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(54) **Title:** CELLS

(57) **Abstract:** The present invention relates to an isolated population of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs for use in the treatment or prophylaxis of an immune-mediated disease in which an immunosuppressive effect is required. The invention also relates to a pharmaceutical composition and a vaccine comprising the isolated population of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs of the present invention in combination with one or more pharmaceutically acceptable excipients. Further, the invention relates to methods involving the administration of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs, and the depletion of tissue CD141⁺ DCs for treating or preventing various disease or conditions, and also relates to methods for monitoring disease and/or therapy progression. Additionally, the invention relates to the production of tissue CD141⁺-like DCs.



Cells

FIELD OF THE INVENTION

The present invention relates to the use of tissue CD141⁺ dendritic cells (CD141⁺ DCs) and tissue CD141⁺-like DCs in the treatment of diseases or conditions associated with inflammation. In particular, the present invention is useful for the treatment of inflammatory diseases in which an immunosuppressive effect is required, but also for the treatment of diseases or conditions in which an immunostimulatory effect is required. The present invention also relates to the use of tissue CD141⁺ dendritic cells and tissue CD141⁺-like DCs for diagnosing a disease or condition associated with inflammation or monitoring the progression thereof. The present invention also relates to the use of tissue CD141⁺ dendritic cells and tissue CD141⁺-like DCs for monitoring the efficacy of an immune therapy involving the use of vitamin D or an analogue thereof. The present invention also relates to the use of vitamin D or an analogue thereof for inducing CD141⁺ dendritic cells.

BACKGROUND OF THE INVENTION

Dendritic cell populations in the blood and skin

Dendritic cells (DCs) represent a heterogeneous population of leukocytes and can be divided based on morphology, functions, surface markers, and gene expression profiles, and anatomical location [1, 2]. In mice, DCs reside in secondary lymphoid tissues can be divided into plasmacytoid DCs (pDCs) and myeloid/conventional DCs (MDCs) subsets. pDCs have unique capacity to produce large amounts of type I interferons and therefore play a key role in antiviral immunity [3]. MDCs can be subdivided based on their reciprocal expression of CD8α⁺ and CD11b⁺ subsets as well as differences in antigen presentation functions. In non-lymphoid tissues such as skin, lung, and gut, MDCs can be divided base on langerin (CD207) and CD103 expression. While CD207⁺ DCs constitute the majority of DCs found in the steady-state tissues, CD207⁺ DCs represent a quantitatively minor population that displays unique functional specialization. Additionally, Langerhans cells (LCs) are specialized subset of DCs primarily reside in the epidermal compartment of the skin [1, 4].

In humans, DC subsets obtained in peripheral blood can be classified based on their surface markers into plasmacytoid DCs (pDCs, CD303 (BDCA2)-positive) and myeloid DCs (MDCs) that includes the CD1c (BDCA1)-positive and the CD141 (BDCA3)-positive subsets [5, 6]. While CD1c⁺ DCs are the major subset of MDCs in blood, CD141⁺ DCs represent a minor subpopulation of MDCs in peripheral blood. Non-lymphoid tissue DC subsets are best studied in the skin. While epidermis hosts only LCs, dermis harbours a complex network of DCs which will be discussed below.

Attention to dermal DCs (DDCs) has risen since a number of early studies reported the existence of resident DCs in the dermis of both murine and human skin [7, 8]. Thus far, several subpopulations of DDCs have been described under both normal and pathologic conditions. Pioneer studies revealed that in healthy human skin, the majority of DDCs are of myeloid origin and express CD1c [8], a surface marker widely used to identify a main subset of myeloid DCs in peripheral blood [6]. Zaba *et al.* extended previous findings and suggested that co-expression of CD1c and CD11c is a useful marker *in situ* to distinguish DDCs from macrophages [9]. These authors further

described the steady-state CD11c⁺CD1c⁺ DDCs as skin “resident DCs” in contrast to the “inflammatory DCs” found merely in the inflamed skin [10].

CD1c⁺ DDCs can be subdivided into at least three discrete subsets base on their surface expression of CD1a and CD14 [8]. It is becoming increasingly appreciated that distinct subsets of DDCs display differential functional specialization. CD1a⁺CD14⁺ DDCs can be distinguished from CD1a⁺ LCs based on their morphology, phenotype, and transcriptomic profiles [11]. *Ex vivo* isolated CD1a⁺CD14⁺ DDCs have a mature phenotype and are potent inducers of allogeneic naïve CD4⁺ and CD8⁺ T-cell proliferation [8, 12-14]. In contrast, CD14⁺ DDCs are less mature than CD1a⁺ DDCs and display a reduced capacity to prime naïve T-cell proliferation [15].

While CD1a⁺ DDCs have strong T-cell stimulatory capacity, other studies suggest that CD14⁺ DDCs are efficient at antigen up-take, potentially through the C-type lectins such as MMR/CD206 and DC-SIGN/CD209 expressed on the cells surface [15, 16]. In addition, Klechevsky *et al.* demonstrates that CD14⁺ DDCs are able to polarize naïve CD4⁺ T cells into follicular helper T cells, which then promote naïve B cells differentiation [13]. Although the significance of this finding needs to be further evaluated in a more physiological experimental setting, these data underline a previously unappreciated function of CD14⁺ DDCs in the activation of humoral immunity.

Another level of functional specialization for DDCs is highlighted by the detailed characterization of dermal-resident CD207⁺CD103⁺ DCs in mice. This population of DDCs has previously been overlooked, mainly due to its low density in the dermis that has led to the assumption that all langerin⁺ DCs found in the dermis are LCs en route to draining lymph nodes [17]. A series of animal studies provide clear evidence suggesting that dermal CD207⁺ DCs are distinct from LCs in both their origin and function [18-20]. Recent studies further revealed a unique functional characteristic of CD207⁺CD103⁺ DDCs to cross-present epidermal-derived viral and self antigens to CD8⁺ T cells [21, 22]. It appears that CD207⁺CD103⁺ DDCs can capture keratinocyte-derived antigens in the skin and transport to cutaneous draining lymph nodes where they exhibit cross-presentation. Further studies are required to understand how dermal resident CD207⁺CD103⁺ DCs gain access to epidermal-derived antigens since LCs seem to be dispensable in this process [22].

The unique functional characteristics of CD207⁺CD103⁺ DDCs make this DC subset an attractive target for therapy. This also raises an important question as to the presence of their human DDC counterparts. Dermal CD207⁺ DCs represent less than 5 % of DDCs in the human skin (unpublished observation). Whether human CD207⁺ DDCs correspond to the CD207⁺CD103⁺ DDCs described in mice remained to be investigated.

Functional role of human CD141⁺ DCs

CD141⁺ DCs represent a minor subpopulation of human MDCs that differ from the CD1c⁺ DCs in terms of Fc receptor expression, Toll-like receptor pattern, cytokine production, and T helper (Th) cell polarization ability [30].

A genome-wide expression profiling study suggested that human blood CD141⁺ DCs may correlate to mouse CD8a⁺ DCs [31]. Characteristics of mouse CD8a⁺ DCs include: 1) expression of transcription factors basic leucine zipper transcriptional factor ATF-like 3 (Batf3) [32] and interferon regulatory factor 8 (IRF-8) [33, 34] that are essential for

their development and differentiation; 2) selective expression of surface molecules Nectin-like protein 2 (Nect2) [35], C-type lectin 9A (CLEC9A) [36], and XC chemokine receptor 1 (XCR1) [37] that regulate their cross-presentation function; 3) differential toll-like receptor (TLR) expression pattern including TLR1, 2, 3, 4, 6, 8, and 9 [38] with TLR3 and 9 stimulation being especially effective for the enhancement of their cross-priming capacity [39].

Sancho *et al.* and others demonstrated that the C-type lectin, CLEC9A, is exclusively expressed by human blood CD141⁺ DCs [36, 40-42]. More recently, several studies expanded this finding and demonstrated that human CD141⁺ DCs are equivalent to mouse CD8 α ⁺ splenic DCs. This is based on similarities in terms of transcription factor expression including Batf3 and IRF8, XCR1 and Nect2 expression, TLR pattern, and Th-1 cell polarization capacity. Most importantly, human blood CD141⁺ DCs have the unique capacity to induce cytotoxic T cell responses through cross-presentation of both soluble and cell-associated antigens [23-25, 43].

Additionally, CD141 molecule *per se* (also known as thrombomodulin) may be involved in immune regulation. Conway *et al.* showed that the lectin-like domain of thrombomodulin can suppress adhesion molecule expression on neutrophils and consequently prevent neutrophil-mediated tissue damage [47]. Interestingly, expression of CD141 on DCs is increased when DCs are exposed to specific environmental factors, such as house dust mite-derived allergen [48] and IL-10 [49]. In contrast to blood CD141⁺ DCs that promote a Th-1-skewed immune response [24], allergen-enhanced CD141⁺ DCs displayed a Th-2 polarizing property [48], while IL-10-induced CD141⁺ DCs demonstrated an overall reduced allostimulatory capacity [49].

Despite CD141⁺ DCs representing a minor DC population in peripheral blood, they are found in various lymphoid and non-lymphoid tissues including lymph nodes, spleen, lung, kidney, the skin [9, 24-29]. The function of CD141⁺ DCs in these tissues is currently unknown.

Immune regulatory roles of DCs

Several studies highlight the critical roles of DCs in the induction of peripheral T-cell unresponsiveness, essentially through constitutive presentation of soluble and tissue-associated self antigens to self-reacting T cells in the steady-state [50]. DCs continuously migrating from peripheral tissues to lymph nodes can capture tissue-associated self antigens and present to circulating naïve T cells. The exogenous antigen acquired by DCs are presented to CD4⁺ T cells via the MHC class II pathway or cross-present to CD8⁺ T cells via the MHC class I pathway. Both antigen presentation mechanisms are thought to be essential for the induction of T cell tolerance. Mechanisms involved in DC-mediated immune regulation are not yet fully understood.

The cross-presentation capacity of DCs is thought to be an important feature for DCs to exert their immunoregulatory properties. DCs with impaired cross-presentation ability due to Rael deficiency [51] failed to control autoreactive CD8⁺ T cells tolerance *in vivo*. Moreover, the fully functional autoreactive CD8⁺ T cells accumulated in the Rael-deficient mice had a greater potential to cause autoimmunity [52]. Since DCs do not produce tissue-associated antigens themselves, cross-presentation of exogenous self-antigens by DCs in a non-

inflammatory condition seems to be a necessary approach to induce unresponsiveness of self-reactive CD8⁺ T cells. This phenomenon is often termed “cross-tolerance” [53].

CD8α⁺ DCs

- 5 In mice, CD8α⁺ DCs have a distinctive function in presenting exogenous antigen obtained from dead or dying cells to both CD4⁺ T cells via the MHC class II pathway and CD8⁺ T cells via the MHC class I pathway (cross-presentation) [54]. While CD8α⁺ DCs are essential to initiate immunity against viral infection and intracellular pathogens, increasing evidence suggests that CD8α⁺ DCs have immunoregulatory properties [55-58].
- 10 Early *in vitro* studies demonstrated that non-activated CD8α⁺ DCs isolated from mouse spleen have a reduced primary and secondary T cell stimulatory capacity compared to the CD8⁺ DCs, even though both subsets express similar levels of co-stimulatory molecules [59-61]. It has been proposed that CD8α⁺ DCs restrict T-cell proliferation through induction of cell apoptosis [62]. *In vivo* evidence suggests that antigens delivering to CD8α⁺ DCs under steady-state condition can promote peripheral tolerance [50]. Delivery of hen egg lysozyme (HEL) to DCs in the
- 15 steady state conditions induces CD4⁺ T cell unresponsiveness to subsequent systemic challenge of HEL. Depletion of HEL-specific T cells was thought to be a potential tolerance mechanism involved in this model system [63]. Liu *et al.* confirms this finding and further showed that *in vivo* delivery of dying cells to DCs led to “deletional tolerance”. In this model, dying cells were captured and cross-presented to CD8⁺ T cells by the CD8α⁺ DC subset. Following cross-presentation, antigen-reactive CD8⁺ T cells were initially driven into cell cycle but then disappeared
- 20 [64]. Later study by Yamazaki *et al.* demonstrated that CD8α⁺ DCs are more potent than CD8⁺ DCs in inducing Tregs, both *in vitro* and *in vivo*, despite expressing similar levels of costimulatory molecules. It was proposed that Treg generation is a potential mechanism involved in CD8α⁺ DC-mediated tolerance [65]. The “non-activated” state of CD8α⁺ DC seemed to be essential for their immunoregulatory properties. One study suggests that TLR9 ligation can reverse the suppressing function of CD8α⁺ DC and promote T-cell immunity [66].

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Human regulatory DCs

- In humans less is known about regulatory DCs and their *in vivo* functions. Jonuleit *et al.* and others demonstrated that allogeneic T cells co-cultured with immature DCs became unresponsive to antigenic re-stimulation. Moreover, immature DCs induce IL-10 producing regulatory T cells that suppress the function of other effector T cells in a
- 30 partially IL-10 dependent manner [67, 68]. Other work has indicated that pro-inflammatory cytokine-matured DCs can efficiently induce regulatory T cells with immune suppressive function [69, 70]. It appears that DC maturation in the absence of PAMPs stimulation may be insufficient for efficient priming of effector T cells responses [71]. Instead, inappropriately matured or “semi-matured” DCs can lead to the induction of regulatory T cells [72]. For example, DCs matured with TNF upregulate MHC class II and costimulatory molecules but remain weak producers
- 35 of proinflammatory cytokines and induce the generation of IL-10 producing Tregs [73]. The physiological significance of these findings in humans remains to be explored. Thus far, it is not known whether certain subset of DCs in the human system have specialized immunoregulatory function during tissue homeostasis.

Role of DCs in pathology

Dysregulation of DC homeostasis can lead to pathology. One intensively studied example is psoriasis, a common skin disease characterized by excessive growth and aberrant differentiation of keratinocytes, as well as infiltration of mononuclear leukocytes (T cells and DCs) into the epidermis[74]. DCs have been shown to play a key role in the pathogenesis of psoriasis. The first evidence was demonstrated by Nestle *et al.* that DDCs obtained from psoriatic plaque possess a strong capacity to stimulate autologous T cell proliferation, leading to the production of IL-2 and IFN γ , when compared with DDCs obtained from normal healthy individuals[75]. Later studies done by Lowes *et al.* further suggested that a subset of inflammatory DCs that produce TNF and inducible nitric oxide synthase (iNOS) (TIP-DCs) found in the skin lesions of psoriasis are the major inflammatory and effector cells in psoriasis[76]. It is proposed that TIP-DCs are potential producers of IL-23 and IL-20, which may activate T cells and keratinocytes respectively. While effector myeloid DDCs are being characterized in psoriasis, Nestle *et al.* found that PDCs secreting IFN α are involved in the initiation of psoriasis[77]. Interference with IFN α production by PDCs could completely inhibit the activation and expansion of pathogenic T cells as well as the development of the psoriatic phenotype. More recently, Lande *et al.* further demonstrated that an antimicrobial peptide, LL37, overexpressed in psoriatic lesions can break tolerance to self-DNA and induces IFN α production by PDCs[78]. Taken together, these works suggest that DCs are key immune cells contributing to the pathogenesis of psoriasis, in which PDCs are the key initiators of the disease while MDCs infiltrating the upper dermis can present antigen to pathogenic T cells thus triggering the adaptive immune response.

While several studies have focused on the pathogenic role of DCs and T cells during skin inflammation, little is known about human tissue resident regulatory DCs and their functional roles during skin homeostasis.

In mice, CD8⁺ DCs are essential to initiate immunity against viral infection and intracellular pathogens, antigens delivered to CD8⁺ DCs under steady-state condition can lead to the induction of Foxp3⁺ regulatory T cells[65] and the deletion of antigen-specific T cells upon antigen restimulation[64]. CD207⁺CD103⁺ mouse skin derived DCs have also been shown capable of mediating both anti-viral immunity[119] and T cell tolerance, possibly in the context of their cross-presentation capability[120].

However, in humans much less is known about regulatory DCs, but immature DCs may control peripheral tolerance by inducing T cell unresponsiveness to antigenic stimulation or through the induction of IL-10 producing regulatory T (Tr1) cells[67-68]. Several studies have also reported that exposure to anti-inflammatory or immunosuppressive agents can induce a regulatory DC phenotype[121]. Common features of human regulatory DCs include reduced expression of co-stimulatory molecules, in particular CD40, CD80, and CD86; reduced T cell stimulatory capacity; impaired maturation status, and induction of regulatory T cells[97].

In human skin, myeloid DCs (MDCs) that reside in the dermal compartment represent a major subset of dermal DCs during tissue homeostasis[122]. Several subpopulations of dermal DCs have been described under both normal and pathological conditions. Classically, dermal DCs are CD1c⁺. They can be subdivided into CD1a⁺CD14⁻, CD1a⁻CD14⁺ and CD1a⁻CD14⁻ subsets[8]. The functional roles of dermal DC subsets are only partly understood. Zaba *et al.* suggested that CD1a⁺CD14⁻ DCs are potent inducers of allogeneic CD4⁺ and CD8⁺ T-cell proliferation[9]. Others

suggested that CD1a⁺CD14⁺ DCs are less immunogenic but are capable of antigen-uptake with the potential to migrate to the epidermis and differentiate into Langerhans cells in response to TGFβ[13,16].

While inefficient tolerance to self-antigen can lead to autoimmunity and chronic inflammation, inappropriate tolerance induction may increase the risk of tumour development. Progressing tumours are tolerated by the immune system despite the presence of tumour-infiltrating DCs (TIDCs). Early works done by Chaux *et al.* suggested that TIDCs have impaired antigen-presenting ability[79]. Vicari *et al.* further demonstrated that TIDCs have an immature phenotype and are refractory to maturation stimuli. This is however reversible by the combined stimulation with CpG and anti-IL-10 receptor, suggesting that IL-10 produced at the site of tumour may play a potential role in the induction of DC tolerance [80].

Vitamin D₃ metabolism and its biological actions

Vitamin D₃ was first identified as an essential nutrient for calcium homeostasis and bone metabolism. However, a variety of research in the past years has revealed a diverse range of important biological effects of vitamin D₃, in particular its pronounced immunomodulatory properties and role in the growth and differentiation of many cell types [81]. In humans, vitamin D₃ is derived from dietary sources or synthesized in the skin from its precursor provitamin D₃ (7-dehydrocholesterol) by the action of sunlight. Exposure to sunlight, specifically ultraviolet (UV) B radiation (wavelength 270-300 nm), promotes the conversion of 7-dehydrocholesterol to pre-vitamin D₃ followed by immediate heat-dependent isomerization into vitamin D₃. Once produced, vitamin D₃ can be stored in adipose tissues before being metabolized in the liver into 25-hydroxyvitamin D₃ (25(OH)D₃) by cytochrome P-450 enzymes including CYP27A1 and CYP2R1 (25-hydroxylase). 25(OH)D₃ is the major circulating biologically inactive metabolite of vitamin D₃, which is transported to the kidney to be converted into the biologically active form, 1α,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) by 1α-hydroxylase CYP27B1. While this step occurs primarily in kidney, extra-renal production of 1,25(OH)₂D₃ by different cell types has been reported and is thought to serve as an autocrine/paracrine factor with cell-specific functions. Finally, 1,25(OH)₂D₃ negatively regulates its circulating level through decreasing parathyroid hormone secretion, suppressing 1α-hydroxylase activity, and inducing 25-hydroxyvitamin D-24-hydroxylase (24-OHase) that catabolizes 1,25(OH)₂D₃ to its inactive metabolite, calcitric acid, which is then excreted in the bile [82, 83].

1,25(OH)₂D₃ exerts most of its functions via interaction with the vitamin D receptor (VDR), a member of the superfamily of nuclear receptors for steroid hormones, thyroid hormones and retinoic acid. VDR acts as a ligand-activated transcription factor that has an extremely high affinity (~10⁻¹⁰M) to its ligand, 1,25(OH)₂D₃. Binding of the 1,25(OH)₂D₃ to VDR promotes heterodimerization with the retinoid X receptor (RXR), which then binds to specific vitamin D response elements (VDREs) within the promoter region of vitamin D responsive genes, such as calcium-binding protein, recruits VDR-interacting nuclear proteins (co-regulators), and ultimately influences the rate of RNA polymerase II-mediated transcription [84].

VDRs are present not only in cells typically involved in calcium and bone metabolism, they are also found in a variety of tissues, such as parathyroid gland, skin and in the immune system [85]. Many immune cells are found to be positive to VDR, including B cells, CD4 and CD8 T cells, monocytes, macrophages and dendritic cells [86]. The

diversity of VDR expression on immune cells suggests a pleiotropic receptor-mediated responses that vitamin D₃ hormone may exert on the immune system.

Cells of the immune system also possess essential enzymes involved in vitamin D₃ biosynthesis. In addition to its conventional metabolic route through the liver and then kidneys, several immune cells including antigen-presenting cells, B cells, and T cells, as well as epidermal keratinocytes have been shown capable of producing key metabolic enzymes (CYP27A1/CYP2R1 and CYP27B1) involved in vitamin D metabolism, allowing local conversion of vitamin D₃ into its full active form, 1,25(OH)₂D₃ allowing for biological function as autocrine/paracrine to regulate immune responses [87-89].

Immunomodulatory role of 1,25(OH)₂D₃ on dendritic cells

DCs express VDR and are important target immune cells for 1,25(OH)₂D₃ [90]. A number of studies have demonstrated that 1,25(OH)₂D₃ and its analogs have profound immunomodulatory effects on the phenotype and function of DCs [87, 91]. 1,25(OH)₂D₃ treatment interferes with DC differentiation and maturation. 1,25(OH)₂D₃-treated DCs are maintained in an immature state, showing reduced expression of MHC class II molecule, maturation-induced molecules CD83 and CD1a, as well as costimulatory molecules CD40, CD80, and CD86 [91]. T-cell stimulatory capacity of DCs is significantly reduced following 1,25(OH)₂D₃ treatment, which may partially due to the immaturity of DCs induced by 1,25(OH)₂D₃.

1,25(OH)₂D₃ also influences DCs cytokine secretion profiles. 1,25(OH)₂D₃ treatment abrogates IL-12 and strongly enhances IL-10 production. Reduced IL-12 production may interfere with downstream Th1 cell development [91]. Moreover, 1,25(OH)₂D₃ inhibits *in vivo* Th17 generation, partially due to its capacity to inhibit Th17-related cytokine production by DCs [92, 93]. This is supported by studies using a mouse model of colitis, in which 1,25(OH)₂D₃ treatment led to the change of Th1/Th17 to Th2/Treg cell, a profile associated with significant reduction of IL-12, IL-23, and IL-6 production by DCs [94].

DCs treated with 1,25(OH)₂D₃ acquire regulatory capacity towards subsequent T cell responses. In addition to inhibiting Th1/Th17 development through the shifting of cytokine production profile, T cells stimulated by 1,25(OH)₂D₃-treated DCs became unresponsiveness to secondary antigenic restimulation and produce reduced amount of IFNγ [91]. A selective induction of autoreactive T cell apoptosis by 1,25(OH)₂D₃-treated DCs has also been reported [95]. Furthermore, 1,25(OH)₂D₃-treated DCs can promote the development of CD4⁺CD25⁺Foxp3⁺ Tregs that are able to control autoimmunity and transplantation tolerance [96, 97]. In a mouse model of type I diabetes, 1,25(OH)₂D₃ treatment led to increased numbers of Tregs and reduced Th1 cell infiltration into the pancreatic islets [98]. Gorman *et al* also demonstrated enhanced hyporesponsiveness of Treg and their suppressive capacity in mice receiving topical 1,25(OH)₂D₃ treatment or UVB irradiation compared to the untreated controls [99].

When comparing the effects of 1,25(OH)₂D₃ on two major subsets of blood DCs, MDCs and pDCs, Penna *et al*. demonstrated selective modulatory effects of 1,25(OH)₂D₃ on MDCs but not pDCs, despite both subsets express similar levels of VDR and respond equally well to 1,25(OH)₂D₃ ligation by up-regulating primary response genes

[100]. It has been shown that $1,25(\text{OH})_2\text{D}_3$ inhibits Th1-inducing capacity of MDCs but have no inhibitory effects on pDCs. In addition, $1,25(\text{OH})_2\text{D}_3$ treatment enhances the capacity of MDCs but not pDCs to induce Tregs activity. It was proposed that pDCs are intrinsically prone to favour tolerance and lack of immunoregulatory modulation by $1,25(\text{OH})_2\text{D}_3$ would leave their tolerogenic potential unmodified [100].

