ICOS-ligand expression on plasmacytoid dendritic cells supports breast cancer progression by promoting the accumulation of immunosuppressive CD4+ T cells

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Abstract

Human breast tumors are infiltrated by memory CD4⁺ T cells along with increased numbers of regulatory T cells (Treg) and plasmacytoid dendritic cells (pDC) that facilitate immune escape and correlate with poor prognosis. Here we report that ICOS, a T cell co-stimulatory molecule of the CTLA4/PD1/CD28 family, is expressed mostly by tumor-associated Treg in primary breast tumors. A large proportion of these ICOS⁺ Treg were Ki67⁺ and this evident proliferative expansion was found relied upon interactions with tumor-associated pDC. Indeed tumor-associated Treg highly expanded in presence of pDC but failed to proliferate under CD3/CD28 signal. In vitro experiments revealed that addition of a neutralizing anti-ICOS antibody blocked pDC-induced Treg expansion and IL-10 secretion by memory CD4⁺ T cells, establishing a pivotal role for ICOS in this process. Supporting these findings, the presence of ICOS⁺ cells in clinical specimens of breast cancer correlated with a poor prognosis.

Together, our results highlight an important relationship between Treg and pDC in breast tumors, and demonstrate that ICOS/ICOS-L interaction is a central event in immunosuppression of tumor-associated memory CD4⁺ T cells.

These findings strongly rationalize antibody-mediated ICOS blockade as a powerful clinical strategy to correct immune escape and promote therapeutic responses in breast cancer.

Precis:

Within breast tumors, interactions between the ICOS receptor on infiltrating T cells with the ICOS ligand on infiltrating plasmacytoid dendritic cells is a pivotal determinant of immune escape and strongly rationalized as a therapeutic target to combine with other treatments.
Introduction

In several cancers, tumor growth and dissemination are associated with perversion of the immune system (1). We and others have shown that primary breast tumor (BT)-associated T cells (TA-T), are highly enriched in CD25^{hi}CD127^{low}FoxP3^{hi}CD4^{+} regulatory T cells (Treg) (2) and that high infiltration of TA-Treg in BT, and more particularly within lymphoid aggregates surrounding tumor correlates with poor prognosis (2). In the past decade, several studies highlighted the negative impact of TA-Treg on Th1 and cytotoxic T lymphocytes mediated anti-tumor immunity (3). Therapeutic targeting of Treg is clearly beneficial in mice as demonstrated in multiple models based on anti-CD25 depletion (for review (3, 4)) or FoxP3 deletion (3, 5). A similar approach developed in human using IL-2-toxin (Denileukin diftitox) (6) showed low efficacy possibly due to its limited specificity and the existence of toxicity. In the objective to increase T cell anti-tumor immunity, early clinical trial with the anti-CTLA4 mAb, recently approved in the treatment of melanoma, and anti-PD-1/PD-L1 demonstrated remarkable long-lasting clinical benefit (7, 8). However, contribution of Treg neutralization in the clinical efficacy of anti-CTLA4 mAb remains uncertain (9, 10). Thus, there is an urgent need of alternative therapeutic strategies to selectively neutralize TA-Treg. A first step along this objective is the understanding of the immunosuppressive networks operating in the BT microenvironment. We therefore undertook to characterize the mechanisms controlling TA-Treg enrichment within BT.

We recently published that human TA-Treg recruitment in BT from blood occurred through CCR4/CCL22 axis (2, 11). BT TA-Treg are strongly activated, proliferate, and express high levels of the inducible co-stimulatory (ICOS) molecule (2) suggesting the local recognition of a tumor-associated antigen, likely through presentation by a DC population. The presence of TA-pDC within tumors has been correlated with poor
prognosis in BT (12), ovarian carcinoma (OC) (13), and melanoma (14). Interestingly, pDC have been reported to strongly favor Treg proliferation in human (15) and rodent models (16). Furthermore, TA-pDC are impaired in their major function of high IFNα secretion levels in response to TLR7/9-L in ovarian (13) and breast cancer (17), and we recently demonstrated that such functional defect strongly favors TA-pDC ability to induce TA-Treg proliferation and increases T-CD4+ IL-10 secretion (17), leading to the establishment of an immunosuppressive Tr1-like response.

Identified in 1999 (18), ICOS, a T cell co-stimulatory molecule of the CTLA4/PD1/CD28 family, plays a non-overlapping function with CD28 (19) on CD4+ T cells. In particular ICOS is critical in the regulation of humoral response (20) through its role on T follicular helper cell activation as illustrated in ICOS KO mice (21) and deficient patients (22). ICOS expression has also been linked with Treg maintenance in mice (23) and mucosal tolerance (24). In human ICOS plays a predominant role in pDC/T cell interaction (25) and participates in naïve CD4+ T cell polarization into IL-10-secreting Tr1-like cells (26). ICOS was also associated with Treg homeostasis in NOD mice (27) and with increased Treg proliferative capacity and immunosuppressive functions (28). All these observations led us to decipher the contribution of ICOS in the establishment of tolerance through TA-Treg amplification in BT mediated by TA-pDC.

