Coexpression of CD163 and CD141 identifies human circulating IL-10-producing dendritic cells (DC-10)

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Tolerogenic dendritic cells (DCs) are key players in maintaining immunological homeostasis, dampening immune responses, and promoting tolerance. DC-10, a tolerogenic population of human IL-10-producing DCs characterized by the expression of HLA-G and ILT4, play a pivotal role in promoting tolerance via T regulatory type 1 (Tr1) cells. Thus far, the absence of markers that uniquely identify DC-10 has limited its in vivo studies. By in vitro gene expression profiling of differentiated human DCs, we identified CD141 and CD163 as surface markers for DC-10. The coexpression of CD141 and CD163 in combination with CD14 and CD16 enables the ex vivo isolation of DC-10 from the peripheral blood. CD14+CD16+CD141+CD163+ cells isolated from the peripheral blood of healthy subjects (ex vivo DC-10) produced spontaneously and upon activation of IL-10 and limited levels of IL-12. Moreover, in vitro stimulation of allogeneic naive CD4+ T cells with ex vivo DC-10 induced the differentiation of alloantigen-specific CD49b+LAG-3+ Tr1 cells. Finally, ex vivo DC-10 and in vitro generated DC-10 exhibited a similar transcriptional profile, which are characterized by an anti-inflammatory and pro-tolerogenic signature. These results provide new insights into the phenotype and molecular signature of DC-10 and highlight the tolerogenic properties of circulating DC-10. These findings open the opportunity to track DC-10 in vivo and to define their role in physiological and pathological settings.

Key words: Dendritic cells; IL-10; T regulatory type 1 (Tr1) cells; Tolerance

INTRODUCTION

Dendritic cells (DCs) are regulators of innate and adaptive immune responses. Different DC subsets drive these responses towards immunity or tolerance. In human blood, two major DC subtypes have been identified as follows: conventional/classical (c)DCs and plasmacytoid (p)DCs.1 Activated cDCs secrete IL-12 and TNF-α and prime naïve CD4+ and CD8+ T cells.2 In steady-state conditions, circulating cDCs have an immature phenotype, maintain immunological homeostasis, and can induce tolerance. pDCs, upon recognition of foreign nucleic acids, produce type I interferons (IFNs) and acquire the capacity to present foreign antigens (AgS). pDCs express lower levels of costimulatory and MHC class II molecules as compared to cDCs and promote T regulatory (Tregs)4–6. In addition to immature cDCs, different subsets of DCs, termed tolerogenic (tol)DCs, play a role in promoting tolerance, thus acting as regulatory cells.7,8 TolDCs can be induced by immunosuppressive compounds, anti-inflammatory cytokines, or genetic modifications.9,10 The regulatory capacity of tolDCs depends on the expression of low levels of costimulatory molecules, expression of inhibitory and/or modulatory receptors, secretion of low amounts of pro-inflammatory cytokines, and high level secretion of anti-inflammatory cytokines. These characteristics lead to inhibition of effector T cell responses and induction of Tregs.11

We identified and characterized a subset of human DCs, termed DC-10, because of their ability to spontaneously secrete IL-10. DC-10 can be differentiated in vitro by culturing peripheral monocytes in the presence of exogenous IL-10.12 DC-10 express CD11c, CD14, and CD16, have a mature phenotype (i.e., express CD83 and CX3CR1), and produce IL-10 in the absence of IL-12, a feature that, together with HLA-G and immunoglobulin-like transcript (ILT)-4 expression, is required for DC-10 mediated induction of T regulatory type 1 (Tr1) cells.12,13 In vitro differentiated DC-10 are currently used to generate Tr1 cells suitable for cell-based therapies.14–16 DC-10 were identified in the peripheral blood and spleen of healthy donors12 and in the decidua of pregnant women17 as CD11c−CD14+CD83+ cells; however, these markers are not ubiquitously expressed by DC-10, and CD83 expression is influenced by activation.

In this study, we investigated the gene expression profile of in vitro differentiated DC-10 to search for specific surface markers allowing for the unequivocal in vivo identification of DC-10. We show that CD141 and CD163 are highly and stably expressed by DC-10 and that the expression of these markers, in combination with CD14 and CD16, allows for the identification and isolation of DC-10 from the peripheral blood and spleen of healthy subjects. Ex vivo isolated DC-10 induce Tr1 cell differentiation in vitro and...
show a transcriptional profile similar to that of in vitro differentiated DC-10.

