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Research Article

# FOXP3 Defines Regulatory T Cells in Human Tumor and Autoimmune Disease

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## Abstract

**Activated T cells may express FOXP3. It is thought that FOXP3 is not a specific marker to determine regulatory T cells (Treg) in humans. Here, we examined the functional phenotype and cytokine profile of the *in vitro* induced FOXP3$^+$ T cells, primary FOXP3$^+$ and FOXP3$^-$ T cells in patients with ulcerative colitis and tumors including colon carcinoma, melanoma, hepatic carcinoma, ovarian carcinoma, pancreatic cancer, and renal cell carcinoma. We observed similar levels of suppressive capacity of primary FOXP3$^+$ T cells in blood, tumors, and colitic tissues. Compared with primary FOXP3$^-$ T cells in the same microenvironment, these primary FOXP3$^+$ T cells expressed minimal levels of effector cytokines, negligible amount of cytotoxic molecule granzyme B, and levels of suppressive molecules interleukin-10 and PD-1. Although the *in vitro* activated T cells expressed FOXP3, these induced FOXP3$^+$ T cells expressed high levels of multiple effector cytokines and were not functionally suppressive. The data reinforce the fact that FOXP3 remains an accurate marker to define primary Tregs in patients with cancer and autoimmune disease. We suggest that the combination of FOXP3 and cytokine profile is useful for further functionally distinguishing primary Tregs from activated conventional T cells.** [Cancer Res 2009;69(9):3995–4000]

## Introduction

CD4$^+$CD25$^+$ regulatory T cells (Treg) play a significant role in suppressing tumor-associated antigen-specific immunity and control the pathogenesis of autoimmune diseases (1–4). FOXP3 is a useful marker to phenotypically define Tregs in mice. Mouse FOXP3$^+$ Tregs can be induced *in vitro* and *in vivo* with transforming growth factor-β (5–7). However, the *in vitro* activated human T cells may express FOXP3 (8–10). It has been suggested that FOXP3 may not be a specific marker to define Tregs in humans (8, 10). In current investigation, we compared multiple effector cytokine expression and functional phenotype in the *in vitro* activated FOXP3$^+$ T cells, primary FOXP3$^+$ versus FOXP3$^-$ T cells in patients with ulcerative colitis and patients with colon carcinoma, melanoma, hepatic carcinoma, ovarian carcinoma, pancreatic cancer, and renal cell carcinoma. Our data have shown that primary CD4$^+$FOXP3$^+$ T cells are functionally suppressive and do not express effector cytokines, and the *in vitro* induced FOXP3$^+$ cells are not functionally suppressive and express effector cytokines. The data indicate that FOXP3 accurately defines primary Tregs in patients with cancers and autoimmune disease and that the combination of FOXP3 and effector cytokine profile may be further helpful to functionally distinguish primary Tregs from *in vitro* activated FOXP3$^+$ conventional T cells.

## Materials and Methods

**Human subjects and human tissues.** We studied previously untreated ulcerative colitis patients ($n$ = 7), patients with epithelial ovarian carcinomas ($n$ = 17), melanoma ($n$ = 12), colon carcinoma ($n$ = 4), renal cell carcinoma ($n$ = 9), hepatic carcinoma ($n$ = 8), and pancreatic cancer ($n$ = 2). Patients gave written informed consent. The study was approved by local institutional review boards. Cells and tissues were obtained from blood, colitic tissues, and tumors as we described (11). The study was approved by the local institutional review boards.

**Flow cytometry analysis (fluorescence-activated cell sorting).** T cells were stimulated for 4 h with leukocyte activation cocktail in the presence of GolgiStop (BD Biosciences). These cells were first stained extracellularly with specific antibodies against human CD3, CD4, CD8, CD25, and PD-1 (BD Biosciences), fixed and permeabilized with Perm/Fix solution (E-Biosciences), and finally stained intracellularly with anti-interleukin (IL)-2, anti-IL-8, anti-IL-10, anti-granulocyte-macrophage colony-stimulating factor, anti-tumor necrosis factor-α, anti-IFN-γ, anti-granzyme B, and anti-Ki-67 (BD Biosciences) and anti-FOXP3 (eBiosciences). Samples were acquired on a LSR II (BD Biosciences) and data were analyzed with DIVA software (BD Biosciences).

