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Angels and demons: Th17 cells represent a beneficial response, while neutrophil IL-17 is associated with poor prognosis in squamous cervical cancer

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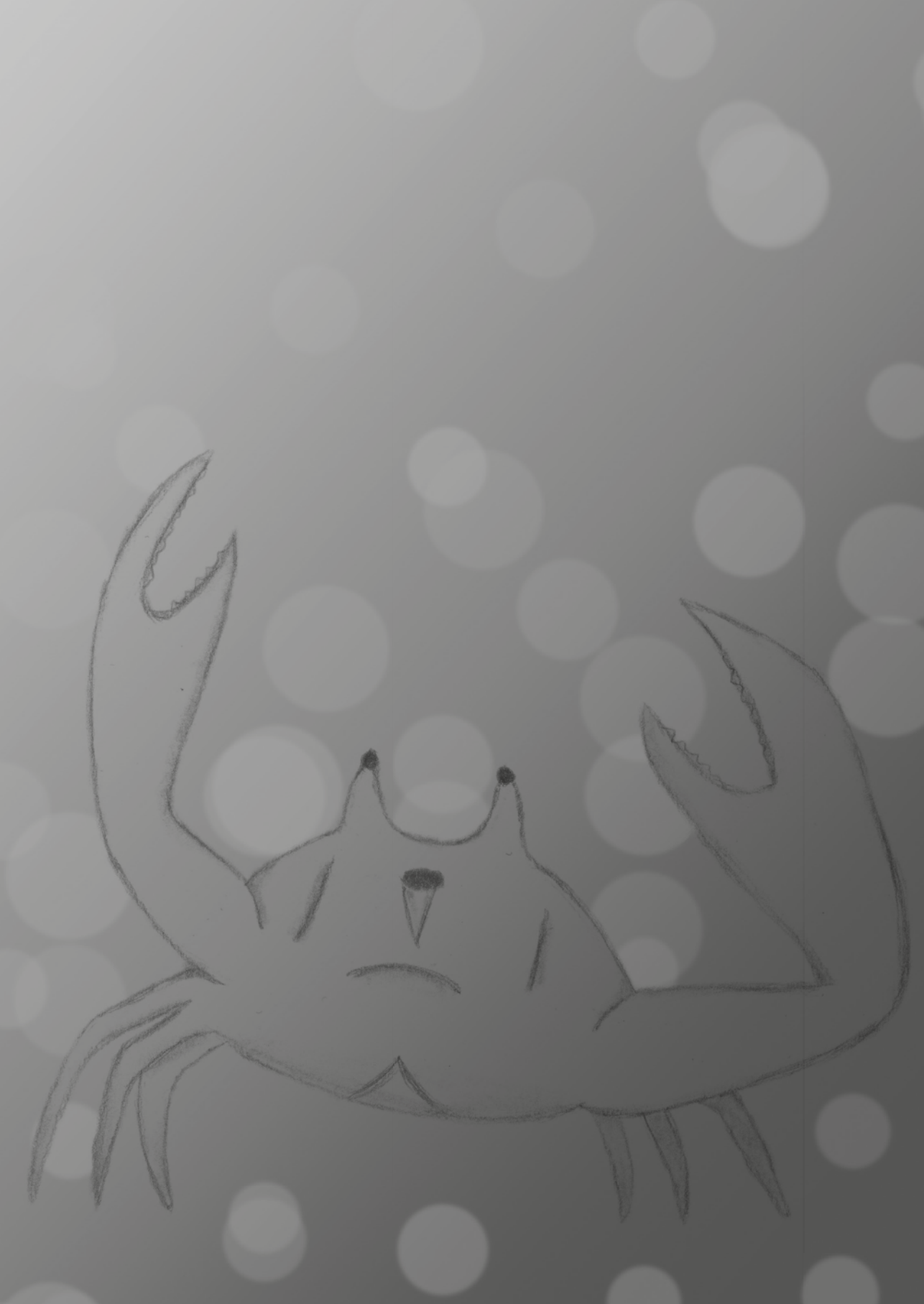


Abstract

The role of IL-17 in cancer remains controversial. In view of the growing interest in the targeting of IL-17, knowing its cellular sources and clinical implications is crucial. In the present study, we unraveled the phenotype of IL-17 expressing cells in cervical cancer using immunohistochemical double and immunofluorescent triple stainings. In the tumor stroma, IL-17 was found to be predominantly expressed by neutrophils (66%), mast cells (23%) and innate lymphoid cells (8%). Remarkably, Th17 cells were a minor IL-17 expressing population (4%). A similar distribution was observed in the tumor epithelium. The Th17 and granulocyte fractions were confirmed in head and neck, ovarian, endometrial, prostate, breast, lung and colon carcinoma. An above median number of total IL-17 expressing cells was an independent prognostic factor for poor survival in early stage cervical cancer ($p=0.016$). While a high number of neutrophils showed a trend toward poor survival, the lowest quartile of mast cells correlated with poor disease-specific survival ($p=0.011$). IL-17 expressing cells and neutrophils were also correlated with the absence of vaso-invasion ($p<0.01$). IL-17 was found to increase cell growth or tightness of cervical cancer cell lines, which may be a mechanism for tumorigenesis in early stage disease. These data suggest that IL-17, primarily expressed by neutrophils, predominantly promotes tumor growth, correlated with poor prognosis in early stage disease. Strikingly, a high number of Th17 cells was an independent prognostic factor for improved disease-specific survival ($p=0.026$), suggesting that Th17 cells are part of a tumor suppressing immune response.

Introduction

Cervical cancer is the second leading cause of death by cancer in young women.[1] Virtually all cervical cancers are initiated by infection with high risk human papilloma virus (HPV).[2] The persistent HPV infection induces an inflammatory response, which is thought to contribute to tumor growth and progression, rather than to induce an effective immune response.[3] This is partly caused by tumor cells regulating the immune response by downregulating Human Leukocyte Antigen (HLA) expression, producing immunosuppressive cytokines, such as interleukin (IL)-10 and transforming growth factor β (TGF-β)[4-6] and attracting regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSCs). Locally produced inflammatory cytokines, chemokines and angiogenic factors also often favor tumor growth and metastasis.[7]

Previously, we have shown that the presence of IL-6 in the tumor microenvironment, especially in combination with a low level of IL-12p40 expressed by tumor cells, probably indicative of the presence of IL-23, is associated with poor disease-specific survival in cervical carcinoma patients.[8] Since IL-6 and IL-23 are both involved in the IL-23/IL-17 pathway, in the present study we aimed to elucidate the role of IL-17 in

cervical cancer. The function of IL-17 is tissue and context dependent and includes activation of nuclear factor-κB (NF-κB),[9] vascular endothelial growth factor (VEGF) production and angiogenesis,[10-14] stimulation of pro-inflammatory cytokine production,[15] neutrophil recruitment[16] and formation of epithelial tight junctions.[17] While it is clear that IL-17 plays a prominent role in both the protection of the host from invading extracellular pathogens and the destruction of host tissues in several chronic inflammatory and auto-immune disorders, there is controversy about its role in cancer.[18] Different studies have shown that IL-17 can favor or counteract tumor growth, depending on tumor type and the balance of other factors in the microenvironment.[19,20] Although IL-17 is regarded as the key cytokine of T helper 17 (Th17) cells, other cell types also have the ability to express IL-17.[21-23] A number of publications on chronic inflammatory and auto-immune disorders have reported that neutrophils and mast cells are the predominant source of IL-17.[23-25] To elucidate the localization and phenotype of IL-17 expressing cells, we performed an extensive analysis of the cell types that express IL-17 in cervical cancer. Subsequently, we investigated the distribution of the main IL-17 expressing cell populations in head and neck, ovarian, endometrial, prostate, breast, lung and colon cancer. The associations between the different IL-17 expressing cell types and clinico-pathological parameters were studied in a cervical carcinoma cohort (n=160). Finally, the effect of IL-17 on cervical cancer cells was assessed in a real time cell analyzer.

