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Human type 1 and type 2 conventional dendritic cells express indoleamine 2,3-dioxygenase 1 with functional effects on T cell priming

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Dendritic cells (DCs) are key regulators of the immune system that shape T cell responses. Regulation of T cell induction by DCs may occur via the intracellular enzyme indoleamine 2,3-dioxygenase 1 (IDO), which catalyzes conversion of the essential amino acid tryptophan into kynurenine. Here, we examined the role of IDO in human peripheral blood plasmacytoid DCs (pDCs), and type 1 and type 2 conventional DCs (cDC1s and cDC2s). Our data demonstrate that under homeostatic conditions, IDO is selectively expressed by cDC1s. IFN-γ or TLR ligation further increases IDO expression in cDC1s and induces modest expression of the enzyme in cDC2s, but not pDCs. IDO expressed by conventional DCs is functionally active as measured by kynurenine production. Furthermore, IDO activity in TLR-stimulated cDC1s and cDC2s inhibits T cell proliferation in settings were DC-T cell cell-cell contact does not play a role. Selective inhibition of IDO1 with epacadostat, an inhibitor currently tested in clinical trials, rescued T cell proliferation without affecting DC maturation status or their ability to cross-present soluble antigen. Our findings provide new insights into the functional specialization of human blood DC subsets and suggest a possible synergistic enhancement of therapeutic efficacy by combining DC-based cancer vaccines with IDO inhibition.

Keywords: cDC1 · cDC2 · plasmacytoid dendritic cells · IDO · epacadostat

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Introduction

Dendritic cells (DCs) are professional antigen-presenting cells that can both induce and regulate T cell priming. One of the mechanisms by which DCs can negatively regulate T cell responses is via the intracellular enzyme indoleamine 2,3-dioxygenase 1 (IDO). IDO catalyzes breakdown of tryptophan into N-formyl-kynurenine, the first and rate-limiting step in tryptophan catabolism. Tryptophan deprivation, sensed by ribosomal kinase GCN2, and tryptophan metabolites such as kynurenine, inhibit effector T cell proliferation and induce anergy or apoptosis [1–3]. In regulatory T cells (Tregs), however, GCN2 signaling leads to activation and induces a strong suppressive phenotype [4]. Furthermore, both IDO-mediated GCN2 activation and binding of kynurenine to the aryl hydrocarbon receptor promote *de novo* Treg differentiation from naïve $CD4^+$ T cells [5,6].

IDO appears to play an important role in inducing tolerance and limiting excessive inflammation, for instance in the placenta [7]. In some settings, however, IDO activity might actually be detrimental to the host. Cancer cells and myeloid-derived suppressor cells often express IDO to inhibit antitumor immunity and high IDO expression is associated with poor prognosis and shorter overall survival in several cancer types, including ovarian cancer [8], endometrial cancer [9], colorectal cancer [10], and melanoma [11]. Primary DCs are currently under investigation for their use in cancer vaccines, which aim at boosting the antitumor-specific T cell response. Because IDO activity inhibits T cell function, expression of IDO by human DC subsets may have important implications for the efficacy of DC-based immunotherapy.

Multiple types of inflammatory signals may induce IDO expression in DCs. IDO1 gene promoters contain several interferon (IFN) response elements, allowing transcription of IDO upon signaling by type I IFNs, and more effectively by type II IFN (IFN-γ) [12,13]. Other inducers of IDO include ligation of CD40 and Toll-like receptors (TLRs), and reverse signaling by B7 molecules upon ligation by CTLA-4 [14–17]. Importantly, IDO expression at the protein level does not necessarily correlate with biological activity of the enzyme. For instance, a combination of different signals was shown to be required to achieve full enzymatic activity in human monocyte-derived DCs (moDCs) [14,18].

Most studies on IDO expression by DCs have been performed with murine DCs and human moDCs. In contrast, less is known about the function of IDO in human primary DCs, which are subdivided into three major subsets: plasmacytoid DCs (pDCs) and type 1 and type 2 conventional DCs (cDC1s and cDC2s). Interestingly, RNA profiling revealed high IDO expression by human steady-state and activated cDC1s [19–22]. Our recent comparative proteomic analysis of steady-state human DC subsets likewise revealed IDO protein to be uniquely expressed by cDC1s [23]. Here, we compared the expression and activity of IDO in immature and stimulated human peripheral blood DCs and studied the effects on T cell proliferation.

Results

Immature cDC1s selectively express high levels of functionally active IDO

High expression of IDO by human cDC1s compared to cDC2s and pDCs was first reported on RNA level by Crozat *et al*. [19], using a dataset generated with RNA microarray [24]. More recently, single-cell RNA-sequencing (RNA-seq) analysis of steady-state human blood DC subsets also demonstrated IDO to be uniquely associated with cDC1s [20,21]. Furthermore, *IDO1* gene expression was higher in cDC1s compared to cDC2s and pDCs in human tonsil [25], and thymus and spleen [26]. We confirm these findings by combining a bulk RNA-seq dataset of blood cDC1s (Mathan *et al*. manuscript in preparation) and a bulk RNA-seq dataset of blood cDC2s and pDCs [27], both generated by our group. Comparing the total prevalence of the genes that Villani *et al*. found to be specific for cDC1s [20] revealed that *IDO1* is, together with *XCR1* and *CADM1*, the most uniquely expressed gene by cDC1s, compared to cDC2s and pDCs (Fig. 1A). Moreover, *IDO1* gene expression in cDC1s is increased upon stimulation with TLR3 or TLR7/8 agonists (Fig. 1B).

Comparative proteomic analysis in steady-state human blood DC subsets revealed that also at the protein level, IDO was highly expressed by cDC1s, while the signal was below detection limit for cDC2s and pDCs (Fig. 2A) [23]. Analysis of immature blood DCs by flow cytometry confirmed significantly higher IDO expression by cDC1s compared to cDC2s and pDCs (Fig. 2B).

