CD103 MARKS A SUBSET OF HUMAN CD34⁺-DERIVED LANGERIN⁺ DENDRITIC CELLS WHICH INDUCE T REGULATORY CELLS VIA INDOLEAMINE 2,3-DIOXYGENASE-1

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Abstract

Indoleamine 2,3-dioxygenase 1 (IDO1) is an immunosuppressive molecule expressed in some subsets of normal and neoplastic cells. Mature human dendritic cells (DCs) have been shown to express IDO1, but little is known about its expression and function during DC differentiation from bone marrow (BM) hematopoietic stem/progenitor cells (HSPCs). Here, we show that during \textit{in vitro} differentiation along myeloid DC lineage CD34\(^+\) HSPCs acquire IDO1 expression, which acts in a tolerogenic manner by inducing a population of fully functional CD4\(^+\)CD25\(^+\) FOXP3\(^+\) T regulatory cells (T\(_{\text{reg}}\)). Phenotypically, CD1a\(^+\)CD14\(^-\) HPSC-derived DCs expressed IDO1, Langerin, CD11b, and CD1c. Cell sorting experiments demonstrated that IDO1 expression is found in a subset of CD1a\(^+\)CD14\(^-\)Langerin\(^+\) cells, expressing CD103, which is capable of inducing T\(_{\text{reg}}\) through an IDO1-dependent manner. In conclusion, DC differentiation from CD34\(^+\) HSPCs results in the expression of a functionally active IDO1 protein in CD1a\(^+\)Langerin\(^+\), CD103-expressing DCs. These data point toward IDO1 expression as part of a tolerogenic signature during DC development.

**Keywords:** Human Dendritic Cells; myeloid differentiation; indoleamine 2,3-dioxygenase; regulatory T cells; immune response
Introduction

Dendritic cells (DCs) are rare, heterogeneous professional antigen-presenting cells (APCs), which regulate the balance between immunity and tolerance, thus critically directing antigen (Ag)-specific immune response [1]. In addition to their classical and well-established function as initiator of immune response, more recent studies focused on the role and function of tolerogenic DCs, which are important in the induction and maintenance of peripheral immune tolerance through a variety of mechanisms, including the generation, de novo, of T regulatory cells (Tregs) [1,2]. Both immunogenic and tolerogenic DCs arise from hematopoietic stem/progenitor cells (HSPCs) in the bone marrow (BM) through specialized myeloid and lymphoid progenitor subsets [3]. Although it is clear that DC function is influenced within peripheral tissues by inflammatory microenvironment, which may switch activatory into immunoregulatory DCs and vice versa [4], it remains to be elucidated whether during DC lineage differentiation BM precursors become committed to tolerogenic or activatory DCs. Particularly, it remains unknown whether specific functional pathways are expressed and activated at the level of BM DC precursors. Human CD34+ HSPCs may in vitro differentiate into DCs through an intermediate BM precursor, which is defined on the basis of CD1a and CD14 expression as CD1a+CD14- for Langerhans cells (LCs) and CD1a-CD14+ for dermal or interstitial DCs (DDC-IDCs).

CD103 (αE) integrin expression marks a population of DCs, which can be found in lymphoid and non-lymphoid organs [5-7]. CD103+ DCs have been shown to play a dual role in Ag presentation, thus contributing to Ag cross-priming to T cells and to the control of inflammation via the conversion of naïve T cells into FOXP3+ Tregs [1,5,8,9]. Altogether, these findings indicate CD103+ DCs as important regulators of immune response [9].

Indoleamine 2,3-dioxygenase 1 (IDO1) catalyzes the initial rate-limiting step of tryptophan degradation along the kynurenine pathway [10]. Both tryptophan starvation and the products of tryptophan catabolism negatively regulate T-cell proliferation and survival [10,12]. The role of IDO1 as immunosuppressive agent in different settings is well-established [12,14]. In particular, IDO1-expressing DCs have a tolerogenic effect on T cell-based adaptive immune response by expanding/inducing Tregs [14,15]. In humans, although several investigators have recently reported about IDO1 expression in monocyte-derived DCs (Mo-DCs) [16-18], no
data are currently available about the expression and function of IDO1 during DC differentiation from human CD34+ HSPCs.

In this report, for the first time, we show that CD103 marks a subset of IDO1-expressing Langerin+DCs obtained during in vitro DC differentiation.

