ORIGINAL RESEARCH

Inflammatory regulatory T cells in the microenvironments of ulcerative colitis and colon carcinoma

Ilona Kryczek, Lin Wang, Ke Wu, Wei Li, Ende Zhao, Tracy Cui, Shuang Wei, Yan Liu, Yin Wang, Linda Vatan, Wojciech Szeli, Joel K. Greenson, Jacek Rolinski, Witold Zgodzinski, Emina Huang, Kaixiong Tao, Guobin Wang, and Weiping Zou

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ABSTRACT
Foxp3^+ CD4^+ regulatory T (Treg) cells are thought to express negligible levels of effector cytokines, and inhibit immune responses and inflammation. Here, we have identified a population of IL-8^+ Foxp3^+ CD4^+ T cells in human peripheral blood, which is selectively increased in the microenvironments of ulcerative colitis and colon carcinoma. Phenotypically, this population is minimally overlapping with IL-17^+ Foxp3^+ CD4^+ T cells, and is different from IL-8^+ Foxp3^+ CD4^+ T cells in the same microenvironment. 40–60% of IL-8^+ Foxp3^+ CD4^+ T cells exhibit naive phenotype and express CD127, whereas IL-8^+ Foxp3^+ CD4^+ cells are basically memory T cells and express minimal CD127. The levels of CXCR5 expression are higher in IL-8^+ Foxp3^+ cells than in IL-8^+ Foxp3^− cells. IL-2 and TGFβ induce IL-8^+ Foxp3^+ T cells. Exogenous Foxp3 expression promotes IL-8^+ Foxp3^+ T cells and inhibits effector cytokine IFNγ and IL-2 expression. Furthermore, Foxp3 binds to IL-8 proximal promoter and increases its activity. Functionally, IL-8^+ Foxp3^+ T cells inhibit T cell proliferation and effector cytokine production, but stimulate inflammatory cytokine production in the colon tissues, and promote neutrophil trafficking through IL-8. Thus, IL-8^+ Foxp3^+ cells may be an "inflammatory" Treg subset, and possess inflammatory and immunosuppressive dual biological activities. Given their dual roles and localization, these cells may be in a unique position to support tumor initiation and development in human chronic inflammatory environment.

Introduce

Appropriate trafficking and retention are indispensable for immune cells to mediate efficient immune responses in vivo. Emerging evidence demonstrates that CD4^+CD25^+ Treg cells traffic to the microenvironments of infectious diseases, autoimmune diseases, tumors, and organ transplantation, where they act to inhibit immunopathogenesis. It is generally thought that Treg cells suppress immune responses 2-7 and temper inflammation. However, large numbers of suppressive Treg cells are often observed in active inflammatory environments such as human ulcerative colitis. We hypothesized that, in addition to their known capacity of inhibiting immune responses, Treg cells might also mediate or support inflammation in the chronic inflammatory environment, where their phenotype and functionality might be altered. To test this hypothesis, we chose patients with chronic ulcerative colitis and colon carcinoma as our research models, and examined the phenotype, cytokine profile, and inflammatory potentials of Treg cells in the diseased tissues. Our results provide the first evidence showing that a functional IL-8^+Foxp3^+ cell population exists in the microenvironments of ulcerative colitis and colon carcinoma. This subset of Treg cells is not only immune suppressive but also induces key inflammatory cytokines and mediates neutrophil trafficking. Therefore, IL-8^+Foxp3^+ cells may be previously unappreciated "inflammatory" Treg cells, and contribute to the pathogenesis of chronic inflammation in patients with ulcerative colitis and in turn possibly promote the initiation and development of ulcerative colitis-associated colon carcinoma.

Results

IL-8^+ CD4^+ Foxp3^+ T cells in the microenvironments of ulcerative colitis and colon carcinoma

In line with our previous observation, we detected high levels of CD4^+CD25^+Foxp3^+ Treg cells in ulcerative colitis, colon carcinoma, and ovarian carcinoma (Fig. 1A).
Limited numbers of Treg cells were also found in the tissues adjacent to active colitis, colon cancer, and ovarian cancer (Fig. 1A). Interestingly, we observed relatively high levels of IL-8^CD4^Foxp3^T cells in the mucosa of colitic colon tissues and colon carcinoma, as well as in their adjacent tissues (Figs. 1B, C). The levels of IL-8^Foxp3^T cells were 3–5-fold smaller in peripheral blood, ovarian carcinoma, renal cell carcinoma, and breast cancer than in colitic colon tissues and colon carcinoma (Figs. 1B, C).

