

Quantitative and Functional Alterations of Plasmacytoid Dendritic Cells Contribute to Immune Tolerance in Ovarian Cancer

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Abstract

In ovarian cancer, the immune system fails to eradicate established tumors partly due to the induction of immune tolerance within tumor microenvironment. In this study, we investigated the contribution of plasmacytoid dendritic cells (pDC) in the establishment of immune tolerance in a cohort of 44 ovarian cancer patients. In the tumor and malignant ascites, CD4⁺CD123⁺BDCA2⁺ pDC were the most abundant dendritic cell subset; however, they were profoundly depleted in peripheral blood. The presence of pDC in primary ovarian cancer, but not ascites, was an independent prognostic factor associated with early relapse. Following chemotherapy, we observed a partial restoration of blood pDC levels in patients in complete remission. These findings show preferential recruitment of pDC into tumors where they express a partially mature phenotype that may reflect an *in situ* activation. Importantly, compared with pDC found in ascites or blood, tumor-associated pDC (TApDC) produced less IFN- α , TNF- α , IL-6, macrophage inflammatory protein-1 β , and RANTES in response to toll-like receptor stimulation, and alterations in pDC functions were mainly mediated through tumor-derived TNF- α and TGF- β . Unlike ascites-derived pDC, TApDC induced IL-10 production from allogeneic naive CD4⁺ T lymphocytes, suggesting the existence of a paracrine immunosuppressive loop. Taken together, our findings indicate that both local and systemic dysfunction of pDC play a critical role in the progression of ovarian cancer via induction of immune tolerance. *Cancer Res*; 71(16); 5423–34. ©2011 AACR.

Introduction

Ovarian cancers are the leading cause of death from gynecologic cancers and the fifth most common overall cause of cancer death among women (1). Most women (~75%) present with advanced stage disease, for which the 5-year survival rate is a dismal 30% or less, and has not changed over 3 decades (2). Thus, understanding mechanisms underlying therapeutic success and failure has important clinical relevance: predicting nonresponse and developing new therapeutic approaches may improve outcome.

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Plasmacytoid dendritic cells (pDC) are one of 2 main subsets of human dendritic cells (DC) in blood. pDC are identified as a CD4⁺, CD11c⁻, lineage marker⁻, and HLA-DR⁺ cells that express CD123/IL-3 receptor alpha chain and/or as BDCA2- and BDCA4-expressing cells (3). At steady state, they are circulating in blood and directly enter in lymph nodes through the high endothelial venules (HEV; ref. 4, 5) and are absent from peripheral tissues. pDC are crucial effector cells in antiviral immunity. Indeed, in response to viral stimulation (6) through toll-like receptors (TLR) 7 and 9 (7), they produce high amounts of type I IFN (α , β , and ω). pDC link innate and adaptive immune responses by promoting the activation and differentiation of natural killer (NK) cells, B cells, myeloid DC (mDC), and T cells (7–9). Consistent with their antigen presentation capabilities, pDC were also shown to mediate tolerance to allogeneic antigens in cardiac allograft (10), oral antigens (11), and airway allergens (12).

Emerging evidence indicates that tumor infiltration by pDC may have clinical importance, as underlined by their identification in tumors including melanoma, head and neck, lung, ovarian, and breast cancers (13–15). Several types of cancers are characterized by impaired function and numbers of pDC (13, 16), but correlations of pDC frequency with disease progression in cancer remain poorly studied. Infiltration of primary breast carcinomas by pDC is variable and their

presence at high density is associated with poor clinical outcome (17) and their depletion in the blood of pancreatic cancer patients is correlated with reduced survival (18).

In ovarian cancer, several studies have shown an accumulation of pDC in malignant ascites, with depletion in blood (14, 19, 20). pDC were reported to be attracted into ascites through SDF-1/CXCL12, to produce high amounts of type I IFN in response to TLR ligand (14, 21), and to induce CD8⁺ regulatory T cells which suppress tumor antigen-specific T cells through the production of IL-10 (21). In addition, ascite pDC were shown to favor tumor angiogenesis *via* the production of TNF- α and IL-8 (20). However, the role of tumor infiltration by pDC [called tumor-associated pDC (TApDC)] in advanced ovarian cancer remains to be addressed.

In this report, we have characterized the tissue distribution, phenotype, and functions of pDC in advanced epithelial ovarian cancer and their impact on patients' clinical outcome. Our findings bring new insights into the physiopathology of pDC in the context of ovarian cancer in humans, showing quantitative systemic and local functional alterations of pDC associated with progression.

Materials and Methods

Patients

We studied 44 untreated patients with International Federation of Gynecology and Obstetrics (FIGO) stage I, II, III, and IV epithelial ovarian cancers. All patients gave written, informed consent. The study was approved by local Institutional Review Boards. Biological samples were collected at Centre Leon Bérard, Lyon, and other institutions in Rhône-Alpes Region, France.

Preparation of tumors and ascites cell suspensions

We collected fresh solid tumors and malignant ascites aseptically. Cells were harvested from ascites by centrifugation over a Ficoll-Hypaque density gradient. Ovarian tumors samples were minced into small pieces in serum-free RPMI medium and digested with collagenase Ia (1 μ g/mL) and DNase I (50 KU/mL; Sigma Aldrich) for 1 hour at 37°C with gentle agitation. The resulting cell suspensions were filtered, washed, and resuspended in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum (FCS; Lonza) and antibiotics (complete RPMI) before isolation of mononuclear cells (MNC) by centrifugation over a Ficoll-Hypaque density gradient for further analyses.

Peripheral blood MNCs

Peripheral blood was collected aseptically from healthy donors (HD; $n = 55$) or ovarian cancer patients ($n = 44$) in a CTAD-coated tube. Peripheral blood mononuclear cells (PBMC) were obtained over a Ficoll-Hypaque density gradient, washed twice, and resuspended in PBS containing 2% FCS (Lonza) and 0.5 mmol/L EDTA for flow cytometry analysis.

Ex vivo mononuclear cell stimulation

We stimulated MNC from tumors, ascites, and peripheral blood from 6 patients and 8 HDs in complete RPMI at 1×10^6 cells per well in duplicate wells with formaldehyde-inactivated

influenza (flu) virus (A/Wisconsin/67/05; generous gift from Aventis Pasteur) and CpG-A/ODN 2336 that were used at 100 HAU/mL and 5 μ g/mL, respectively in 24-well tissue culture plate at 37°C. Cell-culture supernatants were harvested after 18 hours and used for cytokines production quantitation by ELISA.

pDC purification from blood, tonsils, tumors, and ascites

To isolate pDC from biological samples, CD3-, CD8-, CD11b-, CD14-, CD16-, CD19-, CD20-, CD56-, Glycophorin A-, and EpCAM-expressing cells were depleted using specific antibodies and paramagnetic beads (Invitrogen). pDC from blood and HD tonsils were purified from lineage-negative enriched MNC using the BDCA4 cell isolation kit (Miltenyi Biotec) according to the supplier's instructions. Ovarian tumors or malignant ascite pDC were sorted from lineage-negative enriched MNC using FACS (FACSVantage SE DiVa flow cytometer, BD Bioscience) as CD4⁺CD123⁺ cells using APC anti-CD123 (Biolegend) and PE-Cy5 anti-CD4 (Beckman Coulter). Purity was routinely 98% or more.

Preparation of ovarian tumors and ascites-derived supernatants

Single-cell suspensions from ovarian tumors and ascites were incubated at a final concentration of 1.5×10^6 cells/mL in complete RPMI in petri dishes. Culture supernatants were collected after 48 hours, filtrated on 0.2 μ m, and cryopreserved at -80°C until use.

