DNGR-1 is a specific and universal marker of mouse and human Batf3-dependent dendritic cells in lymphoid and non-lymphoid tissues

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ABSTRACT

Mouse CD8α+ dendritic cells (DC) in lymphoid organs and CD103+ CD11b− DC in non-lymphoid tissues share phenotypic and functional similarities, as well as a unique shared developmental dependence on the transcription factor Batf3. Human DC resembling mouse CD8α+ DC in phenotype and function have been identified in human blood, spleen and tonsil. However, it is not clear whether such cells are also present in human non-lymphoid organs and their equivalence to mouse CD8α+ DC has recently been questioned. Furthermore, the identification of “CD8α+ DC-like” cells across different tissues and species remains problematic because of the lack of a unique marker that can be used to unambiguously define lineage members. Here we show that mouse CD8α+ DC and CD103+ CD11b− DC can be defined by shared high expression of DNGR-1 (CLEC9A). We further show that DNGR-1 uniquely marks a CD11b− human DC population present in both lymphoid and non-lymphoid tissues of humans and humanized mice. Finally, we demonstrate that knockdown of Batf3 selectively prevents the development of DNGR-1+ human DC in vitro. Thus, high expression of DNGR-1 specifically and universally identifies a unique DC subset in mouse and human. Evolutionarily-conserved Batf3 dependence justifies classification of DNGR-1hi DC as a distinct DC lineage.
INTRODUCTION

Dendritic cells (DCs) comprise a heterogeneous group of leukocytes with functions in both innate and adaptive immunity. In mouse, rat and human, multiple sub-types of DC can be defined in lymphoid and non-lymphoid tissues and in blood on the basis of differential expression of selected markers, as well as quantitative differences in antigen processing ability and in production of specific cytokines following stimulation. However, the relationship between different DC subsets in different tissues and the equivalence of DC sub-types across species has been difficult to establish and remains controversial. Furthermore, the increased refinement of flow cytometric approaches, allowing cross-correlation of an ever-expanding number of surface markers, has led to a marked increase in the number of putative DC subsets that can be defined phenotypically. For that reason, recent efforts have been directed towards supplementing phenotypic characterization with ontogenetic criteria, as well as using comparative analysis of global gene expression patterns, to define related DC sub-types across tissues and species.

The use of ontogenetic relationships has been successful in formally establishing the relatedness of mouse CD8α+ DC from lymphoid tissues and CD103+ CD11b- DC from non-lymphoid tissues. CD8α+ DC were originally identified as a discrete population of DC that expressed low levels of CD11b and SIRPα, high levels of CD8α, CD24, DEC-205 and DNGR-1 (CLEC9A) and variable levels of CD207 (langerin) and CD103. CD8α-expressing DC have not generally been found outside mouse lymphoid tissues. However, mouse non-lymphoid tissues include a population of CD11b- SIRPα- DC that expresses CD103, CD24 and CD207. These cells are additionally found in lymph nodes, where they are thought to have immigrated from non-lymphoid tissues via the afferent lymph. The phenotypic resemblance, as well as strong dependence on Flt3L for their development, led to the proposition that CD8α+ DC resident in lymphoid tissues and CD103+ CD11b- DC from non-lymphoid tissues and lymph nodes are in fact closely related. Consistent with that notion,
both types of DC have a similar transcriptome\textsuperscript{13} and uniquely require the transcription factors IRF8\textsuperscript{12,14}, Id2\textsuperscript{12,15} and Batf3\textsuperscript{16,17} for their development. The dependence on Batf3 is particularly remarkable as that transcription factor is expressed by other DC subtypes yet appears dispensable for their differentiation. Indeed, Batf3-deficient mice, particularly on a 129S6/SvEv background, lack CD8α\textsuperscript{+} DC from lymphoid tissues and CD103\textsuperscript{+} CD11b\textsuperscript{-} DC from non-lymphoid tissues and lymph nodes while retaining other DC types\textsuperscript{16-18}. Thus, CD103\textsuperscript{+} CD11b\textsuperscript{-} DC and CD8α\textsuperscript{+} can be argued to constitute an ontogenetically distinct lineage of Batf3-dependent “CD8α\textsuperscript{+}-like DC”.

In addition to their common ontogenetic origin, CD8α\textsuperscript{+}-like DC also share a superior ability to cross-present exogenous antigens to CD8\textsuperscript{+} T cells and play a key role in priming of CTL responses against some viruses and tumors\textsuperscript{11,16,17}. However, identification of CD8α\textsuperscript{+}-like DC in mice remains problematic and requires judicious use of flow cytometric markers. For example, CD103 levels on these cells can vary depending on the cytokine environment\textsuperscript{19-21} and loss of CD103 staining can be misinterpreted as absence of the cells in question\textsuperscript{18,22}. In addition, CD103 is highly expressed by a prominent population of CD11b\textsuperscript{+} DC in gut and mesenteric lymph nodes (MLN) that are Batf3-independent and unrelated to the CD8α\textsuperscript{+}-like DC lineage\textsuperscript{16}. Thus, there is at present a dearth of cell surface markers shared between CD8α\textsuperscript{+} DC and CD103\textsuperscript{+} CD11b\textsuperscript{-} DC that can be used to unambiguously identify the family of CD8α\textsuperscript{+}-like DC across mouse tissues.