Macrophages may be the major IL-8 producers. However, based on our knowledge, it is unknown which cell types are the in situ major IL-8 producers in colon mucosa in patients with ulcerative colitis and colon cancer. Multiple color immune fluorescence analysis revealed that Foxp3^T cells and Foxp3^CD3^-cells expressed IL-8 in colitic mucosa (Fig. 1D). However, there were more IL-8^Foxp3^T cells (4.2 ±1.2 per high-power field) than IL-8^Foxp3^-cells (1.3 ± 0.3 per high-power field) (Fig. 1D). We sorted tumor infiltrating CD25^T cells (enriched with Treg cells) and CD25^-immune cells (contained macrophages) in patients with colon carcinoma, and compared their IL-8 production from the same colon cancer tissues with or without in vitro activation. We showed that tumor infiltrating CD25^-immune cells and CD25^Treg cells spontaneously produced IL-8. However, the levels of IL-8 were higher in fresh CD25^Treg cells than fresh CD25^-immune cells (Fig. 1E). After activation with anti-CD3 and anti-CD28 antibodies, CD25^Treg cells released higher levels of IL-8 than CD25^-immune cells (Fig. 1E). The data indicates that colon mucosa Treg cells may be one of the most important sources for IL-8. We further sorted CD4^-CD25^-high T cells from colitic tissues and peripheral blood in patients with ulcerative colitis and colon carcinoma, and IL-8^Foxp3^T cells may affect chronic inflammation and T cell immunity in colon mucosa in humans.
Phenotype and cytokine profile of colon cancer and colitic IL-8\textsuperscript{+}Foxp3\textsuperscript{+} cells

We next compared the phenotype of IL-8\textsuperscript{+}Foxp3\textsuperscript{+} and IL-8\textsuperscript{+}Foxp3\textsuperscript{+} Treg cells. Naïve T cells express minimal amount of functional cytokines. We thought that IL-8\textsuperscript{+}Foxp3\textsuperscript{+} cells might be enriched in memory T cell population. To our surprise, nearly 50% IL-8\textsuperscript{+} Treg cells were CD45RA\textsuperscript{+} and CD127\textsuperscript{+} populations (Figs. 2A, B). We further examined multiple chemokine receptors and integrin molecules which are associated with Treg and Th17 cell tissue trafficking in humans, including CCR4, CCR5, CCR6, CXCR4, CD161, CD49D, and CD49F. IL-8\textsuperscript{+}Foxp3\textsuperscript{+} and IL-8\textsuperscript{+}Foxp3\textsuperscript{+} Treg cells expressed similar levels of these molecules. However, the levels of CXCR5 were higher in IL-8\textsuperscript{+}Foxp3\textsuperscript{+} than IL-8\textsuperscript{+}Foxp3\textsuperscript{+} Treg cells (Fig. 2C). The data indicate that IL-8\textsuperscript{+} cells can be naïve and memory Treg subsets, and CXCR5 may be involved in IL-8\textsuperscript{+} Treg tissue trafficking.

We next analyzed the effector cytokine profile, and the relationship between IL-8 and IL-17 in IL-8\textsuperscript{+} Treg cells. We found that IL-8\textsuperscript{+} and IL-8\textsuperscript{+} Treg cells minimally expressed the effector cytokine IFN\textgreekg (Fig. 2D). Furthermore, IL-8\textsuperscript{+} and IL-17\textsuperscript{+} cells were two distinct Treg subsets in blood and colitis (Fig. 2E). Consistent with their phenotype, we detected moderately higher levels of IL-8 mRNA in naïve than memory Treg cells. However, the levels of IL-17 mRNA were moderately lower in naïve than memory Treg cells (Fig. 2F). Based on the phenotype and cytokine profile, we conclude that IL-8\textsuperscript{+}Foxp3\textsuperscript{+} cells are different from Th17 cells and IL-17\textsuperscript{+}Foxp3\textsuperscript{+} cells, and may be a previously unappreciated Treg subset.