Culture of ovarian tumor-associated or healthy pDC

Freshly isolated TApDC and ascite pDC were cultured at 5×10^5 cells/mL in 96-well flat-bottom plates in complete RPMI for 24 hours in presence of recombinant hIL-3 (PeproTech), formaldehyde-inactivated influenza (flu) virus (A/Wisconsin/67/05) and CpG-A/ODN 2336 that were used at 20 ng/mL, 100 HAU/mL, and 5 μ g/mL, respectively. Freshly isolated healthy blood or tonsil pDC were cultured as previously mentioned in the presence of hIL-3 with or without ovarian tumor (TUMSN) or ascite (ASCSN) supernatants supplied at 25% for 16 hours before addition of TLR ligands for another 24 hours. Cells and supernatants were harvested after 24 hours for TApDC and ascite pDC, and 40 hours for healthy pDC culture to analyze cell surface expression of activation markers and cell viability (by Propidium Iodide Incorporation) by flow cytometry and cytokines production by ELISA.

Flow cytometry analysis

Three-color staining was done by flow cytometry on a FACScan (BD Bioscience) using isotype-matched controls or monoclonal antibodies and data were analyzed with CellQuest software (BD Bioscience). pDC were identified as CD4⁺CD123⁺BDCA2⁺ cells using fluorescein isothiocyanate (FITC) anti-BDCA2 (Miltenyi Biotec), PE anti-CD123 (BD Bioscience), and PE-Cy5 anti-CD4 (Beckman Coulter). mDC were identified as Lin⁻CD4⁺CD11c⁺ using FITC-conjugated lineage (Lin) cocktail (CD3, CD14, CD16, CD19, CD20, and CD56; BD Bioscience), PE anti-CD11c (BD Bioscience),

and PE-Cy5 anti-CD4 (Beckman Coulter) antibodies. CD4 T lymphocytes were identified as CD3⁺CD4⁺ cells using FITC anti-CD3 (BD Bioscience) and PE-Cy5 anti-CD4 antibodies (Beckman Coulter). The phenotype of CD4⁺BDCA2⁺ pDC was analyzed with PE-coupled anti-CD86 (BD Bioscience) and anti-CD40 (Beckman Coulter). Monoclonal antibodies. Viability was assessed by Propidium Iodide Incorporation. Flow cytometry results are expressed as % positive cells and mean fluorescence intensity (MFI). At least 200,000 gated events/condition were analyzed on the total cell population and 5,000 gated events/condition on purified cells.

Allogeneic mixed leukocyte reaction

Allogeneic CD4⁺CD45RA⁺ naive T cells were purified from HD peripheral blood after magnetic depletion using anti-CD8, -CD14, -CD19, -CD16, -CD56, and -glycophorin A antibodies followed by anti-IgG beads (Invitrogen). The purity was routinely more than 95%. Highly purified fluorescence-activated cell sorting (FACS)-sorted pDC from ovarian tumors and ascites from the same patients were differentiated by culture in IL-3 (20 ng/mL) for 16 hours and then cultured with responder T cells in a ratio of 0;3;11;33;111;333;1,111;3,333, and 10,000 per 100,000 T cells for 96 hours in triplicate in round bottom 96-well plates. Finally, cells were pulsed with 0.5 μ Ci per well [3H]thymidine (Amersham Biosciences) and harvested 18 hours later. The [3H]thymidine incorporation was assessed by liquid scintillation spectroscopy (Betaplate scintillation counter, Perkin Elmer). Culture supernatants were collected for cytokine analysis by ELISA.

Determination of cytokines production

The ELISA for hIFN- α (Bender MedSystems), hIL-10 (Bender MedSystems), hIFN- γ (Bender MedSystems), and hIP-10/CXCL10 (BD Biosciences) were used according to manufacturer's instructions. Levels of TNF- α , IL-6, IL-8, macrophage inflammatory protein (MIP)-1 β , IP-10, RANTES, IL-12p40, and IL-12 p70 in culture supernatants were determined using a Milliplex map, based on the Luminex xMAP technology by Millipore.

Statistical analysis

Comparison of independent samples was done by the Student's *t* test or nonparametric tests when appropriate. Correlation analysis of the data was done using the Spearman test. The following clinical variables were collected prospectively: age, performance status (PS), stage, debulking surgery, and the presence of macroscopic residual disease. pDC and mDC levels in tumors and ascites were categorized in upper tertile and lower 2 tertiles.

Progression-free survival (PFS) was defined as the time from pathologic diagnosis to progression. PFS was calculated using Kaplan–Meier method. To evaluate the relationship between survival and biological and/or clinical factors, all potential prognostic factors were included in univariate Cox proportional hazard regression models (22). Candidate prognostic factors with a 0.05 level of significance in univariate analysis were then selected for inclusion in the multivariate analysis. Independent prognostic variables of PFS were identified by a

Cox regression analysis using a backward selection procedure to adjust the time cohort effect on patient's characteristics. All statistical analyses were done using SAS software v.9.2 for Microsoft Windows (SAS Institute). All *P* values for 2-tailed tests were considered significant when *P* < 0.05.

Results

Accumulation of pDC in ascites and depletion in the blood

We evaluated the tissue distribution of CD4⁺CD123⁺BDCA2⁺ pDC (Refs. 3, 23; Supplementary Fig. S1A) and Lineage⁻CD4⁺CD11c⁺ mDC (Supplementary Fig. S1B; ref. 24) as a fraction of total MNC in 44 patients with previously untreated epithelial ovarian cancer. Patients' characteristics are detailed in Supplementary Table S1. Consistent with previous investigations (14), the percentage of both pDC and mDC in total PBMC were significantly reduced (*P* < 10⁻³) in ovarian cancer patients (mDC median% = 0.04, range 0–0.65; pDC median% = 0.11, range 0.01–0.98) compared with HD (mDC median% = 0.18, range 0–1.19; pDC median% = 0.32, range 0.07–0.78; Fig. 1A and B). The depletion of blood pDC tended to be deeper in advanced stages (III/IV; 0.1%) ovarian cancer patients compared with early stages (I/II; 0.19%), although this did not reach statistical significance (*P* = 0.09; Fig. 1C). Similar variations according to stage were observed with mDC (*P* = 0.43; Fig. 1D). These data suggest that pDC are actively recruited from blood to the tumor.

To document variations in pDC number in blood during treatment, we determined pDC levels in patients at diagnosis (*n* = 44), on therapy, that is, after 4 cycles of chemotherapy (*n* = 12), in complete remission (CR) 3 months after the end of treatment (*n* = 10) and at progression (*n* = 12). We observed partial restoration of blood pDC in most of the patients after 4 cycles of chemotherapy (*P* = 0.01; Fig. 1E) which was maintained in patients in CR (*P* < 10⁻²). In contrast, we observed a trend toward decreased pDC levels in patients with progressive disease when compared with those in CR (*P* = 0.22). For mDC, we observed significant lower levels in progressive patients when compared with HD (*P* < 10⁻³; Fig. 1F).

With regard to their tissue distribution, pDC infiltrated some tumors (median% = 0.06, range 0–0.68) and accumulated in the majority of malignant ascites (median% = 0.83, range 0.04–2.93; Fig. 1A). Indeed, ascite pDC were 7.2-fold higher than patient blood pDC (*P* < 10⁻³) and 13.8-fold higher than TApDC (*P* < 10⁻³). MDC were present in malignant ascites (median% = 0.06, range 0–0.86) but in lower proportions than pDC (*P* < 10⁻³) while their presence in tumors was scarce (median% = 0.01, range 0–0.13; Fig. 1B).

