Microenvironment and Immunology

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 inducible costimulatory molecule (ICOS), a T cell costimulatory 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 to rely on 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 the addition of a neutralizing anti-ICOS antibody blocked pDC-induced Treg expansion and interleukin-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 show 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. Cancer Res; 72(23): 6130–41. ©2012 AACR.

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-associated T cells (TA-T), are highly enriched in CD25^+CD127^dimFoxP3^+CD4^ regulatory T cells (Treg; ref. 2) and that high infiltration of TA-Treg in breast tumor, 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 antitumor immunity (3). Therapeutic targeting of Treg is clearly beneficial in mice as shown in multiple models based on anti-CD25 depletion (for review refs. 3, 4) or FoxP3 deletion (3, 5). A similar approach developed in human using interleukin (IL)-2-toxin (Denileukin diftitox; ref. 6) showed low efficacy possibly because of its limited specificity and the existence of toxicity. In the objective to increase T cell antitumor immunity, early clinical trial with the anti-CTLA4 mAb, recently approved in the treatment of melanoma, and anti-PD-1/PD-L1 showed 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 breast tumor microenvironment. We therefore undertook to characterize the mechanisms controlling TA-Treg enrichment within breast tumor.

We recently published that human TA-Treg recruitment in breast tumor from blood occurred through CCR4/CCL22 axis (2, 11). Breast tumor TA-Treg are strongly activated, proliferate, and express high levels of the inducible costimulatory (ICOS) molecule (2), suggesting the local recognition of a tumor-associated antigen, likely through presentation by a dendritic cell (DC) population. The presence of TA-plasmacytoid DC (pDC) within tumors has been correlated with poor prognosis in breast tumor (12), ovarian carcinoma (13), and melanoma (14). Interestingly, pDC have been reported to strongly favor Treg proliferation in human (15) and rodent

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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-12-2409
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models (16). Furthermore, TA-pDC are impaired in their major function of high IFNγ secretion levels in response to TLR7/9-L in ovarian (13) and breast cancer (17), and we recently showed 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 costimulatory molecule of the CTLA4/PD1/CD28 family, plays a nonoverlapping 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 nonobese diabetic (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 breast tumor mediated by TA-pDC.

Using a new blocking mAb against ICOS (clone 314.8), we showed that ICOS blockade inhibited pDC-induced TA-Treg proliferation and reduced IL-10 secretion by memory TA-CD4+ without interfering with mDC-induced TA-CD4+ activation. Finally, we showed that the presence of ICOS+ cells within primary breast tumor 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 breast tumor samples and blood in anticoagulant (CTAD)-coated tubes were obtained from non-pretreated 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.

Isolation of T cells and DC from tumor and healthy donor tissues

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

TA-CD4+ were positively isolated based on their CD4 expression (Life Technologies). HD-CD4+ were obtained as untouched memory CD4+ T cells by depletion. Treg and Tconv (defined as non-Treg memory T-CD4+) from blood and primary breast tumor 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 cocultures and anti-CD3/anti-CD28-coated beads T cells activation

pDC and mDC were preactivated for 24 hours in presence of IL-3 (20 ng/mL; Peprotech) ± granulocyte macrophage colony-stimulating factor (GM-CSF; 10 ng/mL; Schering Plough) with R848 (5 μg/mL; Invivogen) in RPMI medium supplemented with antibiotics, L-glutamin (Life Technologies), and 10% fetal calf serum (PAAG). Activated DC were washed before coculture with T cells.

Cocultures were conducted at a DCT cells ratio (1:5) for 5 days with or without exogenous rhIL-2 (Pro-Immune) in presence of IL-3 (20 ng/mL) ± GM-CSF (10 ng/mL) in RPMI containing 5% AB human pooled serum (EFS). In some experiments, TCD4+ were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; 1 μmol/L) or CellTrace violet (10 μmol/L; Life Technologies) before coculture. In blocking experiment mAbs anti-ICOS (clone 314.8), anti-CD28 (clone CD28,2), or isotype control were used at a concentration of 10 μg/mL.

Patient cohort

For the retrospective immunohistochemistry (IHC) study, tumors from 120 patients with invasive nonmetastatic breast tumors whose clinical and biologic data were available from the regularly updated CLB database were analyzed. Patients’ characteristics are presented in Supplementary Table S1 (column 1). The median follow-up was 11 years [95% confidence interval (CI) 10.6–11.1]. Most of the patients (96%) were treated with postoperative radiotherapy. Note that 80% patients received adjuvant hormonotherapy [tamoxifen, (91.7%), antiaromatase (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).

