The IL-17 and Th17 cell immune response in cervical cancer

Angels or demons: it depends on the context

Simone Punt

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With an open mind,
in combination with the right amount of curiosity,
dreams and the need to explore,
you will find things beyond imagination.

Freddy van der Garde

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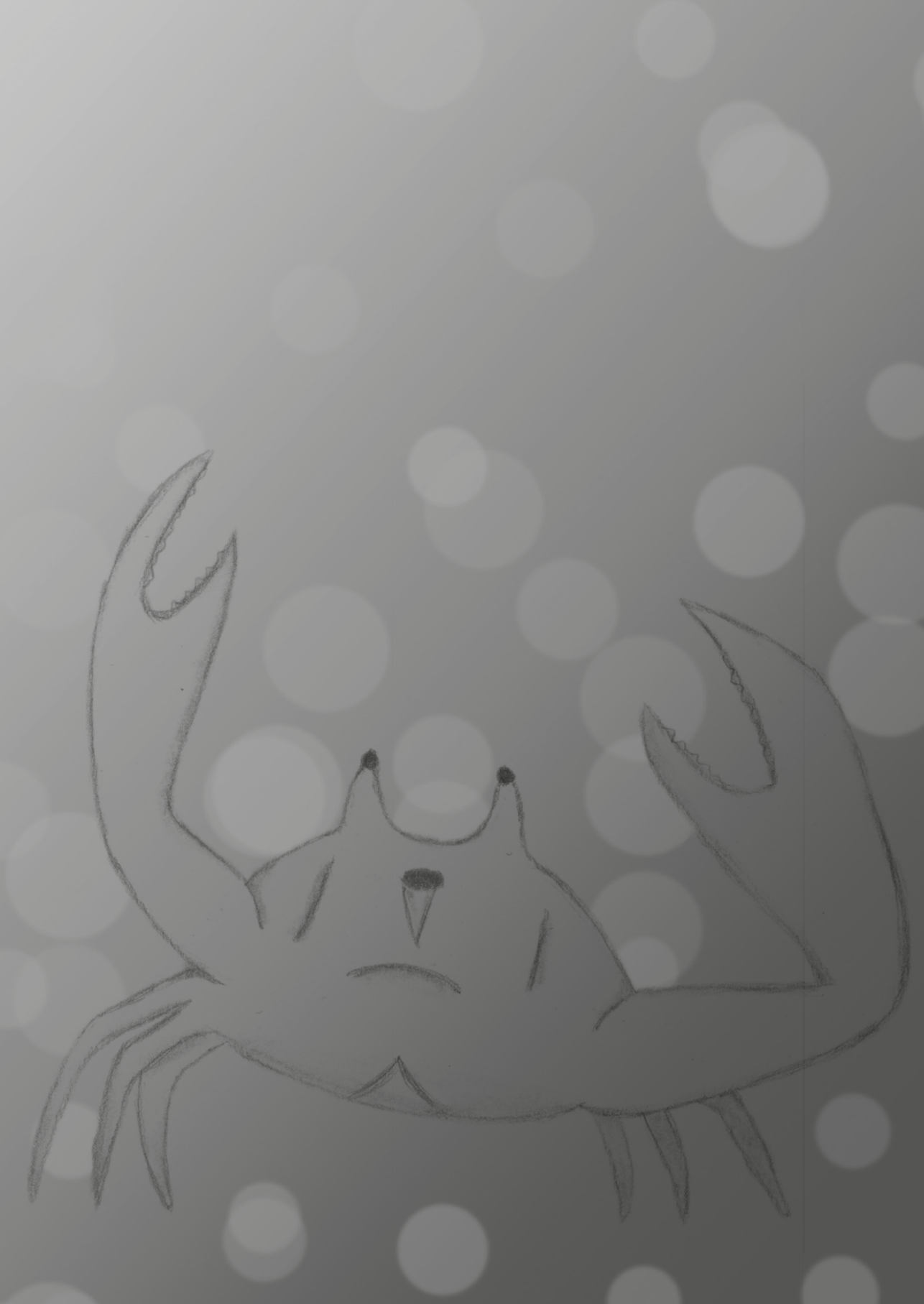

1. Cervical cancer

The cervix is located in the lower part of the female uterus. Cervical cancer most frequently affects women under the age of 50.[1] Cervical cancer is the fourth leading cause of death by cancer in women worldwide.[2] This is mainly attributable to the high incidence in developing countries. In developed countries, the incidence and mortality of cervical cancer have declined by up to 80% between 1930 and 2010 because of widespread screening programs for early detection and prevention.[3,4] Although efforts are being undertaken to screen women for cervical cancer precursor lesions in a cost-effective way in developing countries,[5] cervical cancer is still the third most common cancer type and cause of death by cancer in these countries.[2]
Screening for cervical cancer using the Papanicolaou cytology test (Pap smear) has been introduced in the Netherlands in the 1970s. Cervical cancer is currently the sixth most prevalent cancer type in the Netherlands, with 736 new cases in 2012.[6] The incidence and mortality of cervical cancer in the Netherlands in 2012 were 8.0 new cervical cancer cases and 2.1 deaths per 100,000 age adjusted person years. In Europe, 13.4 new cervical cancer cases and 4.9 deaths per 100,000 years occurred in the same year.[7]