Materials and Methods

Patient material

Formalin-fixed, paraffin-embedded (FFPE) squamous cervical cancer specimens obtained from all patients who underwent primary surgical treatment for cervical cancer between 1985 and 2005 with sufficient material available for analysis were retrieved from the archives of the Department of Pathology, Leiden University Medical Center (n=160). None of the patients had received preoperative anticancer therapy and follow-up data were obtained from patient medical records. Mean follow-up time was 9.4 years (ranged 8.1 – 10.6 ± 2 standard errors). Patient and tumor characteristics are listed in Table 1. Tumor node metastasis (TNM) stages below stage IIA were defined early stage. FFPE specimens from three squamous cell head and neck carcinomas, two serous and one endometrioid ovarian carcinomas, one serous endometrial carcinoma, three prostate adenocarcinomas, five ductal breast adenocarcinomas, four non-small-cell lung adenocarcinomas, three colon adenocarcinomas, three Crohn's disease and three normal colon samples were also retrieved. Patient samples were handled according to the medical ethical guidelines described in the Code of Conduct for Proper Secondary Use of Human Tissue of the Dutch Federation of Biomedical Scientific Societies.

Table 1. Patient and tumor clinico-pathological characteristics

Clinico-pathological parameter	Category	N = 160 (%)
Age	Median	45
	Range	22-87
FIGO stage[1,2]	IB	123 (77)
	IIA	35 (22)
	IIB	1 (1)
TNM stage	Ib1	47 (29)
	Ib	19 (12)
	Ib2	44 (28)
	IIA	29 (18)
	IIB	15 (9)
	IIIA	1 (1)
	IIIB	3 (2)
	IV	2 (1)
Lymph nodes[2]	negative	109 (68)
	positive	50 (31)
Tumor size (mm)[2]	<40	63 (39)
	≥40	77 (48)
Vaso-invasion[2]	Absent	67 (42)
	Present	88 (55)
Infiltration depth (mm)[2]	<15	81 (51)
	≥15	70 (44)
HPV type	16	96 (60)
	18	28 (18)
	other	36 (23)

[1]FIGO=Fédération Internationale de Gynécologie et d'Obstétrique
[2]Data were not available for all patients.

Immunohistochemistry

Immunostainings were performed on 4 µm thick FFPE sections as described before.[8] Deparaffinized sections were treated with 0.3% H_2O_2 in methanol for 20 minutes to block endogenous peroxidase activity. After rehydration, antigen retrieval was performed in citrate, or for double and triple stainings in Tris-EDTA buffer (10 mM TRIS plus 1 mM EDTA pH 9.0). Antibodies were diluted in 1% w/v bovine serum

albumin (BSA) in phosphate buffered saline (PBS), or for stainings involving antibodies conjugated to alkaline phosphatase (AP) in Tris-buffered saline pH=7.6 (TBS). Primary antibodies were incubated at room temperature overnight; secondary antibodies were incubated at room temperature for one hour (listed in Supplementary Table S1). The activity of horseradish peroxidase (HRP) was visualized using 0.5% 3,3'-diamino-benzidine-tetrahydrochloride (DAB) and 0.002% H_2O_2 in TRIS-HCl. Donkey-anti-goat-HRP was visualized by DAB+ (Dako). The activity of AP was visualized using PermaBlue (Diagnostic BioSystems). Antibodies of the same isotype class with an unknown specificity were used as negative controls. Sections single-stained with DAB were counterstained with haematoxylin, while slides stained with PermaBlue were counterstained with nuclear Fast Red. Slides were mounted using CV Mount (Leica Microsystems) or VectaShield mounting medium containing DAPI (Vector Laboratories).

The IL-17 staining was validated on stimulated $CD4^+CD45RO^+$ memory T cells obtained from healthy volunteer peripheral blood samples as described before.[26] The obtained memory T cells including Th17 cells were mixed with HeLa cells, fixed in 4% paraformaldehyde and paraffin embedded. Images were acquired with an LSM700 confocal laser scanning microscope equipped with an LCI Plan-Neofluar 25x/0.8 Imm Korr DIC M27 and C-Apochromat 40x/1.20 W Korr objectives (Zeiss; Supplementary Figure S1). Crohn's tissue, containing a relatively high number of Th17 cells,[27] was used as a positive control for the IL-17/CD3 double staining (Supplementary Figure S2). As a reference, normal colon tissue was stained for IL-17 and CD3 (Supplementary Figure S3). The staining of IL-17 by goat-anti-IL-17 (AF-317-NA, R&D Systems) was furthermore validated by comparative single and double stainings with rabbit-anti-IL-17 (sc-7927, Santa Cruz).

Microscopic analyses

All cervical cancer FFPE tissue specimens were stained for IL-17, CD15 and tryptase and digitized with a Pannoramic Midi automated slide scanner (3DHISTECH). Slides were analyzed using Mirax Viewer (Zeiss). At least 4 but generally 6 random images were taken at a 200x magnification, sampling a total tumor area of 2.5 to 3.7 mm^2 of each slide, comprising vital areas of both tumor epithelium and stroma. Images were analyzed by the open source image processing program ImageJ version 1.44 (http://rsb.info.nih.gov/ij/). DAB and haematoxylin stainings were separated by the imageJ color deconvolution method Haematoxylin, Eosin and DAB (H&E DAB; plugin ImageJ website). Suitable threshold levels for Haematoxylin and DAB were determined on random pictures. The noise was removed by the 'despeckle' command, cells were separated by the 'watershed' command and all stained cells were counted. For statistical purposes, patients were divided in two groups based on the median numbers of positive cells (high and low). The median number of $IL\text{-}17^+$ cells per image was 39 (range 1-

619) for the total cohort, 57 (range 2-619) for the early stage cohort and 43 (range 1-487) for the cohort of samples obtained until 1993. The median number of $CD15^+$ cells was 38 (range 1-939); 59 (range 1-939) for the early stage cohort and 56 (range 2-362) for the cohort until 1993. The median number of $tryptase^+$ cells was 22 (range 1-201); 23 (range 1-201) for the early stage cohort and 20 (range 2-112) for the cohort until 1993.

Four cervical cancer sections were double stained for IL-17 and different phenotype markers and analyzed with a Leica DM4000B spectral microscope equipped with HC PLAN APO 20x/0.70, HCX PLAN APO 40x/0.85 Corr and 63x/1.32-0.60 oil objectives (Leica Microsystems). Spectra between 420 and 720 nm were acquired with an interval of 20 nm and an exposure time of 100 ms per frame. All cells were quantified manually in 6 random high-power fields (40x objective HPF) in vital areas of both the tumor epithelium and tumor stroma, sampling a total area of 0.52 mm^2 of each. Three random images were taken in the other carcinoma types, comprising vital areas of both tumor epithelium and stroma. The spectra for DAB, PermaBlue and nuclear Fast Red were unmixed using the Nuance Fx Multispectral Imaging System version 2.1 (Cambridge Research and Instrumentation). High magnification pictures were taken with the 63x objective and an exposure time of 50 ms per frame for illustration purposes of the different double stainings in the same tissue specimen. A subcohort of 51 consecutive specimens obtained before 1993 was double stained for IL-17 and CD3. Six random images were taken with the 20x objective, sampling a total area of 2.1 mm^2. The median number of Th17 cells per image was 6 (range 1-62).

Immunofluorescence

Immunofluorescent images were acquired with a Zeiss LSM510 confocal laser scanning microscope equipped with a Plan-Apochromat 63x/1.4 Oil Ph3 objective (Zeiss). Pictures were taken in the same tissue sample the immunohistochemical pictures were taken in and analyzed using LSM Image Browser software (Zeiss).