Using a new blocking mAb against ICOS (clone 314.8), we demonstrated that ICOS blockade inhibited pDC-induced TA-Treg proliferation and reduced IL-10 secretion by memory TA-TCD4+ without interfering with mDC-induced TA-TCD4+ activation. Finally, we demonstrated that the presence of ICOS+ cells within primary BT correlated with poor patients’ survival. Thus, supported by the clinical efficacy of anti-
CTLA4 and anti-PD-1/PD-L1 (7, 8, 29), a neutralizing anti-ICOS mAb would represent a potent therapeutic drug to neutralize Treg in breast cancers.
Materials and Methods

Tumor single cell suspensions, CD4+ T cells and pDC isolation

Primary BT samples and blood in CTAD-coated tubes were obtained from non pre-treated patients diagnosed for primary breast carcinoma provided by the Centre Léon Bérard (CLB) tissue bank after written informed consent in accordance with the Declaration of Helsinki. The study was reviewed and approved by the Institutional Review Board of CLB. Human tonsils were obtained anonymously according to the institutional regulations and healthy donor (HD) human blood was purchased anonymously from the EFS (Lyon, France).

Isolation of T cells and DC from tumor and HD tissues

Tumor and tonsil single cell suspensions were generated as previously described (2). HD or patients’ blood mononuclear cells (MNC) were obtained by Ficoll density gradient. Viable HD-pDC (Lin-CD4+CD11c+) and HD-mDC (Lin-CD4+CD11c+) were FACS-sorted from tonsil samples as described previously (13).

TA-TCD4+ were positively isolated based on their CD4 expression (Life Technologies). HD-TCD4+ were obtained as untouched memory CD4+ T cells by depletion. Treg and Tconv (defined as non-Treg memory T-CD4+) from blood and primary BT were obtained using the CD25+ isolation kit (Life Technologies) or by FACS-sorting on the basis of a DAPI,CD4,CD25,CD127 stainings on memory HD-TCD4+.

Allogeneic DC/ T cell co-cultures and anti-CD3/anti-CD28-coated beads T cells activation

PDC and mDC were pre-activated for 24h in presence of IL-3 (Peprotech, 20ng/ml) ± GM-CSF (Schering Plough, 10ng/ml) with R848 (Invivogen, 5μg/ml) in RPMI
medium supplemented with antibiotics, L-glutamin (Life Technologies), and 10 % FCS (PAA). Activated DC were washed before co-culture with T cells.

Co-cultures were performed at a DC:T cells ratio (1:5) for 5 days with or without exogenous rhIL-2 (Pro-Immune) in presence of IL-3 (20ng/ml) ± GM-CSF (10ng/ml) in RPMI containing 5% AB human pooled serum (EFS). In some experiments, TCD4+ were labeled with CFSE (1µM) or Cell trace violet (10µM) (Life Technologies) prior co-culture. In blocking experiment mAbs anti-ICOS (clone 314.8), anti-CD28 (clone CD28.2) or isotype control were used at a concentration of 10µg/ml.

**Patient cohort**

For the retrospective IHC study, tumors from 120 patients with invasive non-metastatic BC whose clinical and biological data were available from the regularly updated CLB database were analyzed. Patients’ characteristics are presented in Table S1 (column 1). The median follow up was 11 years (CI 95% [10.6 - 11.1]). Majority of the patients (96%) were treated with post-operative radiotherapy. 80% patients received adjuvant hormonotherapy (tamoxifen, (91.7%), anti-aromatase (2.1%) or combination (6.3%)). Adjuvant chemotherapy, consisting in anthracyclins (69.4%), anthracyclin+Taxan (26.4%) or other treatments (4.2%), was administered to 59.2% patients (71/120).

**Immuno-histochemistry**

Frozen BT sections (5 µm) were subjected to sequential double IHC analyses with mAbs against BDCA-2 (10µg/ml), FoxP3 (10µg/ml), and cytokeratin (1/50) using ImmPRESS anti-mouse Ig peroxidase kit (Abcys), as previously described (2). After antigenic retrieval by CC1 buffer pH8 (Ventana), BT paraffin embedded tissue microarray (TMA) were incubated for 30min with Anti-ICOS mAb (1:50, Spring Biosciences), then revealed using Ultra View Kit and Hematoxylin-counterstained
(Ventana). Each BT sample was analyzed independently by two pathologists according to the guidelines for HER2/neu amplification, ER/PgR positivity, SBR, and TNM grade. ICOS$^+$ cells were enumerated on six different spots on each tumor sample to obtain ICOS$^+$ cell count (average). Cohort was equally separated in two groups according to the median of their ICOS$^+$ cells number (ICOS$^{\text{low/neg}}$ (< 1.7 cells/spot) and ICOS$^+$ (≥ 1.7 cells/spot)) to have sufficient number of patients in each group for statistical analyses.

**Flow cytometry and cytokine quantification**

All flow cytometry acquisitions were done using Cyan ADP cytometer (Beckman Coulter) and Summit 4.3 Software and analyses were performed on FlowJo 7 Software. FACS-cell sorting had been performed with the Aria III cell-sorter (Becton Dickinson) and DIVA Software. See supplementary Table 1 for antibody list.

Cytokines (IL-10, IFN$\gamma$ and IL-2) secretions were quantified in supernatants by specific ELISA according to manufacturers’ instructions (Bender MedSystems).