1.1 Etiology

Different risk factors for the development of cervical cancer have been identified, including high parity, smoking, co-infection with human immunodeficiency virus (HIV) or other sexually transmitted infections and long-term oral contraceptive use.[8] The only absolute requirement for the development of cervical cancer is a persistent infection with high-risk human papillomavirus (HPV).[9] HPV is a double-stranded DNA virus that encodes for seven early (E1-7) and two late (L1-2) proteins. HPV is the most common sexually transmitted infection, with about 40 of the 150 known HPV types infecting cells at anogenital lesions.[8] Low-risk HPV types are associated with the development of warts. Fifteen high-risk carcinogenic HPV types can cause precancerous lesions, an estimated 65% of which are infected with HPV type 16 or 18.[10]

The endocervix is covered with simple columnar epithelium, which continues toward stratified squamous epithelium covering the ectocervix. Under the influence of hormones during puberty or pregnancy, or in response to physical or chemical stress, the columnar epithelium is replaced by squamous epithelium at the border between the ectocervix and endocervix. This process of metaplasia relocates the epithelium transition area from the endocervix to the ectocervix and is termed the transformation zone. This area is thought to be vulnerable for transformation toward precancerous conditions.[11] The abnormal expansion of immature cells in the transformation zone is termed dysplasia. This can be caused by HPV infection of the basal cells of the squamous epithelium at a local site of micro-trauma in the transformation zone. The

virus replicates episomally in these cells and induces cellular proliferation by the expression of viral proteins E5-7. Upon replication of the basal cells, these move to suprabasal epithelial layers, which induces the expression of the late viral structural proteins. This leads to the assembly and release of complete viruses. The expression of viral proteins also leads to viral antigen expression on the cellular membrane. Recognition by the immune system of these non-self proteins leads to clearance of the infected cells in over 80% of infected women.[12] So although about 80% of women are assumed to be infected by HPV in a lifetime,[13] only about 10-20% of HPV infections progress to cervical intraepithelial neoplasia (CIN).[14] Progression to invasive cancer occurs in approximately 38% of untreated precancerous CIN3 lesions.[15]

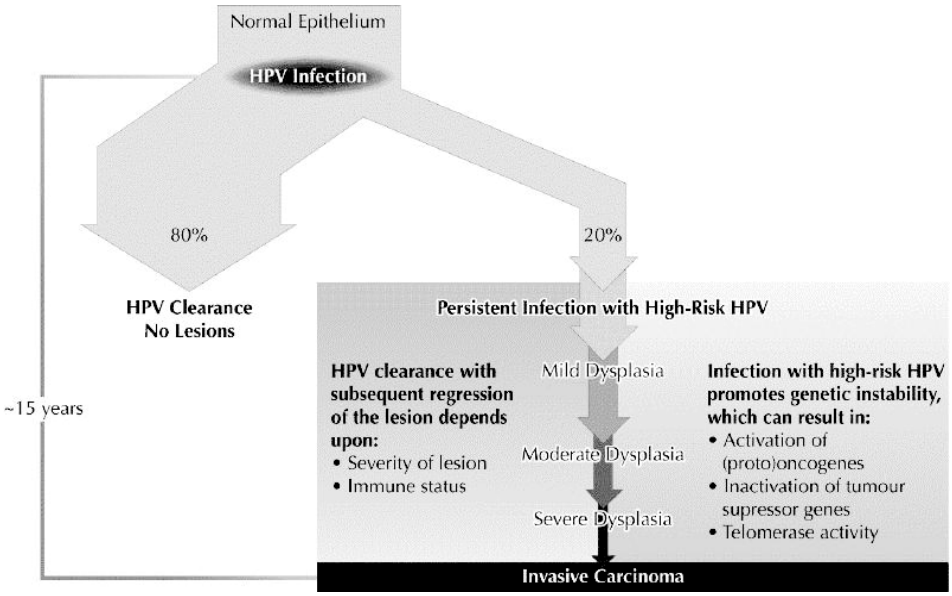

Figure 1. Etiology of cervical cancer

The relationship between infection with high-risk HPV, development of precancerous dysplastic lesions and progression to invasive cancer. From Meijer *et al*.[16]

Upon replication of the basal cell, the viral genome may integrate into the host genome. This typically disrupts the expression of viral gene E2.[17,18] Since the E2 protein suppresses the expression of E6 and E7, this leads to E6 and E7 protein overexpression. The E6 protein binds and causes degradation of the tumor suppressor proteins p53 and Bak.[19,20] Additionally, E6 increases the activity of telomerase.[21] Viral protein E7 binds cyclin related proteins and the tumor suppressor protein family retinoblastoma, leading to their degradation.[22] This deregulates the cell cycle by removing multiple checkpoints, enhancing cell survival and proliferation. This may eventually lead to uncontrolled cell proliferation and carcinogenesis.