METHODS

Cell preparation
Human peripheral blood was obtained from healthy donors in accordance with local committee approval (PERIBLOOD and TIGET09) and with the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation over Lymphoprep™ (Axis-Shield PoC AS, Norway). The monocyte fraction of PBMCs was enriched by a Percoll gradient (GE Healthcare, USA), as previously described. Human spleens were obtained from cadaveric donors through the North Italian Transplant Organization upon informed consent from a first-degree relative in accordance with the local ethical committee approval and with the Declaration of Helsinki. Spleen cells were obtained by mechanical disruption of the organ followed by density gradient centrifugation over a Lymphoprep™ gradient.

DC differentiation
CD14+ cells were isolated from PBMCs by positive selection using CD14 MicroBeads (Miltenyi Biotech, Germany) according to the manufacturer’s instructions. Cells were cultured in RPMI 1640 medium (Lonza, Switzerland) with 10% fetal bovine serum (FBS) (Euroclone, Italy), 100 U/ml penicillin/streptomycin (Lonza, Switzerland), and 2 mM l-glutamine (Lonza, Switzerland), at 10^6 cells/ml at a 1 ml volume in a 24-well culture plate, supplemented with rhGM-CSF (Miltenyi Biotec, Germany) at 100 ng/ml and rhIL-4 (Miltenyi Biotec, Germany) at 10 ng/ml for 7 days at 37 °C with 5% CO2. One milliliter per well of fresh prewarmed medium with density gradient centrifugation over a Lymphoprep™ gradient. CD14+ cells were obtained by mechanical disruption of the organ followed by density gradient centrifugation over a Lymphoprep™ gradient.

Flow cytometry and sorting
Fluorochrome-conjugated antibodies against the following Ags were used for DC staining in PBMCs and spleen: CD1a, CD14, CD16, HLA-DR, CD11c, CD35, FPR1, CD163 (Becton Dickinson, CA, USA), and data were analyzed with FlowJo software (FlowJo, LLC, USA).

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For Ki67 staining, after surface staining with anti-CD4 and anti-CD3, cells were fixed, permeabilized, and stained with anti-Ki67 (Becton Dickinson, CA, USA) using Foxp3/Transcription Factor Staining Buffer Set (eBioscience, USA). Samples were acquired using the FACSVerse II or Fortessa Flow Cytometers (Becton Dickinson, CA, USA), and raw data were analyzed with FlowJo software.

Microarray
Total RNA was isolated using an RNeasy Kit (Qiagen, CA, USA) according to the manufacturer’s instructions. Preparation of terminal-labeled complementary DNA (cDNA), hybridization to the whole-transcript GeneChip Human Gene 1.0 ST Array (Affymetrix, USA) and scanning of the arrays were performed according to the manufacturer’s protocols. Raw data were preprocessed with the robust multichip average (RMA) algorithm.

RT-PCR
Total RNA was extracted using an RNeasy Kit (Qiagen, CA, USA), and cDNA was synthesized with a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) according to the manufacturer’s instructions. cDNA from mDCs, iDCs, and DC-10 was loaded in Low Density TaqMan® cards with Taqman Universal PCR Master Mix (Applied Biosystems, CA, USA), and PCR was performed on an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, CA, USA) following the manufacturer’s instructions. SDS 2.2.1 software was used to analyze the data, using RPLO as an endogenous control. Quantification relative to the endogenous control was carried out using the following formula: $\Delta \Delta C_T = C_{T_{target}}-C_{T_{endogenous}}$.

T cell isolation and proliferation
CD4+ T cells were purified from PBMCs by negative selection using the human CD4+ T cell Isolation Kit II (Miltenyi Biotech, Germany) according to the manufacturer’s instructions. CD4+ T cells were transfected with C4D49RO cells using anti-CD49RO microbeads (Miltenyi Biotec, Germany). CD4+ T cells were consistently >90% of purified cells. CD4+ C4D49RO− T cells were labeled with Cell Proliferation Dye eFluor® 670 (eBioscience, CA, USA) according to the manufacturer’s instructions and stimulated with 10 ng/ml allogeneic sorted DC-10 (ex vivo DC-10) or conventional DCs (ex vivo cDCs) (10:1, T:DCs) in X-VIVO 15 medium (Lonza, Switzerland) supplemented with 5% human serum (Sigma Aldrich, CA, USA) and 100 U/ml penicillin/streptomycin (Lonza, Switzerland). After 5 days, T cells were collected and washed, and their phenotype and proliferation were analyzed by flow cytometry.
T cell differentiation and suppression assay

