Phenotype, distribution, generation, functional and clinical relevance of Th17 cells in the human tumor environments

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Abstract:

Th17 cells play an active role in autoimmune diseases. However, the nature of Th17 cells is poorly understood in cancer patients. We studied Th17 cells, the associated mechanisms and clinical significance in 201 ovarian cancer patients. Tumor infiltrating Th17 cells exhibit a polyfunctional effector T cell phenotype; are positively associated with effector cells, and negatively associated with Tregs. Tumor associated macrophages promote Th17 cells through IL-1β, while Tregs inhibit Th17 cells through an adenosinergic pathway. Furthermore, through synergistic action between IL-17 and IFNγ, Th17 cells stimulate CXCL9 and CXCL10 production to recruit effector T cells to the tumor microenvironment. The levels of CXCL9 and CXCL10 are associated with tumor infiltrating effector T cells. The levels of tumor infiltrating Th17 cells, and the levels of ascites IL-17 are reduced in more advanced diseases and positively predict patient outcome. Altogether, Th17 cells may contribute to protective human tumor immunity through inducing Th1-type chemokines and recruiting effector cells to the tumor microenvironment. Inhibition of Th17 cells represents a novel immune evasion mechanism. This study thus provides scientific and clinical rationale for developing novel immune-boosting strategies based on promoting the Th17 cell population in cancer patients.
**Introduction:**

Adaptive immunity plays a crucial role in tumor immunosurveillance \(^1\)-\(^3\). It has been shown that tumor infiltrating effector T cells are associated with improved prognoses in multiple human cancers \(^4\)-\(^6\), whereas tumor infiltrating regulatory T (Treg) cells are negatively associated with patient outcome \(^6\),\(^7\). Th17 cells are newly identified effector CD4\(^+\) T cells. Th17 cells and IL-17 play an active role in inflammation and autoimmune diseases \(^8\)-\(^{15}\). Th17 cells are found in both mouse and human tumors \(^16\),\(^17\). However, the biological role of Th17 cells is poorly understood in the tumor microenvironment. In this report, we examined the phenotype, cytokine profile, generation, functional relevance, and immunological and clinical predictive values of Th17 cells in 201 patients with ovarian cancers. We provide novel insight into the nature of Th17 cells in the tumor microenvironment in patients with cancer. This information may be useful for designing more effective cancer immunotherapies.
**Materials and Methods:**

**Human subjects.** We studied previously-untreated patients with 201 ovarian carcinomas. Survival data was available for 85 patients (Supplementary Table 1). Patients gave written, informed consent in accordance with the Declaration of Helsinki. The study was approved by the University of Michigan Institutional Review Board.

**Cells and tissues.** Cells and tissues were obtained from ascites, blood, lymph nodes and tumors as we described 16,18,19. Immune cells including monocytes, macrophages, myeloid dendritic cells, plasmacytoid dendritic cells and T cell subsets were enriched using paramagnetic beads (StemCell Technology, Vancouver, Canada) and sorted with FACSaria (Becton Dickinson Immunocytometry Systems, San Jose, CA) as we described 16,18,19. Cell purity was > 98% as confirmed by flow cytometry (LSR II, BD).

**Flow cytometry analysis (FACS).** For cytokine detection the cells were stimulate with PMA (50 ng/ml, Sigma), ionomycin (1 µM, Sigma) for 4 hours prior staining. Cells were first stained extracellularly with specific antibodies against human CD3, CD4, CD8, CD11b, CD11c, CD14, CD15, CD16, CD19, CD25, CD39, CD45, CD45RO, CD49a, CD49c, CD49d, CD49e, CD56, CD123, CD161, PD-1, CCR4, CCR6, CCR7, CXCR4, HLA-DR, and Annexin V (BD Biosciences), CCR2, CXCR3 and CCR5 (R&D System, Minneapolis, MN), EpCam (StemCell Technology), then were fixed and permeabilized with Perm/Fix solution (E-Biosciences) and finally were stained intracellularly with anti-IL-2, anti-IL-10, anti-IL-17, anti-TNFα, anti-IFN-γ, anti-Granzyme A, anti-Ki67 and anti-FOXP3 (all from BD Biosciences, except anti-IL-17, eBioscience). Samples were
acquired on a LSR II (BD Biosciences) and data were analyzed with DIVA software (BD Biosciences).

**Th17 induction and suppression.** Fresh peripheral blood and tumor associated CD14+ macrophages were sorted \(^{19}\) and co-cultured with T cells as indicated for 3-5 days in the presence of anti-CD3 (2.5 - 5 µg/ml) and anti-CD28 (1.2–2.5 µg/ml) mAbs (BD Biosciences). Anti-IL-1 receptor (1 µg/ml) was used as indicated (R&D System, Minneapolis, MN). CD4+CD25\(^{high}\) T cells were sorted from peripheral blood or ovarian cancer tissues \(^{7}\). Different concentrations of tumor associated Treg cells were added into the co-culture. In some cases, ARL67156 (50 µM, Sigma Aldrich) was added into the culture as described. T cell phenotype and cytokine profile were determined by FACS or ELISA (R&D, Minneapolis, MN) as we described \(^{16,18,19}\).