The three predominant histological subtypes of cervical cancer are squamous cell carcinoma, adenosquamous cell carcinoma and adenocarcinoma. Although squamous cell carcinoma accounts for about 75% of all cervical cancer, its incidence rate has decreased over the past 40 years, while the incidence of cervical adenocarcinoma has increased to approximately 20%-25% of cases.[23] The prognosis of patients with cervical adenocarcinoma may be worse than for patients with squamous cell carcinoma,[24-29] although the data on this topic are controversial.[30,31]

1.2 Staging and treatment

Precancerous dysplasia as well as cervical cancer can be detected by analyzing a Pap smear for abnormal cells. If abnormal cells are identified, a biopsy is taken for further assessment. Treatment depends on the stage of the (pre)cancerous lesion. CIN1 is a mild dysplasia characterized by mitotic activity and nuclear atypia in the lower one third of the epithelium. CIN1 regresses in 60% of cases[32-34] and only 2% of cases progresses to severe dysplasia or invasive cancer in two years.[34] Treatment of CIN1 consists of follow-up cytology for two years. In case of moderate (CIN2) or severe (CIN3) dysplasia, the abnormal cells extend toward the middle and upper third of the epithelium, respectively. In the case of CIN2, 16% of cases is expected to progress to a higher stage within two years.[34] The risk of progression from CIN2 and CIN3 to cervical cancer *in situ* or invasive cancer is 2.5 and 4.2 times higher than for CIN1, respectively. CIN2 and CIN3 are usually treated by large loop excision of the transformation zone (LLETZ).

In the case of cervical cancer, cancer cells have invaded the underlying stromal tissue through the epithelial basement membrane. Cervical cancer is staged both according to the Fédération Internationale de Gynécologie et d'Obstétrique (FIGO) and the tumor size, lymph node, distant metastasis (TNM) staging system. The FIGO stage is based on clinical examination of tumor size and invasion into the underlying stroma, parametria, vagina and pelvic wall and distant metastases at the time of diagnosis. The TNM stage also includes metastasis to the lymph nodes and is determined by a pathologist postoperatively. Treatment depends on FIGO stage and for early cancer stages (FIGO stage 1A) usually consists of conization or radical hysterectomy with or without lymphadenectomy (Wertheim procedure).[1] A larger tumor (stages IB and IIA) can be treated with a Wertheim surgery or (concurrent) radiotherapy with or without chemotherapy. Advanced stage cancer (stages IIB to IV) is treated with a combination of radio- and chemotherapy. Clinical criteria that influence prognosis are lymphatic spread, tumor size, vascular invasion and infiltration depth.

FIGO stage	Years after diagnosis				
	1	2	3	4	5
IA	100%	99%	99%	98%	98%
IB	98%	94%	91%	89%	87%
IIA	95%	84%	80%	76%	76%
IIB	92%	79%	70%	67%	64%
IIIA	71%	56%	52%	48%	38%
IIIB	71%	51%	42%	37%	37%
IVA	47%	32%	24%	22%	21%
IVB	35%	17%	12%	9%	7%

Table 1. Prognosis of cervical cancer stages

Survival rates of cervical cancer patients in the Netherlands, stratified for FIGO stage.[1]

2. The immune response to cancer

The immune response developed very early in evolution to protect organisms against pathogens. At the beginning of this millennium, the immune system was also shown to protect the body against cancer formation by a mechanism termed cancer immunosurveillance.[35] The immunosurveillance hypothesis states that T lymphocytes continuously survey the body for tumor cells expressing aberrant proteins. These proteins may be pathogen derived proteins, but also host proteins that are altered due to the high mutation rate of cancer cells. Although leukocytes had already been observed in tumors by Rudolf Virchow in 1863, and immunosurveillance had already been predicted to occur by Paul Ehrlich in 1909,[36] a century passed before this theory was proven. The identification of tumor specific antigens in a variety of tumors transplanted in inbred mouse models[37] and the protection from cancer development by a functional immune response, potentially leading to cancer immune escape,[38] have been crucial to establish the concept of cancer immunoediting. Immunoediting comprises three phases: elimination, equilibrium and tumor escape.[39] Highly immunogenic tumors are thought to be eliminated by the immune system, as was envisioned in the original immunosurveillance hypothesis. The resulting selection pressure may in some cases induce a daughter cell variant to arise that can escape from immune recognition and form a tumor despite the presence of an immune response. Clinically apparent tumors are thus likely to have already adapted to the immune response induced. Tumor immune escape has been recognized as one of the 'hallmarks of cancer' required for tumor development.[40] Tumor infiltrating T lymphocytes have been shown to be an independent predictor for survival in ovarian and colorectal cancer, supporting the immunosurveillance theory.[41,42]

In contrast to an acute pro-inflammatory tumor targeting immune response, chronic inflammation may rather favor tumor growth. All tumors contain chronic inflammatory infiltrate that can promote cancer progression.[43] High expression of the inflammatory cytokine interleukin-6 (IL-6) has for instance been shown to be an independent predictor of poor survival.[44-46] Chronic inflammation can increase the risk of oncogenic transformation through promoting cell survival and suppressing the adaptive immune response.[47] The production of radicals by immune cells may furthermore cause DNA mutations. The other way around, transformed cells induce tumor promoting inflammation by producing cytokines that recruit inflammatory cells.[47] Thus, the composition of the immune response in cancer determines tumor progression and patient prognosis.

