Expression of ICOS on Human Melanoma-Infiltrating CD4\(^+\)CD25\(^{\text{high}}\) Foxp3\(^+\) T Regulatory Cells: Implications and Impact on Tumor-Mediated Immune Suppression

Laura Strauss, Christoph Bergmann, Miroslaw J. Szczepanski, Stephan Lang, John M. Kirkwood and Theresa L. Whiteside

*J Immunol* 2008; 180:2967-2980; doi: 10.4049/jimmunol.180.5.2967

http://www.jimmunol.org/content/180/5/2967

References This article cites 37 articles, 14 of which you can access for free at: http://www.jimmunol.org/content/180/5/2967.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Expression of ICOS on Human Melanoma-Infiltrating CD4⁺CD25highFoxp3⁺ T Regulatory Cells: Implications and Impact on Tumor-Mediated Immune Suppression

Laura Strauss,²⋆ Christoph Bergmann,²⋆ Miroslaw J. Szczepanski, ⋆ Stephan Lang, ⋆ John M. Kirkwood, ⋆ and Theresa L. Whiteside⁴⋆⊗

Objective: Interaction of ICOS with its ligand (ICOSL, B7-H2) promotes T cell responses. As CD4⁺CD25highFoxp3⁺ naturally occurring T regulatory cells in melanoma patients express ICOS, we investigated the impact of ICOS on naturally occurring T regulatory cell function. Methods: Expression of ICOS and T regulatory (Treg) cell markers was determined on CD4⁺CD25high T cells in PBMC and tumor-infiltrating lymphocytes from melanoma patients (n = 10) and PBMC of normal controls (n = 10) by multicolor flow cytometry. Suppression mediated by sorted ICOShigh and ICOSlow Treg was assessed in CFSE-based suppression assays with autologous CD4⁺CD25⁺ responder cells (RC). Transwell inserts separating Treg from RC were used to evaluate suppression mechanisms used by Treg. ICOShigh or ICOSlow Treg were coincubated with RC ± TCR and IL-2 stimulation. ICOShigh and ICOS⁺ Treg were also expanded under conditions previously shown to induce Tr1 from RC. Results: Treg in tumor-infiltrating lymphocytes expressed ICOS (mean fluorescence intensity = 70 ± 10), while Treg in PBMC had low ICOS expression (mean fluorescence intensity = 3.5 ± 2.5, p < 0.001). ICOSlow Treg up-regulated Treg markers (p < 0.0016) and mediated stronger suppression (p < 0.001) relative to ICOSlow Treg. ICOShigh Treg induced Tr1 cells in nonactivated RC and Th2 cells in preactivated RC. ICOShigh Treg exposed to Tr1 cytokines expressed IL-10 and suppressed RC (92 ± 12%) in contrast to ICOSlow Treg, which mediated low suppression (21 ± 15%; p ≤ 0.0028).

Conclusion: ICOShigh Treg can induce diverse immune responses in RC, depending on activation signals and cytokines present. ICOShigh Treg induce Tr1 or Th2 cells depending on the activation state of RC. In a “Tr1” cytokine milieu, ICOShigh Treg transit to Tr1. The Journal of Immunology, 2008, 180: 2967–2980.

The activation and expansion of immune cells is tightly regulated by positive and negative signals that allow for the fine tuning of immune responses. The B7 family ligands and their receptors play a critical role in activation, expansion, as well as contraction of T cell populations. During T cell activation, interaction of B7-1 (CD80) and B7-2 (CD86) with CD28 promotes proliferation, cytokine production, and survival of T cells (1). Additionally, interactions of the ICOS ligand (ICOSL, B7-H2) with the TCR-inducible costimulatory receptor (ICOS) enhances effector T cell responses (2).

CD4⁺CD25highFoxp3⁺ naturally occurring T regulatory (nTreg) cells have emerged in recent years as critical players in the contraction of activated CD4⁺ and CD8⁺ T effector and memory T cells. The absence of nTreg induces severe autoimmunity in mice (3, 4). In contrast, evidence has accumulated demonstrating that the frequency and function of nTreg are increased in the peripheral circulation and in the tumor of patients with cancer (5–7). nTreg are capable of suppressing proliferation of autologous tumor Ag (TA)-specific as well as Ag-nonspecific CD4⁺ and CD8⁺ T cell responses (8). Importantly, nTreg isolated from PBMC or tumors of patients with cancer express higher levels of surface-associated activation molecules and display stronger suppressor function compared with nTreg from normal donors (7, 9). It appears that Treg in tumor-infiltrating lymphocytes (TIL) can mediate suppression of autologous immune cells via distinct mechanisms involving either cell-to-cell contact or inhibitory cytokines, TGF-β and IL-10 (7). In contrast, suppression mediated by Treg in PBMC is exclusively regulated via direct cell-to-cell contact (9).

These results suggest that nTreg in patients with cancer comprise highly activated, differentiated, and cytokine-secreting T cell subsets, whereas nTreg in normal donors are largely nonactivated precursor cells. Furthermore, we and others have shown that signals delivered via the TCR receptor and IL-2R are necessary for nTreg to become active suppressor cells (10, 11). Also, we have previously shown that only “hyperactivated” nTreg obtained from cancer patients induce apoptosis in CD4⁺ and CD8⁺ responder cells (RC) via Fas/Fasl and Granzyme B-mediated pathways (12).

These earlier observations on the activation state of Treg raised the question about the role of ICOS expressed on nTreg. This receptor is exclusively expressed on TCR-activated T cells and is known to induce secretion of IL-10 and TGF-β (Th2 responses) in
human non-Treg cells (2). In cancer patients, nTreg are continuously exposed to strong activation-induced signals originating in the tumor microenvironment or directly mediated by TA. Hence, these cells might be expected to express ICOS. In fact, Miller and colleagues (13) have recently shown that tumor-infiltrating CD4⁺ CD25⁺ Treg accumulating in prostate tumors express higher levels of ICOS than autologous Treg in the peripheral blood. Nevertheless, the role of ICOS expressed on Treg in patients with cancer remains undefined, as are mechanisms ICOS uses to modulate Treg function. The possibility exists that ICOS<sup>high</sup> Treg could cross-link CD4⁺ effector cells, but the impact of such an interaction on anti-tumor immune responses has not been analyzed so far. Furthermore, a relationship between the ICOS<sup>high</sup> Treg subset and the Tr1 regulatory T cell subset comprising cells that express high levels of IL-10 has not been studied. We have recently demonstrated by immunohistochemistry that in human tumor sections, CD4⁺ CD25⁺ IL-10⁺ T cells (Tr1) cluster around CD4⁺ CD25<sup>high</sup> Foxp3⁺ nTreg, suggesting that nTreg might attract, activate, or induce Tr1 cells (7). However, the possible role of ICOS⁺ nTreg in inducing IL-10 or otherwise triggering differentiation of Tr1 cells has not been investigated.