**siRNA knockdown of human IL-23 gene expression.** HEK293 cells were transfected with a Flag-tagged IL-23 expression plasmid and either a nonfunctional scrambled control siRNA or IL-23-specific siRNA using Lipofectamine 2000 (Invitrogen). After the siRNA treatment, the hIL-23 silencing efficiency was measured by Western-blot using anti-Flag tag (not shown). Blood or tumor associated macrophages were transfected with the siRNA or pmaxGFP vector using Nucleofector technology (Macrophages Nucleofector Kit, Amaza, Köln, Germany) as we described \(^{20}\). The transfection efficiency reached 60-80% as confirmed by pmaxGFP vector transfection.

**Cytokine and chemokine detection.** The mRNA levels of cytokines and chemokines were detected by real-time reverse transcriptase polymerase chain reaction (RT-PCR). All experiments were performed using gene-specific primer pairs and SYBR green I (Molecular Probes)
fluorescence detection in an Multiplex instrument (Eppendorff). Data analysis is based on the Ct method with normalization of the raw data to housekeeping gene \(^7,19\). The protein levels of cytokines and chemokines were detected by either intracellular staining or ELISA kits (all from R&D System, Minneapolis, MN).

**Induction of CXCL9 and CXCL10.** Th17 cells were polarized from tumor associated T cells \((10^6/ml)\) for 3 days with tumor associated macrophages \((0.5 \times 10^6/ml)\) in the presence of Th17-inducing cytokine cocktail as we described \(^{20}\). The polarized cells were extensively washed with fresh medium and cultured for additional 40 hours. The polarized Th17 cell supernatants were collected. Primary ovarian cancer cells (OC8) or macrophages \((1 \times 10^5/ml)\) \(^{19}\) were cultured with IL-17 \((10 \text{ ng/ml})\), IFN\(\gamma\) \((0-50 \text{ ng/ml})\), IL-17 plus IFN\(\gamma\) or 100% Th17 cell-polarized culture supernatants for 2-3 days. In some cases, the neutralizing anti-human IFN\(\gamma\) (2 µg/ml, clone 25723, IgG2b) and anti-IL-17 receptor (2µg/ml, clone 133617, IgG) were added into the culture. The culture supernatants were subjected to measuring CXCL9 and CXCL10 with ELISA kits (R & D).

**Migration assay.** CD8\(^+\) T cell migration was assessed as we described \(^{21}\) using ovarian cancer associated CD8\(^+\) T cells \((5-20 \times 10^4)\). T cells were induced to migrate with tumor ascites. In some cases, mouse anti-human CXCR3 (57226.11, IgG2b, 500 ng/ml) were added 2 hours before migration assay. Experiments were performed in triplicate. Migration was expressed as a percentage of migrated cells after subtracting the spontaneous migration (Migration index).
**Tissue immunofluorescence staining.** Immunofluorescence analysis was performed as described. Tissues were stained with monoclonal mouse anti-human CD8 (1/40 dilution, clone HIT8a, IgG2b, BD Biosciences), and mouse anti-human EpCam (1/40 dilution, clone 5E11; IgG1, StemCell Technology) followed by Alexa Fluor 568-conjugated goat anti-mouse IgG2b and Alexa Fluor 488-conjugated goat anti-mouse IgG1 (all 2 µg/ml, Molecular Probes). Positive cells were quantified by ImagePro Plus software and expressed as the mean number of the positive cells per mm² tissue section.

**Statistical calculations.** Pearson coefficient was computed to assess relationships between proteins and immune cell subsets in the tumor environments. Student’s t-tests were used to compare IL-17 expressions across stage (II/III versus IV), grade (0-2 versus 3), histology type (serous, mucinous, endometroid versus clear cells and undifferentiated), and debulking (optimal residual disease versus suboptimal residual disease) categories, with $P < 0.05$ considered significant. Overall patient survival was defined as the interval between date of diagnosis and date of death or last follow-up, whichever occurred earlier. The known tumor-unrelated deaths (e.g. intercurrent disease and accidental death) were excluded from death record for this study. Data were censored at the last follow-up for patients who were disease-free or alive at the time of last follow-up. Univariate association between IL-17, other factors and overall survival was assessed using log-rank test, and survival function estimates were computed using the Kaplan-Meier method. Cox proportional hazards model was used to assess the effect of IL-17 on survival, after adjusting for surgical debulking. All analyses were performed using SAS 9.1 (SAS Institute Inc. Cary, NC) and STATISTIC (StatSoft Inc., Tulsa, OK) software.
**Results**

*Distribution, phenotype and cytokine profile of Th17 cells.*

IL-17⁺CD4⁺ (Th17) cells are found in patients with cancer. However, the distribution, phenotype and cytokine profile of Th17 cells remain poorly defined in human tumors. We first evaluated the tissue distribution of Th17 cells in ovarian cancer patients. The prevalence of Th17 cells was comparable in tumor draining lymph nodes, cancer patient peripheral blood and normal donor peripheral blood (Fig 1A). However, the proportion of Th17 cells was higher in tumors than these compartments (Fig 1A). This suggests that Th17 cells may be induced or/and migrate into the tumor microenvironment.