Correlation of TApDC frequency with poor clinical outcome

The distribution of pDC was skewed with depletion in blood in most of ovarian cancer patients and accumulation in some tumors and especially in malignant ascites. We questioned the impact of the presence of pDC in tumors and ascites on patients' outcome. We adopted a classification by tertile (33rd percentile) to compare the highest tertile subgroup to all

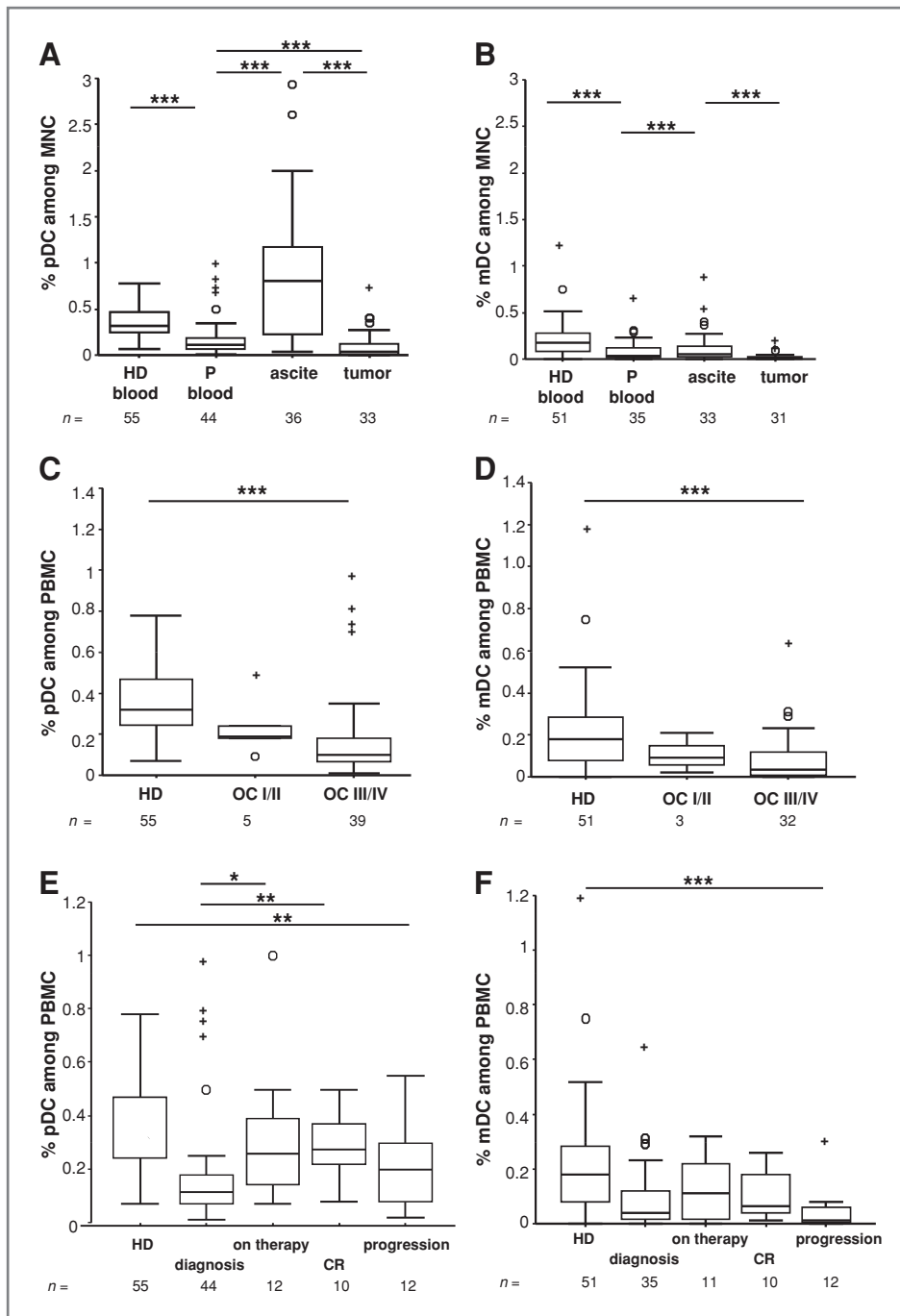


Figure 1. Distribution and frequency of CD4⁺ BDCA2⁺ CD123⁺ pDC and Lin⁻CD4⁺CD11c⁺ mDC obtained by FACS analysis in biological samples. MNC from freshly untreated ovarian cancer, malignant ascites, and peripheral blood from ovarian cancer patients (P) and HDs were prepared. Box plot set of pDC and mDC levels determined as a percentage among total MNC. P values were derived from Mann-Whitney test or Student's test for each comparison. A and B, pDC and mDC levels among MNC in ovarian, ascite, and tumors of ovarian cancer patients and blood of HD. C and D, pDC and mDC levels among PBMC in HD and ovarian cancer patients according to the stage of the disease. E and F, pDC and mDC levels among PBMC in ovarian cancer patients during therapy and comparison with HD. +, extremes; ○, outlier. *, < 0.05; **, <10⁻²; ***, <10⁻³. OC, ovarian cancer.

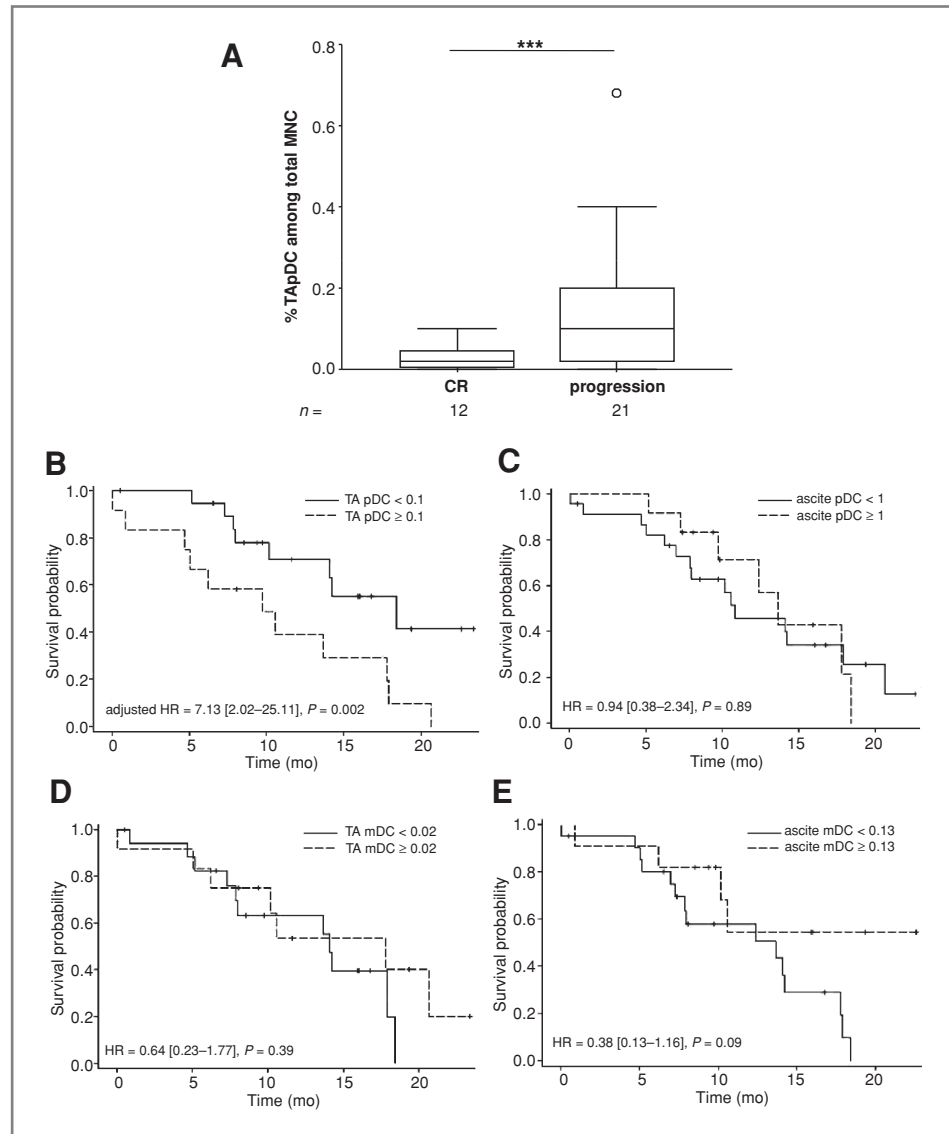
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others. To address this question, we analyzed all relevant clinical and pathologic information's (Supplementary Table S1) that were available on the 44 patients. As our study was prospective with a median follow-up of 14 months, we centred our analysis on PFS. In addition to TApDC levels, 5 clinico-pathologic variables (age, stage, PS, debulking surgery, and the presence of residual disease) were analyzed due to their previously described prognostic significance in ovarian cancer (25). We observed that the subgroup of patients who