**Immunofluorescence analysis.** Immunofluorescence analysis was done as described (11, 12). Tissues were stained with mouse anti-human CD4 (1:40 dilution, RPA-T4; BD Biosciences), mouse anti-human CD8 (1:40 dilution, HIT8a; BD Biosciences), and rat anti-human FOXP3 (1:80 dilution, PCH101; eBiosciences) followed by Alexa Fluor 488-conjugated goat anti-mouse IgG1 and Alexa Fluor 568-conjugated goat anti-mouse IgG2 (all 1:2,000 dilution; Molecular Probes/Invitrogen). Fluorescence images were acquired by fluorescence microscope (Leica) and analyzed by ImagePro Plus software.

**Immunosuppressive assay.** CD4$^+$CD25$^{high}$ T cells were sorted with high-speed sorter (FACSAria; BD Biosciences) from blood, tumors, and colitic tissues. The expression of FOXP3 was initially defined by fluorescence-activated cell sorting (FACS) in the sorted T cells. The number of Tregs added in the immunosuppressive assay was based on the quantification of CD4$^+$FOXP3$^+$ T cells (13). The responder T cells, CD4$^+$CD25$^-$ T cells, antigen-presenting cells, and macrophages were sorted with high-speed sorter from peripheral blood in the same healthy donors to high purity (>99%). The expression of FOXP3 was also examined in the sorted CD4$^+$CD25$^-$ T cells (<0.1% FOXP3$^+$ T cells). The immunosuppressive assay was realized as we described (12, 14–16). Briefly, CD4$^+$FOXP3$^+$ T cells (0-1.2 × 10$^5$/mL) from blood, tumors, and colitic tissues were added into the coculture system containing the same responder T cells (1.8 × 10$^5$/mL) and CD14$^+$

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

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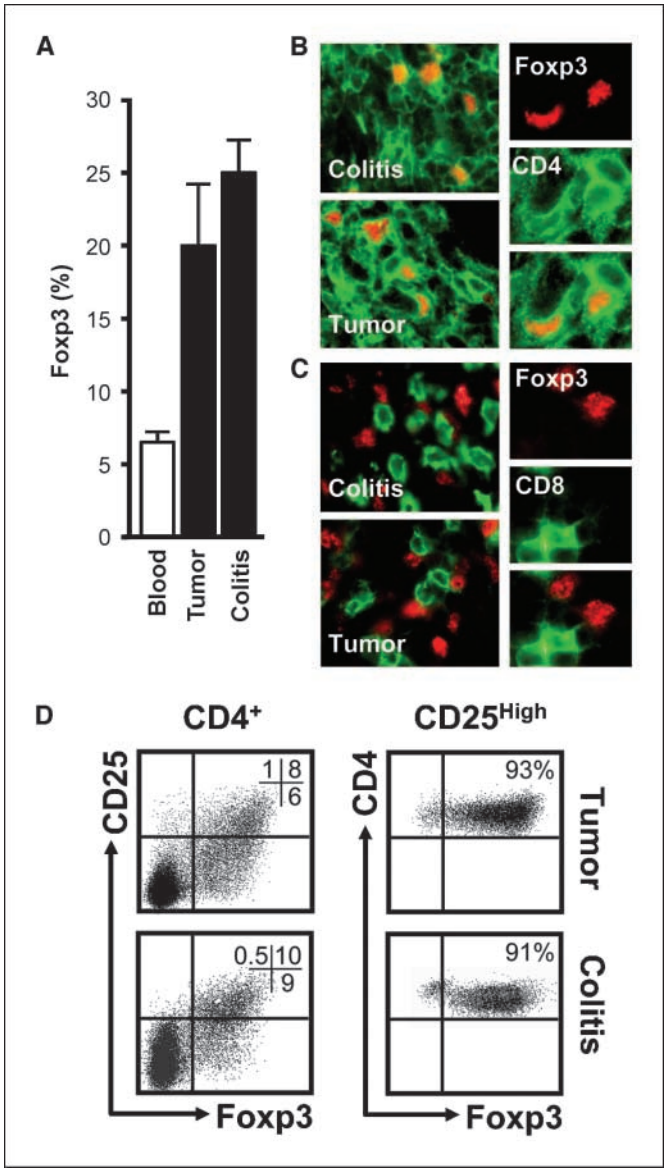