The identification of CD8α\textsuperscript{+}-like DC in humans has been similarly problematic. We and others have recently described a discrete population of DC in human spleen, blood and tonsil that can variably be defined as DNGR-1\textsuperscript{+}, BDCA3\textsuperscript{+} or XCR-1\textsuperscript{+} and that resemble mouse lymphoid tissue CD8α\textsuperscript{+} DC in expression of selected markers and functional properties\textsuperscript{23-26}. On the basis of such correlative analyses, it has been proposed that these cells constitute the long-sought human equivalents of CD8α\textsuperscript{+} DC\textsuperscript{27}. However, this notion has been
put in doubt recently by the observation that some of the supposedly characteristic functions of these cells are shared by other human DC types. For example, a comprehensive comparison of blood and spleen human DC suggests that BDCA3+ DC do not display a higher cross-presentation capability than other human DC subsets, such as BDCA1+ DC\textsuperscript{28}. Moreover, the ontogenetic equivalence of BDCA3+ DC and mouse CD8α+ DC has been questioned by the recent report that patients harboring an autosomal dominant mutation in IRF8 display a loss of BDCA-1+ DC but not BDCA3+ DC in peripheral blood\textsuperscript{29}. Finally, there is at present no characterized equivalent of CD103+ CD11b+ DC in human non-lymphoid tissues. As such, the existence of a Batf3-dependent lineage of CD8α+ -like DC in human lymphoid and non-lymphoid tissues remains unproven.

To address these issues, we compared mouse and human DC across lymphoid and non-lymphoid tissues and tested their Batf3-dependence, as well as their ability to be systematically identified using a limited set of markers. Here we show that high expression of DNGR-1 and low expression of CD11b universally and unambiguously mark mouse and human CD8α+ -like DC in different tissues. We further show that the development of the human cells is Batf3-dependent, as in the mouse. Thus, a Batf3-dependent family of CD8α+ -like DC is found across species and can be identified uniquely and consistently by high DNGR-1 expression.

**MATERIALS AND METHODS**

**Human tissue.** Umbilical cord blood (CB) was collected from mothers attending the Royal London Hospital, London, UK, after informed consent in accordance with the Declaration of Helsinki through a protocol approved by the East London and City Research Ethics Committee. Mononuclear cells obtained by Ficoll density centrifugation and ammonium chloride red cell lysis were depleted of lineage marker positive cells using the StemStep
system (STEMCELL Technologies Inc.) to generate lineage negative (Lin–) HSCs/HPCs.

Healthy terminal ileum and MLN were obtained from patients undergoing cystectomy with neo-bladder reconstruction (n=11). All tissues were obtained with informed patient consent and in accordance with local ethical approval from the Regional Ethics committee in Lund, Sweden.

**Mice and humanized mice.** Animal protocols were approved by the London Research Institute Ethics Committee and UK Home Office (Animal Scientific Procedures Act 1986). C57BL/6 (B6) and Batf3+/− (C57BL/6 background18) and NOD/SCID/IL-2Rγ-null (NSG) mice were bred at the London Research Institute. NSG mice aged 8–12 wk were sublethally irradiated (3.75 Gy) up to 24 h before i.v. injection of 100,000 human Lin– CB cells. Mice were analyzed 7-8 weeks after reconstitution. For lentivirus infections, the Lin– cells were pre-stimulated for 4-6 h with 150 ng/ml SCF, 150 ng/ml Flt3L, 20 ng/ml G-CSF, 10 ng/ml IL-6 and 20 ng/ml TPO, and infected overnight at a multiplicity of infection (MOI) of 30 prior to transplantation.

**Lentivirus (LV) production.** Synthetic oligonucleotides corresponding to the targeting sequences were cloned into the CS-RfA-EG lentivector (a kind gift from Dr A. Miyawaki (RIKEN Tsukuba Institute). The targeting sequences were: sh-CTRL: ACGCTGAGTACTTCGAAAT (targeting luciferase) or GCTCCAAGGTGTACGTGAA (targeting red fluorescent protein); sh1-Batf3: GCTCAGAGAAGTCGGAAGA; sh2-Batf3: GCACTGAAGGGAGCAGAGA. LV were produced as previously described30.

**In vitro-differentiated DC.** Human Lin– CB cells were differentiated into DCs using a two-step protocol, as described previously26, except that round bottom well plates were used
during the differentiation culture. For LV infection, 2.5 x 10⁵ amplified cells were plated and cultured as for the differentiation step. After 1 d, medium was removed and a small volume of LV was added (final MOI of 30) and cells were incubated for 2h before adding back medium and continuing the differentiation culture. Human BMDCs were generated by culturing total bone marrow from humanized mice at 5 x 10⁵ cells/ml in the same conditions as the differentiation culture used for the CB cells.

**Flow cytometry and cell sorting.** Anti-human Abs: anti-HLA-DR (L243), anti-BDCA-3/CD141 (AD5-14H12), anti-CD11c (B-ly6), anti-CD14 (MφP9), anti-CD16 (3G8), anti-CD19 (SJ25C1), anti-CD20 (L27), and anti-CD56 (B159) were purchased from BD Biosciences. Anti-CD3 (OKT3), anti-CD103 (B-Ly7), anti-CD11b (ICRF44), and isotype-matched control antibodies were purchased from eBioscience. Anti-mouse Abs: anti-CD11c (HL3), anti-CD45.2 (104), anti-CD103 (M290), and anti-CD11b (M1/70) antibodies were all purchased from BD Biosciences. Anti-CD207 antibody (929F3) was from Dendritics and the anti-CD45 (30-F11) was from Biolegend. Anti-CD16/CD32 (93), anti-MHC class II (M5/114), and anti-CD8α antibodies were purchased from eBioscience. The anti-human DNGR-1 (8F9) and anti-mouse DNGR-1 (1F6) were described previously². Antibodies were tested for staining against appropriate negative and positive controls.

