
30 within the minor CD303⁻CD141⁻CD117⁺ fraction considered previously (**Fig. 12, C and D**).

[00173] **Pre-DC are functionally distinct from pDC**

[00174] IFN α -secreting pDC can differentiate into cells resembling cDC when cultured with IL-3 and CD40L (33, 34), and have been considered DC precursors (34). However, when traditional ILT3⁺ILT1⁻ (33) or CD4⁺CD11c⁻ (34) pDC gating strategies were used, a “contaminating” CD123⁺CD33⁺CD45RA⁺ pre-DC sub-population in both groups was detected (**Fig. 12, E and F**). This “contaminating” sub-population result raises the question on whether other properties of traditionally-classified “pDC populations” might be attributed to pre-DC. TLR7/8 (CL097) or TLR9 (CpG ODN 2216) stimulation of pure pDC cultures resulted in abundant secretion of IFN α , but not IL-12p40, whereas pre-DC readily secreted IL-12p40 but not IFN- α (**Fig. 2G**, and **Fig. 13**). Furthermore, while pDC were previously thought to induce proliferation of naïve CD4⁺ T cells (32, 35), here only the pre-DC sub-population was found to exhibit this attribute (**Fig. 2H**). Reports of potent allostimulatory capacity and IL-12p40 production by CD2⁺ pDC (35) might then be explained by CD2⁺ pre-DC “contamination” (36) (**Fig. 14**).

[00175] Pitt-Hopkins Syndrome (PHS) is characterized by abnormal craniofacial and neural development, severe mental retardation, and motor dysfunction, and is caused by haplo-insufficiency of TCF4, which encodes the E2-2 transcription factor — a central regulator of pDC development (37). Patients with PHS had a marked reduction in their blood pDC numbers compared to healthy individuals, but retained a population of pre-DC (**Fig. 2I**, and **Fig. 15**), which likely accounts for the unexpected CD45RA⁺CD123⁺CD303^{lo} cell population reported in these patients (57). Taken together, the present data indicate that, while pre-DC and pDC share some phenotypic features, they can be separated by their differential expression of several markers, including CD33, CX3CR1, CD2, CD5 and CD327. pDC are *bona fide* IFN α -producing cells, but the reported IL-12 production and CD4⁺ T-cell allostimulatory capacity of pDC can likely be attributed to “contaminating” pre-DC, which can give rise to both cDC1 and cDC2.

[00176] Identification and characterization of committed pre-DC subsets

[00177] The murine pre-DC population contains both uncommitted and committed pre-cDC1 and pre-cDC2 precursors (38). Thus, microfluidic scRNAseq was used to determine whether the same was true for human blood pre-DC, (**Fig. 16A**: sorting strategy, **Fig. 16, B and C**: workflow and quality control, **Table 4**: number of expressed genes). The additional single cell gene expression data relative to the MARS-seq strategy used for **Fig. 1, A to G** (2.5 million reads/cell and an average of 4,742 genes detected per cell vs 60,000 reads/cell

and an average of 749 genes detected per cell, respectively) was subjected to cMAP analysis, which calculated the degree of enrichment for cDC1 or cDC2 signature gene transcripts (2) for each single cell (**Fig. 3A**). Among the 92 analyzed pre-DC, 25 cells exhibited enrichment for cDC1 gene expression signatures, 12 cells for cDC2 gene expression signatures, and 55 cells showed no transcriptional similarity to either cDC subset. Further Mpath analysis showed that these 55 “unprimed” pre-DC were developmentally related to cDC1-primed and cDC2-primed pre-DC, and thus their patterns of gene expression fell between the cDC1 and cDC2 signature scores by cMAP (**Fig. 3B**, and **Fig. 17**). These data suggest that the human pre-DC population contains cells exhibiting transcriptomic priming towards cDC1 and cDC2 lineages, as observed in mice (38).