Materials and Methods

Cell isolation

All samples were obtained from healthy donors after informed consent was signed. Mononuclear cells (MNCs) were isolated from buffy coats, mobilized peripheral blood (PB), BM and cord blood (CB) by using gradient centrifugation through Lympholyte CL5020 (Cedarlane, Ontario, Canada). CD34+ and CD3+ cells were purified from the MNC fraction by Mini or Large Macs high-gradient magnetic separation column (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of cell populations was always > 90%. CD103+ cells were purified by cell-sorting from the CD34+-derived immature DCs after 7 days of culture, as previously described [19]. The purity of CD103+ cells was greater than 98%.

Cell cultures

CD34+ cells only from mobilized PB were cultured (10^5 cells/ml) in RPMI 1640 (BioWhittaker, Walkersville, MD, USA) containing 10% BSA (Sigma Aldrich, St. Louis, MO, USA) with L-glutamine and antibiotics (10%-RPMI), hereafter referred as medium, supplemented with 50 ng/ml of granulocyte macrophage-colony stimulating factor (GM-CSF; Peprotech, Rocky Hill, NJ, USA), 10 ng/ml of tumor necrosis factor (TNF)-α (Endogen, Rockford, IL, USA) and 50 ng/ml of Fms-related tyrosine kinase 3 ligand (FLT3L; eBioscience, San Diego, CA, USA) for 7 days [20]. CD34+-derived DCs were harvested after 7 days of culture, and used for the purification of CD1a+ cells by using CD1a microbeads (Miltenyi Biotec). CD1a- fraction was used for the positive selection of CD1a-CD14+ cell population by using CD14 microbeads (Miltenyi Biotec).

Flow cytometry
Flow cytometry cell analyses were performed by FACSCanto™ II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). DCs subsets were determined using the following anti-human monoclonal antibodies (mAbs): allophycocyanin (APC)-conjugated CD1a, CD14 and CD86 (Biolegend, San Diego, CA, USA; clones HI149, HCD14, IT2.2 and 67A4); phycoerythrin (PE)-conjugated CD103, CD80 and CD83 (Biolegend; LF61, 2D10 and HB15e); fluorescein isothiocyanate (FITC)-conjugated CD103 and HLA-DR (eBioscience, San Diego, CA, USA; B-Ly7 and L243); phycoerythrin-cyanin 7 (PeCy7)-conjugated CD11b (eBioscience, ICRF44) and CD1c (Biolegend, L161) and PE-conjugated Langerin (R&D systems, Minneapolis, MN, USA; 343828).

T-cell cultures

CD34+ derived DCs (3x10^3/well) were cultured in medium with allogeneic CD3+ T cells (1.5x10^5/well) in 96-well plates with or without the IDO1 inhibitor 1-methyl tryptophan-levo (1-MT-L). After 5 days, T cells were harvested and stained using FOXP-3 intracellular staining kit (eBioscience) including PE-conjugated anti-human FOXP3 (PCH101), FITC-conjugated CD4 (RPA-T4) and APC-conjugated CD25 (BC96) mAbs. CellTiter 96AQueous One Solution Cell Proliferation Assay (Promega, WI, USA) was used to detect the proliferation effect of DCs on CD3+ T cells. Allogeneic CD3+ T cells (1.5x10^5/well) were incubated in 96-well microplates with irradiated (3000 cGy) DCs (3x10^3) with or without 1-MT-L. After 6 days, CellTiter 96AQueous One Solution reagent (Promega) was added to each well and the microplate was incubated for additional 4 h. The OD value was measured in triplicate wells with an ELISA plate reader (Multiskan Ex; Thermo Electron Corporation, MA, USA) at a wavelength of 492 nm. In selected experiments, CD1a-CD14+ and CD1a-CD14+ cells were treated with human IDO1 siRNA, as previously described [21]. After 48 hours of incubation, CD1a-CD14+ and CD1a-CD14+ immature DCs with or without siRNA were mixed with allogenic CD3+ T cells (1:10) for 24 hours and stained for T_{reg} (CD4+CD25+FOXP3+ cells) induction by flow cytometry. The expression of IDO1 after siRNA treatment was evaluated by real-time PCR.
**Tregs suppression assay**