Human and mouse cells were pre-incubated on ice with mouse serum (Jackson ImmunoResearch Laboratories) plus purified IgG2a (BD), or anti-mouse CD16/CD32 (93) (eBiosciences), respectively, to block Fc receptors, and then stained with appropriate antibody combinations. Analysis was performed on an LSRII (BD) or FACSaria (BD) flow cytometer. Doublets and dead cells were excluded by a combination of scatter gating and DAPI exclusion (for unfixed cells) or a LIVE/DEAD Fixable Violet Dead-Cell Stain Kit (Molecular Probes) (for fixed cells; used for some analyses of tissues from humanized mice). Analysis was performed using FlowJo software (Tree Star, Inc.). For cell sorting (FACSaria), cells
from humanized mice were stained and live Lin\(^{-}\) (CD3/14/16/19/20/56) HLA-DR\(^{+}\) cells negative for mouse CD45 were sorted into the indicated subsets.

**Fluorescence microscopy.** Endogenous peroxidase activity of acetone-fixed human ileal cryosections (7\(\mu\)m) was blocked for 10 min with 0.5% \(\text{H}_2\text{O}_2\). Sections were then incubated with donkey serum (10%) in PBS-Tween20 (0.05%) (Sigma) and avidin-biotin blocking kit (Vector Laboratories). Tissue was then stained with mouse anti-human CD11c (10 \(\mu\)g/ml, 3.9, eBioscience) for 45 min followed by DyLight-649 labeled donkey anti-mouse antibody (Jackson ImmunoResearch Laboratories). After washing and blocking with 10% mouse serum for 30 min, sections were stained with biotinylated mouse anti-human DNGR1 (3 \(\mu\)g/ml) and FITC-conjugated mouse anti-human HLA-DR (1.25 \(\mu\)g/ml, LN3, eBioscience) in the presence of 10% mouse serum for 45 min. Slides were visualized using a biotinyl-tyramide signal amplification kit (PerkinElmer Life Science) with Alexa-555 conjugated streptavidin and Alexa-488 conjugated rabbit anti-FITC (Molecular probes). Images were acquired with an Axiovert 200M microscope (Carl Zeiss MicroImaging Inc) and Volocity 5.2.1 software (Improvision Perkin Elmer).

**DC isolation.** Mouse, and human DC, were isolated from lymphoid and non-lymphoid organs as previously described\(^{12,32}\). In brief, the organs were first cut in small pieces and incubated with a mixture of collagenase (type II for the spleen and lymph nodes or type IV for kidney and lung (Worthington Biochemical)) and of DNase (Sigma-Aldrich). After red blood cell lysis (Sigma-Aldrich), total single cell suspensions were stained for analysis.

**RNA isolation and quantitative RT-PCR.** RNA isolation, cDNA preparation and quantitative RT-PCR were as described previously\(^{26}\). Levels of mRNA for the specific gene being measured were divided by those for GAPDH (normalized expression).

**Statistical analyses.** All statistical analyses were performed using Prism 5 software (GraphPad Software, Inc.).
RESULTS

Mouse lymph nodes contain migratory DNGR-1+ CD103+ DC

Like other secondary lymphoid tissues, skin-draining lymph nodes contain resident DC with high expression of CD11c and intermediate expression of MHC class II (Figure 1A, upper left panel, gate I). Phenotypic sub-division of resident DC into CD8α+ and CD11b+ DCs (Figure 1A, upper middle panel, gate A and B), showed that only the CD8α+ subset stained for DNGR-1, as reported for spleen DC31,33 (Figure 1A, upper right panel, gate A and B). Unlike spleen, lymph nodes also contain an additional population of migratory DC (mig-DC) that can be distinguished from resident DC by their lower expression of CD11c and higher expression of MHC class II (Figure 1A, upper left panel, gate II). Mig-DC include the tissue-derived CD103+ CD11b- and CD103- CD11b+ subsets (Figure 1A, lower middle panel, gate C and D). As shown in Figure 1A, CD103+ CD11b- mig-DC also expressed high levels of DNGR-1 (lower right panel, gate C). In contrast, DNGR-1 expression was completely absent from CD103- CD11b+ DC (Figure 1A, lower right panel, gate D). When CD207 was additionally analyzed, DNGR-1+ mig-DC were clearly CD207+ (Figure 1B) even though not all CD207+ DC expressed DNGR-1: a population of CD207+ CD103- CD11b+ DC, likely corresponding to migratory Langerhans cells (LC)34, did not express DNGR-1 (Figure 1B, blue gate), consistent with the fact that anti-DNGR-1 does not stain LC in epidermis (D.S. and C.R.S., unpublished observations, April 2007). When the three markers, CD103, CD207 and DNGR-1, were analyzed simultaneously, there was a perfect correlation between expression of CD103 and DNGR-1 and all double-positive cells were CD11b- (Figure 1B, red gate). Batf3-independent CD103+ CD11b+ mig-DC in MLN did not express DNGR-1 (supplemental Figure 1). In sum, in mouse lymph nodes, high expression of DNGR-1 is found exclusively on resident CD8α+ DC and on migratory CD103+ CD11b- DC, which together comprise the Batf3-dependent CD8α+ -like DC lineage.