[00178] This heterogeneity within the pre-DC population by flow cytometry were further subjected to identification using either pre-DC-specific markers (CD45RA, CD327, CD5) or markers expressed more intensely by pre-DC compared to cDC2 (BTLA, CD141). 3D-PCA analysis of the Lin[−]HLA-DR⁺CD33⁺ population (containing both differentiated cDC and pre-DC) identified three major cell clusters: CADM1⁺ cDC1, CD1c⁺ cDC2 and CD123⁺ pre-DC (**Fig. 3C**, and **Fig. 18A**). Interestingly, while cells located at the intersection of these three clusters (**Fig. 3D**) expressed lower levels of CD123 than pre-DC, but higher levels than differentiated cDC (**Fig. 3C**), they also expressed high levels of pre-DC markers (**Fig. 3D**, and **Fig. 18A**). It is possible that these CD45RA⁺CD123^{lo} cells might be committed pre-DC that are differentiating into either cDC1 or cDC2 (**Fig. 3E**). The Wanderlust algorithm (39), which orders cells into a constructed trajectory according to their maturity, confirmed the developmental relationship between pre-DC (early events), CD45RA⁺CD123^{lo} cells (intermediate events) and mature cDC (late events) (**Fig. 3F**). Flow cytometry of PBMC identified CD123⁺CADM1[−]CD1c[−] putative uncommitted pre-DC, alongside putative CADM1⁺CD1c[−] pre-cDC1 and CADM1[−]CD1c⁺ pre-cDC2 within the remaining CD45RA⁺ cells (**Fig. 3G**, and **Fig. 18B**). These three populations were also present, and more abundant, in the spleen (**Fig. 18C**). Importantly, *in vitro* culture of pre-DC subsets sorted from PBMC did not give rise to any CD303⁺ cells (which would be either undifferentiated pre-DC or differentiated pDC), whereas early pre-DC gave rise to both cDC subsets, and pre-cDC1 and pre-cDC2 differentiated exclusively into cDC1 and cDC2 subsets, respectively (**Fig. 3H**, **Fig. 18D**, and **Fig. 19**).

- [00179] Scanning electron microscopy confirmed that early pre-DC are larger and rougher in appearance than pDC, and that committed pre-DC subsets closely resemble their mature cDC counterparts (**Fig. 3I**, and **Fig. 20A**). Phenotyping of blood pre-DC by flow cytometry (**Fig. 24B**) identified patterns of transitional marker expression throughout the development of early pre-DC towards pre-cDC1/2 and differentiated cDC1/2. Specifically, CD45RO and CD33 were acquired in parallel with the loss of CD45RA; CD5, CD123, CD304 and CD327 were expressed abundantly by early pre-DC, intermediately by pre-cDC1 and pre-cDC2, and rarely if at all by mature cDC and pDC; FcεRI and CD1c were acquired as early pre-DC commit towards the cDC2 lineage, concurrent with the loss of BTLA and CD319 expression; early pre-DC had an intermediate expression of CD141 that dropped along cDC2 differentiation but was increasingly expressed during commitment towards cDC1, with a few pre-cDC1 already starting to express Clec9A; and IRF8 and IRF4 - transcription factors regulating cDC lineage development (40, 41) - were expressed by early pre-DC and pre-cDC1, while pre-cDC2 maintained only IRF4 expression (**Fig. 20C**).
- [00180] Pre-DC and DC subsets were next sorted from blood and microarray analyses were performed to define their entire transcriptome. 3D-PCA analysis of the microarray data showed that pDC were clearly separated from other pre-DC and DC subsets along the horizontal PC1 axis (**Fig. 4A**, and **Fig. 21**). The combination of the PC2 and PC3 axes indicated that pre-cDC1 occupied a position between early pre-DC and cDC1 and, although cDC2 and pre-cDC2 exhibited similar transcriptomes, pre-cDC2 were positioned between cDC2 and early pre-DC along the PC3 axis (**Fig. 4A**). Hierarchical clustering of differentially-expressed genes (DEG) confirmed the similarities between committed pre-DC and their corresponding mature subset (**Fig. 22**). The greatest number of DEG was between early pre-DC and pDC (1249 genes) among which CD86, CD2, CD22, CD5, ITGAX (CD11c), CD33, CLEC10A, SIGLEC6 (CD327), THBD, CLEC12A, KLF4 and ZBTB46 were more highly expressed by early pre-DC, while pDC showed higher expression of CD68, CLEC4C, TCF4, PACSIN1, IRF7 and TLR7 (**Fig. 4B**). An evolution in the gene expression pattern was evident from early pre-DC, to pre-cDC1 and then cDC1 (**Fig. 4C**), whereas pre-cDC2 were similar to cDC2 (**Fig. 4D**, and **Fig. 22**). The union of DEGs comparing pre-cDC1 versus early pre-DC and cDC1 versus pre-cDC1 has 62 genes in common with the union of DEGs from comparing pre-cDC2 versus early pre-DC and cDC2 versus pre-cDC2. These 62 common genes include the transcription factors BATF3, ID2 and TCF4 (E2-2), and the pre-