Real-time cancer cell analysis

Cervical cancer cell lines CC8, CC10a, CC10b and CSCC7 were generated in our department as described previously.[28] These cell lines and the commercially available CaSki, HeLa and SiHa were grown in the xCELLigence RTCA DP system (Roche Applied Science). Cells were seeded at a concentration of 20,000 cells/well, except for HeLa en SiHa, which were seeded at a concentration of 10,000 cells/well in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS, 50 U/ml penicillin, 50 µg/ml streptomycin and GlutaMax (Gibco Life Technologies). Six hrs after seeding, the culture medium was refreshed, and 24 hrs after seeding, the culture medium was replaced by culture medium containing 100 ng/ml recombinant human IL-17A (R&D Systems) dissolved in 40 nM HCl or medium supplemented with 40 nM HCl only. The

electrical impedance imposed on the plate by the cells was measured every 30 minutes and is represented as the cell index, a measure of cell number and status. At least two independent experiments were performed at least in duplicate, but usually in triplicate or quadruplicate, with paired control and test wells equally distributed over the plate.

Statistical analysis

Statistical analyses were performed using SPSS version 20.0 (IBM). Correlations between immunohistochemical data and survival were tested using the Kaplan-Meier and Cox proportional hazards models. Correlations among immunohistochemical data and with clinico-pathological variables were tested using the Spearman's rank correlation rho and Wilcoxon Mann-Whitney tests. All tests were two-sided and p-values below 0.05 were considered statistically significant.

Results

Phenotype of IL-17$^+$ cells in squamous cervical carcinoma

To determine the phenotype of the cell populations expressing IL-17, we double stained four FFPE squamous cervical carcinoma specimens for IL-17 and different phenotype markers: CD1a (Langerhans' cells), CD3 (T cells), CD15 (granulocytes), CD33 (immature myeloid cells), CD79a (B cells), CD127 (innate lymphoid cells), CD163 (type 2 macrophages), S100 (dendritic cells) and tryptase (mast cells) (Figure 1). Since CD127 expressing naïve and memory T cells were expected to represent minor populations in the tumor microenvironment, CD127$^+$ cells are assumed to predominantly represent innate lymphoid cells. Staining for IL-17 was similar to what was observed in cultured Th17 cells and Crohn's tissue (Supplementary Figure S1-3). The IL-17$^+$ cells were primarily present in the tumor stroma. Strikingly, the majority of these IL-17$^+$ stromal cells were granulocytes (mean: 66%) (Figure 2A). Since CD15 is expressed by both neutrophilic and eosinophilic granulocytes, the phenotype of the IL-17$^+$CD15$^+$ population was further investigated by a triple staining for IL-17, CD15 and myeloperoxidase (MPO), a marker for neutrophilic granulocytes (Figure 3). Virtually all (>99%) of the IL-17$^+$CD15$^+$ cells expressed MPO, indicating these cells were neutrophils. The IL-17$^+$ cells also composed a major fraction of the total granulocyte population (mean: 82%) (Figure 2B; Supplementary Table S2). Another large IL-17$^+$ stromal population consisted of mast cells (mean: 23%). The innate lymphoid cells composed the third substantial population of stromal IL-17$^+$ cells (mean: 8%). The IL-17$^+$ cells composed a considerable part of the mast cell (mean: 40%) and innate lymphoid cell (mean: 27%) populations as well.

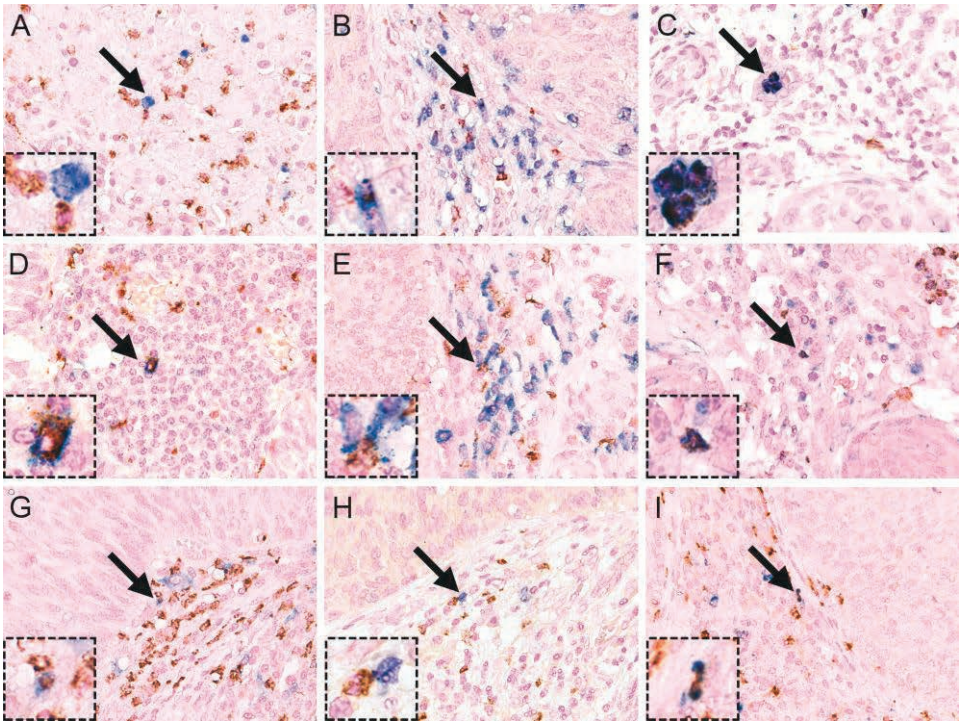
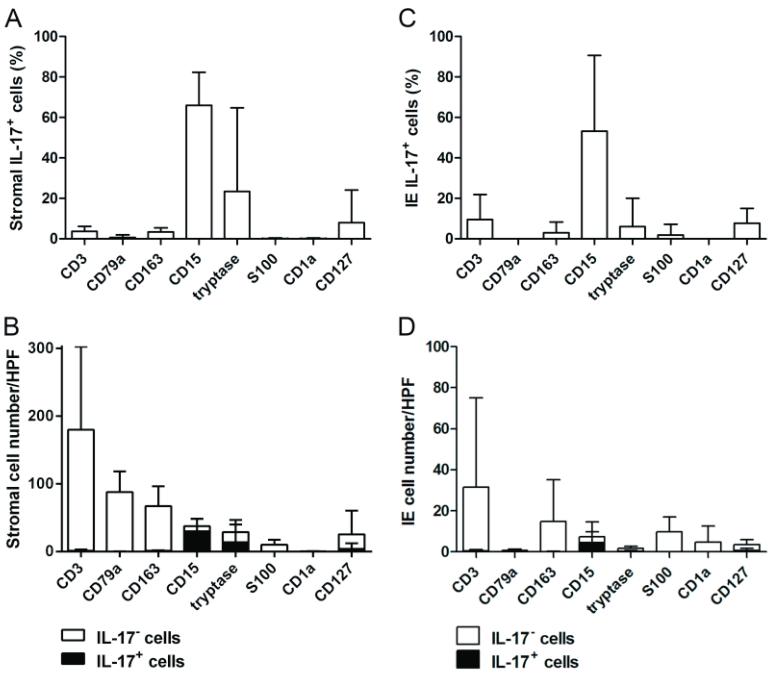


Figure 1. Immunohistochemical double staining of IL-17 and different phenotype markers
Representative images of double stainings for IL-17 (DAB) and CD1a (A), CD3 (B), CD15 (C), CD33 (D), CD79a (E), CD127 (F), CD163 (G), S100 (H) and tryptase (I) (all PermaBlue) at a x630 magnification are shown. Arrows indicate a double positive cell or cells positive for the two different markers in close vicinity, shown enlarged in the insets.

Figure 2. Phenotype of IL-17$^+$ cells in cervical carcinoma
The percentage of IL-17$^+$ cells expressing one of the cellular phenotype markers is shown for both the stromal (A) and intraepithelial (IE) (C) part of the tumor (mean and range). The total number of cells expressing one of the different phenotype markers counted per HPF (mean and range) is represented by the total bars for the tumor stroma (B) and tumor epithelium (D). The number of cells double positive for IL-17 and one of the phenotype markers is represented by the solid bar parts.



