**Title:** Generation and regulation of human CD4+ IL-17-producing T cells in ovarian cancer

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**Abstract:** Despite the important role of Th17 cells in the pathogenesis of many autoimmune diseases, their prevalence and the mechanisms by which they are generated and regulated in cancer remain unclear. Here, we report the presence of a high percentage of CD4+ Th17 cells at sites of ovarian cancer, compared with a low percentage of Th17 cells in peripheral blood mononuclear cells from healthy donors and cancer patients. Analysis of cytokine production profiles revealed that ovarian tumor cells, tumor-derived fibroblasts, and antigen-presenting cells (APCs) secreted several key cytokines including IL-1β, IL-6, TNF-α, and TGF-β, which formed a cytokine milieu that regulated and expanded human IL-17-producing T-helper (Th17) cells. We further show that IL-1β was critically required for the differentiation and expansion of human Th17 cells, whereas IL-6 and IL-23 may also play a role in the expansion of memory Th17 cells, even though IL-23 levels are low or undetectable in ovarian cancer. Further experiments demonstrated that coculture of naive or memory CD4+ T cells with tumor cells, APCs, or both could generate high percentages of Th17 cells. Treatment with anti-IL-1 alone or a combination of anti-IL-1 and anti-IL-6 reduced the ability of tumor cells to expand memory Th17 cells. Thus, we have identified a set of key cytokines secreted by ovarian tumor cells and tumor-associated APCs that favor the generation and expansion of human Th17 cells. These findings should accelerate efforts to define the function of this important subset of CD4+ T cells in the human immune response to cancer.

**Keywords:** cancer immunology | immune regulation | T cell differentiation | tumor microenvironment | Th17 cells

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A growing body of evidence suggests that CD4+ T-helper (Th) cells play a central role in initiating and maintaining immune responses against cancer (1, 2). However, the presence of CD4+ regulatory T (Treg) cells and the newly discovered IL-17-producing Th cells (Th17) may change our view about the role of CD4+ T cells in cancer and many other diseases. Treg cells significantly suppress immune responses, thus inducing immune tolerance at tumor sites (3), whereas Th17 cells have been linked to both autoimmune diseases and cancer (4–6).

Elevated proportions of CD4+CD25+ Treg cells have been identified in the total CD4+ T cell populations in several different human cancers (7–9). We have further demonstrated the presence of antigen-specific CD4+ Treg cells at tumor sites (10, 11). Th17 cells were identified as a lineage of the CD4+ Th2 subpopulation (12, 13). They are distinct from CD4+ Th1 cells by virtue of expressing RORγt as a critical transcription factor (14). IL-23, a critical inducer of experimental autoimmune encephalitis and collagen-induced arthritis (15, 16), promotes the development of Th17 cells in vivo (17). Moreover, several recent studies demonstrate that TGF-β and IL-6, but not IL-23, are critical factors for murine Th17 cell differentiation in vitro (18–20). It appears that TGF-β plays an essential role in dictating whether CD4+ T cells become Treg or Th17 cells. The combination of TGF-β and IL-6 promotes the differentiation of Th17 cells and inhibits T cell differentiation in mice (18–20), whereas TGF-β plus retinoic acid inhibits Th17 cell differentiation and promotes Treg cells (21). IL-1 has also been shown to play a critical role in murine Th17 differentiation (22).

Despite recent advances in our understanding of the differentiation and function of Th17 cells in humans (23–26), very little is known about their prevalence and regulation in human cancer. Here, we report the presence of high percentages of Th17 cells that secrete predominantly IL-17 in the ovarian cancer-infiltrating T cell population. Cytokine profile analysis revealed that tumor cells, tumor-derived fibroblasts, and antigen-presenting cells (APCs) secrete several key cytokines, including IL-1β and IL-6, that may promote or regulate the differentiation and expansion of Th17 cells in the tumor microenvironment. We found that IL-1β was a potent inducer of Th17 cell differentiation and expansion, whereas IL-6 and IL-23 were capable of expanding memory Th17 cells. By coculturing CD4+ T cells with tumor cells, APCs, or both, we were able to modulate the generation and expansion of Th17 cells from naive or memory CD4+ T cells. Here, we provide an insightful mechanism by which Th17 cells are generated and regulated by cytokines secreted from tumor cells and their immune infiltrates.