**Figure 1.** Primary FOXP3$^+$ T cells in tumor and colitic tissues. Single-cell suspensions were prepared from fresh human colon cancer and colitic tissues and stained with relevant antibodies. T-cell subsets were analyzed by FACS or immunofluorescence staining. Results were from 7 patients with colitis and 43 patients with cancer (see Table 1 and Materials and Methods). *A,* FOXP3$^+$ T cells in CD4$^+$ T cells. Mean ± SE percent of FOXP3$^+$ T cells in CD4$^+$ T cells. $P < 0.01$, compared with blood. *B* and *C,* relationship between FOXP3$^+$ and T-cell subsets. Immunofluorescence staining revealed the expression of FOXP3 (*red*), CD4 (*green*; *B*), and CD8 (*green*; *C*) in colon cancer and colitic tissues. One of seven is shown. *D,* relationship between FOXP3$^+$ and CD25$^{high}$ T cells. FACS analysis showed the expression of CD4, CD25, and FOXP3. Results are expressed as the percent of each T-cell subset in CD4$^+$ T cells. *Left,* gated on CD4$^+$ T cells; *right,* gated on CD25$^{high}$ T cells.

monocytes ($1 \times 10^5$/mL) in the presence of 2.5 μg/mL anti-human CD3 and 1 μg/mL anti-human CD28 for 3 days. T-cell proliferation was determined by thymidine incorporation. T-cell cytokines were detected in the supernatants with ELISA kit (R&D Systems).

**Induction of FOXP3 *in vitro*.** Peripheral blood CD4$^+$CD25$^-$ T cells ($5 \times 10^5$/mL) were sorted to high purity with <0.1% FOXP3 expression and activated with macrophages ($2.5 \times 10^5$/mL) and 50 ng/mL IL-2 in the presence of 7 μg/mL anti-human CD3 and 4 μg/mL anti-human CD28 for 3 days. The expression of FOXP3 and cytokines was analyzed by FACS.

**Statistical analysis.** The Wilcoxon rank-sum test was used to determine pairwise differences and the $\chi^2$ test used to determine differences between groups. $P < 0.05$ was considered as significant. Differences in phenotype of T-cell subsets were tested with the paired Student's $t$ test. All statistical analysis was done on Statistica software (StatSoft).

## Results

**Primary FOXP3$^+$ cells in patients with ulcerative colitis and cancer.** We investigated FOXP3$^+$ cells in patients with colon cancer and ulcerative colitis. We observed similar levels of CD4$^+$FOXP3$^+$ T cells in colon cancer (23 ± 6%) and colitic tissues (25 ± 3%; Fig. 1A; Supplementary Table S1). However, the levels of CD4$^+$FOXP3$^+$ T cells were significantly higher in colon cancer and colitic tissues than peripheral blood (Fig. 1*A* and *B*). Consistent with flow cytometry analysis, immunofluorescence staining revealed that, in colon cancer and colitic tissues, 98% to 99% FOXP3$^+$ cells were CD4$^+$ T cells and <1% CD8$^+$ T cells expressed FOXP3 in both tumor and colitic tissues (Fig. 1*B* and *C*; Supplementary Table S1). The expression of CD25 and FOXP3 was overlapping in both colon cancer and colitic tissues (Fig. 1*D, left*). Consistent with our