We next analyzed IDO activity across DC subsets by measurement of L-kynurenine levels in supernatants from 24-hour DC cultures by high-performance liquid chromatography (HPLC). cDC1s converted around 1 μM tryptophan into kynurenine, whereas the same number of pDCs and cDC2s produced less kynurenine (Fig. 2C). Taken together, immature human peripheral blood cDC1s express functionally active IDO at significantly higher levels than cDC2s and pDCs.

IDO is induced in cDC2s and upregulated in cDC1s by TLR ligation or IFN-γ stimulation

Upon activation, DCs acquire the capacity to migrate to the draining lymph node and express co-stimulatory molecules needed to activate T cells. Hence, DC-based vaccines using mature DCs induce superior immunological responses compared to vaccines using immature DCs [28]. We therefore extended our analysis of IDO expression to TLR ligand-matured DCs (Supporting Information Fig. 1). Furthermore, since IFN-γ is a potent inducer of IDO and is highly produced by effector T cells, IFN-γ stimulation is relevant *in vivo* and was therefore also included in the analysis. DC subsets isolated from peripheral blood were cultured overnight with the indicated stimuli and IDO protein expression was analyzed by flow cytometry (Fig. 3A and B), while IDO enzyme activity was determined by measurement

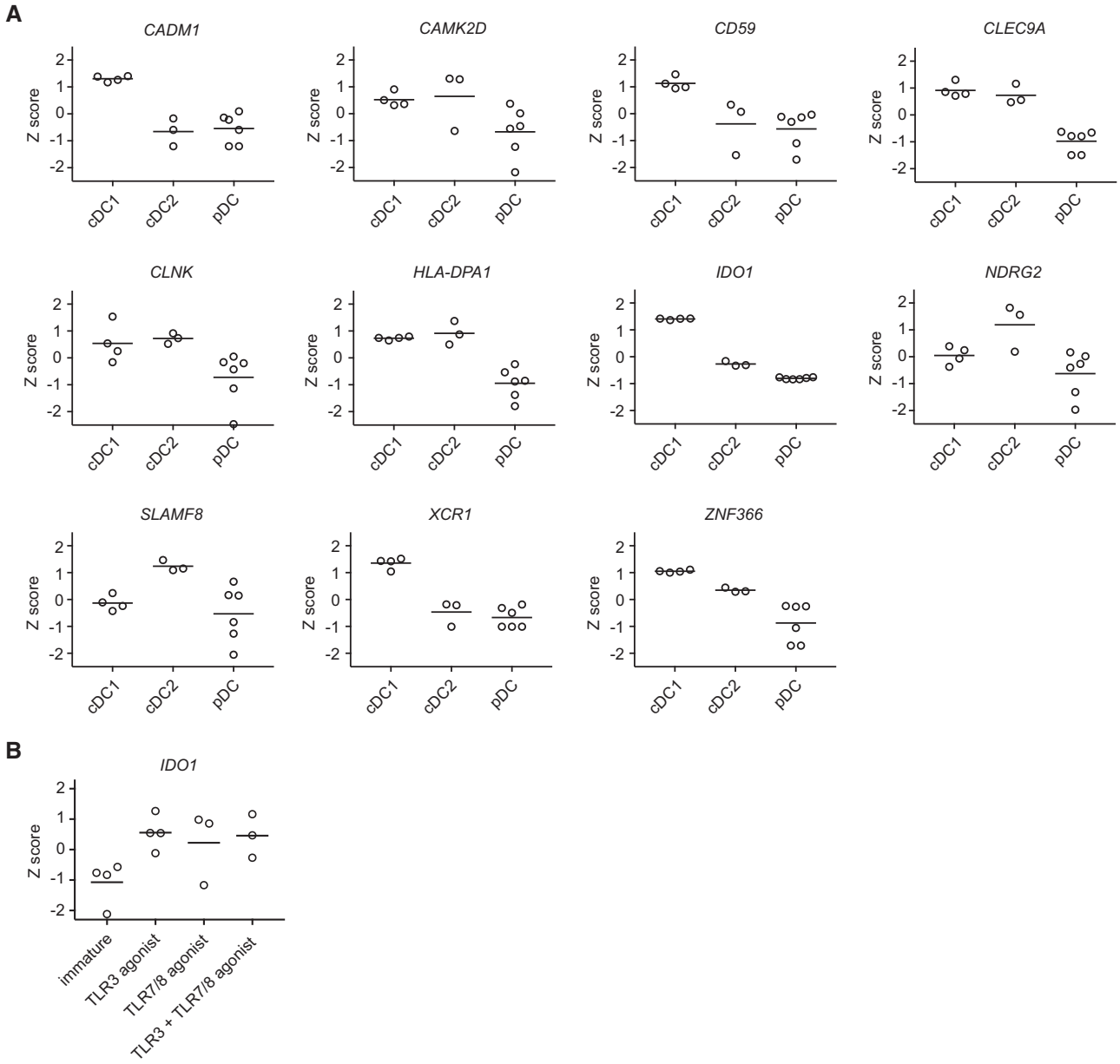

Figure 1. *IDO1* is among the genes that best defines peripheral blood cDC1s under homeostatic conditions and its expression is increased upon stimulation with TLR agonists. Gene expression was analyzed from RNA-seq datasets. Z scores were calculated using normalized and log transformed counts per million values. Means are depicted. (A) Expression of selected genes was compared between immature cDC1s, immature cDC2s, and immature (unstimulated or IL-3-treated) pDCs, of 4 (cDC1) or 3 (cDC2 and pDC) different donors. (B) *IDO1* gene expression among cDC1s of three or four different donors cultured overnight in absence or presence of a clinical grade TLR3 agonist (Hiltonol) and/or TLR7/8 agonist (protamine-RNA).