5

Role of vitamin D₃ in skin immunity

As a molecule primarily derived from the skin, vitamin D₃ is thought to play an important role in skin immunity. Keratinocytes are not only the primary source of vitamin D but also expressing VDR that enables them to respond to $1,25(\text{OH})_2\text{D}_3$. Several $1,25(\text{OH})_2\text{D}_3$ -mediated biological functions in the skin are mediated through its action on
10 keratinocytes. In the steady state, VDR signalling regulate keratinocyte proliferation and differentiation [101]. During wound healing, local increase in $1,25(\text{OH})_2\text{D}_3$ signalling promotes the expression of antimicrobial peptide cathelicidin (LL37), TLR2, and CD14 on keratinocytes. Soluble factors in the wound, such as TGF β , as well as TLR2/6 ligands may promote the production of CYP27B1 in keratinocytes, leading to elevated levels of $1,25(\text{OH})_2\text{D}_3$. It was proposed that vitamin D₃ enhances skin innate immunity during injury to protect against
15 infection [102]. Similar effects of $1,25(\text{OH})_2\text{D}_3$ in promoting innate immunity was also observed during systemic infection. Liu *et al.* demonstrated that TLR2/1 signalling triggers $1,25(\text{OH})_2\text{D}_3$ production by monocytes and macrophages, leading to the induction of LL37 and the killing of intracellular *Mycobacterium tuberculosis* [103, 104].

20 In addition to its role in innate immunity, vitamin D₃ can regulate adaptive immune responses. Sigmundsdottir *et al.* demonstrated that $1,25(\text{OH})_2\text{D}_3$ regulates lymphocyte migration to the skin (mainly epidermis) by promoting the surface expression of CC chemokine receptor 10 (CCR10) on activated/memory T cells. It was proposed that skin DCs can metabolize vitamin D₃ to $1,25(\text{OH})_2\text{D}_3$ and through which they ‘imprint’ T cells with a skin-homing signature [89]. On the contrary, in another study, $1,25(\text{OH})_2\text{D}_3$ was demonstrated as potent inhibitor of CLA
25 expression on T cells. This observation may explain, at least in part, the therapeutic effects of $1,25(\text{OH})_2\text{D}_3$ in the treatment of T cell-mediated inflammatory skin diseases or cutaneous T-cell lymphomas [109]. To reconcile these seemingly opposite effects of $1,25(\text{OH})_2\text{D}_3$ in mediating lymphocytes homing to the skin, it is important to note that $1,25(\text{OH})_2\text{D}_3$ induced CCR10 expression on naïve T cells requires IL-12 that may counteract with the inhibitory effect of $1,25(\text{OH})_2\text{D}_3$. DCs as one of the primary sources of IL-12 may be essential in the CCR10 induction
30 process. Moreover, it is likely that the induction of CCR10 by $1,25(\text{OH})_2\text{D}_3$ takes place after T cells enter the skin via E-selectin binding.

Vitamin D₃ in the treatment of psoriasis

Vitamin D₃ in its active form ($1,25(\text{OH})_2\text{D}_3$) has a widespread clinical application because of its diverse range of
35 biological effects. However, due to its central function in calcium and bone metabolism, the high ability of $1,25(\text{OH})_2\text{D}_3$ to increase serum calcium and phosphate becomes a dose-limiting effect that prevents sustained systemic administration of this compound. To overcome this limitation, chemical modification throughout the $1,25(\text{OH})_2\text{D}_3$ molecule has been performed in order to obtain analogs that retained important therapeutic properties of $1,25(\text{OH})_2\text{D}_3$ but with reduced toxic (i.e. hypercalcemic) side effects [110]. Thousands of different vitamin D
40 analogs have been synthesized worldwide with the aim of achieving enhanced potency, increased tissue specificity,

and reduced calcemic liability. Although vitamin D analogs have been synthesized since the late 1970s, the discovery of the non-classical functions of $1,25(\text{OH})_2\text{D}_3$, in particular its role in immunomodulation, has led to an acceleration in the pace for searching novel vitamin D analogs that may display selective immunoregulatory properties.

5 Vitamin D therapy shows beneficial effects in psoriasis, which is characterized by keratinocytes hyperproliferation and accompanied by large infiltration of inflammatory DCs and T cells.

10 Among many newly developed vitamin D analogs, calcipotriol was the first one to reach the market for the treatment of psoriasis. In Europe, calcipotriol is widely prescribed as a first-line topical treatment for mild-to-moderate form of psoriasis and shows efficacy comparable to topical steroids [111, 112]. Thus far, calcipotriol is thought to be effective in psoriasis because it blocks hyperproliferation and promotes terminal differentiation of keratinocytes. In addition to calcipotriol, other vitamin D analogs have been introduced for treatment of psoriasis, such as tacalcitol and maxacalcitol; both have been shown effective in treatment of psoriasis through their action on 15 keratinocytes proliferation and differentiation [113, 114]. A recent study revealed that calcipotriol treatment selectively inhibits beta-defensin, IL-17A, IL-17F and IL-8, but induces LL-37 in psoriatic lesions [115]. Future advances in the knowledge of $1,25(\text{OH})_2\text{D}_3$ and its immunomodulatory effects on DCs and T cells will bring new insights into the mode of action behind calcipotriol and other vitamin D analog treatments.

20 Despite the higher safety profile of calcipotriol for long-term treatment, it is slower acting and less efficient than other commonly used high-potency topical steroids, such as corticosteroids. This may be due to its reduced capacity of stabilizing VDR activity than the natural hormone [116]. In addition, calcipotriol is ineffective when given orally due to the fact that it is rapidly broken down and transformed into inactive metabolites [85]. However, long-term use of highly effective corticosteroids is associated with the potential for significant side-effects. Therefore, current 25 therapeutic goal is to maximize the short term efficacy of topical agents while minimizing side-effects associated with long-term maintenance therapy.

The use of vitamin D analogs in psoriasis treatment has so far been validated by dermatologists and found well tolerated by patients during long-term treatment. It is hoped that a next generation vitamin D compound that exhibits 30 improved immunomodulatory capacity, particularly in regulation of DCs and T cell functions, high efficacy in topical and systemic usage, and limited side effects will be beneficial to future treatment for psoriasis, as well as other autoimmune disorders.

SUMMARY OF THE INVENTION

35 In accordance with a first aspect of the present invention, there is provided an isolated population of tissue CD141^+ DCs and/or tissue CD141^+ -like DCs for use in the treatment or prophylaxis of an immune-mediated disease in which an immunosuppressive effect is required.

In accordance with a second aspect of the present invention, there is provided the use of an isolated population of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs in the manufacture of a medicament for the treatment or prophylaxis of an immune-mediated disease in which an immunosuppressive effect is required.

- 5 In accordance with a third aspect of the present invention, there is provided a pharmaceutical composition comprising the isolated population of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs of the present invention in combination with one or more pharmaceutically acceptable excipients.

- 10 In accordance with a fourth aspect of the present invention, there is provided a vaccine comprising the isolated population of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs of the present invention.

- 15 In accordance with a fifth aspect of the present invention, there is provided a method of treating or preventing an immune-mediated disease in which an immunosuppressive effect is required, the method comprising administering an isolated population of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs to a subject.

- 20 In accordance with a sixth aspect of the present invention, there is provided a method of treating or preventing an immune-mediated disease in which an immunosuppressive effect is required, the method comprising administering an antigen involved in the development of the immune-mediated disease to tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs.

- 25 In accordance with a seventh aspect of the present invention, there is provided a method of treating or preventing a disease or condition in which an immunostimulatory effect is required, the method comprising depleting tissue CD141⁺ DCs in a subject.

- 30 In accordance with an eighth aspect of the present invention, there is provided a method of predicting or diagnosing a disease or condition associated with an abnormal immune response comprising the steps of determining the level and/or immune activation state of tissue CD141⁺ DCs in a biological sample obtained from a subject, and comparing the determined level and/or immune activation state of tissue CD141⁺ DCs in the subject with a control level and/or control immune activation state of tissue CD141⁺ DCs.

- 35 In accordance with a ninth aspect of the present invention, there is provided a method of monitoring the progression of a disease or a condition associated with an abnormal immune response comprising the steps of determining the level and/or immune activation state of tissue CD141⁺ DCs in a biological sample obtained from a subject at a first time point, determining the level and/or immune activation state of tissue CD141⁺ DCs in a biological sample obtained from a subject at a second time point, and comparing the determined levels and/or immune activation states of tissue CD141⁺ DCs in the subject at the first and second time points.

- 40 In accordance with the tenth aspect of the present invention, there is provided a method of monitoring the efficacy of an immune therapy involving vitamin D or an analogue thereof, the method comprising the steps of determining the level and/or immune activation state of tissue CD141⁺ DCs in a biological sample obtained from a subject before the

therapy, determining the level and/or immune activation state of tissue CD141⁺ DCs in a biological sample obtained from a subject after the therapy, and comparing the determined levels and/or immune activation states of tissue CD141⁺ DCs in the subject before and after the therapy, wherein an increased level and/or immune activation state of tissue CD141⁺ DCs after the therapy is a positive indication of the efficacy of the therapy.

5 In accordance with an eleventh aspect of the present invention, there is provided an *in vitro* method of obtaining tissue CD141⁺-like DCs by treating antigen-presenting cells (APCs) with vitamin D or an analogue thereof and/or means of inducing vitamin D or an analogue thereof.

10 Further aspects and particular embodiments of the invention will now be described in greater detail.

DETAILED DESCRIPTION OF THE INVENTION

15 In accordance with a first aspect of the present invention, there is provided an isolated population of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs for use in the treatment or prophylaxis of an immune-mediated disease in which an immunosuppressive effect is required.

20 The inventors have found that skin tissue-resident CD141⁺ DCs represent the major IL-10 producing DC subset, cross-present self antigens in the absence of inflammatory stimuli and promote antigen specific T cell unresponsiveness. Accordingly, tissue CD141⁺ DCs have an immunosuppressive effect which enhances one's resistance against the production of an inflammatory immune response such that it is less likely for such a response to be elicited. Therefore, tissue CD141⁺ DCs can be used to treat immune-mediated diseases in which an immunosuppressive effect is required.

25 The inventors have also found that when blood CD14⁺ DCs and monocyte-derived DCs are cultured in the presence of vitamin D, in particular, 1 α ,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), a distinct tissue CD141⁺-like DC population co-expressing CD14 and a high level of CD141 is induced. These inducible tissue CD141⁺-like DCs share similar regulatory features with tissue CD141⁺ DCs, e.g., they down-regulate expression of HLA-DR, fail to mature when exposed to pro-inflammatory cytokines, produce high levels of IL-10 and show poor allostimulatory capacity. Accordingly, tissue CD141⁺-like DCs have an immunosuppressive effect which enhances one's resistance against the production of an inflammatory immune response such that it is less likely for such a response to be elicited. Therefore, tissue CD141⁺-like DCs can also be used to treat immune-mediated diseases in which an immunosuppressive effect is required.

30 The term "an isolated population of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs" refers to a cell population which is removed from its natural environment and is enriched with CD141⁺ DCs and/or CD141⁺-like DCs. Such a cell population comprises at least 50% tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs, for example, at least 60%, 70% or 80% CD141⁺ DCs and/or CD141⁺-like DCs. Preferably, the cell population comprises at least 85%, 90% or 95% CD141⁺ DCs and/or CD141⁺-like DCs. More preferably, the cell population comprises at least 96%, 97%, 98% or 99% CD141⁺ DCs and/or CD141⁺-like DCs. Although it is preferable to have a cell population solely

consisting of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs, the cell population may also comprise other cell types.

Tissue CD141⁺ DCs (also known as tissue resident CD141⁺ DCs) are those dendritic cells which are normally found in the tissues of a subject. For example, tissue CD141⁺ DCs may be found in the skin and the inner lining of the nose, lungs, stomach and intestines. An isolated population of tissue CD141⁺ DCs can be obtained by purifying tissue CD141⁺ DCs from other cells using markers specific for tissue CD141⁺ DCs, such as CD141. Methods of obtaining a particular type of cells are well known to those skilled in the art. For example, fluorescence-activated cell sorting (FACS) can be used to isolate CD141⁺ DCs.

Tissue CD141⁺-like DCs are dendritic cells which express CD141⁺ and function in a similar manner to tissue CD141⁺ DCs, for example, they have key functional and phenotypic properties in common with tissue CD141⁺ DCs. An isolated population of tissue CD141⁺-like DCs can be obtained by culturing antigen-presenting cells (APCs), such as blood CD1c⁺ DCs, and monocyte-derived DCs in the presence of vitamin D or analogues thereof.

The properties that are generally displayed by tissue CD141⁺ DCs and tissue CD141⁺-like DCs are as follows:

- 1) Surface expression of CD14;
- 2) Surface expression of CD141;
- 3) Surface expression of macrophage mannose receptor (MMR);
- 4) Surface expression of ILT3;
- 5) Surface expression of CD1c at low levels;
- 6) A stable CD83^{low} immature phenotype after exposure to a potent DC maturation cocktail;
- 7) Production of high amounts of IL-10 after CD40 cross-linking;
- 8) Weak stimulators of CD4⁺ T cell proliferation;
- 9) Cross-presentation ability; and/or
- 10) Suppression of allo/xeno-immunity *in vivo*.

Accordingly, the tissue CD141⁺ DCs of the invention may have one or more of the above properties. Preferably, the tissue CD141⁺ DCs of the invention have 2, 3, 4, 5, 6, 7, 8, 9 or all of the above properties.

Further, the tissue CD141⁺-like DCs of the invention may have one or more of the above properties. Preferably, the tissue CD141⁺ DCs of the invention have 2, 3, 4, 5, 6, 7, 8, 9 or all of the above properties.

The tissue CD141⁺ DCs and the tissue CD141⁺-like DCs of the invention are distinct from blood CD141⁺ DCs and have different properties which differentiate these two groups of DCs. Some of the key differences between the tissue CD141⁺ DCs and the tissue CD141⁺-like DCs relative to blood CD141⁺ DCs are as follows:

- 1) Blood CD141⁺ DCs do not express CD14 and CD1c;
- 2) Blood CD141⁺ DCs produce high amount of IL-12 and polarize Th1 cells; and
- 3) Blood CD141⁺ DCs do not express MMR or ILT3.

In view of this, tissue CD141⁺ DCs and tissue CD141⁺-like DCs generally express one or more of CD14, CD1c, MMR and ILT3.

Further differences between blood CD141⁺ DCs and tissue CD141⁺ DCs are shown in the table below:

5

Molecular markers and functional features	Mouse		Human blood		Human skin	
	CD8a+	CD8a-	CD141	CD1c	CD141	CD1c
BATF3	+	+	+	+	+	+
XCR1	+	-	+	-	+	+/-
Nec12	+	-	+	-	+	+/-
CLEC9A	+	-	+	-	+	-
CD1c	ND	ND	-	+	+/low	+
CD11b	low	++	-	+	+	+
CD11c	+	+	+	+	+	+
CD14	-	-	-	-	+	-
CD103	+/-	-	-	-	-	-
DEC205 (CD205)	++	+/-	+	+/-	-	-
MMR (CD206)	+/-	-	ND	ND	++	-
Langerin (CD207)	+/-	-	+	-	-	+/-
TLR3	+	-	+	+/low	+	+
TLR4	+	+	-	+	+	+
TLR7	-	+	-	+	+	+/low
TLR9	+	+/-	-	-	+	+
IL-12 production (following CD40 cross-linking)	++	-	+	-	-	-
IL-10 production (following CD40 cross-linking)	ND	ND	ND	ND	++	-
Th1 polarization	++	+/-	++	+/-	ND	ND
Treg induction	++	+/-	ND	+/-	++	+/-
Cross-presentation (steady-state)	+	-	-/low	-/low	+	-
Cross-presentation (with exogenous stimuli)	++	-	++	+	+	-
CD4 ⁺ T cell stimulatory capacity	low	++	+	+	low	++

(ND = not determined)

As can be seen above, in particular, from the bottom five properties, tissue CD141⁺ DCs and blood CD141⁺ DCs have different functionality and cannot be considered to be the same or similar to each other.

10

In various embodiments of the invention, the tissue CD141⁺ DCs and tissue CD141⁺-like DCs may express CD14. They may express CD1c at a low level. They may not express DEC205 (CD205). They may express MMR. They may not express Langerin (CD207). They may express TLR4. They may express TLR7. They may express TLR9.

15 Further, the tissue CD141⁺ DCs and tissue CD141⁺-like DCs may induce regulatory T cells. In particular, the tissue CD141⁺ DCs and tissue CD141⁺-like DCs may induce immunosuppressive capabilities in the regulatory T cells such as CD4⁺CD25^{hi} T regulatory cells.

20 “An immune-mediated disease in which an immunosuppressive effect is required” refers to a disease or condition characterised by abnormal immune response and the treatment of such a disease or condition improves one’s tolerance against the immune response. An immune-mediated disease in which an immunosuppressive effect is

required may refer to either tissue or chronic inflammatory diseases. It includes, but is not limited to, hypersensitivities, autoimmune diseases, autoinflammatory diseases, transplant rejections, asthma, chronic prostatitis, glomerulonephritis, inflammatory skin diseases, inflammatory bowel diseases, inflammatory joint diseases, inflammatory lung diseases, inflammatory brain diseases, pelvic inflammatory diseases, systemic inflammatory diseases, thyroiditis, sarcoidosis, vasculitis and interstitial cystitis.

Tissue CD141⁺ DCs and tissue CD141⁺-like DCs can be identified by the presence of cell markers, such as CD141, on the cell surface. Methods for determining the presence of a particular cell marker are well known to those skilled in the art. For example, chromatography, hybridisation (DNA or protein) and mass spectrometry can be used.

In some embodiments, the isolated population of CD141⁺ DCs coexpress at least one of the markers selected from a group consisting CD14, CD80, CD86, CD11c, macrophage mannose receptor (MMR), toll-like receptor 3 (TLR3), TLR4, TLR7, TLR9, immunoglobulin-like transcript 3 (ILT3), CLEC9A, BATF3, XCR1, Necl2, Factor XIIIa and CD163. Preferably, the isolated population of CD141⁺ DCs coexpress at least one of CD14, MMR, ILT3, CLEC9A, BATF3, XCR1, and Necl2. These cell markers can be also identified by standard methods used in the art.

The invention relates to an isolated population of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs. Preferably, the tissue CD141⁺ DCs are dermal CD141⁺ DCs obtained from skin tissues. Alternatively or additionally, the isolated population of CD141⁺ DCs comprises tissue CD141⁺-like DCs obtained by treating APCs with vitamin D or an analogue thereof or a combination thereof, and/or means which induce vitamin D or an analogue thereof.

The APCs may be obtained from peripheral blood or derived from monocytes.

“Monocyte” is a term well known to those skilled in the art. It refers to a type of white blood cells with a single nucleus. They can differentiate into macrophages and DCs.

In particular embodiments, antigen-presenting cells obtained from peripheral blood may be blood CD11c⁺ DCs.

Vitamin D used in the present invention encompasses all forms of vitamin D, i.e., vitamin D1, D2, D3, D4 and D5. Preferably, it refers to vitamin D2 or D3.

Analogues of vitamin D are well known to those skilled in the art. They may be different metabolic forms of vitamin D or synthetic compounds that perform the same function as vitamin D. For example, analogues of vitamin D3 include, but are not limited to, 7-dehydrocholesterol (provitamin D3), 25-hydroxyvitamin D3 (25(OH)D3), 1 α ,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), calcipotriol, tacalcitol and maxacalcitol.

In certain embodiments, the tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs may have been cultured in the presence of an antigen involved in the development of the immune-mediated disease. Accordingly, the antigen may be engulfed and processed by the tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs which will display peptides of the antigen on the cell surface. When these antigen-loaded tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs are

administered to a subject, the tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs will present the antigen to the T cells in the body of the subject. Alternatively, the tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs can be administered in combination with the antigen. In this way, the processing and presentation of the antigen by the tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs will take place after the administration. Using either of these approaches (i.e., a combination of CD141⁺ (-like) DCs and antigens or antigen-loaded CD141⁺ (-like) DCs) may enhance antigen specificity in autoimmune therapy.

When the immune-mediated disease in which an immunosuppressive effect is required refers to an autoimmune disease, the antigen may be an autoantigen which is a self antigen that is capable of eliciting an immune response, for example, through stimulating the production of autoantibodies. Autoantigens involved in the development of various autoimmune diseases are well known to those skilled in the art. For example, if the autoimmune disease is Type 1 Diabetes Mellitus, the autoantigen may be preproinsulin (PPI).

In some embodiments, the immune-mediated disease may be an inflammatory skin disease, such as psoriasis.

In some embodiments, the immune-mediated disease may be transplantation rejections.

The cells of the present invention are suitable for administration by any means known in the art. They can be delivered systemically or locally. Systemic administration can be by intravenous injection, intraarterial injection, intranodal injection, perfusion or infusion. Local administration can be by catheter, intradermal injection, subcutaneous injection, or intralesional injection. The beneficial effects which are observed upon administration of the cells may be due to the cells *per se*, or due to products which are produced by the cells, such as IL-10.

An effective dose of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs will typically be between 1×10^5 and 100×10^6 , preferably between 1×10^6 and 50×10^6 . Depending on the nature of the disease, more or fewer cells can be used. On the basis of body weight of the recipient, an effective dose may be between 1×10^6 and 10×10^6 per kg of body weight, preferably between 1×10^6 and 5×10^6 cells per kg of body weight. Patient age, general condition, and immunological status may be used as factors in determining the dose administered.

In accordance with a second aspect of the present invention, there is provided the use of an isolated population of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs in the manufacture of a medicament for the treatment or prophylaxis of an immune-mediated disease in which an immunosuppressive effect is required.

In accordance with a third aspect of the present invention, there is provided a pharmaceutical composition comprising the isolated population of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs of the present invention in combination with one or more pharmaceutically acceptable excipients.

The pharmaceutical composition of the present invention may also comprise one or more additional therapeutic agents, such as immunosuppressive cytokines consisting of IL-10 and/or immunosuppressive agents consisting of cyclosporine A, rapamycin, anti-TNF, TNF-receptor Fc fusion protein, anti-IL-12/23p40, and JAK kinase inhibitor.

In accordance with a fourth aspect of the present invention, there is provided a vaccine comprising the isolated population of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs of the present invention.

5 Since tissue CD141⁺ DCs and tissue CD141⁺-like DCs have been shown to possess immunosuppressive effects, the vaccine of the present invention may be used to reduce excessive or undesirable immune responses of a subject, thereby improving the efficacy of therapy in immune-mediated diseases, in particular, autoimmune diseases, autoinflammatory diseases, hypersensitivities and transplantation rejection.

10 In accordance with a fifth aspect of the present invention, there is provided a method of treating or preventing an immune-mediated disease in which an immunosuppressive effect is required, the method comprising administering an isolated population of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs to a subject.

15 Since tissue CD141⁺ DCs and tissue CD141⁺-like DCs have been shown to possess immunosuppressive effects, administration of these cells may reduce excessive immune responses of a subject. Therefore, tissue CD141⁺ DCs and tissue CD141⁺-like DCs may be used to treat immune-mediated diseases.

In particular embodiments, the subject is a mammal. Preferably, the subject is human.

20 The terms “an isolated population of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs” and “an immune-mediated disease in which an immunosuppressive effect” for the second to the fifth aspects of the present invention are as defined according to the first aspect.

25 The method according to the fifth aspect may use an administration route and an effective dosage of the isolated population of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs mentioned for the first aspect. It may also comprise administering one or more additional therapeutic agents, such as immunosuppressive cytokines consisting of IL-10 and/or immunosuppressive agents consisting of cyclosporine A, rapamycin, anti-TNF, TNF-receptor Fc fusion protein, anti-IL-12/23p40, and JAK kinase inhibitor.

30 In accordance with a sixth aspect of the present invention, there is provided a method of treating or preventing an immune-mediated disease in which an immunosuppressive effect is required, the method comprising administering an antigen involved in the development of the immune-mediated disease to tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs.