**Results**

**Demonstration of Tumor-Infiltrating Th17 Cells in Ovarian Cancer.** Because inflammation has been linked to cancer development and disease progression (27), it is reasonable to propose that Th17 cells may be present in the tumor microenvironment, where proinflammatory cytokines such as IL-1, IL-6, and IL-23 could be produced by tumor cells and tumor-infiltrating immune cells. Although IL-23 has been linked to tumor development in mice (5), it is not clear whether Th17 cells are present at tumor sites. Thus, we sought to determine the prevalence of Th17 cells within the total tumor-infiltrating T cell population isolated from ovarian cancer tissues. As shown in Fig. L4, there was a high percentage of Th17 cells (7.6–76%, mean 25%) in 5 of the 10 ovarian tumor-derived T cell populations, compared with ~1% among peripheral blood mononuclear cells (PBMCs) from healthy donors. The remaining 5 tumor-derived T cell populations contained the percentage of Th17 cells in a level comparable with that in PBMCs (data not shown). Double staining with anti-IL-17 and anti-IFN-γ antibodies revealed that Th17 cells secreted predominantly IL-17, although a low frequency of T cells produced both IL-17 and IFN-γ (Fig. LB). Further experiments revealed that freshly isolated IL-17-producing T cells were strongly positive for CCR6 molecules and weakly positive

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Author contributions: Y.M. and R.-F.W. designed research; Y.M., W.C., and G.P. performed research; K.O. and J.M. contributed new reagents/analytic tools; Y.M., W.C., and R.-F.W. analyzed data; and R.-F.W. wrote the paper.

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Although a previous study showed a high proportion of CD4+ H11001 PBMCs between healthy donors and cancer patients (Fig. S2), we found that there was no difference in the Th17 frequency in PBMCs from healthy donors and ovarian cancer patients, and we healthy donors, we performed similar experiments by using size (Table S1).

To determine whether the frequency of Th17 cells in PBMCs of ovarian cancer patients is higher than that in PBMCs of healthy donors, we performed similar experiments by using PBMCs from healthy donors (HD) served as controls. (B) Evaluation of IL-17- and IFN-γ-producing T cells in the total tumor-derived T cell population. (C) Phenotypic analysis of ovarian tumor-derived T cells. Bulk T cells generated from OVA1 were stained with antibodies against CD45RA, CD45RO, CCR7, and CD62L molecules and analyzed by FACS. (D) Th17-containing T cells were cultured in the presence of anti-CD3 antibody (OKT3) and IL-2 (300 units/ml) or cultured with tumor cells plus the same concentration of IL-2. After 2 weeks, IL-17-producing T cells in the total T cell population were determined after stimulation with PMA and ionomycin.

Identification of Th17 cells in ovarian tumor-derived T cell population. (A) Prevalence of Th17 cells among ovarian tumor-derived T cells. Bulk T cells isolated from ovarian tumor tissues were stimulated with PMA and ionomycin and stained with anti-CD4 and anti-IL-17 to determine the percentage of IL-17-producing T cells. OVA1–5 denotes T cells isolated from different ovarian tumor specimens. T cells from healthy donors (HD) served as controls. (B) Evaluation of IL-17- and IFN-γ-producing T cells in the total tumor-derived T cell population. (C) Phenotypic analysis of ovarian tumor-derived T cells. Bulk T cells generated from OVA1 were stained with antibodies against CD45RA, CD45RO, CCR7, and CD62L molecules and analyzed by FACS. (D) Th17-containing T cells were cultured in the presence of anti-CD3 antibody (OKT3) and IL-2 (300 units/ml) or cultured with tumor cells plus the same concentration of IL-2. After 2 weeks, IL-17-producing T cells in the total T cell population were determined after stimulation with PMA and ionomycin.

for CCR4, although Th17 cell lines were positive for both CCR4 and CCR6 molecules [supporting information (SI) Fig. S1.A and B]. A comparison of the clinicopathologic characteristics of patients with high or low frequencies of tumor-derived Th17 cells did not reveal any clear trend, presumably because of the small sample size (Table S1).