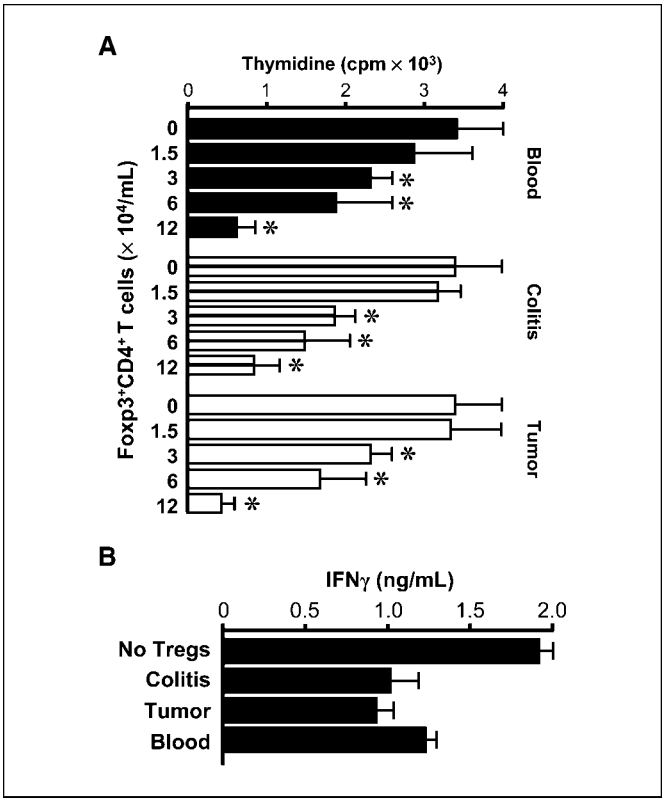


**Figure 2.** Primary FOXP3$^+$ T cells exhibit similar suppressive capacity in tumor and colitic tissues. Tregs were sorted from blood, colon cancer, and colitic tissues and subjected to an immunosuppressive assay (see Materials and Methods). Normal CD4$^+$CD25$^-$ T cells were stimulated with CD14$^+$ monocytes in the presence of anti-CD3 for 3 days with sorted Tregs. Results were from 7 patients with colitis and 4 patients with colon cancer. *A,* primary FOXP3$^+$ T cells inhibited T-cell proliferation cells. T-cell proliferation was detected by [$^3$H]thymidine incorporation on day 3. Treg concentrations were 0, 1.5, 3, 6, and $12 \times 10^4$/mL. The ratios between Tregs and responder T cells were 0, 0.08, 0.16, 0.32, and 0.64. Mean ± SD cpm. $P < 0.01$, for all, compared with controls (no Tregs). *B,* FOXP3$^+$ T cells inhibited T-cell IFN-γ production. IFN-γ was detected in the culture supernatants with ELISA kit. Mean ± SD. Treg concentration was $6 \times 10^4$/mL. $P < 0.01$, for all, compared with controls (no Tregs).

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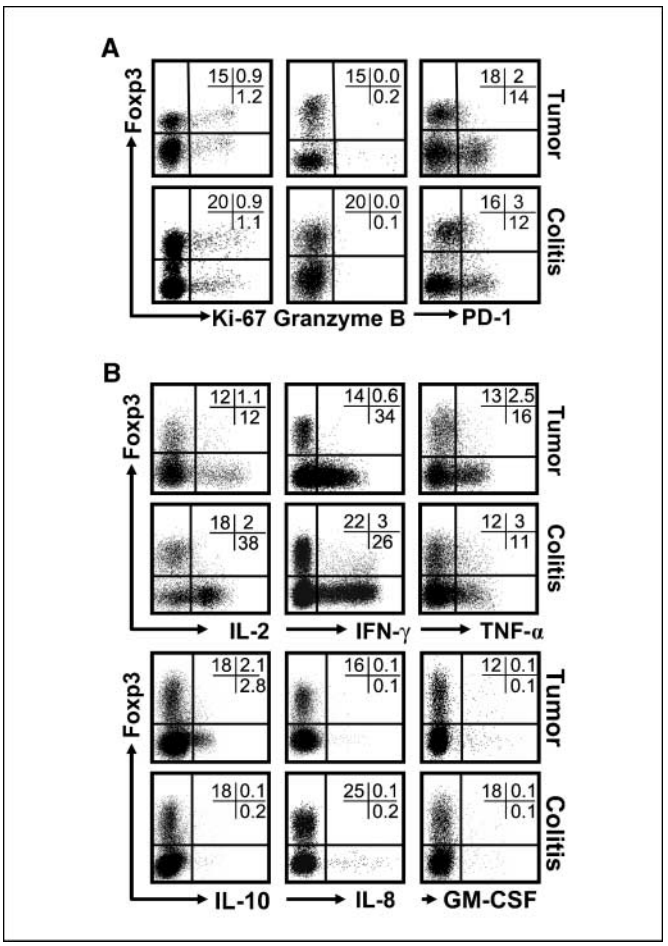


**Figure 3.** Functional phenotype and cytokine profile of primary and induced FOXP3⁺ T cells. Functional markers and cytokine profile of primary FOXP3⁺ T cells. Single-cell suspensions were prepared from fresh human colon cancer and colitic tissues and stained with relevant antibodies to define some T-cell functional markers (*A*) and cytokine profile (*B*). T-cell subsets were analyzed by FACS. Results were expressed as the percent of positive cells in CD4⁺ T cells. One representative is shown from 7 colitic tissues and 43 tumor tissues (see Table 1 and Materials and Methods).

previous observation in human ovarian cancer (12), >90% $CD4^+CD25^{high}$ T cells expressed FOXP3 in tumors and colitic tissues (Fig. 1*D, right*). Similar results were observed in hepatic carcinoma, melanoma, ovarian carcinoma, renal cell carcinoma, and pancreatic cancer (Supplementary Table S1). Altogether, the data indicate that FOXP3 expression is confined to CD4⁺ T cells, not to CD8⁺ T cells, and high expression of CD25 labels majority of the FOXP3⁺ T cells in the microenvironments of human tumor and autoimmune disease.

