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Original Research

Quantitative and qualitative impairments in dendritic cell subsets of patients with ovarian or prostate cancer

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Abstract ***Background:*** Dendritic cells (DCs) are the most efficient antigen-presenting cells, hence initiating a potent and cancer-specific immune response. This ability (mainly using monocyte-derived DCs) has been exploited in vaccination strategies for decades with limited clinical efficacy. Another alternative would be the use of conventional DCs (cDCs) of which at least three subsets circulate in human blood: cDC1s ($CD141^{bright}$), cDC2s ($CD1c^{+}$) and plasmacytoid DCs. Despite their paucity, technical advances may allow for their selection and clinical use. However, many assumptions concerning the DC subset biology depend on

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observations from mouse models, hindering their translational potential. In this study, we characterise human DCs in patients with ovarian cancer (OvC) or prostate cancer (PrC).
Patients and methods: Whole blood samples from patients with OvC or PrC and healthy donors (HDs) were evaluated by flow cytometry for the phenotypic and functional characterisation of DC subsets.
Results: In both patient groups, the frequency of total CD141^{+} DCs was lower than that in HDs, but the cDC1 subset was only reduced in patients with OvC. CD141^{+} DCs showed a reduced response to the TLR3 agonist poly (I:C) in both groups of patients. An inverse correlation between the frequency of cDC1s and CA125, the OvC tumour burden marker, was observed. Consistently, high expression of CLEC9A in OvC tissue (The Cancer Genome Atlas data set) indicated a better overall survival.
Conclusions: cDC1s are reduced in patients with OvC, and CD141^{+} DCs are quantitatively and qualitatively impaired in patients with OvC or PrC. CD141^{+} DC activation may predict functional impairment. The loss of cDC1s may be a bad prognostic factor for patients with OvC.

1. Introduction

Tumour antigens, despite their host-derived origin, can be recognised as targets by T cells [1]. T-cell priming requires dendritic cells (DCs) that are the most potent antigen-presenting cells (APCs). Conventional DCs (cDCs) are important in developing anti-tumour T-cell responses, and they include two main subsets: cDC1s (CD141^{bright}), which are fundamental for CD8 -ell activation, and cDC2s (CD1c^{+}) which are key for CD4 T-cell activation [2,3]. cDC1s are endowed with high cross-presentation capacity and secrete molecules which are able to attract CD8 T cells and thus are particularly important in promoting antigen-specific tumour cell killing [3–5]. Indeed, cDC1 tumour infiltration has been associated with better clinical outcomes in some tumour types [6,7].

To escape immune surveillance, tumours are able to directly suppress tumour-specific immune responses or to affect DC polarisation, activation, generation and tissue infiltration [7,8]. Frequencies and activation capacity of circulating and infiltrating DCs are generally lower in patients with cancer than in healthy individuals. These alterations are associated with poor survival, reduced response to therapies and are likely to be one of the main causes for the limited success of DC-based vaccines in patients with cancer [6,7,9–11]. In patients with breast and pancreatic cancer, the generation of cDC1s is impaired and this correlates with both reduced CD8 T-cell responses and poor clinical outcomes [7]. In patients with prostate cancer (PrC), DC frequency and activation are reduced when compared with healthy individuals, while treatment-induced activation of DCs is associated with better response to immunotherapy [12]. Although in patients with ovarian cancer (OvC), the frequency of DC subsets in ascites is not correlated with their survival [13] and tumour-infiltrating DCs show a regulatory phenotype [14–16]. These data suggest that, although DC-based therapies have a great potential in cancer treatment, they need to be combined with strategies to prevent quantitative and functional defects of DCs [17]. In this regard, it has been shown that immunosuppressive tumour-infiltrating DCs could be converted into APCs capable of priming anti-tumour T-cell responses after treatment with CD40 and TLR3 agonists [6,18]. In addition, because DC subsets have distinct roles in anti-tumour immunity, the use or the targeting of a DC subset particularly efficient in inducing anti-tumour responses may be key for designing powerful immunotherapies. Of note though, most of the assumptions concerning DC subset biology are based on observations made in mouse models. It is, thus, of paramount importance to directly study human DC functions in cancer.

In the present study, we investigated the frequency, the activation status and the capacity of DC subsets to respond to poly (I:C) (TLR3 agonist) stimulation in samples of healthy donors (HDs) and patients with OvC or PrC. In addition, we evaluated possible associations between markers of tumour burden such as cancer antigen 125 (CA125) and prostate-specific antigens (PSAs) and the frequency of DC subsets. Finally, we interrogated The Cancer Genome Atlas (TCGA) databases to identify the impact of CD141 (THBD), CD1c, CD11c (ITGAX), CD123 (IL3RA), BDCA2 (CLEC4C), CLEC9A and XCR1 expression in OvC and PrC tissues on overall survival (OS).

2. Material and methods

2.1. Subjects and specimen preparation

Human blood samples from HDs and patients were collected in accordance with the declaration of Helsinki

Table 1
Clinical data of patients with OvC.