CD4+ CD45RO- T cells (1×10^6) were cultured with 5×10^4 allogeneic sorted DC-10 (ex vivo DC-10) or conventional DCs (ex vivo cDCs) (20:1, T:DCs) in X-VIVO 15 medium (Lonza, Switzerland) supplemented with 5% human serum (Sigma Aldrich, CA, USA) and 100 U/ml penicillin/streptomycin (Lonza, Switzerland). After 10 days, primed T cells were collected, washed, and analyzed. T cells stimulated with DC-10 are referred to as T(DC-10) cells, while those stimulated with cDCs are referred to as T(cDC) cells.

T(DC-10) and T(cDC) cells were plated with in vitro differentiated iDCs that were autologous to ex vivo DC-10 and ex vivo cDCs (10:1, T:DCs), and in some experiments, 100 U/ml of IL-2 (Chiron, Italy) was exogenously added to T(DC-10) cell cultures. As a control, T(DC-10) and T(cDC) cells were stimulated with Dynabeads® Human T-Activator CD3/CD28 (5:1, cells:beads). After 3 days of stimulation, T cells were collected and washed, and cell proliferation was evaluated by Ki67 staining via flow cytometry.

To evaluate the suppressive activity of T(DC-10) cells, T(cDC) cells (responder cells) were stained with Cell Proliferation Dye eFluor® 450 (eBioscience, CA, USA) and activated with iDCs autologous to ex vivo DC-10 and ex vivo cDCs in the presence of T (DC-10) cells at a 1:1 ratio (total T:iDCs ratio was 10:1). After 3 days, the percentages of divided responder T cells were calculated by proliferation dye dilution by flow cytometry.

Cytokine determination

A total of 10^5 FACs-sorted cells were plated in at a 100 μl final volume. Cells were left un-stimulated or activated with 200 ng/ml LPS (Sigma, CA, USA), and supernatants were collected after 48 h. Levels of IL-6, IL-10, IL-12, and TNF-α were determined by a 4-plex Bio-Plex system according to the manufacturer’s instructions (Bio-Rad, CA, USA). The production of IFN-γ and GM-CSF by CD4+ T cells was quantified in coculture supernatants by a BD OptEIA® ELISA Kit (Becton Dickinson, CA, USA).

RNA-Seq analysis and data processing

RNA was isolated using an RNaseasy Micro or RNA MirNeasy Kit (QiAGEN, CA, USA), and 10 ng of total or poly-adenylated RNA was converted to cDNA by random primer amplification. The library was constructed and amplified from cDNA using the Nextera DNA Library Prep Kit with unique dual adapters (Illumina, NY, USA). Samples were multiplexed and sequenced to 1.5×10^7, 2×150-bp reads per sample on a NextSeq500 (Illumina, NY, USA). Raw RNA sequences (≥3×10^7 per sample) were trimmed using Skewer and then aligned against the human reference transcriptome by the Broad Institute’s Human Transcriptome Analysis tool. To identify significantly enriched biological processes, differences were considered significantly higher compared to mDCs. FPR1 expression was higher in DC-10 compared to iDCs; however, the expression of the above markers was low in DC-10 and varied among the donors investigated (Fig. 3a, lower panels, and Figure S3). The expression of CD163 and CD141 were analyzed on DC-10 upon activation with different TLR agonists. The results in Fig. 3b demonstrate that CD141, CD163, and CLEC4G are expressed on DC-10 independently of DC-10-1-LPS revealed the lowest number of DEGs (344), indicating that DC-10 have a stable transcriptional profile (Fig. 1b). Moreover, differences in the transcriptome profile of DC-10 compared to mDCs and iDCs were maintained upon LPS activation (with 1781 and 1034 DEGs, respectively, Fig. 1b). Principal component analysis summarized the observed transcriptional similarity among DC-10, DC-10 + LPS, and mDCs and the divergence from mDCs (Fig. 1c). These results demonstrate that in vitro differentiated DC-10 have a defined transcriptional profile that is stable upon TLR stimulation.