progressed showed significantly higher levels of TApDC than those in CR ($P < 0.05$; Fig. 2A). In univariate analysis, the following factors were associated with prolonged PFS: early stage (I-II), debulking surgery, absence of residual tumors and low tumor infiltration by pDC defined as less than 0.1% of total MNC (Fig. 2B and Supplementary Table S2).

In contrast, high levels of ascite pDC had no impact on ovarian cancer patients' outcome (Fig. 2C). When using a cut-off of ascite pDC 1% or more of total MNC (10-fold

Figure 2. Tumors highly infiltrated by pDC showed early relapse. PFS rates were calculated using Kaplan–Meier method and compared using log-rank test. A, box plot set showing tumor-associated pDC (TApDC) levels in patients in CR and those with progressive disease after a median follow-up of 14 months. B–E, PFS of patients with the highest tertile (33rd percentile) frequency of TApDC ($\geq 0.1\%$), ascite pDC ($\geq 1\%$), TAmDC ($\geq 0.02\%$), and ascite mDC ($\geq 0.13\%$) compared with all others. ***, $<10^{-3}$.



higher than in tumors), median PFS was estimated to 13.6 months compared with 10.8 months in patients with ascite pDC less than 1% [hazard ratio (HR) = 0.94, CI: 0.38–2.34, $P = 0.89$]. To assess whether this effect was specific to pDC or concerned all DCs, we further investigated the impact of mDC accumulation in ascites and tumors on PFS. We did not observe any impact of tumor-associated mDC (TAmDC) nor ascite mDC on PFS of ovarian cancer patients (Fig. 2D and E; Supplementary Table S2).

In multivariate analysis, TApDC remained an independent prognostic factor associated with early relapse. Median PFS for patients with the highest tertile of pDC ($\geq 0.1\%$ of total MNC) was 9.7 months whereas those with TApDC less than 0.1% showed a median PFS of 18.4 months (HR = 7.13, CI: 2.02–25.11, $P = 0.002$; Fig. 2B and Supplementary Table S2) suggesting that infiltration of ovarian tumor by pDC has a deleterious effect on patients' outcome.

Partial phenotypic activation of TApDC

To examine the possibility that tumors alter pDC status, expression levels of markers involved in pDC maturation/activation or in T cell costimulation (24) were measured directly *ex vivo* comparing blood, ascite, and tumor-associated pDC from 17 patients. We found that TApDC expressed a semimature phenotype with significantly higher levels of CD40 and CD86, compared with ascites and blood pDC, which showed classic immature phenotype (Fig. 3). These findings suggest that ovarian tumors induce partial activation of TApDC *in situ*. To assess whether this observation could be explained by the presence of dead cells (26), we investigated by flow cytometry the percentage of dead cells among MNC in ascites and tumors from 13 ovarian cancer patients identified by Propidium Iodide Incorporation. We observed 5-fold higher levels of dead MNC in tumors (median% = 20) than ascites (median% = 4.94, $P < 10^{-3}$).

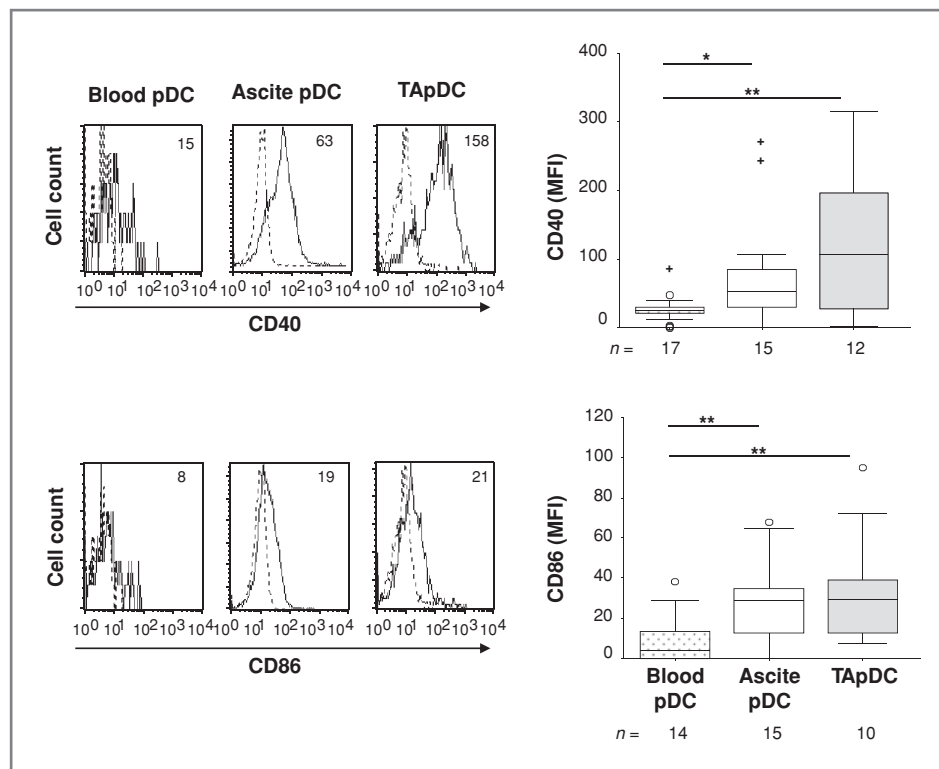


Figure 3. TApDC isolated from ovarian tumors display a partially activated phenotype. Three-color flow cytometry analysis was used to assess the expression of CD40 and CD86 on CD4⁺CD123⁺ pDC from blood, ascites, and tumors from 17 ovarian cancer patients. Statistical analysis was done using Mann-Whitney test. Values are expressed as MFI minus FMO control. +, extremes; ○, outlier. *, < 0.05; **, < 10⁻².