Date of sampling	Code	Date of diagnosis	Age	CA125	Stage FIGO	WBC (x106/ml)	BRCA	Lines of treatment	BEV	CBCDCAs	On treatment
24/03/2017	**0YNN**	01/01/2017	70	6.00	IVb	13.7	Nd	1	yes	yes	yes
25/04/2017	**0XSF**	02/12/2016	71	Nd	IIIC	7.3	Wt	1	no	yes	yes
09/05/2017	**0XQ2**	01/02/2017	73	Nd	IVb	10.7	Nd	1	no	yes	yes
16/08/2017	**0VSZ**	03/06/2014	48	4.00	IIIB	3	Wt	3	yes	no	yes
16/08/2017	**04NP**	14/03/2012	66	197.00	IIIC	5	Wt	5	yes	no	yes
16/08/2017	**0XLZ**	05/01/2017	50	8.00	IIIC	5	Wt	1	no	yes	yes
16/08/2017	**10DE**	30/06/2017	52	101.00	IV	4.1	wt	1	no	yes	yes
16/08/2017	**0N6N**	26/06/2015	69	9.00	IIIB	6.2	Wt	1	no	yes	no
16/08/2017	**0N2Q**	23/06/2015	71	60.00	IVB	10.6	mut	2	yes	yes	yes
16/08/2017	**0XN7**	11/04/2012	66	99.00	IIIC	5.9	Wt	3	yes	no	yes
23/08/2017	**0ZWQ**	14/06/2017	48	15.00	IC	3.8	Wt	1	no	yes	yes
16/08/2017	**0CW3**	21/10/2008	57	33.00	IA	6.7	mut	–	no	yes	no
23/08/2017	**0V0T**	06/04/2017	67	322.00	IIIc	4	mut	1	no	no	yes
23/08/2017	**1169**	25/07/2017	67	15.00	IIIB	6.9	Nd	1	no	yes	yes
23/08/2017	**0Z7B**	31/12/2014	74	30.00	IIIC	5.7	Wt	3	yes	yes	yes
30/08/2017	**0ZPG**	12/04/2017	64	Nd	IVB	6.6	Nd	1	no	yes	yes
30/08/2017	**0YDN**	24/05/2012	49	7.60	IIIC	3.6	mut	4	yes	yes	yes
30/08/2017	**0LZA**	15/01/2015	47	233.00	IIIC	3.9	Wt	2	no	yes	no
30/08/2017	**0S5K**	02/03/2016	77	22.00	IVB	6.6	Nd	1	yes	yes	yes
30/08/2017	**1199**	07.07.2015	66	43.00	IIIC	5	Wt	1	no	yes	no
30/08/2017	**0YJV**	14/02/2013	68	7.00	IA	5.2	Nd	1	no	yes	no
30/08/2017	**0Y7H**	01/02/2013	70	446.00	IIIC	5.9	Wt	3	yes	yes	yes
13/09/2017	**0LMX**	08/04/2015	71	11.00	IVA	6.4	mut	2	yes	yes	yes
13/09/2017	**0YSF**	14/08/2013	83	15.00	IIIC	4.4	Nd	1	no	yes	no
13/09/2017	**11H1**	01/12/2011	79	192.00	IVB	5.1	Wt	5	yes	no	yes
13/09/2017	**0V8Z**	26.09.2016	66	7.00	IVA	5.3	Wt	1	yes	yes	yes
11/10/2017	**0NSO**	29/07/2015	51	20.00	IIIB	7.5	Wt	1	no	yes	no
11/10/2017	**046T**	11/12/2012	77	81.00	IIIC	7.6	Wt	2	yes	yes	no

WBC = white blood cell; BEV = bevacizumab; CBCDCA = carboplatinum; Nd = not determined; Wt = wilde type; mut = mutated; OvC = ovarian cancer; CA125 = cancer antigen 125; FIGO = International Federation of Gynecology and Obstetrics.

Table 2
Clinical data of patients with PrC.

Date of sampling	Code	Age	PSA (ug/ml)	WBC (x106/ml)	Diagnosis	Stage	Gleason score	Previous treatment
21/03/2017	**URO534**	62	11	8.4	Prostate adenocarcinoma	pT2c pN0 R0	9	No
19/04/2017	**URO539**	74	9.7	7.8	Prostate adenocarcinoma	pT2c pN0 R1	7	No
03/05/2017	**URO211**	64	3.8	8.4	Prostate adenocarcinoma	pT2a pN0 Rx	7	No
16/05/2017	**URO543**	66	3.6	5	Prostate adenocarcinoma	cT2c cN0 cM0	9	Yes
04/07/2017	**URO561**	61	6.2	Nd	Prostate adenocarcinoma	pT2c pN0 R0	7	No
19/07/2017	**URO563**	58	2.7	7.7	Prostate adenocarcinoma	pT2c pN0 R0	7	No
10/08/2017	**URO569**	61	6.8	7.5	Prostate adenocarcinoma	pT2c pN0 R0	7	No
11/01/2018	**URO600**	64	11.38	11	Prostate adenocarcinoma	pT2b pN0 R1	7	No
16/01/2018	**URO603**	58	7.7	6.8	Prostate adenocarcinoma	pT2c pN0 R1	7	No
31/01/2018	**URO607**	76	3.14	7	Prostate adenocarcinoma	pT3b pNx R1	9	Yes
20/02/2018	**URO615**	58	8.9	5.9	Prostate adenocarcinoma	pT2b pN0	6	No
26/06/2018	**URO674**	61	4.7	7	Prostate adenocarcinoma	pT2c pN0 R0	7	Yes
07/11/2018	**URO691**	68	23.2	6.2	Prostate adenocarcinoma	pT2c pN0 R0	7	No
28/11/2018	**URO693**	72	3.3	11	Prostate adenocarcinoma	pT2c pN0 R1	7	No
18/10/2018	**URO699**	72	4.6	9.2	Prostate adenocarcinoma	pT2c pN0 R1	7	No
28/11/2018	**URO717**	57	5.5	5.2	Prostate adenocarcinoma		6	Yes

nd = not done; WBC = white blood cell; PSA = prostate-specific antigen; PrC = prostate cancer.

principles. Written informed consent was obtained from all healthy subjects and patients (protocols: 2016–02094 and 2019–00546). Clinical data from patients with OvC or PrC are described in Table 1 and Table 2.