**Statistical analysis**

All statistical analyses were done using the Statistical SPSS 12.0 software. Correlations between clinico-biologic data and ICOS$^+$ cell content in the lymphoid infiltrates were determined using a $\chi^2$ test or a Fisher exact test. Survival curves were plotted using the Kaplan-Meier method and compared using the Log-Rank test.
Results

CD4+FoxP3 hi TA-Treg is the major T cell subset in BT expressing ICOS and proliferating in situ

Within BT, a large majority of TA-Treg (defined as CD25 hiCD127-) expressed high ICOS levels (69.9%) compared to TA-Tconv (23.4%) or TA-TCD8+ (2%) (Fig. 1A). As TA-TCD4+ are of memory phenotype (2), all the present study has been performed on CD45RO+ T cells (TCD4+) isolated from BT or healthy donors. Only weak or no ICOS expression was observed on HD and patients' blood Treg (21.3% and 16.6% respectively), Tconv (3.9% and 1.5% respectively) or T-CD8+ (0.3% and no expression respectively) (Fig. 1A), suggesting that high expression of ICOS on TA-Treg is dependent on tumor environment. Statistical analysis on eleven BT samples demonstrated a higher percentage of ICOS+ cells and a higher MFI of ICOS expression on TA-Treg compared to TA-Tconv (%: p<0.001 and MFI: p<0.01). The percentage of ICOS+ cells was lower in TA-TCD8+ than in TA-Tconv (p<0.05) (Fig. 1B). To evaluate a potential association of ICOS with TA-Treg proliferation, we analyzed the co-expression of ICOS and Ki67 on TA-Tconv and TA-Treg but also on patients’ and HD blood. Within TA-TCD4+ containing 11.1% of FoxP3 hi TA-Treg, 27.6% TA-Treg expressed Ki67 and 80% of them were ICOS+ (22.1% of ICOS+Ki67+ cells) whereas only 6.8% TA-Tconv were found Ki67+ containing 57% of ICOS+ cells (3.9% of ICOS+Ki67+ cells) (Fig. 1C). Only 5.7% Ki67+ cells that weakly or did not express ICOS and FoxP3 were detected in TCD8+ compartment (Fig. S1A). Interestingly, the percentage of Ki67+ cells was higher (p<0.001) among FoxP3 hi TA-Treg than other TA-TCD4+ or TA-TCD8+ on 11 different BT samples (Fig. 1D). Thus, ICOS is selectively expressed on FoxP3 hi Treg in tumor and is associated with their proliferation. The very low expression of Ki67 observed on TA-Tconv suggests that
most of them are in an anergic state consistent with their low expression of activation marker and probably linked to \textit{in situ} Treg-mediated immuno-suppression.

**TA-pDC interacting with TA-Treg \textit{in situ} in BT induce a strong TA-Treg enrichment among TA-TCD4$^+$ \textit{ex vivo}.

To decipher the mechanisms controlling the strong proliferation of TA-Treg \textit{in vivo}, we first compared the proliferative capacity of purified Treg and Tconv from HD blood and BT patients in response to co-stimulation using anti-CD3/anti-CD28-coated beads in the presence of high doses of rhIL-2 (Fig. 2A). Contrasting to the \textit{in vivo} observations, TA-Tconv proliferated as well as HD blood Tconv or Treg, whereas isolated TA-Treg did not proliferate, suggesting that other signals from the BT microenvironment are involved in TA-Treg \textit{in vivo} proliferation.

Through IHC stainings performed on BT frozen sections, we observed FoxP3$^+$ TA-Treg localized in close contact with BDCA2$^+$ TA-pDC within lymphoid aggregates but not in cytokeratin$^+$ tumor area (Fig. 2B), in agreement with our previous work demonstrating only scarce Treg within tumor area (2). We thus investigated the impact of pDC on TA-Treg proliferation among isolated TA-TCD4$^+$, in absence of exogenous cytokines as TA-Tconv can secrete IL-2 favoring TA-Treg proliferation. Whereas anti-CD3/anti-CD28 stimulation was unable to favor TA-Treg maintenance and proliferation (only 1% FoxP3$^{hi}$ cells after CD3/CD28 activation while starting TA-TCD4$^+$ population contained 7.5% FoxP3$^{hi}$ cells) (Fig. 2C), 10% FoxP3$^{hi}$ TA-Treg were detected among TA-TCD4$^+$ cultured with allogeneic TLR7-L-activated pDC. These results demonstrated that activated pDC favored TA-Treg enrichment and maintenance among TA-TCD4$^+$. Importantly, CFSE staining of TA-TCD4$^+$ showed a higher proliferation of FoxP3$^{hi}$ and FoxP3$^{int}$ cells in pDC/TA-TCD4$^+$ co-culture than
under anti-CD3/anti-CD28 stimulation (Fig. 2C). In contrast, a reduced TA-Tconv proliferation was observed with pDC compared to anti-CD3/anti-CD28 stimulation. FoxP3hi Treg amplified in HD-pDC/HD-TCD4+ co-culture do not produce cytokines as shown by IFNγ, and IL-10 staining on PMA/Ionomycine reactivated HD-TCD4+ after five days of co-culture (Fig. S2A). Furthermore, 10 days culture of FACS-sorted HD-Treg with pDC gave rise to immunosuppressive cells reducing by 40% proliferation of allogeneic HD-TCD4+ at the ratio 1:1 (Fig. S2B) and a strongly reduced IL-2 concentration in culture supernatants, directly dependent on the Treg proportion (Fig. S2C). Altogether these results are consistent with the ability of pDC to sustain Treg immunosuppressive functions.