of kynurenine production in supernatants by HPLC (Fig. 3C). Importantly, while immature cDC2s showed only minimal levels of IDO expression and activity, stimulation with IFN-γ or the combination of TLR3-agonist polyinosinic:polycytidylic acid (poly(I:C)) with TLR7/8 agonist resiquimod (R848) increased both protein expression and enzyme activity in this DC subset. cDC1s stimulated with IFN-γ or the combination of poly(I:C) and R848 maintained and further increased IDO protein expression and activity, to levels higher than found in cDC2s (Supporting Information Fig. 2). High kynurenine production by IFN-γ-stimulated cDC1s was reduced upon addition of the IDO1-specific inhibitor epacadostat (Supporting Information Fig. 3), indicating that kynurenine production is IDO-dependent. In contrast, pDCs stimulated with IFN-γ, R848 or TLR9-agonist CpG oligodeoxynucleotide class C (CpG-C) did not upregulate IDO expression or activity (Fig. 3B and C). Thus, TLR ligation or IFN-γ stimulation induces IDO expression and activity in cDC1s and cDC2s, but not pDCs.

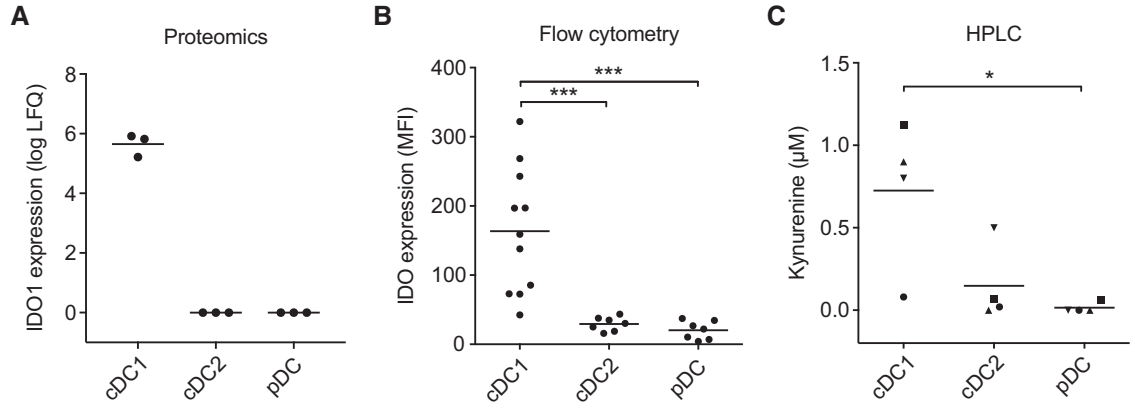

Figure 2. Immature cDC1s, but not cDC2s and pDCs, express functionally active IDO. (A) IDO1 protein expression among steady-state human blood DCs of three different donors was analyzed from a proteomics dataset. Means are depicted. (B) IDO intracellular protein expression was analyzed by flow cytometry in DCs cultured overnight. The graph shows the geometric mean fluorescent intensity (MFI) of IDO expression subtracted by the MFI of the isotype control, with at least seven different donors per condition from 10 independent experiments. Means are depicted. Significance was determined by one-way ANOVA with Bonferroni correction ($^{***}P < 0.001$). (C) IDO activity was analyzed by measuring L-kynurenine in supernatants of 24-h DC cultures by HPLC. Results are from six different donors from four independent experiments; in one experiment (squares), pooled supernatants from 3 different donors were used. Means are depicted and symbols correspond to measurements belonging to the same donors. Significance was determined by repeated measures one-way ANOVA with Bonferroni correction ($^{*}P < 0.05$).

Epacadostat-treatment of cDC1s and cDC2s prevents IDO-mediated inhibition of T cell proliferation

Human blood DC subsets express high levels of stimulatory molecules upon maturation and are known for their T cell stimulatory capacity [29]. However, IDO activity can inhibit T cell responses by depleting tryptophan and by increasing toxic kynurenine metabolites. To test whether IDO expressed by human blood DC subsets affects T cell proliferation, peripheral blood lymphocytes (PBLs) were stimulated with anti-CD3/anti-CD28 antibody-coated beads in DC-conditioned medium. T cell proliferation was inhibited by supernatant of cDC1s or cDC2s matured with poly(I:C) and R848 or stimulated with IFN-γ (Fig. 4A); this inhibition could be partially rescued by specifically blocking IDO activity with epacadostat, with statistically significant differences for the conditions where cDCs were stimulated with TLR ligands. In contrast, treating pDCs with epacadostat did not affect T cell proliferation. These findings are in accordance with the IDO expression and activity data and underline the functional relevance of IDO activity by human cDCs on T cell proliferation.

The effect of IDO on DC-induced T cell proliferation was further studied in a mixed lymphocyte reaction (MLR). All three primary DC subsets induced allogenic T cell proliferation, but inhibition of IDO did not substantially affect T cell proliferation (Fig. 4B), in contrast to the assay with DC-conditioned medium. Yet, addition of epacadostat to moDCs stimulated with LPS and IFN-γ to express high levels of IDO [30] was able to increase allogeneic T cell proliferation (Supporting Information Fig. 4). This suggests that IDO activity in primary cDCs has only limited inhibitory effects in settings were these cells have contact-mediated interactions with T cells. While poly(I:C) and R848 stimulation induces IDO activity (Fig. 3), this potent combination of TLR agonists is also known to strongly upregulate costimulatory molecules and cytokine production [29]. These findings might therefore reflect the complex interplay of inhibitory and stimulatory molecules expressed or secreted by DCs in T cell priming.