In this study, we demonstrate that ICOS is selectively expressed on strongly activated CD4⁺ CD25<sup>high</sup> nTreg expressing Foxp3, IL-10, and TGF-β and endowed with potent suppressor function. This “hyperactivated” nTreg subset is exclusively present in the tumor and is not detected in the peripheral circulation of melanoma patients or normal controls (NC), indicating that the tumor attracts or induces nTreg precursor cells and promotes their differentiation to ICOS<sup>high</sup> active suppressor cells. We also show for the first time that ICOS<sup>high</sup> nTreg are able to differentiate into Tr1 cells, when they are exposed to a cytokine milieu favorable to Tr1. ICOS<sup>high</sup> nTreg not only have the capacity to “transit” to Tr1 cells, but they also can induce Tr1 cells from autologous CD4⁺ CD25⁺ RC. We further demonstrate that ICOS<sup>high</sup> nTreg are only capable of inducing Tr1 cells in RC that are not fully activated. In contrast, CD4⁺ CD25⁺ T cells that are prestimulated via the TCR and IL-2 express a Th2 cytokine profile after interacting with ICOS<sup>high</sup> nTreg and do not mediate suppression.

Materials and Methods

Patients and blood samples

Ten patients diagnosed with metastatic melanoma, who were seen at the University of Pittsburgh Cancer Institute outpatient clinic between November 2001 and January 2006, were included in this study. Tumor tissues and autologous PBMC were obtained from patients with stage III-IV melanoma with in-transit or distant metastases who underwent surgery at the University of Pittsburgh Medical Center. Also, PBMC from 10 healthy volunteers were used in this study as NC. In all experiments, each patient was always tested in parallel with a NC. Patient and control specimens were processed for different experiments, as previously described (13). Briefly, cell surface molecules were detected using anti-CD3-ECED, anti-CD4-PC5, anti-glucocorticoid-induced TNF receptor (GITR)-FITC (clone FAB689F; R&D Systems), anti-CD27-FITC, anti-CD127-FITC, anti-CCR7-FITC, anti-CD62L-FITC, anti-CD45RO-FITC, anti-CD45RA-FITC, anti-CD25-FITC and -PE, anti-CD122-IFITC, anti-CD132-PE, anti-FasL-PE (NOK-1,4, R&D Systems), and anti-ICOS-PE Abs. Isotype controls IgG1, IgG2a, and IgG2b were included in all experiments. For intracellular staining, samples were first incubated with mAbs against surface markers CD3, CD4, and CD25. After extensive washing, cells were fixed with 4% (v/v) formaldehyde in PBS for 20 min at room temperature, washed once with PBS containing 0.5% (v/v) BSA (w/v), and 2 mM EDTA, permeabilized with PBS containing 0.5% BSA and 0.1% (v/v) saponin, and stained with anti-Perforin-FITC, anti-CD152-PE, anti-GranzymeA-PE, anti-GranzymeB-PE, anti-IL-10-PE or its isotype control PE rat IgG2a,κ (BD Pharmingen), anti-Foxp3-FITC (clone PCH101), and anti-IL-4-FITC from eBioscience and anti-TGF-β-FITC (clone TB21) from Antigenix America. Intracytoplasmic expression of TGF-β and IL-10 was assessed before and after stimulation of PBMC or TIL for 12 h with PMA (20 μg/ml) and ionomycin (1 μM/ml) (American Type Culture Collection) and for 4 h with Brefeldin A (1 μg/ml) (Sigma-Aldrich). We also determined the expression of cytokine surface-bound TGF-β(sTGF-β) in nonsaponified cells. Appropriate isotype controls were used in all experiments. Before use, all mAbs were titrated using normal resting or activated PBMC to establish optimal staining dilutions.

Flow cytometry

Flow cytometry was performed using a FACSScan flow cytometer (Beckman Coulter) equipped with Expol32 software (Beckman Coulter). The acquisition and analysis gates were restricted to the lymphocyte gate as determined by characteristic forward and side scatter properties of lymphocytes. Forward and side scatters were set in a linear scale (Fig. 1A). For analysis, at least 1 × 10⁵ lymphocytes were acquired. The analysis gates were restricted to the CD3⁺ CD4⁺, CD4⁺ CD25⁺, and CD4⁺ CD25<sup>high</sup> T cell subsets, as previously described (Fig. 1A). In addition to the above-mentioned gates, we gated on the CD4⁺ CD25<sup>high</sup> ICOS<sup>high</sup>, CD4⁺ CD25<sup>high</sup> ICOS<sup>low</sup>, and CD4⁺ CD25<sup>high</sup> ICOS<sup>low</sup> cells within the CD4⁺ CD25<sup>high</sup> gate (see Fig. 1A). Cells expressing Treg markers and/or “death” molecules were acquired and analyzed in the FL1 or FL2 logarithmic scale using the set gates.

For this analysis, CD4⁺ T cells with a mean fluorescence intensity (MFI) of CD25 expression ≥120 were classified as CD25<sup>high</sup>. We determined this cut off based on our previous data (15) demonstrating that only CD4⁺ CD25<sup>high</sup> T cells with this MFI or higher in PBMC of NC have suppressive activity. CD4⁺ CD25⁺ T cells with a lower MFI for CD25 expression do not suppress autologous or allogeneic responder T cells and are probably activated T effector cells. Therefore, we set the cut off for the ICOS<sup>high</sup> subset within the CD4⁺ CD25<sup>high</sup> gate at the same cut off we have used to define the CD25<sup>high</sup> subset, assuming that real CD4⁺ CD25<sup>high</sup> Treg are “hyperactivated” T cells that might coexpress ICOS.

Multicolor flow single-cell sorting (SCS)

PBMC obtained from patients’ NC and patients’ TIL were SCS using a MoFlo high-speed cell sorter (DakoCytomation). Samples were sorted for separation of the following subsets: CD4⁺ CD25⁻ T cells and CD4⁺ CD25<sup>high</sup> ICOS<sup>high</sup>, CD4⁺ CD25<sup>high</sup> ICOS<sup>low</sup>, and CD4⁺ CD25<sup>high</sup> ICOS<sup>low</sup> T cells. The same cut-offs determined for flow analysis of the Treg phenotype were used for SCS (Fig. 1).
Suppression assays

SCS fresh CD4^+CD25^{hi} (CD4^+CD25^{hi}ICOS^{hi}), or CD^+CD25^{hi}ICOS^{lo} T cells obtained from patients’ TIL or PBMC, from PBMC of NC or CD4^+CD25^+ T cells recovered from TIL cultures, ICOS^{lo} Treg (CD4^+CD25^+ICOS^{lo}), or autologous 

cultures, or control cultures, were tested for regulatory function in CSFE-based assays, as previously described (15). The suppressor to RC (S:R) ratio was 1:1 or 1:5, with at least 0.5 × 10^5 CFSE-labeled autologous CD4^+CD25^+ RC/well. CD4^+ RC were stimulated with soluble OKT3 (10 μg/ml) (ATCC) and IL-2 (150 IU/ml). Cocultures were harvested and the Tr1 phenotype and expression of death molecule T cell populations were classified as suppressors, if they inhibited proliferation of the CD4^+CD25^{lo} T cell subset. To assess whether cell-to-cell contact was necessary for ICOS^+ Treg to function as suppressor cells was determined. CFSE-based assays, as previously described (15). The suppressor to RC (S:R) ratio was 1:1 or 1:5, with at least 0.5 × 10^5 CFSE-labeled autologous CD4^+CD25^+ RC/well. CD4^+ RC were stimulated with soluble OKT3 (10 μg/ml) (ATCC) and IL-2 (150 IU/ml). Cocultures were harvested and the Tr1 phenotype and suppressor function were determined. As positive controls, autologous CD4^+CD25^+ T cells obtained by SCS from TIL or PBMC of patients or NC were also cultured in the Tr1 IVA culture system. Negative controls were cultured in media in the presence of IL-2 alone (10 IU/ml).