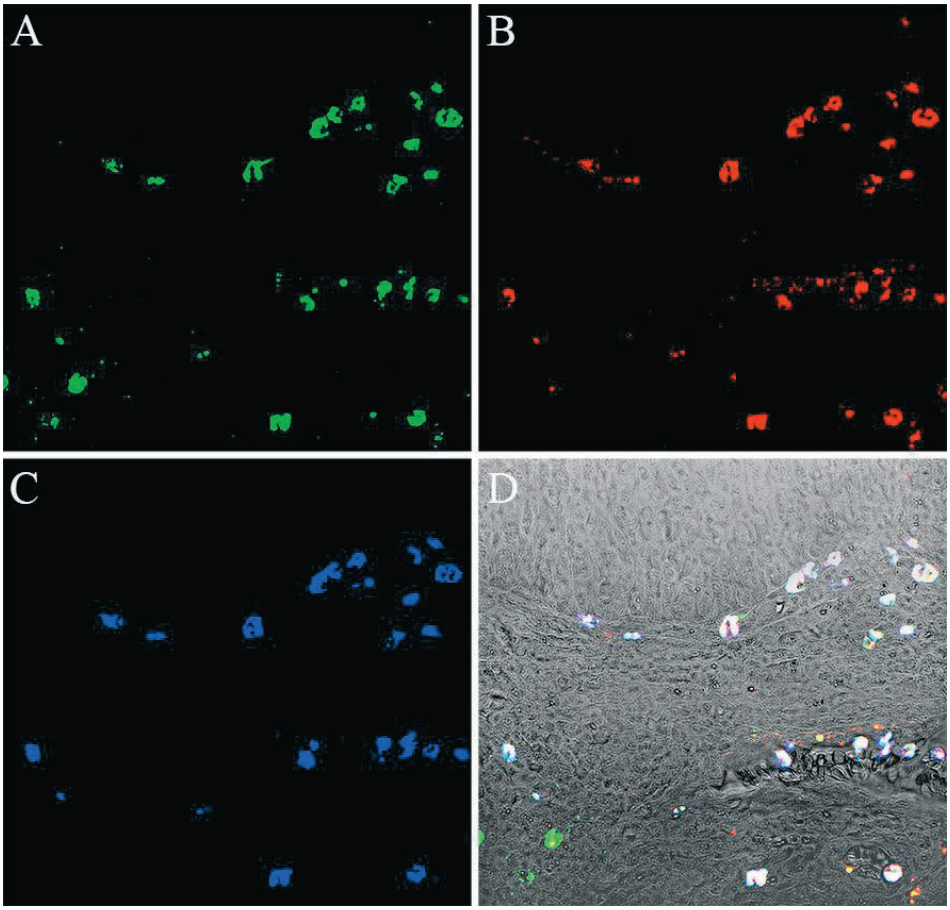


Figure 3. Triple immunofluorescent staining of IL-17$^+$ granulocytes

Representative images for cells positive for IL-17 (A), MPO (B) and CD15 (C) at a x630 magnification. The white cells in the merged picture (D) combined with a Nomarski image are IL-17$^+$MPO$^+$CD15$^+$ cells .

CD33 was used as a marker for immature myeloid cells, including MDSCs. A fraction of these cells was found to express IL-17. However, the large majority of the CD33$^+$ cells was observed to express tryptase and appeared to be mast cells (data not shown). Taken together, the CD33$^+$tryptase$^-$ population composed less than 1% of the total IL17$^+$ population. Other cell types also made minor contributions to the stromal IL-17$^+$ population, including 4% T cells. No CD8$^+$IL-17$^+$ cells were observed in the five samples with most CD3$^+$IL-17$^+$ cells, while CD4$^+$IL-17$^+$ cells were detected (data not shown). We thus designated the CD3$^+$IL-17$^+$ cell population as Th17 cells. Type 2 macrophages comprised 3% of the IL-17$^+$ cells. Of the T cells and type 2 macrophages, 1% and 2% expressed IL-17, respectively. IL-17$^+$ cells were virtually absent in the B cell, dendritic cell and Langerhans' cell populations (<0.5% expressed IL-17). A similar

distribution was observed in the tumor epithelium, with the exceptions of a relative reduction in the number of IL-17$^+$ type 2 macrophages and increase in the number of Th17 cells (Figures 2C and 2D). The relative number of IL-17$^+$ mast cells was reduced as well, but this was due to the near absence of mast cells in the tumor epithelium.

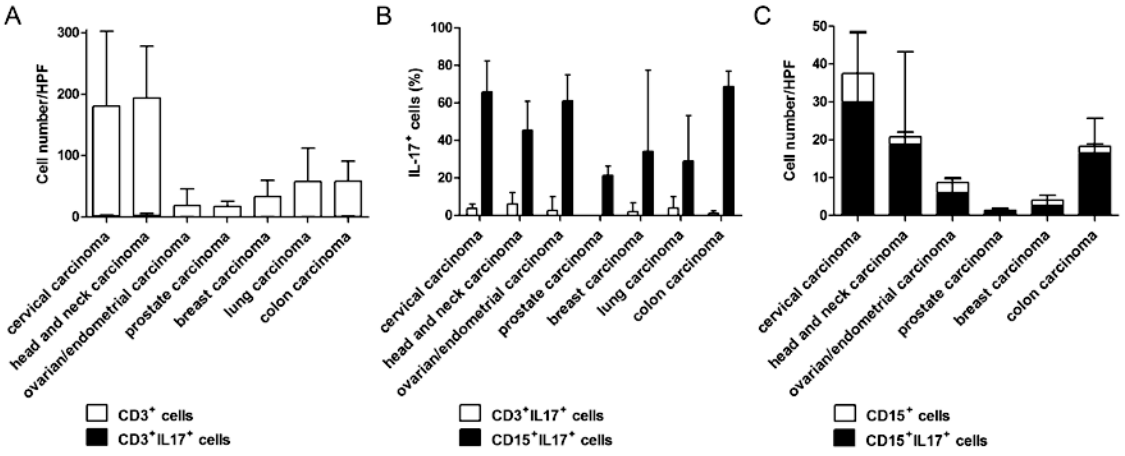


Figure 4. Phenotype of IL-17$^+$ cells in other carcinoma types

The number of cells expressing CD3 (A) or CD15 (C) counted per HPF (mean and range) is represented by the total bars. The number of cells that also express IL-17 is represented by the solid bar parts. The number of cells expressing IL-17 and CD3 (open bars) or IL-17 and CD15 (solid bars) as a percentage of the total number of IL-17$^+$ cells is shown in B.

Phenotype of IL-17$^+$ cells in other carcinoma types

Subsequently, we investigated whether a similar distribution of IL-17$^+$ granulocytes and Th17 cells was present in other types of carcinoma. A double staining for IL-17 and CD3 or CD15 was performed on 22 tumor specimens, including head and neck, ovarian, endometrial, prostate, breast, lung and colon cancer. Since the lung carcinoma samples contained tumor cells expressing CD15, the CD15 single positive cells were excluded from counting in these samples. Analogous to cervical cancer, IL-17 was expressed by a minority of T cells (mean: 0-1%) (Figure 4A; Supplementary Table S3) and Th17 cells were a minor population of the IL-17$^+$ cells (mean: 0-6%) (Figure 4B). Granulocytes composed a substantial fraction of the IL-17$^+$ cells in all tumor types (mean: 21-69%) (Figure 4B). The reverse was also true: the IL-17$^+$ cells composed a substantial fraction of the total granulocyte population (mean: 43-92%) (Figure 4C). Thus in all tumor types studied, Th17 cells were a minor IL-17$^+$ population and granulocytes were a major IL-17 expressing cell type.