Epacadostat is currently under investigation in several clinical trials. Although its effects as a single treatment appear limited, several clinical studies suggest that IDO inhibition might potentiate the effects of other cancer immunotherapies [31]. To achieve a successful combination of epacadostat with DC-based immunotherapy, the inhibitor should not negatively affect DC function. Cross-presentation of exogenous antigens on MHC class I enables DCs to prime $CD8^+$ T cells, which is crucial for induction of anti-tumor immunity. In humans, all blood DC subsets are capable of cross-presenting soluble antigens [32], although cDC1s are superior at cross-presenting cellular antigens [33–36]. We therefore tested the effect of epacadostat on the capacity of cDC1s, cDC2s and pDCs of HLA-A2.1$^+$ donors to cross-present soluble $gp100_{(272\text{-}300)}$ peptide to T cells transduced with a T cell receptor specific for the $gp100_{(280\text{-}288)}$ peptide. Cross-presentation of $gp100_{(272\text{-}300)}$ peptide requires intracellular processing in order to release the HLA-A2.1-restricted $gp100_{(280\text{-}288)}$ epitope. In line with our expectations, all DC subsets effectively cross-presented soluble $gp100_{(272\text{-}300)}$ (Fig. 5). IDO inhibition by epacadostat did not significantly change the ability of any of the three primary DC subsets, nor moDCs (Supporting Information Fig. 5), to cross-present soluble antigen.

The IDO inhibitor 1-methyl-tryptophan has been reported to partially inhibit the maturation of moDCs in response to certain TLR agonists [15]. We, therefore, tested whether epacadostat treatment affects the expression of membrane molecules on DC subsets that are normally upregulated upon maturation, as well as the production of IL-12 by cDC subsets. Epacadostat treatment did not substantially change the expression of MHC-I

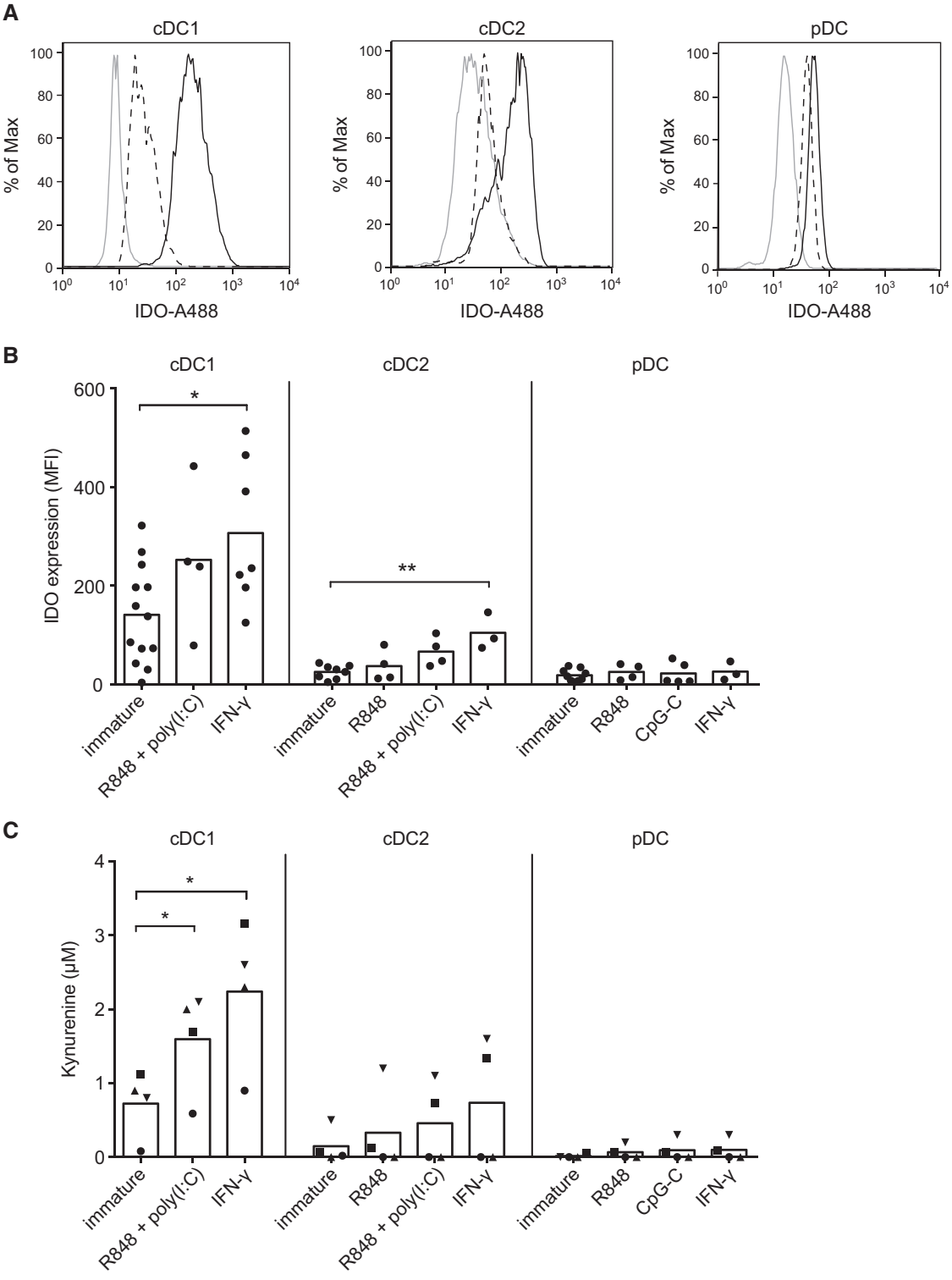

Figure 3. IDO protein expression and activity among stimulated blood DC subsets. (A) Flow cytometry histograms from a representative donor showing isotype control (solid grey line), IDO expression in absence of stimulus (dashed black line), and IDO expression for either IFN-γ or CpG-C stimulation (solid black line) for cDCs or pDCs, respectively. (B) The figure shows the geometric mean fluorescence intensity (MFI) of IDO expression subtracted by the MFI of the isotype control, with at least 3 different donors per condition from 10 independent experiments. Bars represent means. Significance was determined by one-way ANOVA with Bonferroni correction, comparing stimulated DCs with immature control ($^{*}P < 0.05$; $^{**}P < 0.01$). (C) IDO activity was analyzed by measuring L-kynurenine in supernatants of 24-h DC cultures by HPLC. The results are from six different donors from four independent experiments; in one experiment (squares), pooled supernatants from 3 different donors were used. Bars represent means and symbols correspond to measurements belonging to the same donors. Significance was determined by repeated measures one-way ANOVA with Bonferroni correction, comparing stimulated DCs with their immature control ($^{*}P < 0.05$).