DC markers CLEC4C (CD303), SIGLEC6 (CD327), and IL3RA (CD123) (**Fig. 4E**, **Fig. 23** and **Table 5**). The progressive reduction in transcript abundance of SIGLEC6 (CD327), CD22 and AXL during early pre-DC to cDC differentiation was also mirrored at the protein level (**Fig. 4F**). Key transcription factors involved in the differentiation and/or maturation of DC subsets showed a progressive change in their expression along the differentiation path from pre-DC to mature cDC (**Fig. 4G**). Finally, pathway analyses revealed that pre-DC exhibited an enrichment of cDC functions relative to pDC, and were maintained in a relatively immature state compared to mature cDC (**Fig. 24**).

[00181] **Committed pre-DC subsets are functional**

[00182] The present invention then investigated to what extent the functional specializations of DC (42, 43) were acquired at the precursor level by stimulating PBMC with TLR agonists and measuring their cytokine production (**Fig. 5A**). Pre-DC produced significantly more TNF- α and IL-12p40 when exposed to CpG ODN 2216 (TLR9 agonist), than to either LPS (TLR4 agonist) or polyI:C (TLR3 agonist) ($p=0.03$, Mann-Witney test). It was confirmed that pDC were uniquely capable of robust IFN- α production in response to CL097 and CpG ODN 2216. CpG ODN 2216 stimulation also triggered IL-12p40 and TNF- α production by early pre-DC, pre-cDC1, and to a lesser extent pre-cDC2. Although TLR9 transcripts were detected only in early pre-DC (**Fig. 25A**), these data indicate that, contrary to differentiated cDC1 and cDC2, pre-cDC1 and pre-cDC2 do express functional TLR9 and hence can be activated using TLR9 agonists. Interestingly, while pre-cDC2 resembled cDC2 at the gene expression level, their responsiveness to TLR ligands was intermediate between that of early pre-DC and cDC2. Pre-DC subsets also expressed T-cell co-stimulatory molecules (**Fig. 5B**) and induced proliferation and polarization of naïve CD4⁺ T cells to a similar level as did mature cDC (**Fig. 5C**, and **Fig. 25B**).

[00183] **Unsupervised mapping of DC ontogeny**

[00184] To understand the relatedness of the cell subsets, an unsupervised isoMAP analysis (20) was performed of human BM cells, obtained from CyTOF analysis, for non-linear dimensionality reduction (**Fig. 6A**, and **Fig. 26A**). This analysis focused on the Lin⁻CD123^{hi} fraction and identified CD123^{hi}CD34⁺ CDP (phenograph cluster #5), from which branched CD34⁻CD123⁺CD327⁺CD33⁺ pre-DC (clusters #1 and #2) and CD34⁻CD123⁺CD303⁺CD68⁺ pDC (clusters #3 and #4) which both progressively acquired their respective phenotypes. Cells in the pre-DC branch increasingly expressed CD2, CD11c, CD116 and, at a later stage,

CD1c. IsoMAP analysis of Lin⁻CD123⁺ cells in the peripheral blood identified two parallel lineages, corresponding to pre-DC and pDC, in which a CDP population was not detected (**Fig. 6B**). IsoMAP and phenograph analysis of pre-DC extracted from the isoMAP analysis of **Fig. 6A** (BM, clusters #1 and #2) and **Fig. 6B** (blood, cluster #6) revealed the three distinct pre-DC subsets (**Fig. 6C**) as defined by their unique marker expression patterns (**Fig. 26, B and C**).

[00185] In summary, the developmental stages of DC from the BM to the peripheral blood through CyTOF were traced, which shows that the CDP population in the BM bifurcates into two pathways, developing into either pre-DC or pDC in the blood (**Fig. 6, A to C**). This pre-DC population is heterogeneous and exists as distinct subsets detectable in both the blood and BM (**Fig. 6C**, and **Fig. 26, B and C**). Furthermore, an intriguing heterogeneity in blood and BM pDC was uncovered, which warrants further investigation (**Fig. 6C**, and **Fig. 26, D and E**).