2.2. Culture media

RPMI 1640 Glutamax supplement (Thermo Fisher Scientific, Waltham, MA, United States), 10% human serum (BIOWEST, Riverside, MO, United States),

1 mM Na pyruvate (Thermo Fisher Scientific, Waltham, MA, United States), 10 mM/ml HEPES buffer (Thermo Fisher Scientific, Waltham, MA, United States), 1X MEM non-essential amino acids (Thermo Fisher Scientific, Waltham, MA, United States), 0.1% β-mercaptoethanol and 1% penicillin-streptomycin (Bioconcept, Allschwil/BL, Switzerland).

2.3. Antibodies and reagents

Anti-CD11c BV650 (clone 3.9), anti-CD80 PeCy5 (clone 2D10), anti-CD83 Percp Cy5.5 (clone HB15e), anti-CD1c BV510 (clone L161), anti-HLADR BV570 (clone L243), anti-XCR1 PE (clone S15046E), anti-CD40 BV605 (clone 5C3), anti-CD303 PE/Dazzle 594 (201A), anti-CD3 BV421 (clone UCHT1) and anti-CD19 BV421 (clone HIB19) were purchased from BioLegend (San Diego, CA, United States). Anti-Clec9a VioBright-FITC (clone 8F9), anti-CD123 APC-Vio770 (clone AC145) and anti-CD141 APC were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Anti-CD274 PE-Cy7 (clone MIH1), anti-CD86 A700 (clone 2331) and anti-CD14 PB (clone M5E2) were purchased from BD Biosciences (Franklin Lakes, NJ, United States). Red blood cells (RBC) lysis solution was purchased from Qiagen (Hilden, Germany). FcR blocking reagent was purchased from Miltenyi (Bergisch Gladbach, Germany). Poly (I:C) high molecular weight (HMW) was purchased from Invivogen (San Diego, CA, United States).

2.4. Flow cytometry analyses

2.4.1. Blood processing

Fresh anticoagulated blood was diluted at a 1:3 ratio in RBC lysis buffer and incubated for 5 min at RT (during this time, samples were mixed by inverting 3 times). Then, samples were centrifuged for 5 min at 400g. The supernatant was removed by aspiration, and cells were counted with 0.1% of trypan blue (Thermo Fisher Scientific, Waltham, MA, United States).

2.4.2. Poly (I:C) stimulation

When indicated, cells were incubated for 16–18 h in 24-well plates (1–2 x10^6 cells/well) at 37°C with or without 20 μg/ml of poly (I:C). Cells were collected, stained and analysed by flow cytometry.

2.5. Flow cytometry staining and data analysis

For 10 min, 1–2 x 10^6 cells were incubated with the FcR blocking reagent, and then, cells were stained in phosphate buffered solution-ethylene diamine tetra-acetic acid (PBS-EDTA) with the appropriate antibodies.

Flow cytometry acquisition was performed with an LSR Fortessa flow cytometer (BD Biosciences, Franklin Lakes, NJ, United States). Flow cytometry analysis was performed with FlowJo software (version 10.2, Treestar). Data were analysed using Prism, v7.

2.6. Statistical analysis

Statistical analysis was performed with Prism software (version 7, GraphPad). For multiple comparisons, adjusted *p*-values were calculated by one-way ANOVA followed by the Dunn test. Correlations were assessed by the non-parametric Spearman's test to determine *p* and r values, and Bonferroni correction was applied to adjust for multiple testing.

2.7. TCGA analysis

Analyses of TCGA data (https://www.cancer.gov/tcga) were conducted using R (v. 3.5.3). HTSeq-count RNA-sequencing data and patient clinical data were downloaded for ovarian serous adenocarcinoma and prostate adenocarcinoma using the R package TCGAbiolinks (v. 2.10.5) [19]. The raw count data of each tumour type were first filtered to remove genes with less than 1 count per million (cpm) in at least 1 sample. Normalisation factors were then calculated using the trimmed mean of M values method implemented in the edgeR package (v. 3.24.3) [20], and raw counts were converted to normalised $\log_2$(cpm) using the Voom method implemented in the Limma package (v. 3.38.3) [21]. We filtered patients to remove those that had missing clinical data for vital status. This resulted in 373 patients who were retained in the ovary tumour data set, and 496 patients were retained in the prostate tumour data set. The average expression level of each gene was calculated for each tumour type separately, and each patient was categorised into a 'low' or 'high' gene expression group depending on whether the patient's gene expression level was above or below the average. Using the Cox regression model implemented in the survival package (v. 3.1.7) [22], we tested the difference in survival rate between the low- and high-expressing patient groups for each gene, either for all tumour stages combined or within each tumour stage separately.