We next examined the phenotype of IL-17⁺ cells in the tumor microenvironment. We found that IL-17 was exclusively expressed by T cells. Less than 1% tumor infiltrating CD8⁺ T cells expressed IL-17, whereas 99% of the tumor infiltrating IL-17⁺ T cells were IL-17⁺CD4⁺ (Th17) cells (Fig 1B). Tumor infiltrating Th17 cells expressed high levels of CXCR4, CCR6, CD161 (Fig 1C), and multiple CD49 integrins (Fig 1D), but not CCR2, CCR5 and CCR7 (Fig S1). The expressed homing molecules may be associated with Th17 cell migration and retention within tumor.

We also analyzed the markers for T cell activation/effector function and immune suppression. Tumor infiltrating Th17 cells expressed little HLA-DR, CD25, and granzyme B (Fig 1E). This suggests that Th17 cells may not be conventional effector T cells, and may not mediate effector function through the granzyme B pathway. The B7-H1 receptor, PD-1, may be expressed in functionally exhausted T cells. The B7-H1/PD-1 pathway and FOXP3⁺ Treg cells.
contribute to immune suppression in the tumor microenvironment. We found that Th17 cells expressed minimal PD-1 and FOXP3 (Fig 1F). This indicates that Th17 cells are distinct from Treg cells and functionally exhausted PD-1+ T cells.

We further analyzed the cytokine profile of human tumor infiltrating Th17 cells. IL-10+ and IL-10- Th17 cells have been observed in mice\textsuperscript{25,26}. We found that Th17 cells expressed minimal IL-10 (Fig 1F) and high levels of polyfunctional effector cytokines including TNF\textalpha, IL-2 and IFN-\gamma (Fig 1G). Tumor infiltrating T cells including Th17 cells did not express IL-4 (not shown). Similar cytokine profiles were observed in five other human tumor types studied including colon carcinomas, hepatocellular carcinomas, melanoma, pancreatic cancers, and renal cell carcinomas (not shown). These data indicate that Th17 cells exhibit an effector T cell cytokine profile with polyfunctionality as described in infectious diseases\textsuperscript{27,28}.

\textit{Th17 cells and their associations with immune cell subsets in the tumor microenvironment.}

Multiple immune cell populations including T cell subsets and antigen presenting cell (APC) subsets infiltrated the tumor microenvironment. We evaluated the relationships between Th17 cells and immune cell subsets in the same ovarian cancer environment. We first analyzed the correlation between Th17 cells and T cell subsets. We quantified Th17, IFN\textgamma IL-17+ T cells, IFN\textgamma CD8+ and IFN\textgamma CD4+ T cells, and Treg cells in the same tumors. Flow cytometry analysis revealed that Th17 cells were positively correlated with IFN\textgamma expressing T cell subsets including IFN\textgamma CD4+ T cells (Fig 2A), IFN\textgamma CD8+ (Fig 2B), and IFN\textgamma IL-17+ T cells (Fig 2C) in the same
tumor microenvironment. However, the proportion of Th17 and Treg cells were inversely correlated in the same tumors (Fig 2D).

We further analyzed the relationship between Th17 cells and innate immune cells in the same ovarian cancer ascites. Eosinophils were rarely observed (Supplementary Fig 2a). Moderate levels of mast cells (Fig S2B), neutrophils (Fig S2C) and NK cells (Fig 2E) were detected. However, Th17 cells had no correlation with eosinophils, mast cells and neutrophils (Fig S2). We found that the levels of NK cells were higher in patients with high levels of Th17 cells than in patients with low levels of Th17 cells in the same tumor microenvironment (Fig 2E).

Finally, we analyzed the relationship between Th17 cells and APC subsets. Plasmacytoid dendritic cells (PDCs) 21, myeloid dendritic cells (MDCs) and macrophages are the main APC populations in ovarian cancer 19 (Fig S3A). These three APC subsets were found in the tumor ascites and tumor (Fig S3A). However, there were no quantitative correlations between Th17 cells, and myeloid DCs, (Fig S3B), plasmacytoid DCs (Fig S3C) and macrophages (Fig S3D). We further investigated the functional association between Th17 cells and APC subsets in the subsequent studies.

Altogether, the data demonstrate that Th17 cells are quantitatively and positively correlated with NK cell-mediated innate immunity and adaptive T cell immunity.
Induction and suppression of Th17 cell development in the tumor microenvironment.

Th17 cells are basically found in the tumor microenvironment in patients with cancer \(^{16}\). APCs contribute to T cell polarization. We investigated the role of tumor associated macrophages (TAMs), plasmacytoid DCs and myeloid DCs in Th17 cell induction in ovarian cancer. We found that tumor associated plasmacytoid DCs had minimal effects on Th17 cell induction (Fig S4). TAMs and myeloid DCs isolated from ovarian cancers stimulated Th17 cell induction from memory T cells, and not from naïve T cells (Fig S4, and Fig 3A). TAMs were more efficient than normal macrophages (M\(\phi\)s) in eliciting T cell IL-17 production, and the induction was dose dependent (Fig 3B). Macrophages outnumbered myeloid DCs in ovarian cancer \(^{19,21}\) (Fig S3), and were superior to inducing Th17 cells than myeloid DCs (Fig S4 and Fig 3B) \(^{29}\). Our subsequent studies focused on tumor associated macrophages.