35 The antigen may be delivered by pulsing isolated tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs with an antigen *ex vivo*, by co-delivery of an antigen together with tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs, or by targeting an antigen to tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs *in vivo*. Targeting an antigen to tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs *in vivo* may be achieved by, for example, conjugating the antigen to a molecule, such as an antibody, which binds to a cell surface molecule on tissue CD141⁺ DCs and/or tissue CD141⁺-
40 like DCs.

Having processed the antigen, the tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs display peptides of the antigen on the cell surface and present them to T cells. Since tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs have been shown to promote antigen specific T cell unresponsiveness, the presentation of the antigen by the tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs may enhance immunosuppression against the antigen. Therefore, the present invention may be used to treat or prevent an immune-mediated disease associated with the particular antigen.

The method according to the sixth aspect of the present invention may also comprise administering one or more additional therapeutic agents, such as immunosuppressive cytokines consisting of IL-10 and/or immunosuppressive agents consisting of cyclosporine A, rapamycin, anti-TNF, TNF-receptor Fc fusion protein, anti-IL-12/23p40, and JAK kinase inhibitor.

In accordance with a seventh aspect of the present invention, there is provided a method of treating or preventing a disease or condition in which an immunostimulatory effect is required, the method comprising depleting tissue CD141⁺ DCs in a subject.

Since it was found that tissue CD141⁺ DCs and inducible tissue CD141⁺-like DCs have immunosuppressive effects, depleting tissue CD141⁺ DCs in a subject may block immunosuppression and enhance immune response, and therefore be used to treat or prevent diseases or conditions in which an immunostimulatory effect is required.

Depletion of tissue CD141⁺ DCs refers to reducing or abolishing the effect of tissue CD141⁺ DCs in the body. This can be achieved by killing the tissue CD141⁺ DCs, blocking or modulating the cells so that they have little, or at least weakened, immunosuppressive effects in the body. Methods for depleting tissue CD141⁺ DCs are well known to those skilled in the art. They include, but are not limited to, the use of radiation, cytotoxic compounds, antibodies, and RNA interference.

In some embodiments, the method comprises a step of administering an agent selected from antibodies, interfering RNA (such as siRNA) and cytotoxic compounds, and/or delivering radiation to deplete tissue CD141⁺ DCs in a subject.

Suitable antibodies to be used in the present invention are for example cytotoxic antibodies which results in cell lysis, and blocking antibodies which specifically bind to the tissue CD141⁺ DCs, for example through CD141, CD14, CD80, CD86, CD11c, MMR, TLR3, TLR4, TLR 7, TLR9, ILT3, CLEC9A, BATF3, XCR1, Necl2, Factor XIIIa and CD163, to eliminate, or at least reduce, the immunosuppressive effects of the cells.

Similarly, siRNA for any of the above cell surface markers may be used to eliminate, or at least reduce, the immunosuppressive effects of the cells.

A disease or condition in which an immunostimulatory effect is required is clear to those skilled in the art. It refers to a disease or condition whose treatment requires the body to elicit an immune response or a greater immune

response. It includes, but is not limited to, cancer and chronic infection. Depleting tissue CD141⁺ DCs may be particularly useful in enhancing the efficacy of cancer vaccines.

5 The method according to the seventh aspect of the present invention may also comprise administering one or more additional therapeutic agents, such as immunostimulative cytokines, immunostimulative agents and/or cytotoxic agents.

10 In accordance with an eighth aspect of the present invention, there is provided a method of predicting or diagnosing a disease or condition associated with an abnormal immune response comprising the steps of determining the level and/or immune activation state of tissue CD141⁺ DCs in a biological sample obtained from a subject, and comparing the determined level and/or immune activation state of tissue CD141⁺ DCs in the subject with a control level and/or a control immune activation state of tissue CD141⁺ DCs.

15 Since it was found that tissue CD141⁺ DCs have immunoregulatory effects, the level or immune activation state of tissue CD141⁺ DCs can be used as an indication or biomarker of the immunosuppression of a subject, thereby predicting or diagnosing a disease or condition associated with an abnormal immune response.

20 The term “a disease or condition associated with an abnormal immune response” is clear to those skilled in the art. It may refer to an immune-mediated disease in which an immunosuppressive effect is required, as defined in the first aspect of the present invention. In particular, it refers to autoimmune diseases, hypersensitivities or autoinflammatory diseases. In these cases, a lower level of tissue CD141⁺ DCs in the subject than the control level and/or a lower immune activation state of tissue CD141⁺ DCs in the subject than the control immune activation state is a positive indication of the immune-mediated disease or the likelihood of developing such a disease.

25 Alternatively, the term “a disease or condition associated with an abnormal immune response” may refer to a disease or condition in which an immunostimulatory effect is required, as defined in the seventh aspect of the present invention. In particular, it refers to cancer or chronic inflammation. In these cases, a higher level of tissue CD141⁺ DCs in the subject than the control level and/or a higher immune activation state of tissue CD141⁺ DCs in the subject than the control immune activation state is a positive indication of the disease or condition or the likelihood of developing such a disease.

30 In some embodiments, the method further comprises the steps of determining the level of immune-stimulatory cells in the biological sample obtained from the subject, calculating the ratio of tissue CD141⁺ DCs and immune cells, and comparing the calculated tissue CD141⁺ DCs/ immune-stimulatory cells ratio of the subject with a control ratio.

35 In some embodiments, the method further comprises the steps of determining the level of additional immune-regulatory cells in the biological sample obtained from the subject, and comparing the determined level of the additional immune-regulatory cells of the subject with a control level of the additional immune-regulatory cells.

When referring to an immune-mediated disease in which an immunosuppressive effect is required, a lower level of the tissue CD141⁺ DCs/ immune-stimulatory cells ratio in the subject than the control ratio and/or a lower level of the additional immune-regulatory cells in the subject than the control level is a positive indication of the immune-mediated disease or the likelihood of developing such a disease.

5 When referring to a disease or condition in which an immunostimulatory effect is required, a higher level of the tissue CD141⁺ DCs/ immune-stimulatory cells ratio in the subject than the control ratio and/or a higher level of the additional immune-regulatory cells in the subject than the control level is a positive indication of the disease or condition or the likelihood of developing such a disease.

10 In accordance with a ninth aspect of the present invention, there is provided a method of monitoring the progression of a disease or a condition associated with an abnormal immune response comprising the steps of determining the level and/or immune activation state of tissue CD141⁺ DCs in a biological sample obtained from a subject at a first time point, determining the level and/or immune activation state of tissue CD141⁺ DCs in a biological sample
15 obtained from a subject at a second time point, and comparing the determined levels and/or immune activation states of tissue CD141⁺ DCs in the subject at the first and second time points.

The second time point is after the first time point and the two time points are sufficiently spaced to allow the status of the disease/condition to change in such a manner so as to allow progression of the disease or condition to be
20 monitored.

Since it was found that tissue CD141⁺ DCs have immunoregulatory effects, the level and/or immune activation state of tissue CD141⁺ DCs can be used as an indication of the immunosuppressive status of a subject, thereby monitoring the progression of a disease or a condition associated with an abnormal immune response.

25 When the term “a disease or condition associated with an abnormal immune response” refers to an immune-mediated disease in which an immunosuppressive effect is required, a decreased level and/or a decreased immune activation state of tissue CD141⁺ DCs is a positive indication of the progression of the immune-mediated disease.

30 A decreased level and/or a decreased immune activation state of tissue CD141⁺ DCs means that the level and/or immune activation state of tissue CD141⁺ DCs obtained at the second time point is lower than that obtained at the first time point.

35 When the term “a disease or condition associated with an abnormal immune response” refers to a disease or condition in which an immunostimulatory effect is required, an increased level and/or an increased immune activation state of tissue CD141⁺ DCs is a positive indication of the progression of the disease or condition.

An increased level and/or an increased immune activation state of tissue CD141⁺ DCs means that the level and/or immune activation state of tissue CD141⁺ DCs obtained at the second time point is higher than that obtained at the
40 first time point.

In some embodiments, the method further comprises the steps of determining the level of immune-stimulatory cells in the biological sample obtained from the subject at the first time point, determining the level of immune-stimulatory cells in the biological sample obtained from the subject at the second time point, calculating the ratios of tissue CD141⁺ DCs and the immune-stimulatory cells at the first and second time points, and comparing the calculated tissue CD141⁺ DCs/ immune-stimulatory cells ratios of the subject at the first and second time points.

In some embodiments, the method further comprises the steps of determining the level of additional immune-regulatory cells in the biological sample obtained from the subject at the first time point, determining the level of additional immune-regulatory cells in the biological sample obtained from the subject at the second time point, and comparing the determined levels of the additional immune-regulatory cells of the subject at the first and second time points.

When referring to an immune-mediated disease in which an immunosuppressive effect is required, a decreased tissue CD141⁺ DCs/ immune-stimulatory cells ratio and/or an increased level of the additional immune-regulatory cells in the subject is a positive indication of the progression of the immune-mediated disease.

When referring to a disease or condition in which an immunostimulatory effect is required, an increased tissue CD141⁺ DCs/ immune-stimulatory cells ratio and/or a decreased level of the additional immune-regulatory cells in the subject is a positive indication of the progression of the disease or condition.

A decreased ratio means that the tissue CD141⁺ DCs/ immune-stimulatory cells ratio obtained at the second time point is lower than that obtained at the first time point, and vice versa.

An increased level of the additional immune-regulatory cells means that the level of the additional immune-regulatory cells obtained at the second time point is higher than that obtained at the first time point, and vice versa.

In accordance with the eighth and ninth aspects of the present invention, tissue CD141⁺ DCs in the biological sample can be identified using methods for the identification of CD141⁺ DCs mentioned in the first aspect of the present invention. Levels of the tissue CD141⁺ DCs can then be determined using any method well known to those skilled in the art.

The “immune activation state” of tissue CD141⁺ DCs refers to the ability of the CD141⁺ DCs to produce an immunosuppressive effect. It can be determined, for example, by measuring the level of cytokines, such as IL-10, secreted by the cells, and the ability of CD141⁺ DCs to induce T cell unresponsiveness or generation/expansion of regulatory T cells. An increased immune activation state of CD141⁺ DCs means that the CD141⁺ DCs have an enhanced immunosuppressive effect, and vice versa.

A “control level or a control immune activation state of tissue CD141⁺ DCs” refers to the level or the immune activation state of tissue CD141⁺ DCs in a biological sample obtained from a healthy subject who does not suffer

from a disease or condition associated with an abnormal immune response or from an unaffected tissue of a subject who has such a disease/condition.

“Immune-stimulatory cells” refer to immune cells which promote an immune response. They include, but are not limited to effector T cells, such as CD3⁺ effector T cells.

A “control ratio” refers to a tissue CD141⁺ DCs/ immune-stimulatory cells ratio determined from a biological sample of a healthy subject who does not suffer from an immune-mediated disease/condition or from a symptomless tissue of a subject who has such a disease/condition.

“Additional immune-regulatory cells” refer to immune cells, other than CD141⁺ DCs, that have an immunosuppressive effect. They include, but are not limited to regulatory T cells.

A “control level of the additional immune-regulatory cells” refers to the level of the additional immune-regulatory cells in a biological sample obtained from a healthy subject who does not suffer from a disease or condition associated with an abnormal immune response or from an unaffected tissue of a subject who has such a disease/condition.

A “biological sample” refers to any tissue sample containing tissue CD141⁺ DCs, and optionally other immune cells, such as CD3⁺ T cells. It includes, but is not limited to, skin samples.

In accordance with the tenth aspect of the present invention, there is provided a method of monitoring the efficacy of an immune therapy involving vitamin D or an analogue thereof, the method comprising the steps of determining the level and/or immune activation state of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs in a biological sample obtained from a subject before the therapy, determining the level and/or immune activation state of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs in a biological sample obtained from a subject after the therapy, and comparing the determined levels and/or immune activation states of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs in the subject before and after the therapy, wherein an increased level and/or immune activation state of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs after the therapy is a positive indication of the efficacy of the therapy.

Since it was found that vitamin D can induce blood CD1c⁺ DCs and monocyte-derived DCs to acquire tissue CD141⁺ DC-like phenotype and function, the level and immune activation status of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs in a subject may be used as an indication to monitor the efficacy of an immune therapy involving vitamin D or an analogue thereof.

An increased level or immune activation state of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs after the therapy means that the level of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs obtained after the therapy is higher than that obtained before the therapy.

CD141⁺ DCs can be identified using methods mentioned in the first aspect of the present invention and the level of CD141⁺ DCs can be determined using methods well known to those skilled in the art.

The “immune therapy involving vitamin D or an analogue thereof” refers to any immune therapy in which vitamin D or an analogue thereof may have a positive therapeutic effect on the disease in question. It may refer to an immune therapy involving the use of vitamin D or an analogue thereof. Alternatively, it may refer to an immune therapy, such as the use of UVB irradiation, in which vitamin D or an analogue thereof is induced during the course of the therapy.

The term “immune activation state” is as defined in the eighth and ninth aspects of the present invention.

The biological sample used for the tenth aspect of the present invention is preferably a skin sample.

Vitamin D or an analogue thereof is as defined in the first aspect of the present invention.

In some embodiments, the method further comprises the steps of determining the level of immune-stimulatory cells in the biological sample obtained from the subject before the therapy, determining the level of immune-stimulatory cells in the biological sample obtained from the subject after the therapy, calculating the ratios of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs, and immune-stimulatory cells before and after the therapy, and comparing the calculated tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs/ immune-stimulatory cells ratios of the subject before and after the therapy, wherein an increased level of the tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs/ immune-stimulatory cells ratio after the therapy is a positive indication of the efficacy of the therapy.

In some embodiments, the method further comprises the steps of determining the level of additional immune-regulatory cells in the biological sample obtained from the subject before the therapy, determining the level of additional immune-regulatory cells in the biological sample obtained from the subject after the therapy, and comparing the determined levels of the additional immune-regulatory cells of the subject at the first and second time points.

An increased tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs/ immune-stimulatory cells ratio after the therapy means that the tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs/ immune-stimulatory cells ratio obtained after the therapy is higher than that obtained before the therapy.

A decreased level of the additional immune-regulatory cells means that the level obtained after the therapy is lower than that obtained before the therapy.

When referring to a level/ratio being “higher” or “lower” than another in accordance with the eighth to tenth aspects of the present invention, the differences between the levels/ratios are statistically significant.

In accordance with an eleventh aspect of the present invention, there is provided an *in vitro* method of obtaining tissue CD141⁺-like DCs by treating APCs with vitamin D and/or an analogue thereof.

In accordance with a twelfth aspect of the present invention, there is provided an *in vitro* method of obtaining tissue CD141⁺-like DCs by treating a tissue sample with means which induce vitamin D or an analogue thereof.

Means which induce vitamin D or an analogue thereof may be the use of UVB irradiation that results in the generation of vitamin D or an analogue thereof, which can in turn induce APCs into tissue CD141⁺-like DCs. Preferably, the tissue sample is a skin tissue sample.

In accordance with the eleventh and twelfth aspects of the present invention, the terms “APCs” and “vitamin D and/or an analogue thereof” have the same meanings as defined in the first aspect of the present invention.

The present invention is now described by way of example only with reference to the following figures.

Figure 1 shows the characterization of tissue CD141⁺ DCs residing in healthy human dermis. (a,b) Flow cytometry analysis showed a distinct and significant population of CD141⁺ DCs co-expressing CD1c at low levels, CD11c, CD14, and was negative for CD1a. (c) Expression of CD83, costimulatory molecules CD80 and CD86, MHC class I and class II molecules, and MMR by dermal CD141⁺ DCs indicated an immature phenotype comparing to dermal CD1c⁺ DCs. (d) CD141⁺ cells located *in situ* in the upper dermis identified by immunofluorescence staining of normal human skin cryosections for CD141. Scale bar, 50 μm. (e) Dendritic morphology of dermal CD141⁺ DCs was highlighted following F-actin staining on flow cytometry sorted dermal CD141⁺ DCs. Scale bar, 5 μm. (d, e) DAPI was used for nuclear staining. All results are representative of six to eight independent experiments from different skin donors.

Figure 2 shows the extended phenotypic analysis of dermal CD141⁺ DCs. (a) Dermal cells obtained from healthy human dermis were stained for CD45, CD1c and CD141. Cell populations shown in the forward scatter (FSC) and side scatter (SSC) plot includes (1) lymphocytes, (2) DCs / monocytes, (3) and dead / dying cells. Phenotypic analysis of CD1c⁺ and CD141⁺ dermal cells was conducted by successive gating on the DCs / monocytes population (2) and CD45. (b) CD141 co-stained with several myeloid markers revealed that CD141⁺ DCs expressed Factor XIIIa and CD163, but were negative for CD103, DC-SIGN, DEC205, and langerin. All results are representative of four to six independent experiments from different skin donors.

Figure 3 shows that dermal CD141⁺ DCs express skin and lymph node homing receptors and migrate to the draining lymph nodes *in vivo*. (a) *Ex vivo* isolated dermal CD141⁺ DCs expressed CC chemokine receptors CCR7 and CCR6. (b) To identify skin migratory cells, healthy human skin was transplanted onto Rag2^{-/-}γc^{-/-} mice. Cell suspension prepared from spleen and lymph nodes of mice 8-12 weeks post-transplantation (Tx) were co-stained with anti-mouse and anti-human CD45. Percentage of human CD45⁺ and mouse CD45⁺ cells is shown. (c) Immunofluorescence staining for CD141 and CD3 indicates that human CD141⁺ cells localized closely with CD3⁺ cells in lymph nodes of Tx mice. DAPI was used for nuclear staining. Scale bar, 100 μm. (d) mRNA expression for

human CD141, langerin, cyclophilin, and mouse GAPDH in spleen and lymph nodes of Tx mice was analyzed by qPCR and visualized on an agarose gel followed by ethidium bromide staining. Spleen and lymph nodes of mice received no skin transplants (No Tx) was used as negative control. All results are representative of two independent experiments performed with two different skin donors.

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Figure 4 shows that dermal CD141⁺ DCs express ILT3, secrete IL-10, and induce T cell unresponsiveness. Dermal CD1c⁺ and CD141⁺ DCs were isolated from dermal cells by flow cytometry sorting. (a) Flow cytometric and qPCR analysis showed higher ILT3 expression by dermal CD141⁺ DCs comparing to dermal CD1c⁺ DCs. Relative ILT3 mRNA expression was normalized to the amount of human cyclophilin. (b) IL-10 production by dermal CD1c⁺ and CD141⁺ DCs was measured by Multicytokine beads analysis (mean $\pm$ SEM) of supernatant harvested from DCs cultured in the absence or presence of CD40 ligand-transfected L cells (CD40L Tx) for 2 days. High levels of IL-10 secretion by dermal CD141⁺ DCs were observed both with and without CD40 cross-linking. (c) fixed numbers of allogeneic CD4⁺ T cells (5×10^4 cells) were co-cultured with titrated numbers of dermal CD1c⁺ or CD141⁺ DCs. Alloreactive CD4⁺ T cell proliferation is determined by thymidine incorporation (mean $\pm$ SEM) following 5 days of co-culture. (d) IL-2 and IFN- γ production by CD4⁺ T cells (5×10^4 cells) co-cultured with dermal CD1c⁺ or CD141⁺ DCs at 1:1/10 ratio was determined by Multicytokine beads analysis of supernatant harvested from day 4 of DC-T cell co-culture (mean $\pm$ SEM) (e) fixed numbers of allogeneic CD8⁺ T cells (5×10^4 cells) were co-cultured with titrated numbers of dermal CD1c⁺ or CD141⁺ DCs. Alloreactive CD8⁺ T cell proliferation is determined by thymidine incorporation (mean $\pm$ SEM) following 5 days of co-culture. (f) IL-2 and IFN- γ production by CD8⁺ T cells (5×10^4 cells) co-cultured with dermal CD1c⁺ or CD141⁺ DCs at 1:1/10 ratio was determined by Multicytokine beads analysis of supernatant harvested from day 4 of DC-T cell co-culture (mean $\pm$ SEM). (a-f) Results are representative of two to four independent experiments. Wilcoxon matched pair t test (^{##} P<0.01) or two-way ANOVA test (* P<0.05, ** P<0.01, *** P<0.001) was performed.

Figure 5 shows that CD4⁺CD25^{hi}Foxp3⁺ T cells induced by dermal CD141⁺ DCs co-express high levels of CTLA-4 on the cell surface. Allogeneic CD4⁺ T cells (5×10^4 cells) co-cultured with dermal CD1c⁺ or CD141⁺ DCs at 1:1/10 ratio for 5 days were stained for CD3, CD11c, CD25 and (A) Foxp3 or (B) CTLA-4. (A) Both dermal CD1c⁺ and CD141⁺ DCs induced a population of CD4⁺ T cells co-expressing CD25 and Foxp3. Data shown is gated on the CD3⁺ and CD11c⁻ population. Results are representative of two to three independent experiments. (B) CD4⁺CD25^{hi} T cells induced by dermal CD141⁺ DCs showed higher percentage and MFI of CTLA-4 expression on the cell surface compared with CD4⁺CD25^{hi} T cells induced by dermal CD1c⁺ DCs. Data shown is gated on the CD3⁺, CD11c⁻ and CD25⁺ population.

Figure 6 shows that dermal CD141⁺ DCs induce antigenic unresponsiveness of CD4⁺CD25^{hi}Foxp3⁺ T cells during secondary stimulation. CD4⁺CD25^{hi} and CD4⁺CD25^{neg} T cells were isolated from day 5 DC-T cell co-culture (1:10 ratio) by flow cytometry sorting, rested overnight and re-stimulated with dermal CD1c⁺ DCs derived from the same allo-donor at 1:1/10 (T cell : DC) ratio. Proliferation of T cells in response to secondary alloantigen stimulation was measured according to thymidine incorporation (mean $\pm$ SEM) following 5 days of culture. Two-way ANOVA test was performed, ** P<0.01.

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Figure 7 shows that dermal CD141⁺ DCs resemble blood CD141⁺ DCs in gene expression profile and cross-present self antigen preproinsulin (PPI). (a) mRNA expression for Batf3, Necl2, and XCR1 in dermal and blood CD141⁺ DCs isolated by flow cytometry sorting displayed a gene expression profile similar to mouse CD8 α ⁺ DCs. (b) Dermal CD141⁺ DCs expressed mRNA for TLR3, 4, 7, and 9 while blood CD141⁺ DCs were negative for TLR4, 7, and 9. (a, b) Relative mRNA expression was analyzed by qPCR and normalized with human cyclophilin. Results are representative of two to five independent experiments. (c) Flow cytometry sorted dermal CD141⁺ DCs selectively expressed mRNA for CLEC9A compared to dermal CD1c⁺ DCs. Cyclophilin expression was used as positive control. Results are representative of four independent experiments. (d) Dermal CD1c⁺ and CD141⁺ DCs obtained from a HLA-A2 donor were pulsed with PPI or CMVpp65 protein, isolated by flow cytometry sorting, and co-cultured with PPI₁₅₋₂₄-CTL. Dermal DCs pulsed with PPI₁₅₋₂₄ 1 hour before co-culture was used as positive control. Proliferation of PPI₁₅₋₂₄-CTL was measured on day 3 according to thymidine incorporation. Results are representative of two independent experiments. Net CPM (mean $\pm$ SEM) represents proliferation of PPI₁₅₋₂₄-CTL in co-culture with PPI, PPI₁₅₋₂₄- or CMVpp65-pulsed DCs minus proliferation of PPI₁₅₋₂₄-CTL in co-culture with no protein-pulsed DCs. Two-way ANOVA test was performed, * P < 0.05.

Figure 8 shows the phenotypic analysis of DDC-induced Tregs following IL-2 expansion. (a) CD1c⁺ and CD141⁺ DDCs were isolated from healthy human skin and co-cultured with allogeneic CD4⁺ T cells. CD1c Tregs and CD141 Tregs were obtained by cell sorting for CD4⁺ CD25^{hi} T cells from the DDC-T cell co-culture followed by IL-2 expansion. (b) Majority of CD1c Tregs and CD141 Tregs express Foxp3 and CD25 following IL-2 expansion, while a higher percentage of CD141 Tregs maintains CLTA-4 expression when compared to CD1c Tregs.