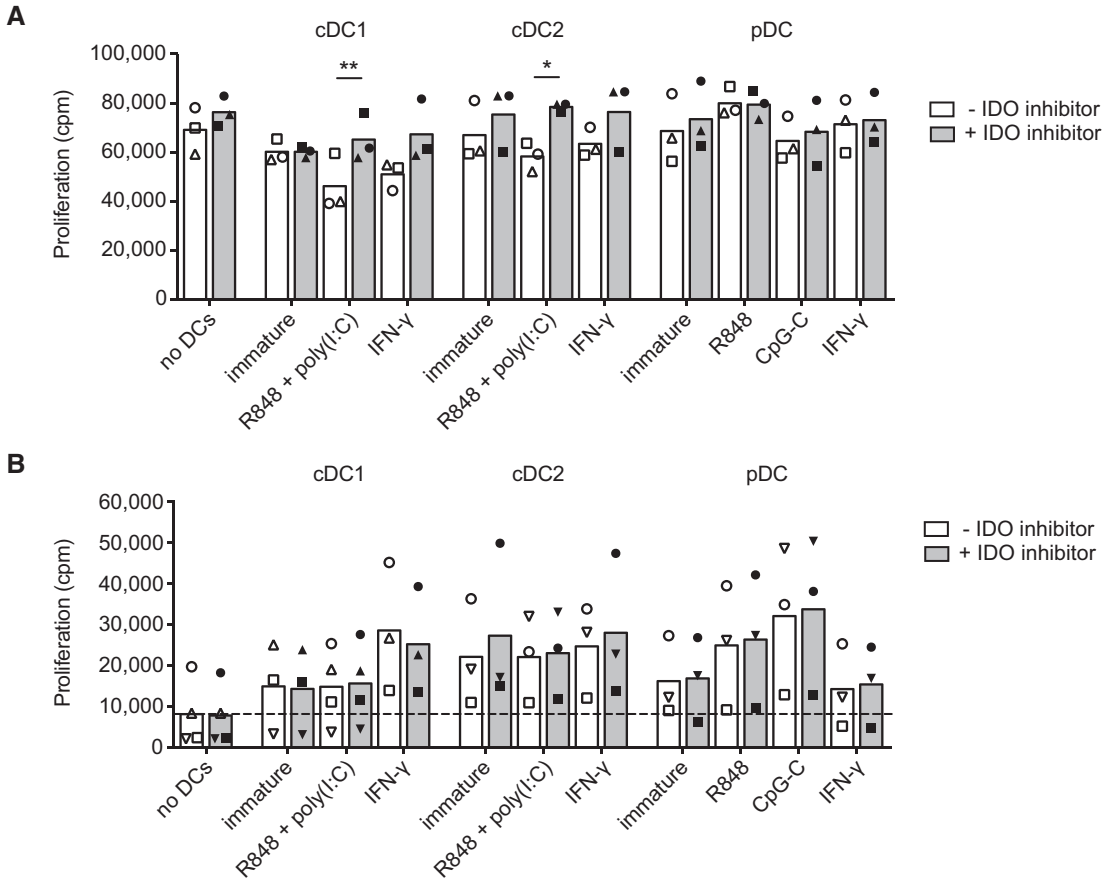

Figure 4. IDO expressed by cDCs inhibits T cell proliferation. Blood DCs were stimulated with indicated stimuli and/or epacadostat in medium containing 10 μM tryptophan. (A) PBLs were stimulated with anti-CD3/CD28-coated beads in supernatants of 48-hour DC cultures and proliferation was measured after three days by tritiated thymidine incorporation. Mean proliferation in counts per minute (cpm) of three different donors from 3 independent experiments with technical triplicates (cDC2, pDC) or duplicates (cDC1) is shown. Symbols correspond to measurements belonging to the same donors. Significance was determined by two-tailed paired *t*-test comparing absence vs presence of IDO inhibitor ($^{*}P < 0.05$; $^{**}P < 0.01$). (B) Allogeneic PBLs were cultured with overnight-stimulated DCs and proliferation was measured after another three days by tritiated thymidine incorporation. Mean proliferation of at least three different donors from four independent experiments with technical triplicates (cDC2, pDC) or duplicates (cDC1) are shown. Symbols correspond to measurements belonging to the same donors. Significance was determined by two-tailed paired *t*-test, comparing absence versus presence of IDO inhibitor.

(HLA-ABC), MHC-II (HLA-DR), CD40, CD80, CD83, CD86, CCR7, PD-L1, or IL-12 by any of the three DC subsets upon maturation by TLR3 and/or TLR7/8 agonists (Supporting Information Fig. 6A and B). Taken together, epacadostat enhances T cell proliferation induced by TCR cross-linking in the presence of supernatant of TLR-stimulated cDCs, while it does not interfere with DC maturation or their ability to cross-present soluble antigen. These properties make this IDO inhibitor an interesting candidate to enhance the efficacy of DC-based cancer immunotherapies.

Discussion

DCs can limit T cell responses via IDO activity. Although IDO is well-characterized in murine DCs and human moDCs, much less is known about its activity and function in human primary DCs. Here, we compared the expression and activity of IDO across the three major human DC subsets found in peripheral blood. We describe a functional role for IDO in cDCs, in particular in cDC1s.