To identify specific markers of DC-10, we focused on DEGs encoding for cell surface proteins. We selected 17 genes significantly (P < 0.01) up-regulated in DC-10, at a steady state and upon LPS activation, compared to mDCs (Fig. 2a). RT-PCR on DC-10 and mDCs from four additional donors confirmed a higher expression of the selected genes in DC-10 compared to mDCs, with the exception of PLXMA2 and ABC2CC (Fig. 2b). To assess the specificity of the 15 validated genes, we analyzed their expression in iDCs, and results showed higher expression of 13 out of 15 genes in DC-10 compared to iDCs (Fig. 2c).

We investigated the protein expression levels of the genes showing at least 10 times higher mean fold change transcript expression in DC-10 compared to iDCs in vitro differentiated additional donors. Flow cytometric data showed that DC-10 expressed significantly higher levels of CD163 (P = 0.039), CD141 (encoded by THBD, P = 0.039), and CLEC4G (P = 0.013) compared to both iDCs and mDCs (Fig. 3a, upper panel, and Figure S3). The expression of CD35 (encoded by CR1), FPR1, and LIR8 (encoded by LILRB5) in DC-10 was significantly higher compared to mDCs. FPR1 and LIR8 expression was higher in DC-10 compared to mDCs; however, the expression of the above markers was low in DC-10 and varied among the donors investigated (Fig. 3a, lower panels, and Figure S3). Finally, CD105 (encoded by ENG) was highly expressed on DC-10 in all donors tested, but it was also expressed at variable levels in iDCs and mDCs, with CD105 expression nearly comparable to that of DC-10 in one donor (Fig. 3a, lower panels, and Figure S3). We selected CD141, CD163, and CLEC4G and analyzed their expression on DC-10 upon activation with different TLR agonists. The results in Fig. 3b demonstrate that CD141, CD163, and CLEC4G are firmly expressed on DC-10 independently
Fig. 1 Transcriptional profile of in vitro differentiated DC-10. Mature (m)DCs, immature (i)DCs, DC-10, and DC-10 activated with LPS (DC-10 + LPS) were differentiated in vitro from peripheral blood monocytes of healthy donors (n = 4) according to the Methods section, and microarray analysis was performed. a Two-dimensional heatmap of significant differentially expressed genes (DEGs) (P < 0.05, absolute fold change (FC) value > 1). The color scale is relative to the mean expression levels. Genes are in columns, and samples are in rows. Data in rows have been hierarchically clustered. b Scatterplots of differential expression between mDCs, iDCs, DC-10, and DC-10 + LPS. Each dot represents the averaged Log2 FC value of a single gene. Dashed diagonal lines indicate the thresholds for FC (absolute FC value > 1). Numbers indicate DEGs. c Principal component analysis plot for expression data in mDCs, iDCs, DC-10, and DC-10 + LPS.
of TLR-mediated activation, indicating that CD141, CD163, and CLEC4G are selectively and stably expressed by in vitro differentiated DC-10.

