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IL-17$^+$ Regulatory T Cells in the Microenvironments of Chronic Inflammation and Cancer

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Foxp3$^+$CD4$^+$ regulatory T (Treg) cells inhibit immune responses and temper inflammation. IL-17$^+$CD4$^+$ T (Th17) cells mediate inflammation of autoimmune diseases. A small population of IL-17$^+$Foxp3$^+$CD4$^+$ T cells has been observed in peripheral blood in healthy human beings. However, the biology of IL-17$^+$Foxp3$^+$CD4$^+$ T cells remains poorly understood in humans. We investigated their phenotype, cytokine profile, generation, and pathological relevance in patients with ulcerative colitis. We observed that high levels of IL-17$^+$Foxp3$^+$CD4$^+$ T cells were selectively accumulated in the colitic microenvironment and associated colon carcinoma. The phenotype and cytokine profile of IL-17$^+$Foxp3$^+$CD4$^+$ T cells was overlapping with Th17 and Treg cells. Myeloid APCs, IL-2, and TGF-β are essential for their induction from memory CCR6$^+$ T cells or Treg cells. IL-17$^+$Foxp3$^+$CD4$^+$ T cells functionally suppressed T cell activation and stimulated inflammatory cytokine production in the colitic tissues. Our data indicate that IL-17$^+$ Foxp3$^+$ cells may be "inflammatory" Treg cells in the pathological microenvironments. These cells may contribute to the pathogenesis of ulcerative colitis through inducing inflammatory cytokines and inhibiting local T cell immunity, and in turn may mechanistically link human chronic inflammation to tumor development. Our data therefore challenge commonly held beliefs of the anti-inflammatory role of Treg cells and suggest a more complex Treg cell biology, at least in the context of human chronic inflammation and associated carcinoma. ***The Journal of Immunology*****, 2011, 186: 4388–4395.**

The relationship between chronic inflammation and cancer has been noted for centuries. It is estimated that at least 15–20% of cancers are associated with inflammation (1, 2). A typical example of this relationship is chronic ulcerative colitis and the associated colon cancer in humans. In the United States, colitis affects 4–6 of 100,000 people. In this population, the risk of colon cancer is 5-fold above the incidence of sporadic colon cancer, reaching upwards of 18% after 30 y disease (3, 4). Despite the clinical relationship between ulcerative colitis and colon cancer, the molecular and cellular links between infiltrating immune cells, inflammation, and cancer development are poorly understood in humans.

In the current study we focused on patients with chronic ulcerative colitis and colon carcinoma. We have investigated the phenotype, cytokine profile, generation, and functions of CD4$^+$ T cell subsets in the microenvironment of chronic ulcerative colitis. To our knowledge, our results provide the first evidence showing that colitic IL-17$^+$Foxp3$^+$ cells are functionally "inflammatory" regulatory T cells in the chronic inflammatory microenvironments. These cells may play unique roles in the pathogenesis of chronic inflammation and the development of inflammation-associated cancer in patients.

Materials and Methods

Human subjects and human tissues

We studied chronic ulcerative colitis patients, patients with colitic-associated colon carcinoma, melanoma, ovarian cancer, and renal cell carcinoma. Ulcerative colitic tissues were from patients with ulcerative colitis presenting for prophylactic colectomy or/and diagnostic biopsy sampling. The diagnosis of ulcerative colitis was determined according to established guidelines based on endoscopic, histopathological, and radiological criteria. Patients who had received no treatment with immunosuppressive agents were recruited for this study. Tissue sampling for inflamed lesions and adjacent tissues was directed toward the same colonic or ileal segment, if possible. There usually is not any "absolutely normal" tissue in ulcerative colitis or colitis-associated colon carcinoma. We took grossly "normal" tissue from at least 5 cm away from active colitic and cancer locations. These normal tissues were ideal inflammatory controls for colitic tissues. Crohn's disease was not included in the study. Psoriatic skin biopsies were obtained from patients with psoriasis, as we previously reported (5). Patients gave written, informed consent. Cells and tissues were obtained from blood and human tissues as we described (6). The study was approved by the local Institutional Review Boards.

Flow cytometry analysis (FACS)

For intracellular cytokine detection, cells were stimulated with PMA (0.5 μg/ml) and ionomycin (0.5 μg/ml) in the presence of monensin (GolgiStop; BD Biosciences) and brefeldin A (GolgiPlug; BD Biosciences) for 4 h. The cells were first stained extracellularly with specific Abs against human CD3, CD4, CD8, CD25, CD45RA, CD45RO, CD49, CD161, CCR4, and CCR6 (BD Biosciences) and then were fixed and permeabilized with Perm/Fix solution (eBioscience) and finally were stained

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Abbreviation used in this article: Treg, regulatory T.

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intracellularly with anti–IL-2, anti–TNF-α, anti–IFN-γ (BD Biosciences), and anti-FOXP3 (eBioscience). Samples were acquired on a LSR II (BD Biosciences) and data were analyzed with Diva software (BD Biosciences).

Immune suppression assay

$CD4^+CD25^+$ T cells were enriched with $CD25^+$ magnetic beads. $CD4^+CD25^{bright}$ 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 FACS in the small aliquot from the sorted $CD4^+CD25^{bright}$ T cells. The number of regulatory T (Treg) cells added in the immune suppression assay was based on the quantification of Foxp3 (not CD25) in the sorted viable $CD4^+CD25^{bright}$ T cells (7). In some cases T cell clones were used. The responder T cells and the APCs, macrophages, were enriched from peripheral blood. The immune suppression assay was performed as we described (8). Briefly, different numbers of tested cells ($CD4^+Foxp3^+$ T cells or T cell clones) were added into the coculture system containing the same responder T cells and APCs for 3 d. T cell proliferation was determined by thymidine incorporation. T cell cytokines were detected in the supernatants with ELISA kit (R&D Systems).