3. Results

3.1. The frequency of cDC1s is reduced in patients with OvC

cDC1s have been described as the APC with the highest cross-presentation capacity, that is, the ability to deliver antigens from the endocytic compartment to the cytosolic pathway of antigen processing and presentation, and are considered the lead DC population to use for cancer immunotherapy. Technical limitations of standard laboratory equipment do not allow the isolation of this population for therapeutic purposes; however, magnetic isolation of the total $CD141^+$ DCs may be

possible in the future using the GMP-compliant Miltenyi's CliniMACS Prodigy® device. To date, however, there are not available data on the amount and the functionality of cDC1s in the peripheral blood of patients with OvC and PrC. We, therefore, evaluated the frequency of cDC1, cDC2 and plasmacytoid DC (pDC) subsets among total DCs in HDs and patients with OvC or PrC (clinical data in Table 1/Table 2 and DCs gating strategy in Fig. S1A) by flow cytometry. In this study, as shown in Fig. S1A, we define the cDC1 subset as HLA-DR$^+$CD141^{bright}. In addition, we evaluate the frequency of the total CD141$^+$ DCs as of future potential interest for vaccine development. The frequency of total CD141$^+$ DCs was lower in patients with OvC or PrC than in HDs, while the cDC1 subset was greatly reduced or completely lost only in patients with OvC (Fig. 1A and B). The frequency of total DCs (HLA-DR$^+$ lymphocytes) was not different among the groups considered (data not shown). The capacity of cDC1s to uptake and cross-present the antigens is dependent upon the expression of CLEC9A and XCR1 that are exclusively expressed by this subset [3,23,24]. The expression of both these markers by cDC1s was reduced only in patients with OvC (Fig. 1C). It is important to note that in 3 of 27 patients with OvC, the cDC1s frequency was below 0.1% of total HLA-DR$^+$; moreover, cDC1s in 7 of 27 patients with OvC expressed neither CLEC9A and/or XCR1. The frequency of cDC2s and pDCs was not altered in patients with OvC or PrC when compared with HDs (Fig. 1D). Finally, the frequency of DC subsets did not correlate with the total leucocyte count (data not shown). These data suggest that the sorting yield of CD141$^+$ DCs from whole blood of patients with OvC or PrC may be low; moreover, in patients with OvC, this population may contain few or no cDC1s, limiting the cross-presenting capacity of the product.

3.2. The activation of CD141$^+$ DCs and cDC2s is increased in patients with OvC or PrC

Mature DCs express high levels of CD40, CD80, CD83 and CD86. These molecules can confer either activatory or inhibitory signalling, depending on the ligand and the interacting cell. Programmed death-ligand 1 (PD-L1) expression by DCs and other cells in the tumour microenvironment suppress effector functions of PD1$^+$ T cells [25]; however, its expression is also a marker of DC maturation and activation [26]. Activation and differentiation status of immune cells may be altered in patients with cancer, affecting their functional response. To evaluate the activation status of DC subsets in patients with OvC or PrC, we monitored the expression of CD40, CD80, CD83, CD86 or PD-L1 by CD141$^+$ DCs and cDC2s (Fig. S1B) and considered each DC expressing any of these markers to belong to the 'activation cluster'. The frequency of cDC1 and pDC subsets was too low to perform this analysis, therefore only total CD141$^+$ DCs and cDC2s were considered. CD141$^+$