**Suppressor activities of primary CD4⁺FOXP3⁺ T cells in peripheral blood, colon cancer, and colitic tissues.** We next examined the suppressive capacity of CD4⁺FOXP3⁺ T cells from different compartments in patients with colon cancer and colitis. We initially sorted $CD4^+CD25^{high}$ T cells and determined the expression of FOXP3 in these sorted $CD4^+CD25^+$ T cells (Fig. 1*D, right*). The suppressive assay was subsequently realized based on the number of CD4⁺FOXP3⁺ T cells by using the responder T cells and antigen-presenting cells from the same healthy donors. We observed similar suppressions on T-cell proliferation (Fig. 2*A*) and IFN-γ production (Fig. 2*B*) mediated by FOXP3⁺ T cells sorted from blood, tumors, and colitic tissues. The suppression was dose-dependent (Fig. 2*A*). Similar results were observed in the six types of human tumors as described above. The data indicate that these primary FOXP3⁺ T cells are Tregs and functionally similar regardless of their tissue and pathological sources.

**Phenotype and cytokine profile of primary CD4⁺FOXP3⁺ T cells in tumor and colitic environment.** We examined the functional markers of primary CD4⁺FOXP3⁺ T cells in tumor and colitic environment. By gating on CD4⁺ T cells, we observed that 1% to 3% primary FOXP3⁺ T cells expressed Ki-67, granzyme B, and PD-1 in colitic and colon cancer tissues (Fig. 3*A*). By gating on primary FOXP3⁺ T cells, we showed that 4% to 6% FOXP3⁺ T cells expressed Ki-67, 2% to 4% FOXP3⁺ T cells expressed granzyme B, and ~10% FOXP3⁺ T cells expressed PD-1 in colitic and tumor tissues (except ovarian cancer). In ovarian cancer, 19% FOXP3⁺ T cells expressed PD-1 (Table 1). The expression levels of granzyme B and PD-1 were significantly higher in primary CD4⁺FOXP3⁻ T cells and CD8⁺ T cells in the same compartments (Fig. 3*A*; Table 1; Supplementary Table S1). The data suggest that granzyme B and PD-1 may not be functionally crucial for Treg-mediated suppression in human tumors and autoimmune disease.

We next compared the effector cytokine profile of primary FOXP3⁺ and FOXP3⁻ T cells in human tumors and colitis. By gating on CD4⁺ T cells, 0.1% to 3% primary FOXP3⁺ T cells expressed

**Table 1.** Phenotype and cytokine profile of CD4⁺Foxp3⁺ T cells in cancer and ulcerative colitis patients

| | Ulcerative colitis | Tumors | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Total | Colon cancer | Hepatic cancer | Melanoma | Ovarian carcinoma | Pancreatic carcinoma | Renal cell carcinoma |
| *n* | 7 | 43 | 4 | 8 | 12 | 17 | 2 | 9 |
| IL-2 | 8 ± 5 | 8 ± 2 | 5 ± 3 | 6 ± 1 | 3 ± 1 | 4 ± 4 | 8 ± 9 | 9 ± 3 |
| IL-10 | 2 ± 1 | 3 ± 1 | 2 ± 1 | 0.2 ± 1 | 3 ± 1 | 2 ± 1 | 1 ± 0 | 5 ± 5 |
| IFN-γ | 9 ± 5 | 6 ± 2 | 6 ± 2 | 5 ± 4 | 5 ± 4 | 5 ± 1 | 8 ± 4 | 6 ± 2 |
| Granzyme B | 2 ± 1 | 4 ± 2 | 2 ± 1 | 6 ± 1 | 3 ± 1 | 5 ± 2 | 2 ± 1 | 5 ± 2 |
| Ki-67 | 4 ± 2 | 6 ± 1 | 5 ± 1 | 3 ± 1 | 5 ± 1 | 5 ± 1 | 7 ± 1 | 7 ± 2 |
| PD-1 | 10 ± 7 | 13 ± 6 | 5 ± 1 | 2 ± 3 | 1 ± 2 | 19 ± 8 | 8 ± 3 | 9 ± 6 |

NOTE: Single-cell suspensions were prepared from fresh cancer and colitic tissues. Cells were stained with relevant antibodies. T-cell subsets were analyzed by FACS. Results are expressed as the percent ± SE of targeted proteins in CD4⁺FOXP3⁺ T cells.

