Research Article

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 (BT) 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 pathophysiological 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 BT biopsies (n=60 patients), we observed through multiparametric flow cytometry increased tumor-associated (TA) pDC (TApDC) rates in aggressive BT, i.e. those with high mitotic index (MI) and the so-called triple negative BT (TNBT). Furthermore, TApDC expressed a partially activated phenotype and produced very low amounts of interferon (IFN)-α following TLR activation in vitro compared to 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.

249 words
INTRODUCTION

Functional alteration of tumor-associated dendritic cells (TADC) that play a critical role in anti-tumor 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 tumours (BT), are highly infiltrated by immune cells. Tumour-resident DC are conditioned by the tumour microenvironment to favour tolerogenic responses that could contribute to disease progression (2). Indeed, we previously showed that plasmacytoid DC (pDC) and Treg infiltrating BT 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 anti-viral immunosurveillance through their massive production of type I interferons (IFN-α/β/ω) in response to DNA or RNA viruses, recognized by Toll Like Receptors (TLR) 9 and 7 respectively (for review, (5)). Beside their direct antiviral properties, type I IFNs produced by pDC activate natural killer (NK) cells, macrophages, and CD11c+ myeloid (m)DCs to elicit anti-microbial/viral/tumour 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 non-infectious autoimmune/inflammatory pathologies (Lupus, Psoriasis) due to uncontrolled production of IFN-α following their chronic activation by self-nucleic acids (8, 9).

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

pDC are also involved in anti-tumor 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 which are associated with a poor prognosis (3), indicating that TATreg can expand in the tumor microenvironment.

In order to understand the negative impact of TApDC on BT patients’ outcome and its possible link with TATreg, we investigated herein, their functional competence within BT. We show that TApDC are impaired for their IFN-α production and consequently promote immune tolerance through TATreg expansion and differentiation of 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 BT 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 anticoagulant) 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, Lyon. 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 Table S1. All clinical and biological data related to breast cancer patients were collected prospectively and included in a regularly updated institutional database at CLB.

Isolation of pDC, naive and memory CD4+ T cells and in vitro generation of MoDC

BT 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 FACS-sorting. MoDC were obtained from blood-purified monocytes. Detailed methods are provided in SI Methods.

Stimulation of pDC

TApDC were cultured at 5x10^5 cells/ml in 96-well flat-bottomed plates in complete medium for 24 h in the presence of IL-3 (20 ng/ml), inactivated Flu virus (100 HAU/ml), CpG-A (5 µg/ml), CpG-B (5 µg/ml), and R848 (1 µg/ml). Healthy pDC were cultured in presence of IL-3 with or without breast TUMSN (25%) for 16 h prior to TLR activation. Cells and supernatants were harvested after 24 h and 40 h 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 co-cultures**

Allogeneic naive 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 pre-treated for 24 h with IL-3, GM-CSF (10 ng/ml), CpG-B, R848 or Flu or purified healthy pDC that were pre-incubated for 40 h in IL-3 with (TUMSN-pDC) or without BT supernatants, and TLR-L. T lymphocytes were added on pre-activated DC subsets (ratio 1:5) and co-cultured for 4 days in triplicate in 96-well round-bottomed plates. Proliferation was assessed by CFSE (Invitrogen) dilution in experiments analysing 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 DAPI exclusion or Live and Dead reagent (Invitrogen) in case of cell permeabilisation. 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 re-stimulated at 10⁵ cells/well in triplicate for 16 h with PMA (50 ng/ml) and ionomycin (2 µg/ml) (Sigma-Aldrich), while for other T/pDC cocultures, supernatants of co-culture 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 (TMA) 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 performed using mouse
anti-BDCA2 (clone 104C12, Dendritic) at 5 µg/ml incubated at 37°C for 30 min, revealed with biotinylated secondary antibody bound to streptavidin peroxidase conjugate (UltraView kit and Amplification kit, Ventana), and revealed with 3,3-diaminobenzidine (DAB; Dako) as substrate. Sections were counterstained with hematoxylin. The density of BDCA2+ cells was assessed semi-quantitatively 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’s t test or non-parametric Wilcoxon tests when appropriate. Comparison of percentages was done by χ² test. Correlation analysis of the data was performed using the Fischer’s exact test, Spearman test, or the Pearson test when appropriate. Data for cytokine production and T cell proliferation are expressed as mean ± SD. Percentages of inhibition of IFN-α production by pDC were compared using a non-parametric 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 BT

