

Impaired IFN- α Production by Plasmacytoid Dendritic Cells Favors Regulatory T-cell Expansion That May Contribute to Breast Cancer Progression

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Abstract

Infiltration and dysfunction of immune cells have been documented in many types of cancers. We previously reported that plasmacytoid dendritic cells (pDC) within primary breast tumors correlate with an unfavorable prognosis for patients. The role of pDC in cancer remains unclear but they have been shown to mediate immune tolerance in other pathophysiologic contexts. We postulated that pDC may interfere with antitumor immune response and favor tolerance in breast cancer. The present study was designed to decipher the mechanistic basis for the deleterious impact of pDC on the clinical outcome. Using fresh human breast tumor biopsies ($N = 60$ patients), we observed through multiparametric flow cytometry increased tumor-associated (TA) pDC (TApDC) rates in aggressive breast tumors, i.e., those with high mitotic index and the so-called triple-negative breast tumors (TNBT). Furthermore, TApDC expressed a partially activated phenotype and produced very low amounts of IFN- α following toll-like receptor activation *in vitro* compared with patients' blood pDC. Within breast tumors, TApDC colocalized and strongly correlated with TA regulatory T cells (TATreg), especially in TNBT. Of most importance, the selective suppression of IFN- α production endowed TApDC with the unique capacity to sustain FoxP3⁺ Treg expansion, a capacity that was reverted by the addition of exogenous IFN- α . These findings indicate that IFN- α -deficient TApDC accumulating in aggressive tumors are involved in the expansion of TATreg *in vivo*, contributing to tumor immune tolerance and poor clinical outcome. Thus, targeting pDC to restore their IFN- α production may represent an attractive therapeutic strategy to overcome immune tolerance in breast cancer. *Cancer Res*; 72(20); 5188–97. ©2012 AACR.

Introduction

Functional alteration of tumor-associated dendritic cells (TADC) that play a critical role in antitumor immunity, as well as mobilization of immunosuppressive regulatory T cells (Treg) that shut down immune responses, have been associated with tumor tolerance (1). Most cancers, including breast

tumors, are highly infiltrated by immune cells. Tumor-resident DC are conditioned by the tumor microenvironment to favor tolerogenic responses that could contribute to disease progression (2). Indeed, we previously showed that plasmacytoid DC (pDC) and Treg infiltrating breast tumors correlate with an adverse clinical outcome (3, 4), suggesting that both pDC and Treg are involved in breast cancer progression.

pDC are well known for their role in antiviral immunosurveillance through their massive production of type I IFN- $\alpha/\beta/\omega$ in response to DNA or RNA viruses, recognized by toll-like receptors (TLR) 9 and 7, respectively (see ref. 5 for review). Beside their direct antiviral properties, type I IFNs produced by pDC activate natural killer (NK) cells, macrophages, and CD11c⁺ myeloid DC (mDC) to elicit antimicrobial/viral/tumor immune responses (5). Moreover, differentiated mature pDC are capable of efficient antigen (cross)-presentation (6, 7) directing T-cell responses with considerable flexibility (5). Of importance, recent works have also established a critical role of pDC in noninfectious autoimmune/inflammatory pathologies (lupus, psoriasis) because of uncontrolled production of IFN- α following their chronic activation by self-nucleic acids (8, 9).

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi: 10.1158/0008-5472.CAN-11-3468

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In addition to immune activation, increasing evidence suggests that pDC also play regulatory functions. Under certain circumstances, the tolerogenic role of pDC as inducers of Treg in the periphery has been clearly illustrated *in vivo* (see ref. 10 for review) as well as their capacity to induce the differentiation (11–13) and expansion (14, 15) of Treg *in vitro*.

pDC are also involved in antitumor immunity, as underlined by their recent identification in several human and murine solid cancers (4, 16–19). Only sparse information are available on TApDC functions showing (i) poor stimulation of CD4 and CD8 T cells (18, 19), (ii) induction of anergic and/or suppressive CD4 and CD8 T cells (19, 20), (iii) promotion of multiple myeloma cell growth, survival, and drug resistance (21), but also (iv) involvement in therapeutic response to TLR7 ligands (22, 23). In breast cancer, we previously reported an accumulation of suppressive and activated Treg that proliferate *in situ* and that are associated with a poor prognosis (3), indicating that TATreg can expand in the tumor microenvironment.

To understand the negative impact of TApDC on breast tumor patients' outcome and its possible link with TATreg, we investigated herein, their functional competence within breast tumors. We show that TApDC are impaired for their IFN- α production and consequently promote immune tolerance through TATreg expansion and differentiation of interleukin 10 (IL-10)-secreting T cells, leading to tumor progression and poor clinical outcome in breast cancer. Thus, restoring the production of IFN- α by pDC within breast tumors emerges as an appealing therapeutic strategy to trigger antitumor immunity.

Materials and Methods

Patients, human tissue samples, and blood

Fresh tumor and blood samples (collected on CTAD anti-coagulant) from 60 patients diagnosed with primary breast carcinoma were obtained before any treatment from the Centre Léon Bérard (CLB) tissue bank after patient informed consent. The study was reviewed and approved by the Institutional Review Board of CLB. Discarded human tonsil material was obtained anonymously according to the institutional regulations in compliance with French law. Healthy human blood was obtained anonymously from the Etablissement Français du Sang (Lyon, France). Written informed consent was obtained from all study participants in accordance with the Declaration of Helsinki. The breast cancer patients' characteristics are detailed in Supplementary Table S1. All clinical and biologic data related to breast cancer patients were collected prospectively and included in a regularly updated institutional database at CLB.

Isolation of pDC, naïve and memory CD4⁺ T cells, and *in vitro* generation of monocyte-derived DC

Breast tumor samples, tonsils, and blood were processed as previously described (3). pDC and naïve or memory CD4⁺ T cells were obtained from tissues after magnetic enrichment or fluorescence-activated cell sorting (FACS)-sorting. Monocyte-derived DC (MoDC) were obtained from blood-purified monocytes. Detailed methods are provided in Supplementary Methods.

Stimulation of pDC

TApDC were cultured at 5×10^5 cells/mL in 96-well flat-bottomed plates in complete medium for 24 hours in the presence of IL-3 (20 ng/mL), inactivated flu virus (100 HAU/mL), CpG-A (5 μ g/mL), CpG-B (5 μ g/mL), and R848 (1 μ g/mL). Healthy pDC were cultured in presence of IL-3 with or without breast TUMSN (25%) for 16 hours before TLR activation. Cells and supernatants were harvested after 24 hours and 40 hours for TApDC and healthy pDC culture, respectively, to analyze cell surface expression of activation markers and cell viability (by propidium iodide exclusion) by flow cytometry and cytokine/chemokine production by ELISA.