2.1 The tumor microenvironment

Besides tumor cells, the tumor microenvironment also comprises blood vessels, different host cell types and extracellular matrix (ECM) proteins and glycans. Most ECM components are synthesized by fibroblasts. The ECM provides a scaffold to organize cellular structure and trafficking, but also mediates cytokine signaling to regulate cell growth and function.[48] The structure and composition of the ECM are altered in cancer and both the biochemical and biomechanical properties support cancer cell growth.

While some resident immune cells will be present, most immune cells are recruited to the tumor via the vasculature. The blood vessels deliver both nutrients and immune cells to the tumor tissue, and are critical for tumors to grow beyond a few millimeters in size. An inverse correlation between tumor growth promoting vessel formation (angiogenesis) and vessel adhesiveness supporting immune cell infiltration (maturation) has been reported.[49] Angiogenesis is represented by a high number of blood vessels, which supports tumor growth and has been correlated with poor survival in cervical cancer.[50] Vessel maturation is characterized by the expression of signaling and adhesion proteins and supports the infiltration of immune cells in the tumor tissue.[49,51]

2.2 The innate immune response

Both innate and adaptive immune cells are recruited to a tissue in response to danger or stress signals. Innate immune cells are cells that respond to common danger signals, for instance dead cell fragments or viral double-stranded RNA. The innate immune response is well conserved in animals, and plants have a similar defense mechanism against pathogenic microorganisms.[52] The innate immune cells act directly upon triggering of their pattern-recognition receptors (PRRs), for instance Toll-like receptors (TLRs) or Nod proteins. Activation involves phagocytosis of pathogens or particles, secretion of toxic molecules, but also production of cytokines and chemokines to recruit and activate an adaptive immune response. The innate immune response provides the

first line of defense at the natural anatomical barriers (i.e. the skin, digestive tract and lungs).

Since cell death is a common phenomenon in cancer, innate immune cells regularly infiltrate tumor tissue. Despite this infiltration of the different types of innate immune cells in tumors, their effect has not been well studied. High frequencies of neutrophilic[53-55] or eosinophilic[56] granulocytes in cancer have been shown to be correlated with poor survival. Mast cells have been ascribed both tumor targeting as well as tumor promoting effects.[57-59] Innate γδ-T and natural killer (NK) cells are generally not found in large numbers, but do seem to have favorable tumor growth suppressing effects.[60-62] Finally, subpopulations of a certain cell type may have different impacts. Macrophages can for instance be categorized as classically activated M1 type macrophages, which promote a tumor targeting immune response and favorable prognosis,[63] or alternatively activated M2 type macrophages, which are correlated with tumor progression and poor prognosis.[64,65] Similarly, inflammation may drive the differentiation of neutrophils toward a tumor promoting inflammatory state by a combination of TGF-β, IL-10 and prostaglandin E2 (PGE_2).[66] Alternatively activated macrophages and neutrophils express different cytokines, are less capable to activate T cells or kill tumor cells and induce more angiogenesis.[67] In line with these cell subpopulations, myeloid derived suppressor cells (MDSCs) are a heterogeneous and difficult to define population of innate immune cells frequently infiltrating tumor tissue. Granulocytic MDSCs are functionally and phenotypically similar to neutrophils, although neutrophils mainly seem to regulate angiogenesis, tumor invasion and metastasis, while MDSCs are predominantly involved in immunosuppression.[68]

In normal tissue, the immune response ultimately induces a repair process, upon which the inflammation resolves. Since tumors can be envisioned as 'wounds that never heal',[69] the immune response results in a chronic inflammation. Both the innate immune cells and the tumor cells then produce a variety of chemokines and pro-inflammatory cytokines that promote tumor growth, such as IL-1β, IL-6, IL-8 (CXCL8) and tumor necrosis factor α (TNFα).[70]

2.3 Cytokines and chemokines

Cytokines are small (glyco)proteins that are mainly produced by immune cells to communicate with other cells.[70] Potential effects induced include cell growth, differentiation and activation. These key regulatory molecules can be pro- or anti-inflammatory, and play a dominant role in the correlation between chronic inflammation and cancer burden.[71] Upon TLR activation, innate immune cells produce cytokines that direct the type and magnitude of the adaptive immune response. Cancer cells can similarly produce cytokines to modulate the immune response to favor tumor growth

and stimulate angiogenesis.[72] Adaptive immune cells also communicate with each other and with target cells through cytokines.
Chemokines are small chemotactic cytokines. Chemotaxis is important to signal specific target cells to infiltrate the tissue along a chemokine gradient. Different cell types express different chemokine receptors. Not only stromal but also tumor cells express chemokines, regulating the type and number of infiltrating immune cells.[73]