To test Tregs function, allogeneic CD3+ T cells were labeled by 5 μM 5-(6-) carboxyfluoresceine diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, Oregon, USA) and activated for 2 days in flat-bottom 96-well microplates (1.5x10^5 / well) pre-coated with anti-CD3 mAb (2 mg/ml, clone UCHT1; Bio-Legend) in presence of soluble anti-CD28 mAb (1 mg/ml; clone CD28.2; BioLegend). After 3 days, Tregs derived from CD1a+CD14- or CD103+ DCs co-cultures were purified by using the CD4+CD25+CD127dim/2 Regulatory T-Cell Isolation Kit (Miltenyi Biotec), and after irradiation added to activated CD3+ T cells (1.5x10^5 / well). CD4+CD25- (0.15x10^5 / well) were used as negative control. After 5 days, T cells were harvested and analyzed by FACS.

**Determination of IDO1 expression using Real-time PCR and functional activity**

Total RNA was extracted from BM, mobilized PB and CB-derived CD34+ cells, CD1a+CD14+ and CD1a+CD14- DCs by using Qiagen RNeasy microplus kit (Qiagen, Valencia, CA, USA). Then, 0.5 μg of RNA was used for retro-transcription with ImProm-II™ Reverse Transcriptase (Promega Corporation) and random hexamers (Invitrogen, Carlsbad, CA, USA). IDO1 copy number was evaluated as previously described [22]. IDO1 enzyme activity was quantified in terms of ability to catalyze L-tryptophan into kynurenine, as described [23].

**Western blot analysis**

Cell lysates were separated by SDS-PAGE, transferred onto nitrocellulose membrane (GE Healthcare, UK) and then, subjected to Western blotting. Membranes were saturated for 1 h at room temperature in blocking buffer (1X PBS, 0.1% Tween 20, 5% non-fat milk) and then, incubated overnight at 4°C with the primary Abs, as described [24].

**Immunofluorescence**

CD34+ derived DCs were fixed for 2’ in cold acetone and frozen at -20°C. Sections were stained with anti-Integrin αE (ber-ACT8), known as CD103 and anti-IDO1 (I-17) (Santa Cruz Biotechnology, CA, USA) and,
then, counterstained with horse Texas Red-conjugated anti-mouse IgG (Vector Laboratories, CA, USA) and FITC-conjugated anti-goat IgG in donkey (Jackson Immuno Research, PA, USA) secondary Abs, respectively. Images were collected and processed with a Axiovert 40 CFL (Carl Zeiss Microscopy-LLC, NY, United States). The immunofluorescence intensity was measured by densitometry (Adobe Photoshop 6.0 software) (n =12 randomly selected cells). The values were corrected for the number of pixels to compare cells of different size. Cells are grouped in classes of fluorescence intensity (fluorescence intensity per pixel) [25].

**Statistical analysis**

The results are expressed as the mean ± SD of at least 8 different experiments. The differences were compared using Student’s t-test, where indicated. The significance was determined at p < 0.05.

**Results**

**Human CD34+ HSPCs give rise to CD1a+ DCs which express IDO1**

Preliminary, IDO1 protein expression in BM, mobilized PB and CB–derived CD34+ cells was under the detection level (Figure 1A) and no enzymatic activity was observed (data not shown). However, during DC differentiation, IDO1 was significantly up-regulated (Figure 1A-C) with a differential expression among CD34+-derived DC subsets, which are commonly distinguished on the basis of CD1a and CD14 expression into CD1a+CD14+ and CD1a−CD14+ cells [26-31]. In particular, as compared to CD1a−CD14+ cells, CD1a+CD14- DCs showed increased IDO1 mRNA (25,366.0 ± 4,727.0 versus 1,400.0 ± 617.0 copies; p = 0.002; Figure 1A) and protein (Figure 1B). CD1a−CD14+ cells expressed IDO1 at very low level (780.0 ± 150.2). Interestingly, upon maturation CD1a+CD14+ DCs up-regulated IDO1 mRNA expression (Figure S1A). CD1a+CD14+ DCs produced larger amount of kynurenine than CD1a−CD14+ counterparts (60.2 ± 17.4 versus 9.2 ± 1.1 μM; p = 0.02) and, accordingly, the addition of the IDO1-inhibitor 1-MT-L reduced tryptophan metabolites production in CD1a+CD14+ DCs (60.2 ± 17.4 versus 24.6 ± 9.1 μM; p = 0.02) and
not in CD1a^CD14^ counterparts (Figure 1C). These data demonstrate that during DC differentiation from CD34^ HSPCs IDO1 is preferentially up-regulated in the CD1a^CD14^- subset.