DC T-cell cocultures

Allogeneic naïve CD4⁺ T cells, Treg, and conventional memory CD4⁺ T cells were cultured in complete medium with or without (i) IL-2 (100 IU/mL) and (ii) purified TApDC, healthy pDC, mDC, and MoDC pretreated for 24 hours with IL-3, granulocyte macrophage colony-stimulating factor (GM-CSF; 10 ng/mL), CpG-B, R848, or flu or purified healthy pDC that were preincubated for 40 hours in IL-3 with (TUMSN-pDC) or without breast tumor supernatants, and TLR-L. T lymphocytes were added on preactivated DC subsets (ratio 1:5) and cocultured for 4 days in triplicate in 96-well round-bottomed plates. Proliferation was assessed by carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) dilution in experiments analyzing FoxP3 expression after gating on CD3⁺ cells or by DNA synthesis analyzed by ³H-TdR uptake (Betaplate scintillation counter, Perkin Elmer). Viable cells were selected by 4', 6-diamidino-2-phenylindole exclusion or Live/Dead reagent (Invitrogen) in case of cell permeabilization. Cytokines secretion was measured by ELISA in the supernatants. At day 4, for naïve T cells and TUMSN-pDC coculture, T cells were harvested, washed, and restimulated at 10^5 cells/well in triplicate for 16 hours with phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (2 μ g/mL; Sigma-Aldrich), while for other T/pDC cocultures, supernatants of coculture were harvested and frozen without any further stimulation of T cells.

Immunohistochemical analysis on tumor tissue

Stainings using CD3 (4) and FoxP3 (3) antibodies were carried out on tissue microarray paraffin sections from 151 patients with invasive nonmetastatic breast cancer using a BenchMark Series automated slide stainer (Ventana) as previously described. After heat-induced Ag retrieval in tris-based buffer pH 8, BDCA2 staining was carried out using mouse anti-BDCA2 (clone 104C12, Dendritics) at 5 μ g/mL incubated at 37°C for 30 minutes, revealed with biotinylated secondary antibody bound to streptavidin peroxidase conjugate (Ultra-View kit and Amplification kit, Ventana), and revealed with 3,3'-diaminobenzidine (Dako) as substrate. Sections were counterstained with hematoxylin. The density of BDCA2⁺ cells was assessed semiquantitatively allowing the stratification of the tumors as positive or negative for BDCA2. FOXP3⁺ cells were enumerated using the ARIOL system (Applied Imaging). To compare the role of high number of FOXP3⁺ cells, we chose as cutoff the highest quartile (≥ 26.7 in non-TNBT and ≥ 61.8 in TNBT).

Statistical analyses

Comparison of independent samples was done by the student *t* test or nonparametric Wilcoxon tests when appropriate. Comparison of percentages was done by χ^2 test. Correlation analysis of the data was conducted using the Fischer exact test, Spearman test, or the Pearson test when appropriate. Data for cytokine production and T-cell proliferation are expressed as mean \pm SD. Percentages of inhibition of IFN- α production by pDC were compared using a nonparametric Friedman test for paired samples. Differences were considered significant for *P* values less than 0.05 and are indicated as * for *P* < 0.05, ** for *P* < 0.01, and *** for *P* < 0.001.

Results

High TApDC infiltration is associated with aggressive breast tumors

We previously reported that infiltration of primary breast tumors by pDC identified as CD123⁺ cells by immunohistochemistry (IHC) correlates with poor prognosis, with a median follow-up of 5 years (4). We updated our clinical database with a median follow-up of 12 years and we observed that the presence of CD123⁺ pDC still represents a major independent adverse prognostic factor for both overall survival (OS; *P* = 0.002) and relapse-free survival (RFS; *P* < 10⁻³; Supplementary Fig. S1). In a prospective study including 79 newly diagnosed breast tumors patients and using flow cytometry, pDC and mDC were identified in primary breast tumor specimens as lineage⁻CD4⁺CD11c⁻CD123⁺BDCA2⁺ and lineage⁻CD4⁺CD11c⁺BDCA1⁺ cells, respectively (Supplementary Fig. S2A). Comparative analyses showed that the mean percentage of DC among total cells in primary tumors was 0.15 \pm 0.18% for pDC versus 0.04 \pm 0.05% for mDC (*P* < 10⁻³; Fig. 1A, left panel). We also observed significant lower pDC and to a lesser extent mDC frequencies in patients' blood (mean pDC: 0.25 \pm 0.22%, *n* = 48), when compared with healthy donors' blood (mean pDC: 0.37 \pm 0.19%, *n* = 48; *P* = 0.006; Fig. 1A, right panel). These observations suggest preferential pDC recruitment within the tumor mass. Because the median follow-up of our prospective cohort was 43 months, there were not enough events to analyze RFS and OS. However, we observed significant increased TApDC in aggressive tumors with high mitotic index (MI) that mirrors the rate at which tumor cells divide (0.22 \pm 0.22 compared with 0.10 \pm 0.11, *P* = 0.03) and triple negative breast tumors (TNBT; hormone-receptors and HER2-neu negative) (0.29 \pm 0.28 compared with 0.12 \pm 0.12, *P* = 0.05) compared with low MI and non-TN tumors, respectively (Fig. 1B). The presence of high numbers of TApDC was correlated only with those parameters that are characteristic of tumor aggressiveness (Supplementary Table S1 and Fig. 1C). These observations were confirmed by IHC analysis as we observed that TApDC infiltration was massive in 50% of TNBT (*n* = 25 tumors), compared with only 19% other tumors (*n* = 162 tumors; *P* = 0.05). Importantly, such correlations were not observed for TAmDC (Supplementary Table S1). Thus, using 2 different methods (flow cytometry and IHC) to identify pDC in 2 independent cohorts, we showed that high numbers of TApDC correlated with tumor aggressiveness,

strengthening our previous report on the deleterious impact of TApDC on breast tumors patients outcome (4).