2.4 Antigen presentation

Intracellular as well as extracellular constituents of the cellular environment are presented on the cell membrane to the immune system by the human leukocyte antigen (HLA) protein family. HLA class I molecules are expressed on all nucleated cells and present peptide fragments from intracellular proteins. HLA class II is expressed on professional antigen presenting cells and present peptides derived from processed material present in the micro-environment, for example peptides derived from pathogens or dead cells. Combined with co-stimulatory molecules and cytokines, this induces an adaptive immune response and ensures that infected cells expressing aberrant non-self peptides are eliminated.[74] Through presentation of HPV derived peptides by HLA molecules, the immune system usually recognizes and destroys HPV infected cells.[12] In some cases though, a small number of infected cells may remain present despite an immune response. This leads to selection pressure for a daughter cell to emerge that can escape from immune recognition and may eventually develop into cancer. Different pathways can be involved in immune escape, including suppression of the tumor targeting immune response, inhibiting apoptosis induced by immune cells or altering HLA expression.[75,76] Altered HLA class I expression was detected in 90% of cervical cancers.[77] Still, the presence and frequencies of different types of immune cells influence tumor progression. The HPV related immunogenicity of cervical cancer cells is already used to prevent cervical cancer by vaccination.[78] However, immunotherapy with the aim to treat cervical cancer has so far yielded limited efficacy.[79] Investigating the local immune response in cervical cancer is thus important to be able to improve the outcome of therapeutic immunotherapies, which offer the potential of treating patients more specifically and effectively, with less accompanying side effects than the currently used radio- and chemotherapies.

2.5 The adaptive immune response

Vertebrates have developed an adaptive immune response. T and B lymphocytes, belonging to the adaptive immune system, require stimulation to proliferate and differentiate, are highly specific and provide long-lasting memory.[80] T cell progenitors mature in the thymus by developing a unique T cell receptor (TCR) through a process of positive and negative selection. Mature naïve T cells circulate through the body until they encounter cells expressing the specific antigen that activates TCR signaling in the

context of HLA. T cells require three so-called signals to be activated: (1) the specific antigen recognized in the context of HLA presentation mediated through TCR triggering, (2) co-stimulatory molecules expressed by antigen presenting cells and (3) cytokine modulation of the type of response. This induces T cell proliferation, differentiation and migration to the tissue. Activated $CD8^+$ cytotoxic T lymphocytes (CTL) are able to directly kill cells that express antigen in the context of HLA class I. A high frequency of cytotoxic T cells has been shown to correlate with improved survival in different cancer types,[81,82] including cervical cancer.[83] Activated naïve $CD4^+$ T cells may differentiate toward T helper 1 (Th1), Th2, Th17 and regulatory T cells (Tregs). Recently, a number of additional T cell subpopulations have been described, like Th9 and Th22 cells.[84] Because of their low frequency and underexplored significance, these cell types will not be discussed in this thesis. T helper cells provide help in the activation of CTL and B cells and produce a variety of cytokines and chemokines that can recruit and activate different cell types.