Quantitative real-time PCR

Total RNA was isolated with Qiagen reagent. The RNA was reverse transcribed into cDNA using oligo(dT) primers and SuperScript II reverse transcriptase (Invitrogen), according to the manufacturer's instructions. The primer sequence combinations spanned contact sequences of subsequent exons. For amplification, the SyberGreen qPCR mix was used (Invitrogen). Each reaction was run in triplicate on the Mastercycler machine (Eppendorf) and was normalized to housekeeping gene GAPDH transcripts.

T cells clones

Peripheral $CD4^+$ T cells were isolated from two independent donors with negative enrichment method through magnetic bead selection (RosetteSep; StemCell Technologies, Vancouver, BC, Canada). T cells were expanded and cloned with 2.5 μg/ml anti-CD3 and 1.25 μg/ml anti-CD28 mAb (BD Biosciences) and 5 ng/ml rhIL-2 (R&D System) in the presence of irradiated allogeneic PBMCs as feeders as previously described (9). The clones were tested for Foxp3 and IL-17 expression by FACS and used for functional experiments.

Inflammatory cytokines in the colitic environments

Single-cell suspensions were made from fresh ulcerative colitis tissues. Colitic $CD4^+CD25^{bright}$ T cells (10^6/ml) were sorted to high purity and activated with anti-CD3 and anti-CD28 beads for 48 h. The supernatants were collected and then cultured with autologous ulcerative colitic colon epithelial cells (2×10^6/ml) for 24 h with or without anti-human IL-17 receptor. Colon epithelial cells were defined by FACS as $lineage^-EpCam^+$ nonimmune cells. The production of cytokines was analyzed by ELISA kits (R&D Systems).

Statistical analysis

The Wilcoxon signed-rank test was used to determine pairwise differences, and the Mann–Whitney *U* test was used to determine differences between groups (10). A p value < 0.05 was considered as significant. All statistical analysis was done on Statistica software (StatSoft, Tulsa, OK) and is further described in the figure legends.

Results

$IL\text{-}17^+Foxp3^+$ cells in ulcerative colitis and colitis-associated colon carcinoma

T cell activation and infiltration has been thought to play a major contribution to the pathogenesis of ulcerative colitis (11). However, the detailed phenotype of T cell subsets and their roles in human ulcerative colitis are poorly understood. A minor population of $IL\text{-}17^+Foxp3^+$ cells was detected in the peripheral blood in healthy humans (12, 13). We analyzed the populations of $CD4^+Foxp3^+$ (Treg), $CD4^+IL\text{-}17^+$ (Th17), and $IL\text{-}17^+Foxp3^+$ T cell subsets in the mucosa and peripheral blood in patients with ulcerative colitis and colitis-associated colon carcinoma and other cancers. In line with previous reports (8, 14), moderate levels of blood Treg cells (Fig. 1*A*) and high levels of tissue Treg cells were detected in the colonic mucosa from patients with ulcerative colitis (Fig. 1*B*) and colitis-associated colon carcinoma (Fig. 1*C*), as well as in the tissues of ovarian cancer, renal cell carcinoma, and melanoma (Fig. 1*D*). Although the levels of Th17 cells were generally lower than Treg cells, we detected higher levels of Th17 cells in the mucosa of colitis and of colon carcinoma (Fig. 1*B*, 1*C*) and moderate levels of Th17 cells in other types of tumor we examined (Fig. 1*D*). Interestingly, a significant population of $IL\text{-}17^+Foxp3^+$ T cells was observed exclusively in the mucosa of colitic colon tissues and colitic-associated colon carcinoma, not in the adjacent tissues (Fig. 1*B*, 1*C*, 1*E*). The levels of $IL\text{-}17^+Foxp3^+$ T cells were minimal (<0.3%) in other types of cancer, including ovarian cancer, melanoma, and renal cell carcinoma (Fig. 1*D*, 1*E*), as well as in normal peripheral blood (<0.3%) (Fig. 1*A*, 1*E*) and blood from colitic patients (<0.3%) (data not shown). To determine whether $IL\text{-}17^+Foxp3^+$ T cells are generally associated with human autoimmune diseases, we further examined $IL\text{-}17^+Foxp3^+$ T cells in patients with psoriasis. We detected minimal $IL\text{-}17^+Foxp3^+$ T cells (<0.3%) in the psoriatic skin tissues (Supplemental Fig. 1). We sorted $CD4^+CD25^{high}$ cells from different tissues to high purity, sampled Foxp3 expression from the sorted

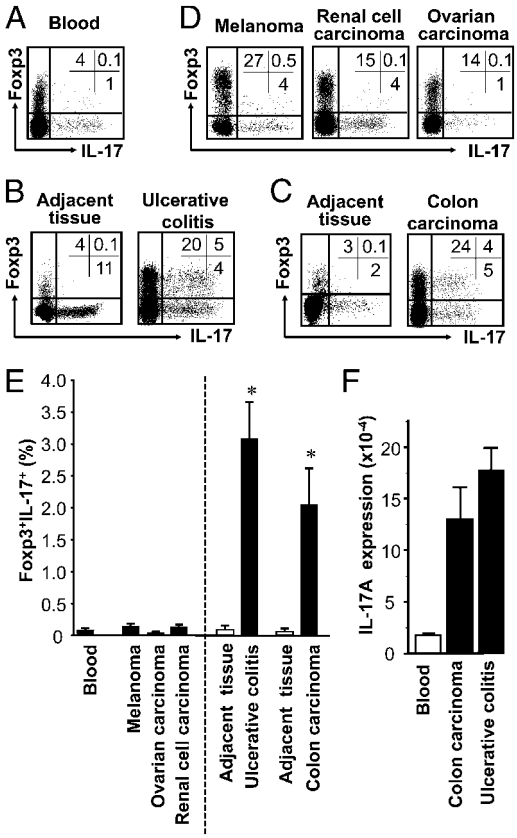

FIGURE 1. $IL\text{-}17^+Foxp3^+$ T cells in colitic environments. *A–E*, Single-cell suspensions were made from fresh healthy donor blood, colitic tissues, and tumor specimens. The cells were subjected to membrane and intracellular staining and analyzed by FACS. Results are expressed as the percentage of each population in $CD4^+$ T cells. *F*, Colitic Treg cells expressed high levels of IL-17 transcripts. Sorted colitic Treg cells were activated with anti-CD3 and anti-CD28 Abs for 48 h and subject to real-time PCR. Results are expressed as the mean values of IL-17A gene expression ± SD from three independent experiments (blood, $n = 10$; ulcerative colitis, $n = 8$; ulcerative colitis-associated colon cancer, $n = 9$; ovarian cancer tissues, $n = 17$; melanoma tissues, $n = 12$; renal cell carcinoma, $n = 9$). *$p < 0.01$ compared with other groups as determined by Mann–Whitney *U* test.