Breast TApDC display a partially activated phenotype and are impaired for IFN- α production in response to TLR ligands

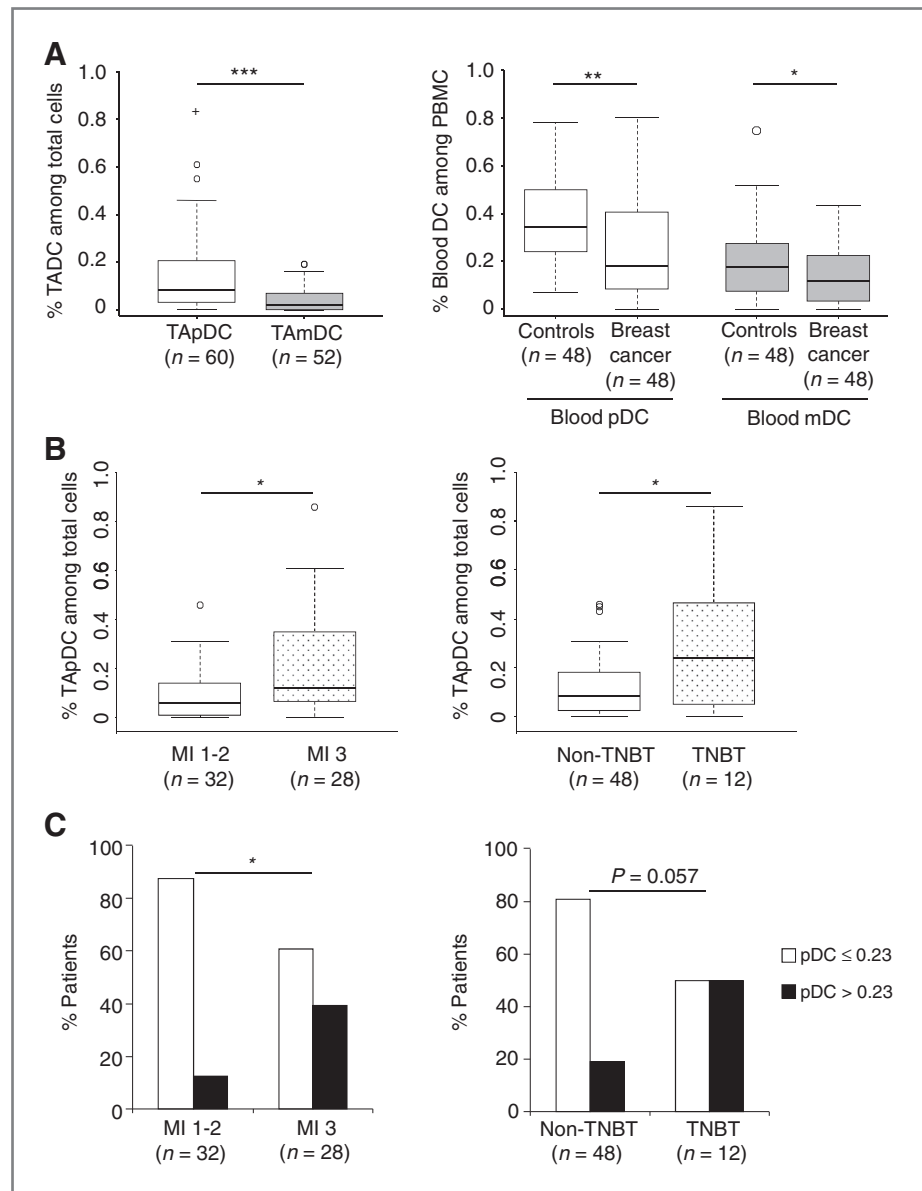
In contrast to tonsil pDC or paired blood pDC, TApDC (gated on CD4⁺CD123⁺ cells; Supplementary Fig. S2B) specifically exhibit a partially activated phenotype with moderate but significantly higher levels of activation markers such as CD40, CD83, CD86, and HLA-DR and reduced BDCA2 expression (Fig. 2A and B), a characteristic resting pDC marker. Thus, the breast tumors environment favors TApDC activation.

pDC are the most potent type I IFNs producing cells in response to TLR7 or 9 ligands (5). IFN- α production by purified TApDC (>98%) was strongly reduced in response to both TLR7 (flu; 76.3% of inhibition, *P* < 0.01) and TLR9 (CpG-A) ligands (89% of inhibition) compared with healthy tonsil used as a control tissue (Fig. 3A, left panel). In contrast, patients' blood pDC produced similar levels of IFN- α than pDC from healthy donor blood in response to TLR activation (Fig. 3B). These results indicate that the capacity of pDC to produce IFN- α is selectively altered at the tumor site. Moreover, this inhibition was specific for IFN- α , because the production of other immune mediators such as IP-10/CXCL10 remained unaffected after TApDC activation (Fig. 3A, right panel). The weak IFN- α response of TApDC suggested that breast tumor cells or other cells present in tumor tissue actively suppress the ability of pDC to produce IFN- α . To study this effect in more detail, healthy pDC were exposed to supernatants derived from cell cultures of single-cell suspensions of primary breast tumors (TUMSN) and 6 different breast cancer cell lines (BCCSN) before TLR stimulation. Most TUMSN (*n* = 25/33; Fig. 3C left panel, *n* = 10 TUMSN shown), but not the BCCSN (Supplementary Fig. S3A), significantly inhibited pDC IFN- α production in response to flu (TLR7-L) and CpG-A (TLR9-L; mean% of inhibition: 60.6 \pm 14%, *P* < 0.05 and 75 \pm 18.6%, *P* < 0.05, respectively), in a dose-dependent manner (Supplementary Fig. S3B). Importantly, IP-10/CXCL10 production remained unaffected (Fig. 3C, right panel). Altogether our observations show that pDC are exposed to soluble factors in breast tumors environment that inhibits their IFN- α production.

Breast tumors' environment conditions pDC to induce selective Treg expansion

BDCA2⁺ TApDC mainly colocalized with CD3⁺ lymphoid aggregates in breast tumors (Fig. 4 and Supplementary Fig. S4A) compared with epithelial cytokeratin⁺ areas (42% vs. 14% of tumors analyzed, *n* = 20; Supplementary Fig. S4B) leading us to investigate TApDC capacity to activate T cells *in vitro*. First, we showed that CD40, CD80, and CD86 expression increased following TLR stimulation in TApDC at levels comparable to tonsil pDC (Fig. 4B and Supplementary Fig. S5). Consistent with the acquisition of a mature phenotype, TLR-stimulated TApDC trigger potent naïve CD4⁺ T-cell proliferation (Fig. 4C) and differentiation into Tr1-like cells producing high levels of IL-10 and IFN- γ (Fig. 4D), as observed for tonsil pDC (Fig. 4C and D). Furthermore, we observed that similarly to TApDC,