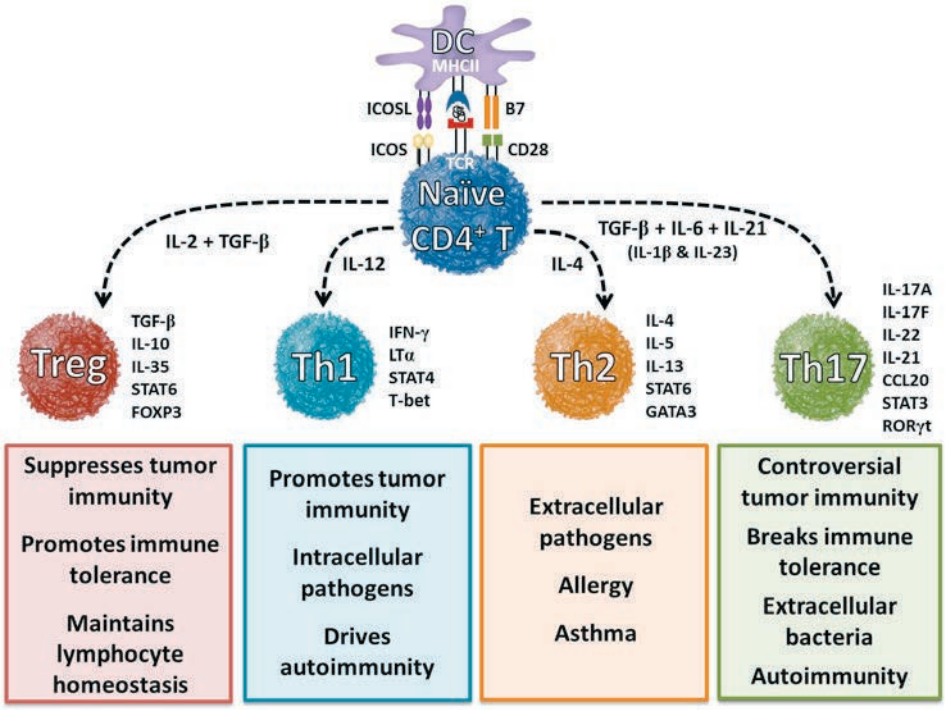

Figure 2. T helper cell subtypes

Upon encountering specific antigen and costimulatory molecules (e.g. B7, ICOSL), different combinations of cytokines induce differentiation of naïve T cells toward different T helper cell subtypes. The role of the different subtypes under normal homeostasis as well as in cancer is indicated in the boxes below each T helper cell type. From Bailey *et al.*[85]

Th1 cells are required to facilitate the clearance of pathogen infected cells. In cancer, Th1 cells are generally appreciated for their potential to induce or stimulate a tumor targeting immune response. An effective tumor targeting immune response is thus characterized by IL-2, IL-12 and interferon-γ (IFNγ). Th2 cells protect against extracellular pathogens, induce allergic reactions and have been shown to support cervical cancer progression,[86] but their general role in cancer is not clear. Th17 cells are essential to protect against extracellular pathogens, particularly those not handled well by a Th1 or Th2 response, and play a dominant role in autoimmune diseases.[87,88] Their role in cancer is unclear, since they have been shown to both be able to promote as well as to counteract tumor growth.[89] The characteristics of Th17 cells are discussed in paragraph 3. Tregs are essential to suppress an immune response when it is not required, for instance to prevent autoimmunity. In cancer, these cells also suppress the activity of other T cells, which may dampen either a tumor suppressing or tumor promoting immune response. Indeed, Tregs have been found to be correlated with poor survival in cervical cancer,[83] but also with less invasion in thyroid cancer and improved recurrence-free survival in head and neck cancer.[90,91] Tumor growth supporting Th2/Treg-mediated chronic immunosuppressive inflammation is induced by a combination of IL-4, IL-5, IL-6, IL-10, IL-13 and transforming growth factor β (TGF-β).[70]

B lymphocytes are the other component of the adaptive immune response. Similar to T cells, B cells mature by developing a unique B cell receptor (BCR) in the bone marrow. The BCR is composed of two identical light and heavy chains, and is identical to an antibody when secreted. Upon binding the specifically recognized antigen combined with T cell stimulation, the B cell is stimulated to proliferate and undergo BCR hypermutation to increase its antigen binding affinity. B cells are typically stimulated by Th2 cells, driving a humoral antibody response. B cell clones with the highest binding affinity are sufficiently stimulated to grow out, undergo antibody class switching and differentiate toward antibody producing plasma cells or memory B cells. Depending on the antibody isotype, antibody binding to a target antigen facilitates phagocytosis, triggers the complement cascade or induces antibody dependent cell-mediated cytotoxicity.

Although it is difficult to assess the role of B cells in immunoediting and the clinical response, circulating antibodies directed against p53 have been shown to be correlated with increased p53 mutational load and accumulation, tumor progression and poor survival.[92] This suggests that circulating antibodies may merely be a reflection of antigenic stimulation that does not necessarily induce an effective immune response. However, a high number of tumor infiltrating B cells has been correlated with improved patient survival in different cancer types.[93,94] Antibodies have furthermore been shown to undergo antigenic selection and affinity maturation in cervical cancer.[95] Differentiation toward regulatory B cells may prevent a tumor targeting immune response,[96] but data on this topic are yet insufficient.

3. The Th17 cell immune response

Human naïve T cells can be differentiated toward Th17 cells by a combination of IL-1β, IL-6 and IL-23, although the exact conditions required are still under debate.[97] Despite otherwise being pleiotropic in nature, these cytokines also have in common that they are generally correlated with poor prognosis in cancer patients. The pro-inflammatory cytokine IL-1β, predominantly produced by macrophages, induces the expression of other signaling molecules like IL-6 and IL-8 and is correlated with an increased risk of developing cancer.[98] After initial chemoattraction of neutrophils by IL-8, IL-6 may subsequently attract monocytes and T cells.[99] Both signaling molecules have been correlated with poor disease-specific survival in cancer. IL-6 may induce signal transducer and activator of transcription 3 (STAT3) expression in tumor cells,[100] epithelial-mesenchymal transition and angiogenesis[101,102] and differentiation of macrophages toward the M2 phenotype, thus supporting tumor growth.[103,104] The cytokine IL-23 belongs to the IL-12 family of heterodimeric cytokines, but antagonizes the functions of IL-12 and IFNγ. IL-23 also induces angiogenesis and infiltration of neutrophils and macrophages.[105] IL-23 is furthermore required to maintain a stable Th17 cell population. Although mouse Th17 cells can be stably induced by the combination of IL-6 and TGF-β, the absolute requirement of TGF-β for Th17 differentiation is still under debate.[97] It seems that IL-1β replaced TGF-β for human Th17 differentiation, while TGF-β probably indirectly favors Th17 differentiation by inhibiting differentiation toward Th1 cells.