cells, and used cells with high levels of Foxp3 expression for further experiments (see *Materials and Methods*). Real-time PCR revealed 6- to 10-fold higher levels of IL-17 transcripts in CD4$^+$ CD25^{high} cells from colon cancer and ulcerative colitis than that from blood (Fig. 1*F*). After activation with anti-CD3 and anti-CD28 in the presence of APCs for 72 h, these colon cancer tissue-derived CD4$^+$CD25^{high} cells (2×10^6/ml) released high levels of IL-17 (119 ± 67 ng/ml). The data indicate that IL-17$^+$ Foxp3$^+$ T cells are selectively associated with chronic colon inflammation and inflammation-associated carcinoma, and they actively express and release high levels of IL-17.

Phenotype and cytokine profile of colitic IL-17$^+$Foxp3$^+$ cells

To investigate the functional relevance of IL-17$^+$Foxp3$^+$ T cells, we initially examined their phenotype and compared their phenotype with that of IL-17$^-$Foxp3$^+$ Treg cellsand IL-17$^+$Foxp3$^-$ Th17 cells in the same colitic environment. Human Treg cells express high levels of CCR4 and CD25 (8). We found that IL-17$^+$Foxp3$^+$ T cells and Treg cells expressed similar levels of CD25 (Fig. 2*A*) and CCR4 (Fig. 2*B*) in the colitic environment (8) ($p > 0.05$). It is not surprising that these two populations expressed significantly higher levels of CD25 and CCR4 than did IL-17$^-$Foxp3$^-$ cells (Fig. 2*A*, 2*B*) ($p < 0.05$). Human tissue Th17 cells express high levels of CD161 and CD49 (15). Similar to Th17 cells, colitic IL-17$^+$Foxp3$^+$ T cells expressed high levels of CD161 and CD49d (Fig. 2*C*, 2*D*) ($p > 0.05$). The levels of CD161 and CD49d were higher in IL-17$^+$Foxp3$^+$ T cells and Th17 cells than in IL-17$^-$ cells (Fig. 2*C*, 2*D*) ($p < 0.05$). The intergrin molecules may be associated with their migration and retention within chronic inflammatory environment (5). Limited IL-17$^+$Foxp3$^+$ T cells were detected in peripheral blood. Hence, we acquired large numbers of cells and analyzed the phenotype of these IL-17$^+$Foxp3$^+$ T cells. We observed a similar phenotype for IL-17$^+$Foxp3$^+$ T cells in peripheral blood (data not shown) as compared with the colitic environment. These data indicate that IL-17$^+$Foxp3$^+$ T cells phenotypically overlap with Treg and Th17 cells in the same chronic inflammatory environment.

We next analyzed the effector cytokine profile of colitic IL-17$^+$ Foxp3$^+$ T cells and compared it with that of Treg and Th17 cells in the same environment. Human tumor-infiltrating Treg cells expressed limited effector cytokines (8), and Th17 cells (15) expressed high levels of polyfunctional effector cytokines, including IFN-γ and IL-2. Similar to Th17 cells, colitic IL-17$^+$Foxp3$^+$ T cells expressed a substantial amount of IFN-γ and IL-2 (Fig. 2*E*, 2*F*). The levels of IFN-γ and IL-2 were higher in IL-17$^+$Foxp3$^+$ T cells than in Treg cells (Fig. 2*E*, 2*F*) ($p < 0.05$). Thus, the cytokine profile of IL-17$^+$Foxp3$^+$ T cells differed from that of Treg cells in the same microenvironment. To determine whether this cytokine profile is tissue specific, we performed identical analyses in peripheral blood IL-17$^+$Foxp3$^+$ T cells. Colitic and blood IL-17$^+$Foxp3$^+$ T cells shared similar cytokine profiles (Fig. 2*G*, 2*H*). These data indicate that IL-17$^+$Foxp3$^+$ T cells express effector cytokines and are distinct from Treg cells in the same inflammatory environment.

APCs, TGF-β, and IL-2 are essential for the induction of IL-17$^+$Foxp3$^+$ cells

We next investigated how IL-17$^+$Foxp3$^+$ T cells were generated. We initially investigated the general conditions to induce IL-17$^+$ Foxp3$^+$ cells from the CD4$^+$ T cell population. We found that myeloid APCs including CD14$^+$ macrophages and CD11$^+$ myeloid dendritic cells induced appreciable levels of IL-17$^+$Foxp3$^+$ T cells without exogenous cytokines. In the absence of myeloid APCs, IL-17$^+$Foxp3$^+$ cells were barely induced (Fig. 3*A*). Furthermore, multiple combinations of the Th17–cytokine mixture failed to stimulate the development of IL-17$^+$Foxp3$^+$ cells in the absence of APCs (Fig. 3*B*). The data indicate that APCs are essential for the development of IL-17$^+$Foxp3$^+$ cells. We further