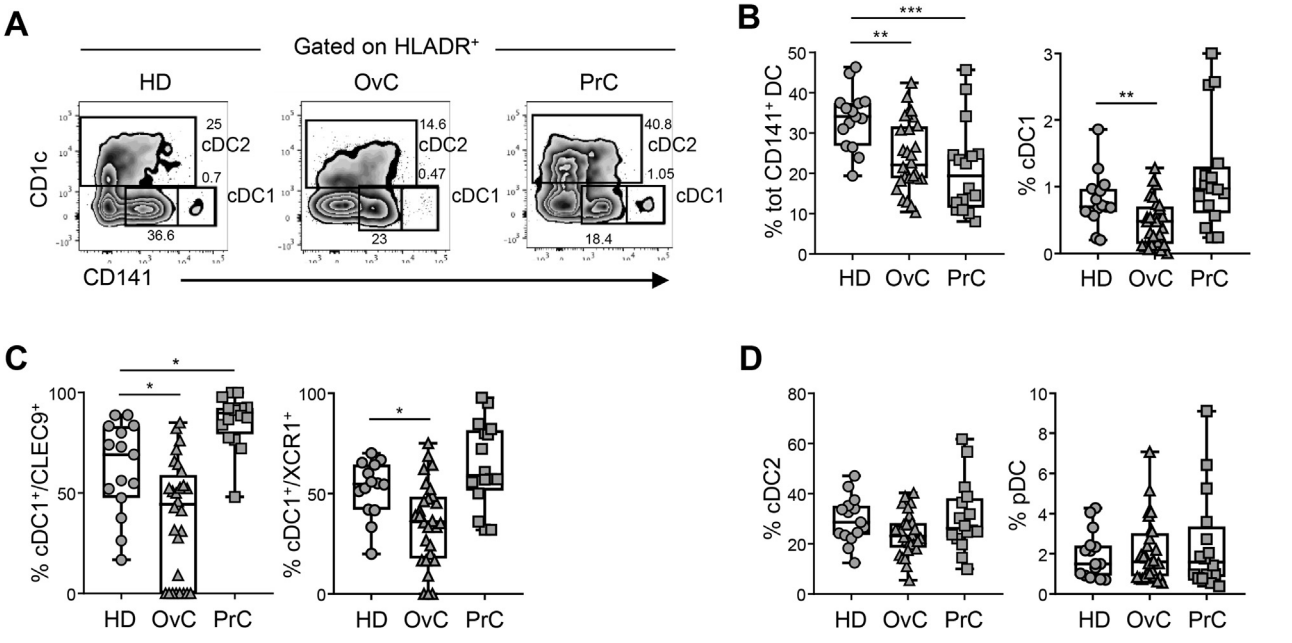

Fig. 1. **Patients with OvC but not PrC present a reduced frequency of cDC1.** (A) Representative example of DC subset frequency in HDs and patients with OvC or PrC. (B) Cumulative data of the frequency of total CD141$^+$ DCs and cDC1 in HDs and patients with OvC or PrC. (C) Cumulative data of the frequency of cDC1 expressing CLEC9A or XCR1 in HDs and patients with OvC or PrC. (D) Cumulative data of cDC2 or pDC frequency in HDs and patients with OvC or PrC. In all box charts, the 25th to 75th percentiles, the median and min–max of the values are represented; HDs n = 15, OvC n = 28, PrC n = 16; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. One-way ANOVA tests. OvC, ovarian cancer; PrC, prostate cancer; DC, dendritic cell; HDs, healthy donors.

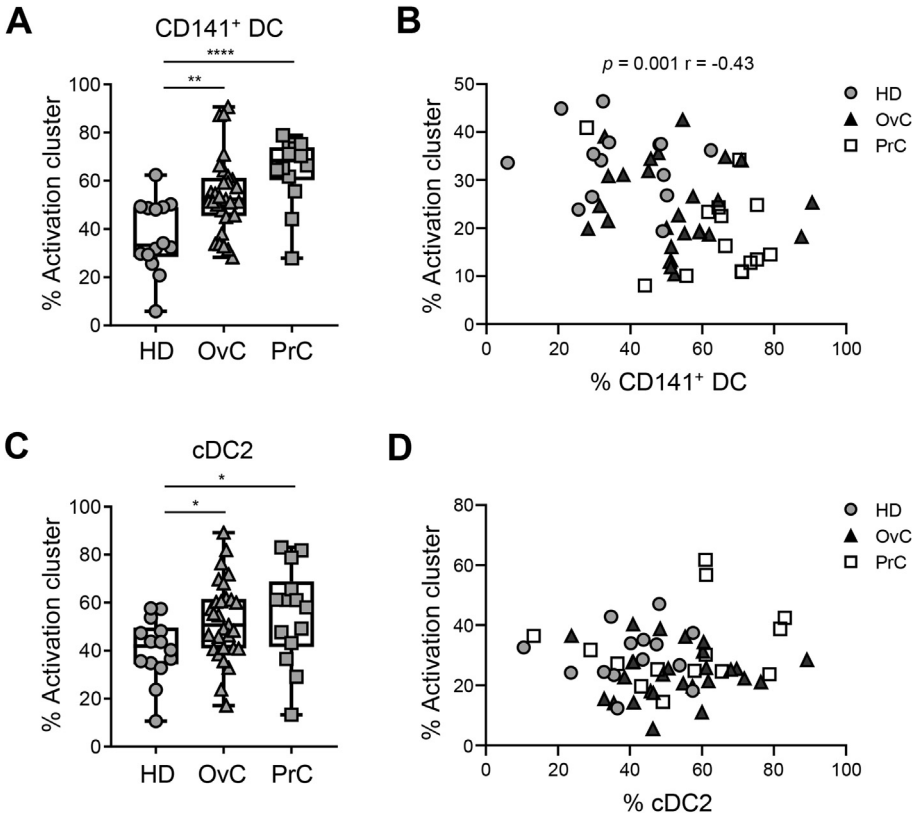

Fig. 2. **Patients with OvC have highly activated cDC1s and cDC2s.** (A) Cumulative data of the frequency of the activation cluster among CD141$^+$ DCs. (B) Correlation between the frequency of total CD141$^+$ DCs and the frequency of the activation cluster among CD141$^+$ DCs. (C) Cumulative data of the frequency of the activation cluster among cDC2. (D) Correlation between the cDC2 frequency and the frequency of the activation cluster among cDC2. In all box charts, the 25th to 75th percentiles, the median and min–max of the values are represented; HDs n = 14, OvC n = 27, PrC n = 14; $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$. One-way ANOVA tests or Spearman tests. OvC, ovarian cancer; PrC, prostate cancer; DC, dendritic cell; HDs, healthy donors.

DCs were more activated in patients with OvC or PrC than in HDs (Fig. 2A). When individual molecules were considered, we observed a higher amount of CD80 and CD83 in patients with OvC or PrC and lower amount of CD86 in patients with PrC in comparison with HDs (Fig. S1C). Of relevance, the activation of CD141$^+$ DCs inversely correlated with their frequency (Fig. 2B). Individually, the expression of CD40, CD80 and CD83 was inversely correlated with the frequency of CD141$^+$ DCs (Fig. S1E). These observations suggest that an increased activation of CD141$^+$ DCs may lead to their progressive reduction.

cDC2s were also more activated in patients with OvC or PrC when compared with HDs (Fig. 2C). The expression of CD80, CD83 and CD86 was also increased in cDC2s in patients with OvC, while in patients with PrC, only CD80 was increased and, in contrast, CD40 was reduced (Fig. S1D). We did not find a correlation between the frequency of cDC2s and their activation status (Fig. 2D). In addition, when individual maturation markers were considered, only the expression of PD-L1 was inversely associated with the frequency of cDC2s (Fig. S1F). These data suggest that, in contrast to CD141$^+$ DCs, the activation of cDC2s may not influence their frequency in patients with OvC or PrC.