Cytokine production assay

For the last 24 h of Tr1 cultures, supernatants (SN) were replaced by fresh complete medium containing anti-CD3 mAb (1 μg/ml), but no exogenous cytokines. The levels of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-17, GM-CSF, IFN-γ, and TNF-α were measured in the SN using immuno-bead-based multiplex assays (Luminex). Panels of capture Ab-coated beads and labeled detection Abs were purchased from BioSource International. The reagents were pretested and qualified by the manufacturer to ensure the absence of cross-reactivity among Ab-coated beads. The assay sensitivity varied from 5 to 15 pg/ml, depending on the analyte. The 10-plex assay and IL-17 Ab bead kit were approved for combination and performed in the same experimental runs. The assays were performed using the Bio-Plex system (the instrument and software for data analysis purchased from Bio-Rad). The assay was performed according to manufacturer’s instructions.

Immunofluorescence of tumor tissues

Tissue samples were embedded in OCT, and 5-μm frozen sections were cut in a cryostat, dried, fixed for 10 min in cold acetone and ethanol (1:1), and again dried in air. The following mAbs were used for staining: mouse anti-human CD4/FTTC (diluted 1/100 in PBS), anti-human CD25/PE (1/50) (BD Pharmingen), rabbit anti-human TGF-β1/β2/β3 (1/50), and polyclonal goat anti-human ICOS (1/50) (Santa Cruz Biotechnology). In addition, polyclonal Abs rabbit anti-human TGF-β1/β2/β3 (1/50) and polyclonal goat anti-human ICOS (1/50) (Santa Cruz Biotechnology) were used. As secondary Abs for Foxp3, IL-10, TGF-β1, and Alexa Fluor 488-labeled goat anti-rat and goat anti-rabbit Abs were used (1/500). To detect ICOS, Cy-5-labeled donkey anti-goat (Jackson ImmuNoResearch Laboratories) Ab was used at 1/1000 dilution. To eliminate nonspecific binding of secondary Abs, tissue sections were initially incubated with 2% BSA (BSA diluted in PBS) for 1 h and washed five times in 0.5% BSA. Sections were incubated with Abs for 1 h at room temperature in a moist chamber. Next, slides were washed in 10% BSA and then incubated with the secondary Abs under the same conditions and in the dark. Primary Abs were omitted in all negative controls. Sections were incubated in a medium with 4',6-diamidino-2-phenylindole (Vector Laboratories) to trace cell nuclei. Slides were evaluated in the Olympus Provis fluorescence microscope under ×400 magnification (mag). For digital image analysis, the software Adobe Photoshop 6.0 version was used.

Statistical analysis

Data were summarized by descriptive statistics (mean and SD for continuous variables; frequency and percentage for categorical variables). All statistical analyses were performed using the Student’s t test, and values of p < 0.05 were considered significant.

Results

ICOS is exclusively expressed on CD4^+CD25^{hi} T cells in TIL from melanoma patients

Flow cytometry analyses of TIL isolated from tumor tissues showed that the CD3^+CD4^+ T cell subset accounted for 45 ± 18% (mean ± SD) of all T cells, with a range of 30–65%. The gating strategy used to identify this cell subset is shown in Fig. 1A. Among these CD4^+ T cells, an average of 15 ± 2.5% were CD25^+ (Table I). This enrichment in CD4^+CD25^+ T cells was significant (p < 0.002) compared with circulating CD3^+CD4^+ T cells (6 ± 3%) in melanoma patients or in NC (2 ± 1.5%). We also determined the proportions of CD25^{hi} cells present in the

primed Tr1 lymphocytes (14). To study the impact of these cytokines on CD4^+CD25^{hi}ICOS^{lo} or CD^+CD25^{hi}ICOS^{hi} Treg separated by SCS from TIL, were cultured in the Tr1 in vitro activated (IVA) culture system, previously described by us (14). Briefly, cells were cultured at 5 × 10^6/well in 96-well plates (Corning Costar). Cytokines IL-2 (10 IU/ml), IL-10 (20 IU/ml), and IL-15 (20 IU/ml), all purchased from PeproTech, were added to these cultures on days 0, 3, and 6. On day 9, culture medium was replaced by fresh medium containing anti-CD3 Ab (1 μg/ml) (ATCC). On day 10, lymphocytes and culture medium were harvested and the Tr1 phenotype and suppressor function were determined. As positive controls, autologous CD4^+CD25^+ T cells obtained by SCS from TIL or PBMC of patients or NC were also cultured in the Tr1 IVA culture system. Negative controls were cultured in media in the presence of IL-2 alone (10 IU/ml).

The Journal of Immunology 2969

Downloaded from http://www.jimmunol.org/ by guest on March 26, 2022

http://www.jimmunol.org/
CD3⁺ CD4⁺ subset in TIL (7.5 ± 3.5%; n = 10) and in PBMC of melanoma patients (3.5 ± 1.5%; n = 10) as well as NC (1 ± 0.75%; n = 10), as shown in Table I. The mean percentages of CD4⁺ CD25⁺ as well as CD4⁺ CD25high T cells were higher in TIL and in PBMC of melanoma patients (p < 0.002 and p < 0.01, respectively) compared with that in PBMC of NC.

For this analysis, CD4⁺ T cells with a MFI of CD25 expression ≥120 were classified as CD25high (Fig. 1A). An average MFI for CD4⁺CD25high cells in TIL was 175 ± 25, while that for CD4⁺CD25high cells in PBMC was 135 ± 15. Thus, CD4⁺ TIL expressed higher levels of CD25 than did circulating CD4⁺ T cells.

Next, we determined ICOS expression on the CD4⁺CD25high and CD4⁺CD25low subsets in PBMC as well as TIL in melanoma patients or in PBMC of NC. As shown in Fig. 1B, ICOS was only weakly expressed on CD4⁺CD25high T cells in PBMC of NC or melanoma patients. In contrast, ICOS was highly expressed on CD4⁺CD25high T cells isolated from TIL in melanoma patients (Fig. 1B). ICOS was not detected on the CD4⁺CD25low T cell subset in PBMC of NC or patients or in TIL (data not shown). Further, we analyzed the % positive cells as well as average MFI of ICOShigh, ICOSint, and ICOSlow T cell subsets within the CD4⁺CD25high T cell population in PBMC from NC and patients or from TIL. As illustrated in Fig. 1C, the CD4⁺CD25high T cell subset in PBMC from melanoma patients was mainly composed by ICOSlow T cells with few ICOSint or ICOShigh T cells, and the MFI in all three subsets was low. Similar data were observed with cells

### Table I. Distribution of CD4⁺ cell subsets in PBMC or TIL in patients with melanoma and PBMC of NCa

<table>
<thead>
<tr>
<th></th>
<th>PBMC</th>
<th>PBMC Melanoma</th>
<th>TIL Melanoma</th>
<th>Valuesb</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3⁺ CD4⁺</td>
<td>50 ± 12</td>
<td>42 ± 20</td>
<td>45 ± 18</td>
<td>NSD</td>
</tr>
<tr>
<td>CD4⁺ CD25⁺</td>
<td>2 ± 1.5</td>
<td>6 ± 3</td>
<td>15 ± 2.5</td>
<td>&lt;0.0002</td>
</tr>
<tr>
<td>CD4⁺ CD25high</td>
<td>1 ± 0.75</td>
<td>3.5 ± 1.5</td>
<td>7.5 ± 3.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD4⁺ CD25⁺</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a The data are mean values ± SD obtained with cells of 10 patients with melanoma and 10 NC.

b The p values are for differences between PBMC and TIL of melanoma patients.