RNA expression profiling of steady-state human DC subsets showed unique *IDO1* expression by cDC1s [19–21]. We confirm that among immature DCs, *IDO1* is one of the most uniquely expressed genes by cDC1s. Using flow cytometry, we demonstrate that IDO is also uniquely expressed at the protein level by immature cDC1s, which corroborates our previous study using proteomics [23]. We expand on this data by showing that IDO in immature cDC1s is biologically active, as it leads to the conversion of tryptophan into kynurenine. Upon stimulation with IFN-γ or TLR agonists, its expression and activity is further increased and can limit T cell proliferation. Of note, human cDC1s have also been reported to specifically express the IDO1-paralog gene *IDO2* [19,22], but its expression at the protein level, activity, and functional role have not been investigated here.

Human cDC1s are considered the equivalent of murine CD8α^{+} DCs [19,33,34,36–38]. Likewise, murine CD8α^{+} DCs can mediate

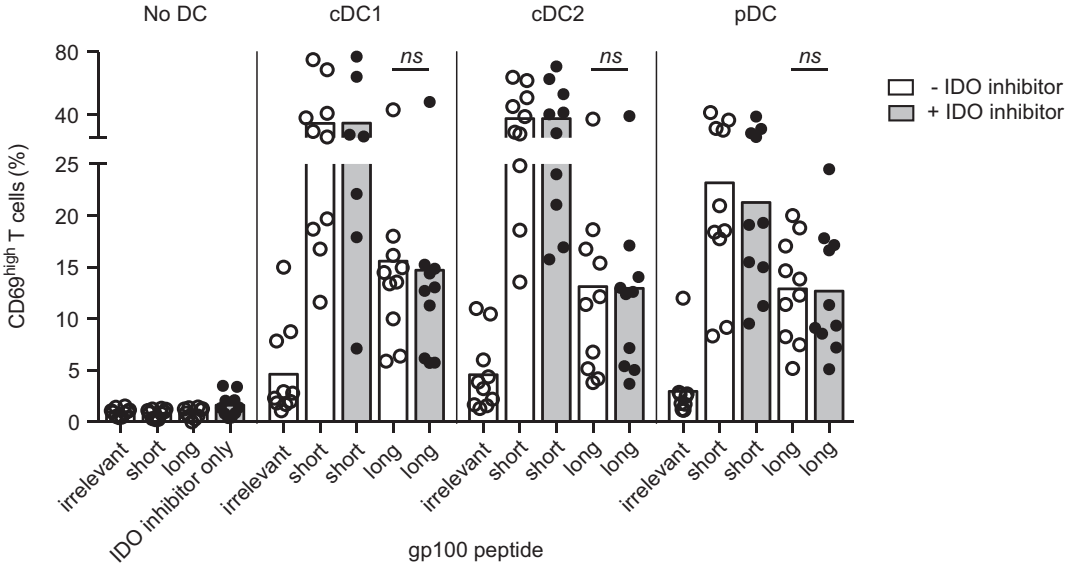

Figure 5. IDO inhibition by epacadostat does not affect the ability of DCs to cross-present soluble antigen to T cells. Blood DCs were stimulated overnight with R848 (pDCs) or R848 and poly(I:C) (cDCs) in medium containing 1 μM tryptophan, with or without epacadostat, and loaded with $gp100_{(154-162)}$ (irrelevant), $gp100_{(280-288)}$ (short), or $gp100_{(272-300)}$ (long) peptide. After overnight culture, T cells expressing the T cell receptor recognizing $gp100_{(280-288)}$ were added at a 1:5 DC:T cell ratio for 24 h. CD69 expression by T cells was used as a readout for DC-induced T cell stimulation and was measured by flow cytometry. Mean frequency of $CD69^{high}$ cells among live $CD3^+$ T cells is shown, with at least seven different donors per condition from 11 independent experiments, with technical duplicates for cDC2 and pDC conditions. Significance was determined by two-tailed paired *t*-test, comparing absence versus presence of IDO inhibitor (*ns*, non-significant).

T cell suppression via IDO [39]. Interestingly, $CD8\alpha^+$ DCs constitutively express IDO, but the enzyme requires additional signaling through IFN-γ or CTLA-4 to become biologically active [40,41]. We show that although signaling via IFN-γ increases IDO expression and activity in human cDC1s, it is not needed per se for its enzymatic activity. Human cDC1s, like their murine counterpart, are excellent cross-presenters and primers of T cells [37]. Expression of immunosuppressive IDO by this potent pro-inflammatory immune cell seems paradoxical. Yet, cDC1s express the C-type lectin receptor CLEC9A to efficiently induce immune responses towards dead cell antigens [33]. It is crucial that during homeostasis, apoptotic self-antigens do not provoke an immune response, for this may lead to systemic autoimmunity. IDO has been reported to regulate tolerance to apoptotic antigens in mice [42]. Hence, preferential expression of IDO in cDC1s could function as a safeguard mechanism, ensuring default immunosuppressive function in absence of pathogen- or danger-associated molecular patterns. In addition, upregulation of IDO by stimulated cDC1s might serve as a negative feedback mechanism to prevent excessive T cell stimulation. These hypotheses should be tested in future studies.

cDC2s do not express IDO when immature, but upregulate it upon exposure to TLR3 and TLR7/8 agonist or IFN-γ. Production of kynurenine by human cDC2s has also been reported in response to LPS, *E. coli*, or prostaglandin E_2 [43,44]. In our assays, the supernatant from TLR-stimulated cDC1s as well as cDC2s had a functional impact on T cell proliferation induced by TCR crosslinking. Inhibition of T cell proliferation was dependent on IDO and independent of cell-cell contact, probably by depleting tryptophan and/or enriching T cell inhibitory breakdown products of tryptophan. Stimulatory cell-cell contact between cDCs and T cells in an MLR seems to overpower IDO-mediated inhibition, as blocking IDO did not boost T cell proliferation in this setting. Still, IDO activity in cDCs might become functionally relevant in the tumor microenvironment, where the enzyme's activity can contribute to other immunosuppressive signals.