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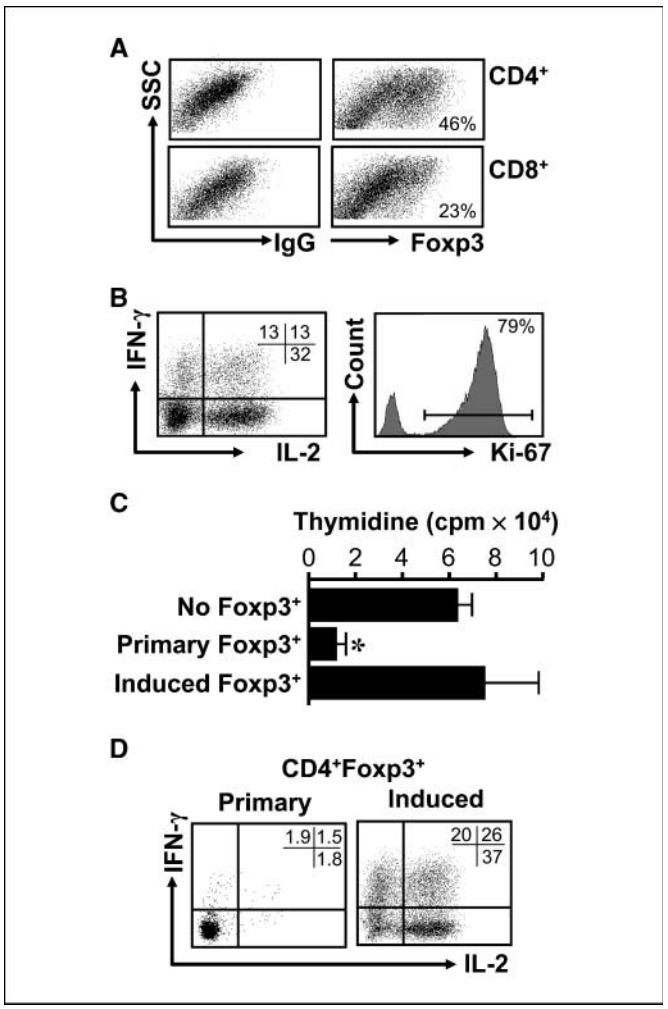


**Figure 4.** Cytokine and functional profile of the *in vitro* induced FOXP3$^+$ T cells. *A* and *B,* human normal CD4$^+$CD25$^-$ T cells or CD8$^+$ T cells were sorted to high purity (<0.1% FOXP3$^+$ cells). T cells were activated as described in Materials and Methods. Expression of FOXP3 and cytokines was detected by intracellular staining and analyzed by FACS. One of five experiments is shown. *A,* FOXP3 expression is induced in T cells. FOXP3 expression was detected by intracellular staining in the activated CD4$^+$ and CD8$^+$ T cells and analyzed by FACS. Results are expressed as the percent of FOXP3$^+$ T cells in CD4$^+$ and CD8$^+$ T cells. *B,* cytokine and Ki-67 expression in the FOXP3$^-$ T cells. Effector cytokine and Ki-67 expression was detected in the activated CD4$^+$ T cells by intracellular staining and analyzed with LSR II by gating on CD4$^+$FOXP3$^-$ T cells. Results are expressed as the percent of cytokine-expressing T cells or Ki-67–expressing T cells in CD4$^+$ T cells. *C,* primary but not the induced FOXP3$^+$ T cells inhibited T-cell proliferation cells. Immunosuppressive assay was done as described in Materials and Methods. The ratio between FOXP3$^+$ and FOXP3$^-$ T cells was 1 to 1. T-cell proliferation was detected by [$^3$H]thymidine incorporation on day 3. Mean ± SD cpm ($n$ = 5). $P$ < 0.01, compared with controls (no Tregs). *D,* cytokine expression in primary and the induced FOXP3$^+$ T cells. Primary colon tumor–infiltrating T cells ($n$ = 7) and the *in vitro* activated conventional T cells ($n$ = 5; see *A, top*) were subject to intracellular staining for FOXP3, IL-2, and IFN-γ. Data were analyzed with LSR II by gating on CD4$^+$FOXP3$^+$ T cells. Results are expressed as the percent of cytokine-expressing T cells in CD4$^+$FOXP3$^+$ T cells.

cytokines including IL-2, IL-8, IL-10, IFN-γ, tumor necrosis factor-α, and granulocyte-macrophage colony-stimulating factor (Fig. 3B). By gating on FOXP3$^+$ T cells, <10% CD4$^+$FOXP3$^+$ T cells expressed these cytokines (Fig. 3*B*; Table 1). High levels of effector cytokines were observed in CD4$^+$FOXP3$^-$ T cells and CD8$^+$ T cells in the same microenvironment. Similar cytokine profile was observed in the six human tumor types (Fig. 3*B*; Table 1; Supplementary Table S1). Notably, although there were 28% IL-10$^+$CD8$^+$ T cells in ovarian cancer, these IL-10$^+$CD8$^+$ T cells did not express FOXP3 (Supplementary Table S1). The data indicate that primary Tregs express limited effector cytokines in tumor and autoimmune environments.