* NSD = No significant difference.
FIGURE 2. Phenotypic characteristics of CD4<sup>+</sup>CD25<sup>high</sup> Treg isolated from PBMC of NC or TIL and PBMC of melanoma patients. A. Percentages of cells positive for various markers within the CD4<sup>+</sup>CD25<sup>high</sup> T cell subset. Flow cytometry-based analysis was performed using PBMC of NC. B. Percentages of cells positive for various markers within CD4<sup>+</sup>CD25<sup>high</sup> T cell subsets in TIL (black bars) or PBMC of melanoma patients. Asterisks indicate significant differences between PBMC and TIL. C. Representative flow dot plots obtained with TIL of one subject for membrane-bound sTGF-β<sub>1</sub>, ITGF-β, GITR, and IL-10. D. MFI for expression of various markers on the surface of CD4<sup>+</sup>CD25<sup>high</sup>ICOS<sup>high</sup> or CD4<sup>+</sup>CD25<sup>high</sup>ICOS<sup>low</sup> T cells in TIL obtained from melanoma patients. E. Representative dot plots obtained with TIL of one subject for expression of Foxp3 and iTGF-β in ICOS<sup>high</sup> or ICOS<sup>low</sup> CD4<sup>+</sup>CD25<sup>high</sup> T cell subsets. Histograms show mean values ± SD from experiments performed with cells of 10 NC and 10 melanoma patients.
of NC (data not shown). In contrast, the CD4+CD25high T cell subset in TIL comprised ICOShigh and ICOSlow T cells (p < 0.01) with few ICOSlow T cells. Of note, the MFI of the ICOShigh and ICOSlow subsets in TIL was greatly enhanced (p ≤ 0.001 and p = 0.01, respectively) compared with the ICOSlow subset (Fig. 1C).

Phenotypic profiles of CD4+CD25high, CD4+CD25highICOSlow, and CD4+CD25highICOSlow T cell subsets

Expression of T cell activation markers, Treg markers, “death” molecules as well as IL-10, sTGF-β1 (membrane-bound), and iTGF-β1 (intracellular) on gated CD4+CD25high T cells was determined in isolated TIL and PBMC of melanoma subjects or NC by multiparameter flow cytometry.

Back-gating on CD4+CD25high T cells, we show in Fig. 2A that in PBMC of NC, only a proportion of the cells expressed markers usually associated with nTreg: CD62L, GTR, Foxp3, or CCLA-4, and only about half were CD45RO+ memory cells expressing Granzyme B. These results suggest that in NC, most CD4+CD25high nTreg are in the precursor stage and only a subset are activated or fully differentiated to perform suppressor function. In contrast, both PBMC and TIL obtained from melanoma patients had CD4+CD25high T cells with an activated/memory phenotype (CD62LlowCD45RO+CD45RA−) (Fig. 2B). These memory T cells in TIL and PBMC of melanoma patients were largely Foxp3+, CCLA-4+, and iTFG-β1+, with the majority expressing Fas (CD95) (Fig. 2B). Few CD4+CD25high T cells were positive for CD45RA (6.8 ± 2.1%) or CD127 (14 ± 10%) (data not shown). Of note, TIL and PBMC obtained from melanoma patients showed strong expression of Granzyme A and Granzyme B (Fig. 2B), whereas Treg in PBMC of NC expressed Granzyme B but not Granzyme A (Fig. 2A).

In TIL, CD4+CD25high T cells expressed a different profile of markers than did this same subset of T cells in PBMC of melanoma patients (Fig. 2B). For example, most CD4+CD25high T cells in TIL were GTR+, FasL−, IL-10−, and sTGF-β1− (Fig. 2, B and C) relative to CD4+CD25high T cells in PBMC (p < 0.0012), only few of which were positive for GTR and all of which were negative for FasL, IL-10, and sTGF-β1 (Fig. 2B). A higher percentage (p < 0.0012) of CD4+CD25high T cells expressed the migration and lymph node homing receptors CCR7 and CD62L in PBMC of melanoma patients than in TIL (Fig. 2A). FasL was absent from the surface of CD4+CD25high PBMC in patients, while the majority of Treg in TIL were FasL+ (Fig. 2B). Although GTR was expressed on 54% of CD4+CD25high cells in TIL, CD4+CD25high T cells in PBMC of melanoma patients expressed little GTR (Fig. 2B). Also, in TIL, close to half of CD4+CD25high Treg had up-regulated CD132 (IL2Rγ) (Fig. 2B).

Next, expression levels of activation markers, Treg molecules, and “death” molecules were compared on CD4+CD25high ICOShigh and CD4+CD25highICOSlow T cell subsets sorted from TIL of melanoma patients (Fig. 2D). Higher expression of markers related to activation, suppressor, and “killer” function of nTreg was evident (p < 0.0015) in a subset of CD4+CD25highICOShigh T cells (Fig. 2, D and E). These results support our assumption that Treg require strong T cell activation signals to become potent suppressor cells.

In aggregate, our data indicate that considerable phenotypic differences exist between the CD4+CD25high populations in TIL and PBMC of melanoma patients. Only Treg in TIL express GTR, IL-10 and TGF-β1 (intracellular and membrane-bound), both granzyme A and B, and up-regulate expression of CD132. Likewise, CD4+CD25high Treg in PBMC and TIL of melanoma patients were phenotypically distinct from those in PBMC of NC.

Suppressor activity of CD4+CD25highICOS+ T cells isolated from PBMC or TIL

To evaluate suppressor activity mediated by sorted CD4+CD25high ICOSlow or CD4+CD25highICOShigh T cell subsets, cocultures were set up with CFSE-labeled autologous CD4+CD25− T cells defined here as RC. The cocultures were established at the ratios of 1:1RC or 1:5RC, using RC proliferating in response to OKT3 in the presence of IL-2. To analyze proliferation of RC, gating was set to include CD4+CFSE− T cells, as previously described. The percentages of inhibition of RC proliferation measured varied, depending on the S:RC ratios used and the ICOS expression level. As illustrated in Fig. 3, CD4+CD25highICOShigh Treg isolated from TIL of melanoma patients almost completely suppressed proliferation (mean ± SD = 98 ± 7.5%) of autologous RC (p < 0.001).
FIGURE 4. CD4⁺CD25highICOShigh Treg isolated from TIL induce Tr1 cells in autologous CD4⁺CD25 T cells isolated from PBMC. A, The average mean fluorescence (MFI ± SD) of cells positive for various markers within the CD4⁺CD25 T cell population isolated from PBMC of melanoma patients (n = 5), before coculture with autologous CD4⁺CD25highICOShigh Treg isolated from TIL, is shown (white bars). B, The average mean fluorescence (MFI ± SD) of cells positive for various markers within the CD4⁺CD25 T cell population isolated from PBMC of melanoma patients (n = 5), after coculture with autologous CD4⁺CD25highICOShigh Treg isolated from TIL in the presence of a TR. C, The average mean fluorescence (MFI ± SD) of cells positive for various markers within the CD4⁺CD25 T cell population isolated from PBMC of melanoma patients (n = 5), after coculture with autologous CD4⁺CD25highICOShigh Treg isolated from TIL in the absence of a TR. D, Representative flow dot plots obtained from one subject with melanoma for expression of CD132, IL-10, and iTGF-β1 on CD4⁺CD25 T cells isolated from the corresponding coculture. All cocultures were performed with nonactivated autologous CD4⁺CD25 T cells.
TABLE II. Th1/Th2 profile and proliferation of PBMC-derived CD4\(^+\)CD25\(^{-}\)RC cells coincubated with autologous CD4\(^+\)CD25\(^{high}\) ICOS\(^{high}\) Treg