To determine whether the frequency of Th17 cells in PBMCs of ovarian cancer patients is higher than that in PBMCs of healthy donors, we performed similar experiments by using PBMCs from healthy donors and ovarian cancer patients, and we found that there was no difference in the Th17 frequency in PBMCs between healthy donors and cancer patients (Fig. S2). Although a previous study showed a high proportion of CD4+ Treg cells in ovarian cancer (9), we did not find a high percentage of Treg cells in these ovarian tumor-derived T cell populations (Fig. S3A). Consistent with this observation, ovarian tumor-derived T cells did not suppress the proliferation of naïve CD4+ T cells (Fig. S3B). To determine the phenotype of the Th17-containing T cell population from tumor samples, we stained them with a panel of antibodies and found that they were positive for CD45RO, but negative for CD45RA, CD62L, and CCR7 (Fig. 1C), indicating that these tumor-derived T cells are memory Th17 cells.

Because tumor cells and immune cells contribute importantly in the induction of immune tolerance and inflammation at tumor sites (27), we asked whether tumor cells can drive the expansion of Th17 cells in the presence of 300 units/ml IL-2. We cultured ovarian tumor-derived bulk T cells (containing 12% Th17 cells) with autologous tumor cells, using the same T cells cultured in OKT3-coated plates as a control (Fig. 1D). The percentage of Th17 cells in OKT3 plus IL-2 decreased from the original finding of 12% to 5%. By contrast, culturing of Th17-containing T cells with tumor cells plus IL-2 increased the percentage of Th17 from 12% to 21% (Fig. 1D). These results indicate that tumor cells may secrete key cytokines required for the expansion of Th17 cells.

High Levels of IL-1β, IL-6, and Other Cytokines in the Ovarian Tumor Microenvironment. All solid tumors are embedded in a stromal microenvironment consisting of immune cells such as macrophages and lymphocytes and nonimmune cells such as fibroblasts. We reasoned that tumor cells, tumor-derived fibroblasts, T cells, and APCs may produce high amounts of IL-1, IL-6, or IL-23, thus forming a proinflammatory cytokine milieu that facilitates the differentiation and expansion of Th17 cells. To test this possibility, we examined cytokine secretion profiles of ovarian tumor cells and tumor-associated fibroblasts, T cells, and APCs, which are typically found in the tumor microenvironment. Ovarian tumor cell lines secreted very high amounts of IL-6, IL-8, interferon-induced protein 10 (IP-10), monocyte-chemoattractant protein 1 (MCP-1), and VEGF, but a low amount of IL-1. Similarly, tumor-associated fibroblasts also secreted IL-6, IL-8, MCP-1, and VEGF, but not IP-10 (Fig. 2). More importantly, tumor-associated APCs obtained from ovarian ascites secreted a large amount of IL-1β, IL-6, and other cytokines, but not IL-1α, after LPS treatment (Fig. 2). Ovarian tumor-derived population of tumor-infiltrating lymphocytes harboring Th17 cells secreted a large amount of IL-17. Neither tumor cells, fibroblasts, nor APCs secreted IL-23 in our assays, although this cytokine was detected in murine colon cancer on...
the basis of IL-23 mRNA expression (5). Whether this discrepancy reflects a difference in tumor type or vertebrate species is unclear. Taken together, the results suggest that tumor cells, tumor-derived fibroblasts, T cells, and APCs produce several key proinflammatory cytokines, including IL-1β and IL-6, that could promote the differentiation and expansion of human Th17 cells in the tumor microenvironment.

**Critical Roles of IL-1 and IL-6 in the Differentiation and Expansion of Human IL-17-Producing T Cells.** We next sought to determine the role of key cytokines secreted by tumor cells and APCs in the generation and expansion of Th17 cells. Naïve CD4+ T cell populations, purified with a commercial kit, were positive for CD45RA, CCR7, and CD62L molecules, but negative for CD45RO (Fig. S4). Purified memory CD4+ T cells, by contrast, were positive for CD45RO, but negative or weakly positive for CD45RA and CCR7 (Fig. S4), consistent with the phenotype of memory T cells (28). Analysis of naïve and memory CD4+ T cells revealed that IL-17-producing T cells are in the memory, but not the naïve CD4+ T cell population (Fig. S4).