Figure 2. Phenotype and cytokine profile of IL-8\textsuperscript{+}Foxp3\textsuperscript{+} T cells. Single cell suspensions were made from fresh blood or ulcerative colitic tissues. The cells were subjected to membrane and intracellular staining and analyzed by FACS. (A–C). IL-8\textsuperscript{+}Foxp3\textsuperscript{+} T cells are in the naïve and memory Treg subset. The expression of CD45RA (A), CD127 (B), and CXCR5 (C), is shown in CD4\textsuperscript{+}Foxp3\textsuperscript{+} T cells. One of six experiments is shown. (D, E). Effector cytokines in IL-8\textsuperscript{+} Treg cells. The expression of IFN\textgreekg (D), IL-8, and IL-17 (E) was determined by intracellular staining in IL-8\textsuperscript{+} Treg cells. Results were expressed as the percentage of cytokine expressing cells in Treg cells. Gated on CD4\textsuperscript{+}Foxp3\textsuperscript{+} cells (A–C). One of eight is shown. (F). IL-8 and IL-17 transcripts in naïve and memory Treg cells. Naïve CD45RA\textsuperscript{+} and memory CD45RA\textsuperscript{−} Treg cells were sorted. The expression of IL-8 and IL-17 was determined by real time PCR in the sorted Treg subsets. Results were expressed as the mean relative values ± SEM. n = 5, p < 0.05.
cells (Fig. 3B, lower panel) to high purity, and determined the expression of Foxp3 in the small aliquots of the sorted cells from different donors. If Foxp3 expression was less than 0.5% in the sorted CD4+CD25− T cells (Fig. 3B, upper panel) and higher than 95% in the sorted CD4+CD25high T cells (Fig. 3B, lower panel), these cells were utilized for functional assays (Fig. S1). The suppressive assay was assessed utilizing CD4+Foxp3+ T cells and responder T cells from the same donors. We observed similar and dose dependent suppressions on T cell proliferation by Foxp3+CD4+ T cells from blood, tumors, and colitic tissues with or without IL-8 neutralization (Fig. 3C). As more than 50% IL-8+Foxp3+ Treg cells were in the CXCR5+ population (Fig. 2C), we sorted CXCR5+ and CXCR5− Treg cells, and compared their suppressive activity. Both CXCR5+ and CXCR5− Treg cells exhibited similar suppressive capacity on T cell proliferation (Fig. 3D) and IFNγ production (Fig. 3E). It suggests that IL-8+Foxp3+ T cells are functionally suppressive. To confirm this possibility, we made IL-8−Foxp3+, IL-8+Foxp3−, and IL-8+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-8+Foxp3+ and IL-8+Foxp3− T cell clones similarly suppressed T cell proliferation. IL-8+Foxp3− T cell clones had no suppressive effects (Fig. 3F). The data strongly suggest that IL-8+Foxp3+ T cells are functional Treg populations.

**Colon cancer and colitic IL-8+CD4+Foxp3+ T cells are functionally inflammatory**

We next examined the inflammatory potential of colitic IL-8+CD4+Foxp3+ T cells. We first observed that colitic Treg cell-derived supernatants induced high levels of IL-1 and IL-6 by colon epithelial cells. IL-8 blockade partially and significantly reduced these effects of colitic Treg cells (Figs. 4A, B). Neutrophils are often recruited into the inflammatory environment including colitic tissues. However, it is poorly understood how neutrophils infiltrate the chronic ulcerative colitic environment. We reasoned that colitic Treg cells might promote neutrophil trafficking. In support of this hypothesis, colitic Treg supernatants, but not blood Tregs, mediated potent neutrophil migration. Importantly, the migration was blocked by neutralization of IL-8 (Fig. 4C). In addition to immune suppression (Fig. 3)9, the current data provides strong evidence that colitic Treg cells promote inflammatory via their induction of key inflammatory cytokines and recruitment of neutrophils into the ulcerative colitic environment through IL-8.