Full maturation of TApDC and ascite pDC after *in vitro* stimulation

Highly purified pDC from ascites, ovarian tumors, and HD blood were cultured with IL-3 and TLR ligands (TLR-L). Changes in expression levels were determined using paired analysis of expression of CD40 and CD86 before and after stimulation. CD40 and CD86 expression increased following IL-3, CpG-A, and influenza virus (flu) stimulation in both ascite and tumor pDC at higher levels than blood pDC (Fig. 4). Interestingly, TApDC seemed to be more prompted than blood and ascite pDC to mature *in vitro* even in the absence of TLR-L as culture in IL-3 induced high expression of both activation markers. Another important observation is the acquisition of a fully mature CD40^{high} CD86^{high} phenotype by both ascite pDC and TApDC but not blood pDC in response to CpG-A. These observations were similar to those obtained with Flu virus. Thus, tumor microenvironment seemed to have altered TLR9 response of ascite pDC and TApDC induced by CpG-A. These findings show that both TApDC and ascite pDC conserve their ability to achieve full maturation following *in vitro* TLR stimulation despite an altered phenotype at baseline.

Amplification of IL-10 production by T cells in response to TApDC

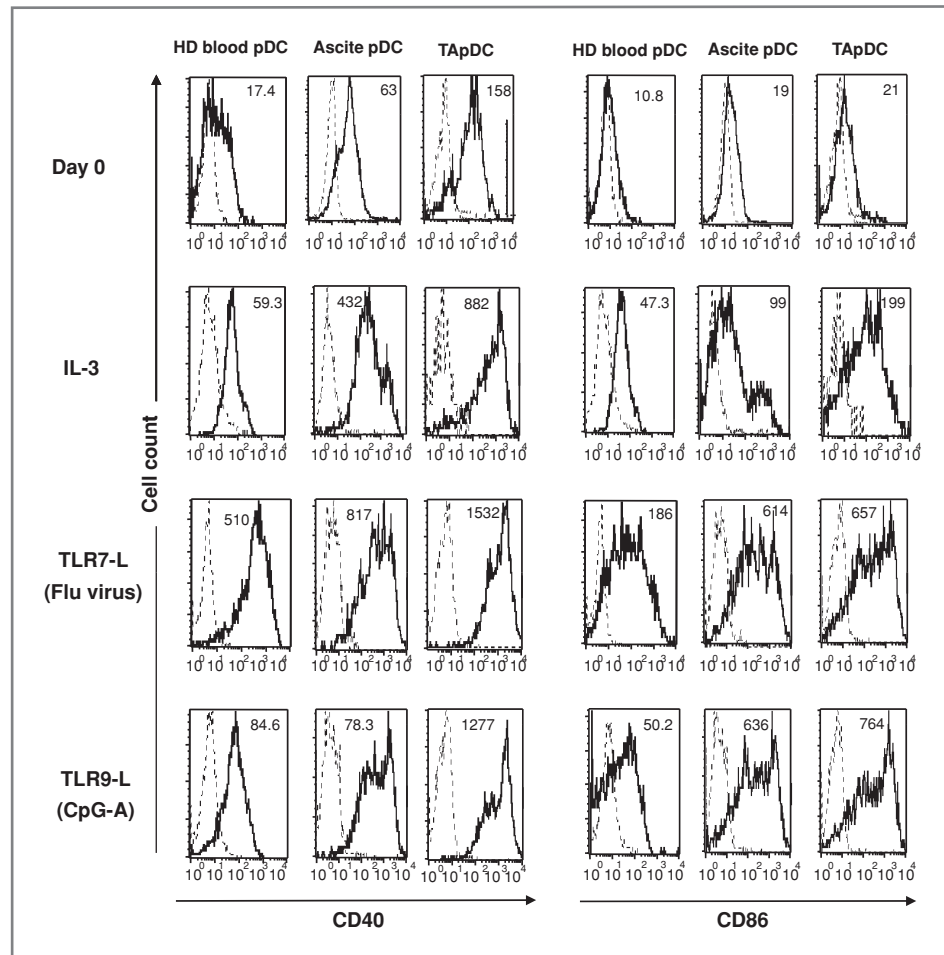
pDC play an important role as antigen-presenting cell by inducing antitumor CD4⁺ T cell responses (27). We observed significant correlation between TApDC and CD4⁺ T lymphocytes infiltration in tumors ($r^2 = 0.657$, $P < 10^{-3}$; Fig. 5A) whereas no significant correlation was observed with mDC

($r^2 = 0.278$, $P = 0.13$). Next, we examined the function of TApDC in priming T-cell responses. Freshly, FACS-sorted pDC from ascites and ovarian tumors from the same patients ($n = 2$) were used to stimulate naive allogeneic CD4⁺ T cells purified from HD blood *in vitro*. Both TApDC and ascite pDC induced proliferation of allogeneic naive CD4⁺ T cells in a dose-dependent manner (Fig. 5B). Strikingly, TApDC induced strong IL-10 ($P < 0.05$) production by primed T cells and slightly higher levels of IFN- γ in comparison to ascite pDC (Fig. 5C). These results lead us to propose that TApDC may contribute to create an immunosuppressive environment through the generation of IL-10-producing T cells.

Inhibition of IFN- α production in TApDC

The production of IFN- α in response to TLR 7 and 9 ligands represents a key mediator in pDC innate function (28). We compared the capacity of blood pDC from HD ($n = 12$), TApDC and ascite pDC from ovarian cancer patients ($n = 5$) to produce IFN- α in response to TLR ligands. Consistent with a previous report (14), ascite pDC produced high levels of IFN- α in response to TLR7 and 9 ligands (Fig. 6A). Although not significant, they produce even more IFN- α than HD blood pDC in response to Flu ($P = 0.1$; Fig. 6A) but not CpG-A ($P = 0.54$; Fig. 6B). In contrast, we observed a severe inhibition of IFN- α production in TApDC compared with ascite and blood pDC mainly in response to TLR9 (CpG-A) and to a lower extent to TLR7 ligands (flu virus). These results indicate that the capacity of pDC to produce IFN- α was selectively altered at the tumor site.

Figure 4. TApDC and ascite pDC acquire a fully mature phenotype after TLR ligands activation *in vitro*. Highly purified TApDC ($n = 5$), ascite pDC ($n = 5$), and HD blood pDC ($n = 2$) were cultured with IL-3 (20 ng/mL), inactivated flu (Influenza virus (100 HAU/mL), and CpG-A (5 μ g/mL) for 40 hours. Activation of pDC was monitored by analyzing CD40 and CD86 expression by flow cytometry. Data are representative of 2 or 5 independent experiments and MFI values of total cells are indicated in each histogram.



pDC were profoundly depleted from the blood of ovarian cancer patients preventing their isolation. As pDC is the main cellular source of IFN- α in response to TLR ligands, we compared the capacity of total MNC (adjusted to the percentage of pDC) of blood, ascite, and tumors from the same patients ($n = 6$) and PBMC from HD ($n = 8$) to produce this cytokine. IFN- α production was reduced in tumor-associated MNC compared with ascite or blood MNC after exposure to CpG-A (Fig. 6D) confirming that only pDC into tumor mass are altered for this innate function. Although pDC are severely depleted in ovarian cancer patient's blood, they are still functional as patients' PBMC produced similar levels of IFN- α in response to TLR ligands than HD (Fig. 6C and D).