[00186] **Validation of down sampling threshold for normalization of MARS-seq single cell transcriptome data**

[00187] High variance in terms of quality of single-cell transcriptomes is expected in a single-cell RNA sequencing experiment due to the low quantity of RNA input material. This caveat necessitates stringent quality control in order to avoid a bias introduced by low quality single-cell transcriptomes. In single-cell transcriptomics it is, therefore, common practice to remove low quality transcriptomes to ensure an unbiased and biologically meaningful analysis (44, 45). Different strategies have been used to filter out low quality cells, including an empirically determined cutoff for cell filtering (45), a down sampling strategy to normalize and filter low quality cells (3), and various filtering cutoffs from 600 UMIs/cell or 400 UMIs/cells (3), <500 molecule counts per cell (46) and <200 UMIs/cell (47). A mathematically determined cut-off was not reported in any of these studies. As these previous studies were performed on murine cells, and quality filters in single-cell data have to be established within the respective dataset, the present approach had adapted the filtering strategy to human cells. To determine the quality threshold for the present dataset, several diagnostics were used to estimate the optimal cutoff for down sampling of molecule counts. Firstly, the cumulative distribution of molecule counts were visualized, where cells on the x-axis were ordered by decreasing UMI count (**Fig. 7C**). Here, in a certain region there was a period of strong decline in the number of molecule counts per cell. This region corresponded

to a range of molecule counts between 400 and 1200 UMIs per cell. The next metric used to judge an objective threshold (**Fig. 7D**) was the molecule count distribution of all cells. Many of the cell barcodes had <650 molecule counts - these cell barcodes most likely represented the background signal of the present MARS-seq data set. The number of cell barcodes with a certain number of molecules decreases with increasing molecule count per cell; through this visualization, natural breakpoints in the distribution that could be used as an objective threshold for filtering and normalization were identified, as these breakpoints mark a change in the data structure and quality, and indicate the transition from background to signal, or from low-quality transcriptomes to high-quality transcriptomes. Here, three notable points were identified (**Fig. 7D**), which corresponded to molecule counts of 650 (left), 1,050 (middle) and 1,700 (right) per cell. To objectively determine which of these points represented a shift in data quality from low to high quality transcriptomes, a turning point needed to be identified (**Fig. 7D**). In the density plot (**Fig. 7D**, top panel), the three lines (left, middle, right) are the breakpoints where the slope of the density function (1st derivative of density, **Fig. 7D**, middle panel) has a sudden change. On the left line, the downward slope (1st derivative) changes from being very steep to less steep, so that the 2nd derivative is the highest at this point. Similarly, on the middle line, the downward slope changes from less steep to more steep, so the 2nd derivative is the lowest. Based on these observations, the three turning points were identified by the 2nd derivative (**Fig. 7D**, bottom panel). When a cutoff of 650 was applied, the number of molecule counts per cell was too low and the three DC populations – plasmacytoid DC (pDC) and conventional DC (cDC) subsets cDC1 and cDC2, could not be distinguished by principal component analysis (PCA; **Fig. 7E**). When a cutoff of 1,700 was applied, the number of cells retained was too low. Therefore, the 1,050 cutoff was an optimal tradeoff between the number of cells analyzed (cells retained after filtering by down sampling normalization) and the number of molecule counts in a cell (gene expression information that remains after discarding molecule counts by down sampling).

[00188] To ensure data reproducibility, stability and independence of the chosen molecule cutoff, the initial analyses were stimulated using cutoffs of 650, 1,050, 1,700 and 2,350 molecule counts (**Fig. 7E**). All four chosen simulation values exhibited the same general data topology if the data were dimensionally-reduced using PCA, thus proving that the biological data structure was robust and independent of filtering thresholds. In addition, the influence of the filtering threshold on the gene loadings within the first two principal components were

correlated. Principal component 1 (PC1) of the dataset down-sampled to 1,050 molecule counts was highly correlated with PC1 of the datasets down-sampled to either 650 or 1,700 molecule counts (Pearson = 0.996 and 0.999, respectively). The same was true for PC2 (Pearson = 0.960 and 0.925, respectively). These results indicated that the chosen filtering cutoff of 1,050 was representative and objectively-derived.