3.3. CD141$^+$ DCs in patients with OvC exhibit a reduced responsiveness to TLR3 agonist poly (I:C)

Toll-like receptor (TLR) agonists are used to mature DCs generated *ex vivo* or *in vivo* for therapeutic vaccination. Human cDC1s express high levels of TLR3 and therefore are mainly targeted by its agonists [3,6]. Poly (I:C) (a TLR3 agonist) showed extraordinary efficacy to activate DCs and to induce pro-inflammatory anticancer immune responses [6,27]. To investigate the ability of CD141$^+$ DCs and cDC2s to respond to poly (I:C), we evaluated their capacity to upregulate activation markers (*i.e.* CD40, CD80, CD83, CD86 or PD-L1) (Fig. S2A). Poly (I:C) activated CD141$^+$ DCs and, to a lesser extent, cDC2s in all groups considered (Fig. 3A and B). cDC2s express less TLR3 than CD141$^+$ DCs [28], and thus, the lower magnitude of cDC2 response to poly(I:C) stimulation was expected. In both, patients with OvC or PrC, the response of CD141$^+$ DCs to poly

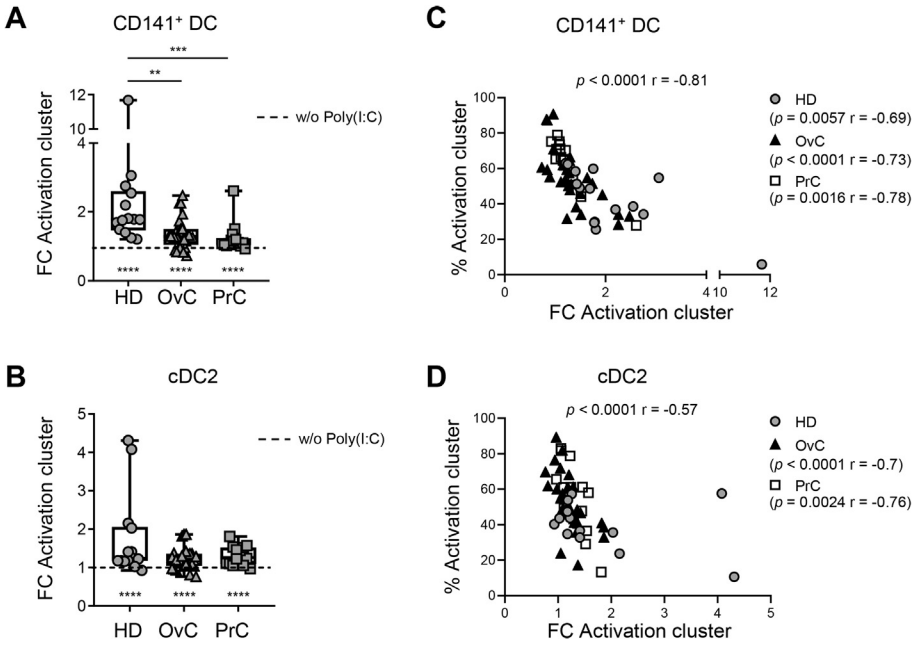

Fig. 3. **CD141$^+$ DCs in patients with OvC have an impaired response to poly (I:C).** (A) Cumulative data of the fold change in the frequency of the activation cluster among CD141$^+$ DCs after poly (I:C) stimulation. (B) Cumulative data of the fold change in the frequency of the activation cluster among cDC2 after poly (I:C) stimulation. In all box charts, the 25th to 75th percentiles, the median and min–max of the values are represented; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. HDs n = 14, OvC n = 27, PrC n = 14. One-way ANOVA tests. (C) Correlation between the frequency of the activation cluster among CD141$^+$ DCs and its fold change after poly (I:C) stimulation. (D) Correlation between the frequency of the activation cluster among cDC2 and its fold change after poly (I:C). (E) Correlation between the frequency of CD141$^+$ DCs expressing CD83 and the fold change in the frequency of the activation cluster among CD141$^+$ DCs after poly (I:C) stimulation. (F) Correlation between the frequency of cDC2 expressing CD83 or CD80 and the fold change in the frequency of the activation cluster among cDC2 after poly (I:C) stimulation. HDs n = 14, OvC n = 27, PrC n = 14. Spearman tests. OvC, ovarian cancer; PrC, prostate cancer; DC, dendritic cell; HDs, healthy donors.

(I:C) was reduced when compared with HDs (Fig. 3A), while no differences were observed in cDC2 responsiveness (Fig. 3B). When individual activation markers were considered, we observed that in patients with OvC, CD40 upregulation by CD141$^+$ DCs was lower than that in HDs, while in patients with PrC, the same impairment was observed for CD86 (Fig. S2B). Of note, CD40 upregulation is particularly relevant for the efficacy of tumour-specific T cells [29]. Thus, in patients with OvC or PrC, CD141$^+$ DCs are unresponsive to TLR3 stimulation. Despite a reduced functionality of CD141$^+$ DCs, we did not find differences in PD-L1 expression or upregulation between HDs and patients with OvC or PrC (Fig. S1C and Fig. S2B). Although we did not find differences in the overall activation of cDC2s, patients with PrC demonstrated an increased upregulation of CD80 and CD83 (Fig. S2C) in cDC2s that may indicate an augmented TLR3 expression or a RIG-I–mediated response [30].