Correlations between IL-17$^+$ cells, survival and clinico-pathological parameters

Finally, we investigated whether the cell types that contributed most prominently to the IL-17$^+$ cell population (total IL-17$^+$ cells, neutrophils, mast cells and Th17 cells) had different effects on survival. To study the potential prognostic correlations of IL-17 and the cell types that express it, a large series of cervical cancer tissues was stained for IL-17 (n=158), CD15 (n=140) and tryptase (n=146), see Supplementary Figure S4. A subcohort was used to identify the Th17 cells by a double staining for IL-17 and CD3 (n=51). The number of IL-17$^+$ cells was significantly correlated with the number of neutrophils (CD15$^+$ cells; r = 0.822, p<0.001), mast cells (tryptase$^+$ cells; r = 0.175, p=0.036) and Th17 cells (IL-17$^+$CD3$^+$ cells; r = 0.623, p<0.001). Although a high number of IL-17$^+$ cells did not significantly correlate with disease-specific survival overall, a high number of IL-17$^+$ cells was significantly correlated with poor survival in early stage disease (p=0.010; Figure 5A). A high number of neutrophils showed a trend toward poor survival in early stage disease (p=0.068; Figure 5B). Although the effect was not statistically significant when the groups were divided based on the median, the group of patients with the lowest number of mast cells based on a quartile division had a significantly worse disease-specific survival, both overall and for early stage disease (p=0.028 and p=0.011 respectively; Figure 5C). Interestingly, having a high number of Th17 cells was significantly associated with improved disease-specific survival (p=0.024; Figure 5D). This supports the hypothesis that the different IL-17$^+$ cell populations contribute differently to survival. Univariate and multivariate Cox analyses including lymph node status, tumor size, vaso-invasion and infiltration depth were performed to study potential prognostic variables. A high number of IL-17$^+$ cells was found to be an independent predictor for poor survival in early stage disease with a hazard ratio of 5.2 (95% CI=1.4-20.2; p=0.016; n=90; Table 2). When the parameters that are accounted for by TNM stage were replaced by the TNM stage, the correlation remained similar (data not shown). A high number of Th17 cells was found to be associated with a significantly decreased hazard ratio of 0.28 (95% CI=0.1-0.9; p=0.034; n=51; Table 3). Since the Th17 staining was performed on a smaller subcohort of patients treated before 1993, multivariate Cox regression analysis was performed by including TNM stage, known for all patients, rather than the separate parameters included before, some of which included missing data. Th17 cells were found to be an independent prognostic factor for improved survival with a hazard ratio of 0.24 (95% CI=0.1-0.9; p=0.026).

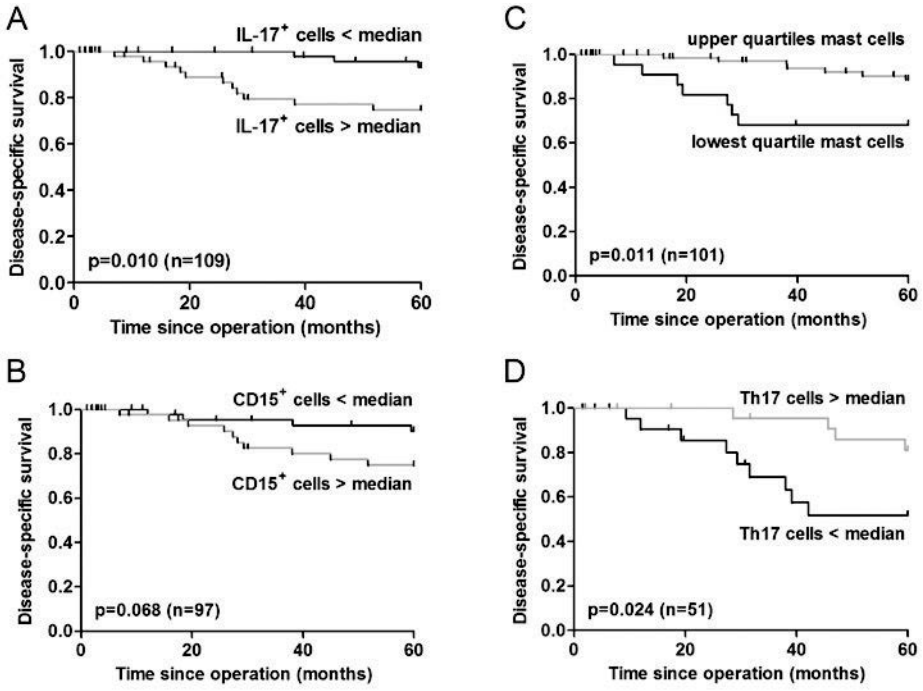


Figure 5. Correlation between IL-17, CD15, tryptase, Th17 and survival

Kaplan-Meier survival curves for the presence of the main IL-17 expressing cell populations in cervical cancer patients. Early stage disease patients with a high number of total IL-17$^+$ (A) and CD15$^+$ (B) cells were compared with patients with a low number of cells. A comparison between early stage disease patients with the lowest versus a higher number of tryptase$^+$ mast cells based on a quartile division is shown in C. A comparison of patients with a high versus a low number of Th17 cells is shown in D.

We studied whether the different cell populations were associated with critical prognostic clinico-pathological parameters (lymph node metastasis, tumor size, vaso-invasion and infiltration depth). A high number of IL-17$^+$ cells and neutrophils were found to be correlated with the absence of vaso-invasion (odds ratio=0.379; 95% CI=0.194–0.739 and odds ratio=0.340; 95% CI=0.166–0.696, respectively). By performing Mann-Whitney U tests, significant correlations with lack of vaso-invasion were found for both IL-17 (p=0.006) and CD15 (p=0.003).

Effect IL-17 on cervical cancer cell index in vitro

Administering IL-17 to the culture medium enhanced the cell index of cervical cancer cell lines CC8, CC10a and HeLa (Figure 6). A significant increase in the number of, or tightness between these cells, or both, was thus observed after administration of IL-17. This effect was not significant for the cell indices of CaSki, CC10b and SiHa. The cell index of CSCC7 decreased after administration of IL-17.

Table 2. Cox regression analysis in early stage disease

Variable	Univariate hazard ratio (95% CI)	p-value	Multivariate hazard ratio (95% CI)	p-value
Lymph node status	5.30 (2.70-10.40)	<0.001	3.33 (0.84-13.14)	0.087
Tumor size	1.04 (1.02-1.05)	<0.001	1.04 (1.00-1.09)	0.039
Vaso-invasion	2.20 (1.08-4.50)	0.031	0.54 (0.13-2.22)	0.395
Infiltration depth	1.03 (1.00-1.06)	0.054	1.05 (0.98-1.12)	0.199
IL-17$^+$ cells	4.61 (1.28-16.52)	0.019	5.24 (1.36-20.18)	0.016
CD15$^+$ cells	2.81 (0.88-8.98)	0.080		
tryptase$^+$ cells	0.28 (0.10-0.80)	0.018		
Th17 cells	0.51 (0.14-1.80)	0.292		

Univariate Cox regression analyses for the categorical clinico-pathological parameters lymph node metastasis and vaso-invasion presence, the continuous variables tumor size and infiltration depth and an above median number of cells positive for the different immunological markers on disease-specific survival in early stage disease. A multivariate Cox analysis is shown for a high number of IL-17$^+$ cells corrected for the clinico-pathological parameters.

Table 3. Cox regression analysis Th17 cohort

Variable	Univariate hazard ratio (95% CI)	p-value	Multivariate hazard ratio (95% CI)	p-value
TNM stage	1.62 (1.26-2.07)	<0.001	1.72 (1.21-2.45)	0.003
IL-17$^+$ cells	1.05 (0.35-3.13)	0.932		
CD15$^+$ cells	1.30 (0.41-4.11)	0.652		
tryptase$^+$ cells	0.32 (0.10-1.06)	0.063		
Th17 cells	0.28 (0.09-0.91)	0.034	0.24 (0.07-0.85)	0.026

Univariate and multivariate Cox regression analyses for TNM stage and an above median number of cells positive for the different immunological markers in a cohort of patients treated between 1985 and 1993.