Figure 9 demonstrates the immunoregulatory effects of CD4⁺CD25^{hi} T cells induced by CD141⁺ DDCs (CD141 Treg). (a) IL-2 expanded CD141 Treg are unresponsive to secondary polyclonal stimulation and are (b) superior to CD1c Treg in suppressing CD4⁺CD25⁻ effector T cell (Teff) proliferation. Results are representative of two to five experiments. Two-way ANOVA (b), or unpaired t test (a), *P<0.05, **P<0.01, ***P<0.001, #P<0.05.

Figure 10 shows that CD141⁺ DDC induced Tregs inhibit human alloimmune cell mediated skin inflammation. (a) A schematic demonstration of the experimental set up. Rag2^{-/-} γ_c ^{-/-} mice transplanted with healthy human skin were injected with PBS only, allogeneic PBMCs only, in combination with CD141⁺ DDC induced regulatory T cells (CD141 Tregs) or CD1c⁺ DDC induced regulatory T cells (CD1c Tregs). Skin grafts were analyzed after 4 weeks using immunohistological staining. (b) Epidermal CD3⁺ T cell infiltration, (c) epidermal keratinocyte expression of Ki67 and (d) intact human CD31 superficial dermal microvasculature (all images are at x100 magnification). (e) Quantitative histological analysis of at least 3 independent visual fields per skin graft. Lines represent the mean (n=3 animals per treatment group). CD141 Tregs are significantly superior to CD1c Tregs in reducing PBMC mediated skin inflammation *in vivo*. One-way ANOVA test, ***P<0.001.

Figure 11 shows the human leukocyte analysis in the alloimmune cell mediated skin inflammation model. (a) Harvested human skin grafts showed no gross differences in the human CD45⁺ cell infiltrates as determined by immunofluorescent tissue staining. (b) There was also no significant difference in engraftment levels across treatment groups as shown by human CD45⁺ chimaerism in splenocytes isolated from skin transplant mice.

Figure 12 shows that $CD4^+CD25^{hi}$ T cells induced by dermal $CD141^+$ DCs are highly potent in suppressing $CD4^+$ T cell proliferation. $CD4^+CD25^{hi}$ T cells were isolated from day 5 co-culture with dermal $CD1c^+$ DCs ($CD4^+CD25^{hi}[CD1c]$) or $CD141^+$ DCs ($CD4^+CD25^{hi}[CD141]$) by flow cytometry sorting, rested overnight, and then co-cultured at titrated numbers (regulators) with a fix number of autologous $CD4^+$ T cells (effectors, 5000 cells) under the stimulation of dermal $CD1c^+$ DCs (500 cells, 1:1/10 (effector : DC) ratio) derived from the same allo-donor. A more profound suppression of $CD4^+$ T cells was observed with $CD4^+CD25^{hi}[CD141]$ T cells compared with $CD4^+CD25^{hi}[CD1c]$ T cells, as measured according to thymidine incorporation (mean $\pm$ SEM) following 5 days of culture. Two-way ANOVA test was performed, ** $P < 0.01$.

Figure 13 shows that vitamin D_3 induces dermal $CD141$ -like phenotype in blood DCs. (a) $1,25(OH)_2D_3$ (VitD3) treatment induces $CD141$, $CD14$, $ILT3$, and MMR expression in blood $CD1c^+$ DCs. (b) $1,25(OH)_2D_3$ -treated $CD1c^+$ MDCs were matured with TNF , $IL-1\beta$, $IL-6$, and PGE_2 overnight. Expression of $CD83$ and $HLA-DR$ was compared by gating on the $CD141^-$ and $CD141^+$ populations. (c) $IL-10$ production was measured by Multicytokine beads analysis (mean $\pm$ SEM) of supernatant harvested from flow cytometry sorted $CD141^-$ and $CD141^+$ DCs cultured in the absence or presence of $CD40L$ Tx for 2 days. (d) $CD141^+$ MDCs are less capable than $CD141^-$ MDCs in stimulating $CD4^+$ T cell proliferation when co-cultured at titrated amount with allogeneic $CD4$ T cells purified from peripheral blood. Proliferation of alloreactive T cells was measured according to thymidine incorporation (mean $\pm$ SEM) following 5 days of co-culture. (e) A schematic showing the experimental set up of the xeno-GvHD experiment. (f) $CD141^{hi}$ VitD3 moDCs (triangles pointing down) are superior to $CD141^{dim}$ VitD3 moDCs (triangles pointing up) and control moDC (circles) in prolonging survival from human PBMCs mediated xeno-GvHD. (g) A schematic showing the experimental set up of the allo-PBMC-mediated anti-tumour immunity model in NSG mice. (h) Animals co-injected with PBMCs and $CD141^{hi}$ VitD3 moDCs displayed a significantly increased tumour size compared to injection of PBMCs only and had (i) decreased tumour $CD45^+$ infiltration compared to injection of PBMCs only. (j) Representative pictures of human $CD45^+$ infiltrates in tumours. Results are pooled from two independent experiments ($n=3-6$ animals per treatment group). One-way ANOVA (c, i) or two-way ANOVA (d, h), ** $P < 0.01$, *** $P < 0.001$, $^{###}P < 0.01$.

Figure 14 shows that vitamin D_3 induces dermal $CD141$ -like phenotype in monocyte- derived DCs (moDCs). MoDC were cultured in the presence of 100 nM $1,25(OH)_2D_3$ for 2 days before flow cytometry sorting for the $CD141^{dim}$ ($1,25(OH)_2D_3[CD141^{dim}]$) and $CD141^{hi}$ ($1,25(OH)_2D_3[CD141^{hi}]$) populations. (a) $IL-10$ production was measured by Multicytokine beads analysis (mean $\pm$ SEM) of supernatant harvested from $1,25(OH)_2D_3[CD141^{dim}]$ and $1,25(OH)_2D_3[CD141^{hi}]$ moDCs cultured in the absence or presence of $CD40L$ Tx for 2 days. (b) $1,25(OH)_2D_3[CD141^{hi}]$ moDCs are less capable than $1,25(OH)_2D_3[CD141^{dim}]$ moDCs in stimulating $CD4^+$ T cell proliferation when co-cultured at titrated numbers with allogeneic $CD4$ T cells purified from peripheral blood. Proliferation of alloreactive T cells was measured according to thymidine incorporation (mean $\pm$ SEM) following 5 days of co-culture. Results are representative of two to three independent experiments. Two-way ANOVA test was performed, ** $P < 0.01$, *** $P < 0.001$.

Figure 15 shows that vitamin D₃ induces mRNA expression for CLEC9A and promotes DC cross-presentation. (a) mRNA expression for CLEC9A in blood and dermal CD1c⁺ DCs was induced after 100 nM of 1,25(OH)₂D₃ treatment. CLEC9A expression was analyzed by qPCR and normalized with human cyclophilin. (b) Autologous moDCs generated in the absence or presence of 1,25(OH)₂D₃ were pulsed on day 5 with PPI protein or PPI₁₅₋₂₄, matured on day 6 with poly (I:C) cocktail containing poly (I:C), IFN α , IFN γ , TNF, and IL-1 β , and co-cultured on day 7 with PPI₁₅₋₂₄-CTL at 1 to 10 ratio. Proliferation of PPI₁₅₋₂₄-CTL was measured on day 3 according to thymidine incorporation. Net CPM (mean $\pm$ SEM) represents proliferation of PPI₁₅₋₂₄-CTL in co-culture with PPI- or PPI₁₅₋₂₄-pulsed DCs minus proliferation of PPI₁₅₋₂₄-CTL in co-culture with no protein-pulsed DCs. Two-way ANOVA test was performed, * P < 0.05.

Figure 16 shows the infiltration of DCs and T cells in psoriatic lesions. (a) Immunofluorescent staining of psoriatic lesional and peri-lesional skin cryosections for CD11c and CD3 reveals infiltration of DCs and T cells in the psoriatic lesions. (b) CD141⁺ cells were identified along with CD3⁺ cells in both psoriatic lesional and peri-lesional skin by immunofluorescent staining for CD141 and CD3. DAPI was used for nuclear staining. Scale bar, 150 μ m. Results are representative of six independent experiments.

Figure 17 shows that the numbers of CD141⁺ DCs per CD3⁺ T cell are reduced in psoriatic lesions. (a, b) Reduced CD141⁺ / CD3⁺ ratio was found in psoriatic lesions as compared with normal and peri-lesional skin. (c, d) No difference in CD11c⁺ / CD3⁺ ratio was observed among normal, psoriatic lesional and peri-lesional skin. (A-D) Percentage was calculated by dividing total numbers of CD141⁺ or CD11c⁺ cells by CD3⁺ cells in dermis acquired from the same confocal images. Each symbol corresponds to an average value of two independent images obtained from an individual and counted by two independent researchers. Horizontal bars represent median. (A, B) Mann-Whitney non-parametric t test (* P < 0.05) or (c, d) Wilcoxon matched pairs test (# P < 0.05) was performed.

Figure 18 shows that vitamin D₃ induces dermal CD141⁺ILT3⁺ DCs in psoriatic lesions. Dermal cells were harvested from dermis of lesional psoriatic skin after 2 days treatment with or without 100nM 1,25(OH)₂D₃. Cells obtained from 1,25(OH)₂D₃- treated dermis showed increased percentage of dermal CD141⁺ILT3⁺ DCs compared with the non-treated skin. Results are representative of two independent experiments performed with two different skin donors.

Figure 19 shows that vitamin D₃-induced CD141⁺ DCs protect against PBMC-mediated xenograft-versus-host disease (GvHD). Rag2^{-/-} γ c^{-/-} mice were injected on week 0 with no human cells (Control, 3 mice), 10⁷ CD14-depleted PBMCs (PBMC(CD14⁻) alone, 4 mice), or co-injected with 10⁶ immature moDCs (PBMC(CD14⁻)+iDCs, 3 mice) or 10⁶ vitamin D₃-induced CD141⁺ moDCs (PBMC(CD14⁻)+CD141⁺DCs, 3 mice). Mice injected with PBMC(CD14⁻) alone developed GvHD at week 12 (1 out of 4). Mice co-injected with PBMC and iDCs developed GvHD (1 out of 3) 6 weeks earlier than mice injected with PBMCs alone, while no GvHD has been observed (until week 20) with mice co-injected with PBMCs and CD141⁺ DCs.

Figure 20 shows the human leukocyte analysis in human xeno-GvHD and human melanoma xenotransplantation model. There was no gross difference in the human CD45⁺ cell engraftment levels across treatment groups as shown by human CD45⁺ chimaerism in splenocytes isolated from (a) xeno-GvHD, and (b) tumour bearing mice.

- 5 Figure 21 shows the mechanisms of dermal CD141⁺ DCs in the regulation of skin immune responses. In humans, vitamin D₃ is produced mainly in the skin by the action of sunlight. Under homeostasis, dermal DCs capable of producing 25-hydroxylase and 1 α -hydroxylase can convert vitamin D₃ locally into its active form, 1,25(OH)₂D₃. 1,25(OH)₂D₃ further supports the development of dermal CD141⁺ DCs in the skin. Dermal CD141⁺ DCs promote skin immune tolerance via secretion of regulatory cytokine, IL-10; induction of T cell antigenic unresponsiveness, 10 as well as cross-presentation of self-antigens under steady state conditions.

METHODS

Isolation of dermal DCs

- 15 Normal human skin was obtained from abdominal plastic and mammoplasty surgery after informed consent and ethical approval by the institutional review board of Guy's Hospital in accordance with the Helsinki Declaration. Epidermis and dermis was separated following 2 hours digestion at 37°C with 5mg/mL Dispase (StemCell Technologies Inc.). Dermis was sliced into 1-2mm thin strips and then cultured in RPMI 1640 medium supplemented with 2mM L-glutamine, 50IU/mL penicillin, 50 μ g/mL streptomycin (all from Invitrogen) and 10% 20 (volume/volume) human AB serum (Sigma) (complete medium). Non-plastic adherent cells that had migrated out from the dermis were harvested after 48-72 hours of culturing. In some cases, dermal migratory cells were stained with monoclonal antibodies to CD45-PECy7 (H130; eBioscience), CD11c-PE (AD5-8E7) and CD141-APC (AD5-14H12; both from Miltenyi Biotec), and dermal CD11c⁺ or CD141⁺ DCs were sorted from the CD45⁺FSC^{hi}SSC^{hi} population with a FACS Aria II cell sorter (BD).

- 25 *Mice and skin grafting*
- RAG2 γ ^{-/-} mice 8-12 weeks old were used in all experiments and were maintained under specific pathogen free conditions. Human skin 400-500 μ m kermatome sheet (Zimmer Hand Held Dermatome; Zimmer, UK) was cut into 0.5-cm squares and transplanted orthotopically onto the mice. After 8-12 weeks of transplantation, spleens and 30 lymph nodes from transplanted mice were harvested for FACS and qPCR analysis. Spleens from non-transplanted mice were used as negative controls. Cell suspensions were obtained by mashing spleens and lymph nodes through 70 μ m nylon mesh strainers (BD). To identify human leukocytes, cell suspensions prepared from spleens and lymph nodes were pre-incubated with Mouse BD Fc Block™ (BD) and then co-stained with monoclonal antibodies against human CD45-PECy7 (H130) and mouse CD45-APC/eFluor780 (30-F11; both from eBioscience).

- 35 *Flow Cytometry*
- For flow cytometry (FACS) analysis, the following antibodies were used in different combinations: CD11c-PE (AD5-8E7) and CD141-APC (AD5-14H12; both from Miltenyi Biotec); CD45-PECy7 (H130) and CD205-PE (MG38; both from eBioscience); CD11c-PerCp/Cy5.5 (Bu15) and CD163-PerCp/Cy5.5 (GHI/61; both from BioLegend); CCR7-FITC (FAB19F) and purified CD208 (goat polyclonal; both from R&D Systems); Factor XIIIa
- 40

(sheep polyclonal; Enzyme Research Laboratories); CD14-FITC (TUK4), CD80-PE (MEM-233), CD83-FITC (HB15e), CD86-FITC (BU63), HLA-DR-PE (TU36), and Alexa Fluor 488-conjugated donkey anti-goat IgG (all from Invitrogen); CD1a-PE (SK9), CD16-PE (5D2), CD206-PECy5 (19.2), CD209-PE (DCN46), CCR6-PerCp/Cy5.5 (11A9) and HLA-ABC (DX17; all from BD); Langerin-PE (DCGM4), ILT3-PC5 (ZM3.8) and CD103-FITC (2G5; all from Beckman Coulter). Intracellular staining was performed after surface staining followed by fixation and permeabilization in Fixation/Permeabilization and Permeabilization Buffers (eBioscience) according to manufacturers' instructions. Analysis of FACS data was performed by FlowJo (TreeStar) software.

Microscope

Laser confocal microscopy was performed using a confocal microscope (TCS SP2; Leica). The following antibodies were used to stain OCT embedded cryosections: CD141 (AD5-14H12; Miltenyi Biotec); CD11c (CBR-p150/4G1; AbD Serotec); CD3 (rabbit polyclonal, Dako); human CD45 (eBioscience); Ki-67 (Novocastra); CD31 (Abcam). Alexa Fluor 488-conjugated goat anti-mouse IgG, and Alexa Fluor 555-conjugated goat anti-rabbit IgG were purchased from Invitrogen and used as secondary detection reagents. For morphology study, FACS sorted dermal CD141⁺ DCs were immobilized on poly-L-lysine coated coverslips (BD BioCoat), and stained with Fluorescent phallotoxins (Invitrogen) according to manufacturers' specification. ProLong Gold antifade reagent with DAPI (Invitrogen) was used in all cases for nuclear staining.

DC, monocyte-derived DCs (moDCs) and T cell isolation and culturing

To obtain CD1c⁺ myeloid DCs (MDCs), peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats (NBS Totting, London) by density gradient centrifugation over Lymphocyte Separation Medium (PAA). DC populations were further enriched using Dynal DC-enrichment kit according to manufacture's specification (Invitrogen). CD1c⁺ MDCs were further purified from the enriched DC populations by FACS sorting of lineage negative (CD3, CD14, CD19, and CD56 negative) and CD1c positive population. To induce dermal CD141-like DCs, CD1c⁺ MDCs were cultured in the presence of 100nM 1,25(OH)₂D₃ (Sigma) for 2 days in RPMI (Invitrogen), supplemented with 50IU/mL penicillin, 50ug/mL streptomycin, and 2mM L-glutamine (all from Invitrogen, complete medium) and 10% heat-inactivated human AB serum (HS, Sigma). For maturation of DCs, a cocktail of cytokine containing TNFα (10ng/mL), IL-1β (10ng/mL), and IL-6 (1000U/mL) (all from R&D Systems) plus prostaglandin E₂ (1μg/mL; Sigma) was added to the culture on day 1. DCs with different treatments were harvested on day 2 for phenotypic analysis. To generate monocytes-derived DCs (moDCs), monocytes were firstly isolated from PBMCs using CD14 microbeads (Miltenyi Biotec) according to manufacture's instructions. Monocytes were cultured in RPMI plus 1% single donor plasma (NBS Totting, London), 500IU/mL GM-CSF (Peprotech) and 500IU/mL IL-4 (R&D system). Fresh GM-CSF and IL-4 was added on day 2 and day 5 of culturing. Cells were cultured for 7 days either without treatment or treated on day 5 with 100nM 1,25(OH)₂D₃. For functional studies, CD141^{brigh} or CD141^{dim} DCs were isolated from 1,25(OH)₂D₃-treated DC culture by FACS sorting.

To access allostimulatory capacity of DCs, allogeneic CD4⁺ and CD8⁺ T cells were prepared from buffy coats using RosetteSep human CD4⁺ or CD8⁺ T cell enrichment cocktail (StemCell Technologies Inc.) according to manufacturers' instructions. CD4⁺ or CD8⁺ T cells were cultured in 96-well round-bottom plates (5x10⁴ cells per well) with graded numbers of allogeneic dermal CD1c⁺ / CD141⁺ DCs, or 1,25(OH)₂D₃-induced CD141^{brigh} /

CD141^{dim} DCs. Proliferation of alloreactive T cells was assessed by [³H]thymidine incorporation (1μCi/well, Amersham Bioscience) during the last 18 hr of 5 day cultures.

DDC-induced CD4⁺CD25^{hi} T cells were isolated by cell sorting CD11c⁺CD4⁺CD25^{hi} T cells from DDC co-culture, rested overnight and re-stimulated with CD11c⁺ DDCs derived from the primary allogeneic skin donor. To generate CD4⁺CD25^{hi} T regulatory cell lines (Treg), CD4⁺CD25^{hi} T cells were cell sorted directly into 96 well plates containing RPMI 10% HS and left to rest overnight. Subsequently low dose IL-2 (R&D Systems) was added at 250 IU/mL and replenished every 2-3 days. Following adequate expansion for a period of 4-5 weeks, Tregs were harvested. To assess *in vitro* suppressive capacity of the expanded Tregs, titrated numbers of Tregs were co-cultured with 5x10⁴ autologous CD4⁺CD25⁻ effector T cells for 5 days in the presence of CD3/CD28 T Cell Expander DynalBeads (Invitrogen). Cell proliferation was measured as described above. The *in vivo* suppressive capacity of Tregs was investigated by co-injecting Tregs with autologous PBMCs into mice bearing healed allogeneic human skin grafts at a ratio of 1:10 (Tregs : CD3 composition of PBMCs inoculum); typically 3x10⁶ human PBMCs alone or in combination with 1.5 x 10⁵ Tregs.

Xeno-graft models of GvHD and melanoma

NOD/scid/*IL-2Rγ*^{-/-} (NOD.cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (obtained from The Jackson Laboratory, abbreviated as NSG) mice were used between 8-11 weeks of age. Xeno-Graft-versus-host-disease (GvHD) was induced by intravenous transfer of 10x10⁶ human PBMCs and animals were monitored for body weight and other GvHD symptoms (hunched back, fur loss, skin inflammation). For tumour experiments, 5x10⁵ human A375 melanoma tumour cells (obtained from ATCC) were injected subcutaneously into the flank of NSG mice 5 days prior to transfer of 10x10⁶ human PBMCs. Tumour size was measured at day 15 and day 25 post PBMC transfer and calculated using the formula: (short diameter)²×(long diameter)/2.

To assess the immunoregulatory potential of VitD3 moDC in xeno-GvHD and melanoma, syngeneic human PBMCs were either injected alone or co-transferred with 5x10⁵ (ratio of 20:1) cell sorted CD141^{hi} VitD3 moDC, CD141^{dim} VitD3 moDC, or control moDC.

All humanised mice used in the study were bled at 3 weeks post human PBMC transfer for the assessment of human cell engraftment where any mice with human CD45 chimaerism lower than 1% were excluded from further analyses.

Determination of IL-10 production

To measure cytokine production, 2x10⁴ of DCs from different preparation were cultured in 96-well flat-bottom plates with 100μL of complete medium containing 10% HS for 2 days in the absence or presence of CD40 ligand-transfected L cells (5x10⁴ cells/well). IL-10 cytokine levels in the supernatant were assayed by using the MILLIPLEX MAP Human Cytokine Kit (Millipore) and acquired on a Luminex 100 flow-based sorting and detection analyzer (Luminex Corporation).

RNA extraction and quantitative RT-PCR (qRT-PCR)

RNA extraction was carried out using NucleoSpin RNA XS Kit (Macherey-Nagel GmbH & Co, Duren, Germany) according to manufacturers' instructions and retro transcribed into cDNA. Human CD141, Langerin, CLEC9A, ILT3, BATF3, Necl2, XCR1, TLR3, TLR4, TLR7, and TLR9 expression was assessed by multiplex real-time quantitative RT-PCR by using Taqman assays (Applied Biosystems) according to manufacturers' instructions. For each sample, mRNA abundance was normalized to the amount of human Cyclophilin. Data analysis was performed using the $\Delta\Delta C_t$ method: results were expressed either as relative mRNA levels in arbitrary units. Where indicated, RT-PCR product was run on a 2% agarose gel and followed by ethidium bromide staining to visualize mRNA expression.

Cross-presentation

For cross-presentation assays, 20 $\mu\text{g/mL}$ of PPI protein (prepared in house; 117) or recombinant CMV protein (Miltényi Biotech) as negative control were added to the dermal culture prepared from a HLA-A2 donor. After 48 hours of migration period, CD1c⁺ and CD141⁺ DCs were isolated by FACS sorting, rested overnight, and then co-cultured with 4x10⁴ PPI-specific CD8⁺ T cell (PPI PPI₁₅₋₂₄-CTL) clone at different ratio [117]. Dermal DCs pulsed with PPI₁₅₋₂₄ peptides 1 hour before co-culture were used as positive control. Proliferation of PPI₁₅₋₂₄-CTL clone was assessed by [³H]thymidine incorporation (1 $\mu\text{Ci/well}$) during the last 18 hr of 3 day cultures.

Statistical analysis

Statistic analysis was performed using Prism version 4.0 (GraphPad Software). Results were assessed for normal Gaussian distribution and then analyzed by Mann-Whitney non-parametric *t* test, Wilcoxon matched pairs test, one-way ANOVA test, or two-way ANOVA, as appropriate. For mRNA expression analysis qRT-PCR was performed in triplicates, mean $\pm$ SD was calculated, results were assessed for normal Gaussian distribution, and then analyzed by Mann-Whitney or unpaired two-tailed *t* test, as appropriate. For studying the ratio of CD141⁺ DCs or CD11c⁺ DCs versus CD3⁺ T cells in the skin, percentage of CD141 vs CD3 or CD11c vs CD3 was calculated by dividing total amount of CD141⁺ or CD11c⁺ cells by CD3⁺ cells acquired from the same confocal image (20x). Two independent images were acquired from each individual and each image was counted by two independent researches. Values of $P < 0.05$ were considered significant.