<table>
<thead>
<tr>
<th></th>
<th>CD25</th>
<th>Foxp3</th>
<th>IL-10</th>
<th>IL-2</th>
<th>IL-4</th>
<th>% Proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(MFI ± SD)</td>
<td>(MFI ± SD)</td>
<td>(pg/ml)</td>
<td>(pg/ml)</td>
<td>(pg/ml)</td>
<td></td>
</tr>
<tr>
<td>Nonactivated RC coincubated with:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4(^+)CD25(^{high})ICOS(^{high})</td>
<td>12 ± 8</td>
<td>15 ± 12</td>
<td>3850 ± 250*</td>
<td>52 ± 85*</td>
<td>22 ± 13*</td>
<td>25 ± 11%*</td>
</tr>
<tr>
<td>CD4(^+)CD25(^{low})ICOS(^{low})</td>
<td>18 ± 12</td>
<td>23 ± 12</td>
<td>95 ± 550</td>
<td>2338 ± 4428</td>
<td>169 ± 3895*</td>
<td>96 ± 5%*</td>
</tr>
<tr>
<td>Preactivated RC (OKT3 and IL-2) coincubated with:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4(^+)CD25(^{high})ICOS(^{high})</td>
<td>20 ± 15</td>
<td>13 ± 10</td>
<td>2888 ± 1445.6</td>
<td>3897 ± 6110*</td>
<td>950 ± 4760*</td>
<td>92 ± 7.8%*</td>
</tr>
<tr>
<td>CD4(^+)CD25(^{low})ICOS(^{low})</td>
<td>10 ± 5</td>
<td>19 ± 13</td>
<td>120 ± 380</td>
<td>2252 ± 7224</td>
<td>140 ± 2351</td>
<td>94 ± 5%*</td>
</tr>
</tbody>
</table>

* The data are from experiments testing ICOS\(^{+}\) Treg subsets obtained from TIL of five patients with melanoma, as described in Materials and Methods.

** Indicates significant differences in the phenotype compared to that before co-culture.

(see left panel). In contrast, CD4\(^+\)CD25\(^{high}\)ICOS\(^{low}\) Treg isolated from TIL of melanoma patients only partially suppressed proliferation of RC (68 ± 15%) at the same S:RC ratio of 1:1 (see left panel). Similarly, CD4\(^+\)CD25\(^{high}\)ICOS\(^{high}\) Treg isolated from PBMC of melanoma patients had higher suppression activity (82 ± 12%) than CD4\(^+\)CD25\(^{high}\)ICOS\(^{low}\) Treg isolated from PBMC of the same patients (38 ± 10%) (data not shown). The CD4\(^+\)CD25\(^{high}\)ICOS\(^{high}\) as well as CD4\(^+\)CD25\(^{high}\)ICOS\(^{low}\) Treg subsets isolated from PBMC of NC had weak suppressor activity (26 ± 12%) (data not shown). Further, only the CD4\(^+\)CD25\(^{high}\)ICOS\(^{high}\) Treg isolated from TIL suppressed RC proliferation at a S:RC ratio of 1:5 (68 ± 12%), whereas TIL- or PBMC-derived CD4\(^+\)CD25\(^{high}\)ICOS\(^{low}\) Treg obtained from patients or NC had no suppressor activity at this ratio (data not shown). These results support our hypothesis that only activated Treg expressing ICOS and receiving costimulatory signals up-regulate activation molecules and become potent suppressor cells. Thus, in patients with cancer, nTreg that are in direct contact with the tumor are “hyper-activated” suppressor cells, whereas Treg in PBMC express significantly less ICOS and are activated to a lesser degree. In contrast, the CD4\(^+\)CD25\(^{high}\) Treg subset in NC is mainly composed by activated CD25\(^{+}\) non-Treg, which may or may not serve as Treg precursors, as previously reported.

Mechanisms of suppressor activity mediated by TIL-derived CD4\(^+\)CD25\(^{high}\)ICOS\(^{high}\) T cells

A large majority (75–96%) of CD4\(^+\)CD25\(^{high}\) T cells in TIL of melanoma patients were positive for iIL-10 and iTGF-β1 as well as sTGF-β1 by flow cytometry (Fig. 2A). Importantly, as described above (Fig. 2C), CD4\(^+\)CD25\(^{high}\)ICOS\(^{high}\) T cells in TIL expressed the highest levels of inhibitory cytokines and mediated stronger suppression relative to CD4\(^+\)CD25\(^{high}\)ICOS\(^{low}\) Treg, which expressed low levels of iIL-10 and TGF-β1. Therefore, we hypothesized that Treg function of CD4\(^+\)CD25\(^{high}\)ICOS\(^{high}\) T cells in TIL was mediated via IL-10 and TGF-β1 secretion and required cell-to-cell contact. To test this hypothesis, CD4\(^+\)CD25\(^{high}\)ICOS\(^{high}\) or CD4\(^+\)CD25\(^{high}\)ICOS\(^{low}\) Treg isolated from TIL were coincubated at the S:RC ratio of 1:1 with CFSE-labeled autologous CD4\(^+\)CD25\(^{-}\) RC responding to OKT3 and IL-2 in the presence or absence of TR that permit diffusion of soluble factors but prevent cell-to-cell contact.
Coincubation of CD4⁺CD25⁺ICOS⁺ T cells isolated from TIL in the presence of TR inhibited suppression of RC proliferation by ~50% (Fig. 3, upper right panel). In contrast, incubation of CD4⁺CD25⁺ICOS⁻ T cells isolated from TIL in the presence of TR almost completely abrogated suppression of RC proliferation (Fig. 3, lower right panel). These results confirm our previously published results (6), indicating that cytokine-negative nTreg depend entirely on direct cell-to-cell contact for suppression of RC proliferation, whereas in those nTreg that secrete inhibitory cytokines, direct cell-to-cell contact is not required for suppression of RC proliferation but it might enhance cytokine-mediated suppression. This is especially evident with ICOS⁺ Treg; these cells express high levels of membrane-bound TGF-β₁, and direct cell-to-cell contact might facilitate suppression of RC proliferation by this cytokine.

TIL-derived ICOS⁺CD4⁺CD25⁺ Treg induce differentiation of Tr1 cells from autologous CD4⁺CD25⁻ T cell precursors

We and others have previously shown that Treg require activation via the TCR and IL-2/Stat5b signaling to develop and mediate effective suppression (11, 17–20). However, the mechanisms responsible for suppression of immune cell proliferation by strongly activated Treg are not clear. We have also shown that both nTreg as well as Tr1 cells infiltrate human solid tumors and that Tr1 cells...
form cell clusters with nTreg in the tumor microenvironment (7). Based on this observation, we hypothesized that highly activated ICOS

+ nTreg can induce Tr1 cells in autologous CD4

+ CD25

− T cells that are attracted from the peripheral circulation into the tumor. 

