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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Abbreviations: DAB; 3; 3'-diamino-benzidine-tetrahydrochloride; FFPE; formalin-fixed; paraffin-embedded; HPF; high-power field; HPV; human papillomavirus; MDSCs; myeloid-derived suppressor cells; MPO; myeloperoxidase; TNM; tumor node metastasis stage.

The role of interleukin (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, T-helper 17 (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 disease-specific survival in early stage disease (p < 0.016). While a high number of neutrophils showed at trend toward poor survival, the lowest quartile of mast cells correlated with poor 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 survival (p = 0.026), suggesting Th17 cells are part of a tumor suppressing immune response.

Introduction

Cervical cancer is worldwide the second leading cause of death by cancer in young women.1 Virtually all cervical cancers are initiated by infection with high risk human papillomavirus (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 IL-10 and transforming growth factor β (TGF-β)4,6 and attracting regulatory T cells 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. 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. Although IL-17 is regarded as the key cytokine of Th17 cells, other cell types also have the ability to express IL-17. 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. 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 clinicopathological 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.

**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) (Fig. 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 (Fig. 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%) (Fig. 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 (Fig. 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%) (Fig. 2B; Table S1). 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.

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 (Figs. 2C, D). 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.

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%) (Fig. 4A; Table S2) and Th17 cells were a minor population of the IL-17+ cells (mean: 0–6%) (Fig. 4B). Granulocytes composed a substantial fraction of the IL-17+ cells in all tumor types (mean: 21–69%) (Fig. 4B). The reverse was also true: the IL-17+ cells composed a substantial fraction of the total granulocyte population (mean: 43–92%) (Fig. 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 clinicopathological 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 Fig. 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; Fig. 5A). A high number of neutrophils showed a trend toward poor survival in early stage disease (p = 0.068; Fig. 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 had a significantly worse disease-specific survival, both overall and for early stage disease (p = 0.028 and p = 0.011 respectively; Fig. 5C). Interestingly, having a high number of Th17 cells was significantly correlated with improved disease-specific survival (p = 0.024; Fig. 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 tumor node metastasis stage (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).

We studied whether the different cell populations were associated with critical prognostic clinicopathological 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
When the parameters accounted for by TNM stage were replaced by the TNM stage, the correlation remained similar (data not shown).

IL-17

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. 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.14,26 We observed a heterogeneous 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.27,28 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 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.

**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.14,26 We observed a heterogeneous 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.27,28 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 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 1. Patient clinicopathological characteristics**

| Clinicopathological parameter | Category | N = 160 (%) |
|-------------------------------|----------|-------------|
| **Age**                       | Median   | 45          |
| Range                        |          | 22–87       |
| **FIGO stage**<sup>a,b</sup>  | I       | 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)      |
|                              | IIbA     | 1 (1)       |
|                              | IIIB     | 3 (2)       |
|                              | IV       | 2 (1)       |
| **Lymph nodes**              | negative | 109 (68)    |
|                              | positive | 50 (31)     |
| **Tumor size (mm)**<sup>b</sup> | <40   | 63 (39)     |
|                              | >40      | 77 (48)     |
| **Vaso-invasion**<sup>b</sup> | Absent | 67 (42)     |
|                              | Present  | 88 (55)     |
| **Infiltration depth (mm)**<sup>b</sup> | <15 | 81 (51) |
|                              | >15      | 70 (44)     |
| **HPV type**                 | 16       | 96 (60)     |
|                              | 18       | 28 (18)     |
|                              | other    | 36 (23)     |

<sup>a</sup>FIGO, International Federation of Gynecologists and Obstetricians  
<sup>b</sup>For some variables, data were not available for all patients.