Co-expression of CD141 and CD163 in combination with CD14 and CD16 identifies functional DC-10 in vivo
We analyzed the expression of CD141, CD163, and CLEC4G on peripheral blood CD14+CD16+ cells, which contain DC-10, CD14+CD16− (classical) and CD14−CD16+ (non-classical) monocytes served as controls. The vast majority of CD14+CD16+ cells and classical monocytes were CD163+ while a significantly \(P = 0.008\) lower frequency of non-classical monocytes expressed CD163. Within CD14+CD16+ cells, a significantly \(P = 0.004\) higher proportion of cells expressed CD141 compared to both classical and non-classical monocytes. Conversely, CLEC4G was expressed at low levels in all populations (Fig. 3c and S4). Thus, CD163 expression can be used to discriminate CD14+CD16+ DC-10 from non-classical monocytes, whereas coexpression of CD16...
Fig. 3 Co-expression of CD14, CD16, CD141, and CD163 identifies in vitro differentiated DC-10 and putative DC-10 in vivo. A DC-10, mDCs, and iDCs were differentiated in vitro from peripheral blood monocytes of healthy donors \((n = 5–7)\) according to the Methods section. Expression levels of the indicated markers were measured by flow cytometry. The following gating strategy was applied: doublet exclusion, followed by alive cells and gating on DC11c\(^+\) cells. Each dot represents a single donor, lines indicate medians, and whiskers are minimum and maximum levels. B Expression levels of CD141, CD163, and CLEC4G were evaluated in DC-10 activated with the indicated TLRs at day 5 of differentiation \((n = 4)\). The following gating strategy was applied: doublet exclusion, followed by alive cells and gating on DC11c\(^+\) cells. Each dot represents a single donor, lines indicate medians, and whiskers are minimum and maximum levels. C Expression levels of CD141, CD163, and CLEC4G were evaluated on CD14\(^+\)CD16\(^+\) cells that contain DC-10, on CD14\(^+\)CD16\(^-\) (classical), and on CD14\(^{low}\)CD16\(^+\) (non-classical) monocytes \((n = 7–9)\). The following gating strategy was applied: doublet exclusion, followed by alive cells and indicated gating. Each dot represents a single donor, lines indicate medians, and whiskers are minimum and maximum levels. D CD14\(^+\)CD16\(^+\)CD141\(^+\)CD163\(^+\) cell frequencies in peripheral blood of healthy donors and in splenic CD45\(^+\) cells of cadaveric donors are shown \((n = 7)\). Each dot represents a single donor, lines indicate medians, and whiskers are minimum and maximum levels. *\(P \leq 0.05\), **\(P \leq 0.01\) (Wilcoxon matched pairs test, two-tailed)
and CD141 segregates CD14+CD16+ DC-10 from classical monocytes. In accordance with our previous study, CD14+CD16−CD141+CD163+ DC-10 express HLA-DR, CD80, and CD86 at levels similar to those expressed by ex vivo DC-10 (Figure S6A-S6B). Cytokine profile of ex vivo cDC used is shown in Figure S6C. The ability of ex vivo DC-10 and ex vivo cDCs to stimulate allogeneic naïve CD4+ T cells was compared (Fig. 5a). The results showed that naïve CD4+ T cells cultured with ex vivo DC-10 expressed significantly (P < 0.03) lower levels of CD25 and HLA-DR, proliferated significantly (P < 0.008) less, and secreted significantly (P < 0.0008) lower levels of IFN-γ and GM-CSF compared to T cells stimulated with cDCs (Fig. 5b, c). Thus, similar to in vitro differentiated DC-10, ex vivo DC-10 promote hypo-responsive in allogeneic CD4+ T cells.

To investigate the induction of alloantigen-specific Tr1 cells, allogeneic naïve CD4+ T cells were stimulated for 10 days with ex vivo DC-10 or ex vivo cDCs at a 1:2 ratio. After culturing, CD4+ T cells primed with ex vivo DC-10 (T(DC-10) cells) or ex vivo cDCs (T(cDC) cells) were restimulated with cells from the allogeneic donor in the priming (Fig. 6a). At the end of 10 day culture, the percentage of CD4+CD45RA−CD49b+LAG-3+ cells was assessed as bona fide Tr1 cells. In all donors tested, CD4+ T cells primed with ex vivo DC-10 (T(DC-10) cells) contained a significantly (P < 0.002) higher proportion of Tr1 cells compared to T(cDC) cells, with an average of 17.9% and 3.7% of CD49b+LAG-3+ Tr1 cells, respectively (Fig. 6b, c and S7). Upon secondary stimulation with IDCs from the allogeneic donor used in the priming, T(DC-10) cells were anergic, as demonstrated by the significantly (P = 0.0002) lower percentage of Ki67+ cells in T(DC-10) cells compared to T(cDC) cell cultures (Fig. 6c, d). Conversely, upon polyclonal stimulation, T(DC-10) cells showed a proliferative response similar to T(cDC) cells (data not shown). The addition of exogenous IL-2 during in vitro alloantigen restimulation restored T (DC-10) cell proliferation (from 32.2 ± 25.9% to 76.6 ± 6.4% of Ki67+ cells), similar to that of T(cDC) cells (76 ± 14.15% of Ki67+ cells, Fig. 6d). In agreement with the presence of a high frequency of Tr1 cells, T(DC-10) cells efficiently suppressed the proliferation of T(cDC) cells activated with cells from the allogeneic donor used for priming (Fig. 6a, e). These results demonstrate that ex vivo isolated CD14+CD16−CD141+CD163+ DC-10 promote