**IDO1-expressing CD34^+-derived DCs reduce allogeneic T-cell proliferation and induce a population of functionally active FOXP3^+ Tregs**

To assess IDO1 immunological function in CD34^+-derived DCs, CD1a^CD14^- DCs were compared to CD1a^-CD14^+ counterparts as stimulators of allogeneic T-cell proliferation and as inducers of Tregs. Figure 2A shows that IDO1-expressing CD1a^CD14^- DCs have lower allostimulatory capacity of T-cell proliferation than CD1a^-CD14^+ cells (fold – change 1.93 ± 0.12 versus 5.99 ± 2.00; p = 0.04). Again, the addition of 1-MT-L resulted in increased T-cell proliferation only when IDO1-expressing CD1a^-CD14^- DCs were used (fold – change 1.93 ± 0.12 versus 3.71 ± 0.51; p = 0.01). A similar pattern was observed as for Tregs induction (Figure 2B). In particular, CD1a^-CD14^- DCs increased the percentage of CD4^CD25^FOXP3^+ T cells (37.40 % ± 11.60) as compared to their CD1a^-CD14^+ counterparts (22.87 % ± 10.38) (p = 0.005) and the addition of 1-MT-L reduced such effect only when IDO1-expressing CD1a^-CD14^- DCs were used (37.40 % ± 11.60 % versus 26.40 % ± 12.59 % with and without 1-MT-L, respectively; p = 0.005). In agreement with data about IDO1 expression, mature CD1a^-CD14^- DCs are capable of inducing higher number of Tregs as compared to immature CD1a^-CD14^- DCs (Figure S1B). Accordingly, CD4^CD25^+ T cells generated from co-culture with CD1a^- CD14^- DCs, and not with CD1a^-CD14^+ cells, had inhibitory capacity on T-cell proliferation (Figure 2C and Figure S2). To confirm these data, we treated CD1a^-CD14^- and CD1a^-CD14^+ DCs with IDO1 siRNA and we observed that the reduction of Tregs induction only when IDO1-expressing CD1a^-CD14^- were used (Figure 2D,E). These data demonstrate that CD34^+-derived CD1a^-CD14^- DCs reduce T-cell proliferation and induce *bona fide* FOXP3^+ Tregs via IDO1 expression.

**CD1a^-DCs express Langerin, CD11b, CD1c and CD103**

To characterize the phenotypical pattern of IDO1-expressing CD1a^-CD14^- DCs obtained during the differentiation of CD34^+ cells, DCs were analysed for the surface expression of different markers. CD1a^-CD14^- and CD1a^-CD14^+ DCs represented 21% and 36% of the whole cell population, respectively (Figure
The resting cells are represented by double negative CD1a-CD14- (41%) and double positive CD1a+CD14+ cells (2%), as shown in Figure 3A. Phenotypically, both CD1a+CD14- and CD1a+CD14+ DCs may be considered as immature DCs, since they expressed co-stimulatory molecules and HLA-DR at comparable level and showed low expression of CD83 (Table 1; Figure S3 and S4). CD1a+ CD14- DCs highly expressed CD11b and CD1c (86.9 ± 8.2 % and 86.3 ± 7.1 %, respectively), whereas only a minority of CD1a-CD14+ cells, which expressed CD11b at the same level as CD1a+CD14+ DCs, expressed CD1c (25.9 ± 12.1 %). As expected [31, 32], Langerin+ cells were found only within CD1a+CD14- subset (20.0 ± 3.4 %) as compared to CD1a-CD14- counterparts (0.70 ± 0.10%). Interestingly, as shown in Figure 3B, a subpopulation of CD1a+CD14- cells, and not of CD1a-CD14+ cells, concomitantly expressed Langerin and CD103 (20.6 ± 3.0 %; Figure S5). CD103+Langerin+CD1a-, which were shown to lack CD14 expression, were also negative for the monocyte-linked marker, CD68 (data not shown).