**Cytokine regulation of the development of IL-8+Foxp3+ T cells**

We further investigated how IL-8+Foxp3+ T cells were generated. As TGFβ and IL-2 induce and maintain human Foxp3
expression,\textsuperscript{15,16} we tested the effects of these two cytokines on the development of IL-8\textsuperscript{+}Foxp3\textsuperscript{+} T cells. The percentage of IL-8\textsuperscript{+}Foxp3\textsuperscript{-} T cells was minimal (<1\%) in fresh blood T cells (Fig. 5). Activation with anti-CD3 and anti-CD28 moderately increased the percentage of IL-8\textsuperscript{+}Foxp3\textsuperscript{+} cells as compared to fresh T cells (Fig. 5). However, TGF\beta and IL-2, but not IL-1, IL-6, IL-21, and IL-23, independently induced IL-8\textsuperscript{+}Foxp3\textsuperscript{+} cells in a dose dependent manner, as demonstrated by increased percentages of IL-8\textsuperscript{+}Foxp3\textsuperscript{+} cells in CD4\textsuperscript{+} T cells (Figs. 5A, B). It is well known that TGF\beta and IL-2 induce and maintain Foxp3 expression in T cells. It is possible that IL-8\textsuperscript{+}Foxp3\textsuperscript{+} cells are increased due to increased Foxp3 expression in T cells. However, we showed that the percentages of IL-8\textsuperscript{+} cells in Foxp3\textsuperscript{+} cells were also increased by TGF\beta and IL-2 (Figs. 5C, D). It suggests that IL-8 is induced in Foxp3\textsuperscript{+} T cells. The data indicate that TGF\beta and IL-2 induce IL-8\textsuperscript{+}Foxp3\textsuperscript{+} cells.

**Mechanisms controlling the development of IL-8\textsuperscript{+} Foxp3\textsuperscript{+} T cells**

Finally, we examined whether Foxp3 directly regulated IL-8 expression in T cells. To this end, we transfected CD4\textsuperscript{+}Foxp3\textsuperscript{-} T cells with a lentiviral vector expressing Foxp3, and examined the resulting cytokine production. We showed that ectopic Foxp3 expression increased IL-8\textsuperscript{+}Foxp3\textsuperscript{+} T cells (Fig. 6A) and reduced the production of IL-2, IFN\gamma, and TNF\alpha (Fig. 6B). Interestingly, ChIP assays demonstrated that Foxp3 was capable of binding to IL-8 proximal promoter (Fig. 6C). Furthermore, luciferase experiments revealed that Foxp3 increased IL-8 promoter activities (Fig. 6D). We further closely examined the relationship between the levels of Foxp3 and IL-8 in fresh colitic Treg cells. The levels of IL-8 and Foxp3 expression were correlated per single cell basis (Fig. 6E). The data indicate that Foxp3 may promote IL-8 expression in Treg cells.

**Discussion**

Treg cells have been thought to be broadly immune suppressive and anti-inflammatory.\textsuperscript{3,4,17} To our surprise, we have observed for the first time substantial numbers of IL-8\textsuperscript{+} Treg cells in the colon mucosa of patients with ulcerative colitis and colon carcinoma. This population is detectable in normal peripheral blood.\textsuperscript{18} IL-8\textsuperscript{+} Treg cells release high levels of IL-8, induce inflammatory cytokines, and promote neutrophil migration. Increased inflammatory cytokine production and neutrophil infiltration are two features of the pathogenesis of ulcerative colitis.\textsuperscript{14,19-22} In addition to neutrophil, IL-8 may recruit myeloid derived suppressor cells (MDSCs) into the colon cancer microenvironment. Our data challenges the generally held view that Treg cells suppress inflammation, and suggests that Treg cells may accelerate inflammation in the microenvironments of colitis and colon carcinoma. Additionally, we have demonstrated that colitic and colon cancer Treg cells\textsuperscript{9} and IL-8\textsuperscript{+} Treg cells inhibit adaptive T cell immunity. Altogether, the data indicates that Treg cells possess dual biological activities: inhibiting adaptive immunity and stimulating inflammation. Given the obvious link between inflammation and cancer development,\textsuperscript{23} and the critical immune suppressive roles of Treg cells in the tumor microenvironment in humans,\textsuperscript{2,17} one may reason that IL-8\textsuperscript{+} Treg cells may play a complex and unique role in tumor initiation and development in chronic inflammatory environments. In support of this possibility, it has been reported that IL-8,\textsuperscript{24} Th22 cells\textsuperscript{25} and MDSCs\textsuperscript{26} promote human cancer stem cell development. We speculate that IL-8\textsuperscript{+} Treg cells may potentially affect colon cancer stem cells through IL-8 production. Future studies will determine the relationship between IL-8\textsuperscript{+} Treg cells and patient pathological and clinical outcome including disease stage, metastasis, and therapeutic efficacy.