Batf3-dependent DNGR-1+ CD103+ CD11b- DC are found in mouse non-lymphoid tissues
To investigate DNGR-1 expression by mouse DC in peripheral tissues, we analyzed mouse kidney (Figure 2A) and lung (Figure 2B). Kidney DC were defined as CD45+ leukocytes expressing high levels of CD11c and MHC class II (Figure 2A, upper left) and were subdivided into CD103+ CD11b- DC (Figure 2A, upper middle panel, gate A) and CD103- CD11b+ DC (Figure 2A, upper middle panel, gate B)\(^12\). Consistent with the data obtained in lymph nodes, only the CD103+ CD11b- kidney DC expressed DNGR-1 (Figure 2A, upper panels). Similarly, lung DC (defined as CD45+ CD11c+ MHCII+ cells bearing low levels of autofluorescence (auto\(^{low}\)) only showed DNGR-1 staining in the CD103+ CD11b- DC subset, as recently reported\(^{35}\) (Figure 2B, upper panels). No staining was detected in DC from kidneys or lungs of Batf3\(^{-/-}\) mice, in agreement with the absence of CD103+ CD11b- DC in those animals (Figure 2A-B, lower panels). Thus, DNGR-1 specifically marks Batf3-dependent CD103+ CD11b- mouse DC in non-lymphoid tissues.

**Human DNGR-1\(^+\) BDCA3\(^+\) DCs are found in non-lymphoid tissues of humanized mice**

We have previously reported that DNGR-1 marks a human DC population in spleens of humans and in spleens of humanized mice that resembles murine spleen CD8\(^{alpha}\)+ DC in phenotype and function\(^{26}\). Given the results above showing that DNGR-1 additionally marks non-lymphoid tissue Batf3-dependent mouse DC, we analyzed a number of non-lymphoid organs from humanized mice for the presence of human DNGR-1\(^+\) DC. Dual expression of human CD11c (hCD11c) and HLA-DR was used to identify human DC in single cell suspensions from lungs (Figure 3A, upper panels) and kidneys (Figure 3A, lower panels) of mice transplanted with human cord blood (CB) hematopoietic stem cells/ hematopoietic progenitor cells (HSCs/HPCs) (Figure 3A, left panels). To refine our analysis, we used one empty channel to exclude autofluorescent cells and another channel (called “dump”) to exclude any cells that stained with human lymphoid cell-lineage markers (CD3/CD19/CD56), or that expressed mouse CD45 or that failed to exclude a cell viability fluorescent dye (Figure 3A, middle panels). Using this approach, we could find two prominent subsets of human DC in both tissues that were clearly defined by mutually exclusive expression of BDCA3 and CD11b (Figure 3A, right panels). Notably, DNGR-1 staining was found in the BDCA3\(^+\) DC
subset but not in the CD11b+ DC (Figure 3B), reminiscent of the situation in the mouse where
DNGR-1 expression is similarly restricted to the CD11b- subset (see above). Interestingly,
CD103 was not detected on either DNGR-1+ BDCA3+ or DNGR-1- BDCA3- DC (Figure 3C,
upper panel) despite the fact that the anti-CD103 mAb could clearly mark other human cell
types in the same preparation (Figure 3C, lower panel). In sum, the non-lymphoid tissues of
humanized mice contain two discrete populations of human DC that can be defined as
DNGR-1+ BDCA3+ CD11b- or DNGR-1- BDCA3- CD11b+ DC, the former of which likely
represent the human equivalent of mouse CD103+ CD11b- DC.

*Human DNGR-1+ DCs are found in human small intestine lamina propria and in gut-
associated lymphoid tissue*

To extend these results to human tissues, we analyzed samples of human small
intestine, gut-associated lymphoid tissue and mesenteric lymph node. Because of scarcity of
tissue, analysis was restricted to staining of frozen tissue sections, which permitted cross-
correlation of a more limited set of markers as compared to flow cytometry. Nevertheless, as
shown in Figure 4A-B, this approach allowed identification of discrete DNGR-1+ cells in the
sub-epithelial dome of human Peyer’s patch and the T cell areas of MLN (Figure 4A-B).
DNGR-1+ cells co-expressed HLA-DR and CD11c but, as expected, only a fraction of
CD11c+ HLA-DR+ DC labeled with anti-DNGR-1 (Figure 4A-B, arrows). Notably, DNGR-1+
CD11c+ HLA-DR+ DC were also readily observed within the small intestinal lamina propria
(Figure 4C). Therefore, DNGR-1+ DC are found in both human lymphoid and non-lymphoid
tissues.

*In vitro generated DNGR-1+ BDCA3+ CB-derived DC are Batf3-dependent*

On the basis of the above results, DNGR-1 would appear to act as a marker for the
CD8α+-like DC family across species and across tissues. This family is currently best defined
in the mouse on the basis of Batf3-dependence but this has not been established in the human.
The ability to grow DNGR-1+ BDCA3+ DC from CB HSCs/HPCs in a two-step culture (cord
blood-derived DCs; CBDCs) allowed us to investigate their ontogenetic equivalence with
the putative mouse counterparts. First, we screened ten Batf3-silencing shRNA lentivirus (LV) vector constructs bearing a puromycin selection marker and found two that appeared to selectively impair the development of puromycin-resistant DNGR-1+ DC (data not shown). We sub-cloned these two Batf3-silencing hairpins, as well as two scrambled sequence controls (CTRL), into another lentiviral vector, in which the puromycin resistance locus is replaced with a gene encoding GFP, which acts as a marker of LV-transduced cells and their progeny. We produced shRNA LVs, used them to infect CBDC differentiation cultures and monitored the development of the DNGR-1+ BDCA3+ subset. The control viruses (sh-CTRL) were used to optimize the transduction and culture protocols. We found that LV infection at 24 h after initiation of the differentiation culture reliably allowed generation of GFP+ DC, identified as live cells that lacked lineage-specific markers and expressed both GFP and HLA-DR (Figure 5A, left column). Among these DC we could identify a discrete DNGR-1+ BDCA3+ CD11b- subset (Figure 5A, upper panel; only one sh-CTRL depicted). Notably, when cultures infected with control vs. Batf3-silencing shRNA LV were compared, we found a significant and reproducible decrease in the frequency of DNGR-1+ BDCA3+ CD11b- DC among the GFP+ DC in cultures infected with the latter (Figure 5A, middle and lower panels, sh1-Batf3 and sh2-Batf3; and B, left panel). Other uncharacterized DNGR-1- BDCA3- CD11b+ DC that developed in the cultures were not impaired by Batf3-silencing and, consequently, their frequency was increased compared to the controls (Figure 5A-B, right panel). As an internal control, all cultures contained DNGR-1+ BDCA3+ DC in the GFP fraction (data not shown). In addition, LV-mediated Batf3 knockdown did not affect the growth of M-CSF- or IL-34-derived human macrophage-like cells (data not shown). In conclusion, shRNA-mediated knockdown of Batf3 selectively impairs the generation of DNGR-1+ BDCA3+ DC in vitro, establishing that these human cells are ontogenetically similar to their putative mouse counterparts.