CD103 expression in CD34+-derived DCs identifies IDO1-expressing cells

Gut CD103+ DCs have been shown to express IDO1 [33]. We, then, evaluated whether CD103 may identify the subset of IDO1-expressing DCs also among CD34+-derived DCs. As shown in Figure 4A (upper panel), immunofluorescence studies demonstrated that only CD103+ cells expressed IDO1 at high level. These data have been confirmed by testing IDO1 expression on CD103+ and CD103- CD1a+CD14- DCs (Figure S6, upper panel). Indeed, as shown in Figure 4A (lower panel), the quantification of IDO1 signal intensity revealed that in the class of very high intensity (30-40 fluorescence intensity) only CD103+ cells, and not CD103- cells, could be observed. Moreover, the majority of CD103+ cells were located in the class of intense signal intensity (20-30 fluorescence intensity) (CD103+/CD103- = 4/1) while CD103- cells were mainly located in the class of intermediate intensity (10-20 fluorescence intensity) (5/1=CD103-/CD103+) (Figure S6, lower panel). A representative field used for quantification was shown in Figure 4A (upper panel). To confirm these data, CD103+ cells were sorted from CD1a+ DCs obtained after 7 days of culture and tested for IDO1 expression by Western blotting. In agreement with immunofluorescence results, IDO1 protein expression was observed in CD103+ cells, whereas it was almost absent in their CD103- counterparts (Figure 4B).
Taken together, these results indicate CD103 as the cell-surface marker for IDO1-expressing CD34+‐derived CD1a+ CD14+ DCs.

**CD103+ DCs induce Tregs via IDO1**

To investigate the role of IDO1 expression by CD103+ DCs on Treg-cell development, we co‐cultured IDO1-expressing CD103+ DCs with purified CD3+ T cells with or without 1-MT-L. Cell viability after culture with 1-MT-L was not different from that cultured in medium alone (85.0 ± 7.0 % and 80.0 ± 8.0 %, respectively), as well as CD4/CD8 T cell ratio was not modified by 1-MT-L (data not shown). As shown in Figure 5A, co-culture of T cells with IDO1-expressing CD103+ DCs increased the percentage of CD4+CD25+FOXP3+ T cells as compared to CD3+ T cells alone: 22.03 ± 3.84 % versus 4.23 ± 0.76 % (p = 0.002). Although also CD103 DCs were capable of inducing Tregs, the addition of 1-MT-L reduced such effect only when IDO1-expressing CD103+ DCs were used (22.03 ± 3.84 % versus 6.80 ± 1.40 %; p = 0.01) and, importantly, to the level observed before culture (Figure 5A). Accordingly with recent reports on normal IDO1-expressing Mo-DCs [17, 18], these data strongly suggest that IDO1 represents the main mechanism by which CD103+ DCs induce CD4+CD25+FOXP3+ T cells. Conversely, CD103 DCs induce Tregs in a IDO-independent manner. To validate the Treg nature of the cells induced by IDO1-expressing CD103+ DCs, allogeneic CD3+ T cells were stimulated by anti-CD3 and anti-CD28 mAbs in the presence of CD4+CD25+ or CD4+CD25 T cells obtained from co-culture with CD103+ DCs. T-cell proliferation was significantly reduced when CD4+CD25+ T cells, generated from co-culture with CD103+ DCs, were added to cell cultures, as compared to their CD4+CD25 counterparts (Figure 5B; p=0.01). These data support the hypothesis that CD4+CD25+FOXP3+ T cells induced by IDO1-expressing CD103+ DCs may be considered *bona fide* Tregs.

**Discussion**

Human DCs arise from HSPCs and may be divided into different cell subsets, known as conventional or “myeloid” DCs and defined by their *in vitro* cytokine-mediated differentiation pathways [34]. Among these,
LCs and DDC-IDCs, which localize to skin epithelia and dermis, respectively, develop in vitro from CD34+ HSPCs upon exposure to a cytokine combination, including GM-CSF and TNF-α [35-37] through an intermediate BM precursor, which is defined on the basis of CD1a and CD14 expression as CD1a⁺CD14⁻ for LCs and CD1a⁺CD14⁺ for DDC-IDCs [26].