We investigated the mechanism by which TAMs induce Th17 cells. We found that TAMs expressed higher levels of IL-1\(\beta\) and IL-23p19 mRNA, as compared to normal macrophages (Fig 3C). Blockade of IL-1, but not IL-6 and TGF\(\beta\) consistently and largely reduced TAM-mediated induction of Th17 cells (Fig 3D, and not shown). Blocking IL-23 with specific siRNA further helped reduce Th17 cell induction (Fig 3D). Our data suggest that IL-1\(\beta\) plays a predominant role in TAM-mediated Th17 cell induction in patients with ovarian cancer.

Since TAMs are potent Th17 cell inducers (Fig 3A, B, D), we examined why there were limited numbers of Th17 cells in the tumor microenvironment (Fig 1). We hypothesized that tumor associated Treg cells might suppress Th17 cell development. To test this hypothesis, we first
stimulated T cells with TAMs in the presence of tumor associated Treg cells. Treg cells suppressed Th17 cells and T cell IL-17 production in a dose dependent manner (Fig 3E, F).

We further studied the mechanism by which Tregs suppressed Th17-induction. Tumor associated Treg cells highly expressed CD39 (Fig S5A, B), an ectonucleotidase which converts ATP into adenosine. Mouse Treg cells may mediate T cell suppression through adenosine induction \textsuperscript{30,31}. We found that ARL67156, a structural analogue of ATP and an ectonucleotidase inhibitor, partially but significantly recovered T cell IL-17 production suppressed by tumor associated Treg cells (Fig 3G). These data indicate that Th17 cell development is partially suppressed by tumor associated Treg cells through the adenosinergic pathway.

\textit{Th17, and Th1, Th2 type-cytokines and chemokines.}

To further examine the relationships between Th17 cells and the types of immune responses in the ovarian cancer microenvironment, we quantified numerous representative cytokines and chemokines associated with Th17, Th1 and Th2 type-responses in the ovarian cancer ascites.

Th17 cells were the only cell type expressing IL-17 in the ovarian cancer ascites. We detected variable levels of IL-17 in ascites fluid. Interestingly, the levels of IL-17 were positively correlated with IL-1\textbeta and IL-1\textalpha (Fig S6A, B), but not with TGF\textbeta, IL-6 (Fig S6C and not shown), IL-21 (Fig S6), IL-23 (Fig S6E) and PGE2 (Fig S6F). IL-23 protein was barely detectable in most of the samples tested (Fig S6E). All these molecules have been reported to be associated with Th17 cell
development. Given that the levels of IL-1α were less than 5 pg/ml (Fig S6B), the data further support that IL-1β plays a selective and crucial role in Th17 cell induction in the ovarian cancer microenvironment (Fig 3C, D).

Cytokines associated with Th1 and Th2-type responses including IL-12, IL-2 and IL-4 were less than 10 pg/ml in ovarian cancer ascites. IL-17 has been reported to induce tumor angiogenesis. Consistent with previous reports, high levels of angiogenic factors including IL-8 and VEGF were detected in the ascites. However, IL-17 was not correlated with these angiogenic molecules (Fig S7).

In addition to cytokines, we further evaluated the relationship between IL-17 and chemokines associated with Th1-type response including CXCL9, CXCL10, and with Th2-type response including CXCL12 and CCL22. Interestingly, we observed a significant positive correlation between IL-17, CXCL9 and CXCL10 (Fig 4A, B). Although we detected high levels of CXCL12 and CCL22, IL-17 had no association with these chemokines (Fig S8). The data indicate that in addition to Th1-type effector T cells and NK cells (Fig 2), Th17 cells and IL-17 are correlated with Th1-type chemokines in the ovarian cancer microenvironment.

In addition, we examined the mechanistic relationship between Th17 cells and tumor immunity. Th17 cells or IL-17 had no direct effects on primary ovarian cancer cell proliferation and apoptosis (Fig S9). As Th17 cells are positively correlated with Th1-type chemokines and effector T cells, we hypothesized that Th17 cells induce Th1-type chemokines, and in turn recruit Th1-type effector T cells into tumor microenvironment. To test this hypothesis, we initially studied the effects of Th17
cells on Th1-type chemokine production. We found that IFNγ and IL-17 synergistically induced the production of CXCL9 and CXCL10 by primary ovarian cancer cells and macrophages (Fig 4C, D, and not shown). Consistent with this observation, real-time PCR revealed that the levels of IL-17 were positively correlated with that of CXCL9 and CXCL10 in the same tumor tissues (Fig S10). In further support, the supernatants derived from Th17 cells induced high levels of CXCL10 production. This production was blocked by neutralizing anti-human IFNγ and anti-IL-17 (Fig 4E). These data indicate that Th17 cells induce Th1-type chemokine production.