RESULTS

Human skin contains a distinct population of CD141⁺ DCs

Comprehensive phenotypic characterization of skin DCs revealed a significant percentage of CD141⁺ DCs in dermis of human skin. Percentage of CD141⁺ cell population ranged from 14.13 % to 53.36 % (mean = 30 %, SD = 12.08, $n = 9$) (Fig. 1a and Fig. 2a). Dermal CD141⁺ DCs co-expressed CD1c at low levels, CD11c, and CD14, but were negative for CD1a (Fig. 1b). Further phenotypic analysis revealed that dermal CD141⁺ DCs expressed other skin-relevant myeloid markers such as Factor XIIIa and CD163. Expression of CD103 and C-type lectin receptors such as DC-SIGN, DEC205, and langerin were undetectable on dermal CD141⁺ DC (Fig. 2b). Dermal CD141⁺ DCs spontaneously migrated out from human dermis in tissue culture and maintained an immature phenotype, indicated by the absence of CD83 expression (Fig. 1c), but expression of significant levels of co-stimulatory molecules, CD80 and CD86, as well as MHC class I and class II molecules. High levels of macrophage mannose receptor (MMR)

expression was uniquely detected on the dermal CD141⁺ DCs, supporting their proposed role in antigen recognition and cross-presentation [123] (Fig. 1c). CD141⁺ cells were located mainly in the upper dermal compartment immediately under the epidermis (Fig. 1d). In agreement with the literature, some CD141 expression was also detected on keratinocytes [118]. Immunofluorescence staining for filamentous-actin (F-actin) of flow cytometry sorted dermal CD141⁺ cells demonstrated a typical morphology with multiple dendritic processes (Fig. 1e).

Dermal CD141⁺ DCs express a different set of markers which distinguish them from mouse CD8α⁺ DCs and human blood CD141⁺ DCs as shown in the table below:

Molecular markers and functional features	Mouse		Human blood		Human skin	
	CD8a ⁺	CD8a ⁻	CD141	CD1c	CD141	CD1c
BATF3	+	+	+	+	+	+
XCR1	+	-	+	-	+	+/-
Nec12	+	-	+	-	+	+/-
CLEC9A	+	-	+	-	+	-
CD1c	ND	ND	-	+	+/low	+
CD11b	low	++	-	+	+	+
CD11c	+	+	+	+	+	+
CD14	-	-	-	-	+	-
CD103	+/-	-	-	-	-	-
DEC205 (CD205)	++	+/-	+	+/-	-	-
MMR (CD206)	+/-	-	ND	ND	++	-
Langerin (CD207)	+/-	-	+	-	-	+/-
TLR3	+	-	+	+/low	+	+
TLR4	+	+	-	+	+	+
TLR7	-	+	-	+	+	+/low
TLR9	+	+/-	-	-	+	+
IL-12 production (following CD40 cross-linking)	++	-	+	-	-	-
IL-10 production (following CD40 cross-linking)	ND	ND	ND	ND	++	-
Th1 polarization	++	+/-	++	+/-	ND	ND
Treg induction	++	+/-	ND	+/-	++	+/-
Cross-presentation (steady-state)	+	-	-/low	-/low	+	-
Cross-presentation (with exogenous stimuli)	++	-	++	+	+	-
CD4 ⁺ T cell stimulatory capacity	low	++	+	+	low	++

(ND = not determined)

Dermal CD141⁺ DCs migrate from skin to draining lymph nodes

Dermal CD141⁺ DCs spontaneously migrated out of dermal tissue explants and expressed both lymph node (CCR7) and skin (CCR6) homing CC chemokine receptors (Fig. 3a). To address *in vivo* migratory capacity of dermal CD141⁺ DCs, the inventors established a humanized skin-transplantation mouse model by grafting healthy human skin onto Rag2^{-/-}γc^{-/-} mice, a mutant strain that lacks T, B, and NK cells thus allowing high engraftment of human tissues/cells[124]. To identify human skin migratory cells, spleen and skin draining lymph nodes were harvested from mice 8-12 weeks post-transplantation and co-stained with antibodies specific for human CD45 and mouse CD45 to distinguish human leukocytes. In mice that had received skin transplants, the inventors detected human CD45⁺ leukocytes in lymph nodes but not in spleen (Fig. 3b). Immunofluorescence double staining revealed that both human CD3⁺ and CD141⁺ cells were present in skin draining lymph nodes of mice receiving skin transplants

(Fig. 3c). PCR analysis using human specific primers further confirmed that CD141⁺ cells were detected in mouse lymph nodes (Fig. 3d). Since the skin was the only source of human leukocytes in this model system, the data show that skin-resident CD141⁺ cells are capable of migrating from skin into draining lymph nodes *in vivo*.

5 *Dermal CD141⁺ DCs have immunoregulatory capacity*

To investigate the immunoregulatory capacity of dermal CD141⁺ DCs, the inventors isolated both CD1c⁺ and CD141⁺ dermal DCs by flow cytometry sorting and analyzed their phenotype, cytokine profile as well as their capacity to induce T cell tolerance/unresponsiveness. Dermal CD141⁺ DCs expressed immunoglobulin-like transcript 3 (ILT3), a cell surface inhibitory receptor that has been shown to be involved in immunoregulation [125].

10 Quantitative PCR (qPCR) analysis confirmed this expression and showed that ILT3 mRNA level is consistently higher in dermal CD141⁺ DC compared to CD1c⁺ DC (Fig. 4a). In agreement with their immunoregulatory phenotype, dermal CD141⁺ DCs, but not CD1c⁺ DC, spontaneously secreted high level of IL-10 in culture in the absence of any further stimulation. CD40 cross-linking enhanced IL-10 production by dermal CD141⁺ DCs (Fig. 4b). Next, T cell priming capacity of dermal CD1c⁺ and CD141⁺ DCs was examined by co-culturing fixed numbers of allogeneic CD4⁺ or CD8⁺ T cells purified from PBMCs (5×10⁴ cells) with titrated numbers of dermal CD1c⁺ and CD141⁺ DCs. In agreement with their immunoregulatory phenotype, dermal CD141⁺ DCs were less efficient than dermal CD1c⁺ DCs in stimulating alloreactive T cell responses. Allogeneic CD4⁺ T cells co-cultured with dermal CD141⁺ DCs showed reduced proliferation (Fig. 4c) as well as decreased IL-2 and IFN γ production (Fig. 4d) as compared with the same CD4⁺ T cells co-cultured with dermal CD1c⁺ DCs. Likewise, dermal CD141⁺ DCs failed to stimulate allogeneic CD8⁺ T cell proliferation as well as IL-2 and IFN γ production when compared with dermal CD1c⁺ DCs (Fig. 4e & f).

Dermal CD141⁺ DCs induce CD4⁺CD25^{hi}Foxp3⁺ T cells with high levels of surface CTLA-4 expression

During DC-T cell co-culture, alloreactive T cells became activated and upregulated CD25 expression on the cell surface. Both dermal CD1c⁺ and CD141⁺ DCs induced a population of T cells that expressed high levels of CD25. Activated CD4⁺CD25^{hi} T cells, induced by either dermal CD1c⁺ or CD141⁺ DCs coexpressed transcription factor Foxp3 (Fig. 5a) However, CD4⁺CD25^{hi} T cells induced by dermal CD141⁺ DCs showed higher surface expression of cytotoxic T lymphocyte antigen-4 (CTLA-4) compared to CD4⁺CD25^{hi} T cells induced by dermal CD1c⁺ DCs (Fig. 5b).

30 *CD4⁺CD25^{hi}Foxp3⁺ T cells induced by dermal CD141⁺ DCs are anergic to secondary antigenic re-stimulation*

To investigate whether CD4⁺CD25^{hi}Foxp3⁺ T cells induced by dermal CD141⁺ DCs are resistant to secondary antigenic stimulation, CD4⁺CD25^{hi} and CD4⁺CD25^{neg} T cells as control were isolated from day 5 DC-T cell co-culture (1:10 ratio) by flow cytometry sorting and re-challenged with dermal CD1c⁺ DCs derived from the same allo-donor. CD4⁺CD25^{hi} T cells induced by dermal CD141⁺ DCs were unresponsive to secondary alloantigen stimulation in contrast to CD4⁺CD25^{hi} T cells induced by dermal CD1c⁺ DCs that proliferate actively. On the contrary, CD4⁺CD25^{neg} T cells that were not activated during primary co-culture with either CD1c⁺ or CD141⁺ dermal DCs responded actively to secondary alloantigen stimulation independent from the primary co-culture with dermal CD1c⁺ and CD141⁺ DCs (Fig. 6).

Dermal CD141⁺ DCs induce CD4⁺CD25^{hi} T cells with profound immune suppressive capacity

Naturally-occurring CD4⁺CD25^{hi} Tregs (nTregs) obtained from peripheral blood constitutively express transcription factor Foxp3 and CTLA-4 and are anergic to TCR stimulation *in vitro*. While both dermal CD1c⁺ and CD141⁺ DCs induced a population of CD4⁺CD25^{hi}Foxp3⁺ T cells during DC-T cell co-culture, only CD4⁺CD25^{hi} T cells induced by dermal CD141⁺ DCs resemble nTregs expressing high levels of surface CTLA-4 and were anergic to antigenic restimulation (Fig. 5b & Fig. 6). To determine immune suppressive capacity of CD4⁺CD25^{hi} T cells induced by dermal CD141⁺ DCs compared with CD1c⁺ DCs, titrated numbers of flow cytometry isolated CD4⁺CD25^{hi} T cells (regulators) were co-cultured with a fixed number of autologous CD4⁺ T cells (effectors, 5000 cells) under the stimulation of dermal CD1c⁺ DCs (500 cells) derived from the same allo-DC donor used in the primary DC-T cell culture. CD4⁺CD25^{hi} T cells induced by both dermal CD1c⁺ and CD141⁺ DCs were able to suppress CD4⁺ T cell proliferation in response to dermal CD1c⁺ DCs stimulation. However, CD4⁺CD25^{hi} T cells induced by dermal CD141⁺ DCs displayed better suppression than CD4⁺CD25^{hi} T cells induced by dermal CD1c⁺ DCs, particularly when CD4⁺CD25^{hi} T cells were added at ratio as low as 1:1/4 (effectors : regulators) ratio (Fig. 7).

CD141⁺ DDCs induce T regulatory cells (Treg) with potent immunosuppressive capability in vitro and in vivo

In preliminary experiments the inventors observed suppressive activity in CD4⁺CD25^{hi} T cells induced by both CD141⁺ DDCs (CD141 Treg) and CD1c⁺ DDCs (CD1c Treg). The inventors then expanded the cells in the presence of IL-2 to obtain sufficient cell numbers for *in vivo* Treg cell therapy (18) (Fig. 8a). Following cell expansion, CD141 Treg and CD1c Treg maintained their expression of Treg associated markers CD25 and Foxp3 (Fig. 8b). CD141 Treg were unresponsive to secondary TCR stimulation (Fig. 9a), and suppressed CD4⁺CD25⁺ T effector (Teff) cell proliferation, while CD1c Treg enhanced T-cell proliferation in a dose-dependent manner (Fig. 9b).

To determine the *in vivo* immunoregulatory function of DDC-induced Treg, the inventors took advantage of a recently described model of alloimmune cell-mediated skin inflammation [126]. *In vivo* function was determined by transfer of allogeneic PBMCs alone or in combination with allo-specific CD141 Treg or CD1c Treg into mice engrafted with healthy human skin (Fig. 10a). Skin grafts from animals receiving allogeneic PBMCs showed typical inflammatory skin pathology with increased numbers of epidermal T cells, proliferating keratinocytes in the basal epidermal layer, and loss of intact CD31⁺ dermal microvasculature, as compared to control animals (Fig 10b-d). Co-transfer of CD141 Treg protected from the induction of skin pathology in a significant manner, restoring markers of pathology to levels observed in control grafts (Fig. 10e), while no significant protective effect was seen in mice treated with CD1c Treg. Protection conferred by CD141 Treg was not due to differences of splenic human cell engraftment or dermal human CD45⁺ infiltrate (fig. 11a and b). Together, these findings demonstrate that CD141⁺ DDCs display both phenotypic and functional characteristics of immunoregulatory DCs and induce Treg cells with potent immunosuppressive capability *in vitro* and *in vivo*.

Dermal CD141⁺ DCs cross-present self-antigen

DC induce peripheral tolerance based on their capacity to capture, process, and present tissue derived antigens to self-reactive T cells in the periphery under steady state conditions[64]. In mice, CD8⁺ DC are the primary cross-presenting DC subset in spleen. Recent studies suggested similarities between human blood CD141⁺ DCs and mouse

CD8⁺ DCs based on both transcriptomic and functional studies[23-25,31,43]. Dermal CD141⁺ DCs expressed mRNA for BATF3, Necl2, and XCR1 (Fig. 12a), in a pattern similar to mouse CD8 α ⁺ DCs. In addition dermal CD141⁺ DCs expressed TLR3, 4, 7 and 9. This is in contrast to blood CD141⁺ DCs that do not express TLR4, 7 and 9 (Fig. 12b). Markers associated with cross-presentation such as MMR[123] (Fig. 1c), and CLEC9A, which is expressed by mouse CD8 α ⁺ DCs and human blood CD141⁺ DCs[36], were selectively expressed by dermal CD141⁺ DCs and not by CD1c⁺ DCs (Fig. 12c). Thus on a phenotypic level, dermal CD141⁺ DCs share important molecular markers of cross-presentation with mouse CD8 α ⁺ DCs and human blood CD141⁺ DC but have a distinct profile of TLR expression.

To investigate the capacity of dermal CD141⁺ DCs to cross-present self-antigens, the inventors examined their ability to process and present the selfantigen pre-proinsulin (PPI) to PPI specific CD8⁺ T cells. Dermal DCs were isolated from healthy dermis of HLA-A2⁺ donors and cultured in the presence of PPI protein or recombinant CMVpp65 protein as negative control for 48 hours. Dermal CD1c⁺ and CD141⁺ DCs were then sorted and co-cultured with a PPI-specific CD8⁺ T-cell clone (PPI₁₅₋₂₄-CTL) that recognizes PPI₁₅₋₂₄, an HLA-A2-bound epitope derived from the leader sequence of PPI³². Dermal CD141⁺ DCs pulsed with PPI protein but not CMVpp65 protein induced significantly higher proliferation of PPI₁₅₋₂₄-CTL when compared to PPI-pulsed dermal CD1c⁺ DCs (P < 0.05). Both dermal CD1c⁺ and CD141⁺ DCs induced comparable PPI₁₅₋₂₄-CTL proliferation when pulsed with PPI₁₅₋₂₄ peptide. (Fig. 12d). Cross-presentation of PPI by dermal CD141⁺ DC took place in the absence of proinflammatory stimuli such as poly (I:C), suggesting an intrinsic and unique capacity of dermal CD141⁺ DCs to cross-present self-antigens under steady state conditions.

Vitamin D₃ induces dermal CD141-like DCs from blood-derived DCs

The inventors next asked whether phenotypic and functional characteristics of dermal CD141⁺ DCs are inducible by skin-relevant molecules. Vitamin D₃ is a key skin derived and sunlight induced factor. To address whether vitamin D₃ can promote dermal CD141⁺ DCs from circulating DC populations, the inventors isolated CD1c⁺ DCs from peripheral blood and cultured the cells in the presence of 1,25(OH)₂D₃, the active form of vitamin D₃. After 2 days of culture, a distinct dermal CD141-like DC population co-expressing CD14 and high levels of CD141 was induced, which co-expressed ILT3 and up-regulated MMR (Fig. 13a). 1,25(OH)₂D₃-induced CD141⁺ DCs had a stable immature DC phenotype, as indicated by low to absent expression of CD83 after exposure to a potent DC maturation cocktail consisting of TNF- α , IL-1 β , IL-6, and prostaglandin E₂. This was in contrast to the CD141 negative DC population from the same 1,25(OH)₂D₃-treated culture which readily up-regulated CD83 (Fig. 13b).

Next, the inventors showed that 1,25(OH)₂D₃-induced CD141⁺ DCs produced significantly higher amounts of IL-10 after CD40 ligand cross-linking compared to the CD141⁻ DC population (Fig. 13c). When co-cultured with allogeneic CD4⁺ T cells, 1,25(OH)₂D₃-induced CD141⁺ DCs were less potent than the CD141⁻ DCs in priming alloreactive CD4⁺ T cell proliferation (Fig. 13d). Similar findings were also observed with 1,25(OH)₂D₃ treated monocyte-derived DCs (moDCs) (Fig. 14a and 14b). Furthermore, 1,25(OH)₂D₃ treatment induced the expression of CLEC9A on both blood-derived and dermal CD1c⁺ DCs (Fig. 15a). In agreement with this observation, 1,25(OH)₂D₃-treated moDCs cultured with PPI protein followed by poly (I:C) stimulation showed enhanced capacity to cross-present PPI protein to PPI₁₅₋₂₄-CTL when compared with non 1,25(OH)₂D₃-treated moDCs (Fig.

15b). These results reveal vitamin D₃ as a major sunlight induced skin factor able to induce characteristics of dermal regulatory CD141⁺ DCs in circulating DCs.

Decrease of the CD141⁺ DCs/effector T cell ratio in inflammatory skin disease

5 To investigate dermal CD141⁺ DCs in an inflammatory pathology, the inventors focussed the attention on psoriasis, an inflammatory skin disease characterized by abnormal keratinocyte proliferation and differentiation, as well as infiltration of immune cells, including T cells and DCs [74]. Skin cryosections obtained from psoriatic lesions and the normal appearing skin obtained at the edge of the lesions (peri-lesions) were stained for CD3, CD11c and CD141. Large numbers of both CD3⁺ and CD11c⁺ cells infiltrated lesions of psoriatic skin were observed while
 10 much less CD3⁺ and CD11c⁺ cells were found in the peri-lesions (Fig. 16a). CD141⁺ DCs were also detected in both psoriatic lesions and peri-lesions (Fig. 16b). When calculating the numbers of infiltrating CD141⁺ DCs against CD3⁺ T cells, significantly reduced CD141⁺ DC/CD3⁺ T cell ratio was found in psoriatic lesions compared with normal skin (Fig. 17a). Peri-lesional psoriatic skin showed a CD141⁺ DC/CD3⁺ T cell ratio that was significantly higher than lesional psoriatic skin and comparable to normal skin (Fig. 17b). In contrast, no difference in CD11c⁺
 15 DC/CD3⁺ T cell ratio was observed in psoriatic lesion when compared with either normal (Fig. 17c) or psoriatic peri-lesions (Fig. 17d). Decreased numbers of CD141⁺ DCs per CD3⁺ T cell in lesional psoriatic skin support a potential immunoregulatory role of dermal CD141⁺ DCs in inflammatory skin pathologies.

Vitamin D₃ treatment induces dermal CD141⁺ DCs in psoriasis

20 To investigate whether vitamin D₃ treatment could induce CD141⁺ DCs in psoriasis, dermis obtained from psoriatic lesions were treated with 1,25(OH)₂D₃ for 2 days and non-adherent dermal cells were harvested for flow cytometric analysis. Dermal cells obtained from 1,25(OH)₂D₃ treated psoriatic dermis showed increased percentage of CD141⁺ DCs with the majority of the cells co-expressing ILT3 (Fig. 18). This confirms the findings shown above using blood-derived DCs and further indicates the potential benefit of vitamin D₃ treatment in the induction of regulatory
 25 CD141⁺ DC in psoriasis.

Vitamin D₃-induced CD141⁺ DCs protect against PBMC-mediated xenograft-versus-host disease (GvHD)

To investigate whether vitamin D-induced CD141⁺ DCs have therapeutic effects in transplantation rejection, the inventors tested the effects of vitamin D₃-induced CD141⁺ DCs in GvHD, a condition which can occur following a
 30 bone marrow transplant, or a peripheral blood stem cell transplant, from a donor. The inventors established a mouse model of xeno GvHD in which CD14⁺ monocyte-depleted human PBMCs (PBMC(CD14⁻)) were injected into Rag2^{-/-}γc^{-/-} mice. While mice injected with PBMC(CD14⁻) alone developed GvHD at week 12, co-injection of vitamin D₃-induced CD141⁺ DCs with PBMC(CD14⁻) prevented the development of PBMC-mediated GvHD. On the contrary, mice co-injected with PBMC and immature DCs (iDCs) developed GvHD 6 weeks earlier than mice
 35 injected with PBMCs alone (Fig. 19). This shows that vitamin D-induced CD141⁺ DCs can protect against transplantation rejection.

Vitamin D₃-induced CD141⁺ DCs derived from monocytes have in vivo immunoregulatory roles

To investigate the *in vivo* regulatory potential of VitD3-induced moDCs highly expressing CD141 (CD141^{hi} VitD3 moDCs), the inventors first evaluated their immunomodulatory capacity in a human GvHD model [127] (Fig. 13e). Injection of human PBMCs induced GvHD in NOD/SCID/ $\gamma c^{-/-}$ (NSG) mice [median survival time (MST) = 32]. Co-injection of CD141^{hi} VitD3 moDCs with PBMCs significantly prolonged survival time ($P < 0.001$ vs. PBMCs alone; MST=62), while no significant protection was conferred by transfer of CD141^{dim} VitD3 moDCs (MST=39; Fig. 13f). Mice co-injected with immunoregulatory immature control moDCs also improved survival time ($P < 0.05$ vs. PBMCs alone; MST=50).

The inventors next assessed the immunoregulatory role of CD141^{hi} VitD3 moDCs in an immune cell dependent human melanoma xenograft model (Fig. 13g). Injection of PBMCs reduced tumour size significantly compared to PBS injected mice. However, tumour size was significantly increased in mice injected with PBMCs plus CD141^{hi} VitD3 moDCs but not CD141^{dim} VitD3 moDCs or control moDCs (Fig. 13h), suggesting suppression of anti-tumour immunity. In line with these findings, the inventors observed a significantly reduced human CD45⁺ cell infiltrate in tumours recovered from CD141^{hi} VitD3 moDCs injected mice (Fig. 13i and 13j). There was no significant difference in human CD45⁺ splenic cell engraftment between treatment groups (Fig. 20a and 20b).

All documents cited herein are hereby incorporated in their entirety within this disclosure.

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Claims

1. An isolated population of tissue CD141⁺ dendritic cells (DCs) and/or tissue CD141⁺-like DCs for use in the treatment or prophylaxis of an immune-mediated disease in which an immunosuppressive effect is required.
2. An isolated population of DCs for use according to claim 1, wherein the isolated population is of dermal CD141⁺ DCs.
3. An isolated population of DCs for use according to claim 1, wherein the isolated population is of tissue CD141⁺-like DCs.
4. An isolated population of DCs for use according to claim 3, wherein the tissue CD141⁺-like DCs are obtained by culturing antigen-presenting cells (APCs) in the presence of vitamin D or an analogue thereof.
5. An isolated population of DCs for use according to claim 4, wherein the APCs are selected from blood CD1c⁺ DCs and monocyte-derived DCs.
6. An isolated population of DCs for use according to any preceding claim, wherein the DCs coexpress one or more of the markers selected from the group consisting of CD14, CD1c, MMR and ILT3.
7. An isolated population of DCs for use according to any preceding claim, wherein the DCs coexpress one or more of the markers selected from the group consisting of CD14, CD1c, MMR, ILT3, TLR4, TLR7 and TLR9.
8. An isolated population of DCs for use according to any preceding claim, wherein the DCs do not express DEC205 (CD205) and/or Langerin (CD207).
9. An isolated population of DCs for use according to any preceding claim, wherein the DCs coexpress the markers CD14, CD1c, MMR and ILT3 and do not express DEC205 (CD205) and Langerin (CD207).
10. An isolated population of DCs for use according to any preceding claim, wherein the DCs coexpress at least one of the markers selected from a group consisting of CD14, CD80, CD86, CD11c, MMR, TLR3, TLR4, TLR7, TLR9, ILT3, CLEC9A, BATF3, XCR1, Necl2, Factor XIIIa and CD163.
11. An isolated population of DCs for use according to any preceding claim, wherein the immune-mediated disease is selected from hypersensitivities, autoimmune diseases, autoinflammatory diseases, transplant rejections, asthma, chronic prostatitis, glomerulonephritis, inflammatory skin diseases, inflammatory bowel diseases, inflammatory joint diseases, inflammatory lung diseases, inflammatory brain diseases, pelvic inflammatory diseases, systemic inflammatory diseases, thyroiditis, sarcoidosis, vasculitis and interstitial cystitis.