We investigated whether the excessive level of basal activation (Fig. 2) may also influence the functionality of DCs. We found an association between poly (I:C) induced activation and the basal activation levels of CD141$^+$ DCs and cDC2s (Fig. 3C and D). When we considered the distinct activation markers separately, we found multiple negative correlations between the fold change in CD40, CD80, CD86 or PD-L1 expression and their basal expression levels in CD141$^+$ DCs (Fig. S3A, Table S1, Table S2) or cDC2s (Fig. S3B Table S3, Table S4). These associations were in some cases found only in a specific group of samples (Table S2, Table S4). Overall, these data may indicate that basal DC activation is associated with a defect in the function of DCs and may predict their capacity to respond to TLR3 agonists.

3.4. A reduced frequency of cDC1s may be associated with worse prognosis in patients with OvC

The immune system composition is dynamic and can be influenced by several factors such as environment and ageing. We evaluated whether there was an association between patients' age and the relative frequency of DC subsets. There was no association found neither when considering the overall samples nor when considering each group separately (Figs. S4A and B and data not shown).

Tumour progression in patients with OvC can often be predicted in the absence of symptoms or radiographic findings by the CA125 value increase, especially when more than twice an augmentation from the nadir level is

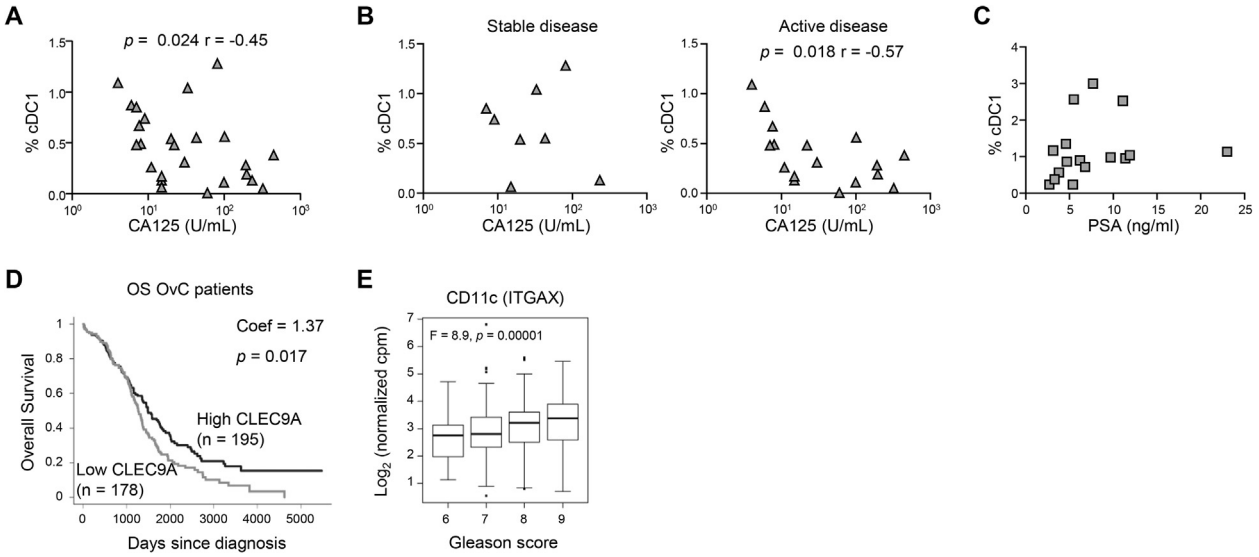

Fig. 4. **The infiltration of cDC1 in patients with OvC is inversely associated with disease progression.** (A) Correlation between cDC1 frequency and CA125 levels in patients with OvC. N = 25. Spearman test. (B) Correlation between cDC1 frequency and CA125 levels in patients with OvC with stable (left panel, n = 8) or active (right panel, n = 17) disease. Spearman test. (C) Correlation between cDC1 frequency and PSA levels in patients with PrC. N = 16. Spearman test. (D) TCGA-derived survival curves of patients with OvC having high or low CLEC9A expression in tumours. N = 373. Cox regression model. (E) Expression of CD11C (ITGAX) in prostate cancer tissues of patients with Gleason score 6 (n = 46), 7 (n = 245), 8 (n = 64) and 9 (n = 141). One-way ANOVA test. OvC, ovarian cancer; PrC, prostate cancer; DC, dendritic cell; HDs, healthy donors; PSA, prostate-specific antigen.

observed [31]. Furthermore, PSA elevation from the usually low concentrations measured in serum (<4.0 ng/ml) is regarded as an important indicator of PrC progression [32] and its evaluation contributes to the early detection of PrC [33]. We investigated whether the frequency of DC subsets was associated with CA125 or PSA levels. The frequency of cDC1s in patients with OvC was inversely associated with levels of CA125 (Fig. 4A). This correlation was stronger when only patients with active disease were considered (Fig. 4B). In contrast, PSA levels did not correlate with cDC1 frequency (Fig. 4C). None of the other DC populations considered were associated with CA125 or PSA levels (data not shown). The frequency of DC subsets was not different among patients with OvC or PrC at different disease stages (Figs. S4C and D); however, the latter analysis did not have enough statistical power due to low and unequal number of patients per group (Tables 1 and 2). Among the patients with OvC analysed, only one did not have any previous treatment, while among the patients with PrC, the vast majority (14/16) did not receive any radiotherapy, chemotherapy or hormonotherapy (Tables 1 and 2). Thus, the impact of chemotherapy cannot be evaluated in any of the groups considered.