**Th17, Th1 type-chemokines and effector T cell trafficking.**

Tumor associated effector CD8+ T cells highly expressed CXCR3, the receptor for CXCL9 and CXCL10 (Fig 5A). Tumor associated effector CD8+ T cells efficiently migrated toward tumor ascites in a dose dependent manner. The migration was reduced by neutralizing anti-CXCR3 (Fig 5B). We also quantified the number of tumor infiltrating CD8+ T cells by immunofluorescence staining. The mRNA levels of CXCL9 and CXCL10 were positively correlated with tumor infiltrating CD8+ T cells in the same tumor (Fig 5C, D). Furthermore, when we divided tumor tissues into two groups based on the median levels of IL-17, we observed that the levels of tumor ascites IL-17 were positively associated with tumor infiltrating CD8+ T cells (Fig 5E, F). Altogether, the data support the notion that Th17 cells induce Th1-type chemokines through IL-17 and IFNγ, and in turn recruit Th1-type effector T cells and NK cells into tumor microenvironment.
Increased tumor associated IL-17 predicts improved patient survival.

Our current data suggest that Th17 cells may contribute to protective tumor immunity in ovarian cancers. IL-17 is released into the tumor environment consisting of the abdominal cavity. IL-17 was detectable in all the ovarian cancer ascites we evaluated (Fig 4A, B). We analyzed the impact of IL-17 levels in the ascites on patient survival.

There was a significant association between ascites IL-17 levels and survival in the group as a whole ($n = 85$, $P = 0.0001$), and also for patients in stage II/III ($n = 57$, $P = 0.01$) and stage IV ($n = 28$, $P = 0.005$). Tumor ascites IL-17 was a significant predictor of death hazard (95% CI, $P = 0.0003$) even after controlling for surgical debulking and other parameters using a Cox proportional hazards model (Fig 6, Table 1 and S Table 1).

As an alternative analysis, patients were divided into two groups based on the median values of IL-17 (220 pg/ml). Survival functions were significantly different for the 2 groups (Fig 5A) ($P < 0.0001$). The median survival in the high IL-17 group was 78 months, compared to 27 months in the low IL-17 group. Tumor ascites IL-17 was a significant predictor of death even after controlling for surgical debulking. Patients in the high IL-17 group had a significantly lower death hazard compared to those in the low IL-17 group (hazard ratio = 0.08, 95% confidence interval: 0.03 to 0.20, $P < 0.0001$).

Furthermore, when the analyses were stratified by stage, we found significant association between ascites IL-17 and survival for patients in stage III ($n = 52$, $P = 0.01$) (Fig 6B) as well as
stage IV disease (n = 28, \( P = 0.005 \)) (Fig 5C). We additionally found that patients in stage IV had significantly reduced IL-17 in ascites compared to those in stage III (Fig 6D) \((P = 0.03)\).

Th17 cells are the IL-17 producers in the tumor. Therefore, decreased tumor ascites IL-17 or/and Th17 cells are a significant predictor of increased risk for reduced survival in ovarian cancer.
Discussion:

In this study, we have applied multiple complementary strategies to map out the phenotype, mechanisms of induction, biological function, and clinical relevance of Th17 cells in the tumor microenvironment of patients with ovarian cancer.

We have shown that tumor infiltrating Th17 cells highly express effector cytokines, but little in the way of molecules associated with immune suppression. This cytokine profile reveals a phenotype for polyfunctional effector T cells similar to that observed in patients with infectious diseases. This phenotype was universally found in six different human cancer types that we examined. It suggests that tumor associated Th17 cells may be functional effector T cells. In line with this possibility, we found that Th17 cells are negatively associated with the presence of Treg cells and are positively associated with effector immune cells including IFNγ effector T cells, CD8+ T cells and NK cells in the same tumor microenvironment. The data are consistent with several lines of evidence. (i) Transgenic T cells polarized with TGFβ and IL-6 can induce tumor eradication in mice. (ii) Forced expression of IL-17 ectopically in tumor cells can suppress tumor progression through enhanced anti-tumor immunity in immune competent mice. (iii) IL-17-deficient mice exhibit accelerated tumor growth and lung metastasis. (iv) Both blocking indoleamine 2,3-dioxygenase (IDO) and adjuvant IL-7 treatment result in improved anti-tumor immunity, which is associated with marked CD8+ T cell activation and Th17 cell enhancement. (v) In patients with
prostate cancer, a significant inverse correlation is found between Th17 skewing and tumor grade.

Along this line, we have detected IL-17 in tumor associated ascites, and the levels of IL-17 positively predict patient survival. Th17 cells are the sole cellular source for IL-17 in the human tumor microenvironment. Hence, the data provide evidence that Th17 cells may contribute to protective tumor immunity in humans with advanced tumors. In addition to CD8+ effector T cells, our data indicate that Th17 cells are an important immune component in tumor immunosurveillance.