**Cytokine and functional profiles of the *in vitro* induced FOXP3$^+$ T cells.** To examine the cytokine and functional profiles of the induced FOXP3$^+$ T cells derived from FOXP3$^-$ T cells, we sorted CD4$^+$CD25$^-$ T cells from healthy donors to high purity and checked the expression of FOXP3. If the expression of FOXP3 was <0.1%, these cells were used for furthering experiments. We showed that high levels of FOXP3 expression were induced in the activated CD4$^+$ and CD8$^+$ T cells (Fig. 4*A*). The *in vitro* induced FOXP3$^+$ T cells were not a distinct population (Fig. 4*A*). In the presence of 46% induced FOXP3$^+$ T cells (Fig. 4*A, top*), FOXP3$^-$ T cells highly expressed multiple effector cytokines, including IL-2 and IFN-γ (Fig. 4*B, left*), and Ki-67, a proliferating marker (Fig. 4*B, right*). The data suggest that the induced FOXP3$^+$ T cells are not able to inhibit FOXP3$^-$ T-cell activation in the culture system. In further support of this notion, we showed that 50% primary tumor FOXP3$^+$ T cells (Fig. 2*A*), but not the same amount of the *in vitro* induced FOXP3$^+$ T cells, inhibited thymidine incorporation in the effector T cells (Fig. 4*C*). We next quantified and directly compared the effector cytokine expression in primary tumor FOXP3$^+$ T cells and the *in vitro* induced FOXP3$^+$ T cells. The induced FOXP3$^+$ T cells expressed higher levels of effector cytokines, including IL-2 and IFN-γ, than primary FOXP3$^+$ T cells (Fig. 4*D*). More than 80% induced FOXP3$^+$ T cells expressed the effector cytokines (IL-2 and IFN-γ) and <6% primary FOXP3$^+$ T cells expressed these two effector cytokines (Fig. 4*D*). The data indicate that primary FOXP3$^+$ T cells and the induced FOXP3$^+$ T cells are functionally different.

## Discussion

Tregs inhibit tumor-associated antigen-specific immunity and control the pathogenesis of autoimmune diseases (1–4). FOXP3 is a useful marker to define Tregs in mice. Given that the *in vitro* activated human T cells express FOXP3 (8, 10, 17), it is questioned if FOXP3 is a marker for human Tregs. In this report, we have investigated and compared primary FOXP3$^+$ T cells, FOXP3$^-$ T cells, and the *in vitro* induced FOXP3$^+$ T cells in tumor and autoimmune disease, two typical pathologic scenarios in humans.

Our first observation is that 98% to 99% primary FOXP3$^+$ T cells are CD4$^+$ T cells and <1% CD8$^+$ T cells express FOXP3 in colitic tissues and six types of human cancers. These primary FOXP3$^+$ T cells are functionally suppressive Tregs. It is thought that FOXP3$^+$CD8$^+$ T cells may be CD8$^+$ Tregs in humans (17, 18). Although FOXP3$^+$CD8$^+$ T cells can be induced *in vitro* (this report; refs. 8, 17), our data indicate that this subset is a minor population *in vivo* in patients with cancer and colitis. We have described previously that plasmacytoid dendritic cells induce IL-10$^+$CD8$^+$ Treg in patients with ovarian cancer (19, 20). However, although substantial amount of primary IL-10$^+$CD8$^+$ Treg is detected in patients with ovarian cancer (this report; ref. 20), these primary IL-10$^+$CD8$^+$ Treg do not express FOXP3 (this report). Primary IL-10$^+$FOXP3$^-$CD8$^+$ T cells may be a functionally relevant CD8$^+$ Treg population in human ovarian cancers (19, 20). The biological significance of the minor FOXP3$^+$CD8$^+$ T-cell population remains to be clarified in human tumors.

We have reported previously that human Tregs sorted from tumors, peripheral blood, and tumor draining lymph nodes exhibit