Figure 1. pDC infiltrate highly aggressive tumors. **A**, box plot set of the frequencies of lineage-negative CD4⁺ CD11c⁻ pDC and CD4⁺ CD11c⁺ mDC (see Supplementary Fig. S2A for DC identification) among total cells of primary tumors ($n = 60$ or $n = 52$) are indicated (left). The frequencies of pDC and mDC within peripheral blood mononuclear cell (PBMC) of breast cancer patients ($n = 48$) and healthy donors ($n = 48$) are indicated (right). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; paired Student t test. Box plot set of pDC levels (**B**) determined as in **A**, and percentages of breast cancer patients with low- or high-pDC numbers (**C**; the highest quartile $>0.23\%$ was used as cutoff value) among patients with breast tumors with a MI 1–2 versus 3 and with TNBT versus non-TNBT. MI was established by counting mitoses in 10 high-power ($\times 400$) fields (HPF) per tumor section and then averaging the number by HPF (1.96 mm^2). Mitotic cell counts between 1 and 9, between 10 and 19, and more than 20 were defined as MI 1, MI 2, and MI 3, respectively. *, $P < 0.05$; nonparametric Wilcoxon test in **B** and Fisher exact test in **C**.



higher numbers of FoxP3⁺ TATreg infiltrate TNBT than non-TNBT (3rd quartile = 61.8 compared with 26.6 FoxP3⁺ cells, respectively). Importantly, we observed a significant positive correlation between TApDC and TATreg frequencies in TNBT ($r^2 = 0.749$, $P = 0.002$, $n = 14$), and to a lower extent in non-TNBT ($r^2 = 0.413$, $P = 0.004$, $n = 48$; Fig. 5A). Such correlations (i) were not as significant for TAmDC ($r^2 = 0.672$, $P = 0.05$ in TNBT and $r^2 = 0.291$, $P = 0.08$ in non-TNBT; not shown) and (ii) were confirmed by IHC analysis as we observed that 50% of TNBT and 42% of non-TNBT containing TApDC also contained high numbers of FoxP3⁺ TATreg in lymphoid areas ($P = 0.02$) although tumors lacking pDC were poorly or not infiltrated by TATreg (17.7% and 0% for non-TNBT and TNBT, respectively; Fig. 5B). Thus, because (i) TApDC infiltrates are associated with TATreg infiltrates, (ii) TApDC and TATreg are in close contact within lymphoid infiltrates (Fig. 4A and Faget, manuscript in preparation), and (iii) both cell subsets are

associated with poor prognosis in breast tumors (3, 4), we consequently investigated whether TApDC showing reduced capacity to secrete type I IFN may favor TATreg accumulation. Importantly, we observed that in absence of exogenous IL-2, R848-activated pDC have the highest capacity to promote the proliferation of purified allogeneic Treg while all DC subsets (pDC, mDC, and MoDC) induced similar proliferation of purified memory conventional T cells (Fig. 5C). Interestingly, TUMSN-pretreated pDC, in IL-3 alone or in TLR-7L (flu or R848), potentially increased by 2.8- to 4.6-fold the percentage of FoxP3^{high} T cells ($9.3 \pm 1.1\%$, $8.6 \pm 0.5\%$, and $7.3 \pm 0.5\%$, respectively) among CD4⁺CD45RO⁺ T cells compared with absence of TUMSN (2% for IL-3 and $2.6 \pm 0.5\%$ for both TLR-L; Fig. 5D). Furthermore, IL-3-treated TApDC and TUMSN-conditioned healthy donor pDC were more potent than IL-3-treated healthy donor pDC to favor Treg expansion among CD4⁺CD45RO⁺ T cells (9% vs. 1.5% of CD3⁺ T

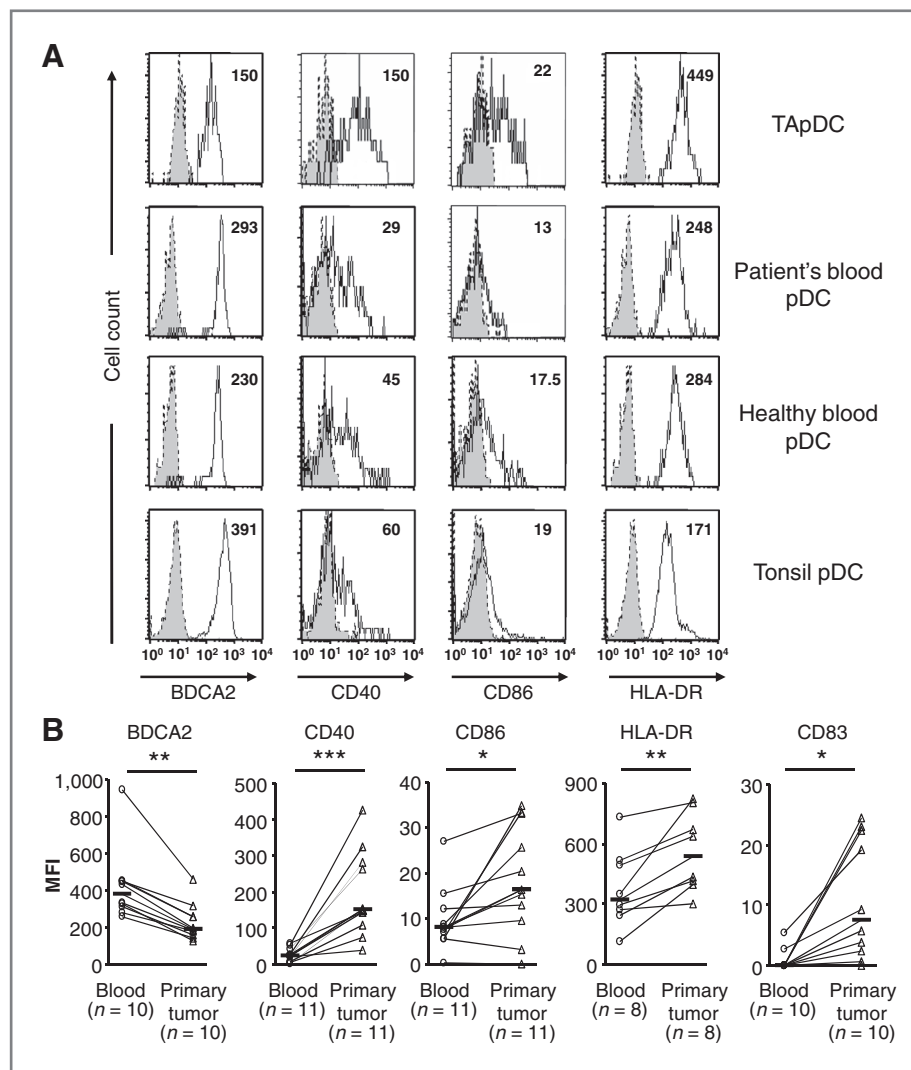


Figure 2. TApDC isolated from human primary breast tumors display a partially activated phenotype. A, pDC from primary breast tumors, patient or healthy blood, and tonsils were compared for BDCA2, CD40, CD83, and CD86 expression after gating on CD4⁺ CD123⁺ cells [corresponding to >98% BDCA2⁺ pDCs (Supplementary Fig. S2B)] without any purification steps. Dotted line represents specific isotype controls. Mean fluorescence intensity (MFI) values of total cells are indicated in each histogram. Results are representative of 5 independent experiments. B, phenotype of pDC from primary tumor and paired blood was compared for different breast cancer patients. Each symbol represents a single sample ($n > 8$ patients) and mean values are represented by horizontal lines in each series. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; nonparametric Wilcoxon test.

cells; Fig. 6A). However, TUMSN-conditioned healthy pDC did not induce the differentiation of FoxP3⁺ Treg from naïve CD4⁺ T cells (Supplementary Fig. S6). Thus, TApDC mainly favored Treg expansion rather than differentiation. Collectively, these observations show that breast tumors environment amplified pDC unique capacity to stimulate Treg expansion.