The next question is how Th17 cells mediate anti-tumor immunity in patients with cancer. Th17 cells do not express granzyme B and perforin, and have no direct effects on primary ovarian cancer cell proliferation and apoptosis. Th17 cells may not mediate a direct tumor cytotoxic activity against tumor cells. Recent compelling evidence demonstrates that trafficking properties and location of effector T cells play a central role in the control of tumor growth and recurrence. In line with this notion, we found that IL-17 was positively associated with tumor infiltrating IFNγ+ effector T cells, and with Th1-type chemokines CXCL9 and CXCL10, but not with Th2-type chemokines CXCL12 and CCL22. Mechanistically, Th17 cell-derived IL-17 and IFNγ synergistically induced the production of CXCL9 and CXCL10, and in turn promoted effector T cell migration towards tumor. The levels of CXCL9 and CXCL10 were directly correlated with tumor infiltrating CD8+ T cells and NK cells. The data suggests that Th17 cells may play a role in promoting effector T cell and NK cell tumor trafficking and retention, and the polyfuctional cytokine profile (IFNγ+IL-17+) of Th17 cells is essential for synergistically inducing Th1-type
chemokines. In support of this notion, human psoriatic environmental Th17 cells express both IL-17 and IFNγ, and synergistically induce β-defensin, a functional marker for human psoriasis. Furthermore, polarized Th17 cells mediate tumor regression in an IFNγ-dependent manner in mice.

Notably, the role of IL-17 and IL-23 in tumor is controversial in the murine system. Earlier studies have shown that exogenous IL-17 either enhances anti-tumor immunity or promotes tumor growth by inducing tumor vascularization in tumor bearing mice. Recent studies have also revealed opposite roles of IL-23 in mouse tumors. It is worthwhile to point out that the potential role of endogenous IL-17 (or Th17 cells) has not been examined in tumor initiation in spontaneous mouse tumor models including those induced by infectious pathogens and chemical carcinogens or in humans with preclinical diseases. It is possible that endogenous IL-17 (or Th17 cells) may play distinct roles in tumor initiation versus established tumor growth. In addition to IL-17, Th17 cells express a polyfunctional cytokine profile in human tumors. This polyfunctional cytokine profile may not be observed in specific mouse system. The collaborative effects among these cytokines including IL-17 and IFNγ may be decisive in determining the biological activities of Th17 cells in human tumors as demonstrated in this and other human studies. Further, the roles of exogenous and endogenous IL-17 may potentially be distinct due to local biological levels of IL-17 and environment. In patients with ovarian cancer, IL-17 is quantitatively and mechanistically associated with CXCL9 and CXCL10 but not with the well defined angiogeneic factors IL-8 and VEGF in ovarian cancer. In addition to attracting effector T cells, CXCL9 and CXCL10 are two potent anti-angiogeneic cytokines. IL-17 is also not associated with IL-23, and IL-23 plays a minor role, if any, in Th17 cell development in human ovarian cancer (see discussion below). The
data does not rule out the potential angiogenic and proinflammatory roles of IL-17 derived from Th17 cells in human tumors. However, these potential effects may possibly be outweighed by the anti-tumor immunity and anti-angiogenic activities mediated by Th17 cell-induced CXCL9 and CXCL10 in patients with cancer.

We have further demonstrated that tumor associated macrophages are capable of inducing Th17 cell development in vitro. IL-1β, but not IL-1α, IL-6, TGFβ and IL-23 is crucial for Th17 cell induction, and is positively associated with IL-17 in ovarian cancer ascites. Consistent with this observation, the levels of IL-1α and IL-23 are negligible in ovarian cancer ascites. It suggests that IL-1α and IL-23 play a minor role in Th17 cell development in human ovarian cancer. However, IL-1α, IL-1β and IL-23 are involved in memory Th17 cell expansion in patients with psoriasis. It is possible that the molecular mechanisms are distinct in inducing Th17 cells in patients with tumors versus autoimmune diseases. The role of IL-6 and TGFβ in Th17 cell development remains controversial in humans. High levels of IL-6 and TGFβ are often detected in the tumor microenvironment. If IL-6 and TGFβ have played potent roles in promoting Th17 cells, one may expect substantial numbers of Th17 cells in human tumors. However, it is evident that the numbers of Th17 cells are limited, as compared to Treg cells and other T cell subsets in the tumor microenvironment. Blockade of IL-1, rather than IL-6 and TGFβ, albeit Th17 cell induction. Furthermore, IL-17 and Th17 cells are not quantitatively associated with IL-6 and TGFβ. Therefore, at least these two cytokines are not crucial for Th17 cell development in the ovarian cancer microenvironment. The role of IL-1β is relatively selective in Th17 cell development in the human tumor microenvironment.
We have also investigated the underlying mechanisms by which Th17 cells are limited in the tumor microenvironment. Interestingly, the levels of Treg cells and Th17 cells are inversely associated in the same tumors. Tumor associated Treg cells highly express CD39, an ectonucleotidase which converts ATP into adenosine, and suppress Th17 cell development through the adenosinergic pathway. Although it has been reported that mouse Treg cells may apply this pathway to suppress T cell activation \(^{30,31}\), we demonstrated for the first time that human tumor associated Treg cells inhibit Th17 cells with a similar molecular mechanism. In addition to multiple modes of immune suppressive mechanisms demonstrated in the tumor microenvironment \(^{49-53}\), as human Th17 cells likely mediate protective tumor immunity, inhibition of Th17 cell development may be a novel immunoediting mechanism for tumor to escape tumor immunity.

In summary, we have extensively defined the nature of Th17 in the human tumor microenvironment. Our data provides immunological and clinical evidence linking Th17 cells to immune protection in human cancer, and suggests that inhibition of Th17 cell development is a novel immune evasion mechanism. This study thus provides the rationale for developing novel immune-boosting strategies based on promoting the Th17 cell population in cancer patients.
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IK, RL and WZ: designed research, analyzed data, and wrote the paper.
MB: Analyzed data.
PC, EH, EF, DS, THW, AC, GC and RL: provided specimen and clinical information, and reviewed the paper.
IK, LV, WS, SW: performed research.