similar suppressor capacity in the *in vitro* functional assay (12). $CD4^+CD25^+$ T cells isolated from patients with inflammatory bowel diseases, including ulcerative colitis, are functionally suppressive (21–24). Early studies suggest that Tregs may be numerically reduced and functionally defective *in vitro* in patients with certain autoimmune diseases (25–27). Interestingly, our data indicate that, regardless of the tissue sources, Tregs manifest similar suppressive activities *in vitro* in patients with ulcerative colitis and cancer. In addition to different disease settings, this apparent discrepancy may be partially due to technical evolution. The initial *in vitro* suppressive assay was based on the purified $CD4^+CD25^+$ T cells (25–27). It is understandable that there may be sizable amount of $CD4^+CD25^+$ effector T cells in patients with autoimmune diseases. Therefore, these autoimmune disease-associated $CD4^+CD25^+$ T cells may exhibit a reduced suppressor activity (25–27). Now, the specific antibody against human FOXP3 is available. After sorting $CD4^+CD25^{high}$ T cells, we are able to examine the expression of FOXP3 in these sorted T cells, and our suppressive assays are subsequently realized based on the number of $CD4^+FOXP3^+$ T cells. Further, to minimize the potential variations among responder T cells from different donors, we have used the responder T cells from the same healthy donors with minimal levels of FOXP3 expression. Thus, our modified assay may be more reliable to define the "true" Treg suppressor activity. Of course, it remains arguable whether these primary $FOXP3^+$ T cells are equally suppressive *in vivo* in the different microenvironments including human tumors and autoimmune diseases.

To further define these Tregs in the microenvironments of human tumor and autoimmune disease, we have examined their functional markers and cytokine profiles. Approximately 5% Tregs express Ki-67, a proliferating marker, indicating active but limited Treg proliferation in the microenvironments of human tumor and autoimmune disease. Tregs may induce apoptosis of effector T cells and antigen-presenting cells by granzyme B pathway (28), and B7-H1/PD-1 pathway inhibits tumor immunity (29, 30). As most of primary Tregs do not express granzyme B and PD-1, the data suggest that these two pathways may not be crucial for Treg functionality in these patients.

Conventional T cells, but not Tregs, are thought to express effector cytokines. Polyfunctional cytokine profile (IL-2, IFN-γ, and tumor necrosis factor-α) has been described in effector T cells in infectious diseases (31). However, multiple effector cytokines have not been examined in combination with intracellular FOXP3 expression per single-cell basis in human colitic and tumor tissues. Further, it remains unknown if the cytokine profile has been altered in Tregs in pathologic environment in humans including tumors and autoimmune diseases. We have compared the effector cytokine profile of primary $FOXP3^+$ and $FOXP3^-$ T cells in human tumors and colitis. In contrast to $CD4^+FOXP3^-$ T cells and $CD8^+$ T cells, primary $CD4^+FOXP3^+$ T cells expressed minimal amount of effector cytokines. This observation is consistent with a recent report on patients with melanoma (32). Although the effector cytokine-expressing $FOXP3^+$ T cells are a minor population among primary $CD4^+FOXP3^+$ T cells, it may be important to further examine whether this minor population is functionally relevant in tumor and autoimmune disease. Nonetheless, the data indicate that primary Tregs differed from conventional T cells in the context of effector cytokine profile in tumor and autoimmune environments.

Intracellular FOXP3 has been used as a surrogate marker to define Tregs in humans and mice (33–35). Recent studies suggest that FOXP3 may not be a specific marker to define Tregs in humans. *In vitro* activated T cells express FOXP3 (8, 10). Our work has shown three differences between primary $FOXP3^+$ T cells and the *in vitro* induced $FOXP3^+$ T cells: (*a*) primary $FOXP3^+$ T cells are a distinct $CD4^+$ T-cell population. Less than 1% primary $CD8^+$ T cells express FOXP3 *in vivo* in patients with tumors and colitis. However, high levels of FOXP3 can be equally induced in $CD4^+$ T cells and $CD8^+$ T cells *in vitro*. The induced $FOXP3^+$ T cells are not a distinct population. (*b*) Less than 10% primary $FOXP3^+$ T cells express polyfunctional cytokines, whereas >80% induced $FOXP3^+$ T cells express polyfunctional cytokines. (*c*) Primary $FOXP3^+$ T cells are functionally suppressive, whereas the induced $FOXP3^+$ T cells are not. Therefore, although FOXP3 alone can not be used to define human Treg cells in the *in vitro* settings, FOXP3 remains an useful marker to investigate primary Tregs in human pathology including tumor and autoimmune disease.

In summary, our current report has examined primary $FOXP3^+$ T cells in patients with colitis and tumors. Our data show that primary $FOXP3^+$ T cells are functionally suppressive and barely express effector cytokines per single-cell basis in the microenvironments of human tumor and autoimmune disease. This feature is distinct from the *in vitro* activated $FOXP3^+$ T cells. The data reinforce the fact that FOXP3 remains an useful marker to define primary Tregs in patients with cancer and autoimmune disease. We suggest that the combination of FOXP3 and multiple cytokine profile is useful for further functionally distinguishing Tregs from activated conventional T cells.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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