We previously reported that infiltration of primary BT by pDC identified as CD123⁺ cells by IHC correlates with poor prognosis, with a median follow-up of five years (4). We updated our clinical database with a median follow-up of twelve 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 BT patients and using flow cytometry, pDC and mDC were identified in primary BT 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±0.18% for pDC versus 0.04±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 ± 0.22%, n=48), when compared to healthy donors’ blood (mean pDC: 0.37 ± 0.19 %, n=48) (p=0.006) (Fig. 1A, right panel). These observations suggest preferential pDC recruitment within the tumor mass. Since the median follow-up of our prospective cohort was 43 months, there were not enough events to analyse 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±0.22 compared to 0.10±0.11, p=0.03) and triple negative (TN) (hormone-receptors and Her2-neu negative) BT (0.29±0.28 compared to 0.12±0.12, p=0.05) compared to 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 triple negative BT (TNBT) (n=25 tumors), compared to only 19% other tumors (n=162 tumors) (p=0.05). Importantly, such correlations were not observed for TAmDC (Table S1). Thus, using two different methods (flow-cytometry and IHC) to identify pDC in two independent cohorts, we showed that high numbers of TApDC correlated with tumor aggressiveness, strengthening our previous report on the deleterious impact of TApDC on BT 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 BT environment favours 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 to 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 since 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 BT (TUMSN) and six different breast cancer cell lines (BCCSN) prior to 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±14%, p<0.05 and 75±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 demonstrate that pDC are exposed to soluble factors in BT environment that inhibits their IFN-α production.

**BT environment conditions pDC to induce selective Treg expansion**

BDCA2+ TApDC mainly colocalized with CD3+ lymphoid aggregates in BT (Fig. 4 and Fig. S4A) compared to epithelial cytokeratin+ areas (42% vs 14% of tumors analyzed, n=20) (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 Fig. S5). Consistent with the acquisition of a mature phenotype, TLR-stimulated TApDC trigger potent naive 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, higher numbers of FoxP3+ TATreg infiltrate TNBT than non-TNBT (third quartile=61.8 compared to 26.6 FoxP3+ cells respectively). Importantly, we observed a significant positive correlation between TApDC and TATreg frequencies in TNBT (r²=0.749, p=0.002, n=14), and to a lower extent in non-TNBT (r²=0.413, p=0.004, n=48) (Fig. 5A). Such correlations i) were not as significant for TAMDC (r²=0.672, p=0.05 in TNBT and r²=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) while 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 BT (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), potently increased by 2.8 to 4.6 fold the percentage of FoxP3^{high} T cells (9.3±1.1%, 8.6±0.5%, and 7.3±0.5% respectively) among CD4^+CD45RO^+ T cells compared to absence of TUMSN (2% for IL-3 and 2.6±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 favour Treg expansion among CD4^+CD45RO^+ T cells (9 % vs 1.5% of CD3^+ T cells) (Fig. 6A). However, TUMSN-conditioned healthy pDC did not induce the differentiation of FoxP3^+ Treg from naive CD4^+ T cells (Supplementary Fig. S6). Thus, TApDC mainly favored Treg expansion rather than differentiation. Collectively, these observations demonstrate that BT environment amplified pDC unique capacity to stimulate Treg expansion.