We further investigated the production of 8 cytokines/chemokines (TNF- α , IL-6, IL-8, IL-12p40, IL-12p70, MIP-1 β , IP-10, and RANTES) by FACS-sorted TApDC and ascite pDC that were activated *in vitro* by TLR ligands (Supplementary Fig. S2A and B). We observed, similar to IFN- α , a significant inhibition of proinflammatory cytokines (IL-6 and TNF- α) and chemokines (MIP-1 β and RANTES) production by TApDC mainly in response to TLR9 ligand (Supplementary Fig. S2A). By contrast, there was no significant difference for the production of IL-8 and IP-10 by TLR-activated TApDC and ascite pDC (Supplementary Fig. S2B).

Altogether, the substantial differences between TApDC and ascite pDC in term of frequencies, phenotype, response to TLR ligands, and impact on patients' outcome suggest that pDC are differentially affected in the microenvironment of ascites and tumors suggesting that malignant ascites do not closely mirror the tumor microenvironment of the tumor mass.

Inhibition of IFN- α production of healthy pDC by ovarian tumor-derived supernatants mainly through TGF- β and TNF- α dependent mechanism *in vitro*

The weak IFN- α production of TApDC compared with ascite and blood pDC suggests that the tumor microenvironment actively and specifically inhibits TApDC ability to produce IFN- α . To decipher the mechanisms involved in TApDC dysfunction, pDC isolated from blood and/or tonsils of HD were preincubated in presence of IL-3 and ovarian tumor-derived supernatants (TUMSN, $n = 5$) for 16 hours and then stimulated during 24 hours with TLR ligands (flu virus or CpG-A). Most of TUMSN inhibited IFN- α production of pDC to both flu virus (median% of inhibition = 60.8%) and CpGA (median% of inhibition = 53.2%; Fig. 7A). In contrast, IP-10 production remained unaffected (Fig. 7A) and no effect on pDC viability was detected (data not shown), excluding that the negative effect of TUMSN on IFN- α production by

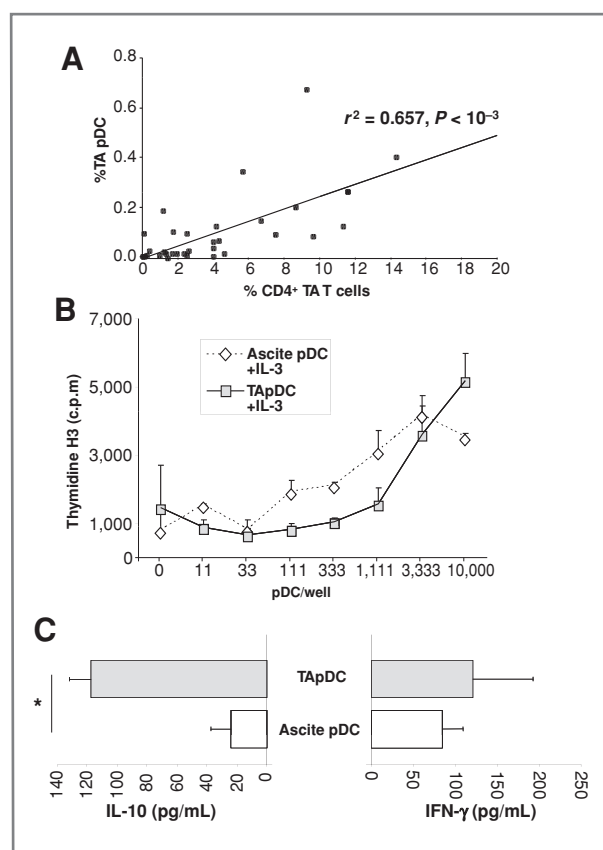


Figure 5. TApDC prime allogeneic naïve CD4⁺ T cells and induce IL-10 T cell response. **A**, Spearman rank correlations between the proportion of CD4⁺BDCA2⁺CD123⁺ pDC and CD3⁺CD4⁺ T lymphocytes in ovarian tumors. **B**, FACS-sorted TApDC and ascite pDC were cultured with IL-3 (20 ng/mL) for 24 hours and then incubated at graded doses with naïve allogeneic CD4⁺CD45RA⁺ T cells for 5 days. T cell proliferation was determined by 3H thymidine incorporation [mean and s.e.m (error bars) of triplicate cultures]. **C**, culture supernatants were collected and IL-10 and IFN- γ were analyzed by ELISA. Data are one of 2 independent experiments. *, <0.05. TA, tumor-associated.

TLR-activated pDC involved induction of cell death. These results suggest that soluble factors present within the tumor environment contribute to the functional inhibition of TLR-activated pDC. Of most importance, supernatants derived from tumors (TUMSN) were more efficient than those derived from ascites (ASCSN; 48%–67% compared with 14%–23% of inhibition, respectively; $P < 10^{-2}$) to block IFN- α production by CpG-A-activated pDC (Supplementary Fig. S3).

We next investigated the involvement of several well-known immunomodulators including TGF- β (29), TNF- α , and IL-10 (30) to inhibit IFN- α production by pDC. We observed that TUMSN contained significantly higher levels of TNF- α (median = 470 pg/mL, range 279–2,503 pg/mL) than ASCSN (median = 218 pg/mL, range 64–476 pg/mL; $P = 0.03$; Supplementary Fig. S4A). IL-10 was also higher in TUMSN (median = 2,900 pg/mL, range 263–5,862 pg/mL) than ASCSN (median = 1,638 pg/mL, range 530–2,864 pg/mL) but this difference was not significant ($P = 0.07$; Supplementary

Fig. S4B) whereas active TGF- β 1 was present at similar levels in both TUMSN and ASCSN (Supplementary Fig. S4C).

To investigate the role of IL-10, TGF- β , and TNF- α in pDC alteration by the tumor microenvironment, we blocked these cytokines in ovarian TUMSN using neutralizing antibodies (Ab). Blocking TNF- α and TGF- β independently, but not IL-10, induced a partial reversion of ovarian TUMSN-mediated inhibitory effect on IFN- α production of healthy pDC (Fig. 7B). TUMSN-mediated inhibitory effect on IFN- α production was totally reversed when TNF- α and TGF- β were simultaneously blocked, while blocking IL-10 together with either TNF- α or TGF- β has only minor additional effect. Collectively, these observations show that the specific inhibitory effect associated to the soluble fraction of the ovarian tumor environment on pDC innate functions is mainly mediated through TGF- β and TNF- α cooperation.