In agreement with previous reports [26, 31, 32], our data show that CD1a⁺CD14⁻ cells express Langerin, CD11b, CD1c and are immature DCs. Interestingly, we report for the first time that a significant fraction of these CD1a⁺Langerin⁺CD14⁻ DCs also expresses CD103. CD103-expressing DCs may be found in lymphoid and non-lymphoid organs and are derived from BM committed DC progenitors, which give rise to lymphoid tissue DCs and non-lymphoid tissue CD103⁺ DCs [5, 38]. In particular, fully mature non-lymphoid CD103⁺ DCs have been observed in the dermal layer of the skin, where they are Langerin⁺CD11b<sub>low</sub> (CD103⁺ DDCs) and in nearly all non-lymphoid tissues, including gut, kidney, lung and liver [5]. At the level of progenitor cells, few reports have addressed the expression of CD103 on BM hematopoietic DC precursors, especially in humans [8]. However, the demonstration of CD103 on human CD34⁺-derived CD1a⁺Langerin⁺ DCs has not been previously reported and may suggest a specific lineage commitment of a subset of CD34⁺-derived DCs, defined by CD103 expression.

Mature non-lymphoid tissue CD103⁺ DCs are important regulators of the immune response through a dual, both activatory and tolerogenic, role in Ag presentation [5]. While they facilitate Ag cross-priming to T cells [2, 38], they have been also demonstrated to contribute to the control of inflammation via the conversion of naïve T cells into FOXP3⁺ T<sub>regs</sub> [5]. Such functional plasticity, which certainly depends on different signals to CD103⁺ DCs deriving from tissue microenvironment [39], raises the question of whether, at the level of progenitor cells, committed CD103⁺ DCs may have expression pathways corresponding to specific functions. Indeed, although some authors have recently demonstrated that the development program of human DCs is operated independently of lineage commitment [40], some others suggest that different DC subsets, i.e. plasmacytoid DCs [41], possess distinct characteristics and functions as a consequence of a different origin. In this scenario, it is noteworthy that fully mature and resident gut CD103⁺ DCs acquire tolerogenic functions, thus contributing to oral tolerance induction, via IDO1 expression [33]. In the present paper, we demonstrate that among human in vitro generated CD34⁺-derived DCs, CD103 marks a subset of
CD1a⁺ Langerin⁺ DCs with a specific expression pathway and function, as defined by IDO1. Given the critical role of IDO1 in the regulation of tolerance, we may speculate that at the level of HSPCs, CD103⁺ DCs are committed to tolerance induction, independently of tissue-derived signals. Accordingly, at the functional level, CD103⁺ DCs are in vitro capable of inducing a population of FOXP3⁺ Tregs.

The in vivo relevance of our in vitro findings needs to be better investigated. Results from BM transplant in the mouse model have demonstrated that CD103⁺ non-lymphoid DCs localize to dermis directly from the blood stream and are of hematopoietic BM origin [7, 38]. Our data characterize a population of human hematopoietic origin DCs, expressing CD103, which may act as tolerogenic cells via IDO1 expression. To investigate the localization of these cells in vivo, specific experiments in the mouse model and in human skin biopsies are highly warranted and are in progress.

In conclusion, a subset of DCs obtained from human CD34⁺ HSPCs induces Tregs through IDO1. These cells, are phenotypically defined by CD103 expression. Given the role of IDO1 in regulating immune tolerance, CD34⁺-derived CD103⁺DCs may be intrinsically committed to function as regulatory DCs via IDO1 expression, which may be part of a tolerogenic signature during DC development.

Support and Financial Disclosure Declaration

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Conflict of interest statement

The authors declare that they have no potential conflicts of interest.
References


Figure legends

Figure 1. Expression and enzymatic activity of IDO1. (A) The expression of IDO1 transcript by BM, mobilized PB and CB–derived CD34+ cells before culture as well as CD1a⁺CD14⁻ and CD1a⁻CD14⁺ DCs was evaluated by using RT-PCR. CD1a⁺CD14⁻ and CD1a⁻CD14⁺ DCs were derived only from mobilized PB. Results represent the mean ± SD of 8 different experiments. (B) Western blot analysis of IDO1 protein in CD1a⁺CD14⁻ and CD1a⁻CD14⁺ DCs. β-actin was used as loading control. The results are representative of 8 independent experiments. (C) IDO1 enzyme activity was quantified in terms of its ability to catalyze L-tryptophan into kynurenine. 1-MT-L was used as inhibitor of IDO1 activity. Results represent the mean ± SD of multiple replicates of 8 different experiments. * p < 0.05, ** p < 0.03.