The purified naïve and memory T cells were cultured in the presence of different cytokines, and the percentages of IL-17- and/or IFN-γ-producing T cells were determined after intracellular staining of T cells with anti-IL17 and IFN-γ. We first selected IL-1α, IL-1β, IL-6, and IL-23 as key cytokines and evaluated their contribution to the generation and expansion of Th17 cells from naïve and memory T cells. Fig. 3A shows that IL-1α and IL-1β could promote the differentiation (5%) of IL-17-producing cells from the naïve CD4+ T cell population, compared with 0.2–0.3% of Th17 cells in the presence of IL-6 or IL-23. The combination of IL-1β plus IL-6 or IL-23 slightly increased the percentage of Th17 cells in the naïve CD4+ T cell population, but no additional stimulation was observed with the combination of IL-6 and IL-23 (data not shown). Furthermore, IL-1α, IL-1β, IL-6, and IL-23 each expanded the IL-17-producing T cells in the memory T cell population (Fig. 3A). Notably, there was a high percentage of T cells producing IL-17 and IFN-γ in both the treated naïve and memory T cell populations, consistent with several recent studies on human Th17 cells (28, 29), but the frequency of such cells is much less than that in tumor-derived T cell population (Fig. 1).

To demonstrate that the results in Fig. 3A could be generalized to CD4+ T cells isolated from other donor PBMCs, we tested the effects of IL1α, IL-1β, IL-6, IL-23, and other cytokines, such as IL-12, IL-27, and TNF-α, on Th17 differentiation and expansion by using T cells from another five healthy donors. IL-2-containing medium provided a baseline for comparison. Analysis of naïve and memory CD4+ T cells from multiple donors revealed that IL-1α and IL-1β play a more prominent role in differentiation and expansion of IL-17-producing T cells than do IL-6 and IL-23. By contrast, IL-12, IL-27, and TNF-α inhibited IL-17-producing T cells (Fig. 3B). To determine the kinetics of Th17 differentiation from naïve T cells and Th17 cell expansion in memory T cell population, we labeled the purified naïve and memory T cells with carboxyfluorescein succinimidyl ester (CFSE) and then cultured them on OKT-coated plates in the presence of IL-1. The frequency of Th17 cells was monitored at the indicated time points.

**TGF-β Inhibits the Expansion of Human CD4+ Th17 Cells from Memory T Cell Population.** To determine whether ovarian tumor cells secreted TGF-β, we found that ovarian tumor cells secreted a high amount of latent TGF-β (inactive form), but the level of active form of TGF-β was very low (∼30 pg/ml) or undetectable because of its short half-life (Fig. 4A). These results are consistent with a recent study showing that ovarian tumor cells secretes latent (inactive) TGF-β but little or low active TGF-β (30). Because tumor-infiltrating T cells are memory T cell phenotype and the role of TGF-β in Th17 cell differentiation from naïve T cells have been extensively studied (23–25, 31–33), we focused on the effect of TGF-β on Th17 in the memory CD4+ T cell population. Results in Fig. 4 show that IL-1β, IL-6, and IL-23 alone and various combinations strongly increased the percentage (20–28%) of Th17 cells in the CD4+ memory T cell population compared with IL-2 alone. Surprisingly, TGF-β alone or in combination with other cytokines markedly reduced the percentage of IL-17-producing T cells in the memory T cell population (Fig. 4B). Titration experiments further demonstrated that TGF-β reduced the percentage of Th17 cells in a dose-dependent manner. Similar results were obtained with multiple memory CD4+ T cells freshly isolated.
from different healthy donors (Fig. 4C). By contrast, TGF-β increased the frequency of Foxp3+ T cells (Fig. 4D), whereas IL-1β, IL-6, and IL-23 did not. Interestingly, IL-6, but not IL-1β and IL-23, inhibited TGF-β-mediated conversion of Foxp3+ T cells from memory T cells (Fig. 4D). TGF-β-induced Foxp3+ T cells showed variable suppressive activity, but they gained strong suppressive function after multiple stimulations (data not shown), consistent with recent studies on TGF-β-induced Foxp3+ T cells in human (34, 35). Taken together, these findings suggest that high concentrations of TGF-β reduce the percentage of Th17 cells in memory T cell population but promote the generation of Foxp3+ T cells.