Batf3 silencing is not sufficient to impair DNGR-1+ BDCA3+ DCs development in humanized mice
To assess the Batf3-dependence of human DNGR-1⁺ BDCA3⁺ DC in vivo, we generated humanized mice with human HSCs/HPCs that had been transduced with the different shRNA LV. To assess the stability of transduction in vivo, we isolated bone marrow from these mice at 7-8 weeks after transplantation and used it as a source of progenitors for in vitro DC differentiation (pseudo-“bone marrow-derived DCs”; BMDCs). We found that this approach allowed generation of a large fraction of GFP⁺ DC from all mice, indicating that transduced human HSCs/HPCs had successfully engrafted and retained DC-generating potential. Notably, there was a marked decrease in the frequency of DNGR-1⁺ BDCA3⁺ DC among GFP⁺ cells grown from bone marrow of mice that received human HSCs/HPCs transduced with either of the two shRNA LV targeting Batf3 displayed compared to the shRNA CTRL LV (Figure 6A-B). These results indicate that Batf3 silencing can be maintained in vivo by transduction of human HSCs/HPCs with shRNA LV and further confirm that Batf3 is required to grow DNGR-1⁺ BDCA3⁺ DC from human progenitor cells, in this case obtained from the bone marrow of humanized mice.

Given the above results, we fully expected to see a decrease in the frequency of DNGR-1⁺ BDCA3⁺ DC among the GFP⁺ DC in spleen, lung and kidney of humanized mice transplanted with Batf3-silenced human HSCs/HPCs. Surprisingly, this was not the case (Figure 7). Although there was a slight trend towards reduction (Figure 7A), pooled analyses of individual animals revealed no statistically significant difference in the overall frequency of GFP⁺ DNGR-1⁺ BDCA3⁺ DC between mice reconstituted with HSCs/HPCs transduced with sh1-Batf3 LV vs. sh-CTRL LV (Figure 7B and data not shown). To ensure that this was not due to in vivo selection of cells resistant to Batf3 knockdown, we sorted human DC from the spleens of these animals and confirmed that there was a significant reduction of Batf3 mRNA in the GFP⁺ (Figure 7C) but not the GFP⁻ (data not shown) fraction. Furthermore, we confirmed that the knockdown was functional by showing that the bone marrow of these mice still failed to generate GFP⁺ DNGR-1⁺ BDCA3⁺ DC upon ex vivo culture (see Figure 6). We conclude that Batf3 knockdown prevents development of DNGR-1⁺ BDCA3⁺ in vitro but that this can be by-passed in our humanized mouse model.
DISCUSSION

DCs are potent antigen-presenting cells involved in priming and regulating adaptive immune responses\textsuperscript{36}. In addition, DC can also play a key role in innate immunity, acting as a potent source of cytokines such as IFN-α/β or IL-12\textsuperscript{11,30}. In recent years, the heterogeneity of the DC system has become increasingly apparent\textsuperscript{2,6,37}. An attempt to make sense of DC heterogeneity and to map equivalent DC subsets across species has led to efforts to define the ontogeny and transcriptome of phenotypically-distinct DC types in the hope that they might help define related DC sub-types\textsuperscript{5,7,8,10}. This approach has led to the definition of a Batf3-dependent CD8α\textsuperscript{+} -like DC family in mouse, which encompasses the CD8α\textsuperscript{+} DC of lymphoid tissues and the CD103\textsuperscript{+} CD11b\textsuperscript{-} DC found in non-lymphoid organs. An XCR1\textsuperscript{+} DNGR-1\textsuperscript{+} BDCA-3\textsuperscript{+} subset of DC in human blood, spleen, lymph nodes and tonsil was recently described as the putative equivalent of mouse lymphoid tissue CD8α\textsuperscript{+} DC on the basis on phenotypic and functional similarity\textsuperscript{23-26}. However, whether related human DC also exist in non-lymphoid organs has not been established and the actual equivalence of DNGR-1\textsuperscript{+} human DC and mouse CD8α\textsuperscript{+} DC awaits demonstration that the former, like the latter, depend on Batf3 for their development. Furthermore, a common marker for this lineage across species has not been reported, leading to the ambiguous use of “CD8α\textsuperscript{+} -like DC” to refer to the global Batf3-dependent family of mouse DC, as well as their putative counterparts in human lymphoid organs and blood. Here, we demonstrate that high DNGR-1 expression identifies the “CD8α\textsuperscript{+} -like DC” family in both lymphoid and non-lymphoid tissues of mouse, human and humanized mouse and show that human DNGR-1\textsuperscript{+} DC require Batf3 for their development in vitro. We propose that high expression of DNGR-1 uniquely marks a Batf3-dependent DC lineage across species, which might therefore be referred to as “DNGR-1\textsuperscript{hi} DC”.