**Exogenous IFN-α reverts immunosuppressive T cell responses induced by TApDC and BT environment**

We hypothesized that the defect in IFN-α production by TApDC’s could favour 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 vs 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 towards Th1 polarization (Fig. 6B). Collectively, these data indicate that TApDC capacity to promote immuno-suppressive 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 BT (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 BT. 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 demonstrate that TApDCs altered for their IFN-α production contributes to establish immune tolerance through Treg expansion leading to tumour progression and poor clinical outcome in breast cancer.

pDC are well recognized for their role in anti-viral 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 non-infectious auto-immune/inflammatory pathologies (8, 9). In contrast, in BT 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) tumours have been shown to express LL37 (25) and ii) endogenous danger signals such as self nucleic acids (26) are released from dying tumour cells, it is tempting to speculate that, upon [LL37/self nucleic acids] complexes recognition TApDC might contribute to tumour immunosurveillance through type I IFNs production. This is consistent with the partially activated phenotype of breast TApDC. Thus, the tumour has evolved mechanisms to inhibit type I IFNs secretion by TApDC to prevent an effective antitumour response and favour tumour progression. This hypothesis is currently under investigation but it is supported by recent works showing that i)
type I interferon 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 BT 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 BT environment cooperate to inhibit IFN-α secretion by TApDC (24). Our ongoing study is pointing to similar mechanisms in BT (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 BT environment are the immune cells and/or stromal cells (fibroblasts and mesenchymal stem cells) since breast cancer cell 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 BT 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).

While TApDCs’ innate functions are strongly impaired by BT, they exhibit similarly to ovarian cancers (24) exacerbated ability to induce allogeneic naive 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+ regulatory T cells in ovarian cancer (20), these findings suggest that pDC will favour 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 BT 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 (for review, (10)), by either promoting Treg or Tr1 cells or directly suppressing effector T cell responses. While in other contexts pDC-driven Treg generation from naive T cells has been reported both in vitro (12, 13, 35-38) and in vivo (39, 40), BT environment-conditioned pDC do not induce FoxP3+ Treg differentiation from naïve CD4+ T cells (Supplementary Fig. S6). These results provide evidence that TApDC may contribute to tumour progression through Treg expansion. This unique capacity of TApDC is due to ICOS/ICOS-L interaction between TApDC and TATreg respectively (Faget, manuscript in preparation and (41)).

Of most importance, the inhibition of type I IFNs production by TApDC is required for this tolerogenic TApDC function since 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 (42, 43). Thus, in vivo local interactions between IFN-α-deficient TApDC and TATcell ((3) and Faget, in preparation) 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 immuno-pathological 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 tumour aggressiveness as observed in TNBT strengthens our previous
observations showing that infiltration of primary BT 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, 44). 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 (45). Mechanisms beyond this predominant TApDC infiltration in TNBT is an important question that is currently under investigation. Of note, due to 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 TNBC.

In conclusion, we identify in human breast cancer a TApDC defect in IFN-α production leading to TATreg expansion and contributing to BT progression. These findings uncover the mechanisms that mediate the deleterious impact of pDC infiltration in BT and would provide new therapeutic approaches targeting pDC, as in skin cancers (22, 23, 46, 47). We recently obtained evidences in a mouse mammary tumours model that reactivation of TApDC leads to tumour regression and anti-tumour immune responses (Le Mercier et al., manuscript in preparation). Thus, restoring TApDC innate function might represent an attractive therapeutic strategy for localized BT.
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REFERENCES


FIGURE LEGENDS

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 panel). The frequencies of pDC and mDC within PBMC of breast cancer patients (n=48) and healthy donors (n=48) are indicated (right panel). ***P < 0.001, **P < 0.01, and *P < 0.05; paired Student’s t-test. (B) Box plot set of pDC levels determined as in (A), and (C) Percentages of breast cancer patients with low or high pDC numbers (the highest quartile >0.23% was used as cutoff value) among patients with BT with a mitotic index (MI) 1-2 vs 3 and with TNBT vs non-TNBT. MI was established by counting mitoses in 10 high power (400×) fields (HPF) per tumor section and then averaging the number by HPF (1.96mm²). Mitotic cell counts between 1 and 9, between 10 and 19, and more than 20 were defined as MI 1, MI2, and MI3 respectively. *P<0.05; non-parametric Wilcoxon test in (B) and Fisher’s exact test in (C).