SCS ICOS

high or ICOS

low CD4

+ CD25

high T cells obtained from TIL were coincubated with autologous PBMC-derived CD4

+ CD25

RC at the S:RC ratio of 1:1. As described above, the assays were performed in the presence or absence of TR and using CD4

+ CD25

RC activated or not activated with OKT3 and IL-2. In the assays that were performed in the absence of TR, CD4

+ CD25

RC were labeled with CFSE to differentiate them from the cocultured Treg. On day 5 of coculture, expression of activation markers, Treg markers, and “death” molecules was determined using flow cytometry and gating on CD4

+ CD25

CFSE

− vs CD4

+ CD25

CFSE

− T cell subsets. Fig. 4A shows that before any culture, ex vivo-harvested CD4

+ CD25

RC were largely negative for Foxp3, CTLA-4, IL-10, or TGF-β and only 20–30% expressed CD132 and ICOS. When ICOS

high CD4

+ CD25

high Treg isolated from TIL were coincubated with autologous nonactivated CD4

+ CD25

− RC in the absence of TR, the cells generated in these cocultures expressed the phenotype of Tr1 cells, which is characterized by expression of CD132 and IL-10 in the absence of CD25 (Fig. 4B). These Tr1 cells induced by ICOS

high Treg expressed a memory T cell phenotype (CD4

+ CD25

CD62L

low CD45RO

+ CD45RA), low levels of Foxp3 and CTLA-4, and had significantly higher (p < 0.006) levels of “suppressor” cytokines IL-10 and TGF-β (intracellular and membrane-bound) than those in ex vivo-tested CD4

+ CD25

precursors (Fig. 4A, B, and D). These “induced” Tr1 cells also expressed higher levels of ICOS (p < 0.006) and CD132 (p < 0.001) and high levels of the cytokotins Granzyme A and Granzyme B. 

In contrast, when ICOS

high CD4

+ CD25

high Treg isolated from TIL were coincubated with nonactivated CD4

+ CD25

− RC in the presence of TR, the shift to the Tr1 profile was not significant, indicating that direct cell-to-cell contact is necessary for the induction of Tr1 cells from autologous CD4

+ CD25

− T cell precursors (Fig. 4, C and D). Of note, when ICOS

high CD4

+ CD25

high Treg were coincubated with CD4

+ CD25

− T cells that were previously activated via the TCR and IL-2, no Tr1 phenotype was induced. Instead, proliferation of the CD4

+ T cells was induced and cells with a characteristic “Th2” cytokine profile emerged (Table II). When ICOS

low Treg were coincubated with autologous preactivated or nonactivated CD4

+ CD25

− RC in the absence or presence of TR, no phenotypic changes compared with their phenotype before coculture were observed (Table II). In aggregate, these results suggest that ICOS might participate in the induction and differentiation of CD4

+ CD25

− RC into immunosuppressive Tr1 cells or active Th2 cells, depending on the activation state of the responder population.

We next analyzed whether the phenotype of Tr1 cells induced by ICOS

high Treg isolated from TIL corresponds with their suppressor function. The Tr1 cells induced by ICOS

high Treg in the presence or absence of TR were coincubated with autologous CFSE-labeled CD4

+ CD25

RC at the IS:1RC ratio as described in Materials and Methods. The Tr1 cells that were induced by ICOS

high Treg in the absence of TR and had a “characteristic” Tr1 phenotype partially suppressed proliferation of CD4

+ CD25

RC (65 ± 12%) (Fig. 5), whereas Tr1 cells that were induced in the presence of TR and expressed a weak Tr1 phenotype had low (30 ± 15%) suppressor activity (Fig. 5). Thus, cell-to-cell contact between inducer and RC was necessary for the generation of Tr1 cells in this coculture system.

ICOS

high Treg convert into IL-10

+ Tr1 cells endowed with potent suppressor activity in the presence of IL-2, IL-15, and IL-10

In the above-described experiments, we showed that CD4

+ CD25

high ICOS

high T cells obtained from TIL promoted induction and differentiation of Tr1 cells from autologous CD4

+ CD25

− precursors. As these experiments involved coincubation and then separation of the coincubated cell populations, a possibility had to be considered that CD4

+ CD25

high ICOS

high Treg convert into Tr1 cells. To test the hypothesis that CD25

high ICOS

high Treg in TIL might act as precursors of Tr1, sorted CD4

+ CD25

RC, ICOS

high CD25

high T cells, and ICOS

low CD25

high T cells from TIL of melanoma patients were incubated for 10 days in media supplemented with low doses of IL-2, IL-10, and IL-15 every 3 days under conditions previously reported to favor outgrowth of CD4

+ CD25

IL-10

+ TGF-β

+ T cells (16). As shown in Fig. 6, Tr1 cells were induced in CD4

+ CD25

RC populations after 10 days in culture, confirming the results we recently reported with cells obtained from head and neck squamous cell carcinoma patients. These Tr1 cells had up-regulated marker expression of Tr1-associated surface or intracellular molecules, as shown in Fig. 6A, upper row. ICOS

low CD25

high TIL did not up-regulate expression of the same markers, as shown in Fig. 6A, middle row, and Fig. 6B. However, ICOS

high CD25

high TIL converted into cells with a phenotype similar to Tr1.
cells (Fig. 6B), with down-regulated expression of IL2Ra (CD25) (MFI < 5) and up-regulated expression of IL2Rγ (CD132; MFI >152; Fig. 6A, lower row). Furthermore, these Tr1 cells remained positive for IL-10 (MFI: 63 ± 12.5) and TGF-β, expression (MFI: 38 ± 7.5), in contrast to ICOSlowCD25high TIL (MFI: 3 ± 4.5 and 11 ± 5.2, respectively) (Fig. 6B). Also, T cells, which outgrew from CD25−CD4+ TIL in the Tr1 culture, retained a high expression of IL7Ra (CD127), whereas ICOShigh and ICOSlow TIL remained low for IL7Ra expression (Fig. 6B). Phenotypic analysis of these different groups of Tr1 cells expanded in the presence of the Tr1 cytokines for apoptosis-related molecules revealed a reciprocal relationship between these molecules and ICOS expression (Fig. 6C): while non-Tr1 cells expanded from the ICOSlow CD4+ TIL, in contrast to ICOSlowCD25high TIL (MFI: 30 ± 3.1), ICOShigh and ICOSlow high T cell subsets had a significantly higher expression of Fas and down-regulated Granzyme B and FasL. The data suggest that these cells might be able to differentially regulate T cell activation-induced cell death.

We also evaluated the ability of T cells expanded from different TIL subsets to suppress autologous T cell proliferation. T cells outgrowing from the three different types of Tr1 cultures were titrated into CFSE-labeled autologous CD4+CD25−RC at different ratios and stimulated with OKT3 and IL-2 for 5 days. Fig. 7A shows that T cells that outgrew in Tr1 cultures established from CD4+CD25−TIL, mediated greater suppression (67% ± 7.7) than T cells outgrowing from CD4+ICOSlowCD25high TIL in the presence of Tr1 cytokines (23% ± 9.1). T cells from cultures with CD4+ICOShighCD25high starting population and cytokines showed even greater suppressor activity (92% ± 5.2) and the difference was highly significant at p < 0.001.