Expression of DNGR-1 in mouse spleen has previously been shown to be restricted to CD8α\textsuperscript{+} DC and pDC\textsuperscript{31,33}. The latter express lower levels of the receptor\textsuperscript{31,33}. Expression of
DNGR-1 on splenic DC is stable and the gene is not induced on other splenocytes even after LPS injection into mice\textsuperscript{38}. Here, we extended those observations to show that lymph node CD8\(\alpha^+\) DC also express high levels of DNGR-1 but that, in addition, the receptor also marks the Batf3-dependent extended CD8\(\alpha^+\) DC family, including the CD103\(^+\) CD11b\(^-\) DC in non-lymphoid tissues and their progeny among lymph node immigrant DC. As such, DNGR-1 appears similar to XCR-1, which was very recently also shown to selectively identify the Batf3-dependent DC lineage in mice\textsuperscript{13}.

We previously exploited DNGR-1 as a marker to identify human DC in human spleen and in the spleens of humanized mice that resemble mouse CD8\(\alpha^+\) DC in phenotype and function\textsuperscript{26}. Here, we show that DNGR-1 additionally marks a CD11c\(^+\) HLA-DR\(^+\) cell population in human lymph nodes and Peyer’s patches. These cells represent conventional DC, as human pDC do not express DNGR-1\textsuperscript{26,31,33,39} or CD11c\textsuperscript{40,41}. In Peyer’s patches, which have no afferent lymph supply, DNGR-1\(^+\) DC cells likely correspond to the CD11b\(^-\) resident DC in the sub-dome area that were first reported in the mouse and are thought to represent the equivalent of spleen CD8\(\alpha^+\) DC\textsuperscript{42}. In contrast, in MLN, DNGR-1 staining is expected to mark the human equivalents of both the resident and the migratory CD11b\(^-\) DC. The origin of the latter remains unclear. It has been argued that CD11b\(^-\) DC identified by flow cytometry in murine intestinal lamina propria cell preparations arise from contaminating gut associated lymphoid tissues including Peyer’s patches and isolated lymphoid follicles\textsuperscript{43}. In turn, this would suggest that DNGR-1\(^+\) CD11b\(^-\) mig-DC found in MLN immigrate from these sites. However, it remains possible that some CD11b\(^-\) DC reside in the intestinal lamina propria, an issue that cannot be resolved by flow cytometry alone. Our immunohistochemical analysis demonstrated that DNGR-1\(^+\) DC are readily identified within the intestinal lamina propria, situated within the villous core. Thus, similar to other non-lymphoid tissues, gut lamina propria contains a discrete population of DNGR-1\(^+\) CD11b\(^-\) DC. These DC may serve to sample luminal antigens or apoptotic epithelial cells and may give rise to the migratory CD8\(\alpha^+\) -like DC that subsequently drain through the afferent lymph to the MLN\textsuperscript{44}.

The presence of mouse or human DNGR-1\(^+\) DC in multiple lymphoid and non-lymphoid organs of mice, humans and humanized mice, suggests that DNGR-1 marks a
discrete population of DC that is conserved across species and tissues. Notably, we were able to show that Batf3 is required for growing human DNGR-1+ DC populations in vitro, formally establishing that Batf3 is required in human, as in mouse. This finding helps cement the equivalence between DNGR-1+ human DC and mouse CD8α+ DC. However, Batf3-dependence could not be established in vivo as DNGR-1+ DC were seen to develop normally in humanized mice from progenitors transduced with Batf3 shRNA LV. Because of scarcity of material, we did not test the functionality of the cells and therefore cannot establish if they were functionally impaired, for example in their cross-presenting activity. Nevertheless, their presence in normal frequencies may indicate that the level of transcription factor knockdown achieved with shRNA LV is not sufficient to block development of human DNGR-1+ DC in vivo, even though it suffices in vitro. An alternative explanation is that Batf3-dependence may be reduced or by-passed altogether in humanized mice under the influence of factors absent from our DC differentiation cultures. It is notable that, even in mouse, CD8α+ DC in some organs can develop independently of Batf3, a fact that became especially apparent when Batf3-deficient mice, originally made in a 129/SvEv background, were backcrossed to the C57BL/6 strain. In addition to Batf3, CD8α+ DC development in the mouse depends on NFIL3/E4BP4, a transcription factor that acts upstream of Batf3, as well as on Id2 and IRF8. Interestingly, BDCA-3+ DC are still found in the blood of two patients bearing an autosomal dominant mutation in IRF8. Thus, it is possible that partial abrogation of IRF8 function caused by the human mutation, which likely generates a dominant negative IRF8, as well as partial Batf3 knockdown, as shown here, is not sufficient to abrogate development of DNGR-1+ DC in vivo even if it does so under more stringent conditions in vitro. It would be interesting to combine knockdown of Batf3 with knockdown of NFIL3/E4BP4, IRF8 and Id2 to see if any such combination abrogates DNGR-1+ DC development in humanized mice.