Our data show that immature human pDCs exhibit no or very low IDO expression, which is in accordance with a study by Trabanelli *et al*. [44], as well as RNA expression profiling of steady-state human DC subsets [19]. We also found very low expression and activity of IDO in pDCs stimulated with CpG-C, R848, or IFN-γ. Nevertheless, this does not rule out the possibility that human pDCs are able to express functional IDO in other settings. Indeed, IDO expression by human pDCs has been reported upon stimulation by HIV, CpG-A or CpG-B [45,46]. Gerlini *et al*. identified IDO-expressing pDCs in metastatic sentinel lymph nodes of melanoma patients [47]. pDCs from control lymph nodes and the blood of these patients did not express IDO, suggesting that expression of the enzyme by human pDCs can be induced by tumors.

By using the IDO1-selective inhibitor epacadostat, we were able to increase T cell proliferation in conditioned medium of cDC1s or cDC2s. Epacadostat was demonstrated to be safe in phase I/II clinical trials, and is currently being tested in multiple phase III trials in combination with checkpoint blockade [31,48]. Our results suggest that epacadostat could alternatively be used in combination with DC-based cancer immunotherapies to increase inflammatory responses. Besides combining systemic epacadostat treatment with DC-based cancer therapies, epacadostat might also

be used during DC vaccine preparation. Treating conventional DCs *ex vivo* with epacadostat prior to injection into the patient could potentially enhance efficacy of immunostimulatory DC vaccines.

Materials and methods

RNA sequencing and proteomic analysis

RNA-seq analysis was performed on a dataset of cDC2s and pDCs deposited at the Gene Expression Omnibus (accession number: GSE89442) [27] and a dataset of cDC1s (Mathan *et al*. manuscript in preparation). Data were analyzed using the R platform for statistical computing. Count values from both datasets were normalized and log-transformed, before calculating *Z* scores using the edgeR package [49]. Proteomics analysis was performed on a dataset of cDC1s, cDC2s and pDCs deposited at the ProteomeXchange Consortium (dataset identifier: PXD004678) [23].

Cell isolation and culture

Human primary DCs were isolated from buffy coats or apheresis products (Sanquin, Amsterdam, The Netherlands) obtained from healthy volunteers after written informed consent and according to institutional guidelines. Peripheral blood mononuclear cells were obtained via ficoll density gradient centrifugation. DCs were isolated by FACS sorting after enrichment with Dynabeads Human DC enrichment kit (Invitrogen, Carlsbad, CA). The remaining cells were incubated with anti-Lin1-FITC, anti-HLA-DR-PE-Cy7 (both BD Biosciences, Franklin Lakes, NJ), anti-CD1c-BV421 (Biolegend, San Diego, CA), anti-CD141-APC and anti-CD304-PE (both Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were sorted on a BD Aria II from lineage negative, HLA-DR^{high} cells into three different populations, based on the expression of CD141, CD1c or CD304 to obtain cDC1s, cDC2s, or pDCs, respectively. In some experiments as indicated, DC subsets were isolated from PBLs with magnetic microbeads by MACS sorting (Miltenyi Biotec). PBLs were prepared by depleting monocytes via anti-CD14-conjugated microbeads or adherence to plastic culture flasks. To generate immature moDCs, monocytes were cultured in X-VIVO 15 medium supplemented with 2% human serum, 300 IU/mL IL-4 and 450 IU/mL GM-CSF (both ImmunoTools, Friesoythe, Germany) for 7 days. For mature moDCs, a maturation cocktail was added for the last day of the culture period.

Cells were cultured in either X-VIVO 15 medium (Lonza, Basel, Switzerland) supplemented with 2% human serum (Sigma–Aldrich, Saint Louis, MO), hereinafter described as complete X-VIVO medium, or in custom-made tryptophan-free RPMI 1640 (Gibco/Thermo Fisher Scientific, Waltham, MA) supplemented with 2 mM ultraglutamine (Lonza), 1% antibiotic-antimycotic (Gibco), 10% human serum albumin (Sanquin) and the indicated concentration of L-tryptophan (Merck, Darmstadt, Germany), hereinafter described as complete RPMI medium. DCs were incubated with stimuli as indicated: 4 μg/mL R848, 20 μg/mL poly(I:C), 5 μg/mL CpG-C (all Enzo Life Sciences, Farmingdale, NY), 100 ng/mL LPS (Sigma–Aldrich), or 10 ng/mL IFN-γ (Thermo Scientific). In the absence of activation stimulus, 10 ng/mL recombinant human IL-3 (CellGenix) was added to pDC cultures as survival factor.

Flow cytometry

Flow cytometry experiments were performed according to the guidelines by Cossarizza *et al*. [50]. To analyze IDO expression, DCs were isolated by MACS sorting and cultured for 16 h in complete X-VIVO medium with indicated stimuli. The next day, cells were stained extracellularly with anti-CD141-PE for cDC1s, anti-CD1c-PE for cDC2s, or anti-CD304-PE (all Miltenyi Biotec) for pDCs. Cells were subsequently fixed, permeabilized, and stained intracellularly with anti-IDO-A488 (clone #700838; R&D systems, Minneapolis, MN). Cells were measured on a BD FACS Calibur and data were analyzed by FlowJo software (see Supporting Information Fig. 7 for the gating strategy).