Levels of cytokines in culture SN

We analyzed the SN of different types of Tr1 cultures for levels of cytokines using Luminex technology as described in Materials and Methods. The objective was to identify the type of response that is promoted in each starting population cultured in a Tr1-favoring milieu. As opposed to SN from the control cultures, which consisted of CD4+CD25−TIL cultured in the presence of IL-2 (50 IU/ml) for 10 days, SN from T cell cultures containing CD4+CD25−TIL and “Tr1 cytokines” showed very low levels of proinflammatory cytokines IFN-γ, IL-1β, IL-4, TNF-α, and IL-17 but high levels of IL-10 and IL-2. Surprisingly, SN from T cell cultures with ICOShigh or ICOSlow Treg in the presence of “Tr1 cytokines” showed a comparable anti-inflammatory cytokine profile (Table III). However, as shown in Fig. 7, the MFI for expression of these cytokines in Tr1, which originated from ICOSlow Treg, is significantly higher and these cells are stronger suppressors, compared with Tr1 originated from ICOSlow Treg. These findings suggest that Tr1 cells originating from ICOShigh Treg are able to create a stronger immunosuppressive environment.

In situ analysis of ICOS+ Treg in melanoma tissues

Fig. 8a shows that using immunofluorescence, numerous ICOS+ CD4+ T cells can be visualized in a representative melanoma tissue. In Fig. 8a2, pink CD4+CD25+ICOS+ nTreg are localized in the proximity of CD4+CD25−ICOSlow/neg T cells (see inset), implying that these cell subsets can interact in situ. We have also examined distribution in the tumor of ICOS+ cells (data not shown). As shown in Fig. 8c, ICOS+ cells are located adjacent to CD4+CD25+ICOS+ T cells (inset). These cells are yellow and cells that are CD25lowFoxp3low are pink. Note that CD4+CD25+ ICOS+ Treg are located adjacent to CD4+CD25+ICOS+ T cells (inset). A section stained for CD25, Foxp3, and ICOS; in b1, CD25+ cells are red, Foxp3 cells are green, and CD25+Foxp3low cells appear yellow; and cells that are CD25lowFoxp3low are pink (inset). ICOS+ cells are blue and nuclei are pseudocolored white; and in b3, an overlay showing that ICOS+ cells (CD25lowFoxp3low) express ICOS (blue). A section stained for CD25, IL-10, and ICOS; in c1, CD25+ cells are red, IL-10+ cells are green, and CD25+IL-10+ cells are yellow; in c2, ICOS+ cells are blue and nuclei are pseudocolored white; and in c3, an overlay showing that CD25+IL-10+ cells are pink, i.e., ICOS+ and that nearly all of IL-10+ cells are also ICOS+. Mag. ×400. Inset in 8a, mag. ×600.

### Table III. Levels of cytokines in SN of Tr1 cell cultures generated from various starting subsets of TIL

<table>
<thead>
<tr>
<th>TIL subset</th>
<th>IFNγ</th>
<th>IL-1β</th>
<th>IL-2</th>
<th>IL-4</th>
<th>TNFα</th>
<th>IL-17</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+CD25− control</td>
<td>16684 ± 1554</td>
<td>63 ± 20.8</td>
<td>26831 ± 1445.6</td>
<td>97 ± 21.0</td>
<td>22688 ± 2760</td>
<td>487 ± 85.5</td>
<td>402 ± 32.3</td>
</tr>
<tr>
<td>CD4+CD25−</td>
<td>364 ± 72</td>
<td>198 ± 33.1</td>
<td>2341 ± 171.7</td>
<td>52 ± 22.4</td>
<td>2140 ± 335.1</td>
<td>83.7 ± 56.7</td>
<td>3605 ± 450.0</td>
</tr>
<tr>
<td>CD4+CD25−ICOSlow</td>
<td>23.3 ± 29.1</td>
<td>23 ± 21.7</td>
<td>2396 ± 780</td>
<td>21 ± 28.2</td>
<td>35.3 ± 32.9</td>
<td>7.3 ± 6.7</td>
<td>5754 ± 405.4</td>
</tr>
<tr>
<td>CD4+CD25−ICOShigh</td>
<td>20.3 ± 19.6</td>
<td>38 ± 37</td>
<td>2533 ± 510</td>
<td>32 ± 30</td>
<td>31.7 ± 31.5</td>
<td>34.7 ± 44.6</td>
<td>5781 ± 462.7</td>
</tr>
<tr>
<td>p Value (vs control)</td>
<td>&lt;0.0002</td>
<td>&lt;0.02</td>
<td>&lt;0.00001</td>
<td>&lt;0.01</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* Treg subsets were SCS from TIL of 10 patients with melanoma and cultured in the presence of “Tr1 cytokines” to generate Tr1 cells in vitro, as previously described (14). SN of Tr1 cells were tested for cytokine levels by Luminex.
shown). In addition to ICOSL + melanoma cells, which were localized at the border between the lymphocytic infiltrates and tumor nests, numerous infiltrating CD4 + T cells expressed ICOSL. These observations suggest that ICOSL is abundantly expressed in the tumor microenvironment. In Fig. 8b, the white circles denote the areas of distribution of CD25 high Foxp3 + nTreg relative to a cluster of CD25 dim Foxp3 dim cells, which could be either Tr1 cells or nTreg “transiting” to Tr1 cells in the tumor microenvironment. Finally, Fig. 8c shows that numerous IL-10 + lymphocytes accumulating in the tumor are ICOS + and include both CD25 + and CD25 neg populations. Multicolor immunofluorescence in situ confirms that ICOS + Treg have the characteristics of nTreg and Tr1 cells accumulate in melanoma tissues and are in direct contact with each other.

**Discussion**

The generation of effective immune responses to T cell-dependent Ags, including TA, is a dynamic process involving the coordinated action of cytokines and costimulatory molecules expressed on APC and T cells (21). During this process, the type and level of costimulatory signals delivered to T cells will ultimately govern the outcome of Ag presentation in the context of MHC molecules (22). Among major costimulatory receptor-ligand pairs identified to date, the CD28/B7 pathway has been most extensively studied (23, 24). More recently described, the ICOS/ICOSL (ICOS/B7-H2) pathway appears to play a key role in the control of T cell-mediated immune responses and tolerance. Unlike CD28, which is constitutively expressed on the surface of T cells, ICOS requires up-regulation delivered via TCR stimulation (25). Evidence suggests that B7/CD28 interactions are important in clonal expansion and effector functions of naive CD4 + T cells, whereas ICOS/ICOSL interactions control the responses of already activated T cells. Although CD28/B7 interactions are important for IL-2-driven responses, costimulation through ICOS is particularly effective in enhancing IL-10 production, and in direct comparisons, ICOS is more potent than CD28 in inducing the production of IL-10 (26).

Recently reported data indicate that ICOS may be expressed not only on effector T cells but also on Treg. Storkus and colleagues (27) show that murine CD4 + TIL with an activated/memory phenotype (ICOS + CD62L - CD45RB + ) suppressed allo-specific T cell proliferation and IFN-γ production from cross-primed anti-CMS4 CD8 + T cells via a mechanism at least partially dependent on IL-10 and TGF-β1. In patients with prostate cancer, Treg in TIL were found to express higher levels of ICOS and GITR compared with Treg in PBMC (13). In addition, recent studies show that the development of IL-10-secreting CD4 + CD25 + Treg is mediated by mature dendritic cells and allergens in a process that requires T cell costimulation via the ICOS/ICOSL pathway (28). Importantly, these “ICOS-induced” Treg suppressed the proliferation of allergen-specific RC via IL-10, supporting the hypothesis that Treg and IL-10-mediated suppression requires signaling via CD28 costimulation (29–31). De Jong and colleagues (30) also demonstrated that induction of IL-10 in Treg requires ICOS and signaling via CD28 in mice with colitis. Other results extend these findings by demonstrating that ICOS expression on Treg, defined as CD4 + CD25 high cells, in TIL of patients with melanoma correlates with high expression levels of Foxp3, CTLA-4, IL-10, and TGF-β1, the molecules that have been associated with nTreg suppressor function (7, 20, 32–34). Treg appear to comprise ICOS high and ICOS low subsets, and ICOS high Treg in TIL express the highest levels of Foxp3, CTLA-4, IL-10, and intracellular as well as membrane-bound TGF-β1. In contrast, Treg in the patient’s PBMC expressed low levels of ICOS and GITR, no IL-10, and only moderate levels of TGF-β1.