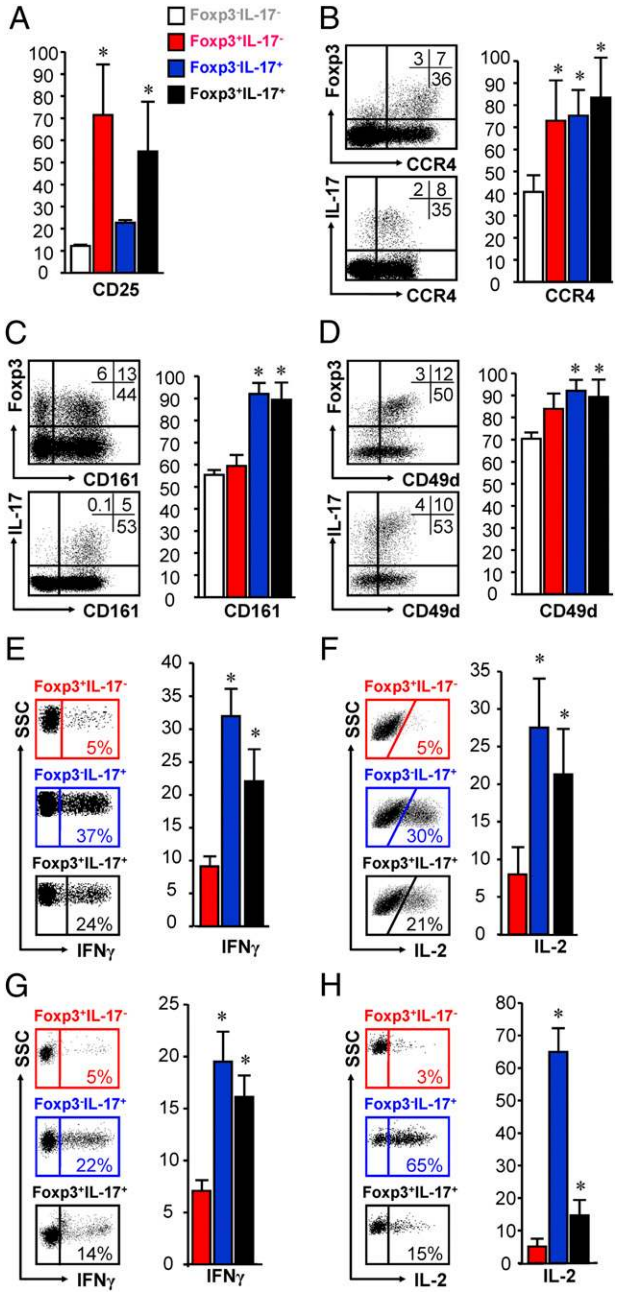

FIGURE 2. Phenotype and cytokine profile of IL-17$^+$Foxp3$^+$ T cells. Single-cell suspensions were made from fresh blood or ulcerative colitic tissues. The cells were subject to membrane and intracellular staining and analyzed by FACS. Results are expressed as the percentage of each population ± SEM in the given CD4$^+$ T cells. Foxp3$^-$IL-17$^-$ (open bars), Foxp3$^+$IL-17$^-$ (Treg cells, red bars), Foxp3$^-$IL-17$^+$ (Th17 cells, blue bars), and Foxp3$^+$IL-17$^+$ (inflammatory Treg cells, black bars) are shown. *A–D*, Phenotype of Foxp3$^+$IL-17$^+$ cells. The expression of CD25 (*A*), CCR4 (*B*), CD161 (*C*), and CD49 (*D*) was analyzed and compared among different T cell populations. *E–H*, The cytokine profile was analyzed by intracellular staining. Colitic T cells ($n = 8$) (*E*, *F*) and healthy donor blood T cells ($n = 17$) (*G*, *H*) are shown. For *A–D*, $*p < 0.01$ compared with Foxp3$^-$IL-17$^-$ cells as determined by Wilcoxon signed-rank test ($n = 10$). For *E–H*, $*p < 0.05$ compared with Foxp3$^+$IL-17$^-$ cells as determined by Wilcoxon signed-rank test.

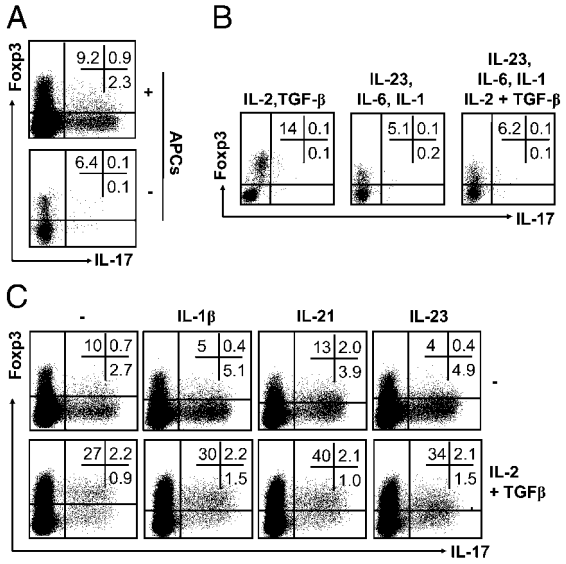

FIGURE 3. Induction of IL-17$^+$Foxp3$^+$ T cells in the presence of APCs, TGF-β, and IL-2. *A* and *B*, APCs induced IL-17$^+$Foxp3$^+$ T cells. Normal blood CD4$^+$ T cells (5×10^5/ml) were stimulated with anti-CD3 and anti-CD28 in the presence or absence of blood CD14$^+$ monocytes (2.5×10^5/ml) for 3 d (*A*) or with different cytokine cocktails in the absence of APCs (*B*). The stimulated T cells were analyzed by FACS. Results are expressed as the percentage of IL-17$^+$Foxp3$^+$ T cells in CD4$^+$ T cells. Similar results were observed in 10 donors. *$p < 0.01$ between the groups with and without APCs as determined by Wilcoxon signed-rank test. *C*, TGF-β and IL-2 induced maximal IL-17$^+$Foxp3$^+$ T cells in the presence of APCs. Blood CD4$^+$ T cells were stimulated with anti-CD3, anti-CD28, APCs, and cytokine combinations for 3 d. The stimulated T cells were analyzed by FACS. Results are expressed as the percentage of given T cell subset in CD4$^+$ T cells ($n = 8$ donors; controls, no TGF-β and IL-2). *$p <$ 0.01 between the groups with and without TGF-β and IL-2 as determined by Wilcoxon signed-rank test for IL-17$^+$Foxp3$^+$ T cells; $p > 0.05$ between the groups with APCs plus TGF-β plus IL-2 and the groups APCs plus TGF-β plus IL-2 plus IL-1 or IL-21 or IL-23.