Role of Tumor Cells and APCs in the Generation and Expansion of Th17 Cells. To determine whether tumor cells, APCs, or both are capable of converting or expanding Th17 cells from purified naïve and memory T cells, we cultured T cells with medium alone, tumor cells, LPS-stimulated APCs, or tumor cells plus LPS-stimulated APCs for 8 days. We found a low percentage (0.1–0.8%) of Th17 cells from either naïve or memory T cells in medium alone (Fig. 5A). However, the percentages of Th17 cells generated from memory CD4+ T cells were increased to ≈30% after being cultured with irradiated tumor cells. Similar effects were observed when memory T cells were cultured with LPS-pretreated APCs or tumor cells plus APCs (Fig. 5B). By comparison, the increased percentages of Th17 cells differentiated from naïve T cells were small (0.2–2.3%) when being cultured with either tumor cells alone or APCs alone. The combination of tumor cells and APCs dramatically improved differentiation of Th17 cells (3.3–7.2%) from naïve T cells, while still retaining their ability to expand Th17 cells from the memory T cell population (Fig. 5A and B). Addition of anti-IL-1 alone or combination of anti-IL-1 protein and anti-IL-6 antibody inhibited the expansion of Th17 cells in the memory T cell population (Fig. 5C), suggesting that IL-1 is critical for memory Th17 expansion. Taken together, these results suggest that tumor cells and APCs play an important role in promoting Th17 cell differentiation from naïve T cells and that IL-1 secreted by tumor cells and APCs is at least in part if not fully, responsible for the observed expansion of memory Th17 cells.

Discussion

Given the fact that inflammation is linked to cancer development and progression, it is reasonable to predict that proinflammatory...
Th17 cells may accumulate in the tumor microenvironment. In a previous study, IL-17 mRNA was detected in 11 (65%) of 17 ovarian tumors (36), but it is not clear whether IL-17 detected in tumor tissues is produced by Th17 cells. The results presented in this article clearly demonstrate the high percentages of Th17 cells in 5 (50%) of the 10 human ovarian cancers tested. By contrast, we did not observe a high percentage of Foxp3+ T cells in the same set of T cell lines, suggesting that Th17 and Foxp3+ Treg cells may be reciprocally regulated by cytokines present in the tumor microenvironment. However, the molecular mechanisms underlying the generation and regulation of Th17 cells in the tumor microenvironment remain largely unknown.

In this work, we have identified several key cytokines secreted by tumor cells and APCs that appear to be responsible for the generation and expansion of Th17 cells. Among the cytokines secreted by tumor cells, tumor-associated fibroblasts, and APCs, IL-1β and IL-6 were found to play a dominant role in the differentiation and expansion of Th17 cells. IL-1α promoted the differentiation of naive CD4+ T cells to Th17 cells and expanded human memory Th17 cells, whereas IL-6 stimulated the expansion of human Th17 cells in the memory T cell population. Importantly, our data and another recent study (23) demonstrate the differentiation of naive T cells to Th17 cells, and two other studies reported the failure to induce Th17 cells from naive T cells when monocytes or dendritic cells were stimulated with various TLR and NOD2 ligands (26, 37). One caveat of these studies is the use of potential TGF-β-containing serum in the culture medium, although we did not detect any TGF-β in serum we used. Therefore, we could not determine whether TGF-β is required for the differentiation of Th17 cells from naive T cells. Recently, three groups demonstrated that TGF-β is required for Th17 cell differentiation from naive T cells (31–33). These discrepancies may reflect the serum level of TGF-β, different stimulation systems used, cell sources, and perhaps the biological variability of human samples among individual donors. Nonetheless, the differentiation of Th17 cells from naive T cells remains relatively low (~10%) in all reported experimental systems even when the best cytokine combinations were used, raising the possibility that additional factors may be needed to achieve a more efficient differentiation of Th17 cells from naive T cells. As we demonstrated, IL-1 and IL-6 produced by tumor cells, fibroblasts, and APCs are potent in expanding memory Th17 cells. Thus, it is likely that these key cytokines are, at least in part, responsible for high percentages of tumor-infiltrating Th17 cells in ovarian cancer.

Besides the role of TGF-β on Th17 cell differentiation from naive T cells as discussed above, it is of particular interest to know the effect of TGF-β on the generation and expansion of memory Th17 cells. Although the tumor-derived latent (inactive) form of TGF-β could be readily detected in ovarian cancer, we detected no or a very low amount of the active form of TGF-β secreted by tumor cells. Our results indicate that high concentrations of TGF-β inhibit the expansion of memory Th17 cells but increase the frequency of Foxp3+ T cells, which can be reversed by IL-6. Consistent with this observation, it has recently been reported that TGF-β induces RORγt at low concentrations but inhibits its expression and function and favors Foxp3+ Treg cells at high concentrations (38). Thus, the relative concentration of TGF-β, IL-1, and IL-6 in the tumor microenvironment may be a critical factor for the generation of Th17 and Foxp3+ T cells. Based on these findings, we predict that cytokine milieu (low amounts of TGF-β and high amounts of IL-1 and IL-6) in ovarian cancer favors the generation and expansion of Th17 cells, although further studies are needed to validate this concept.