3.1 Phenotype and function of Th17 cells

Th17 lymphocytes were first described as a unique T helper cell subpopulation in 2000[106] and further characterized in 2005.[107] These cells were termed Th17 cells because of their production of IL-17, which is generally used as a marker to characterize Th17 cells.[108] Th17 cells are also characterized by the lineage specific transcription factors retinoic acid receptor-related orphan receptor γ t (RORγt) and RORα,[109,110] which are induced by IL-6 signaling through STAT3 activation.[111] STAT3 induces IL-23R expression, while IL-23 signaling stabilizes the Th17 cell phenotype and induces IL-22 secretion.[88,112,113] IL-23 expression has been associated with tumor development.[105,114]

Th17 cells also produce IL-21, which in an autocrine manner inhibits forkhead box P3 (FoxP3)[115] and IFNγ[116] expression and further supports the Th17 cell phenotype.[117] Th1 or Th2 cell inducing cytokines (IFNγ, IL-4) rather inhibit differentiation toward Th17 cells.[118] The effect of TGF-β has been shown to depend on its local concentration: while low levels of TGF-β facilitate Th17 differentiation, high TGF-β levels induce the expression of the Treg lineage specific transcription factor FoxP3.[119] FoxP3 inhibits the

transcriptional activity of RORγt, leading to differentiation toward Tregs. IL-6 abrogates this inhibition and induces Th17 differentiation.[120] Although TGF-β does not seem to be required for Th17 cell differentiation, Th17 cells generated in the presence of TGF-β *in vitro* have been reported to be less pathogenic in an autoimmune mouse model.[121] Counteracting this effect, IL-23 suppresses IL-10 expression by Th17 cells.[122] Besides inhibiting Th17 cell differentiation, Tregs may also regulate the pathogenicity of Th17 cells.[123] Finally, through consuming the IL-2 present in the microenvironment, Tregs may even promote Th17 differentiation.[124]

Th17 cells have been described to display a high degree of plasticity and to be able to differentiate to Th1 cells in vivo.[125] An intermediary phenotype comprising Th17/Th1 cells that co-produce IL-17 and IFNγ has also been described.[116] On the other hand, Th17 cells have been shown to potentially originate from Tregs.[126-128] $CD8^+$ CTL and $FoxP3^+$ Tregs can also express IL-17, although the specific characteristics of these Tc17 and $IL\text{-}17^+$ Treg cells are unclear.[129,130]

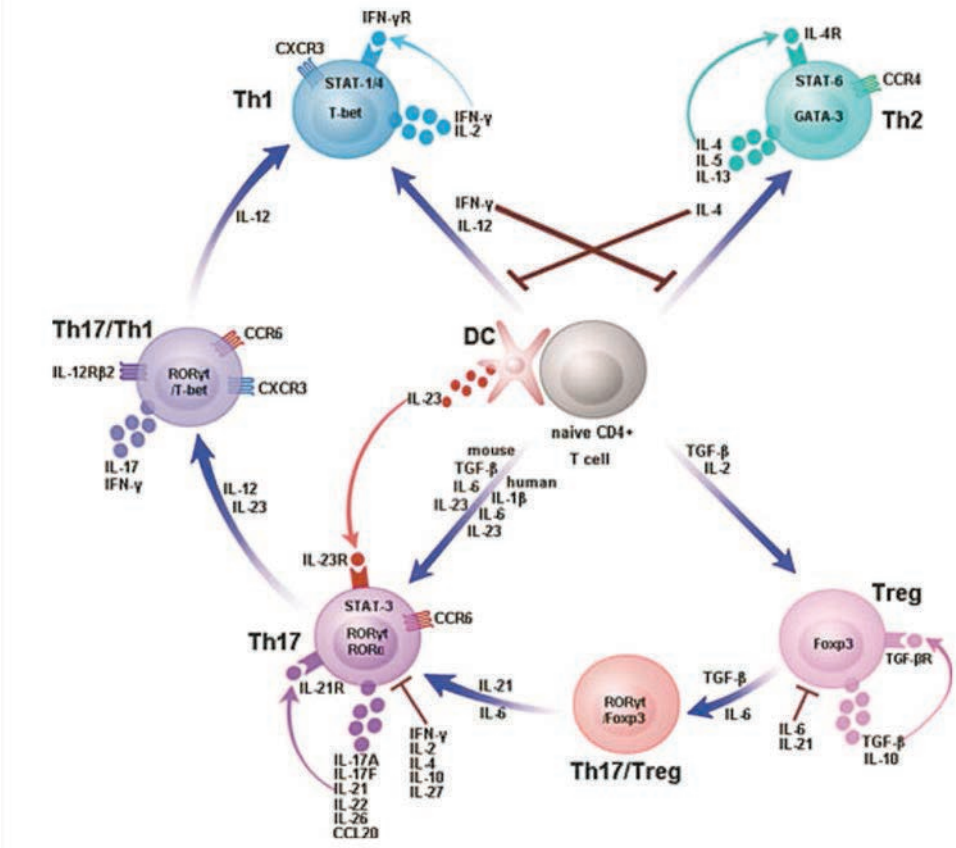

Figure 3. Th17 cell phenotype

Human Th17 cell differentiation is induced by the combination of IL-1β, IL-6 and IL-23. This induces the expression of STAT3, RORγt and different cytokines including IL-17. Th17 cells may derive from naïve $CD4^+$ T cells or Tregs, and they may also be stimulated to differentiate toward Th1 cells. Adapted from Ji *et al.*[97]