12. An isolated population of DCs for use according to claim 11, wherein the immune-mediated disease is an autoimmune disease, an autoinflammatory disease or hypersensitivity.
- 5 13. An isolated population of DCs for use according to claim 12, wherein the DCs have been cultured in the presence of an antigen involved in the development of the immune-mediated disease.
14. An isolated population of DCs for use according to any preceding claim, wherein the immune-mediated disease is psoriasis.
- 10 15. An isolated population of DCs for use according to any preceding claim, wherein the isolated population of DCs is suitable for administration systemically.
16. An isolated population of DCs for use according to claim 15 wherein systemic administration is via an administration route selected from the group consisting of intravenous injection, intraarterial injection, intranodal
15 injection, perfusion and infusion.
17. An isolated population of DCs for use according to any one of claims 1 to 14, wherein the isolated population of DCs is suitable for administration locally.
- 20 18. An isolated population of DCs for use according to claim 17, wherein said local administration is via an administration route selected from the group consisting of catheter, intradermal injection, intralesional injection and subcutaneous injection.
- 25 19. A pharmaceutical composition comprising an isolated population of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs in combination with one or more pharmaceutically acceptable excipients.
20. The pharmaceutical composition of claim 19 which additionally comprises one or more additional therapeutic agents.
- 30 21. A vaccine comprising an isolated population of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs.
22. A method of treating or preventing an immune-mediated disease in which an immunosuppressive effect is required, the method comprising administering an isolated population of tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs to a subject.
- 35 23. A method according to claim 22, wherein the DCs coexpress at least one of the markers selected from a group consisting CD14, CD80, CD86, CD11c, MMR, TLR3, TLR4, TLR7, TLR9, ILT3, CLEC9A, BATF3, XCR1, Nec12, Factor XIIIa and CD163.

24. A method according to claim 22 or 23, wherein the DCs coexpress one or more of the markers selected from the group consisting of CD14, CD1c, MMR and ILT3.

25. A method according to claim 22, 23 or 24, wherein an isolated population of dermal CD141⁺ DCs is administered to a subject.

26. A method according to claim 22, 23 or 24, wherein an isolated population of tissue CD141⁺-like DCs is administered to a subject.

27. A method according to claim 26, wherein the tissue CD141⁺-like DCs are obtained by culturing antigen-presenting cells (APCs) in the presence of vitamin D or an analogue thereof.

28. A method according to claim 27, wherein the APCs are selected from blood CD1c⁺ DCs and monocyte-derived DCs.

29. A method according to any one of claims 22 to 28, wherein the immune-mediated disease in which an immunosuppressive effect is required is selected from hypersensitivities, autoimmune diseases, autoinflammatory diseases, transplant rejections, asthma, chronic prostatitis, glomerulonephritis, inflammatory skin diseases, inflammatory bowel diseases, inflammatory joint diseases, inflammatory lung diseases, inflammatory brain diseases, pelvic inflammatory diseases, systemic inflammatory diseases, thyroiditis, sarcoidosis, vasculitis and interstitial cystitis.

30. A method according to claim 29, wherein the immune-mediated disease is an autoimmune disease, an autoinflammatory disease or hypersensitivity.

31. A method according to claim 30, wherein the DCs have been cultured in the presence of an antigen involved in the development of the immune-mediated disease or the DCs are administered together with the antigen.

32. A method according to any one of claims 22 to 31, wherein the immune-mediated disease is psoriasis.

33. A method according to any one of claims 22 to 32, wherein the isolated population of DCs is administered systemically.

34. A method according to claim 33, wherein systemic administration is via an administration route selected from the group consisting of intravenous injection, intraarterial injection, intranodal injection, perfusion and infusion.

35. A method according to any one of claims 22 to 32, wherein the isolated population of DCs is administered locally.

36. A method according to claim 35, wherein said local administration is via an administration route selected from the group consisting of catheter, intradermal injection, intralesional injection and subcutaneous injection.

37. A method of treating or preventing an immune-mediated disease, the method comprising administering an antigen involved in the development of the immune-mediated disease to tissue CD141⁺ DCs and/or tissue CD141⁺-like DCs.

38. A method of treating or preventing a disease or condition in which an immunostimulatory effect is required, the method comprising depleting tissue CD141⁺ DCs in a subject.

39. A method according to claim 38, wherein the method comprises a step of administering an agent selected from antibodies, cytotoxic compounds and interfering RNA to deplete tissue CD141⁺ DCs in a subject.

40. A method according to claim 38 or 39, wherein a disease or condition in which an immunostimulatory effect is required is cancer.

41. A method of predicting or diagnosing a disease or condition associated with an abnormal immune response comprising the steps of determining the level and/or immune activation state of tissue CD141⁺ DCs in a biological sample obtained from a subject, and comparing the determined level and/or immune activation state of tissue CD141⁺ DCs in the subject with a control level and/or control immune activation state of tissue CD141⁺ DCs.

42. A method according to claim 41, further comprising the steps of determining the level of immune-stimulatory cells in the biological sample obtained from the subject, calculating the ratio of tissue CD141⁺ DCs and immune-stimulatory cells, and comparing the calculated tissue CD141⁺ DCs/ immune-stimulatory cells ratio of the subject with a control ratio.

43. A method according to claim 41, further comprising the steps of determining the level of additional immune-regulatory cells in the biological sample obtained from the subject, and comparing the determined level of the additional immune-regulatory cells of the subject with a control level.

44. A method according to any one of claims 41 to 43, wherein the disease or condition associated with an abnormal immune response is an immune-mediated disease in which an immunosuppressive effect is required, and wherein a lower level and/or immune activation state of tissue CD141⁺ DC in the subject than the control level and/or the control immune activation state of tissue CD141⁺ DC, a lower level of the tissue CD141⁺ DCs/ immune-stimulatory cells ratio in the subject than the control ratio, and/or a higher level of the additional immune-regulatory cells in the subject than the control level of the additional immune-regulatory cells is a positive indication of the immune-mediated disease.

45. A method according to any one of claims 41 to 43, wherein the disease or condition associated with an abnormal immune response is a disease or condition in which an immunostimulatory effect is required, and wherein

a higher level and/or immune activation state of tissue CD141⁺ DC in the subject than the control level and/or the control immune activation state of tissue CD141⁺ DC, a higher level of the tissue CD141⁺ DCs/ immune-stimulatory cells ratio in the subject than the control ratio, and/or a lower level of the additional immune-regulatory cells in the subject than the control level of the additional immune-regulatory cells is a positive indication of the disease or condition.

46. A method of monitoring the progression of a disease or condition associated with an abnormal immune response comprising the steps of determining the level and/or immune activation state of tissue CD141⁺ DCs in a biological sample obtained from a subject at a first time point, determining the level and/or immune activation state of tissue CD141⁺ DCs in a biological sample obtained from a subject at a second time point, and comparing the determined levels and/or immune activation states of tissue CD141⁺ DCs in the subject at the first and second time points.

47. A method according to claim 46, further comprising the steps of determining the level of immune-stimulatory cells in the biological sample obtained from the subject at the first time point, determining the level of immune-stimulatory cells in the biological sample obtained from the subject at the second time point, calculating the ratios of tissue CD141⁺ DCs and Immune-stimulatory cells at the first and second time points, and comparing the calculated tissue CD141⁺ DCs/ Immune-stimulatory cells ratios of the subject at the first and second time points.

48. A method according to claim 46, further comprising the steps of determining the levels of additional immune-regulatory cells in the biological sample obtained from the subject at the first time point, determining the levels of additional immune-regulatory cells in the biological sample obtained from the subject at the second time point, and comparing the determined levels of the additional immune-regulatory cells of the subject at the first and second time points.

49. A method according to any one of claims 46 to 48, wherein the disease or condition associated with an abnormal immune response is an immune-mediated disease in which an immunosuppressive effect is required, and wherein a decreased level and/or immune activation state of tissue CD141⁺ DCs, a decreased level of the tissue CD141⁺ DCs/ immune-stimulatory cells ratio, and/or an increased level of the additional immune-stimulatory cells ratio is a positive indication of the progression of the immune-mediated disease.

50. A method according to any one of claims 46 to 48, wherein the disease or condition associated with an abnormal immune response is a disease or condition in which an immunostimulatory effect is required, and wherein an increased level and/or immune activation state of tissue CD141⁺ DC, an increased level of the tissue CD141⁺ DCs/ Immune-stimulatory cells ratio, and/or a decreased level of the additional immune-stimulatory cells is a positive indication of the progression of the disease or condition.

51. A method according to claim 44 or 49, wherein the immune-mediated disease in which an immunosuppressive effect is required is selected from hypersensitivities, autoimmune diseases, autoinflammatory diseases, transplant rejections, asthma, chronic prostatitis, glomerulonephritis, inflammatory skin diseases,

inflammatory bowel diseases, inflammatory joint diseases, inflammatory lung diseases, inflammatory brain diseases, pelvic inflammatory diseases, systemic inflammatory diseases, thyroiditis, sarcoidosis, vasculitis and interstitial cystitis.

5 52. A method according to claim 45 or 50, wherein the disease or condition in which an immunostimulatory effect is required is cancer.

10 53. A method of monitoring the efficacy of an immune therapy involving vitamin D or an analogue thereof, the method comprising the steps of determining the level and/or immune activation state of tissue CD141⁺ DCs in a biological sample obtained from a subject before the therapy, determining the level and/or immune activation state of tissue CD141⁺ DCs in a biological sample obtained from a subject after the therapy, and comparing the determined levels and/or immune activation states of tissue CD141⁺ DCs in the subject before and after the therapy, wherein an increased level and/or immune activation state of tissue CD141⁺ DCs after the therapy is a positive indication of the efficacy of the therapy.

15 54. A method according to claim 53, further comprising the steps of determining the level of immune-stimulatory cells in the biological sample obtained from the subject before the therapy, determining the level of immune-stimulatory cells in the biological sample obtained from the subject after the therapy, calculating the ratios of tissue CD141⁺ DCs and immune-stimulatory cells before and after the therapy, and comparing the calculated tissue CD141⁺ DCs/ immune-stimulatory cells ratios of the subject before and after the therapy, wherein an increased level of the tissue CD141⁺ DCs/ immune-stimulatory cells ratio after the therapy is a positive indication of the efficacy of the therapy.

20 55. A method according to claim 53, further comprising the steps of determining the levels of additional immune-regulatory cells in the biological sample obtained from the subject before the therapy, determining the levels of additional immune-regulatory cells in the biological sample obtained from the subject after the therapy, and comparing the determined levels of the additional immune-regulatory cells of the subject before and after the therapy, wherein a decreased level of the additional immune-regulatory cells is a positive indication of the efficacy of the therapy.

30 56. A method according to any one of claims 41 to 55, wherein the biological sample is a skin sample.

57. A method according to any one of claims 53 to 55, wherein the immune therapy involving vitamin D or an analogue thereof is an immune therapy involving the use of vitamin D or an analogue thereof.

35 58. A method according to any one of claims 53 to 55, wherein the immune therapy involving vitamin D or an analogue thereof is an immune therapy involving the use of means which induce vitamin D or an analogue thereof.

40 59. An *in vitro* method of obtaining tissue CD141⁺-like DCs by treating APCs with vitamin D and/or an analogue thereof.

60 An *in vitro* method of obtaining tissue CD141⁺-like DCs by treating a tissue sample with means which induce vitamin D or an analogue thereof.

5 61. An *in vitro* method of obtaining tissue CD141⁺-like DCs according to claim 60, wherein means which induce vitamin D or an analogue thereof comprises the use of UVB irradiation.

62. An *in vitro* method according to claim 59, 60 or 61, wherein the APCs are selected from blood CD1c⁺ DCs and monocyte-derived DCs.

Figure 1.

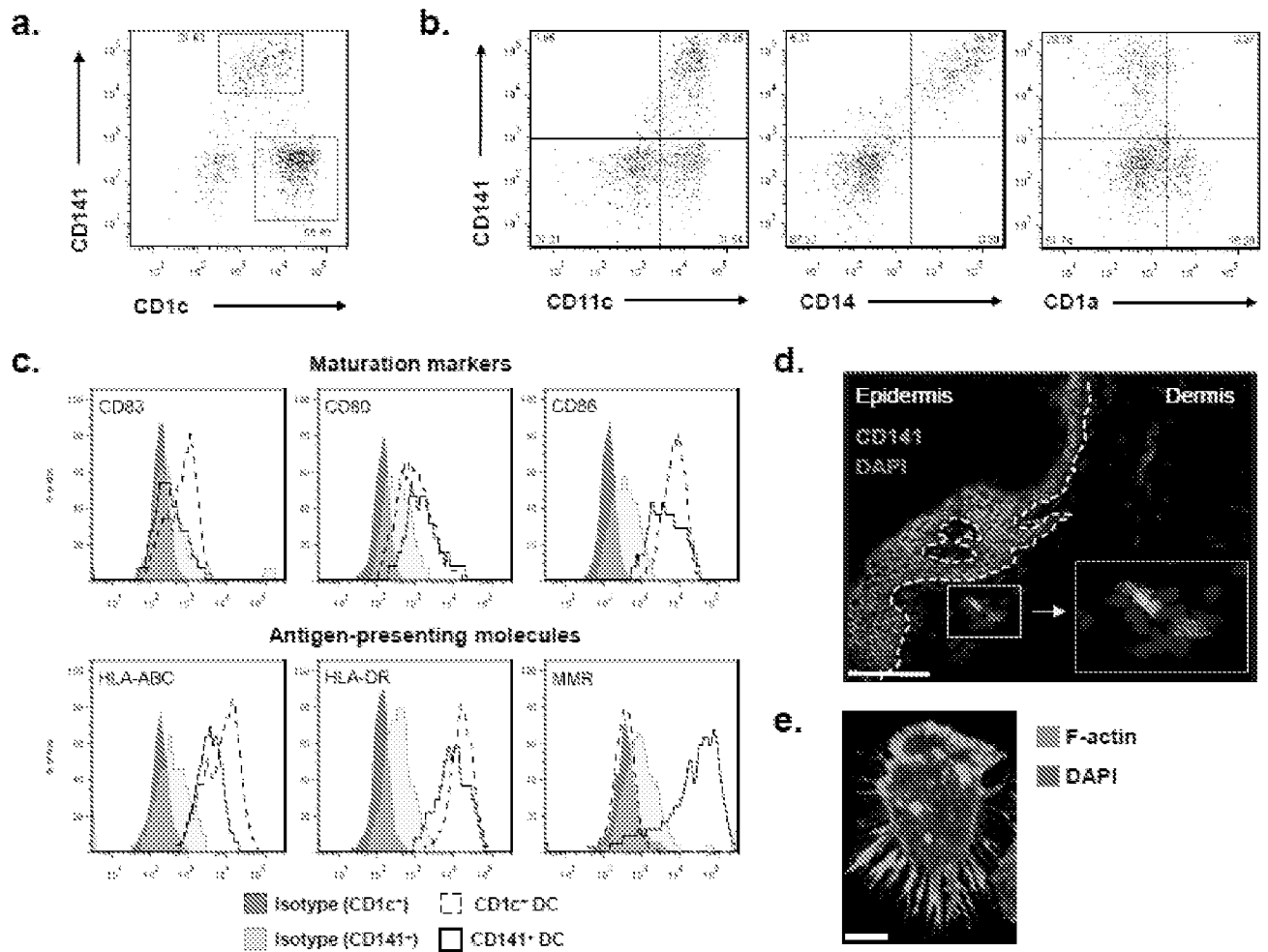


Figure 2.

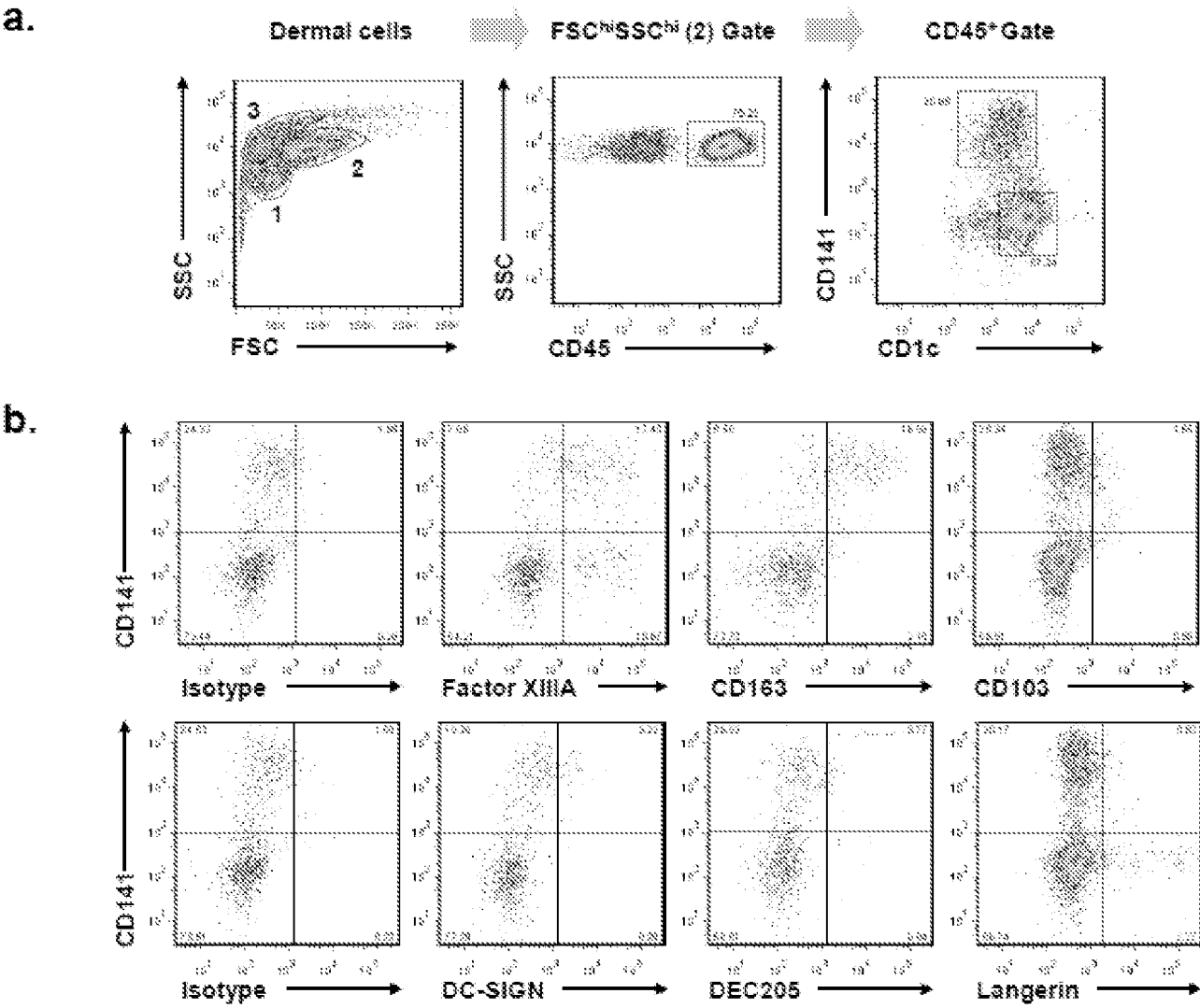


Figure 3.

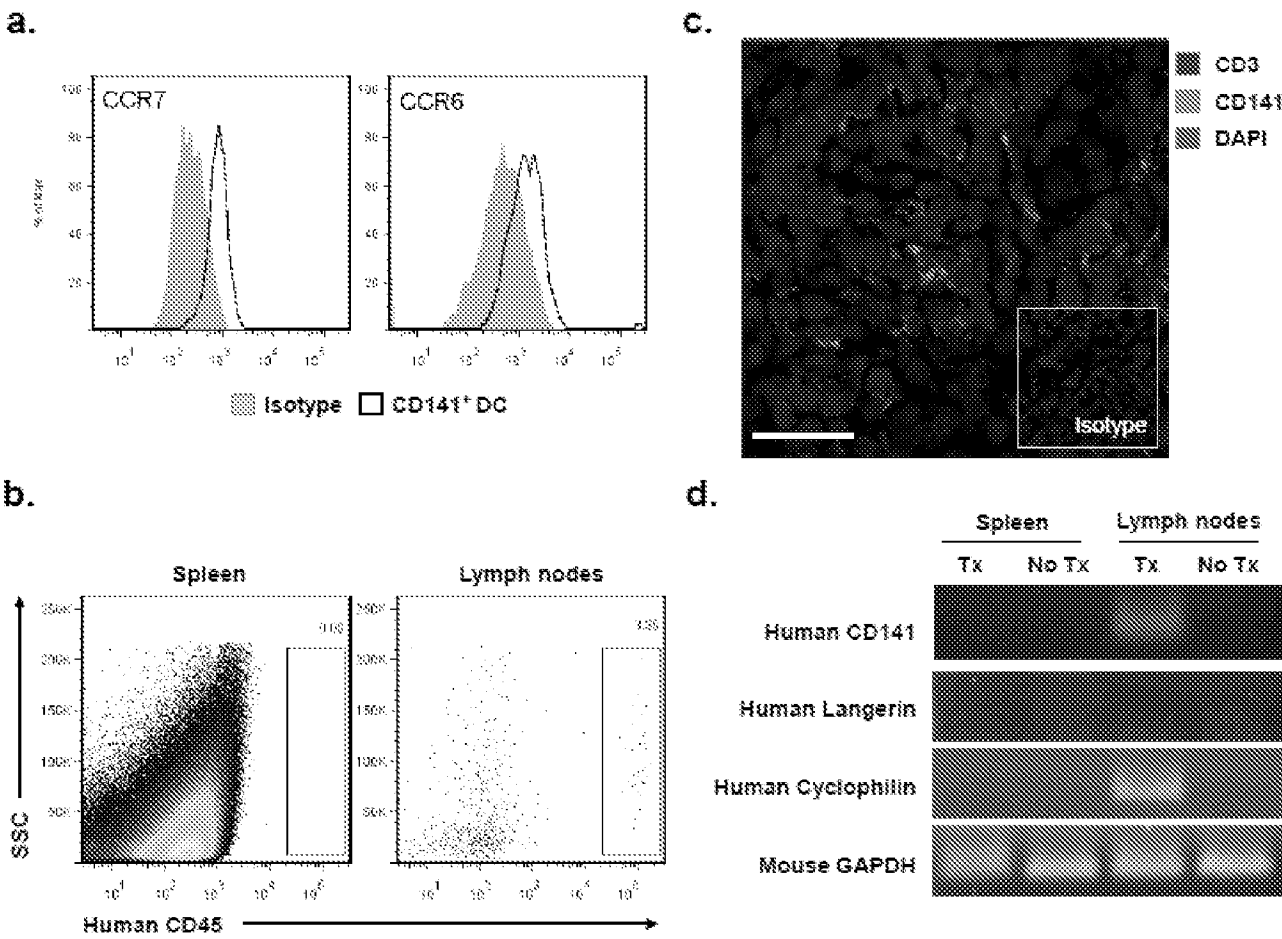


Figure 4.

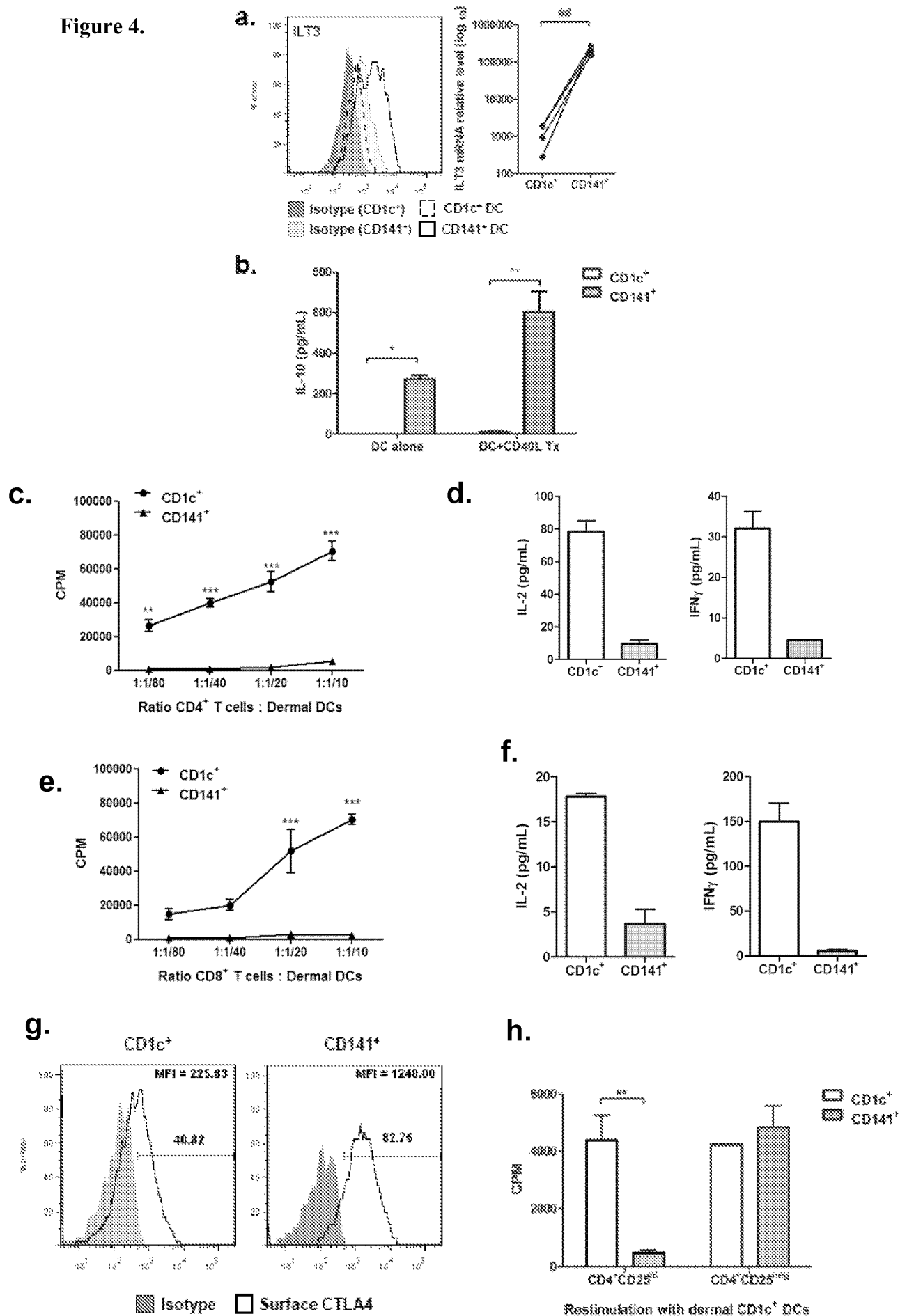


Figure 5.