It is generally thought that macrophages are the major source of IL-8. Notably, it has been demonstrated that the levels of IL-8 are enhanced in the microenvironments of inflammatory and malignant colorectal tissues. However, viable immune cell subsets including Treg cells have not been sorted from colitic and colon cancer tissues for detailed phenotypic and functional experiments in humans. Conventional immunohistochemical staining and in situ hybridization have been utilized to detect IL-8 expressing cells in colon tissues. These studies have failed to conclusively reveal the phenotype of IL-8\textsuperscript{+} cells and the functionality of IL-8\textsuperscript{+} cells in the gut.\textsuperscript{27,28} Interestingly, colon cancer-associated macrophages, if not activated by LPS (lipopolysaccharides), produce limited IL-8.\textsuperscript{29} The data suggests that in addition to macrophages, other cells may be important producers of IL-8 in the colon mucosa. In line with this possibility, to our surprise, we have shown that the levels of IL-8 are dramatically higher in Treg cells than the Treg-depleted mononuclear cells (containing macrophages) in the microenvironments of colitis and colon cancer. TCR engagement stimulates high levels of IL-8 production from colitic Treg cells, but not blood Treg cells. Altogether, our data indicate that Treg cells are important IL-8 producers in the gut.
Why do Treg cells produce IL-8 and where are the IL-8+ Treg cells from in the gut? Recent studies suggest considerable levels of plasticity between different T cell lineages, and point toward potent peripheral regulation of effector T cell subset development in specific microenvironments. For instance, peripheral Treg cells can be converted into Th17 cells, an event favored by inflammation in the context of IL-6 production. In support of this possibility, a minor population of IL-17+Foxp3+ cells can be observed in the tonsils, and peripheral blood in humans as well as in the small intestine in mice. Our study indicates that in the presence of TGFβ and IL-2, T cells can become IL-8+Foxp3+ cells. Foxp3 appears to positively regulate IL-8 expression. We have shown that Foxp3 binds IL-8 promoter and stimulates IL-8 promoter activities. IL-17+Foxp3+ cells usually do not express IL-8. It is possible that IL-17+Foxp3+ cells may have Th17 cell property and express high levels of RORγ. RORγ may antagonize the stimulatory effect of Foxp3 on IL-8 expression. Notably, although Foxp3 positively regulates IL-8 expression, peripheral Treg cells express minimal IL-8. It indicates that in addition to Foxp3, additional key factor(s) may control the expression of IL-8 in Treg cells. We suggest that the nature of IL-8 expression in Treg cells may be endowed in the specific microenvironment. TGFβ and IL-2 may be extrinsic signal, and Foxp3 may be

Figure 5. Induction of IL-8+Foxp3+ T cells. IL-2 and TGFβ induced IL-8+Foxp3+ T cells. Normal blood CD4+ T cells were stimulated with anti-CD3 and anti-CD28 in the presence of IL-2 (A, C) and TGFβ (B, D) for 3 d. The fresh and stimulated T cells were analyzed by FACS. Results were expressed as the percent of IL-8+Foxp3+ T cells in CD4+ T cells (A, B) or in Foxp3+ T cells (C, D). n = 5. *p < 0.05, compared to medium or fresh cells.
Figure 6. Foxp3 regulates the development of IL-8\(^+\) Treg cells. (A, B). Ectopic Foxp3 expression promotes IL-8 expression and suppresses effector cytokine expression. CD4\(^+\)CD25\(^-\) T cells were transfected with Foxp3 expressing lentiviral vector and control vector, and were stimulated with anti-CD3 and anti-CD28 for 3 d. The stimulated T cells were analyzed by FACS. Results were expressed as the percent of IL-8\(^+\)Foxp3\(^+\) T cells in CD4\(^+\) T cells (A) or the percent of cytokine-expressing T cells in CD4\(^+\) T cells (B). n = 3 donors. \(p < 0.01\) as compared to controls (scramble). Solid bars, lentiviral vector; Empty bars, control vector. (C). Foxp3 directly binds the IL-8 proximal promoter area. ChIP analysis was performed in Foxp3\(^+\) Treg cells and general T cells. Anti-human Foxp3, anti-human AcH3 (positive control), and IgG (negative control) were used. Results were shown that Foxp3 occupied at \(-4200\) bp region of the predicted region of IL8 promoter in Treg cells. One of four experiments is shown. (D). Foxp3 increases IL-8 promoter Luciferase activity. Luciferase assay was carried out in Jurkat cells. Foxp3 or pcDNA3 vector control was expressed with IL-8-luc reporter and Renilla-pLTK-luc. Results were expressed as the mean values of relative luciferase activity ± SD from three individual experiments with duplication (\(p < 0.01\), compared to control). (E). IL-8 and Foxp3 expression in Treg cells. Colitic T cells were stained for CD3, CD4\(^+\), Foxp3, and IL-8, and analyzed by FACS. The levels of Foxp3 expression were positively associated with that of IL-8. One of four experiments is shown.
intrinsic signal controlling IL-8 expression in Treg cells. Therefore, our data provide new insights into the plasticity of human T cell development.