Figure 2. **TApDC isolated from human primary BT display a partially activated phenotype.** (A) pDC from primary BT, 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. 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
Figure 3. Specific inhibition of IFN-α production by TApDC in response to TLR activation is mediated by soluble factors from BT environment. (A) Purified pDC from BT and healthy tonsil and (B) PBMC from breast cancer patients and healthy donors were cultured with IL-3, flu, and CpG-A for 24 h. IFN-α and IP-10/CXCL10 production was quantified by ELISA. Results were standardized to ng/ml for 1x10^5 pDC for each sample. Each symbol represents a single sample and mean values are represented by horizontal lines. ***P < 0.001; non-parametric Wilcoxon Test. (C) Healthy pDC were pretreated with IL-3 ± TUMSN (25%) derived from ten patients for 16 h before flu activation for another 24 h. IFN-α and IP-10/CXCL10 production was measured by ELISA. Data are expressed as mean ± SD, by subtracting the quantity of each cytokine detected in TUMSN alone with no pDC. Data are representative of more than four independent experiments, representing more than thirty TUMSN from individual patients. *P < 0.05; non-parametric Wilcoxon test.

Figure 4. TLR-activated TApDC acquire a fully mature phenotype and induce naive CD4^+ T cell proliferation and differentiation of IL-10-secreting T cells. (A) IHC analysis on paraffin-embedded BT sections was performed using anti-BDCA2, anti-CD3 or anti-FoxP3 (brown) (x40). (B) Activation phenotype of 40 h differentially stimulated TApDC and tonsil pDC was monitored by analyzing CD86 expression by flow cytometry. Data are representative of five independent experiments and MFI values of total cells are indicated in each histograms. (C) Purified tonsil pDC and TApDC were cultured with IL-3, flu, or CpG-B for 24 h and then incubated at graded doses with allogeneic naive CD4^+ T cells for 5 days as specified in Supplemental Material and methods. T cell proliferation was determined by ^3H
thymidine incorporation. Data are expressed as mean ± SD and are representative of three independent experiments. (D) Purified tonsil pDC and TApDC were cultured with IL-3 or CpG-B for 24 h and then incubated with allogeneic naive CD4⁺ T cells for 5 days as specified in Supplemental Material and methods. T cell cytokine production was determined in coculture supernatant by ELISA. Data are expressed as mean ± SD and representative of two independent experiments.

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’s exact test. (C) pDC, mDC, and MoDC were preactivated for 24 h with IL-3 or GM-CSF and R848, washed, and cocultured for five days with CFSE-labeled conventional memory CD4⁺CD25 Low/Neg/CD127⁻/⁺ T cells or CD4⁺CD25 high/CD127⁺ Treg in the absence of IL-2. At day 5, CFSE dilution was analysed on CD3⁺ T cells. Percentages of CFSE-diluted cells are indicated. (D) Healthy pDC were pretreated for 16 h with IL-3 with or without 25% TUMSN before activation or not with R848 or Flu for 24 h. Memory CD4⁺ T cells were then added with IL-2 (100 IU/ml) at 25% v/v for five days. Anti-CD3/anti-CD28 expandbeads® were used as control. FoxP3 expression was analysed on CD3⁺ viable cells.