Taking advantage of publicly available databases, we evaluated the impact of CD141 (THBD), CD1C, CD11c (ITGAX), CD123 (IL3RA), BDCA2 (CLEC4C, expressed only in PrC), CLEC9A and XCR1 expression in OvC (n = 373) and PrC (n = 496) tissues on the OS of patients with OvC or PrC (Tables S5 and S6). We found the expression of CLEC9A (a cDC1s marker) in OvC tissues to be associated with better OS (Fig. 4D and Table S5). None of the DC markers considered significantly increased along with the increase of stage in patients with OvC. On the other hand, no correlation was found for OS in patients with PrC (Table S6), probably due to the low mortality rate. However, CD11c expression (a cDC2s marker) significantly increased along with PrC progression as depicted by its Gleason score (Fig. 4E). Overall, these data suggest that the reduction of cDC1s may be a bad prognostic factor in patients with OvC, while it may have no impact on PrC progression.

4. Discussion and conclusions

cDC1s have been shown to have the highest cross-priming capacity in both mice and humans. Therefore, cDC1s are the best candidates to induce a potent immune anti-tumour response. In clinical practice, the isolation of pure cDC1s in adequate quantities is not currently feasible. A valid alternative that may become available in the close future would be to magnetically isolate the total $CD141^+$ DC population that is enriched in cDC1s to test them in a phase I clinical trial. So far, only pilot clinical studies as part of the 'Professional cross-priming for ovarian and prostate cancer' collaborative European project are under evaluation [17,34]. Although recently published preclinical mouse data suggest that the use of cDC1s is promising in terms of efficacy [5], in patients with cancer, DCs may

be functionally impaired or absent rendering a challenge in their isolation and consequently in their clinical use [7,35]. For the first time, our data demonstrate that cDC1s in patients with OvC and total CD141$^+$ DCs in patients with OvC or PrC are reduced in frequency. Thus, we predict potential limitations in the isolation of cross-presenting DCs in sufficient number for subsequent vaccine development. We also unveil the need of monitoring this population and defining a range in the frequency of CD141$^+$ DCs as inclusion criteria during clinical trials aimed at testing CD141$^+$ DCs vaccines as it is crucial not to subject patients to unnecessary invasive aphaeresis procedures. Moreover, the monitoring of the cross-presentation capacity of the final product would allow the evaluation of the impact on the vaccine immunogenicity and the clinical response. A longitudinal evaluation of the frequency of CD141$^+$ DCs in patients with cancer would be informative to discriminate between the effect of the disease process and that of the treatment.

In the present work, we also demonstrate that CD141$^+$ DCs have a reduced functionality compared with HDs based on their reduced ability to respond to the TLR3 agonist poly (I:C). We also observed an increased basal level of CD141$^+$ DC activation that was related to a reduction in both frequency and responsiveness to poly (I:C). These data indicate that the higher the basal activation of CD141$^+$ DCs, the lower is their capacity to respond to TLR3. Thus, our work indicates that the activation profile may be a good biomarker to predict CD141$^+$ DC functionality in future therapeutic vaccine trials. The reduced functionality of cross-presenting DCs represents an additional challenge for designing therapeutic vaccines. To overcome systemic immune exhaustion and to increase DC-induced responses *in vivo*, many trials are either combining DC-based vaccines with agents inducing immunogenic cell death or recruiting patients at earlier disease stages [17,34].

Our study also reveals an interesting correlation between the frequency of cDC1s and CA125 levels. Although these data need to be further confirmed by longitudinal evaluation of CA125 and cDC1s, they suggest that the loss of cDC1s may correspond to an increased tumour burden. Consistently with observations in other tumour types [7,36], analysis of TCGA databases demonstrated that cDC1 infiltration (defined as CLEC9A expression) in OvC tissue is a good prognostic factor, increasing OS; this highlights the relevance of cDC1s in generating potent anti-tumour responses, despite the technical challenges to isolate them. Further studies are needed to understand whether current or novel immunotherapeutic molecules may improve the frequency and the function of CD141$^+$ DCs. Clinical trials have shown the safety of mobilising DC progenitors using FLT3L [37,38]. However, recently, contrasting data have been published regarding the capacity of these progenitors to efficiently differentiate into cDC1s [5,7,9]. Additional immunological studies in humans are needed to better elucidate this point. An alternative would be to generate cDC1-like *in vitro* [39]; however, cDC1-like enrichment and yield are not adequate yet for clinical translation, and several groups are attempting to improve cultures outcomes [40].

Overall, our study highlights important quantitative and functional defects in cross-presenting DCs in patients with OvC. Although the cDC1 subset is not quantitatively affected in patients with PrC, the observed reduced frequency of CD141$^+$ DCs may limit the potential of isolating this subset for vaccination purposes. Further studies are needed to clarify the mechanisms behind these alterations. Preclinical and clinical tests will also be crucial in determining whether the number and function of cross-presenting DCs may be restored to increase the efficacy of immunotherapy.

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Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2020.04.036.

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