In summary, contrasting the established view, certain Treg cell subsets may be inflammatory, rather than anti-inflammatory. Colitic and colon cancer IL-8+ Treg cells may simultaneously inhibit local immunity and support local inflammation, and in turn contribute to the complexity of chronic inflammation. The dual functionalities place IL-8+ Tregs in a unique position to support tumor initiation and development in the chronic inflammatory environment in humans, such as ulcerative colitis, through inhibiting protective tumor immunity and stimulating/maintaining inflammation.

**Materials and methods**

**Human subjects and human tissues**

Peripheral blood, ulcerative colitis tissues, colon carcinoma, and ovarian carcinoma tissues were collected for this study. Because the tissues associated with ulcerative colitis cannot objectively be called “normal”, we took grossly adjacent tissues from at least 5 cm away from active colitic and colon cancer locations. Patients received no treatment with immunosuppressive agents were recruited for this study. Tissue sampling for inflamed lesions and adjacent tissues was aimed in the same colonic or ileal segment, if possible. Crohn’s disease was not included in the study. Patients gave written, informed consent. The study was approved by the local Institutional Review Boards. Single cell suspensions were made from these tissues. Colon mucosa mononuclear lymphocytes and colon epithelial cells were used for phenotyping and functional assays.

**Phenotypes and cytokine profile of immune cells**

Phenotypes and cytokine profile of immune cells were analyzed with flow cytometry analysis (FACS). Cells were first stained extracellularly with specific antibodies against human CD3, CD4+, CD8+, CD25, CD45RA, CD45RO, CD49, CD127, CD161, CXCR4, CXCR5, CCR4, CCR5, CCR6 (BD Biosciences), then were fixed and permeabilized with Perm/Fix solution (E-Biosciences) and finally were stained intracellularly with anti-IL-2, anti-IL-8, anti-IFNy (BD Biosciences) and anti-FOX3 (eBiosciences). Samples were acquired on a LSR II (BD Biosciences) and data were analyzed with DIVA software (BD Biosciences).

**Immunoﬂuorescence analysis**

Immunoﬂuorescence analysis was performed as described.70 Tissues were stained with polyclonal rabbit anti-human-CD3 (Dako), polyclonal mouse anti-human IL-8 (Abcam), and rat anti-human Foxp3 (e-Biosciences) followed by NL-493-conjugated donkey anti-goat IgG (R&D System). After washing tissues were stained with Alexa Fluor 647-conjugated goat anti-rabbit IgG and Alexa Fluor 568-conjugated goat anti-rat IgG (Molecular Probes). Fluorescence images were acquired by fluorescence microscope (Leica) and analyzed by ImagePro Plus software.

**Migration assay**

Migration assay was performed as we previously described.70,38 CD4+CD25high T cells (10⁶/mL) were electronically sorted. Sampled staining showed >95%Foxp3 expression. The cells were stimulated with anti-CD3 and anti-CD28 beads for 40 hours. Supernatant was collected, and added to the lower chamber of a 3 μm pore size transwell plate (Corning). Neutrophils were isolated using a Ficoll separation followed by a 6% dextran gradient, and 50,000 cells were added to the upper chamber of the transwell plate. In some cases, anti-human IL-8 (CXCL8) mAb (100 ng/mL, BD) was added to lower chamber for 2 h prior to neutrophil addition. After 8 h, the numbers of neutrophils in the lower chamber were recorded. Migration index was calculated as the percent of migrated cells.