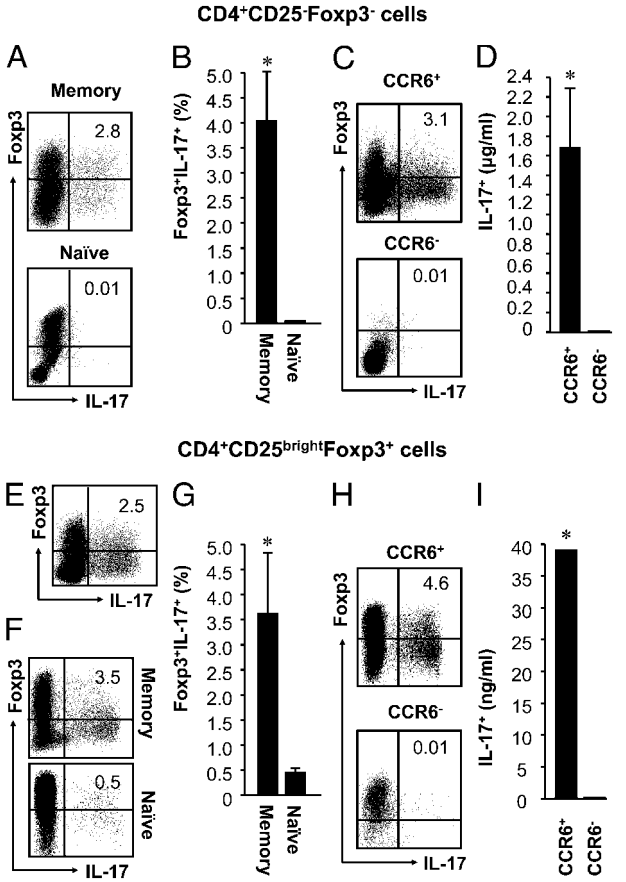

FIGURE 4. Induction of IL-17$^+$Foxp3$^+$ T cells with conventional T cells (*A–D*) and Treg cells (*E–I*). Conventional CD25$^-$Foxp3$^-$CD4$^+$ T cells were sorted into memory versus naive T cell subsets (*A*, *B*) or CCR6$^+$ versus CCR6$^-$ cells (*C*, *D*). CD25^{bright}Foxp3$^+$ Treg cells (*E*) were also sorted into memory versus naive cells (*F*, *G*) or CCR6$^+$ versus CCR6$^-$ cells (*H*, *I*). All of these T cell subsets were stimulated with optimal stimuli for IL-17$^+$Foxp3$^+$ T cells for 3 d. The stimulated T cells were analyzed by FACS. Results are expressed as the percentage of IL-17$^+$Foxp3$^+$ T cells in CD4$^+$ T cells ($n = 10$–15 donors). *$p < 0.01$ as compared with each control as determined by Mann–Whitney *U* test.

tested the potential stimulatory effects of a variety of Th17-associated cytokine combinations on the induction of IL-17$^+$ Foxp3$^+$ cells in the presence of APCs. We observed that TGF-β and IL-2 were the optimal combination to maximize the yield of IL-17$^+$Foxp3$^+$ cells (Fig. 3*C*) (*$p < 0.01$ compared with no TGF-β and IL-2). Addition of IL-1, IL-21, and IL-23 did not increase the yield of IL-17$^+$Foxp3$^+$ cells and of IL-17-Foxp3$^+$ cells (Fig. 3*C*, Supplemental Fig. 2*A*). However, in the presence of IL-1, IL-21, and IL-23, TGF-β and IL-2 were able to retain the high levels of Foxp3 expression (Supplemental Fig. 2*B*) (*$p < 0.01$), but they reduced the generation of Th17 cells (IL-17$^+$Foxp3$^-$ cells) (Supplemental Fig. 2*C*) (*$p < 0.01$ compared with no TGF-β and IL-2). The data indicate that myeloid APCs, TGF-β, and IL-2 are the optimal stimuli for the induction of IL-17$^+$Foxp3$^+$ cells.

IL-17$^+$Foxp3$^+$ cells are induced from memory CCR6$^+$ T cells

We next examined the potential differentiation of IL-17$^+$Foxp3$^+$ cells from multiple T cell subsets. Conventional CD25$^-$Foxp3$^-$ CD4$^+$ T cells (Fig. 4*A–D*) were sorted into naive versus memory CD4$^+$ T cells as we described (see *Materials and Methods*). We showed that in the presence of optimal IL-17$^+$Foxp3$^+$ cell polarization conditions (Fig. 3*C*), IL-17$^+$Foxp3$^+$ cells were induced from memory but not naive T cells (*$p < 0.01$) (Fig. 4*A*, 4*B*).

Th17 cells are enriched in CCR6$^+$ T cells (5, 15–17). It is possible that CCR6$^+$ T cells are the precursors of IL-17$^+$Foxp3$^+$ cells. To test this possibility, conventional CCR6$^+$CD4$^+$ and CCR6$^-$CD4$^+$ T cells were sorted from conventional CD25$^-$ T cells. We showed that IL-17$^+$Foxp3$^+$ cells were preferentially induced from CD4$^+$CCR6$^+$ T cells, but not from CCR6$^-$ cells (*$p <$ 0.01) (Fig. 4*C*). The induced IL-17$^+$Foxp3$^+$ cells produced large amounts of IL-17 (Fig. 4*D*). CCR6$^+$ cells were basically memory T cells (not shown). The data support that CCR6$^+$ memory conventional T cells are the preferential precursors of IL-17$^+$Foxp3$^+$ T cells.