The authors declare that they have no competing financial interests.
References:


Table 1. Relationship between IL-17 and clinical pathological characteristics in ovarian cancer patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unadjusted HR (95% CI)</th>
<th>Adjusted** HR (95% CI)</th>
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<td>Stage*</td>
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<tr>
<td>Grade*</td>
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<td>Histology type*</td>
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<td>Debulking</td>
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<td>0.189 (0.078 – 0.458)</td>
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<tr>
<td>IL-17</td>
<td>0.994 (0.992 – 0.997)</td>
<td>0.994 (0.991 – 0.997)</td>
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*Age is continuous (in years), Stage is binary (II/III vs. IV), Grade is binary (0-2 vs. 3), Histotype is binary (Serous/Mucinous/Endometrial vs. Clear cells & undifferentiated), Debulking is binary (optimal vs. suboptimal residual disease), and IL-17 is continuous (in pg/ml).

**Adjusted HRs are based on a multivariable Cox proportional hazards model with Debulking (binary: optimal vs. suboptimal) and IL-17 (continuous) as covariates.
Figure legends

Fig 1. Distribution, phenotype and cytokine profile of Th17 cells. Single cell suspensions were made from fresh tumor specimens. The cells were subjected to membrane and intracellular staining and analyzed by FACS. One representative tumor specimen of 73 is shown in b-g.

(A) The distribution of Th17 cells in patients with ovarian cancer. Results are expressed as the percentage of Th17 cells in CD4+ T cells in different tissues by gating on IL-17+CD4+CD3+ cells. Normal blood: n = 41. TDLN: Tumor draining lymph nodes, n = 53. Cancer patient blood: n = 61. Ovarian cancer tissues: n = 73 (P < 0.001, compared to blood and TDLNs).

(B) IL-17 expression in CD4+ and CD8+ T cells. IL-17 expression was analyzed in tumor infiltrating CD45+ cells.

(C) The expression of CXCR4, CCR6, and CD161 in tumor infiltrating Th17 cells.

(D) The expression of CD49C, CD49D, and CD49E in tumor infiltrating Th17 cells.

(E, F) The markers associated with T cell activation/effector function and suppression. The expression of activation/effector molecules (CD25, HLA-DR and granzyme B) (E) and of suppression associated molecules (PD-1, FOXP3 and IL-10) (F) were analyzed in tumor infiltrating Th17 cells.
(G) The effector cytokine profile of Th17 cells. The cytokine profile was analyzed in tumor infiltrating Th17 cells.

**Fig 2. Th17 cells and their associations with immune cell subsets.**

(A-D) The correlation between Th17 cells and T cell subsets in the same tumor environment. Multiple tumor infiltrating T cell subsets were defined with specific staining and analyzed by FACS. The percentages of Th17 cells in CD4+ T cells, IFNγ+CD8+ T cells in CD8+ T cells and IFNγ+IL-17+CD4+ T cells in IL-17+CD4+ T cells (Th17 cells) were quantified in tumor tissues. The correlations between the percentages of Th17 cells and IFNγ+CD4+ T cells (A), IFNγ+CD8+ (B), IFNγ+IL-17+ T cells (C) and FOXP3+CD4+ T cells (D) were evaluated. Correlation coefficients were computed to assess relationship between Th17 cells and T cell subsets in the same tumor environments.

(E) The relationship between Th17 and NK cells in the same tumor environment. Th17 cells and NK cells were defined with specific staining and analyzed by FACS. Results are expressed as the percentage of NK cells in CD45+ cells. NK cells were quantified as the percentage of CD16+CD56+ cells in CD45+ cells in tumor ascites by gating on CD45+, non-T, B and myeloid cells. The samples were divided into two groups based on median percentage of Th17 cells.

**Fig 3. Induction and suppression of Th17 cell development.**

(A) Tumor associated macrophages (TAMs) induced Th17 cells. Normal blood T cells (5 x 10^5/ml) were stimulated with blood macrophages or TAMs (2.5 x 10^5/ml) from three ovarian cancer
patients (OC17, OC20 and OC38). Th17 cells were analyzed by FACS. Results are expressed as the percent of Th17 cells in CD4\(^+\) T cells. Similar results were observed in 8 ovarian cancer patients (\(P < 0.01\), compared to control).

(B) TAMs induced T cell IL-17 production. Normal T cells (5 x 10\(^5\)/ml) were stimulated with different concentrations of normal macrophages (M\(\phi\)) or TAMs from donor OC20. IL-17 was detected by ELISA in the culture supernatants. Results are expressed as mean ± SEM, \(n = 5\). \(P < 0.01\).

(C) The cytokine expression in TAMs. TAMs were isolated from ovarian cancer. Expression of IL-1\(\beta\) and IL-23p19 was detected by real-time PCR. Results are expressed as mean ± SEM, \(n = 5\). \(P < 0.01\).