**T cells clones**

Peripheral CD4+ T cells were isolated from two independent donors with negative enrichment method through magnetic bead selection (Rosettesep StemCells Technology, Canada). T cells were expanded and cloned with 2.5 μg/mL anti-CD3 and 1.25 μg/mL anti-CD28 monoclonal antibody (BD Biosciences) and 5 ng/mL rhIL-2 (R&D System) in the presence of irradiated allogeneic peripheral blood mononuclear cells as feeders as previously described.42 The clones were tested for Foxp3 and IL-8 expression by FACS and used for functional experiments.

**Immune suppressive assay**

CD4+CD25+ T cells were enriched with CD25+ magnetic beads. CD4+CD25bright T cells were sorted with high speed sorter (FACSAria, BD) from blood, tumors, and colitic tissues. The expression of Foxp3 was initially defined by FACS from the sorted CD4+CD25bright T cells. The number of Treg cells added in the immune suppressive assay was based on the quantification of Foxp3 (not CD25) in the sorted viable CD4+CD25bright T cells (Fig. S1). In some cases, T cell clones were used. The responder T cells and antigen presenting cells (APCs), macrophages were enriched from peripheral blood. The immune suppressive assay was performed as we described.10 Briefly, different numbers of suppressor cells (CD4+Foxp3+ T cells or Foxp3+ T cell clones) were added into the co-culture 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 by intracellular staining and ELISA kit (R&D System).

**ChIP assay**

ChIP assay was performed as described43,44 with modifications. 1.4 x 10⁷ Treg or T cells were used in each IP. Cells were lysed in lysis buffers and sonicated. Protein A/G beads (2:1) were applied to pull down the antibody. After reverse-crosslinking, the DNA samples were purified with a PCR Purification kit (Qiagen). The −1400 bp region of the transcription start site (TSS) of human IL-8 promoter was amplified by real-time PCR with primers 5’GCATTGAAAATGGCATTCCCCCTC3' and 5’AGTAAAAGTCTGTTCGCTTTGCGC3’.
**IL-8 promoter Luciferase assay**

Human IL-8 promoter luciferase plasmid contained IL-8 promoter region −1481 to +40 bp of TSS that drives a luciferase reporter.\(^\text{45,46}\) Foxp3-HA expression plasmid was described previously.\(^\text{47}\) Luciferase assay was carried out in 24-well plate with 5 × 10^4 of Jurkat cells per well. Foxp3 or pcDNA3 vector control (0.5 or 1 μg) was expressed with IL-8-luc reporter (1 μg) and Renilla-pLTK-luc (5 ng) using Lipo 2000 reagents according to the manufacture instruction (Invitrogen). 48 h after transfection, cells were harvested for luciferase assay using Dual Luciferase kit (Promega).

**Forkhead box P3-expressing lentivirus transfection**

Normal CD4^+CD25^- T cells (10^6/mL) were transfected with Foxp3-expressing lentivirus vector and control vector as we previously described.\(^\text{47}\) Cells were activated with anti-CD3 (2.5 μg/mL) and anti-CD28 (1.25 μg/mL) mAbs for 3 d. The cytokine profile was determined by intracellular staining.\(^\text{9,38-41}\)

**Inflammatory cytokines in the cancer and colitic environments**

Single-cell suspensions were made from fresh ulcerative colitis tissues. Colon cancer or colitic CD4^+CD25^bright T cells (10^6/mL) were sorted to high purity and activated with anti-CD3 and anti-CD28 beads for 40 h. Supernatant was collected, and cultured with colon epithelial cells (2 × 10^6/mL) for 48 h with anti-human IL-8. Colon epithelial cells were from colon tissues adjacent to colon carcinoma. Cytokine production was analyzed by ELISA kits (R & D System).

**Detection of cytokine mRNA expression**

The mRNA levels of cytokines and chemokines were detected by real-time reverse-transcriptase polymerase chain reaction (PCR). All experiments were performed using gene-specific primer pairs and SYBR green I (Invitrogen) fluorescence detection in a Multiplex instrument (Eppendorf). Data analysis is based on the Ct method with normalization of the raw data to housekeeping gene.\(^\text{9,38-41}\)

**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.\(^\text{46}\) p < 0.05 was considered as significant. All statistical analysis was done on Statistica software (StatSoft Inc., Tulsa, OK) and further described in the figure legends.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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