Previous studies have shown that human Treg cells can be converted into Th17 cells after stimulation under various conditions (18–20). To test whether Treg cells can be induced into IL-17$^+$ Foxp3$^+$ cells, CD4$^+$CD25^{high} T cells were sorted to high purity. If the sorted cells expressed >95% Foxp3 in the small samples, the cells were used in the experiments. We showed that IL-17$^+$ Foxp3$^+$ cells could be efficiently generated from CD4$^+$CD25^{high} Foxp3$^+$ Treg cells (Fig. 4*E*). We further divided the Treg cells into CD45RO$^+$ memory versus CD45RA$^+$ naive populations. We showed that IL-17$^+$Foxp3$^+$ cells could be generated from memory and naive Treg cells (Fig. 4*F*, 4*G*). However, memory Treg cells were more efficiently induced into IL-17$^+$Foxp3$^+$ cells (*$p < 0.01$) (Fig. 4*F*, 4*G*). We further examined CCR6$^+$ versus CCR6$^-$ Treg cells. CCR6$^+$ but not CCR6$^-$ Treg cells were induced into IL-17$^+$ Foxp3$^+$ cells (*$p < 0.01$) (Fig. 4*H*). The induced IL-17$^+$Foxp3$^+$ cells produced high levels of IL-17 (*$p < 0.01$) (Fig. 4*I*).

Collectively, the data indicate that IL-17$^+$Foxp3$^+$ cells are preferentially generated from memory CCR6$^+$ T cells, including Treg and conventional T cells.

Colitic Treg cells including IL-17$^+$Foxp3$^+$ cells are immunosuppressive

We next examined and compared the suppressive capacity of Treg cells from different compartments in patients with colon cancer and colitis. We initially enriched and sorted CD4$^+$CD25^{high} T cells to high purity and determined the expression of Foxp3 and IL-17 in the sorted CD4$^+$CD25^{high} T cells (Fig. 5*A*). The suppressive assay was assessed utilizing CD4$^+$Foxp3$^+$ T cells and responder T cells from the same donors. We observed similar suppressions on T cell proliferation (Fig. 5*B*) and IFN-γ production (Fig. 5*C*) by Foxp3$^+$CD4$^+$ T cells from blood, tumors, and colitic tissues. The suppression was dose-dependent (Fig. 5*B*). Given that there were 30% IL-17$^+$ cells in the colitic Foxp3$^+$ population, the data suggest that colitic Treg cells including IL-17$^+$Foxp3$^+$ T cells are immunosuppressive. To further support this possibility, we made IL-17$^+$Foxp3$^+$, IL-17$^-$Foxp3$^+$, and IL-17$^+$Foxp3$^-$ T cell clones from the same donors. We tested the suppressive capacity of these T cell clones using the identical T cell responders. We observed that IL-17$^+$Foxp3$^+$ and IL-17$^-$Foxp3$^+$ T cell clones similarly suppressed T cell proliferation. IL-17$^+$Foxp3$^-$ T cell clones had no suppressive effects ($^*p < 0.01$ compared with control) (Fig. 5*D*). The data strongly suggest that IL-17$^+$Foxp3$^+$ T cells are functional Treg cell populations.

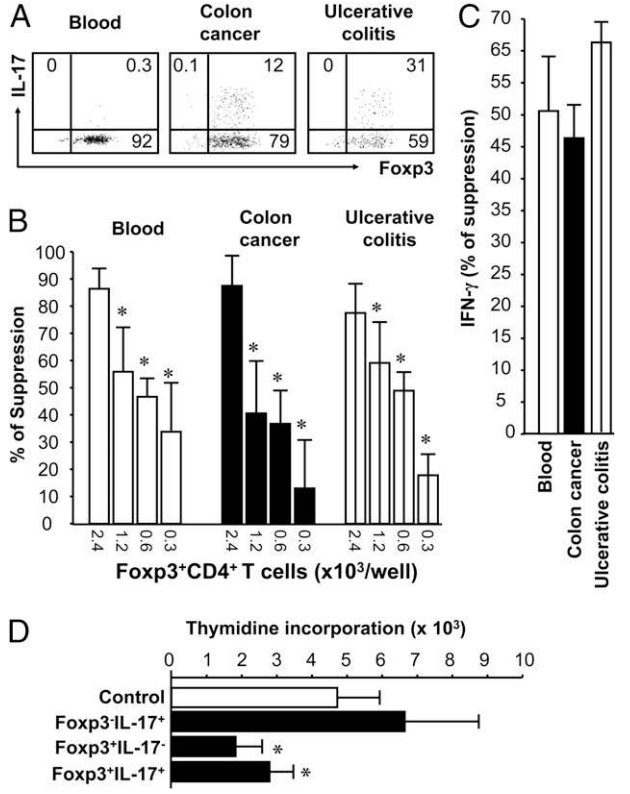

FIGURE 5. Foxp3$^+$ T cells including IL-17$^+$Foxp3$^+$ T cells are immunosuppressive. *A*, Sorted Foxp3$^+$ and IL-17$^+$Foxp3$^+$ T cells. CD25^{high} cells were sorted from bead-enriched CD4$^+$CD25$^+$ T cells, and the levels of Foxp3 and IL-17 expression were determined by intracellular staining. The sorted Treg cells were used for the immunosuppressive assay. *B* and *C*, Foxp3$^+$ T cells are equally immunosuppressive regardless of the sources and the levels of IL-17 expression. T cell suppression assay was performed in the presence or absence of different concentrations of Treg cells as we described (8). T cell activation was determined by thymidine incorporation (*B*, *D*) and IFN-γ production (*C*). Results are expressed as the percentage of suppression in thymidine incorporation (*B*) and IFN-γ production (*C*). $^*p < 0.05$ compared with no Treg cells as determined by Wilcoxon signed-rank test ($n = 6$). The suppressed IFN-γ production was shown when the Treg cell/T cell ratio was 1:1. *D*, Inhibitory activity of IL-17$^+$Foxp3$^+$ T cell clones. IL-17$^+$Foxp3$^-$, IL-17$^-$Foxp3$^+$, and IL-17$^+$Foxp3$^+$ T cell clones were generated from peripheral blood cells in two donors. Suppressive assays were conducted in triplicates with normal responder T cells from three donors. Results are expressed as the mean values ± SEM. $^*p < 0.05$ compared with control as determined by Wilcoxon signed-rank test. The ratio between T cell clones/responder T cells was 1:2.