[00189] The MARS-seq data obtained in this disclosure were generated by two independent experiments (run1 and run2), which were combined for further data analysis. After normalization, the correlation between the average molecule count of all genes in run1 vs run2 was assessed (**Fig. 7F**, which shows the high correlation between the average molecular counts in both runs ($r=0.994$)). When assessing for a batch effect, it is important to ensure that runs do not determine the clustering itself. The t-distributed stochastic neighbor embedding (tSNE) values were plotted (**Fig. 7G**) (cells of run1 and run2 in equal proportions) together with their density estimates. This analysis showed that the general distribution and, therefore, the clustering was not governed by the run, which is in line with the observation that the present clustering identified biologically reasonable groups that clearly corresponded to the three DC populations (pDC, cDC1 and cDC2) (**Fig. 1A**). Consequently, the observed clusters were not explained by the variance between the runs, but by biology.

[00190] The frequencies of cell types were compared, as determined by the clustering, within the two runs (**Fig. 7H**). This showed that the ratio between the cells in different clusters was comparable between the two runs. Of note, the ratio does not need to be identical in both runs (46). In addition, this analysis showed that no cluster dominated a single run. Due to the fact that we are taking relatively small samples from a large total population, the frequencies of cell types are expected to show natural variation between runs, which could explain slight shifts in cellular frequencies.

[00191] Discussion

[00192] Using unsupervised scRNAseq and CyTOF analyses, the complexity of the human DC lineage at the single cell level was unraveled, revealing a continuous process of differentiation that starts in the BM with CDP, and diverges at the point of emergence of pre-DC and pDC potentials, culminating in maturation of both lineages in the blood. A previous study using traditional surface marker-based approaches had suggested the presence of a minor pre-DC population in PBMC (32), but the combination of high-dimensional techniques

and unbiased analyses employed here shows that this minor population had been markedly underestimated: as the present results reveal a population of pre-DC that overlaps with that observed by Breton and colleagues (32) within the CD117⁺CD303⁺CD141⁻ fraction of PBMC, but accounts for >10 fold the number of cells in peripheral blood than was originally estimated, and is considerably more diverse (**Fig. 12C**).

[00193] Recent work in mice found uncommitted and subset-committed pre-DC subsets in the BM (38, 43). Here, similarly, three functionally- and phenotypically- distinct pre-DC populations in human PBMC, spleen and BM were identified which are: uncommitted pre-DC and two populations of subset-committed pre-DC (**Fig. 27** and **Fig. 28**). In line with the concept of continuous differentiation from the BM to the periphery, the proportion of uncommitted cells was higher in the pre-DC population in the BM than in the blood. Altogether, these findings support a two-step model of DC development whereby a central transcriptomic subset-specific program is imprinted on DC precursors from the CDP stage onwards, conferring a core subset identity irrespective of the final tissue destination; in the second step of the process, peripheral tissue-dependent programming occurs to ensure site-specific functionality and adaptation (38, 43). Future studies will be required to reveal the molecular events underlying DC subset lineage priming and the tissue-specific cues that regulate their peripheral programming, and to design strategies that specifically target DC subsets at the precursor level. In addition, how the proportions of uncommitted pre-DC versus committed pre-DC are modified in acute and chronic inflammatory settings warrants further investigation.

[00194] An important aspect of unbiased analyses is that cells are not excluded from consideration on the basis of preconceptions concerning their surface phenotype. Pre-DC was found to express most of the markers that classically defined pDC, such as CD123, CD303 and CD304. Thus, any strategy relying on these markers to identify and isolate pDC will have inadvertently included CD123⁺CD33⁺ pre-DC as well. While this calls for reconsideration of some aspects of pDC population biology, it may also explain earlier findings including that: pDC cultures possess cDC potential and acquire cDC-like morphology (33, 34), as recently observed in murine BM pDC (48); pDC mediate Th1 immunity through production of IFN- α and IL-12 (33, 49-53); pDC exhibit naïve T-cell allostimulatory capacity (35, 51); and pDC express co-stimulatory molecules and exhibit antigen-presentation/cross-presentation capabilities at the expense of IFN- α secretion (49, 1). These observations could be attributed