The ICOS high Treg subset had enhanced suppressor function compared with autologous ICOS low or ICOS − Treg, which might be related to higher expression of inhibitory cytokines by the former. ICOS high Treg also express very high levels of Fas, Fasl, and Granzyme B, indicating that they might be able to act as “killer” cells of CD4 + and/or CD8 + target cells. We have recently reported that human CD4 + CD25 high Foxp3 + Treg in cancer patients can induce apoptosis in autologous CD8 + T cells via the Fas/FasL pathway and in autologous CD4 + T cells via a Granzyme B-mediated pathway (12). Thus, ICOS expression on Treg in TIL appears to be associated with increased functional potential of these cells.

The biologic importance of ICOS expression on Treg in human TIL has not been evaluated so far. It now appears that the modulation of immune responses by ICOS-expressing nTreg in the tumor depends on the costimulatory signal delivered to RC and on the local cytokine milieu. We showed for the first time that CD4 + CD25 high ICOS high nTreg can induce Tr1 cells (CD4 + CD25 + CD132 IL-10 ) in autologous CD4 + CD25 + RC. In contrast, when ICOS high nTreg were co-cultured with CD4 + CD25 + RC preactivated via TCR and IL-2, proliferation of responder with a Th2 cytokine profile ensued and no Tr1 cells were detected in the cocultures. These results confirm that nonactivated CD4 + CD25 + RC are precursors of Tr1 (16) and that ICOS costimulation may be critical for their differentiation to Tr1 cells.

Cytokines are another critical component. We have reported (16) and further show in this manuscript that Tr1 precursor cells (CD4 + CD25 + ) obtained from patients with cancer are activated by IL-15 and IL-10 in the absence of TCR and/or CD28 engagement and, in contrast to CD4 + CD25 high ICOS high Treg and CD4 + RC, require only low dose of IL-2 for their differentiation and expansion. ICOS costimulation induces low levels of IL-2 and IL-10 that may be sufficient to accomplish such differentiation and expansion of naive CD4 + CD25 high T cells in the Tr1 pathway. But if the CD4 + CD25 + precursors are activated via TCR and/or CD28 engagement, ICOS reinforces this interaction by supporting signaling triggered by TCR and CD28 costimulation, as previously suggested (29). This leads to differentiation and expansion of activated CD4 + RC into effector, rather than regulatory, cells. However, in the tumor microenvironment, activated CD4 + RC that encounter ICOS may not proliferate because of the paucity of IL-2 and the excess of inhibitory cytokines. Instead, the microenvironment enriched in ICOS high Treg, which express high levels of IL-10, favors induction of Tr1 in CD4 + CD25 − T cells.

IL-10 suppresses proliferation of CD4 + RC via Stat5 up-regulation. However, Taylor and colleagues (29) have recently shown that IL-10 inhibits CD4 + T cell proliferation only in a defined range of TCR triggering, where T cells require costimulation. These findings suggest that CD4 + T cells that differentiate into Tr1 cells upon encountering ICOS high CD4 + CD25 high Tr1 cells in the tumor are suboptimally stimulated. In fact, the absence or paucity of costimulation in the presence of abundant TA at tumor sites is known to lead to tolerance (35–37). Our data are consistent with this paradigm.

This selective expansion of Tr1 from CD4 + CD25 − precursors by ICOS high nTreg in the suboptimal range of TCR engagement and IL-2 might be an important mechanism for the regulation of T lymphocyte homeostasis. When CD4 + CD25 high Tr1 have prevented an excessive expansion of T effector cells, and the immune response is declining after IL-2 has been consumed by activated T effector and nTreg, Tr1 cells might be induced as a secondary
response that prevents an excessive induction of new immune responses upon re-challenge with the Ag. This possibility is supported by our finding that when CD4+ CD25highFoxp3+ ICOShighTreg are cultured in Tr1-favorable conditions, they differentiate into CD25+ Tr1 cells that produce IL-10. Of note, Tr1 cells differentiated from CD4+ CD25highFoxp3+ ICOShighTreg have stronger suppressor activity than Tr1 induced from CD4+ CD25lowFoxp3+ ICOSlow Treg. Surprisingly, levels of cytokines in the SN of Tr1 cells originated from ICOShigh Treg were not increased in comparison to those in the SN of Tr1 cultures originated from ICOSlow Treg (Table III). However, the MFI for expression of “suppressor” cytokines in Tr1 cells that had originated from ICOShigh Treg was significantly higher compared with Tr1 cells from ICOSlow Treg. This observation suggests that direct cell-to-cell contact, even in the case of Tr1 cells that mediate suppression via cytokines, might be required to maximize the suppressor function of human Treg cells in cancer.

The observation that highly activated human nTreg present in the tumor microenvironment can “transit” to Tr1 or induce Tr1 cells in activated CD4+ CD25+ RC might explain our recent findings indicating that Tr1 cells accumulate in tumors of cancer patients and mediate strong suppression of anti-tumor immunity (7). In contrast to nTreg, few Tr1 are detected in the peripheral circulation of patients with cancer. We hypothesize that Tr1 precursor cells might be induced by or differentiate from activated nTreg during the contraction phase of an immune response, when the microenvironment provides suboptimal activation signals for CD4+ effector cells. Tr1 cells might be continuously induced in necrotic tumor areas that are poor in IL-2 and, thus, partially contribute to the autocrine-feedback loop of chronic immunosuppression in cancer patients. In tumor sections obtained from melanoma patients (Fig. 8), CD4+ CD25+ Foxp3+ ICOS+ nTreg colocalized with CD4+ CD25+ IL-10+ Tr1 cells, emphasizing their mutual involvement. Our data show that Tr1 cells originating from ICOShigh Treg express high levels of Fas but not FasL or Granzyme B, whereas Tr1 cells originating from ICOSlow Treg do not express Fas but are Fasl− and Granzyme B−. Such a distinct profile suggests that Tr1 cells originating from ICOSlow Treg might control cytokines in Tr1 cells or those differentiated from CD4+ CD25highFoxp3+ ICOSlow Treg. This observation suggests that direct cell-to-cell contact, even in the case of Tr1 cells that mediate suppression via cytokines, might be required to maximize the suppressor function of human Treg cells in cancer.

In summary, our data suggest that the modulation of the immune responses by ICOS-expressing nTreg present in the tumor depends upon costimulatory signals delivered to the RC and the cytokine milieu. Targeting of costimulatory and co-inhibitory molecules on human Treg and effector T cells with mAbs in combination with cytokine-based therapy might be a promising strategy for enhancing or decreasing peripheral tolerance in patients with autoimmune disease or cancer, respectively.

Acknowledgments
The authors have no financial conflict of interest.

Disclosures

References