Collectively these data demonstrated that in contrast to CD3/CD28 co-stimulation, TLR7-L-activated HD-pDC induced and maintained TA-Treg enrichment among TA-TCD4+. Such enrichment resulted at least in part from a higher proliferation of FoxP3hi TA-Treg, rising the hypothesis that another co-stimulatory signal different from CD28 required for TA-Treg expansion is delivered during pDC/TA-TCD4+ interaction.

**TA-pDC and TA-Treg are found in close vicinity in tumor mass consistent with ICOS/ICOS-L interaction**

Activated pDC are known to express high ICOS-L levels (25, 26) and we previously described a partly activated status of TA-pDC within BT environment (17). Consistently, TA-pDC expressed higher CD80 and CD40 levels than blood pDC and a similar up-regulation was observed on TA-mDC. Contrasting to such activated phenotype, ICOS-L was undetectable on freshly isolated TA-pDC whereas HD or patients’ blood pDC expressed ICOS-L upon isolation and upregulated levels upon activation (Fig. 3A & S3A). ICOS-L may be down-regulated on TA-pDC membrane
after engagement with ICOS highly expressed by TA-Treg. In line with this, ICOS-L expression on TLR7-L activated HD-pDC was prevented in the presence of high numbers of allogeneic activated blood HD-TCD4⁺ and this blockade was reverted in presence of a neutralizing anti-ICOS mAb (Fig. 3B). To confirm the relevance in BT, ICOS-L expression was analyzed, on TA-pDC and TA-mDC, after 48 h culture of whole tumor cell suspension in IL-3 favoring TA-pDC survival and the impact of blocking ICOS was assessed. Interestingly, TA-pDC highly expressed CD86 in all culture conditions (Fig. S3B) but ICOS-L was detectable at high level on TA-pDC only after culture with neutralizing anti-ICOS mAb and not with anti-CD28 mAb (Fig. 3C). As reported previously (26), high expression of ICOS-L was restricted to HD-pDC as ICOS-L was only marginally detected on mDC from HD blood or BT (Fig. 3A), even in the presence of TLR7-L (Fig. S3A) or anti-ICOS mAb (Fig. 3C) while they strongly expressed CD86 (Fig. S3B).

Collectively, these observations strongly suggest that ICOS/ICOS-L is involved in TA-Treg and TA-pDC interaction in BT leading to the down-regulation of ICOS-L expression on TA-pDC.

**pDC are strong inducers of Treg enrichment among CD4⁺ T cells through ICOS/ICOS-L co-stimulation.**

We analyzed the impact of ICOS blockade in HD-TCD4⁺ allogeneic reactions either with HD-pDC or HD-mDC. HD-Treg enrichment occurred with IL3+TLR7-L-pre-activated HD-pDC (13.8±2.4% of FoxP3ʰⁱ HD-TCD4⁺, compared to 4.5%±0.25% at day 5 with HD-mDC) (Fig. 4A and 4B). In HD-TCD4⁺/HD-pDC co-cultures in absence of exogenous rhIL-2, ICOS inhibition reduced by 42% FoxP3ʰⁱ Treg proliferation induced by HD-pDC (division index = 2.29±0.21 and 1.33±0.24 respectively in presence of control and anti-ICOS mAb) and did not impact HD-Tconv
proliferation. HD-Treg and HD-Tconv proliferation induced by mDC was also not affected by anti-ICOS mAb (Fig. S4A & S4B). To assess the impact of ICOS/ICOS-L neutralization on isolated Treg proliferation, CFSE labeled HD-Treg or HD-Tconv purified from blood were cultured with TLR7-L+IL-3-pre-activated HD-pDC purified from tonsil with exogenous rhIL-2 and neutralizing anti-ICOS mAb. HD-pDC induced proliferation of allogeneic FoxP3^{hi} cells (23.3% of diluted CFSE FoxP3^{hi} cells in presence of control mAb) that is almost completely blocked by anti-ICOS mAb (2.8%). In contrast, the anti-ICOS mAb decreased only moderately the proportion of proliferating HD-Tconv (78.1% and 52.2% in control and anti-ICOS mAb respectively) (Fig. 4C). Interestingly ICOS neutralization in HD-Treg/HD-pDC co-culture decreased the proportion of FoxP3^{hi} Treg from 19±2.4% to 3.3±1% (6 fold, (Fig. 4D) while the proliferation of HD-Tconv was only moderately affected.

The efficacy of anti-ICOS mAb to reduce Treg proportion was evaluated on TA-TCD4^{+}, even in the presence of exogenous rhIL-2. In accordance with results on HD-TCD4^{+}, the FoxP3^{hi} sub-population induced by HD-pDC decreased in the presence of anti-ICOS mAb (13.5±2% vs 4±1% in control and anti-ICOS mAb respectively) (Fig. 5A & 5B). In reverse experiments, purified IL-3±TLR7-L pre-activated TA-pDC favored a strong Treg enrichment among HD-TCD4^{+} (containing initially 1.7% of FoxP3^{hi} Treg) at day 5 (7.1 and 6.4 fold increase with IL-3 and IL-3+TLR7-L-pre-activated TA-pDC respectively). As expected, such Treg enrichment was strongly reduced by ICOS neutralization (57-83% reduction in FoxP3^{hi} cells compared to control condition (Fig. 5C & 5D). Taken together these results demonstrated the critical role of ICOS/ICOS-L interaction in TA-pDC-mediated TA-Treg amplification in BT.
ICOS neutralization inhibits pDC-induced IL-10 secretion by T-CD4⁺