IHC

Frozen breast tumor sections (5 μm) were subjected to sequential double IHC analyses with mAbs against BDCA-2 (10 μg/mL), FoxP3 (10 μg/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), breast tumor paraffin-embedded tissue microarray (TMA) were incubated for 30 minutes with Anti-ICOS mAb (1:50, Spring Biosciences), then revealed using Ultra View Kit and Hema3. Sequential double IHC analyses with mAbs against BDCA-2 (10 μg/mL), FoxP3 (10 μg/mL), and cytokeratin (1/50) using ImmPRESS Anti-Mouse Ig peroxidase kit (Abcys), as previously described (2).

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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 conducted on FlowJo 7 Software. FACS-cell sorting had been conducted with the Aria III cell-sorter (Becton Dickinson) and DIVA Software. See Supplementary Table S2 for antibody list.

Cytokines (IL-10, IFNγ, 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 SAS V9.2 package (Cay) 12.0 software. Correlations between clinicobiologic data and ICOS+ cell content in the lymphoid infiltrates were determined using a χ2 test or a Fisher exact test. Survival curves were plotted using the Kaplan–Meier method and compared using the Log-rank test.

Results
CD4+FoxP3hi TA-Treg is the major T-cell subset in breast tumor expressing ICOS and proliferating in situ
Within breast tumor, a large majority of TA-Treg (defined as CD45RO+CD4+CD27+) expressed high ICOS levels (69.9%) compared with TA-Tconv (23.4%) or TA-TCD8+ (2%; Fig. 1A). As TA-TCD4+ are of memory phenotype (2), all the present study has been conducted on CD45RO+ T cells (TCD4+) isolated from breast tumor or healthy donors. Only weak or no ICOS expression on TA-Treg compared with TA-Tconv (%: and MFI: 0.3% and 1.5%, respectively, or T-CD8+ (22.1% of ICOS+ and MFI: 0.3% and no expression, respectively; Fig. 1B). As TA-Tconv were found Ki67 expression on TA-Treg compared with TA-Tconv (%: < 0.001). 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 coexpression of ICOS and Ki67 on TA-Tconv and TA-Treg but also on patients’ and healthy donor blood. Within TA-TCD4+ containing 11.1% of FoxP3+ 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-TCD4+ 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 (Supplementary Fig. S1A). Interestingly, the percentage of Ki67+ cells was higher (P < 0.001) among FoxP3+ TA-Treg than other TA-TCD4+ or TA-TCD8+ on 11 different breast tumor samples (Fig. 1D). Thus, ICOS is selectively expressed on FoxP3+ 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 in vivo Treg-mediated immunosuppression.

TA-pDC interacting with TA-Treg in situ in breast tumor induce a strong TA-Treg enrichment among TA-TCD4+ ex vivo
To decipher the mechanisms controlling the strong proliferation of TA-Treg in vivo, we first compared the proliferative capacity of purified Treg and Tconv from healthy donor blood and breast tumor patients in response to costimulation using anti-CD3/anti-CD28-coated beads in the presence of high doses of rhIL-2 (Fig. 2A). Contrasting to the in vivo observations, TA-Tconv proliferated as well as healthy donor blood Tconv or Treg, whereas isolated TA-Treg did not proliferate, suggesting that other signals from the breast tumor microenvironment are involved in TA-Treg in vivo proliferation.

Through IHC stainings conducted on breast tumor 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% FoxP3hi cells after CD3/CD28 activation although starting TA-TCD4+ population contained 7.5% FoxP3hi cells; Fig. 2C). 10% FoxP3hi TA-Treg were detected among TA-TCD4+ cultured with allogeneic TLR7-L-activated pDC. These results showed that activated pDC favored TA-Treg enrichment and maintenance among TA-TCD4+. Importantly, CFSE staining of TA-TCD4+ showed a higher proliferation of FoxP3hi and FoxP3hi cells in pDC/TA-TCD4+ coculture than under anti-CD3/anti-CD28 stimulation (Fig. 2C). In contrast, a reduced TA-Tconv proliferation was observed with pDC compared with anti-CD3/anti-CD28 stimulation.

FoxP3hi Treg amplified in HD-pDC/HD-TCD4+ coculture do not produce cytokines as shown by IFNy, and IL-10 staining on PMA/ionomycin reactivated HD-TCD4+ after 5 days of coculture (Supplementary Fig. S2A). Furthermore, 10 days culture of FACS-sorted HD-Treg with pDC gave increase to immunosuppressive cells reducing by 40% proliferation of allogeneic HD-TCD4+ at the ratio 1:1 (Supplementary Fig. S2B) and a strongly reduced IL-2 concentration in culture supernatants, directly dependent on the Treg proportion (Supplementary Fig. S2C). Altogether, these results are consistent with the ability of pDC to sustain Treg immunosuppressive functions.