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Ex vivo DC-10 were evaluated for their effect on naïve CD4+ T cells. As a control, we used FACS-isolated CD11c+CD11c+ cells (ex vivo cDCs) that expressed HLA-DR, CD80, and CD86 at levels similar to those expressed by ex vivo DC-10 (Figure S6A-S6B). Cytokine profile of ex vivo cDC used is shown in Figure S6C. The ability of ex vivo DC-10 and ex vivo cDCs to stimulate allogeneic naïve CD4+ T cells was compared (Fig. 5a). The results showed that naïve CD4+ T cells cultured with ex vivo DC-10 expressed significantly (P < 0.03) lower levels of CD25 and HLA-DR, proliferated significantly (P < 0.008) less, and secreted significantly (P < 0.0008) lower levels of IFN-γ and GM-CSF compared to T cells stimulated with cDCs (Fig. 5b, c). Thus, similar to in vitro differentiated DC-10, ex vivo DC-10 promote hypo-responsive in allogeneic CD4+ T cells.

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Ex vivo DC-10 secretes IL-10 spontaneously and upon activation. Ex vivo DC-10 (CD14+CD16−CD141+CD163+) and non-classical (CD14+CD16+CD163+) monocytes were FACS-isolated from the peripheral blood of healthy donors and left unstimulated or stimulated with LPS for 48 h (n = 9). Concentration levels of IL-10, IL-12, TNF-α, and IL-6 in culture supernatants were evaluated by a multiplex microarray cytokine array. Each dot represents a single donor, lines indicate medians, and whiskers are minimum and maximum levels. *P < 0.05, **P < 0.01 (Wilcoxon matched pairs test, two-tailed).
Fig. 5  Ex vivo DC-10 promote hypo-responsiveness in allogeneic naïve CD4+ T cells. a Scheme of the experiment: naïve CD4+ T cells were cultured with ex vivo DC-10 (CD14+CD16−CD141+CD163+) or ex vivo cDCs (CD1c+CD11c−) FACS isolated from the peripheral blood of healthy donors (ratio 10:1) for 5 days. b Expression of the activation markers CD25 and HLA-DR on CD4+ T cells stimulated with ex vivo cDCs (red) or ex vivo DC-10 (blue) were evaluated by flow cytometry (n = 6). The following gating strategy was applied: doublet exclusion, followed by alive cells and gating on CD3+CD4+ cells. Left, each dot represents a single donor; right, flow cytometry histograms from one representative donor. c Naïve CD4+ T cell proliferation was evaluated by proliferation dye dilution (n = 8). The following gating strategy was applied: doublet exclusion, followed by alive cells and gating on CD3+CD4+ cells. The percentage of proliferated cells is shown for each single donor (left panel) and in representative dot plots (right panels). d IFN-γ and GM-CSF in culture supernatants were measured by ELISA (n = 8). Each dot represents a single donor. *P ≤ 0.05 **P ≤ 0.01 (Wilcoxon matched pairs test, two-tailed)
Fig. 6  Ex vivo DC-10 induce anergic alloantigen-specific CD49b+LAG-3+CD4+ T cells. a  Scheme of the experiment: naïve CD4+ T cells were cultured with allogeneic ex vivo DC-10 (CD14+CD16+CD141+CD163+) [T(DC-10) cells] or ex vivo cDCs (CD1c+CD11c+) [T(cDC) cells] FACS-isolated from peripheral blood of healthy donors (ratio 20:1) for 10 days. After culturing, primed CD4+ T cells were restimulated with iDCs in vitro differentiated from monocytes from the allogeneic donor used in priming, separately or mixed at a 1:1 ratio. b, c The percentages of Tr1 cells within T(cDC) and T(DC-10) cell cultures were evaluated by CD49b and LAG-3 expression on CD45RA−CD4+ T cells (n = 10). The following gating strategy was applied: doublet exclusion, followed by alive cells and gating on CD3+CD4+CD45RA− cells. The percentage of Tr1 cells in each donor tested (b) and dot plots from one representative donor tested (c) are shown. d After 10 days, T cells were restimulated with in vitro differentiated iDCs, which are autologous to cDCs and DC-10 used for priming (n = 8), and in some experiments exogenous IL-2 was added to T(DC-10) cell cultures (n = 3). Proliferative responses were evaluated by Ki67 staining with flow cytometry. The following gating strategy was applied: doublet exclusion, followed by alive cells and gating on CD3+CD4+ cells. The percentage of Ki67+ T cells in each donor tested (left and middle panel) and histograms from one representative donor tested (right panel) are shown. The red histogram shows proliferation of T(cDC) cells, while the blue histogram corresponds to proliferation of activated T(cDC) cells in the presence of T(DC-10) cells, and the gray shaded histogram shows unstimulated T(cDC) control. **P ≤ 0.01 ***P ≤ 0.001 (Wilcoxon matched pairs test, two-tailed)
alloantigen-specific Tr1 cell differentiation in vitro and are functionally superimposable to in vitro differentiated DC-10.