Exogenous IFN- α reverts immunosuppressive T-cell responses induced by TApDC and breast tumor environment

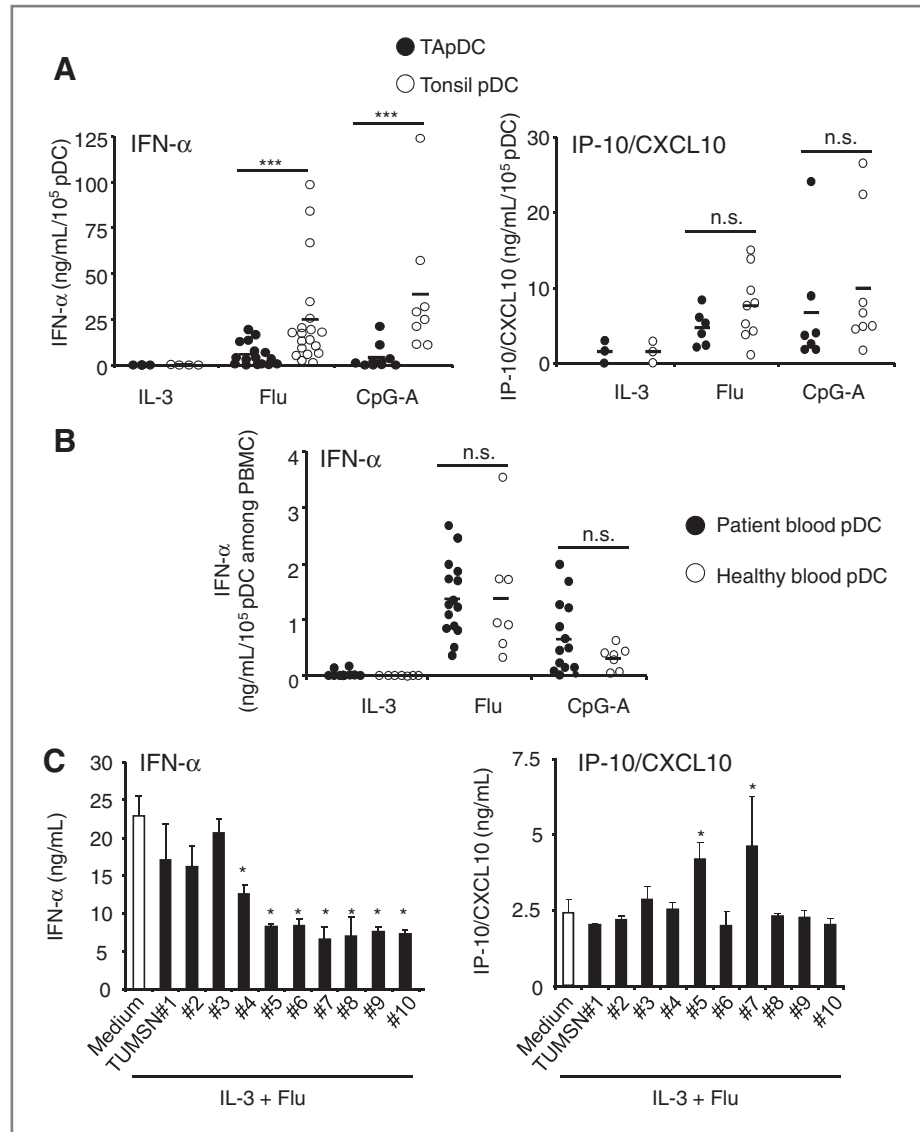
We hypothesized that the defect in IFN- α production by TApDC could favor Treg amplification. Addition of 1,000 IU/mL exogenous IFN- α (Fig. 6A) strongly reduced by 78% Treg amplification from CD4⁺CD45RO⁺ T cells induced by IL-3-treated TApDC. Similar results were obtained using healthy pDC cultured in IL-3 or IL-3 + TUMSN with respectively 1.5% and 7.7% of FoxP3^{high} T cells without IFN- α versus 0.9% and 4.8% in the presence of IFN- α (Fig. 6A). Furthermore, IFN- α inhibited IL-10 and enhanced IFN- γ secretion by total CD4⁺ T cells induced by IL-3-treated TApDC suggesting a switch in T-cell response toward Th1 polarization (Fig. 6B).

Collectively, these data indicate that TApDC capacity to promote immunosuppressive T-cell responses through FoxP3^{high} Treg expansion and IL-10-secreting T cells is strongly amplified in tumors as a result of their impaired IFN- α production.

Discussion

The negative prognostic value of pDC and Treg infiltration in human breast tumors (3, 4) prompted us to examine the contribution of pDC and their interaction with Treg in breast cancer immune evasion mechanisms. We show herein that TApDC are preferentially infiltrating aggressive breast tumors. Moreover, TApDC are highly repressed for their IFN- α production after TLR stimulation. Such IFN- α -deficient TApDC strongly correlate with TATreg infiltrate in TNBT, promote TATreg expansion, and prime IL-10-secreting CD4⁺ T cells. Finally, these tolerogenic properties of TApDC are reverted by exogenous IFN- α . Altogether, our observations show that TApDC altered for their IFN- α production contributes to establish immune tolerance through Treg expansion leading to tumor progression and poor clinical outcome in breast cancer.