We evaluated IL-10 and IFNγ production by HD-Tconv and HD-Treg co-cultured with pre-activated pDC. In HD-pDC/HD-Tconv co-culture with exogenous rhIL-2, ICOS blockade strongly inhibited IL-10 (by 94%) and in a smaller proportion, IFNγ secretion (by 33%) (Fig. 6A) whereas no IL-10 and IFNγ were detectable in co-cultures with HD-Treg (Fig. 6A). ICOS blockade strongly reduced IL-10 production in TA-TCD4⁺/HD-pDC (90% inhibition) (Fig. 6B, left panel) as well as in HD-TCD4⁺/TA-pDC (83–72% inhibition) co-cultures (Fig. 6B, right panel). IFNγ secretion was also significantly, but to a lesser extent, inhibited upon ICOS neutralization in all culture conditions (59% to 63%). Consistent with the strong IL-10 secretion induced by pDC, we detected high IL-10 levels in 48h-culture supernatants of BT single cell suspensions (Fig. S5) in 7/13 tumors tested (median = 646.8 pg/ml, range [3.2 – 6915] pg/ml) while IL-2, IL-17, and IFNγ were never observed.

Finally, we investigated the impact of ICOS blockade on mDC, pDC or mDC+pDC/T cell co-cultures. In presence of exogenous rhIL-2 HD-TCD4⁺ co-cultured with HD-pDC produced more IL-10 but less IFNγ than with HD-mDC (respectively 596±103pg/ml and 181±28pg/ml for IL-10 and 279±52pg/ml and 641±9pg/ml for IFNγ) (Fig. 6C). Culture of HD-TCD4⁺ with mixed DC subsets resulted in lower IL-10 (232±9pg/ml) but higher IFNγ secretion (574±80pg/ml) compared to [pDC+TCD4⁺] co-cultures. ICOS neutralization strongly reduced IL-10 levels (82% for pDC, 50% for mDC and 62% for pDC+mDC) without significantly interfering with IFNγ secretion (+28% for pDC, -19% for mDC and -24% for pDC+mDC) contrasting with observations done with cells from BT origin (Fig. 6C). In absence of exogenous rhIL-2, except the lower levels of IL-10 and IFNγ, similar effects of ICOS neutralization
were observed. Furthermore, ICOS blockade increased IL-2 concentration in coculture with HD-pDC (p=0.035, Fig. 6D).

Overall these results demonstrated that ICOS blockade leads to a strong inhibition of pDC-induced IL-10 secretion by HD-TCD4⁺ that is accompanied by increased levels of IL-2, without significantly affecting IFNγ production in particular in response to mDC.

**Detection of ICOS⁺ cells within primary BT correlates with poor prognosis**

IHC analyses were performed with anti-ICOS mAb on paraffin-embedded tumor section using TMA cores specific for tumor area or lymphoid infiltrates. High numbers (the median defined as cutoff = 1.7 cells/spot) of ICOS⁺ cells were detectable within lymphoid aggregates but not within tumor bed (Fig. 7A-C). The presence of ICOS⁺ cells within lymphoid infiltrates (≥ median ICOS⁺ cells/spot) was significantly correlated with high SBR grade (p=0.001), luminal A/B molecular subtypes (p<0.001), absence of ER expression (p=0.025) and Her2/neu overexpression (p=0.017) by tumor cells, triple negative status (p=0.02), and lymphatic emboles (p<0.001) (Table S1). Importantly, in univariate analysis the presence of ICOS⁺ cells in lymphoid-enriched areas was associated with an increased risk of relapse (PFS, Log rank p-value=0.0285) (Fig. 7D) and death (OS, Log rank p-value=0.0465) (Fig. 7E). However, when introduced in a multivariate analysis together with other significant clinical and biological parameters, high ICOS expression was no more significant.
Discussion

In addition to our previous reports demonstrating that TA-Treg frequency within tumor lymphoid infiltrates correlates with poor prognosis in primary BT (2, 30), we identify herein ICOS engagement as a major pathway contributing to their local accumulation through direct interaction with TA-pDC.

We confirm that TA-Treg within primary BT express high ICOS and Ki67 levels in a specific manner compared to other TA-TCD3+ subsets or blood Treg. Interestingly TA-Tconv and TA-TCD8+ contain a low minority of Ki67+ cells consistent with observations that Treg suppress Th1 and CD8+ T cell cytotoxic activity in the context of human tumors, such as colon carcinoma (31), melanoma (9), and OC (32). Early clinical trial with mAbs neutralising CTLA4 or PD-1/PDL1 inhibitory receptors are showing promising clinical activity in melanoma, renal and lung carcinoma (20-30% of objective responses) (7-9, 33).