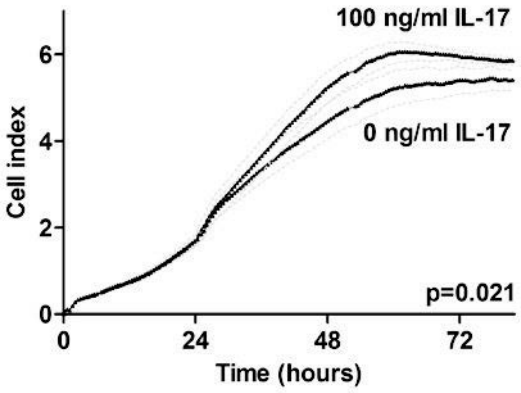


Figure 6. Effect of IL-17 on cervical cancer cells *in vitro*

Mean (black dots) and standard error of the mean (grey lines) of a representative experiment of the cell index of four wells per condition of cervical cancer cell line CC10a seeded at a concentration of 20,000 cells/well at time 0 is shown. At 6 hours, culture medium was refreshed, and at 24 hours, the culture medium was replaced by culture medium with or without 100 ng/ml IL-17A. The slope of the lines between 24 en 60 hours was found to differ significantly between the conditions (p=0.021) using a Mann-Whitney U test.

Discussion

In the present study, formalin-fixed tissue was used to determine the phenotype and location of IL-17 expressing cells. By using immunohistochemistry to investigate the different cell types in the original tissue morphology, we showed for the first time that the predominant cell type expressing IL-17 in squamous cervical cancer is the neutrophilic granulocyte. In addition, mast cells and innate lymphoid cells composed substantial IL-17 expressing cell populations. Minor IL-17$^+$ cell populations or cell types that did not express IL-17 were Th17 cells, macrophages, B cells, dendritic cells, Langerhans' cells and MDSCs. Subsequently, we showed that the predominant IL-17$^+$ cell type in head and neck, ovarian, endometrial, prostate, breast, lung and colon cancer was the granulocyte as well, while Th17 cells were a minor IL-17 expressing population. A limited number of studies described the phenotype of IL-17$^+$ cells in solid cancer. In colorectal carcinoma, IL-17 was found in Th17 cells and macrophages, but the two cell populations were not quantified.[13] Two studies in non-small cell lung cancer and advanced epithelial ovarian cancer described that most IL-17$^+$ cells were lymphocytes, but IL-17 expression was also observed in polymorphonuclear cells.[14,29] We observed a heterogenous pattern in the cell types expressing IL-17 in the different tumor types we analyzed, with 21-69% being granulocytes. Although we have not been able to measure IL-17 at RNA level in neutrophils *in vitro*, this might be caused by the short-lived and terminally differentiated state of neutrophils, which have been described to contain no or very low mRNA levels for the granule proteins they express.[30,31] The frequent large size of the other IL-17$^+$ cells observed, suggested that mast cells composed another substantial population. In accordance with this, Wang et al. recently described that Th17 cells and mast cells represented 2% and 72% of IL-17$^+$ cells in esophageal squamous cell carcinoma, respectively, and 19% of total mast cells expressed IL-17.[32] Our findings that 23% of the IL-17$^+$ cells were mast cells and 40% of mast cells expressed IL-17 in cervical cancer, suggests that the relative numbers of mast cells and granulocytes varies in different types of cancer, and probably even in different patients. Thus there appears to be heterogeneity in the cell types expressing IL-17 between different types of cancer. Overall, we showed that a predominance of IL-17 expressing granulocytes and mast cells and limited numbers of Th17 cells seem to be a general feature in different types of carcinoma. This suggests that the correlations that have been described between IL-17, neutrophils and angiogenesis might be more tightly linked than previously suspected, since the neutrophils can produce IL-17 themselves and are also strongly associated with angiogenesis.

We further studied the association between the different IL-17$^+$ cell populations and clinico-pathological parameters. The total number of IL-17$^+$ cells was significantly correlated with poor disease-specific survival in early stage squamous cervical carcinoma. The fact that we did not find this correlation for all TNM stages, suggests that IL-17$^+$ cells are mainly effective in early disease stages. This might be correlated

with their negative association with vaso-invasion, since both higher TNM stages and a low number of IL-17$^+$ cells were correlated with vaso-invasion. In higher TNM stages, where vaso-invasion is more frequently present, IL-17$^+$ cells do not seem to have a distinct effect. This heterogenous function corresponds with the literature that has reported on both tumor promoting[14,33] and tumor suppressing[29,34,35] effects of IL-17. In the present study, we show that this may also be the case because IL-17 is expressed by different cell populations, which have different effects in the tumor microenvironment. Their contributions might change during disease progression. One of the mechanisms through which IL-17 might be correlated with poor survival, is by directly contributing to tumor growth. The effect of IL-17 on tumor growth has been controversial, partly because IL-17 has indirect effects by stimulating other cells to produce a diversity of cytokines. We studied the effect of IL-17 on tumor cell growth *in vitro* and observed an enhanced cell index in three (CC8, CC10a and HeLa) out of seven cervical cancer cell lines. Since we measured changes in electrical impedance, this might reflect an increase in either cellular growth, or cellular tightening, or both. IL-17 has been described to increase the number of tight junctions in epithelial cells,[17] supporting the hypothesis that cellular tightening may play a role. Previous studies did not find an effect of IL-17 on HeLa and IC1 cervical cancer cell growth.[36,37] These results suggest that IL-17 may increase cellular tightness in cervical cancer cells. Additionally, both a high number of IL-17$^+$ cells and a high number of neutrophils were significantly correlated with the absence of vaso-invasion in cervical cancer. These findings are also in accordance with the observation that IL-17 is able to increase the number of tight junctions in epithelial cells,[17] further supporting the hypothesis that cellular tightening may play an important role. A similar finding was reported by Cunha et al., who showed that the absence of CD4$^+$IL-17$^+$ cells correlated with invasion into the underlying tissue in a multivariate analysis in differentiated thyroid carcinoma patients.[38] Together, this suggests that IL-17 may stimulate cellular tightening and decrease the occurrence of vaso-invasion.

Corresponding to the majority of IL-17$^+$ cells expressing CD15 and the majority of CD15$^+$ cells expressing IL-17, the number of IL-17$^+$ cells strongly correlated with the number of CD15$^+$ neutrophils. These CD15$^+$ neutrophils did not show a significant effect on disease-specific survival in cervical cancer. This corresponds to the finding that most of these cells express IL-17, which overall did not have an association with survival either. This does suggest that it is primarily the production of IL-17 that is associated with poor survival in early stage disease, since its main cell source is not significantly correlated but does show a trend. However, the presence of neutrophils in the tumor microenvironment was associated with an N2 phenotype and both IL-17 and neutrophils were correlated with angiogenesis and poor survival in, among others, colorectal and hepatocellular cancer.[13,39,40] The reason for this discrepancy is unclear. Part of the explanation may be the heterogeneity of the CD15$^+$ cell population. This population may include both tumor suppressing N1 and tumor promoting N2 type neutrophils, the former for instance by promoting the Th17 pathway. Activated murine

neutrophils were shown to produce CCL2 and CCL20, ligands for CCR2 and CCR6, respectively, expressed by Th17 cells.[41] This might be a mechanism for neutrophils to drive the Th17 pathway.[42] Although circulating $CD15^+$ neutrophils have been shown to be an important source of IL-17 in humans, and mouse liver infiltrating neutrophils were shown to express both IL-17 and the transcription factor crucial for IL-17 differentiation retinoic acid receptor-related orphan receptor-γt (RORγt),[43] the functional properties of neutrophils expressing IL-17 are not clear yet.

Although not as strongly as for CD15, the number of $IL\text{-}17^+$ cells also significantly correlated with the number of $tryptase^+$ cells. The lowest quartile of mast cells was significantly correlated with poor disease-specific survival in our study. Although total mast cells are frequently associated with angiogenesis and tumor progression, as was described for cervical cancer,[44] the intratumoral rather than the peritumoral mast cells were described to be associated with improved survival in prostate and breast cancer.[45,46] The latter observations are in agreement with our findings.