to the undetected pre-DC in the pDC populations described by these studies, and indeed it has been speculated that the IL-12 production observed in these early studies might be due to the presence of contaminating CD11c⁺ cDC (53). The present disclosure addressed this possibility by separating CX3CR1⁺CD33⁺CD123⁺CD303⁺CD304⁺ pre-DC from CX3CR1⁻CD33⁻CD123⁺CD303⁺CD304⁺ “pure” pDC and showing that pDC could not polarize or induce proliferation of naïve CD4 T cells, whereas pre-DC had this capacity; and that pDC were unable to produce IL-12, unlike pre-DC, but were the only cells capable of strongly producing IFN- α in response to TRL7/8/9 agonists, as initially described (54). Thus, it is of paramount importance that pre-DC be excluded from pDC populations in future studies, particularly when using commercial pDC isolation kits. Finally, if pDC are stripped of all their cDC properties, it raises the question as to whether they truly belong to the DC lineage, or rather are a distinct type of innate IFN-I-producing lymphoid cell. It also remains to be shown whether the BM CD34⁺CD123^{hi} CDP population is also a mixture of independent *bona fide* cDC progenitors and pDC progenitors.

[00195] Despite their classification as precursors, human pre-DC appear functional in their own right, being equipped with some T-cell co-stimulatory molecules, and with a strong capacity for naïve T-cell allostimulation and cytokine secretion in response to TLR stimulation (**Fig. 2, Fig. 5, Fig. 13, and Fig. 15**). Pre-DC produced low levels of IFN- α in response to CpG ODN 2216 exposure, and secreted IL-12 and TNF- α in response to various TLR ligands. Hence, it is reasonable to propose that pre-DC have the potential to contribute to both homeostasis and various pathological processes, particularly inflammatory and autoimmune diseases where dysregulation of their differentiation continuum or their arrested development could render them a potent source of inflammatory DC ready for rapid recruitment and mobilization.

[00196] Beyond the identification of pre-DC, the present data revealed previously-unappreciated transcriptional and phenotypic heterogeneity within the circulating mature DC populations. This was particularly clear in the case of cDC2 and pDC, which were grouped into multiple Mpath clusters in the single-cell RNAseq analysis, and showed marked dispersion in the tSNE analysis of the CyTOF data with phenotypic heterogeneity. IsoMAP analysis of the CyTOF data also revealed another level of pDC heterogeneity by illustrating the progressive phenotypic transition from CDP into CD2⁺ pDC in the BM, involving intermediate cells that could be pre-pDC. Whether a circulating pre-pDC population exists

remains to be concluded. Finally, defining the mechanisms that direct the differentiation of uncommitted pre-DC into cDC1 or cDC2, or that maintain these cells in their initial uncommitted state in health and disease could lead to the development of new therapeutic strategies to modulate this differentiation process.

5 [00197] In summary, the present invention revealed the complexity of human DC lineage at the single cell level. DC in the bone marrow start as common CDP and diverge at the point of emergence into pre-DC and pDC potentials, culminating in maturation of both lineages in the blood. Furthermore, three functionally and phenotypically distinct pre-DC populations were identified in the human PBMC, spleen and bone marrow: uncommitted pre-DC and two
10 populations of subset-committed pre-DC (pre-cDC1 and pre-cDC2). Importantly, the present invention revealed a novel activation pathway of pre-DC that unlike mature DC subsets, committed pre-DC subsets respond to TLR9 stimulation. PBMC was stimulated with TLR agonists and their cytokine production was measured. Pre-DC produced significantly more TNF- α and IL-12p40 when exposed to CpG ODN 2216 (TLR9 agonist), than to either LPS
15 (TLR4 agonist) or polyI:C (TLR3 agonist) ($p=0.03$, Mann-Witney test) (**Fig. 5**). CpG ODN 2216 stimulation also triggered IL-12p40 and TNF- α production by early pre-DC, pre-cDC1, and to a lesser extent pre-cDC2. The application of the TLR9 stimulation of pre-DC may include using a combination of one or more TLR9 agonists (such as CpG) and an antigen delivery system that specifically targets pre-DC and committed pre-DC (for example, by
20 inclusion of an antibody that specifically targets pre-DC and committed pre-DC) to (i) mobilize and activate pre-DC, (ii) deliver the antigen to pre-DC for presentation of antigenic peptides to T cells, and (iii) activate antigen specific T cells. The design strategy could be used in immunotherapy for cancer and other diseases.

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