Colitic Treg cells express functional IL-17 and promote inflammation

To further define the inflammatory functionality of IL-17$^+$Foxp3$^+$ T cells, we sorted and activated colitic Treg cells. Autologous colitic cells were exposed to culture medium (control) or supernatants from colitic Treg cells for a short time. In the absence of colitic Treg cells, colitic colon epithelial cells produced sizable IL-1β and IL-6 levels (Fig. 6*A*, 6*B*). Blood Treg cells expressed minimal IL-17 (Fig. 1*A*). Blood Treg cells failed to induce inflammatory cytokines (not shown). The levels of IL-1β and IL-6 were increased in the presence of coltic Treg cell supernatants (Fig. 6*A*, 6*B*). Blockade of IL-17 receptor reduced the production of IL-1β and IL-6 stimulated by colitic Treg cells (Fig. 6*A*, 6*B*). Consistent with this observation, we showed that colitic Treg cells expressed high levels of IL-17 (Fig. 1*A*). Furthermore, higher levels of IL-17 were detected in ulcerative colitis tissues, as compared with "normal" colon tissues (Fig. 1*C*). We detected low levels of IL-17 in the sera in patients with ulcerative colitis, which were slightly higher than that in the sera of healthy donors (not shown). The data indicate that Th17 and IL-17$^+$Foxp3$^+$ T cells may play an active role in the local environment.

Discussion

In the current study, we have investigated the nature of IL-17$^+$ Foxp3$^+$ T cells in patients with ulcerative colitis.

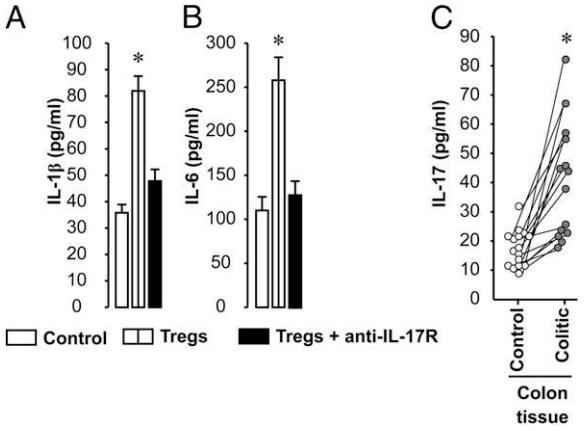

FIGURE 6. Coltic Treg cells express IL-17 and are functionally inflammatory. *A* and *B*, Treg cells induced IL-1 and IL-6 production by primary colitic cells through IL-17. Primary colitic cells were cultured with colitic Treg cell-derived supernatants for 12 h in the presence or absence of anti–IL-17 receptor. IL-1β and IL-6 were detected in the cell supernatants by ELISA. Results were expressed as the mean values of ± SEM. $^*p < 0.05$ compared with controls by Wilcoxon signed-rank test ($n = 8$). *C*, IL-17 in colitic environment in patients with ulcerative colitis. Single-cell suspensions were made from colitic and control tissues. The cells were cultured for 24 h. IL-17 was measured in the supernatants. Results are expressed as IL-17 value in each individual donor $p < 0.05$, compared with controls by Wilcoxon signed-rank test ($n = 17$).

$CD4^+Foxp3^+$ Treg cells play a significant role in suppressing tumor-associated Ag-specific immunity, tempering inflammation, and controlling the pathogenesis of autoimmune diseases (14, 21–24). Foxp3 in combination with cytokine profiles are useful markers to phenotypically define primary human Treg cells in tumor and inflammatory tissues (7, 25, 26). In contrast, Th17 cells represent a different inflammatory component and play an active role in inflammation (27–35) and cancer (36). Interestingly, recent studies suggest considerable levels of plasticity between different T cell lineages, and they point toward potent peripheral regulation of effector T cell subset development in the specific microenvironment (37, 38). For instance, peripheral mature Treg cells can be converted into Th17 cells, an event favored by inflammation in the context of IL-6 production (13, 38–40). In support of this possibility, a minor population of $IL\text{-}17^+Foxp3^+$ cells can be observed in the tonsils (13) and peripheral blood (12, 13, 41, 42) in humans, as well as in the small intestine in mice (43). However, it is unknown whether $IL\text{-}17^+Foxp3^+$ cells can be detected in the pathological setting, and, if so, what is their phenotype, cytokine profile, generation requirements, and functional relevance in humans?

To our knowledge, we have observed for the first time that substantial numbers of $IL\text{-}17^+$ regulatory T cells selectively accumulate in the mucosa of ulcerative colitis and colon cancer carcinoma. This population is barely visible in the peripheral blood and in multiple human tumors, including ovarian cancer, breast cancer, melanoma, and renal cell carcinoma. The distribution suggests that this T cell population may play a role in the chronic inflammatory environment of mucosa and colon.

We have analyzed the phenotype and cytokine profile of $Foxp3^+$ $IL\text{-}17^+CD4^+$ T cells. $Foxp3^+IL\text{-}17^+CD4^+$ T cells share the trafficking phenotype with Treg and Th17 cells. Such analysis suggests that $Foxp3^+IL\text{-}17^+$, Th17, and Treg cells may apply similar mechanisms for their homing to and retention in the microenvironment. However, different from Treg cells, $Foxp3^+IL\text{-}17^+CD4^+$ T cells express moderate levels of effector cytokines. This suggests that these cells are not a typical Treg subset and may play a distinct role in the chronic environment as discussed below. It has been reported that human Treg cells express CD39 and suppress Th17 cell development through an adenosinergic pathway (15, 42). Interestingly, the levels of CD39 expression are similar between $IL\text{-}17^+Foxp3^+$ and $IL\text{-}17^-Foxp3^+$ cells in patients with ulcerative colitis (data not shown). If $IL\text{-}17^+Foxp3^+$ cells were converted from $IL\text{-}17^-Foxp3^+$ cells, CD39 might not suppress this conversion.