Herein we report the major role of ICOS, a member of the CTLA4/PD1/CD28 family, in TA-Treg proliferation and accumulation in BT. First, we accumulate evidences that strongly support the hypothesis of ICOS+ TA-Treg and TA-pDC interaction in situ interaction by IHC on primary BT frozen sections and ICOS/ICOS-L engagement in BT as illustrated by reversion of ICOS-L down-regulation on TA-pDC upon ex vivo culture of whole tumor cell suspension in presence of blocking anti-ICOS mAb. Secondly, we also demonstrate that activated-pDC expressing ICOS-L, but not mDC or CD3/CD28 co-stimulation, favor allogeneic FoxP3hi TA-Treg enrichment among TA-TCD4+ that is abolished by ICOS blockade in agreement with data recently published in ovarian carcinoma (34). In agreement with this, thymic ICOS+ Treg needs ICOS/ICOS-L co-stimulation to proliferate (15). Nevertheless, others
molecules present in tumor environment like IDO, OX40-L (35), TNFα (36) or TGFβ (37) could also participate to TA-Treg enrichment within BT.

TA-pDC-induced FoxP3\textsuperscript{hi} Treg amplification explains the positive correlation observed between TA-pDC and TA-Treg in BT (Fig. S6A) as well as their negative impact on patients’ survival in BT (Fig. S6B, 17) and OC (13, 38). Furthermore, in contrast to recent works in melanoma (39) and glioblastoma (40), ICOS\textsuperscript{+} TA-Treg expansion in BT is not mediated through direct interaction with tumor cells as i) ICOS-L is not expressed either on primary BT cells or on BT cell lines (not shown) and ii) Ki67\textsuperscript{+} proliferating Treg are only detected within lymphoid aggregates and not within tumor nests (2).

To date TA-Treg origin is not fully elucidated. pDC are known to favor immunosuppressive T cell induction on both TCD4\textsuperscript{+} (15, 41) and TCD8\textsuperscript{+} (42) subsets and we cannot formally exclude the differentiation of Treg from naive TCD4\textsuperscript{+}, as previously reported for both human and mouse pDC (41, 43, 44). However, we recently demonstrated i) a specific recruitment of CCR4\textsuperscript{+} Treg from the blood to BT through the tumor cells CCL22 production (2, 11), and ii) that all of TA-TCD4\textsuperscript{+} are of memory phenotype, consistent with a recruitment of TA-Treg from the periphery followed by their local expansion through ICOS/ICOS-L interaction with pDC in BT environment.

In accordance with results in NOD type 1 diabetes murine model (27), ICOS\textsuperscript{+} TA-Treg display high immunosuppressive function (2) through not yet characterized mechanisms that may involve FASL, CD39/Adenosine, perforin, CTLA4 or PD-1 known to participate in Treg immuno-suppressive functions (for review (4)).

High IL-10 levels are detected in BT environment and TA-TCD4\textsuperscript{+} secrete large amounts of IL-10 in co-culture with pDC but not with mDC. ICOS co-stimulation of
naive CD4+ T cells is already known to induce the differentiation of IL-10-secreting cells (26). Of importance, our results show that pDC preferentially activate pre-existing IL-10 secreting cells among memory HD-Tconv. Furthermore mDC-induced T cell responses are not affected by ICOS blockade as they do not over-express ICOS-L after activation. Interestingly, we notice that ICOS blockade leads to IL-2 accumulation in co-cultures with pDC suggesting that ICOS engagement favors the enrichment of TCD4+ subpopulation that do not secrete and/or consume high amounts of IL-2 in culture. This correlates with previous results (45) showing that T cell responses under ICOS stimulation depend on exogenous rhIL-2.

Of most importance, IHC staining on 120 primary BT with more than 10 years clinical follow-up allows to demonstrate that presence of high numbers of ICOS+ cells infiltrating primary BT is associated with poor prognosis in univariate analysis but is no more significant when introduced in the multivariate analysis together with other significant clinical and biological parameters.

Collectively our results suggest that abrogation of ICOS/ICOS-L interaction using neutralizing anti-ICOS mAb may reduce Treg expansion, IL-10 secretion and IL-2 consumption by TA-TCD4+ that can favor anti-tumor immunity through ICOS-independent TCD8+ and TCD4+ activation.

This role of ICOS in TA-Treg biology is supported by the fact that i) ICOS+ Treg have a stronger suppressive function in melanoma (46) and murine models (27, 28) and ii) ICOS deletion in human (22) and mice (23) correlates to a decreased Treg proportion.

On the other hand ICOS could be expressed on activated T cells (47) and ICOS expression is upregulated on IFNγ secreting T cells during anti-CTLA4 treatment in phase III trials in melanoma patients (for review (33)). However there is no evidence
in these clinical trials that ICOS contributes to anti-tumor immunity and ICOS may simply represent a T cell effector marker. This could suggest that treatment with neutralizing anti-ICOS mAb needs to be restricted to a short time period to abrogate Treg amplification without impacting on potential restoration of effector cells expressing ICOS. The treatment by the anti-ICOS 314.8 mAb may be particularly relevant in neo-adjuvant settings combined to therapies inducing antitumor immunity by favoring tumor cell death such as therapies targeting tumor molecular alterations (Herceptin, lapatinib) (48) or immunogenic chemotherapies (anthracyclins) (49).
Acknowledgements:
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**Figures legend**

**Figure 1:** TA-Treg but neither TA-Tconv nor Treg and Tconv from blood strongly co-express ICOS and Ki67.
(A) ICOS expression was analyzed on viable Treg (CD3^+^CD4^+^CD8^−^CD25^{hi}CD127^{low}), Tconv (CD3^+^CD4^+^CD8^−^CD25^{low/neg}) and TCD8^+^ (CD3^+^CD4^−^CD8^+) in HD blood, patients’ blood and BT single cells suspensions, one representative experiment out of five. (B) Dot plots represent percentage of ICOS^+^ cells among TA-Treg, TA-Tconv or TA-TCD8^+^ (top panel) and ICOS MFI of the total population (bottom panel). (C) Ki67 and ICOS expression were analyzed together by multicolor flow cytometry on BT single cell suspensions. T cell sub-populations were identified as in B. (B and D) Each symbol represents an independent donor (— = median). Statistical analysis were performed using a Mann-Whitney U-test ** p<0.01, * p<0.05.