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

BDCA, Blood Dendritic Cell Antigen; BT, breast tumor; ODN, oligodeoxynucleotide; pDC, plasmacytoid dendritic cells; TApDC, Tumor-Associated pDC; TATreg, Tumor-Associated Treg; TN, triple negative; TNBT, triple negative breast tumor; TLR, Toll-like receptor; TUMSN, tumor supernatant

Figure 1

A

% TApDC among total cells

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=48)</th>
<th>Breast Cancer (n=48)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood pDC</td>
<td>TApDC (n=52)</td>
<td>TAmDC (n=60)</td>
</tr>
</tbody>
</table>

B

% TApDC among total cells

<table>
<thead>
<tr>
<th>MI 1-2 (n=32)</th>
<th>MI 3 (n=28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-TNBT</td>
<td>TNBT</td>
</tr>
</tbody>
</table>

C

% Patients

<table>
<thead>
<tr>
<th>MI 1-2 (n=32)</th>
<th>MI 3 (n=28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-TNBT</td>
<td>TNBT</td>
</tr>
</tbody>
</table>

p=0.057

pDC≤0.23

pDC>0.23

Research.
**Figure 2**

(A) Histograms showing the cell count of different types of plasmacytoid dendritic cells (pDC) from patient's blood, healthy blood, and tonsil. The expression of BDCA2, CD40, CD86, and HLA-DR is indicated.

(B) Graphs illustrating the mean fluorescence intensity (MFI) of BDCA2, CD40, CD86, HLA-DR, and CD83 in blood and primary tumor samples.

*Statistical significance: **p < 0.01, ***p < 0.001, *p < 0.05*
Figure 3

A

IFN-α (ng/ml/10^5 pDC)

IL-3  Flu  CpG-A

IFN-α (ng/ml/10^5 pDC)

IP-10/CXCL10 (ng/ml/10^5 pDC)

IL-3  Flu  CpG-A

B

IFN-α (ng/ml/10^5 pDC among PBMC)

IL-3  Flu  CpG-A

Patient blood pDC
Healthy blood pDC

C

IFN-α (ng/ml)

IL-3 + Flu

IP-10/CXCL10 (ng/ml)

IL-3 + Flu
Figure 4

A

BDCA2
CD3
FoxP3

BT#1

BT#2

B

before activation

IL-3
IL-3+Flu
IL-3+R848
IL-3+CpG-A
IL-3+CpG-B

Cell count

CD86

TApDC

Tonsil pDC

C

TApDC

Tonsil pDC

H thymidine incorporation (cpm x 10^3)

0/50,000
1/50,000
1/1,000
1/10,000
1/100,000
1/1,000
1/10

Ratio pDC/Naive CD4^+ T cells

D

IL-10 (ng/ml)

IFN-γ (ng/ml)

Black

TApDC

IL-3
IL-3+CpG-B

White

Tonsil pDC

IL-3
IL-3+CpG-B
Figure 5

A

Non-TNBT

\( r^2 = 0.413, p = 0.004 \)

TNBT

\( r^2 = 0.749, p = 0.002 \)

B

\[ \begin{array}{cc}
\text{Treg}^+ & \text{Treg}^{\text{neg}} \\
\text{Non-TNBT} & \text{TNBT} \\
\end{array} \]

\( ** \)

\( * \)

% patients

% TApDC

Non-TNBT

TNBT

C

No DC

pDC\(^{R848}\)

mDC\(^{R848}\)

MoDC\(^{R848}\)

Treg

Tconv

CFSE

D

FoxP3\(^{\text{high}} \) (% among CD3\(^+\))

medium

pDC\(^{-}\) No TRL

pDC\(^{R848}\)

pDC Flu
Figure 6

A

B

IL-2
IL-2 + IFN-α

No pDC
medium
TUMSN
TApDC

0.1
1.5
7.7
9

0
0.9
4.8
1.9

FoxP3

IFN-γ (pg/ml)

No pDC TApDC

IL-10 (pg/ml)

Medium
IFN-α 1000 UI/ml
Impaired IFN-α production by Plasmacytoid dendritic cells favors regulatory T cell expansion and contributes to breast cancer progression

Vanja Sisirak, Julien Faget, Michael Gobert, et al.

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