To analyze maturation marker expression upon treatment with epacadostat, DCs were isolated by FACS sorting and cultured at 1×10^4 DCs/well for 16 hours in complete RPMI medium with 1 or 10 μM L-tryptophan, indicated stimuli and with or without 10 μM epacadostat (INCB024360; Selleck Chemicals, Houston, TX). After overnight culture, cells were labeled with fixable viability dye eFluor-780 (eBioscience), anti-CD80-FITC (Biolegend), anti-CD83-APC, anti-CD86-PE-Cy7, anti-HLA-ABC-V450, anti-HLA-DR-BV510 (all BD Biosciences), anti-PD-L1-APC (Biolegend), CD40-PE (Immunotech/Beckman Coulter, Brea, CA), and anti-CD1b/c-FITC (Diaclone, Besancon Cedex, France) or anti-CD303-FITC (Miltenyi Biotec). Expression of maturation markers on live DCs was determined on a BD FACSVerse and data were analyzed by FlowJo software.

Cytokine detection

Supernatants of DCs cultured at 1×10^4 DCs/200μL/well with indicated stimuli were analyzed with standard sandwich ELISAs detecting IL-12p70 (Biolegend).

Kynurenine quantification

DCs were isolated by FACS sorting and 80×10^3 DCs were cultured at 0.5×10^6 cells/mL for 24 h in complete X-VIVO medium and indicated stimuli. Conditioned media were analyzed for L-kynurenine by HPLC. Samples were pretreated with perchloric acid to precipitate proteins. Samples were pretreated (5:1) with 20% formic acid. 200 μL of samples was mixed with 200 μL buffer (0.77 g/L NH_4Ac, 100 mg/L EDTA-Na_2, 375 mg/L NaCl, 37.5 mL/L MeOH; pH 5.35). Pure kynurenine (Sigma-Aldrich) was used for calibration. Samples were injected into an

Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters, Milford, MA), consisting of an autosampler, injector, column (Acquity UPLC HSS T3 1.8 μm; 2.1 × 100 mm) and UV detector (Waters) at 360 nm. Chromatographic separation was performed at an elution rate of 0.3 mL/min buffer.

T cell proliferation assays

For the proliferation assay with conditioned medium, DCs were isolated by FACS sorting and 2.5-3 × 10^4 DCs were cultured for 48 hours in 100 μL complete RPMI medium with 10 μM L-tryptophan, indicated stimuli and with or without 10 μM epacadostat. DCs from three different donors were pooled at the same ratio for each subset to obtain a sufficient number of cells and to even out donor variation. After 48 h, 75 μL of this conditioned medium was added to 75 μL complete RPMI medium containing 1.5 × 10^5 PBLs and 3 × 10^4 anti-CD3/CD28 beads (Gibco). After 3 days, proliferation was assessed by adding 1 μCi [0.037 MBq]/well of tritiated thymidine (MP Biomedicals, Irvine, CA) to the cells. Tritium incorporation over 16 h was measured with a scintillation counter. All experiments were performed in triplicate for cDC2s and pDCs and in duplicate for cDC1s.

For classical MLR assays, DCs were isolated by FACS sorting and 2.5-4 × 10^4 DCs (always the same numbers across subsets) were cultured for 16 h in 100 μL complete RMPI medium with 10 μM L-tryptophan, indicated stimuli and with or without 10 μM epacadostat. Then, 1.5 × 10^5 PBLs were added and incubated with the DCs for 3 days. Proliferation was assessed by tritiated thymidine incorporation as above. All experiments were performed in triplicate for cDC2s and pDCs and in duplicate for cDC1s.

Antigen cross-presentation assay

Jurkat T cells transduced with CD8 and the α and β chains of a T cell receptor specific for the HLA-A2.1-restricted $gp100_{(280\text{-}288)}$ peptide (JE6.1 fl296; described previously [51]) were cultured in RPMI supplemented with 2 mM ultraglutamine, 1% antibiotic-antimycotic and 10% fetal bovine serum (Greiner Bio-One, Kremsmünster, Austria). Positivity for the gp100-specific TCR was monitored by flow cytometry with anti-TCR-V-β14-PE (ImmunoTools).

DCs were isolated from buffy coats or apheresis products of HLA-A2.1$^+$ donors by FACS sorting and cultured at 1 × 10^4 DCs/well in complete RPMI with 1 μM L-tryptophan, indicated stimuli and with or without 10 μM epacadostat. In addition, DCs were loaded with 1 μM $gp100_{(154\text{-}162)}$, 1 μM $gp100_{(280\text{-}288)}$, or 50 μM $gp100_{(272\text{-}300)}$ peptide (all JPT peptide technologies, Berlin, Germany). After 16 h of culture, 5 × 10^4 JE6.1 fl296 T cells were added directly to the DCs and cultured for another 24 h. The cells were labeled with anti-CD3-FITC (BD Biosciences), anti-CD69-APC (eBioscience) and fixable viability dye eFluor-780 to determine the percentage of $CD69^{high}$ cells among live CD3$^+$ T cells. All experiments were performed in singlicate for conditions with cDC1s and in duplicate for conditions with cDC2s and pDCs.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software version 7 (GraphPad Software Inc, San Diego, CA). Details on the performed statistical tests can be found in the figure legends. Statistical significance was defined as: ns, $P > 0.05$; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

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Abbreviations: **cDC1s**: type 1 conventional dendritic cells · **cDC2s**: type 2 conventional dendritic cells · **CpG-C**: CpG oligodeoxynucleotide class C · **DCs**: dendritic cells · **HPLC**: high-performance liquid chromatography · **IDO**: indoleamine 2,3-dioxygenase 1 · **IFN**: interferon · **LPS**: lipopolysaccharide · **MLR**: mixed lymphocyte reaction · **moDCs**: monocyte-derived dendritic cells · **PBLs**: peripheral blood lymphocytes · **pDCs**: plasmacytoid dendritic cells · **Poly(I:C)**: polyinosinic:polycytidylic acid · **R848**: resiquimod · **RNA-seq**: RNA-sequencing · **TLRs**: Toll-like receptors · **Tregs**: regulatory T cells

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