**Figure 2:** TA-Treg are amplified in response to pDC but not under CD3/CD28 activation ex vivo.
(A) FACS sorted CD4^+^CD25^{hi}CD127^{−} Treg, and CD4^+^CD25^{−}CD127^{+} Tconv from HD blood or BT samples were cultured with anti-CD3/anti-CD28-coated beads in presence of rhIL-2 (500IU/ml). Numbers and viability of cells were quantified every 3 days. Results are representative of 3 independent HD blood and BT samples. (B) TA-pDC stained in brown (anti-BDCA2 mAb) are localized in lymphoid infiltrate in BT and not in tumor mass (anti-cytokeratin mAb) revealed in green (left panel). BDCA2^+^ cells (green) are in close contact with FoxP3^+^ Treg (brown) in BT (right panel). IHC stainings were done on BT frozen sections and observed with a 10 or 20 fold magnification (and 40x fold in insets). (C) Purified and CFSE labeled TA-TCD4^+^ encompassing 7.5% of FoxP3^{hi} TA-Treg after purification were co-cultured 5 days with R848-pre-activated HD-pDC or anti-CD3/anti-CD28-coated beads without
exogenous rhIL-2. FoxP3 detection and CFSE dilution were assessed on CD3+ viable cells by flow cytometry. Results are representative of 3 patient samples.

**Figure 3: ICOS-L engagement by ICOS during TA-pDC and TA-Treg interaction leads to ICOS-L down-regulation on pDC membrane.**

(A) BT single cell suspension or PBMC from HD blood were stained with Lin, CD4, CD11c and CD123 mAbs to identify TA-pDC (Lin–CD4+CD11c–CD123hi) and TA-mDC (Lin–CD4+CD11c–CD123neg). ICOS-L, CD40, HLA-DR or appropriate controls were used to assess expression of activation markers. Results are representative of 4 independent HD blood and tumor samples. (B) Freshly isolated and R848+IL-3-pre-activated HD-pDC were cultured for 48h with allogeneic HD-TCD4+ from blood at different pDC:Tcells ratio in the presence of control or anti-ICOS mAb (314.8) and analyzed for their expression of ICOS-L by flow cytometry. (C) After 48h culture period of BT single cell suspensions in presence of IL-3 20ng/ml and anti-ICOS 314.8, anti-hCD28 (CD28.2) mAb or isotype control, cells were then stained for ICOS-L to assess expression on Lin–CD4+CD11cnegCD123hiBDCA2+ TA-pDC or Lin–CD4+CD11c–CD123neg TA-mDC.

**Figure 4: HD-pDC induce a strong Treg enrichment among HD-TCD4+ compared to mDC under the dependence of ICOS signaling.**

(A & B) Overnight activated (IL-3+GM-CSF+R848) FACS-sorted tonsil HD-pDC and HD-mDC were co-cultured for 5 days with purified HD CD4+CD45RO+ T cells (HD-TCD4+) (containing 1.3% of FoxP3+ Treg at day 0). (C & D) CFSE labeled CD25hi HD-Treg and CD25neg HD-Tconv from blood were co-cultured with R848+IL-3-pre-activated HD-pDC in presence of rhIL-2 (100IU/ml) with anti-ICOS or control mAbs. At day 5, CFSE dilution and FoxP3 expression were analyzed by flow cytometry after gating on CD3+ viable cells. (B & D), histograms represent the frequency of FoxP3hi
Treg among viable HD-TCD3+ of one representative experiment from 4 blood samples.

**Figure 5: ICOS is predominant in Treg enrichment during pDC/CD4+ T cell co-culture with cells from BT origin**

(A & B) R848+IL-3-pre-activated HD-pDC were co-cultured with purified TA-TCD4+ cells (encompassing initially 11% FoxP3hi TA-Treg). (C & D) HD CD4+CD45RO+ T cells (HD-TCD4+) containing 1.7% FoxP3hi Treg after isolation were co-cultured with IL-3±R848 pre-activated TA-pDC. In A and B co-cultures were performed during 5 days in presence of rhIL-2 (100IU/ml) and anti-ICOS or control mAbs. Foxp3 expression was analyzed by flow cytometry on viable CD3+ cells, (B & D) histograms represent one out of 3 experiments for B and 2 experiments for D carried out in triplicate, error bars represent standard deviation.