Discussion

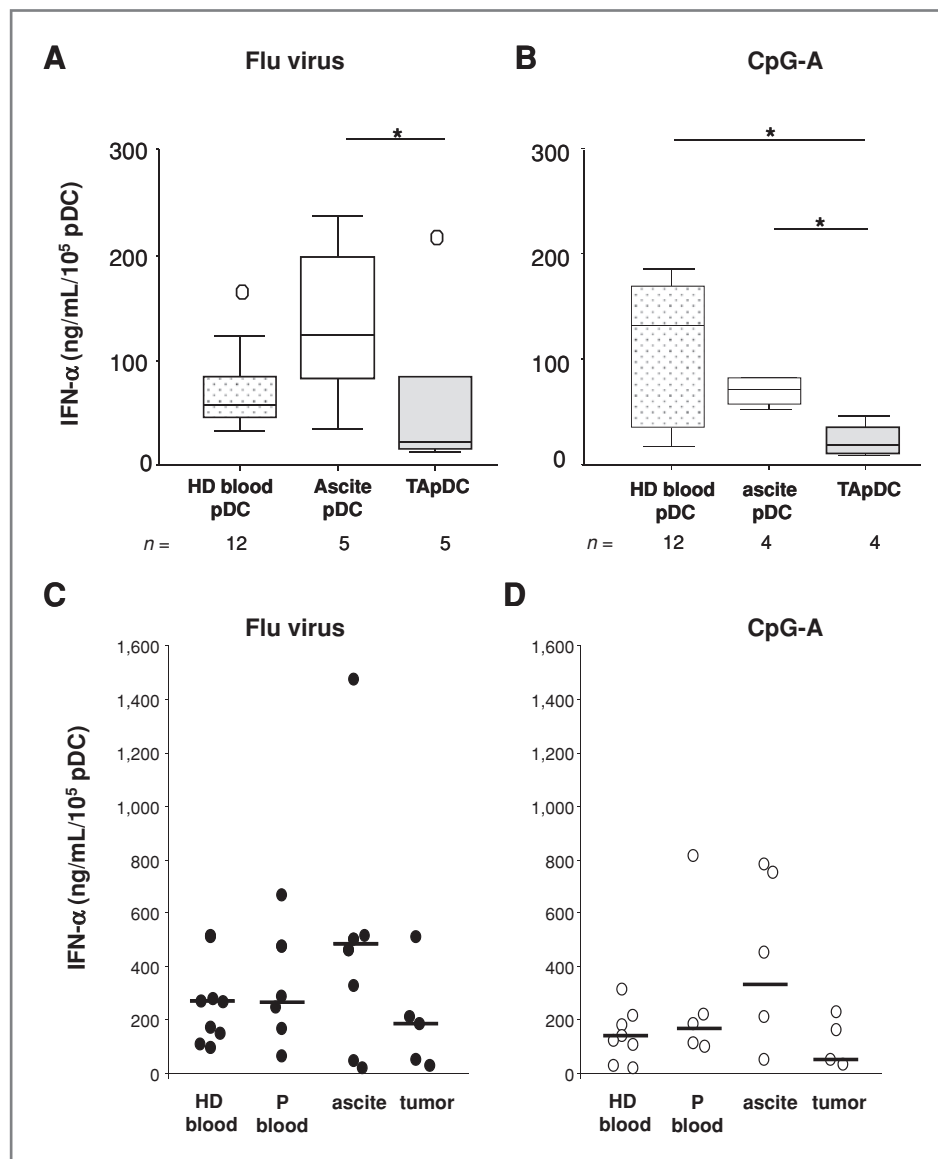
In this prospective study on 44 patients presenting ovarian cancer and using *ex vivo* analysis, we showed that TApDC are different from ascite and blood pDC in terms of distribution, phenotype, innate and adaptative functions, and impact on patients' outcome. Indeed, pDC accumulate mainly in malignant ascites whereas their frequencies were variable in ovarian tumors. Of most importance, only accumulation of TApDC was an independent prognostic factor associated with early relapse whereas their presence in ascites was not deleterious for patients. The presence of mDC in ascites and tumors had no impact on patients' outcome highlighting the specific pejorative role of pDC in the context of tumor microenvironment.

To further investigate the clinical significance of the presence of pDC in tumors and ascites, we conducted a systematic comparison of TApDC and ascite pDC. TApDC showed a semimature phenotype as illustrated by higher levels of activation molecules CD40 and CD86. This partial activation of TApDC might be due to endogenous danger signals such as self-nucleic acids released by dying cells in the tumor microenvironment (26) which were in higher proportions in tumors than ascites. We also observed increased levels of TNF- α in ovarian TUMSN when compared with ASCSN, a cytokine previously shown to induce pDC maturation (31). In addition to their baseline semimature phenotype, TApDC were more potent than ascite or blood pDC to mature *in vitro* in response to both TLR-independent and TLR-dependent signals.

In contrast, TApDC but not ascite pDC were strongly affected for their capacity to produce IFN- α upon TLR stimulation, in particular in response to CpG-A, a TLR9 ligand known to induce type I IFN secretion in pDC (32). Collectively, these results showed that TApDC seemed to respond to signals by maturing rather than by producing IFN- α , even in response to IFN- α inducers.

Moreover, ovarian tumor-derived supernatants blocked specifically IFN- α production by TLR-activated pDC *in vitro*. These results suggest that soluble factors present in the tumor microenvironment are involved in TApDC dysfunction. In this context, we showed that cytokines such as TGF- β and TNF- α

Figure 6. TApDC are inhibited for their IFN- α production following TLR ligands stimulation. A and B, FACS-sorted TApDC, ascite pDC, and blood pDC from HDs were cultured with inactivated flu virus (100 HAU/mL) and CpG-A (5 μ g/mL) for 24 hours. IFN- α production in culture supernatants was determined by ELISA. Results were standardized by calculating the production of IFN- α for 1×10^5 pDC for each sample. C and D, total MNC (1×10^6 cells/well) from blood, ascites, and tumors of the same patients ($n = 6$) and from blood of HD ($n = 8$) were activated in duplicate with TLR-7 (flu) and 9 (CpG-A) ligands for 18 hours in 24-well tissue culture plates. IFN- α production was measured by ELISA and standardized to the percentage of pDC contained in each sample as determined by FACS analysis. The production of IFN- α was adjusted to 1×10^5 pDC. Median values are represented by horizontal lines in each series.



detected in ovarian tumor derived-supernatants, were involved in this pDC dysfunction. The inhibitory role of TGF- β is consistent with a previous study showing that increased intratumoral IFN- α levels correlates with reduced TGF- β 1 in breast cancer patients (33). Moreover, recent reports in mice and human showed that this cytokine contributes to peyer patches, splenic stromal, and tumor environments mediated inhibition of pDC capacity to produce IFN- α (29, 34, 35).

The deleterious prognostic effect of TApDC in ovarian cancer could be related to the alteration of IFN- α production. This cytokine is indeed active against ovarian cancer *in vitro* (36, 37) and *in vivo* (38, 39). Inhibition of IFN- α production by TApDC might confer a selective advantage for ovarian tumors, as IFN- α has direct antitumor activities, by inhibiting tumor cell proliferation and neoangiogenesis, but also by promoting

immunosurveillance through the activation of B cells, NK cells and macrophages (reviewed in reference 40) and inhibiting tumor stem cell-like population (41).

In this study, we observed that both TLR-activated TApDC and ascite pDC were able to induce CD4⁺ T cell proliferation consistently with the acquisition of a fully mature phenotype. Importantly, T cell proliferation induced by TApDC correlated with high IL-10 production, confirming the role of TApDC in inducing immune tolerance in ovarian cancer. Taken together, the differences observed between TApDC and ascite pDC suggest that the role of these immune cells are different and possibly opposite according to the tissue. pDC play a proinflammatory role in malignant ascites, whereas they are immunosuppressive in tumors. IFN- α produced primarily from pDC, in addition to having direct antitumoral activity (36, 37), provides an important signal for T helper precursor differentiation