We have also addressed how $Foxp3^+IL\text{-}17^+CD4^+$ T cells are generated. APCs induce T cell polarization. We have demonstrated that myeloid APCs are essential for the induction of $Foxp3^+IL\text{-}17^+CD4^+$ T cells. In support of this, psoriatic (5) and tumor environmental myeloid APCs (15) promote Th17 cells from memory T cells. We have reasoned that similar to Th17 cell polarization, Th17-associated cytokine cocktails may promote $Foxp3^+IL\text{-}17^+CD4^+$ T cells. We have observed that the combination of TGF-β, IL-2, and APCs is the optimal stimulus to induce $Foxp3^+IL\text{-}17^+CD4^+$ T cells. Although IL-1, IL-21, and IL-23 promote Th17 cell development, these cytokines have no additional effects on the induction of $Foxp3^+IL\text{-}17^+CD4^+$ T cells. As expected, TGF-β and IL-2 also induce Treg cells and retain Foxp3 expression (7, 44, 45), and they reduce Th17 cells (46). This may explain why $Foxp3^+IL\text{-}17^+CD4^+$ T cells are phenotypically and functionally overlapping with Treg cells.

Given that $Foxp3^+IL\text{-}17^+CD4^+$ T cells may possibly be differentiated in the chronic inflammatory environment, we have thoroughly examined the precursor cells of this population. We have first shown that memory but not naive T cells are the precursors of $Foxp3^+IL\text{-}17^+CD4^+$ T cells. As human Th17 cells highly express CCR6 (15, 47), and CCR6 cells are thought to be the precursors of Th17 cells (47), we have further demonstrated that $Foxp3^+IL\text{-}17^+CD4^+$ T cells can be preferentially differentiated from $CCR6^+$ T cells. Consistent with previous reports (12, 13), highly purified $Foxp3^+$ Treg cells can be differentiated into $Foxp3^+IL\text{-}17^+CD4^+$ T cells. Furthermore, memory Treg cells or $CCR6^+$ Treg cells are significantly more efficiently induced into $Foxp3^+IL\text{-}17^+CD4^+$ T cells. As true naive T cells may not exist in the colitic environment, although $Foxp3^+IL\text{-}17^+CD4^+$ T cells may be recruited into local inflammatory environments from peripheral blood and other organs, the data strongly support the possibility that $Foxp3^+$ $IL\text{-}17^+CD4^+$ T cells are induced from the memory compartment in the inflammatory environment. The data also raise certain concerns about the potential application of Treg cells used in the treatment of patients with autoimmune diseases. If Treg cells are able to be converted into $Foxp3^+IL\text{-}17^+CD4^+$ T cells in the inflammatory environment, these cells may accelerate, rather than dampen, autoimmune diseases. Our functional studies support this possibility.

Although it is challenging to investigate the functional relevance of $Foxp3^+IL\text{-}17^+CD4^+$ T cells in vivo in patients with ulcerative colitis and colon carcinoma, we have primarily addressed this important issue in ex vivo and in vitro functional assays. First, we found that colitic $Foxp3^+IL\text{-}17^+CD4^+$ T cells express moderate levels of multiple effector cytokines, including IL-2, IFN-γ, and TNF-α. This cytokine profile reveals a phenotype for polyfunctional effector T cells similar to that observed in patients with infectious diseases (48, 49). We have observed this phenotype for tumor-infiltrating Th17 cells in multiple human cancer types (15). It suggests that colitic $Foxp3^+IL\text{-}17^+CD4^+$ T cells may be a "special" effector T cell population expressing multiple effector cytokines. These cytokines may directly and indirectly enhance inflammation in the colitic environment. Second, we have sorted these cells with high purity and tested their effects on the signature inflammatory gene expression described in ulcerative colitis. To our surprise, we have found that Treg cells induce, rather than suppress, the production of IL-1 and IL-6 by colitic cells. Blocking IL-17 receptor signaling results in a significant inhibition of IL-1 and IL-6 production in colitic cells. The data indicate that functional IL-17 is derived from $Foxp3^+IL\text{-}17^+CD4^+$ T cells as an importation portion of the Treg cell population. In line with this possibility, elevated inflammatory cytokines are detected in the fresh colitic environment. It has been reported that recombinant IL-17 induces IL-6 expression in other systems (50–53). As IL-1 and IL-6 play crucial roles in ulcerative colitis (11, 54–57), it is likely that IL-17–expressing T cells, including Th17 cells and $Foxp3^+IL\text{-}17^+CD4^+$ T cells, promote the production of inflammatory cytokines and potentially contribute to early tumorigenesis associated with immunopathogenesis of ulcerative colitis in patients.

In support of this possibility, recent mouse studies demonstrate that $CD4^+$ T cells may promote mammary epithelial cell malignancy (58). Notably, although the link between inflammation and cancer has been noted for >100 y, only recently have investigators started to address the cellular, molecular, and genetic causal relationships between inflammation and cancer. Compelling evidence has demonstrated that inflammation orchestrates the microenvironment around tumors, contributing to the proliferation, migration, and survival of cancer cells that can result in tumor invasion, migration, and metastasis (1, 2). However, inflammatory reactions in the tumor microenvironment are an important component of the tumor-associated immune response. Inflammatory

(immune) cells and molecules may have crucial roles in initiating and maintaining protective anti-tumor immunity (22, 36). As we have demonstrated in patients with chronic colitis in the current study, the specific nature of the inflammatory (immune) response and the tissue context may determine the beneficial versus the detrimental impact of inflammation on tumor pathology. Therefore, in addition to colitis-associated colon cancer, our study may be important for understanding the pathogenesis of other inflammation-associated human cancers, including hepatitis-associated hepatocellular carcinoma, atrophy gastritis-associated gastric cancer, and pancreatitis-associated pancreatic carcinoma.

In summary, human IL-17$^+$Foxp3$^+$ cells induce proinflammatory cytokines and suppress T cell function, exhibiting the dual inflammatory and regulatory function. These inflammatory regulatory T cells are induced from memory CCR6$^+$ T cells and Treg cells, and they may contribute to promoting active inflammation and tumor development through multiple modes of action in the local environment.

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Disclosures

The authors have no financial conflicts of interest.

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