Figure 3. Specific inhibition of IFN- α production by TApDC in response to TLR activation is mediated by soluble factors from breast tumor environment. Purified pDC from breast tumors and healthy tonsil (A) and peripheral blood mononuclear cell (PBMC) from breast cancer patients and healthy donors (B) were cultured with IL-3, flu, and CpG-A for 24 hours. IFN- α and IP-10/CXCL10 production was quantified by ELISA. Results were standardized to ng/mL for 1×10^5 pDC for each sample. Each symbol represents a single sample and mean values are represented by horizontal lines. ***, $P < 0.001$; nonparametric Wilcoxon test. C, healthy pDC were pretreated with IL-3 \pm TUMSN (25%) derived from 10 patients for 16 hours before flu activation for another 24 hours. IFN- α and IP-10/CXCL10 production was measured by ELISA. Data are expressed as mean \pm SD by subtracting the quantity of each cytokine detected in TUMSN alone with no pDC. Data are representative of more than 4 independent experiments, representing more than 30 TUMSN from individual patients. *, $P < 0.05$; nonparametric Wilcoxon test. n.s., not significant.



pDC are well recognized for their role in antiviral immunosurveillance driven by type I IFNs production (5). In addition, excessive production of IFN- α by pDC that are chronically activated by [LL37/self-nucleic acids] complexes participated to the development/maintenance of noninfectious autoimmune/inflammatory pathologies (8, 9). In contrast, in breast tumors we observe that TApDC are strongly inhibited for their IFN- α production upon stimulation with exogenous TLR-L *in vitro*, in agreement with a previous study in head and neck cancer (16) and our recent work in ovarian carcinoma (24). As (i) tumors have been shown to express LL37 (25) and (ii) endogenous danger signals such as self-nucleic acids (26) are released from dying tumor cells, it is tempting to speculate that, upon (LL37/self-nucleic acids) complexes recognition TApDC might contribute to tumor immunosurveillance through type I IFNs production. This is consistent with the partially activated phenotype of breast TApDC. Thus, the tumor has evolved mechanisms to inhibit type I IFNs secretion

by TApDC to prevent an effective antitumor response and favor tumor progression. This hypothesis is currently under investigation but it is supported by recent works showing that (i) type I IFN is selectively required by DC for immune rejection of tumors (27, 28) and (ii) the type I IFN signature is predictive of responses to anthracyclines in breast cancer patients (29).

Consistent with TApDC defect in IFN- α production, we show that soluble factors derived from breast tumors environment block type I IFNs production by healthy activated pDC *in vitro*. We recently showed in ovarian cancer that TGF- β and TNF- α from breast tumors environment cooperate to inhibit IFN- α secretion by TApDC (24). Our ongoing study is pointing to similar mechanisms in breast tumors (Sisirak, submitted) but also to cell-associated molecules such as BST-2 that (i) is expressed by BCC lines and (ii) inhibits TLR-triggered IFN- α secretion by healthy pDC (30). It is likely that the main source of inhibitory factors in the breast tumors environment are the immune cells and/or stromal cells

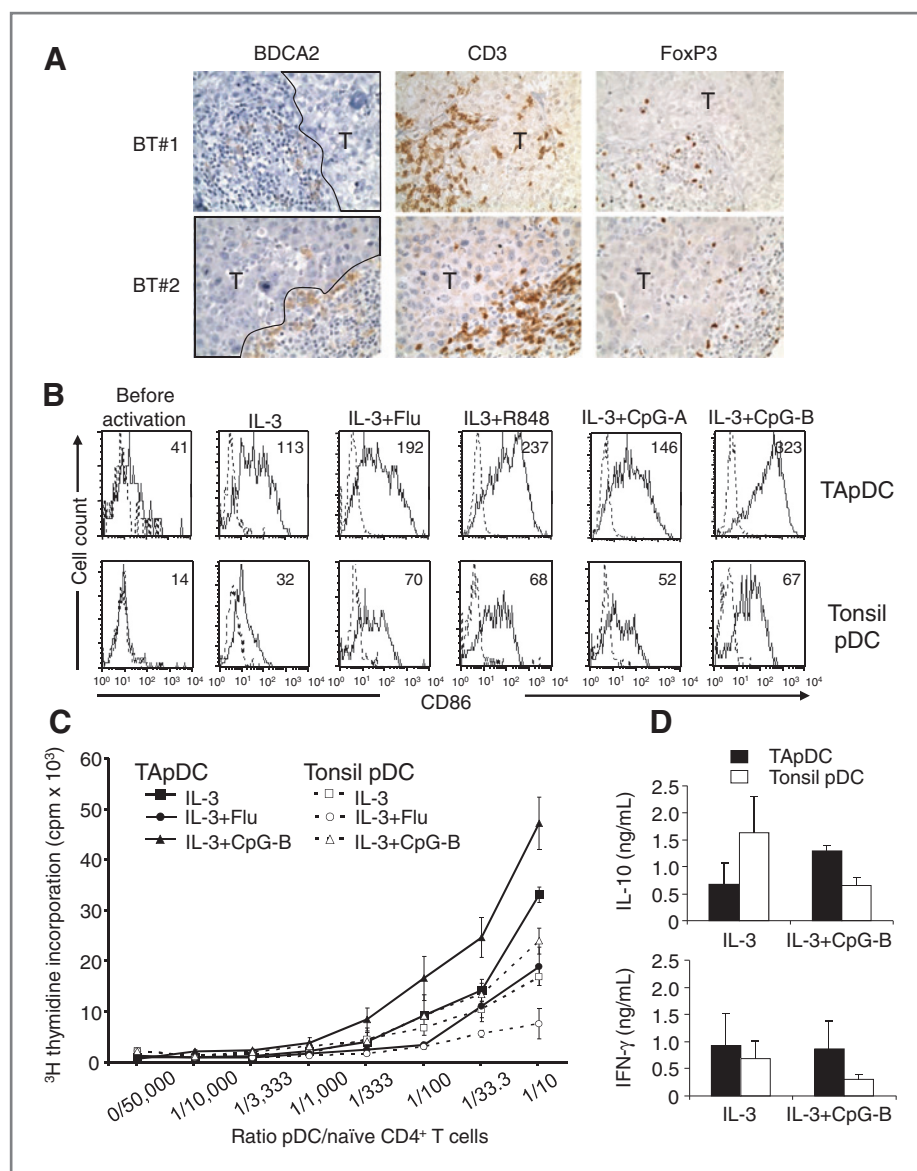


Figure 4. TLR-activated TApDC acquire a fully mature phenotype and induce naïve CD4^+ T-cell proliferation and differentiation of IL-10-secreting T cells. **A**, IHC analysis on paraffin-embedded breast tumor sections was conducted using anti-BDCA2, anti-CD3, or anti-FoxP3 (brown; $\times 40$). T, tumor mass. **B**, activation phenotype of 40-hour differentially stimulated TApDC and tonsil pDC was monitored by analyzing CD86 expression by flow cytometry. Data are representative of 5 independent experiments and MFI values of total cells are indicated in each histogram. **C**, purified tonsil pDC and TApDC were cultured with IL-3, flu, or CpG-B for 24 hours and then incubated at graded doses with allogeneic naïve CD4^+ T cells for 5 days as specified in Supplementary Materials and Methods. T-cell proliferation was determined by ^3H thymidine incorporation. Data are expressed as mean \pm SD and are representative of 3 independent experiments. **D**, purified tonsil pDC and TApDC were cultured with IL-3 or CpG-B for 24 hours and then incubated with allogeneic naïve CD4^+ T cells for 5 days as specified in Supplementary Materials and Methods. T-cell cytokine production was determined in coculture supernatant by ELISA. Data are expressed as mean \pm SD and representative of 2 independent experiments.