Figure 2. T-cell proliferation, Tregs induction and function. (A) CellTiter Proliferation Assay was used to evaluate T-cell proliferation. Allogeneic CD3⁺ T cells were cultured with both CD1a⁺CD14⁻ and CD1a⁻CD14⁺ DCs with and without 1-MT-L. Fold change was calculated by using CD3⁺ T cells alone as reference. (B) Induction of CD4⁺CD25⁺FOXP3⁺ Tregs from allogeneic CD3⁺ T cells by CD1a⁺CD14⁻ and CD1a⁻CD14⁺ was evaluated. 1-MT-L as inhibitor was used. (C) CD4⁺CD25⁺⁻ T cells derived from CD1a⁺CD14⁻ DCs were tested for their capacity to inhibit the proliferation of allogeneic CD3⁺ T cells, previously labelled with CFSE. The cell proliferation was evaluated using flow cytometry. CD3⁺ T cells were activated by anti-CD3 and anti-CD28 mAbs. Activated CD3⁺ T cells alone were used as control samples. * p < 0.05, ** p < 0.03. Results represent the mean ± SD of multiple replicates of 8 different experiments. D) Silencing of IDO1 gene after siRNA treatment is reported as RT-PCR analysis. E) Induction of CD4⁺CD25⁺FOXP3⁺ Tregs from allogeneic CD3⁺ T cells by CD1a⁺CD14⁻ and CD1a⁻CD14⁺, previously treated with IDO1 siRNA, was evaluated. * p < 0.05, ** p < 0.03. Results represent the mean ± SD of multiple replicates of 3 different experiments.

Figure 3. Phenotype of CD34-derived immature DCs. (A) CD1a and CD14 expression in whole culture after 7 days of CD34⁺ cells differentiation (representative experiment). (B) Surface expression of Langerin and
CD103 in CD1a⁺CD14⁻ and CD1a⁻CD14⁺ DCs. CD103 and Langerin expression was evaluated in CD1a⁺CD14⁻ and CD1a⁻CD14⁺ DCs by using flow cytometry. Results are representative of 8 independent experiments.

Figure 4. IDO1 protein expression in CD103⁺ and CD103⁻ DCs. (A) Immunofluorescence staining of IDO1 (FITC) and CD103 (Texas Red) in CD34⁺-derived DCs (upper panel). Representative field is used for quantification. Bar 100 μm. The graph shows quantification of IDO1-specific signal in CD103⁺ and CD103⁻ DCs (lower panel). IDO1 fluorescence intensity was measured by densitometry (n=12; randomly selected cells). Cells are grouped in classes of fluorescence intensity and plotted relative to CD103 expression. B) Western blot analysis of IDO1 protein in CD103⁺ and CD103⁻ DCs. β-actin was used as loading control. Results are representative of 8 independent experiments.

Figure 5. Induction of Tregs derived from CD103⁺ DCs and their functional activity. (A) The induction of CD4⁺CD25⁺FOXP3⁺ Tregs from allogeneic CD3⁺ T cells by CD103⁺ DCs was evaluated. 1-MT-L as inhibitor was used. CD103⁺ DCs were purified from CD34⁺-derived immature DCs. Results represent the mean ± SD of 8 different experiments. (B) CD4⁺CD25⁺/- T cells derived from CD103⁺ DCs were tested for their capacity to inhibit the proliferation of allogeneic CD3⁺ T cells, previously labelled with CFSE. Cell proliferation was evaluated by using flow cytometry. CD3⁺ T cells were activated by anti-CD3 and anti-CD28 mAbs. Results represent the mean ± SD of multiple replicates of 8 different experiments. * p < 0.05, ** p < 0.03.
Table 1. Immunophenotypic characterization of CD1a⁺ CD14⁻ and CD1a⁻CD14⁺ DCs.

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<td>CD14</td>
<td>7,70 ± 1,50</td>
<td>92,30 ± 2,20</td>
</tr>
<tr>
<td>Langerin</td>
<td>20,00 ± 3,40</td>
<td>0,70 ± 0,10</td>
</tr>
<tr>
<td>CD103</td>
<td>25,20 ± 3,80</td>
<td>1,50 ± 0,20</td>
</tr>
<tr>
<td>CD11b</td>
<td>86,90 ± 8,20</td>
<td>84,90 ± 7,80</td>
</tr>
<tr>
<td>CD1c</td>
<td>86,30 ± 7,10</td>
<td>25,90 ± 12,10</td>
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<tr>
<td>Lang/CD103</td>
<td>20,60 ± 3,00</td>
<td>1,00 ± 0,30</td>
</tr>
</tbody>
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Results represent the mean ± SD of 8 different experiments.