Humanized mice offer an unprecedented tool for studying the human immune system in vivo. However, the development of human DC in such mice may be limited by particular mouse factors that do not cross-react with human receptors. For example, human GM-CSF receptor cannot respond to mouse GM-CSF. As GM-CSF induces CD103 expression by mouse CD8α+ -like DC, this could explain why we do not detect CD103 on DNGR-1+
human DC in humanized mice. Flow cytometric analysis of the same cells isolated from human tissues will be necessary to establish whether CD103 can act as a marker for the CD8α⁺-like DC lineage in humans. Similarly, whether DNGR-1⁺ human DC can express CD8α in some situations remains to be determined. Independently of additional markers, our current data clearly identify DNGR-1 as a specific and universal identifier of the Batf3-dependent CD8α⁺ DC family across mouse and human. The ability to use anti-DNGR-1 to identify these cells in situ thus opens the door to further characterizing these cells in different human tissues, both in normal and pathological conditions. Finally, the identification of a common marker and the ontogenetic verification of a common DC lineage in both mice and humans underscores the usefulness of mouse models of DC function and reinforces the notion that antigen targeting to DNGR-1, which has been so successful in mice⁴⁻¹, may yet serve as a useful strategy for immune modulation in Man⁴⁹.

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AUTHORSHIP

L.F.P., Y.R, H.U-H., and B.S. performed the experiments and analyzed data; L.F.P, Y.R., H.

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**REFERENCES**

FIGURE LEGENDS

Figure 1. Mouse lymph nodes contain resident and migratory DNGR-1⁺ CD8α⁺-like DC. (A) Flow cytometry analysis of cutaneous draining lymph nodes cell suspensions from B6 mouse. Live cells were analyzed for the expression of CD11c versus MHCII (upper right panel). Two populations were defined (gate I: CD11c<sup>high</sup> MHCII<sup>inter</sup> tissue-resident DC; gate II : CD11c<sup>inter</sup> to <sup>high</sup> MHCII<sup>high</sup> migratory DC) and analyzed for the expression of, respectively, CD8 versus CD11b (for gate I), or CD103 versus CD11b (for gate II) (upper left panels). Four populations were defined (gate A: CD8⁺, CD11b⁻; gate B: CD8⁻, CD11b⁺; gate C: CD103⁺, CD11b⁺; gate D: CD103⁻, CD11b⁺) and analyzed for the expression of DNGR-1 (red) or for an isotype control Ab (blue). (B) CD11c<sup>inter</sup> to <sup>high</sup> MHCII<sup>high</sup> migratory DCs were analyzed for the expression of CD207 versus DNGR-1 (upper left panel). Two populations were defined (blue gate: CD207⁺, DNGR-1⁻; red gate: CD207⁺, DNGR-1⁺) and analyzed for the expression of CD103 versus CD11b. Migratory DC were analyzed also for the expression of CD103 versus DNGR-1 (lower left panel). Two populations were defined (blue gate: CD103⁻, DNGR-1⁺; red gate: CD103⁺, DNGR-1⁻) and analyzed for the expression of CD207 and CD11b. Numbers indicate percentage of cells in each of the indicated gates. Arrows show gating strategy. Data in A and B are representative of two independent experiments.

Figure 2. DNGR-1⁺ CD103⁺ CD11b⁻ DC are found in mouse non-lymphoid tissues. (A) CD11c⁺ MHCII⁺ CD45⁺ live kidney cells from Batf3-sufficient mice (B6) (upper left panel) or Batf3⁻/⁻ mice (lower left panel) were analyzed as in Figure 1 for the expression of CD103 versus CD11b. For the Batf3⁺/⁺ mice two populations were defined (gate A: CD103⁺, CD11b⁻; gate B: CD103⁻, CD11b⁺), and for the Batf3⁻/⁻ mice only one dendritic cell gate was defined encompassing gate A and B. Cells within these gates were analyzed for staining with anti-
DNGR-1 (red) or an isotype-matched control Ab (blue). (B) CD11c+ MHCII+ CD45+ auto^low live lung cells were analyzed as described for the kidney. Numbers indicate percentage of cells in each of the indicated gates. Arrows show gating strategy. Data in A and B are representative of two independent experiments.

**Figure 3. DNGR-1^+ BDCA3^+ DCs are found in non-lymphoid tissues of humanized mice.**
(A) hCD11c^+ HLA-DR^+ lung (upper left panel) or kidney (lower left panel) cells were analyzed as shown for the expression of BDCA3 versus CD11b (right panel), after having gated out Dump^+ auto^+ cells (middle panels). Two populations were defined (gate I: BDCA3^+, CD11b^−; gate II: BDCA3^−, CD11b^+). The Dump channel contains the following stainings grouped together: a Lin cocktail (anti-human CD3/CD19/CD56), an anti-mouse CD45, and a viability marker. (B) Lung (upper panels) and kidney (lower panel) BDCA3^+ (left panels) or CD11b^+ (right panels) cells, as defined in (A) by gates I and II, were analyzed for the expression of DNGR-1 (red) or for an isotype control Ab (blue). (C) hCD11c^+ HLA-DR^+ BDCA3^+ lung cells, as gated in (A), expressing DNGR-1^+ (upper left) or not (upper right) were analyzed for CD103 expression (red) or for an isotype control Ab (blue). As an internal control, total lung cells were also analyzed for these markers versus the Dump channel (bottom). Numbers indicate percentage of cells in each of the indicated gates. Arrows show gating strategy. Data in A and B are representative of at least 3 independent experiments.

**Figure 4. DNGR-1^+ DCs are found in human small intestinal lamina propria, MLN and Peyer’s patches.**

Three-color immunofluorescence staining for DNGR1, CD11c and HLA-DR in cryosections of human intestinal tissue. The arrowheads indicate DNGR1^+ CD11c^+ HLA-DR^+ cells in (A)
Peyer’s patches n=3, (B) mesenteric lymph nodes n=3 and (C) intestinal lamina propria n=6.