According to expectations, a strong correlation was observed between the numbers of $IL\text{-}17^+$ cells and Th17 cells. Surprisingly, despite the small fraction the Th17 cells represent, high Th17 numbers were found to be significantly correlated with improved disease-specific survival. A high number of Th17 cells proved to be an independent prognostic factor for survival. We conclude that this association is at least a reflection of a beneficial immune response in cervical cancer. The role of Th17 cells in cancer is controversial with both tumor promoting and tumor suppressing functions being reported. For instance, Tosolini et al. found that a Th17 gene expression profile was correlated with poor survival in colorectal cancer.[47] However, in agreement with our results, Kryczek et al. reported that a high amount of Th17 derived IL-17 in the ascites of ovarian cancer patients was correlated with improved survival.[48] Based on these results, Th17 cells are suggested to be part of a tumor suppressing immune response. Indeed, Th17 cells have been shown to be correlated with interferon-gamma production and infiltration of cytotoxic T cells.[49]

To conclude, we found that IL-17 in different types of carcinoma was primarily expressed by granulocytes and mast cells. These granulocytes were shown to be neutrophils in squamous cervical cancer and while total $IL\text{-}17^+$ cells were independently associated with poor survival in early stage disease, neutrophils showed a trend toward association with poor survival. Since we showed that IL-17 enhanced the cell index in three out of seven cervical cancer cell lines, IL-17 seems to directly contribute to tumorigenesis. Additionally, both $IL\text{-}17^+$ cells and $CD15^+$ cells were significantly correlated with the absence of vaso-invasion in cervical cancer. These data suggest that IL-17 may primarily function by inducing tumor cell tightness, which might play an important role in early stage disease, where vaso-invasion less frequently occurs. A high number of mast cells was correlated with improved disease-specific survival. Th17 cells were an independent prognostic factor for improved disease-specific survival in squamous cervical cancer. These data support our hypothesis that the different cell types

expressing IL-17 play different roles in the tumor microenvironment and have different effects on survival.

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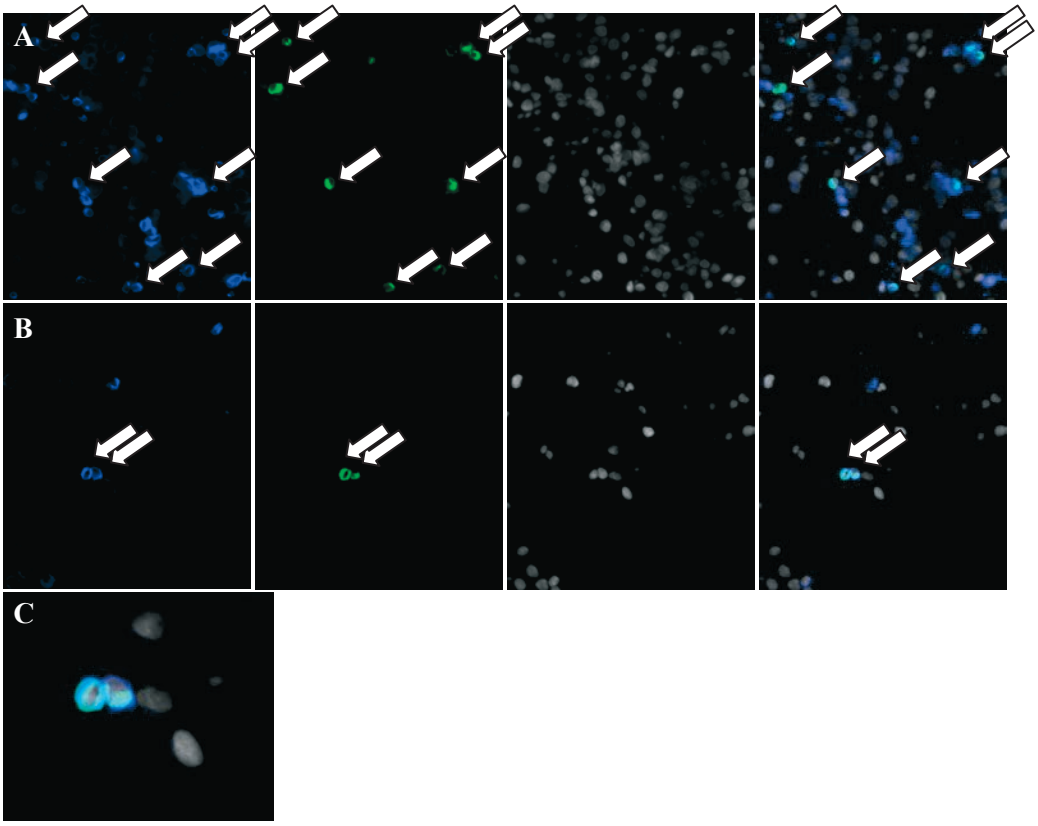
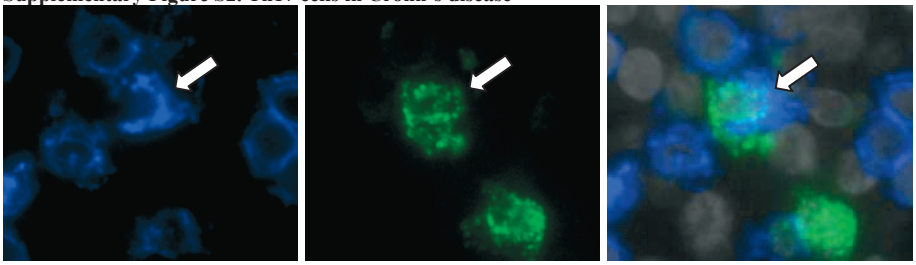
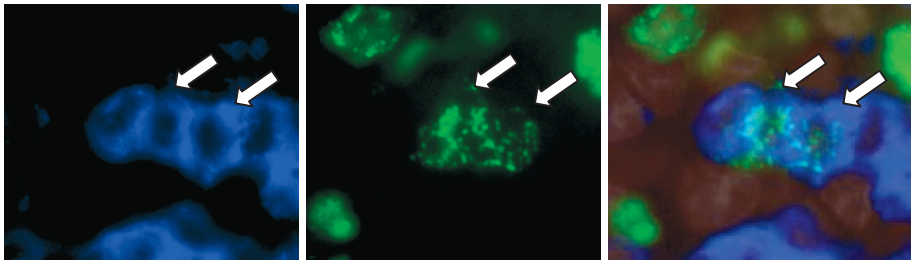
Supplementary Figure S1. Confocal analysis of activated memory T cells including Th17 cells



Stimulated $CD4^+CD45RO^+$ memory T cells were mixed with HeLa cervical cancer cells, formalin fixed and embedded in paraffin. Cells positive for CD3 (blue), IL-17 (green) and DAPI (grey) are shown for two donors in A and B. Arrows indicate $CD3^+IL\text{-}17^+$ double positive cells (all $IL\text{-}17^+$ cells were positive for CD3). The double positive cells from B are shown enlarged (at original magnification 250x) in C.

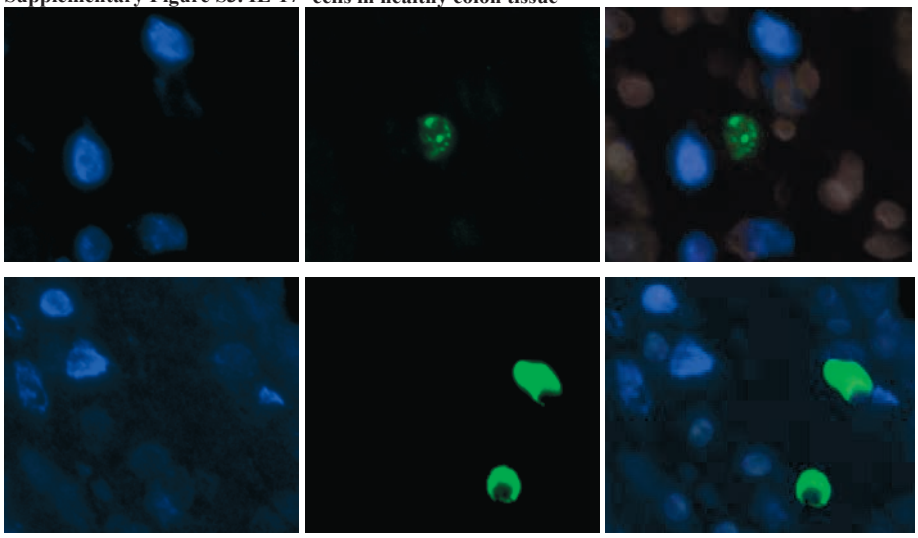
Supplementary Figure S2. Th17 cells in Crohn's disease