(D) The importance of IL-1\(\beta\) in TAM-mediated Th17 cell induction. T cells were stimulated for 5 days with TAMs with or without the indicated neutralizing antibodies. IL-23 was blocked by specific IL-23 siRNA as we reported \(^{20}\). Th17 cells were detected by FACS. Results are expressed as the mean of Th17 cells in CD4\(^+\) T cells ± SEM, \(n = 5\), *\(P < 0.05\) compared to control.

(E, F) Treg cells suppressed Th17 and T cell IL-17 production induced by TAMs. T cells (5 x 10\(^5\)/ml) were stimulated with TAMs (2.5 x 10\(^5\)/ml) in the presence or absence different concentrations of tumor associated Treg cells. Th17 cells were analyzed by FACS (E). Results are expressed as the percent of Th17 cells in CD4\(^+\) T cells. IL-17 was detected by ELISA in the culture supernatants (F). \(n = 6\), *\(P < 0.05\) compared to control.
(G) The relevance of the adenosinergic pathway in Treg cell-mediated Th17 suppression. In the culture system described (E, F), ARL67156 was added. IL-17 was detected by ELISA in the culture supernatants. n = 6, *P < 0.05 compared to control.

**Fig 4. Th17 cells induce Th1-type chemokines.**

(A, B) The correlations between IL-17 and Th1-type chemokines CXCL9 and CXCL10 in ovarian cancer ascites. IL-17, CXCL9 and CXCL10 were detected by ELISA in ovarian cancer ascites. The correlations between IL-17 and CXCL9 (A), and CXCL10 (B) were analyzed.

(C, D) IL-17 and IFNγ synergistically induced CXCL9 and CXCL10 production by primary ovarian tumor cells. Primary ovarian cancer cells (OC8) were cultured with IL-17 in the presence of variable concentrations of IFNγ. CXCL9 and CXCL10 were detected in the cell supernatants by ELISA. Results are expressed as the mean values of ± SEM (P < 0.05).

(E) Th17 cells induced CXCL10 production by primary ovarian tumor cells through IL-17 and IFNγ. Primary ovarian cancer cells were cultured with Th17-derived supernatants in the presence or absence of anti-IFN-γ and anti-IL-17. CXCL10 was detected in the cell supernatants by ELISA. Results are expressed as the mean values of ± SEM (P < 0.05).

**Fig 5. Relationship between IL-17, Th1-type chemokines and effector T cell tumor trafficking**
(A) Effector CD8+ T cells expressed CXCR3. Blood and tumor associated T cells were stained for CXCR3. (n = 8, P < 0.01).

(B) Effector CD8+ T cells migrated toward tumor ascites through CXCR3. Tumor associated CD8+ T cells were subject to migration to different concentrations of tumor ascites with or without anti-CXCR3. Results are expressed as the mean migration index of ± SEM (n = 8, P < 0.01).

(C, D) The correlation between the mRNA levels of CXCL9 (H), CXCL10 (I) and CD8+ T cells in the same tumors. The mRNA levels of CXCL9 and CXCL10 were quantified by real-time PCR. Tumor infiltrating CD8+ T cells were defined by immunofluorescence staining and were quantified as described in the methods.

(E, F) The correlation between the levels of IL-17 and CD8+ T cells in the same tumors. Tumor infiltrating CD8+ T cells were defined by immunofluorescence staining and were quantified as described in the methods. Representative images showed CD8+ T cell infiltration in low versus high levels of IL-17 (E). The numbers of tumor effector CD8+ T cells in patients with low versus high levels of tumor ascites IL-17 were compared (P = 0.009) (F).

**Fig 6. Increased tumor associated IL-17 predicts improved patient survival**
(A-C) Kaplan-Meier curve for overall survival by the levels of IL-17 in 85 patients in stage II-IV (A), stage III (B), and stage IV (C). Samples were divided into two groups based on the median levels of IL-17 in tumor ascites (see Methods). Survival was significantly increased as a function of IL-17.

(D) The levels of IL-17 in different stages. The levels of IL-17 in different stages were compared.
Figure 1 Weiping Zou

P < 0.001, N = 73

(a) Th17 (%)

Normal blood  TDLN  Blood  Tumor  Cancer patients

(b) IL-17  CD8  CD4  CD3

(c) IL-17  CXCR4  CCR6  CD161

(d) IL-17  CD49c  CD49d  CD49e

(e) IL-17  HLA-DR  CD25  Granzyme B

(f) IL-17  PD-1  Foxp3  IL-10

(g) IL-17  TNF-α  IL-2  IFN-γ
Figure 4 Weiping Zou

(a) $P < 0.001$, $N = 104$, $R = 0.8$

(b) $P < 0.001$, $N = 71$, $R = 0.9$

(c) Control vs. IL-17

(d) Control vs. IL-17

(e) CXCL10 (pg/ml)
Figure 5 Weiping Zou

(a) Blood vs Tumor

(b) Migration index vs Ascites (%)

(c) CXCL9 (U) vs CD8 (/mm²)

(d) CXCL10 (U) vs CD8 (/mm²)

(e) Low IL-17 vs High IL-17

(f) CD8+ (/mm²)
Phenotype, distribution, generation, functional and clinical relevance of Th17 cells in the human tumor environments

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