Scale bars: (A) 63\(\mu\)m (B-C) 31\(\mu\)m

**Figure 5. In vitro generated DNGR-1\(^+\) BDCA3\(^+\) cord blood-derived DC are Batf3-dependent.** CBDC were generated and infected with different sh-RNA LVs as described in the Materials and Methods. (A) Lin\(^-\) (CD3/14/16/19/20/56) live cells were analyzed for the expression of HLA-DR and GFP. HLR-DR\(^+\) GFP\(^+\) cells were analyzed for the expression DNGR-1 versus CD11b, BDCA3 versus DNGR-1, and BDCA3 versus CD11b. (B) Frequency of DNGR-1\(^+\) BDCA3\(^+\) GFP\(^+\) (left panel) and of CD11b\(^+\) GFP\(^+\) cells (right panel) is depicted. Data in A-B are representative of multiple CBDC cultures with 2 independent pools of cord blood-derived HSCs/HPCs.

**Figure 6. Ex vivo generated DNGR-1\(^+\) BDCA3\(^+\) from humanized mice are Batf3-dependent.** (A) BMDCs were generated as described in the Material and Methods from bone marrow of humanized mice and analyzed as in Figure 5. (B) Frequency of DNGR-1\(^+\) BDCA3\(^+\) GFP\(^+\) (left panel) and of CD11b\(^+\) GFP\(^+\) cells (right panel) is depicted. Numbers indicate percentage of cells in each of the indicated gates. One representative experiment out of two is shown in A, with 4 mice for each sh-RNA. Data in B are mean ± SEM of two independent experiments. *P<0.05, **P<0.01 ***P<0.001; Mann-Whitney test. sh-CTRL, control.

**Figure 7. Batf3-silencing is not sufficient to impair DNGR-1\(^+\) BDCA3\(^+\) DCs development in humanized mice.** (A) CD11c\(^+\) HLA-DR\(^+\) live cells (same as in Figure 3) were analyzed for the expression of BDCA3 versus CD11b (upper panel) among the GFP\(^+\) cells. Two
populations were identified (gate I: BDCA3⁺, CD11b⁻; gate II: BDCA3⁻, CD11b⁺). Numbers indicate percentage of cells in each of the indicated gates. (B) Frequency of BDCA3⁺ GFP⁺ (upper panel) and of CD11b⁺ GFP⁺ cells (lower panel) is depicted. (C) Normalized expression of Batf3 within Lin⁻ (CD3/14/16/19/20/56) HLA-DR⁺ GFP⁺ live cells, purified from the spleen of the humanized mice. In A and C, data are representative of 2 independent experiments, with 4 mice for each sh-RNA. Data in B are mean ± SEM of two independent experiments. *P<0.05, Mann-Whitney test. sh-CTRL, control. Sp, spleen; Lg, lung; and K, kidney.
mouse cutaneous LNs

A

Tissue-resident

MHC-II

CD11c

CD8

CD11b

0.619

0.7

II

I

B

Migratory DCs

CD103

CD11b

19.5

B

25.3

23

DNGR-1

CD11b

CD8

CD103

CD11b

22.2

16.3

25.8

64.4

CD11b

CD103

CD207

DNGR-1

CD11b

CD103

CD207

DNGR-1

CD11b

CD8

CD103

CD11b

25.3

33

DNGR-1

isotype

DNGR-1

isotype
mouse tissues

A Kidney

Batf3\(^{+/+}\)

CD45\(^{+}\)

CD11c

CD11b

CD103

CD11c\(^{+}\) MHCII\(^{+}\)

16.2

28.2

16.8

10.8

33.4

CD11b

Dendritic cells

Batf3\(^{-/-}\)

CD45\(^{+}\)

CD11c

CD11b

CD103

CD11c\(^{+}\) MHCII\(^{+}\)

16

28.7

1.02

28.5

Dendritic cells

B Lung

Batf3\(^{+/+}\)

CD45\(^{+}\) CD11c\(^{-}\)

MHCII

auto

16.2

45.9

CD11b

Dendritic cells

Batf3\(^{-/-}\)

CD45\(^{+}\) CD11c\(^{-}\)

MHCII auto\(^{low}\)

CD11b

Dendritic cells

CD11b

CD103

A

B

CD103

CD11b

CD45

CD11c

MHCII

autolow

Dendritic cells

CD11b

CD103

A

B

CD11b

CD45

CD11c

MHCII

autolow

Dendritic cells

CD11b

CD45

CD11c

MHCII

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autolow

Dendritic cells

CD11b

CD45

CD11c

MHCII

autolow

Dendritic cells

CD11b

CD45

CD11c

MHCII

autolow

Dendritic cells

CD11b
humanized tissues

A

Lung

hCD11c+ HLA-DR+

BDCA3+

CD11b+

auto

CD11b

hCD11c+ HLA-DR+

BDCA3+

CD11b+

auto

CD11b

Kidney

hCD11c+ HLA-DR+

BDCA3+

CD11b+

auto

CD11b

BDCA3+

BDCA3- DNGR-1-

Lung

hCD11c+ HLA-DR+

BDCA3+

CD103

hCD11c+ HLA-DR+

BDCA3+

CD103

isotype

hCD11c

BDCA3

isotype

BDCA3

DNGR-1

DNGR-1

Total lung cells

isotype

0.394

0.39e-3

CD103

isotype

0.394

0.39e-3

For personal use only.on July 14, 2017. by guest
in vitro CBDCs

A

Live cells Lin−

sh-CTRL

HLA-DR+ GFP+

DNGR-1+ BDCA3+ GFP+

sh1-Batf3

sh2-Batf3

B

Frequency of DNGR-1+ BDCA3+ GFP+

Frequency of CD11b+ GFP+
DNGR-1 is a specific and universal marker of mouse and human Batf3-dependent dendritic cells in lymphoid and non-lymphoid tissues

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