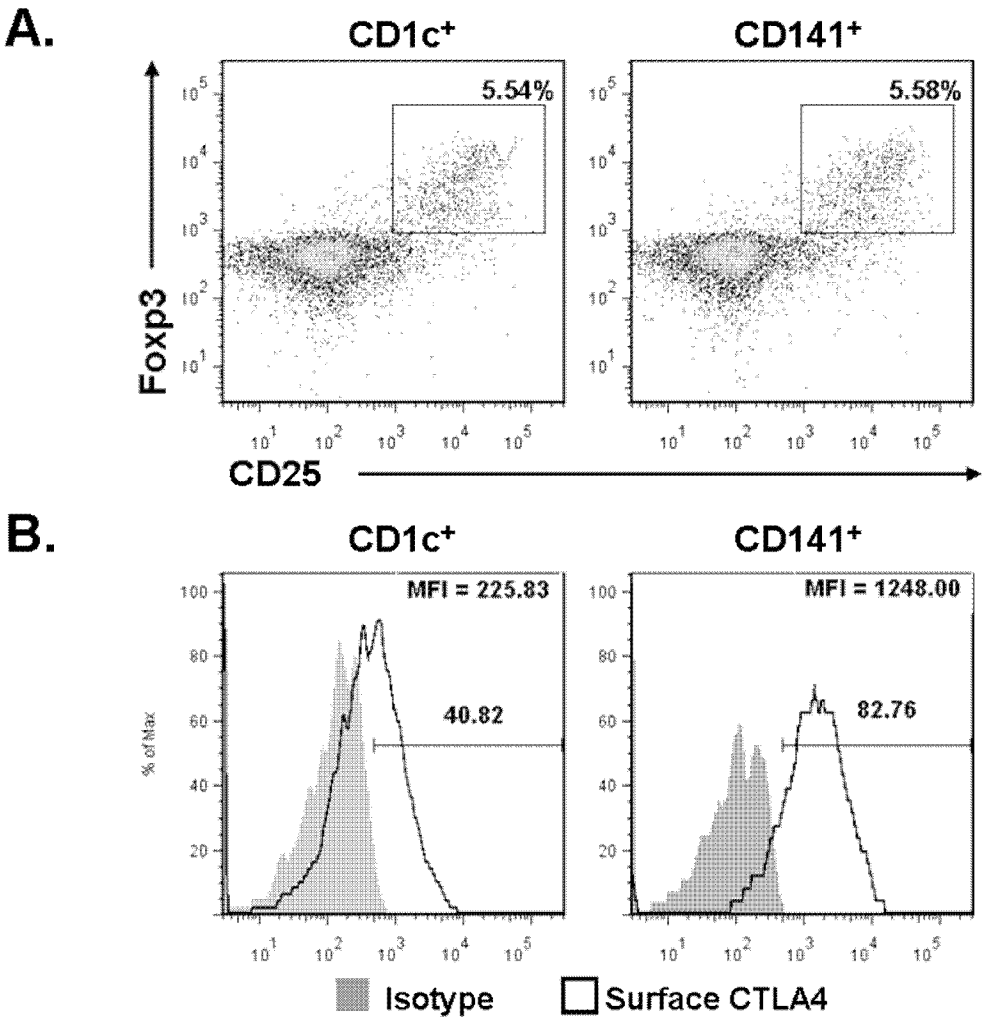


Figure 6.

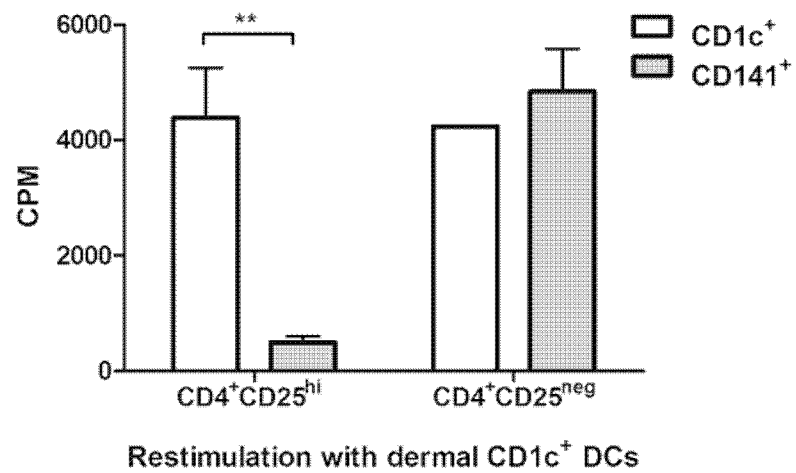


Figure 7.

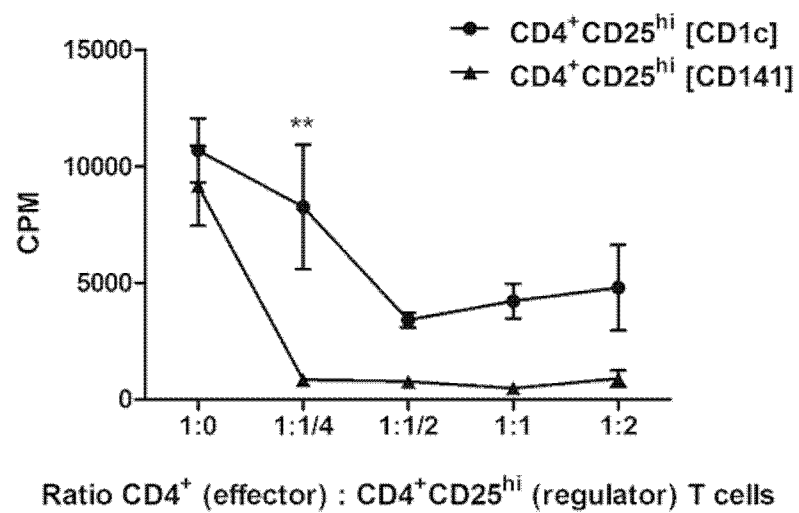


Figure 8.

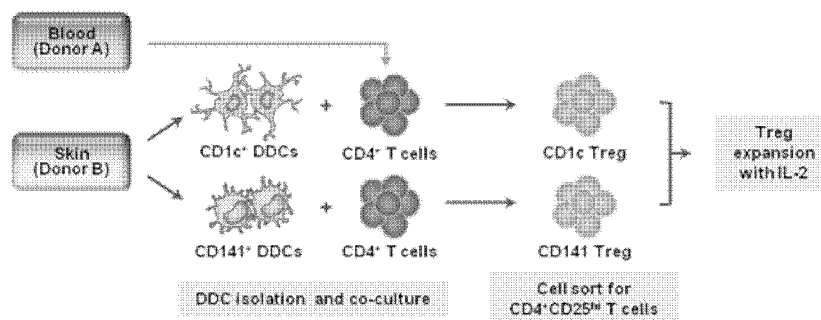
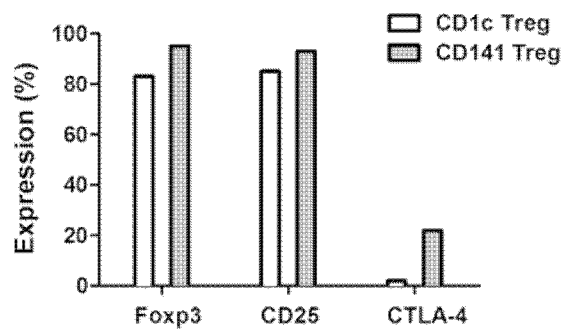
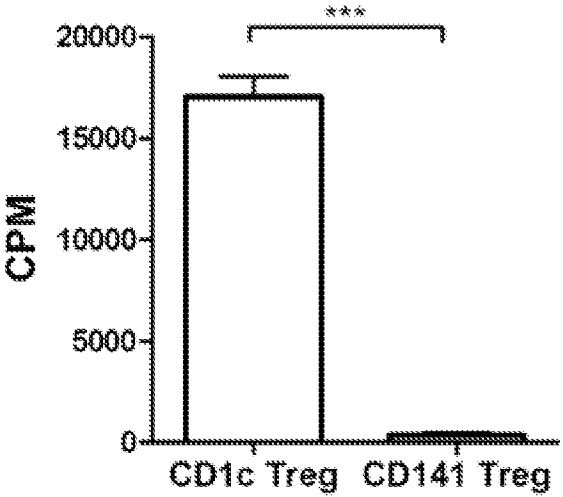
A**B**

Figure 9.

A.



B.

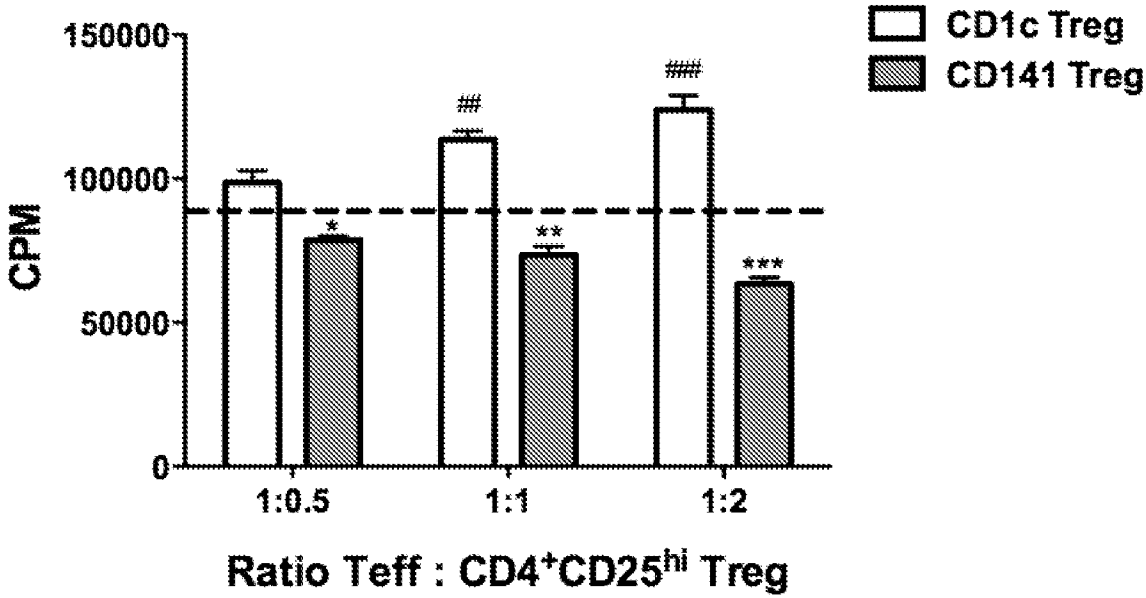


Figure 10.

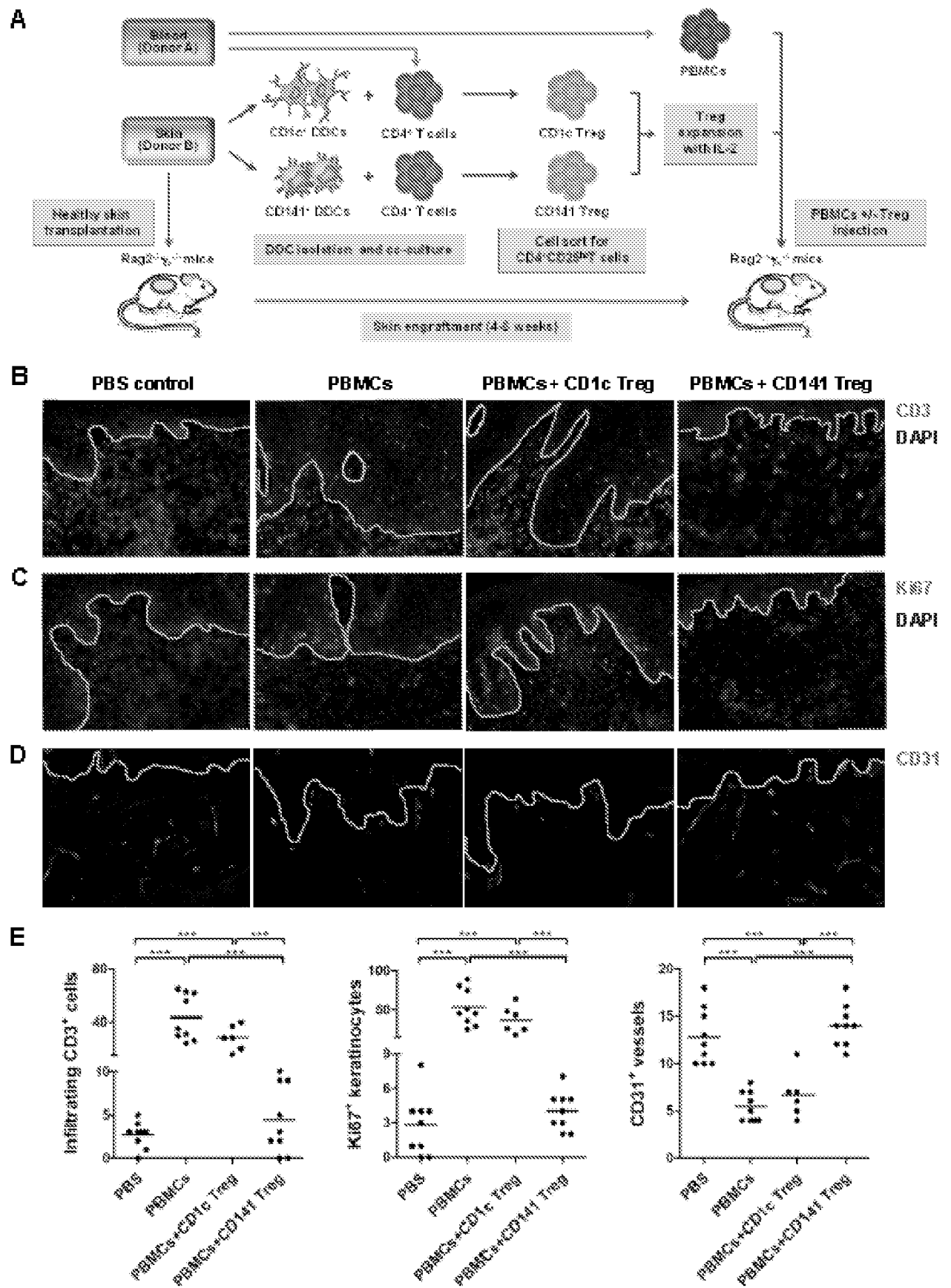


Figure 11.

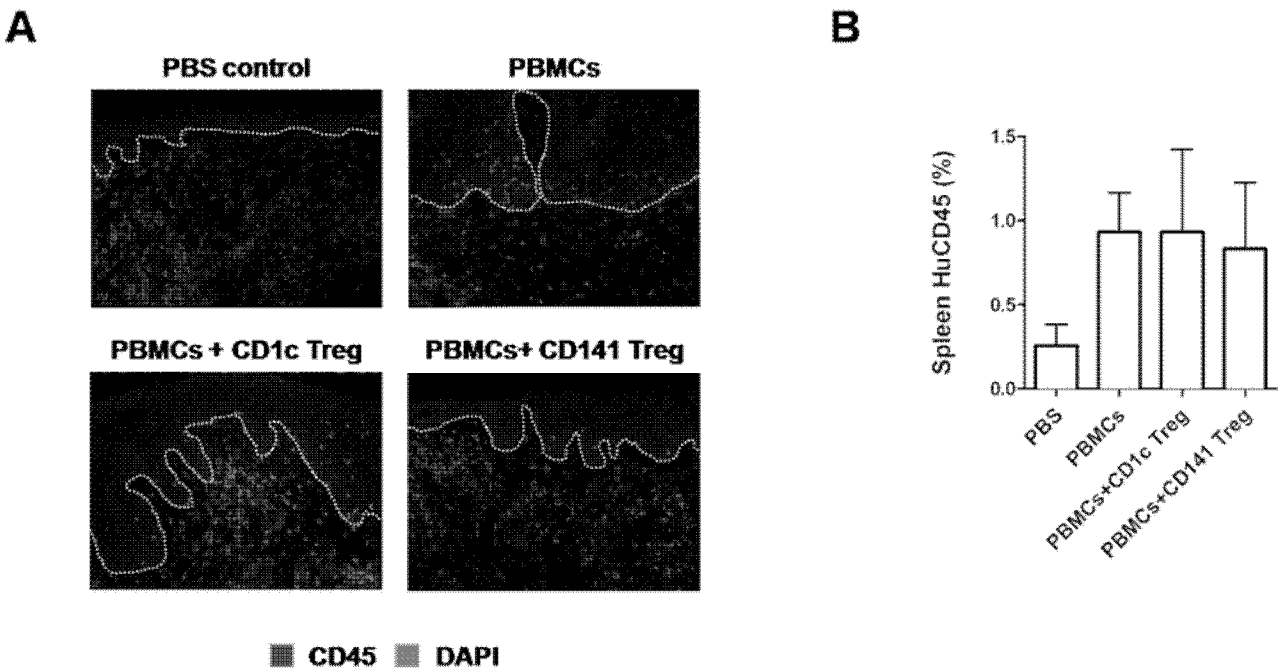


Figure 12.

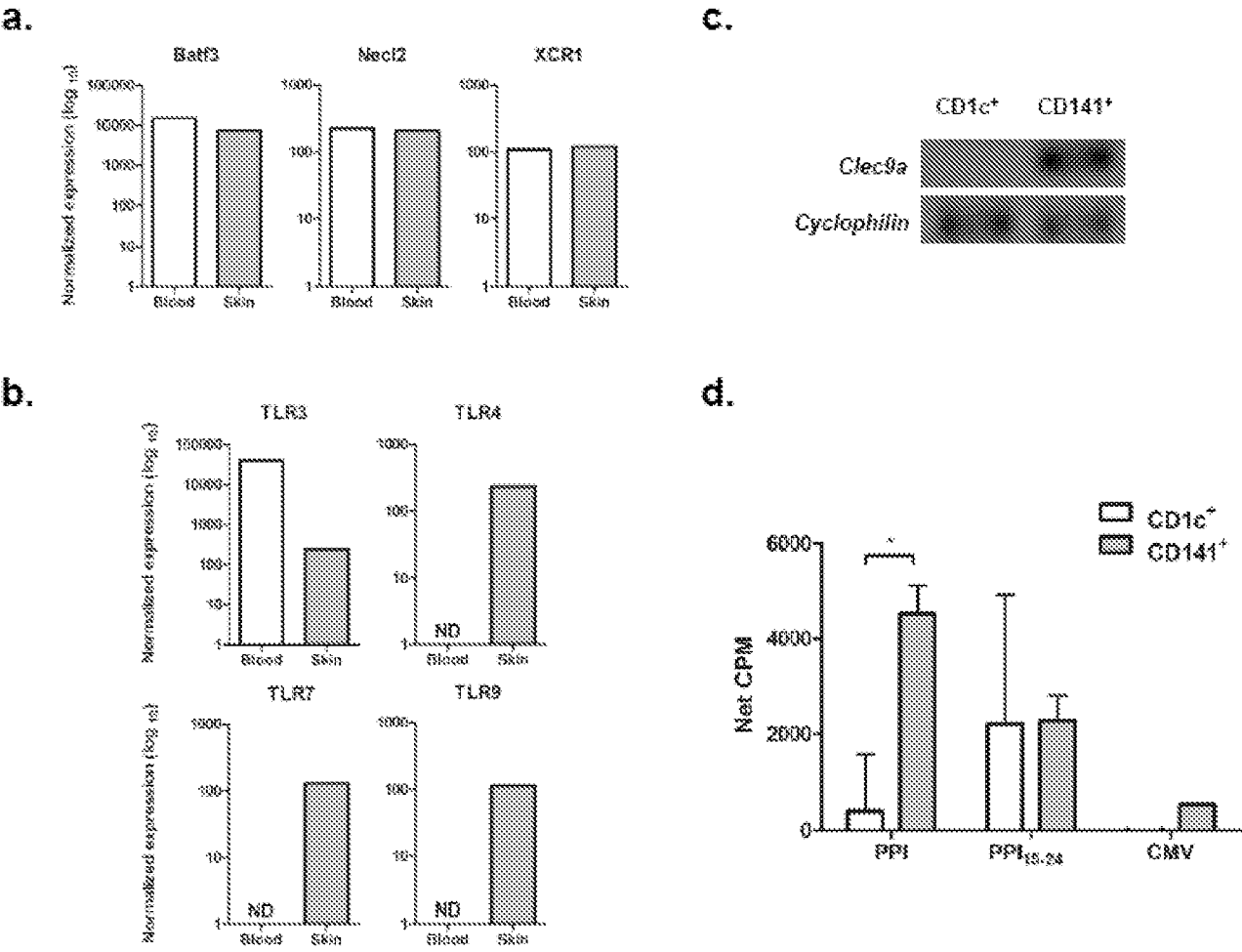


Figure 13.