(fibroblasts and mesenchymal stem cells) because BCC lines supernatants did not block pDCs' innate functions.

The absence of inhibition of pDC maturation and production of IP-10/CXCL10 formally excludes a general downregulation of TLR expression by the breast tumors environment in contrast to what has been observed in head and neck cancer (16). Our ongoing studies are identifying the molecular mechanisms involved in TApDC dysfunction (Sisirak, submitted).

Although TApDCs' innate functions are strongly impaired by breast tumors, they exhibit similarly to ovarian cancers (24) exacerbated ability to induce allogeneic naïve CD4^+ T-cell proliferation and differentiation into IL-10-producing cells, that were shown to be suppressive cells *in vitro* (11). Taken together with the study showing that pDC induce IL-10-producing CD8^+ Treg in ovarian cancer (20), these findings suggest that pDC will favor an immunosuppressive IL-10-rich

environment. Our FACS and IHC analysis show strong correlation between TATreg and TApDC infiltrate as well as their *in situ* colocalization in breast tissues. In line with these observations, we observe that breast TApDC are specialized in promoting the expansion of preexisting natural Foxp3^+ Treg *in vitro*, in agreement with other studies showing that pDC induce Treg proliferation (14, 15, 31–34). We also report herein, that soluble factors from the breast tumors environment condition pDC to become tolerogenic through their ability to promote Treg expansion. A tolerogenic role for pDC was also recently reported *in vivo* in different mouse models (see ref. 10 for review), by either promoting Treg or Tr1 cells or directly suppressing effector T-cell responses. Although in other contexts, pDC-driven Treg generation from naïve T cells has been reported both *in vitro* (12, 13, 35–38) and *in vivo* (39, 40), breast tumors environment-conditioned pDC do not induce Foxp3^+ Treg differentiation from naïve CD4^+ T cells (Supplementary

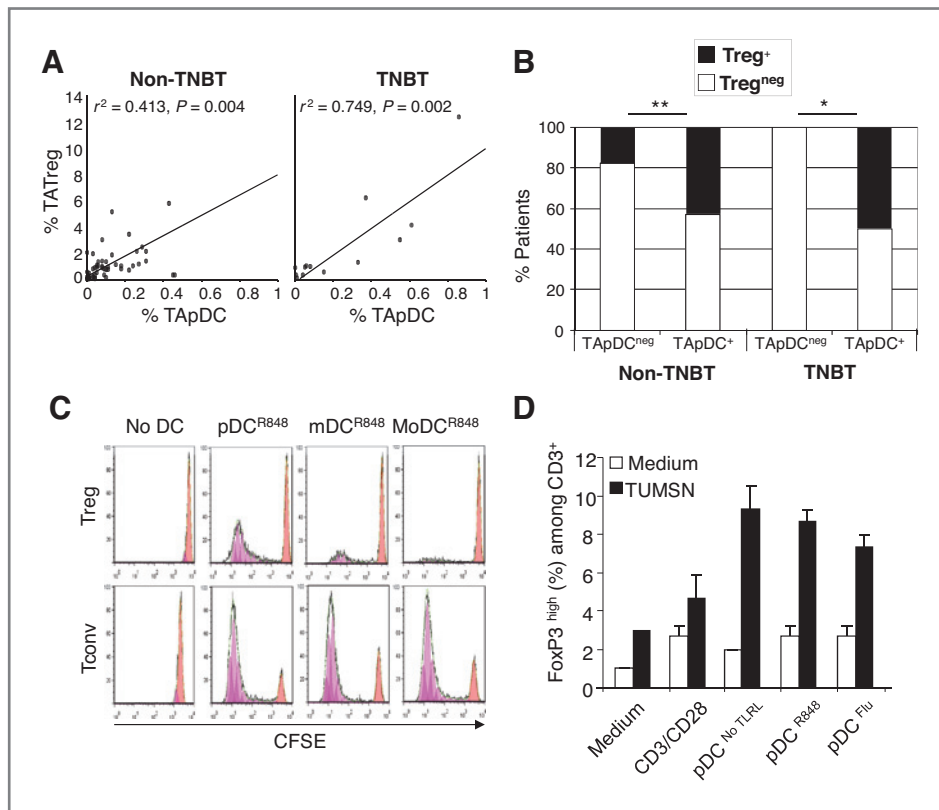


Figure 5. IFN- α -deficient TApDC increase Treg expansion. **A**, Spearman rank correlations between the proportions of CD4⁺BDCA2⁺CD123⁺ pDC and CD4⁺CD45RO⁺CD25^{high} Treg as determined by flow cytometry in non-TNBT ($n = 48$) and TNBT ($n = 12$). **B**, percentages of breast cancer patients with low or high numbers of TATreg [the highest quartile ≥ 26.7 and 61.8 FoxP3⁺ cells for non-TNBT and TNBT, respectively, was used as cutoff value as defined in a retrospective study by IHC (3)] among patients with or without TApDC in non-TNBT ($n = 133$) versus TNBT ($n = 18$). *, $P < 0.05$; **, $P < 0.01$; Fischer exact test. **C**, pDC, mDC, and MoDC were preactivated for 24 hours with IL-3 or GM-CSF and R848, washed, and cocultured for 5 days with CFSE-labeled conventional memory CD4⁺CD25^{Low/Neg}CD127^{+/-} T cells (Tconv) or CD4⁺CD25^{high}CD127⁻ Treg in the absence of IL-2. At day 5, CFSE dilution was analyzed on CD3⁺ T cells. Percentages of CFSE-diluted cells are indicated. **D**, healthy pDC were pretreated for 16 hours with IL-3 with or without 25% TUMSN before activation or not with R848 or flu for 24 hours. Memory CD4⁺ T cells were then added with IL-2 (100 IU/mL) at 25% v/v for 5 days. Anti-CD3/anti-CD28 expandbeads were used as control. FoxP3 expression was analyzed on CD3⁺ viable cells.