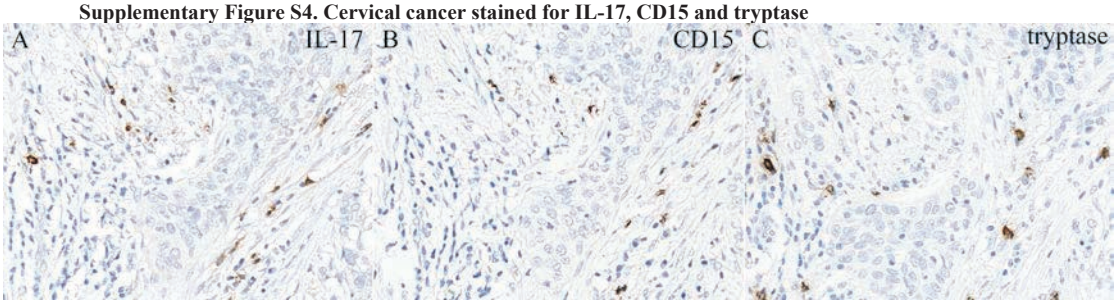
Cells double positive for CD3 (blue) and IL-17 (green) were observed in FFPE tissue from colon biopsies from Crohn's disease patients. Nuclei are stained by DAPI, shown in grey in the merged image on the right. Arrows indicate double positive cells. Representative pictures from two random samples are shown (400x magnification).

Supplementary Figure S3. IL-17$^+$ cells in healthy colon tissue



A different distribution of cells positive for CD3 (blue) and IL-17 (green) was observed in FFPE tissue from healthy colon tissue samples. Nuclei are stained by DAPI, shown in grey in the merged image on the right. Relatively more large IL-17 single positive cells and no Th17 cells seemed to be present. Representative pictures from two random samples are shown (250x magnification).

Supplementary Figure S4. Cervical cancer stained for IL-17, CD15 and tryptase



Representative immunohistochemical stainings (200x magnification) identifying IL-17 (A), CD15 (B) and tryptase (C) at a similar position in sections of the same tumor specimen.

Supplementary Table S1. Antibodies and reagents used for immunohistochemistry and immunofluorescence

Antigen	Cellular specificity	Antibody supplier	Isotype	Antibody reference	Secondary reagent	Reagent supplier	Reagent reference
CD1a	Langerhans' cells	Dako	Mouse IgG1	clone 010	RatαM IgG1-AP	Southern Biotech	clone SB77E
CD3	T cells	Dako	Mouse IgG1	clone F7.2.38	RatαM IgG1-AP	Southern Biotech	clone SB77E
CD4	T helper cells, macrophages	Santa Cruz	Rabbit IgG	H-370	DαR-A546	Invitrogen, Life Technologies	A10040
CD8	CTL	Dako	Mouse IgG1	Clone C8/144B	DαM-A647	Invitrogen	A31571
CD15	Granulocytes and monocytes	Dako	Mouse IgM	clone C3D-1	RatαM IgM-AP	Southern Biotech	1B4B1
					DαM-A647	Invitrogen	A31571
CD33	Immature myeloid cells	Leica Micro-systems	Mouse IgG2b	clone PWS44	RatαM IgG2b-AP	Southern Biotech	clone SB74g
CD79a	B cells	Dako	Mouse IgG1	clone JCB117	RatαM IgG1-AP	Southern Biotech	clone SB77E
CD127	Innate Lymphoid Cells	Santa Cruz	Rabbit IgG	sc-662	DαR-AP	Abcam	ab7084
CD163	Macrophages type 2	Leica Micro-systems	Mouse IgG1	clone 10D6	RatαM IgG1-AP	Southern Biotech	clone SB77E
IL-17		R&D Systems	Goat IgG	AF-317-NA	Goat HRP-polymer kit	Biocare Medical	GHP516
					DαG-HRP	Abcam	ab97120
					DαG-A488	Invitrogen	A11055
IL-17		Santa Cruz	Rabbit IgG	sc-7927	Bright-Vision	Immunologic	DPVO-HRP
					GαR-A546	Invitrogen	A11010
S100	Dendritic cells	Dako	Rabbit IgG	Z0311	DαR-AP	Abcam	ab7084
Tryptase	Mast cells	Millipore	Mouse IgG1	clone G3	RatαM IgG1-AP	Southern Biotech	clone SB77E
Myelo-peroxidase	Neutrophilic granulocytes	Dako	Rabbit IgG	A398	DαR-A546	Invitrogen	A10040

The characteristics of the primary and secondary antibodies and reagents used to visualize each antigen are listed. Abbreviations: D=Donkey; G=Goat; R=Rabbit; M=Mouse; AP=alkaline phosphatase; DAB=3,3'-diamino-benzidine-tetrahydrochloride

Supplementary Table S2. Phenotype of IL-17$^+$ cells in cervical carcinoma

	Stromal part		Epithelial part	
Phenotype marker	**Total cell nr**	**IL-17$^+$ cells**	**Total cell nr**	**IL-17$^+$ cells**
CD1a (LC)	0.3 (0-1)	0 (0-0.2)	5 (1-13)	0 (0-0)
CD3 (T cell)	180 (88-303)	2 (0-3)	31 (6-75)	1 (0-1)
CD15 (PMN)	38 (26-67)	30 (19-49)	7 (1-20)	5 (0-15)
CD79a (B cell)	88 (36-119)	0.2 (0-1)	1 (0-1)	0 (0-0)
CD127 (ILC)	26 (13-58)	4 (0-12)	4 (0-5)	1 (0-2)
CD163 (Mφ2)	67 (41-97)	1 (1-2)	15 (2-35)	0.1 (0-0.3)
S100 (DC)	10 (5-18)	0 (0-0.2)	10 (5-17)	0 (0-0.2)
tryptase (MC)	29 (11-73)	14 (3-40)	2 (0-3)	0.3 (0-1)

The mean and range of the total number of cells positive for each of the different phenotype markers are shown for the tumor stroma as well as for the tumor epithelium. The mean and range of these cells also expressing IL-17 is shown in an additional column. Abbreviations: LC=Langerhans' cells; PMN=polymorphonuclear cell; ILC=innate lymphoid cell; Mφ2=macrophage type 2; DC=dendritic cell; MC=mast cell

Supplementary Table S3. Phenotype of IL-17$^+$ cells in other carcinoma types

Cancer type	**total IL-17$^+$ cells**	**total CD3$^+$ cells**	**CD3/IL17 positive cells**	**total CD15$^+$ cells**	**CD15/IL17 positive cells**
cervical	42 (15-59)	180 (88-303)	2 (0-3)	38 (26-67)	30 (19-49)
head/neck	31 (14-74)	194 (61-281)	2 (0-6)	21 (4-47)	19 (3-43)
ovar/endo	8 (2-17)	18 (7-46)	0.1 (0-0.3)	9 (4-14)	6 (3-10)
prostate	4 (1-6)	17 (4-26)	0 (0-0)	1 (0-2)	1 (0-2)
breast	9 (2-24)	33 (7-60)	0.2 (0-1)	4 (0-8)	3 (0-6)
lung	8 (3-14)	57 (20-112)	0.3 (0-1)	NA	3 (1-5)
colon	29 (7-54)	59 (40-91)	1 (0-1)	24 (20-27)	17 (6-26)

In the different types of carcinoma, the total number of IL-17$^+$ cells (mean and range), the total number of CD3$^+$ and CD15$^+$ cells, and of these cells, the number of cells expressing both IL-17 and CD3 or CD15 are shown. Abbreviations: ovar/endo=ovarian and endometrial carcinoma; NA=not applicable since part of the lung tumor cells expressed CD15