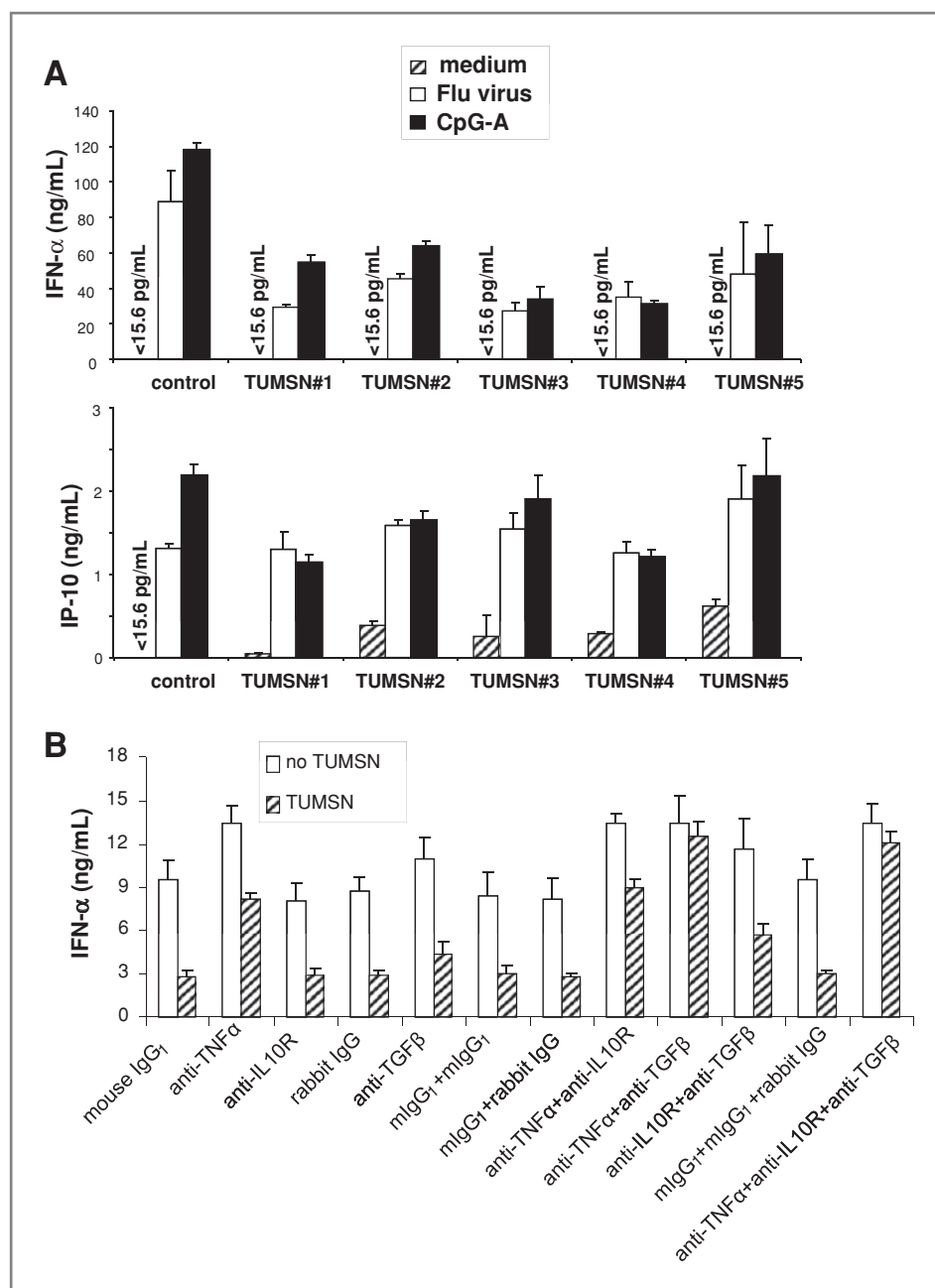


Figure 7. Ovarian tumor microenvironment inhibits IFN- α production by healthy pDC through TGF- β and TNF- α cooperation. Supernatants of single-cell suspensions from ovarian tumors (TUMSN) were collected after 48 hours and frozen. A, purified blood pDC from HD were preincubated with 20 ng/mL IL-3 either alone or with 25% of TUMSN derived from 5 different ovarian cancer patients for 16 hours and then activated or not for another 24 hours with inactivated fu virus (100 HAU/mL) or CpG-A (5 μ g/mL). After 40 hours, the production of IFN- α and IP-10 was measured by ELISA. No IFN- α was detected in media conditions (no TLR activation) and IP-10 was detected in some ovarian tumor-derived supernatants at low levels. B, purified tonsil pDC were preincubated with IL-3 either alone or in combination with 25% of ovarian TUMSN in the presence or absence of specific control isotype antibodies, anti-TNF- α , anti-IL-10 receptor, and anti-TGF- β blocking antibodies (10 μ g/mL) for 16 hours. Flu virus was then added for another 24 hours. IFN- α was measured in culture supernatants by ELISA. Data are expressed as mean \pm SD and are representative of 3 independent experiments.

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in favor of a T helper type 1 immune response (42). The dramatically decreased secretion of IFN- α by TApDC associated with IL-10 T-cell response would have serious consequences on the induction of efficient antitumoral immune response and explain the deleterious impact of the presence of pDC into tumors. Therefore, tumors may deregulate immunity by attracting and manipulating pDC behaviour.

Our data also clearly show that malignant ascites could not serve as a model for tumor microenvironment in the context of ovarian cancer. Indeed, TApDC and ascite pDC from ovarian cancer patients have clearly distinct phenotype, functions, and impact on survival. Our observations are in agreement with the

work published by Zou and colleagues (14, 21) showing that pDC isolated from malignant ascites are functional in terms of IFN- α production. We further provide here novel information showing that the accumulation of pDC in ascites (up to 10-fold higher than in tumors) had no impact on patients' outcome whereas their presence in tumors was deleterious. Moreover, we showed that TApDC were strongly inhibited for their innate response. Thus, functional inhibition of TApDC is strictly dependent on ovarian tumor microenvironment.

In our study, we found that blood pDC and mDC were significantly decreased compared with HDs, consistent with prior studies in breast (13) and pancreatic cancer (18)

patients. This imbalance in the circulating pDC pool is not just exclusively a finding in cancers, but is also observed in patients with autoimmune diseases, such as systemic lupus erythematosus (43) and in chronic HIV infections (44, 45). Importantly, during primary HIV infection, numbers of blood pDC and levels of IFN- α production have been shown to be severely reduced, leading to speculation that HIV disease progression may result in part from the failure of pDC to limit viral replication (45–47). Upon initiation of effective antiretroviral therapy in HIV-infected individuals, the levels of pDC have been shown to partially recover (48, 49).

We reported in this work several similarities between ovarian cancer patients and HIV patients. Indeed, we observed severe reduction in blood pDC in patients with advanced stages ovarian cancer, similarly to HIV patients (44, 45, 50), in comparison with those with early stages, and to HDs. We have also shown severe alteration of IFN- α production in TApDC in comparison with HD blood pDC in response to TLR ligands. Similarly, to HIV patients undergoing antiretroviral therapy (48), levels of blood pDC partially recovered in patients undergoing chemotherapy or in CR whereas there was a trend toward decrease levels in patients with progressive disease. Taken together, these data suggest that the solid tumors exert systemic modulatory effects on the immune system, in addition to their effects in the tumor microenvironment. These similarities observed between HIV and ovarian cancer patients suggest similar mechanisms implicated in the disturbance of blood pDC homeostasis in both diseases.

To our knowledge, this work represents the first systematic comparison of pDC phenotype and function in blood, ascites,

and tumors from cancer patients. We showed that pDC's functional alterations occurred in tumors whereas they seem to have a proinflammatory role in malignant ascites. The correlation of TApDC with poor outcome confirms their participation in immune tolerance. Likewise, identifying molecular pathways implicated in the inhibition of IFN- α production by TApDC will help to develop targeted therapies reversing the deleterious role of TApDC in the context of ovarian cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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