**Figure 6: pDC but not mDC enhance IL-10 secretion and IL-2 consumption by memory TCD4+ though ICOS engagement**.

(A) IL-10 and IFNγ were quantified by ELISA in 5 days co-culture supernatants of IL-3+R848-pre-activated HD-pDC with FACS-sorted memory HD-Treg or HD-Tconv in presence of IL-2 (100IU/ml), anti-ICOS or control mAbs, one representative out of 3 independent experiments. (B) IL-10 and IFNγ were measured by ELISA in 5 days co-culture supernatants of purified TA-TCD4+ cells with IL-3+R848-pre-activated-HD-pDC (n=3), (left panel) or purified HD-TCD4+ with IL-3±R848-pre-activated TA-pDC in presence of rhIL-2 (100IU/ml) and anti-ICOS or control mAbs (n=2) (right panel). (C) HD-pDC and HD-mDC were pre-activated as in Fig. 4A and co-cultured with HD-TCD4+. When pDC and mDC were mixed at a ratio 1:1, the same number of total DC was co-cultured with T cells in presence of anti-ICOS or control mAbs, ± rhIL-2 (100IU/ml). After 5 days, supernatants were harvested and were quantified for IL-10.
and IFNγ by ELISA (n=4 independent experiments) (D) IL-2 secretion was measured by ELISA in supernatant of HD-TCD4⁺/HD-pDC co-cultures and anti ICOS or control mAbs. Histograms represent the results of 6 independent experiments.

**Figure 7:** High number of ICOS⁺ cells within primary BT correlates with reduced patients' survival.

(A) 120 paraffin-embedded primary BT samples with more than 10 years clinical follow-up were stained with anti-ICOS mAb and counterstained with hematoxylin. The number of positive cells was manually enumerated on 6 different cores for each tumor sample. Representative pictures of tumors non-infiltrated (A), weakly infiltrated (B), and highly infiltrated (C) by ICOS⁺ cells are shown (magnification x10). (C) Log-rank analysis of progression free (PFS) and overall (OS) survival of patients from the cohort according to the presence of ICOS⁺ cells (the median was chosen as cut-off ≥ 1.7 positive cells).
References


Figure 1

A

Healthy blood

Patients' blood

Tumor

Tconv  Treg  CD8+

B

ICOS+ among gated cells (%)

**  *

ICOS on gated cells (MFI)

*  NS

C

TA-CD4+ T cells

TA-Tconv  TA-Treg  TA-CD8+

D

Ki67+ cells among gated cells (%)

**  NS
Figure 2

A

Expansion (x)

TA-Treg

HD-Treg

TA-Tconv

HD-Tconv

Days

B

C

TA-TCD4^+

Total CD3^+

FoxP3^{Neg}

FoxP3^{Low}

FoxP3^{Hi}

HD-pDC

CD3/CD28

FoxP3

CFSE

FoXP3^{Hi} (%)
Figure 3

A

<table>
<thead>
<tr>
<th></th>
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<th>mDC</th>
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<tbody>
<tr>
<td>DH Blood</td>
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<tr>
<td>ICOS-L</td>
<td>MFI: 9.3</td>
<td>MFI: 10.6</td>
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<td>MFI: 2.75</td>
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<td>CD40</td>
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<tr>
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<td>MFI: 9.7</td>
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<td>CD40</td>
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- tested Ab
- Isotype Ctrl

B

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<tr>
<td>Anti-ICOS</td>
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</tbody>
</table>

- Isotype Ctrl
- HD-pDC
- HD-pDC + HD-TCD4+

C

Gated on TA-pDC

- Ctrl Ab
- Anti-ICOS
- Anti-CD28

Gated on TA-mDC

- Ctrl Ab
- Anti-ICOS
- Anti-CD28
Figure 4

A

HD-TCD4+T (no rhIL-2)

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<th>HD-pDC</th>
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B

HD-TCD4+T (no IL-2)

Bars show percent of CD3+ cells.

C

rhIL-2

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<tr>
<th>HD-Treg</th>
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D

HD-Tconv or HD-Treg + rhIL-2

Bars show percent of CD3+ cells.

Legend:
- Open square: HD-Tconv
- Filled square: HD-Treg
Figure 5

A. TA-TCD4+ + rhIL-2

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B. HD-pDC + rhIL-2 (100IU/ml)

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<td>FoxP3hi (%)</td>
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<tr>
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C. HD-TCD4+ rhIL-2

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<td>TA-pDCR848</td>
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D. HD-TCD4+ + rhIL-2

<table>
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<th>Anti-ICOS</th>
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<td>FoxP3hi (%)</td>
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<td></td>
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<td>10</td>
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</table>

Research.
Figure 6

A

B

HD-TCD4+ cells + rhIL-2

TA-TCD4+ cells + rhIL-2

HD-TCD4+ cells

No pDC

HD-pDC

HD-pDC

+ Anti-ICOS

D

HD-CD4+ + HD-pDC

Cytokine (ng/ml)

IL-10

IFNγ

HD-Treg + rhIL-2

HD-Tconv + rhIL-2

IgG1

Anti-ICOS

C

HD-TCD4+ + rhIL-2

HD-TCD4+ no rhIL-2

D

HD-CD4+ + HD-pDC

Cytokine (ng/ml)

IL-10

IFNγ

IgG1

Anti-ICOS

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Figure 7

A. ICOS neg

B. ICOS low

C. ICOS high

D. Log-rank p-value = 0.0465

E. Log-rank p-value = 0.0285

Cumulated survival vs OS (years)

Cumulated survival vs PFS (years)

+ censored

ICOS<med

ICOS>med
ICOS-ligand expression on plasmacytoid dendritic cells supports breast cancer progression by promoting the accumulation of immunosuppressive CD4+ T cells

Julien Faget, Nathalie Bendriss-Vermare, Michael Gobert, et al.

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