Fig. S6). These results provide evidence that TApDC may contribute to tumor progression through Treg expansion. This unique capacity of TApDC is because of ICOS/ICOS-L interaction between TApDC and TATreg, respectively (Faget; submitted for publication).

Of most importance, the inhibition of type I IFNs production by TApDC is required for this tolerogenic TApDC function because exogenous IFN- α (i) blocks TApDC-mediated Treg expansion and (ii) potentiates IFN- γ and inhibits IL-10 production by memory CD4⁺ T cells highlighting a shift toward Th1 phenotype instead of tolerogenic memory CD4⁺ T-cell activation. Our findings are supported by recent publications showing that through a direct effect on Ag-presenting cells and by affecting Treg proliferation and differentiation, IFN- α sustains and drives CD4⁺ effector functions (41, 42). Thus, *in vivo* local interactions between IFN- α -deficient TApDC and TAT cell (ref. 3 and Faget; submitted for publication) lead to TATreg and Tr1 expansion, favoring an immunosuppressive environment that may contribute to tumor immune escape and progression.

Overall, our data provide direct evidence that TApDC have an important immunopathologic role through Treg expansion

in human breast cancer that likely explain their deleterious impact on the clinical outcome. The positive correlation between TApDC and TATreg content and tumor aggressiveness as observed in TNBT strengthens our previous observations showing that infiltration of primary breast tumors by pDC correlates with poor prognosis (4). It also corroborates our recent findings in ovarian cancer and from others in melanoma showing that TApDC accumulation correlates with early relapse (24, 43). TNBT represent about 15% of all breast cancers but they have the severest prognosis. It represents an important clinical challenge and little is known about their biology (44). Mechanisms beyond this predominant TApDC infiltration in TNBT is an important question that is currently under investigation. Of note, because of the low incidence of TNBT, we could not analyze the impact of TN status on the functional alteration of *ex vivo* purified TApDC nor the impact of pDC on the clinical outcome of TNBT.

In conclusion, we identify in human breast cancer a TApDC defect in IFN- α production leading to TATreg expansion and contributing to breast tumor progression. These findings uncover the mechanisms that mediate the deleterious impact of pDC infiltration in breast tumors and would provide new

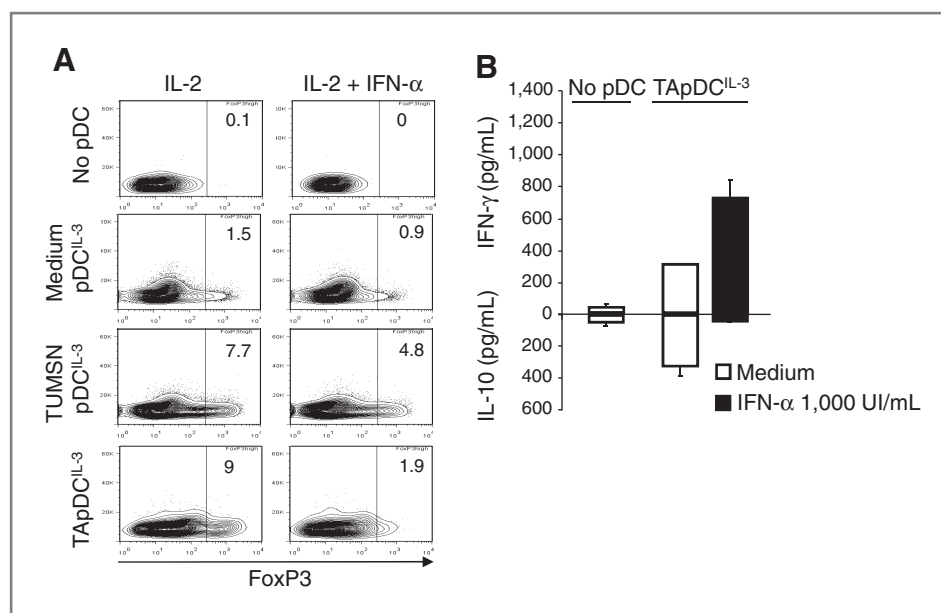


Figure 6. Exogenous IFN- α reverts tolerogenic properties of TApDC. A, healthy pDC or TApDC were pretreated with IL-3 \pm 25% TUMSN for 24 hours and then cocultured with memory CD4⁺ T cells in the presence of IL-2 (100 IU/mL) \pm IFN- α (1,000 IU/mL) for 5 days. FoxP3 expression was analyzed on CD3⁺ viable cells. B, 24-hour IL-3- or IL-3+R848-activated TApDC were cocultured with healthy CD4⁺ T cells during 5 days in presence of IL-2 (100 IU/mL) \pm IFN- α (1,000 IU/mL). IL-10 and IFN- γ secretion was measured by ELISA. Data are representative of 3 independent experiments.

therapeutic approaches targeting pDC, as in skin cancers (22, 23, 45, 46). We recently obtained evidences in a mouse mammary tumors model that reactivation of TApDC leads to tumor regression and antitumor immune responses (Le Mercier and colleagues, manuscript in preparation). Thus, restoring TApDC innate function might represent an attractive therapeutic strategy for localized breast tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V. Sisirak, J. Faget, N. Goutagny, S.I. Labidi-Galy, A. Bajard, J.-Y. Blay, C. Caux, N. Bendriss-Vermare

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V. Sisirak, S. Renaudineau, A. Puisieux, J.-Y. Blay, N. Bendriss-Vermare

Study supervision: J.-Y. Blay, C. Caux, N. Bendriss-Vermare

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Performing technical experiments: M. Gobert

Contributed new reagents or analytic tools: I. Durand

Provided helpful discussions: I. Le Mercier

Acknowledgments

The authors are grateful to the breast cancer patients who consented to participate in this study and to the medical staff and the Centre de Ressources Biologiques from the CLB who provided us with breast tumor specimens. We thank doctors and colleagues from clinics and hospitals who provide us with blood and tonsils samples. We thank X.-N. N'Guyen and C. Rigal for early technical contribution and A. Besse for help with statistical analyses.

Grant Support

This work was funded by institutional grants from Breast Cancer Research Foundation, Association pour la Recherche sur le Cancer (ARC), Comité départemental de Saône-et-Loire et du Rhône de la Ligue nationale contre le cancer, Lyon Biopole DEMINAP and PLATINE projects, and Institut National du Cancer grant ACI-63-04, ACI 2007-2009, and Met-escape 2007. V. Sisirak is a recipient of a grant from the Région Rhône-Alpes and from ARC. J. Faget and M. Gobert are recipients of a grant from the Ligue Nationale contre le cancer.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Received October 21, 2011; revised June 20, 2012; accepted July 12, 2012; published OnlineFirst July 25, 2012.

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