Collectively these data showed that in contrast to CD3/CD28 costimulation, 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 costimulatory signal different from CD28 required for Treg-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 breast tumor environment (17). Consistently, TA-pDC expressed higher CD80 and CD40 levels than blood pDC and a similar upregulation was observed on TA-mDC. Contrasting to such activated phenotype, ICOS-L was undetectable on freshly isolated TA-pDC whereas healthy donor or patients' blood pDC expressed ICOS-L upon isolation and upregulated levels upon activation (Fig. 3A and Supplementary Fig. S3A).

ICOS-L may be downregulated 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 breast tumor, ICOS-L expression was analyzed on TA-pDC and TA-mDC, after 48 hours 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.

**Figure 1.** TA-Treg but neither TA-Tconv nor Treg and Tconv from blood strongly coexpress ICOS and K67. A, ICOS expression was analyzed on viable Treg (CD3⁺ CD4⁺ CD8⁻ CD25⁻/CD127low), Tconv (CD3⁺ CD4⁺ CD8⁻ CD25low/neg), and TCD8⁺ (CD3⁺ CD4⁻ CD8⁺) in healthy donor blood, patients' blood, and breast tumor single cells suspensions, 1 representative experiment out of 5. B, dot plots represent percentage of ICOS⁺ cells among TA-Treg, TA-Tconv, or TA-TCD8⁺ (top) and ICOS MFI of the total population (bottom). C, K67 and ICOS expression were analyzed together by multicolor flow cytometry on breast tumor single cell suspensions. T-cell subpopulations were identified as in B. B and D, each symbol represents an independent donor (--, median). Statistical analysis was conducted using a Mann-Whitney U test. ***, P < 0.01; **, P < 0.05.

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enrichment occurred with IL3+TLR7-L-preactivated HD-pDC (13.8 ± 2.4% of FoxP3hi HD-TCD4⁺, compared with 4.5 ± 0.25% at day 5 with HD-mDC; Fig. 4A and B). In HD-TCD4⁺/HD-pDC cocultures in absence of exogenous rhIL-2, ICOS inhibition reduced by 42% FoxP3⁺ 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 (Supplementary Fig. S4A and 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 cocultured 5 days with R848-preactivated 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.

pDC are strong inducers of Treg enrichment among CD4⁺ T cells through ICOS/ICOS-L costimulation

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-preactivated HD-pDC (13.8 ± 2.4% of FoxP3hi HD-TCD4⁺, compared with 4.5 ± 0.25% at day 5 with HD-mDC; Fig. 4A and B). In HD-TCD4⁺/HD-pDC cocultures in absence of exogenous rhIL-2, ICOS inhibition reduced by 42% FoxP3⁺ 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 (Supplementary Fig. S4A and 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 cocultured 5 days with R848-preactivated 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.
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 coculture decreased the proportion of FoxP3hi Treg from 19/2.4% to 3.3/1% (6-fold, Fig. 4D) although 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 FoxP3hi subpopulation induced by HD-pDC in the presence of anti-ICOS mAb (13.5 ± 2% vs. 4 ± 1% in control and anti-ICOS mAb, respectively; Fig. 5A and B). In reverse experiments, purified IL-3/TLR7-L preactivated TA-pDC favored a strong Treg enrichment among HD-TCD4+ (containing initially 1.7% of FoxP3hi Treg) at day 5 (7.1- and 6.4-fold increase with IL-3 and IL-3+TLR7-L-preactivated TA-pDC, respectively). As expected, such Treg enrichment was strongly reduced by ICOS neutralization (57%–83% reduction in FoxP3hi cells compared with control condition; Fig. 5C and D). Taken together, these results showed the critical role of ICOS/ICOS-L interaction in TA-pDC-mediated TA-Treg amplification in breast tumor.

ICOS neutralization inhibits pDC-induced IL-10 secretion by T-CD4+

We evaluated IL-10 and IFNγ production by HD-Tconv and HD-Treg cocultured with preactivated pDC. In HD-pDC/HD-Tconv coculture with exogenous rhIL-2, ICOS blockade...
strongly inhibited IL-10 (by 91%) and in a smaller proportion, IFNγ secretion (by 33%; Fig. 6A) whereas no IL-10 and IFNγ were detectable in cocultures 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) cocultures (Fig. 6B, right panel). IFNγ secretion was also significantly, but to a lesser extent, inhibited upon ICOS neutralization in all culture conditions (59%–63%). Consistent with the strong IL-10 secretion induced by pDC, we detected high IL-10 levels in 48-hour-culture supernatants of breast tumor single cell suspensions (Supplementary 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 cocultures. In presence of exogenous rhIL-2 HD-TCD4+ cocultured with HD-pDC produced more IL-10 but less IFNγ than with HD-mDC (respectively, 596 ± 103 pg/mL and 181 ± 28 pg/mL for IL-10 and 279 ± 52 pg/mL and 61 ± 9 pg/mL for IFNγ; Fig. 6C). Culture of HD-TCD4+ with mixed DC subsets resulted in lower IL-10 (232 ± 9 pg/mL) but higher IFNγ secretion (574 ± 80 pg/mL) compared with pDC+TCD4+ cocultures. 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 breast tumor 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 showed 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 breast tumor correlates with poor prognosis