The ex vivo DC-10 transcriptome resembles that of monocyte-derived DC-10
To study similarities between ex vivo and in vitro differentiated DC-10, we performed RNA-sequencing analyses. As a control, we used donor-matched in vitro differentiated mDCs (in vitro mDCs) and published RNA-seq data of FACS-isolated cDCs (ex vivo cDCs, GSE70106). The large majority of transcriptional variance (55%) was linked to the origin of cell populations, with in vitro DC-10 and mDCs divided from ex vivo DC-10 and cDCs by PC1 (Fig. 7a).

Interestingly, PC2 (23% of variance) segregated ex vivo and in vitro DC-10 from ex vivo cDCs and in vitro mDCs (Fig. 7a), thus suggesting similarities between ex vivo and in vitro DC-10. High-ranking DEGs obtained by in vitro DC-10 versus in vitro mDCs comparison were then used to perform a Gene Set Enrichment Analysis in ex vivo DC-10 and cDC transcripts. The top 500 up-regulated genes in in vitro DC-10 were highly expressed by ex vivo DC-10 but not by cDC (Fig. 7b, left panel). Conversely, the top 500 down-regulated genes in in vitro DC-10 were not expressed by ex vivo DC-10, and their expression was significantly upregulated in ex vivo cDCs (enriched at nominal P-value <1%; Fig. 7b, right panel).

The Gene Ontology Biological Process (GOBP) enrichment analysis performed on up-regulated and down-regulated genes by both ex vivo isolated and in vitro differentiated DC-10 showed that DC-10 were enriched in genes associated with anti-inflammatory responses as well as negative regulation of T cell proliferation and production of pro-inflammatory cytokines (i.e., IL-12, IL-18, and IL-23). Conversely, responses to type I IFNs and pro-inflammatory cytokines (i.e., IL-12 and IFN-γ), and the pro-inflammatory responses were GOBP-enriched for down-regulated genes. Accordingly, KEGG analysis identified the NF-kB signaling pathway as the most significantly (P = 0.006) down-regulated pathway in DC-10 compared to pro-inflammatory DCs (i.e., cDCs and mDCs, data not shown). These pathway analyses highlight the common pro-tolerogenic and anti-inflammatory signatures of ex vivo isolated and in vitro differentiated DC-10 cells, demonstrating an overlap between their transcriptomes and confirming that CD14+CD16+CD141+CD163+ cells represent the in vivo counterpart of in vitro-generated DC-10.

DISCUSSION
In this study, we show that the combined expression of CD141 and CD163 with CD14 and CD16 identifies human DC-10. Ex vivo isolated DC-10 from the peripheral blood of healthy donors secrete IL-10 spontaneously at variable levels and consistently upon in vitro activation with limited amounts of IL-12, poorly stimulate allogeneic naïve CD4+ T cells, and induce alloantigen-specific anergic Tr1 cells. The co-expression of CD14, CD16, CD141, and CD163 is specific for DC-10 as classical and non-classical monocytes and cDCs do not co-express these markers. The combined expression of CD14, CD16, CD141, and CD163 can be used to isolate and track human DC-10 in vivo.

The expression of CD141 characterizes a population of circulating cDCs, BDCA-3+ DCs. Our data show that, although DC-10 and BDCA-3+ DCs share CD141 expression, they differ in several aspects: BDCA-3+ DCs are lineage-negative cells, while DC-10 express CD14 and CD16; BDCA-3+ DCs are TLR4+ cells, while we show here that DC-10 respond to LPS, a known TLR4 agonist; BDCA-3+ DCs preferentially secrete IL-12,2 with an IL-10/IL-12 ratio2 opposite to that of DC-10. Moreover, BDCA-3+ DCs promote allogeneic CD4+ T cell proliferation and IFN-γ secretion, with higher IFN-γ production compared to that induced by CD1c+ DCs. Conversely, DC-10 promote hypo-responsiveness and low IFN-γ production by allogeneic CD4+ T cells. Chu et al.3 described a population of CD141+ DCs in human skin that play an essential role in maintaining skin homeostasis and in regulating systemic and anti-tumor immunity. These DCs share some features with DC-10, including constitutive IL-10 secretion and the ability to induce T cell hypo-responsiveness and Tregs. It still remains to be defined whether skin-resident CD141+ DCs may represent a population of tissue-resident DC-10.