To understand further how tumor cells and APCs contribute to the generation and expansion of Th17 cells, we found that either tumor cells alone or LPS-treated APCs alone can potently expand Th17 cells from memory CD4+ T cells but could not effectively induce naive T cells to become Th17 cells. However, the combination of tumor cells and APCs enhanced the differentiation of Th17 cells from naive T cells while retaining their ability to expand memory Th17 cells. Blocking experiments identify IL-1α as a critical factor driving memory Th17 cell expansion. Our findings clearly support the emerging concept that tumor environmental factors drive the generation and expansion of Th17 cells. This knowledge should accelerate efforts to define the biological function of these tumor-infiltrating T cells in tumor pathogenesis and progression.

Materials and Methods
Preparation of Bulk CD4+, Naïve, and Memory CD4+ T Cells. Peripheral blood, ascitic fluid, and tumor specimens were obtained from patients undergoing surgery for ovarian cancer at Roswell Park Cancer Institute, Buffalo, NY. Blood samples were also obtained from normal healthy volunteers as controls. All specimens were collected according to a protocol approved by the Institutional Review Board. PBMCs were isolated from buffy-coat by centrifugation on a Ficoll–Hypaque gradient, washed with Ca2+/Mg2+-free PBS, and suspended in PBS with 2% FCS. Bulk CD4+ T cells were isolated from PBMCs by magnetic separation, using CD4 beads and magnetic separation columns (MACS; Miltenyi Biotech) according to the manufacturer’s instructions. Human naïve (CD45RA+/CD45RO−) and memory (CD45RA−/CD45RO+) CD4+ T cells were further purified by EasySep enrichment kits (StemCell Technologies), according to the manufacturer’s instructions. The purity of naive and memory CD4+ T cells was ~97%, as determined with a flow cytometer (Becton Dickinson).

In Vitro Stimulation and Cytokines for Th17 Cell Differentiation and Expansion. Cells (5 × 105) were cultured in 1 ml of complete medium containing human 300 units/ml IL-2 and 10% AB serum in 48-well plates and stimulated with plate-bound anti-CD3 (4 µg/ml). Half of the culture medium was replaced with fresh medium containing cytokines on day 3 or 6. The exogenous cytokines used were TGF-β (3 ng/ml), IL-6 (100 ng/ml), IL-1α (10–40 ng/ml), IL-1β (10–40 ng/ml), IL-2 (10–40 ng/ml), and IL-23 (10–40 ng/ml). Recombinant human TGF-β1, IL-6, IL-23, and TNF-α were purchased from R&D Systems. Recombinant IL-1α, IL-1β, and IL-12 were purchased from PeproTech.

Intracellular Staining and Flow Cytometry and Antibodies. Eight days after culture, CD4+ T cells were washed with PBS and stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 5 h. After incubated with protein transport inhibitor (BD Pharmingen) to prevent cytokine secretion, the cells were stained with anti-CD4-FITC (BD Pharmingen). After washing, intracellular IL-17A and IFN-γ were stained with anti-IL-17A-PE (eBioscience) and anti-IFN-γ-PECy7 (BD Pharmingen). After final washes, the cells were analyzed on a FACScalibur flow cytometer. The following human mAbs were purchased from BD Pharmingen and used for staining of cell surface molecules: anti-CD45RA, anti-CD45RO, anti-CC chemokine recep- tor 7 (CCL7), anti-CD62L. Mouse IgG2b, mouse IgG2a, and rat IgG2a served isotype controls.

Cytokine Release Assay. Tumor cell lines or fibroblasts cell lines (2.5 × 106 per well in 6-well plates) were cultured in 2% FCS RPMI 1640 medium for 2 days, and then supernatants were collected for cytokine assays. Tumor-derived APCs were obtained by plating single cells from ovarian cancer ascites in 7T5 flasks in RPMI medium 1640 containing 10% FCS for 2 h and then washed with PBS to remove T cells and tumor cells. These cells (5 × 106 per well in 24-well plates) were stimulated with LPS (10 µg/ml) for 5 h followed by 5 mM ATP for 30 min, and the supernatants were used for cytokine assays. Ovarian tumor-derived bulk T cells that harbored Th17 cells were stimulated with OKT3 (2 µg/ml) for 18 h in 2% human AB serum containing medium without IL-2, and the supernatants were then collected for cytokine assays. The cytokines released from T cells, tumor cells, fibroblasts, and APCs were determined by the Bio-Plex cytokine assay kit (Bio-Rad) or by ELISA.