IHC analyses were conducted 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 emboli (P < 0.001; Supplementary Table S1). Importantly, in univariate analysis the presence of ICOS+ cells in lymphoid-enriched areas was associated with an increased risk of relapse (progression-free survival, Log-rank P value = 0.0285; Fig. 7D) and death (overall survival, Log-rank P value = 0.0465; Fig. 7E). However, when introduced in a multivariate analysis together with other significant clinical and biologic 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 breast tumor (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 breast tumor express high ICOS and Ki67 levels in a specific manner compared with 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 ovarian carcinoma (32). Early clinical trial with mAbs neutralizing CTLA4 or PD-L1/PD1L1 inhibitory receptors are showing promising clinical activity in melanoma, renal, and lung carcinoma (20%–30% of objective responses; refs. 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 breast tumor. First, we accumulate evidences that strongly support the hypothesis of ICOS+ TA-Treg and TA-pDC interaction in situ interaction by IHC on primary breast tumor frozen sections and ICOS/ICOS-L engagement in breast tumor as illustrated by reversion of ICOS-L downregulation on TA-pDC upon ex vivo culture of whole tumor cell suspension in presence of blocking anti-ICOS mAb. Second, we also show that activated-pDC expressing ICOS-L, but not mDC or CD3/CD28 costimulation, favor allogeneic FoxP3+ 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 need ICOS-ICOS-L costimulation to proliferate (15). Nevertheless, others molecules present in tumor environment such as IDO, OX40-L (36), or TGFβ (37) could also participate to TA-Treg enrichment within breast tumor.

TA-pDC-induced FoxP3+ Treg amplification explains the positive correlation observed between TA-pDC and TA-Treg in breast tumor (Supplementary Fig. S6A) as well as their negative impact on patients’ survival in breast tumor (Supplementary Fig. S6B; ref. 17) and ovarian carcinoma (13, 38). Furthermore, in contrast to recent works in melanoma (39) and glioblastoma (40), ICOS+ TA-Treg expansion in breast tumor is not mediated through direct interaction with tumor cells as (i) ICOS-L is not expressed either on primary breast tumor cells or on breast tumor cell lines (not shown) and (ii) Ki67+ 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+ (15, 41) and TCD8+ (42) subsets and we cannot formally exclude the differentiation of Treg from naive TCD4+, as previously reported for both human and mouse pDC (41, 43, 44). However, we recently showed (i) a specific recruitment of CCR4+ Treg from the blood to breast tumor through the tumor cells CCL22 production (2, 11), and (ii) that all of TA-TCD4+ 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 breast tumor environment.

In accordance with results in NOD type 1 diabetes murine model (27), ICOS+ 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 immunosuppressive functions (for review 4).

High IL-10 levels are detected in breast tumor environment and TA-TCD4+ secrete large amounts of IL-10 in coculture with pDC but not with mDC. ICOS costimulation 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 preexisting IL-10 secreting cells among memory HD-Tconv. Furthermore, mDC-induced T-cell responses are not affected by ICOS blockade as they do not overexpress ICOS-L after activation. Interestingly, we note that ICOS blockade leads to IL-2 accumulation in cocultures with pDC suggesting 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 breast tumors with more than 10 years clinical follow-up allows to show that presence of high numbers of ICOS$^+$ cells in infiltrating primary breast tumors 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 biologic 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 antitumor 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 antitumor 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; ref. 48) or immunogenic chemotherapies (anthracyclins; ref. 49).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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Figure 7. High number of ICOS$^+$ cells within primary breast tumor correlates with reduced patients’ survival. A, 120 paraffin-embedded primary breast tumor 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 noninfiltrated (A), weakly infiltrated (B), and highly infiltrated (C) by ICOS$^+$ cells are shown (magnification, ×10). 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 cutoff ≥1.7 positive cells).
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Received June 19, 2012; revised September 13, 2012; accepted September 13, 2012; published OnlineFirst October 1, 2012.
ICOS-Ligand Expression on Plasmacytoid Dendritic Cells Supports Breast Cancer Progression by Promoting the Accumulation of Immunosuppressive CD4+ T Cells

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