The scavenger receptor CD163 expression is induced by IL-10, and IL-10 secretion is elicited by DC-10 engagement. CD163 is commonly expressed at high levels in various tissue-resident macrophages polarized towards an M2-like phenotype. DC-10, similar to M2-like macrophages, express CD163 and up-regulate genes encoding for anti-inflammatory factors. We demonstrate that DC-10 are blood circulating DCs distinct from the described CD163+ macrophages or DCs. DC-10 indeed expressed TLR3, barely expressed CD206 (data not shown), and, once ex vivo isolated from the peripheral blood, prime naïve CD4+ T cells in vitro. In contrast to circulating CD163+CD11c+ DCs, DC-10 express CD14 and are CD16+hight. Moreover, DC-10 are CD141+CD16+, a characteristic that distinguishes them from a recently described population of human intestinal CD14+CD163+ DCs. A more in-depth analysis of the presence of DC-10 within the gut mucosa is needed to define whether they may represent a distinct population of DCs that cooperate with other cells to maintain tolerance.
Fig. 7  The Ex vivo DC-10 transcriptome resembles that of their in vitro counterpart. RNA was extracted from ex vivo DC-10 ($n=4$) and from in vitro differentiated DC-10 and mDCs ($n=2$) and sequenced. Published RNA-seq data of FACS-isolated cDCs (ex vivo cDCs, GSE70106) were used ($n=3$). a Principal component analysis. PC variances are indicated on the axis, and each dot represents a donor and each subset is differently colored. b Gene Set Enrichment Analysis in ex vivo DC-10 and cDC transcriptomes of upregulated DEGs (left panel) and downregulated DEGs (right panel) in in vitro differentiated DC-10 compared to mDCs was performed. The enrichment profile is shown by the green line, while the hits are indicated by the black line. c The enrichment of biological process GO terms was analyzed with EnrichR and summarized using REVIGO. For each significant class ($P<0.01$), enriched terms passing the redundancy reduction control are represented as scatterplots. Bubble colors indicate the $P$-value. The Y-axis indicates the semantic similarity between GO terms, whose units have no intrinsic meaning: semantically similar GO terms should cluster together in the plot. Pathways that are upregulated (left panel) and downregulated (right panel) are shown.
tolerance in vivo. It still remains to be defined the origin of ex vivo DC-10, whether these cells are derived from cDC precursors or are monocyte-derived cells. We observed in our RNA-seq analysis that ex vivo DC-10 did not express ZBTB46 (data not shown, see GSE117945). This result is an indirect indication that ex vivo DC-10 are derived from monocytes, as ZBTB46 is specifically expressed by cDCs and committed cDC precursors, but not by monocytes, pDCs, or other immune cell populations.3,35 Future studies will investigate the origin of DC-10 in vivo.

In summary, we discover that the combination of CD14, CD16, CD141, and CD163 allows unambiguous identification of human DC-10 in vivo and represents a significant improvement of the previously described strategy, which relies on the use of the activation-dependent marker CD83. DC-10 isolated with the newly described strategy shows tolerogenic activity, IL-10 secretion and Tr1 cell induction. The definition of this marker combination makes it now feasible to study the role of DC-10 in vivo, which could be crucial for maintaining immunological homeostasis or detrimental in tumor settings.

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AUTHOR CONTRIBUTIONS

M.C. performed the experiments, collected and analyzed data, and wrote the manuscript; M.C., D.A. and M.F. carried out the bioinformatic analyses; F.S.D.S. performed some experiments and contributed to the analysis of RNAseq data; D.T. prepared samples for microarray analysis; M.V. performed RT-PCR validation; M.J.U. conceived the idea, supervised the project, and wrote the manuscript.

ADDITIONAL INFORMATION

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