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 (Fig. 6). A significant increase in the number of, or tightness between these cells, or both, was thus observed after

**Table 2. Cox regression analysis in early stage disease**

| Variable          | Univariate Cox regression | Multivariate Cox regression |
|-------------------|---------------------------|-----------------------------|
|                   | Hazard ratio (95% CI)     | p-value                     | 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<sup>c</sup> cells | 4.61 (1.28–16.52)    | 0.019                       | 5.24 (1.36–20.18)         | 0.016   |
| CD15<sup>c</sup> cells | 2.81 (0.88–8.98)       | 0.080                       |                            |         |
| tryptase<sup>c</sup> 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 clinicopathological 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 are shown. A multivariate Cox analysis is shown for a high number of IL-17+ cells corrected for the clinicopathological parameters. When the parameters accounted for by TNM stage were replaced by the TNM stage, the correlation remained similar (data not shown).
Table 3. Cox regression analysis Th17 cohort.

| Variable           | Univariate Cox regression | Multivariate Cox regression |
|--------------------|---------------------------|----------------------------|
|                    | Hazard ratio (95% CI)     | p-value                    | 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 Cox regression analyses for TNM stage and an above median number of cells positive for the different immunological markers on disease-specific survival in a cohort of patients treated between 1985 and 1993 are shown. A multivariate Cox analysis is shown for a high number of Th17 cells corrected for TNM stage.

19% of total mast cells expressed IL-17.\(^{29}\) 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 associations between the different IL-17\(^+\) cell populations and clinicopathological 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\(^{14,30}\) and tumor suppressing\(^{26,31,32}\) 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.\(^{33,34}\) 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.\(^{35}\) 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.\(^ {13,36,37}\) 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-type neutrophils 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.\(^{38}\) This might be a mechanism for neutrophils to drive the Th17 pathway.\(^{39}\) 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), the functional properties of neutrophils expressing IL-17 are not yet clear.

Although not as strongly as for CD15, the number of IL-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, the intratumoral rather than the peritumoral mast cells were described to be correlated with improved survival in prostate and breast cancer. The latter observations are in agreement with our findings.

According to expectations, a strong correlation was observed between the numbers of IL-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. 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. 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.

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-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-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.
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 y (ranged 8.1–10.6 ± 2 standard errors). Patient and tumor characteristics are listed in Table 1. TNM stages below stage 2A 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.

Immunohistochemistry

Immunostainings were performed on 4 μm thick FFPE sections as described before. Deparaffinized sections were treated with 0.3% H2O2 in methanol for 20 min 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 in phosphate-buffered saline, or for stainings involving antibodies conjugated to alkaline phosphatase in Tris-buffered saline pH = 7.6. Primary antibodies were incubated at room temperature overnight; secondary antibodies were incubated at room temperature for one hour (listed in Table S3). The activity of horseradish peroxidase (HRP) was visualized using 0.5% 3,3′-diamino-benzidine-tetrahydro-chloride (DAB) and 0.002% H2O2 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 hematoxylin, 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. 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 25 × /0.8 Imm Korr DIC M27 and C-Apochromat 40 × /1.20 W Korr objectives (Zeiss; Fig. S1). Crohn’s tissue, containing a relatively high number of Th17 cells, was used as a positive control for the IL-17/CD3 double staining (Fig. S2). As a reference, normal colon tissue was stained for IL-17 and CD3 (Fig. 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 four but generally six random images were taken at a 200× magnification, sampling a total tumor area of 2.5–3.7 mm² of each slide, comprising vital areas of both the 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 hematoxylin stainings were separated by the imageJ color deconvolution method Hematoxylin, Eosin, and DAB (H&E DAB; plugin ImageJ website). Suitable threshold levels for hematoxylin 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-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 20×/0.70, HCX PLAN APO 40×/0.85 Corr, and 63×/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 six random high-power fields (40× objective HPF) in vital areas of both the tumor epithelium and tumor stroma, sampling a total area of 0.52 mm² of each. Three random images were taken in the other carcinoma types, comprising vital areas of both the 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 63× 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 20× objective, sampling a total area of 2.1 mm². 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 63×/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.49 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 and 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 hours after seeding, the culture medium was refreshed, and 24 h 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 min and is represented with 40 nM HCl only. The electrical impedance imposed on the plate by the cells was measured every 30 min 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 clinicopathological variables were tested using the Spearman’s rank correlation rho (r) and Wilcoxon Mann–Whitney tests. All tests were two-sided and p-values below 0.05 were considered statistically significant.
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Supplemental Material

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