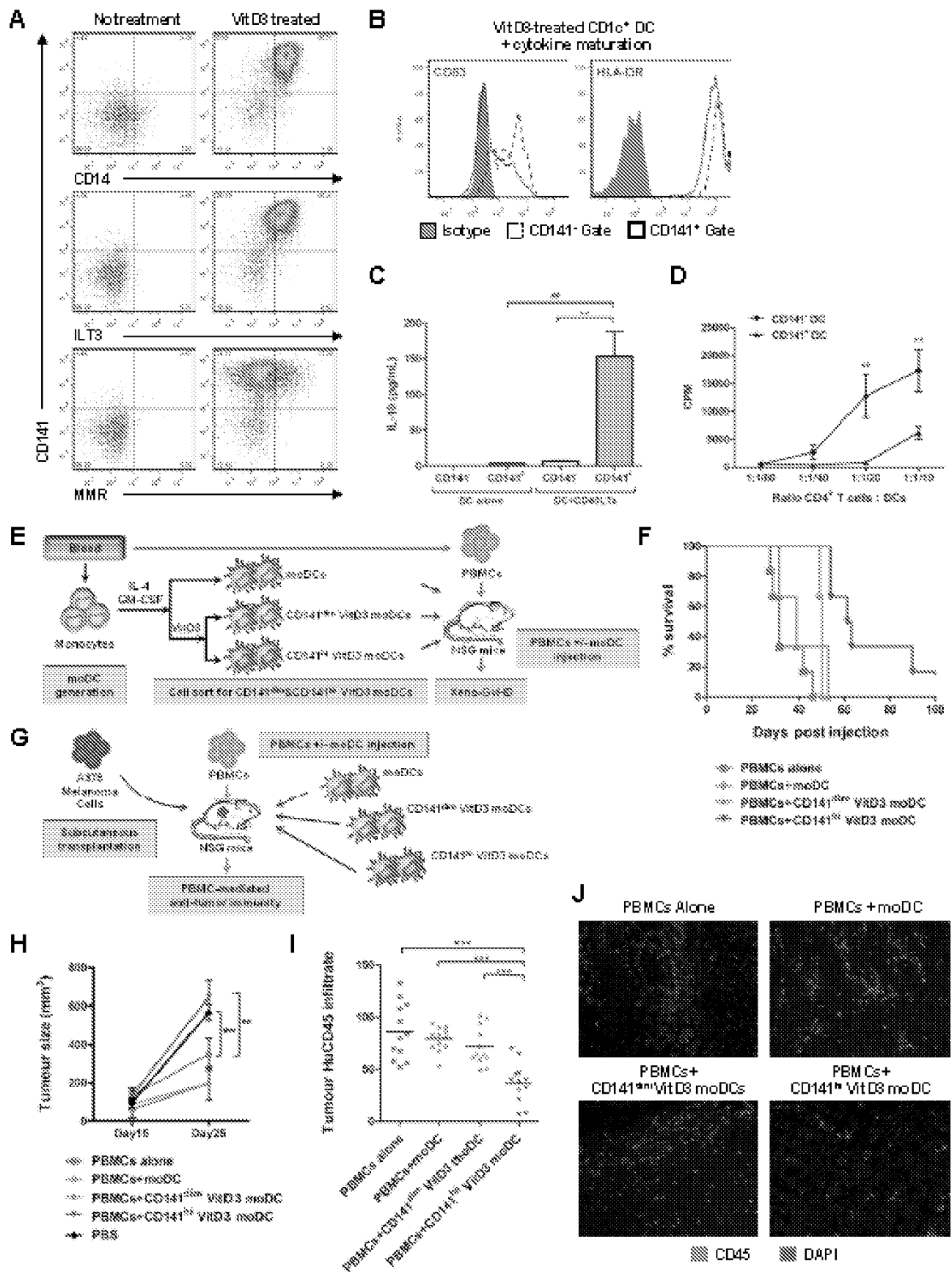


Figure 14.

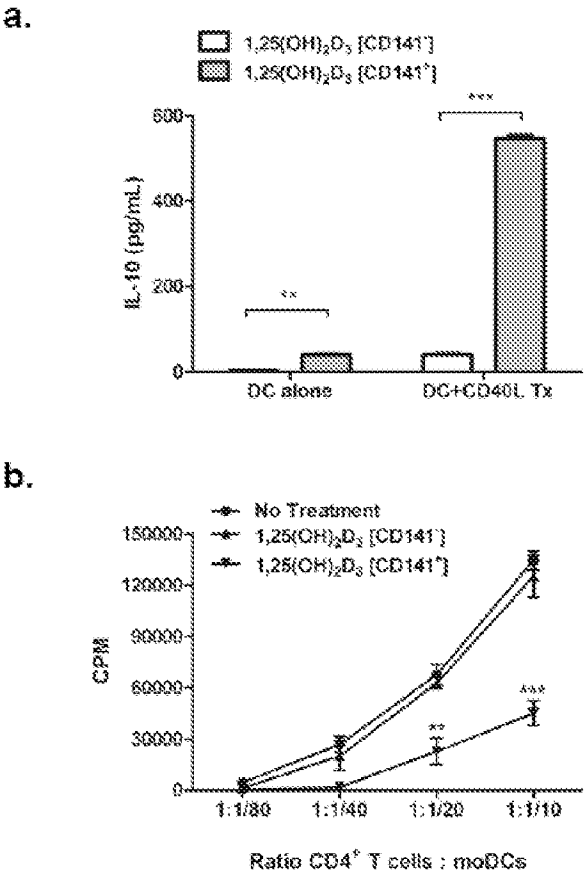


Figure 15.

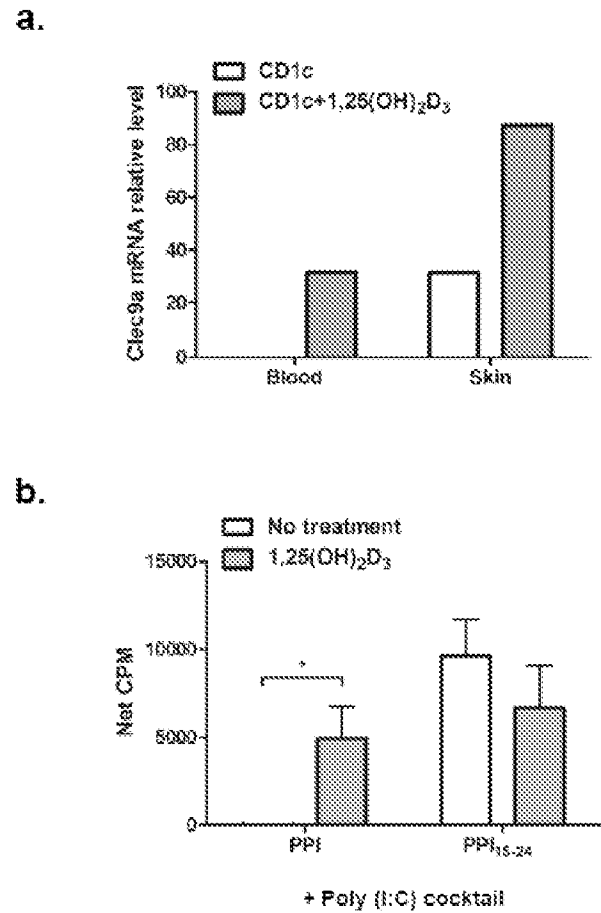


Figure 16.

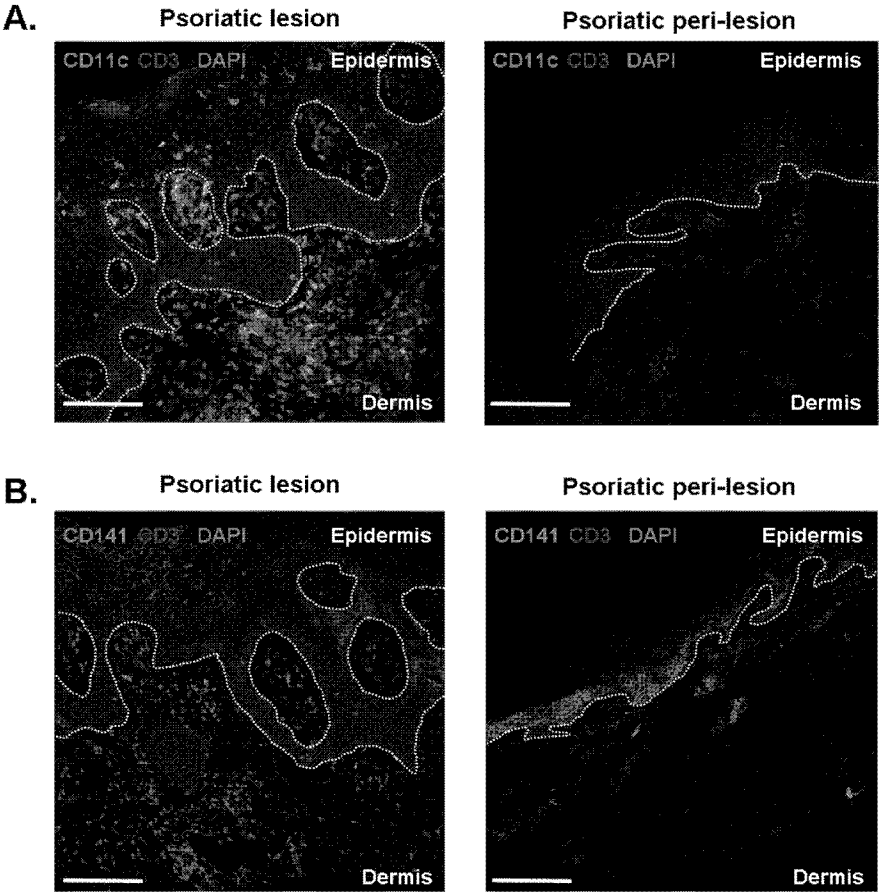


Figure 17.

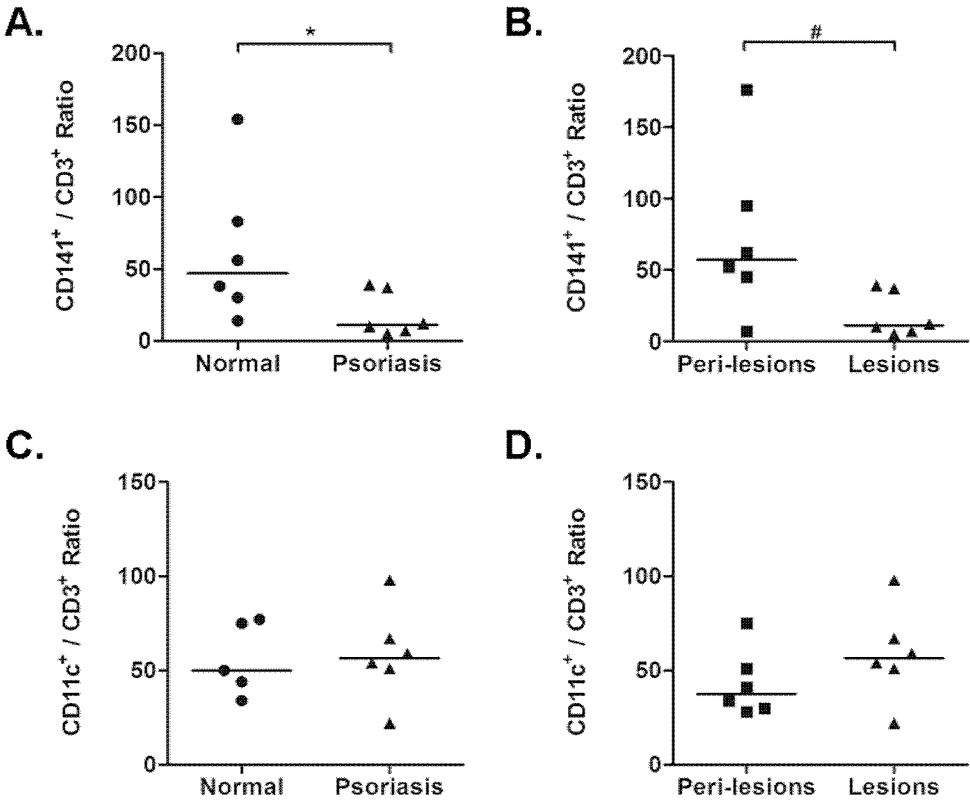


Figure 18.

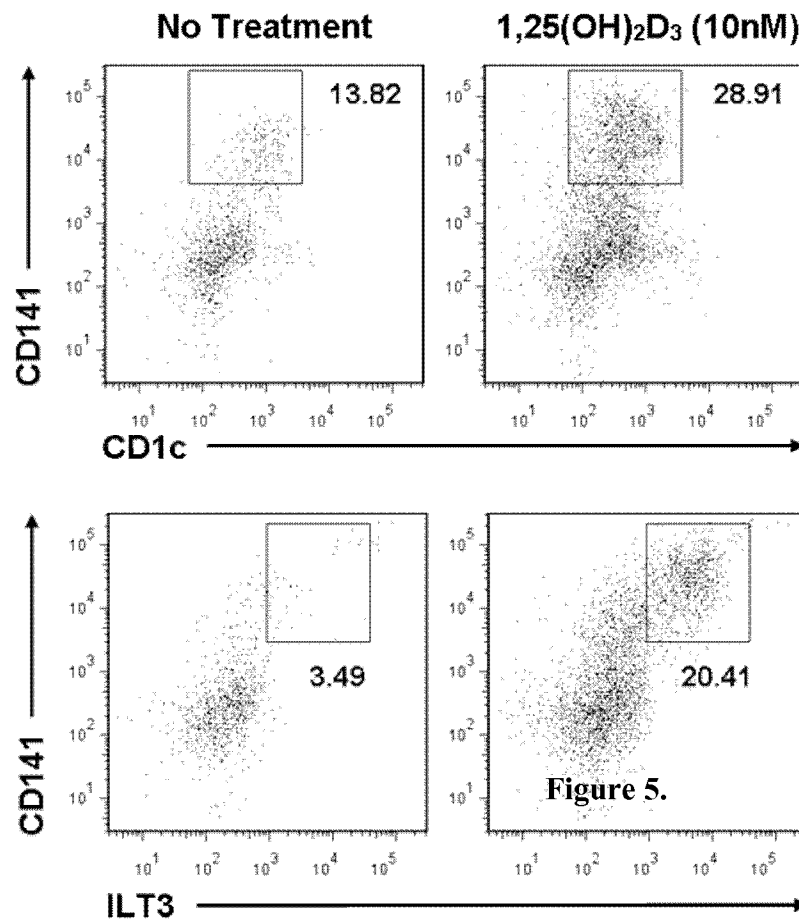


Figure 19.

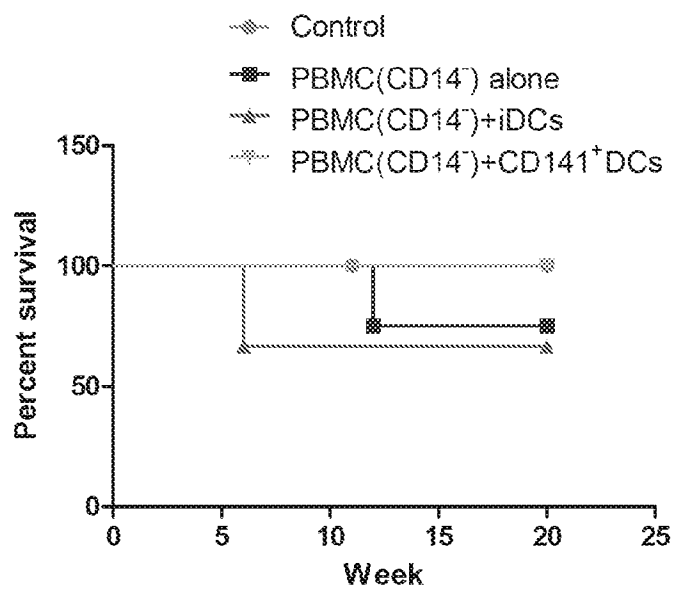


Figure 20.

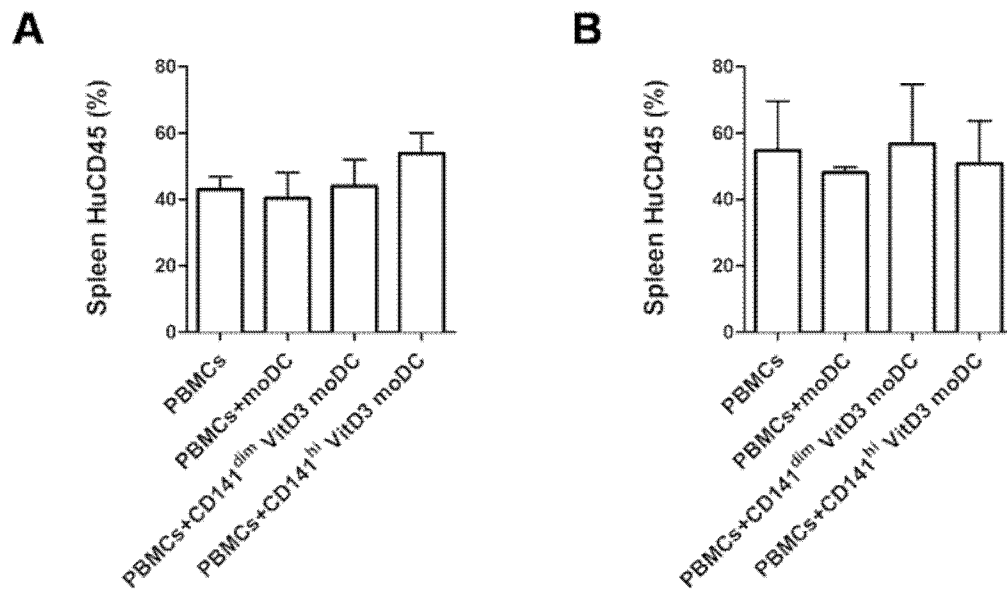
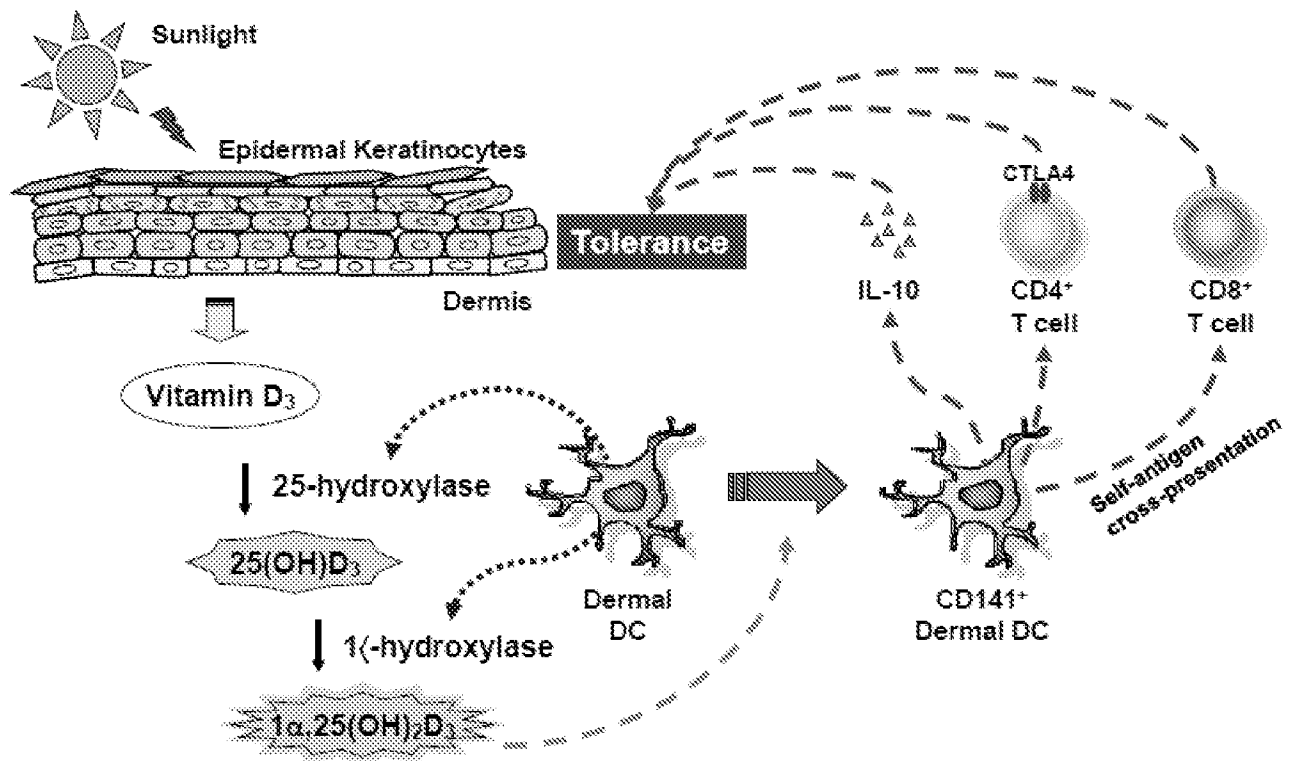


Figure 21



INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2011/052403

A. CLASSIFICATION OF SUBJECT MATTER INV. C12N5/0784 A61K35/14 A61K35/36 ADD.				
According to International Patent Classification (IPC) or to both national classification and IPC				
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C12N				
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched				
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EP0-Internal, BIOSIS, EMBASE, WPI Data				
C. DOCUMENTS CONSIDERED TO BE RELEVANT				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.		
X	PEDERSEN A W ET AL: "Phenotypic and functional markers for 1alpha,25-dihydroxyvitamin D(3)-modified regulatory dendritic cells.", CLINICAL AND EXPERIMENTAL IMMUNOLOGY JUL 2009 LNKD- PUBMED:19659770, vol. 157, no. 1, July 2009 (2009-07), pages 48-59, XP55020567, ISSN: 1365-2249 the whole document ----- -/--	1-37		
☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.				
* Special categories of cited documents : <table border="0"> <tr> <td> "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed </td> <td> "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family </td> </tr> </table>			"A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family
"A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family			
Date of the actual completion of the international search 27 April 2012		Date of mailing of the international search report 15/05/2012		
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Manu, Dominique		

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2011/052403

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PEDERSEN ANDERS ELM ET AL: "Dexamethasone/lalpha-25-dihydroxyvitamin D3-treated dendritic cells suppress colitis in the SCID T-cell transfer model.", IMMUNOLOGY JUL 2009 LNKD- PUBMED:19019085, vol. 127, no. 3, July 2009 (2009-07), pages 354-364, XP55020661, ISSN: 1365-2567 the whole document	1-37
A	----- ZABA LISA C ET AL: "Resident and "inflammatory" dendritic cells in human skin.", THE JOURNAL OF INVESTIGATIVE DERMATOLOGY FEB 2009 LNKD- PUBMED:18685620, vol. 129, no. 2, February 2009 (2009-02), pages 302-308, XP55020605, ISSN: 1523-1747 the whole document	1-37
X	----- JACOBS JOANNES F M ET AL: "Dendritic cell vaccination in combination with anti-CD25 monoclonal antibody treatment: a phase I/II study in metastatic melanoma patients.", CLINICAL CANCER RESEARCH : AN OFFICIAL JOURNAL OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH 15 OCT 2010 LNKD- PUBMED:20736326, vol. 16, no. 20, 15 October 2010 (2010-10-15), pages 5067-5078, XP55025865, ISSN: 1078-0432 the whole document	38-40
A	----- JONGBLOED SARAH L ET AL: "Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens.", THE JOURNAL OF EXPERIMENTAL MEDICINE 7 JUN 2010 LNKD- PUBMED:20479116, vol. 207, no. 6, 7 June 2010 (2010-06-07), pages 1247-1260, XP55005001, ISSN: 1540-9538 cited in the application the whole document	1-40
	----- -/--	

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2011/052403

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	CHU C -C ET AL: "Phenotypic and functional characterization of a novel subset of skin-resident regulatory CD141(+) DCs that spontaneously produce high levels of IL-10", IMMUNOLOGY, vol. 131, no. Suppl. 1, December 2010 (2010-12), pages 41-42, XP002670733, & ANNUAL CONGRESS OF THE BRITISH-SOCIETY-FOR-IMMUNOLOGY; LIVERPOOL, UK; DECEMBER 06 -10, 2010 ISSN: 0019-2805 abstract -----	1-37
X,P	CHU C ET AL: "Characterization of a novel skin resident IL-10 producing regulatory dendritic cell subset", JOURNAL OF INVESTIGATIVE DERMATOLOGY, vol. 131, no. Suppl. 1, April 2011 (2011-04), page S102, XP002670734, & ANNUAL MEETING OF THE SOCIETY-FOR-INVESTIGATIVE-DERMATOLOGY; PHOENIX, AZ, USA; MAY 04 -07, 2011 abstract -----	1-37

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2011/052403

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

1-40
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-37

Tissue(-derived) CD141+ dendritic cells (DCs) and to so called tissueCD141+-like DCs for use in treating diseases requiring immunosuppression. A method of treating or preventing an immune-mediated disease, the method comprising administering an antigen involved in the development of the immune-mediated disease to tissue CD 14 1 DCs and/or tissue CD141+- like DCs. Pharmaceutical composition or a vaccine comprising tissue CD141+ DCs or tissue CD141+-like DCs

2. claims: 38-40

A method of treating a disease requiring immunostimulation comprising depleting tissue CD141+ CDs in a subject

3. claims: 41-58

A method of predicting or diagnosing or monitoring the progression of a disease or condition associated with an abnormal immune response or a method of monitoring the efficacy of an immune therapy involving vitamin D or an analogue thereof, every method comprising the steps of determining and comparing the level and/or immune activation state of tissue CD141+ DCs in a biological sample

4. claims: 59-62

An in vitro method of obtaining tissue CD141+-like DCs by treating APCs with vitamin D and/or an analogue thereof or by treating a tissue sample with means which induce vitamin D or an analogue thereof