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Fig. S1. CCR4 and CCR6 expression in IL-17-producing ovarian tumor-derived T cells. (A) Two freshly isolated T cells from ovarian tumor samples were stimulated by phorbol 12-myristate 13-acetate (PMA) and ionomycin for 5 h in the presence of protein transport inhibitor. After stimulation, these cells were stained with PE-Cy5-anti-human CD4 mAb, FITC-anti-human CCR4 mAb, or FITC anti-human CCR6 mAb, and followed by staining PE-anti-human IL-17 mAb. The cells were analyzed with a FACSCalibur. (B) Two different ovarian tumor-derived Th17 lines were stained with PE-anti-human CCR4 mAb (R&D Systems) and biotinylated anti-human CCR6 mAb and then stained with streptavidin-APC (BD PharMingen). The cells were analyzed with a FACSaria.
Fig. S2. No differences in the frequency and in the proliferative activity of IL-17-producing CD4⁺ T cells in peripheral blood mononuclear cells (PBMCs) between ovarian cancer patients and healthy donors. (A) PBMCs from healthy donors (n = 13) and ovarian cancer patients (n = 8) were stimulated by PMA and ionomycin for 5 h in the presence of protein transport inhibitor. After stimulation, these cells were stained with FITC-anti-human CD4 mAb and PE-anti-human IL-17 mAb and analyzed on a FACSCalibur. (B) Bulk CD4⁺ T cells were isolated from PBMCs of healthy donors (n = 6) and ovarian cancer patients (n = 4). These cells were stimulated with plate-bound OKT3 in the presence or absence of IL-1β (20 ng/ml). Eight days later, cells were harvested and stained with specific antibodies. The percentage of IL-17-secreting T cells detected in the presence of IL-2 alone served as baseline for determining fold increases observed in IL-2 plus IL-1β. The bar represents the average fold increases of IL-17-producing cells.
Fig. S3. Foxp3 expression and functional analysis of ovarian tumor-derived tumor-infiltrating lymphocytes (TILs). (A) FACS analysis of ovarian tumor-derived TILs. (Upper) Ovarian tumor-derived TILs were stained with FITC-anti-human CD4 mAb and PE-anti-human IL-17 mAb after stimulation with PMA and ionomycin for 5 h in the presence of protein transport inhibitor. (Lower) Cells were also stained with FITC-anti-human CD4 mAb and Allophycocyanin (APC)-anti-human Foxp3 mAb (236A/E7; eBioscience) without stimulation. (B) Ovarian tumor-derived TILs was tested for their ability to suppress naïve T cell proliferation. Ovarian tumor-derived TILs (2 × 10^5) were cultured with or without purified naïve CD4^-CD25^- T cells (1 × 10^5) in U-bottomed 96-well plates coated with OKT3 for 60 h. Cells were harvested, and the incorporation of [3H]thymidine was measured with a scintillation counter.
Fig. S4. FACS analysis of purified CD4⁺ naive and memory T cells. Naïve and memory CD4⁺ T cells were purified with specific purification kits and analyzed by FACS after staining with specific antibodies for naïve and memory T cells. Using the purified T cells, Th17 cells were detected only in the memory T cell population and not in the naïve T cell population, after stimulation with PMA and ionomycin.
**Table S1. Relationship between Th17 frequency and clinicopathological parameters**

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<th>Clinical response†</th>
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<td>8</td>
<td>&lt;1.0 (L)</td>
<td>IV</td>
<td>Serous</td>
<td>NA</td>
<td>DOD</td>
<td>5.27</td>
</tr>
<tr>
<td>9</td>
<td>&lt;1.0 (L)</td>
<td>IIIb</td>
<td>Serous</td>
<td>CR</td>
<td>ANED</td>
<td>&gt;15.77</td>
</tr>
<tr>
<td>10</td>
<td>&lt;1.0 (L)</td>
<td>IIIc</td>
<td>Serous</td>
<td>CR</td>
<td>AWD</td>
<td>&gt;16.83</td>
</tr>
</tbody>
</table>

*H, high; L, low.
†CR, complete response; PR, partial response; PD, progressive disease; DOD, dead of disease; ANED, alive no evidence of disease; AWD, alive with disease; NA, not applicable.