Th17 cells are thought to provide a first line of defense, particularly against pathogens that are not handled well by a Th1 or Th2 response.[88] Job's syndrome or hyperimmunoglobulin E syndrome patients, classically characterized by a dominant STAT3 mutation and thus inability to produce IL-17 or Th17 cells, suffer from recurrent staphylococcal and candidal infections.[131,132] A mutation in the IL-17 receptor A (IL-17RA) or a mutation causing aberrant IL-17 cytokines predominantly lead to an inability to clear *Candida albicans* infections.[133]
Th17 cells can induce the production of a variety a pro-inflammatory cytokines, as described in the next paragraph. IL-2, IL-10 and IL-27 can regulate the Th17 response and suppress the production of these pro-inflammatory cytokines.[118,123,134] IL-17 production may however be crucial to mount an immune response in the presence of pathogen-induced IL-10.[135] The effect of IL-2 may have a temporal nature by inhibiting early differentiation of Th17 cells, but promoting the expansion of differentiated Th17 cells.[136]

3.2 Interleukin-17

The cytokine IL-17 was discovered in 1993 and originally named cytotoxic T lymphocyte-associated-8 (CTLA-8).[137] IL-17, also known as IL-17A, is a member of the IL-17 family. The IL-17 cytokine family also comprises IL-17B through IL-17F.[138] IL-17F shows some homology in structure and functions with IL-17A. The other family members are produced by different cell types and have distinct functions. The IL-17 cytokines are homodimeric disulfide-linked proteins, although IL-17A and IL-17F also form a heterodimeric complex. IL-17 can be produced by Th17 cells, but also by innate immune cell types including neutrophils, macrophages, mast cells, γδT cells, invariant natural killer T cells and innate lymphoid cells.[139-141] It may thus create a bridge between the innate and adaptive immune response.[142]
The affinity of the IL-17RA receptor is high for IL-17A, weaker for IL-17F and intermediate for IL-17A/F. The affinities for IL-17B-D are much weaker. Binding of IL-17A or IL-17F to IL-17RA induces conformational changes that inhibit the binding of another IL-17RA molecule.[143] Both IL-17A and IL-17F preferentially subsequently engage an IL-17RC molecule.

IL-17RA signaling activates tumor necrosis factor receptor-associated factor-6 (TRAF-6) and nuclear factor-κB (NF-κB) activator protein 1 (Act1) to activate NF-κB and mitogen-activated protein kinases.[112,115,144] This leads to the production of a variety of pro-inflammatory cytokines and chemokines in target cells, including G-CSF, GM-CSF, IL-1β, IL-6, IL-8, PGE_2, TNFα, CC chemokine ligand 20 (CCL20), CXC chemokine ligand 1 (CXCL1), CXCL2, CXCL5, CXCL10 and matrix metalloproteinases (MMPs).[87,112,145-147] Target cells are predominantly epithelial and endothelial cells, fibroblasts and hematopoietic cells, but practically all cell types express the IL-17

receptor. Through inducing IL-8 expression, IL-17 stimulates neutrophil recruitment.[148] Besides inducing neutrophil infiltration, IL-17 also enhances the activity of neutrophil elastase and myeloperoxidase.[112] Another well-known effect of IL-17 signaling is the induction of vascular endothelial growth factor (VEGF) production and angiogenesis.[146,149-152] Finally, IL-17 can induce the formation of epithelial tight junctions.[153]

3.3 Th17 cells in autoimmunity

Th17 cells play a dominant role in a variety of autoimmune diseases, including psoriasis, asthma, rheumatoid arthritis (RA), encephalomyelitis, multiple sclerosis (MS), systemic lupus erythematosus (SLE), Crohn's disease, type 1 diabetes and Sjögren syndrome.[87,134] Psoriatic skin lesions as well as the peripheral blood of psoriasis patients contain increased Th17 cell frequencies.[154] IL-17 has been shown to induce the expression of antimicrobial peptides and neutrophil chemoattractants in psoriasis patients.[155] Th17 cells are increased in asthma patients,[156] and the sputum IL-17 expression level has been found to be correlated with the neutrophil frequency and disease severity.[157,158] The development of RA has been shown to be correlated with an increased circulating Th17 cell frequency and IL-17 level.[159] An increased Th17 or IL-17^+ cell frequency has also been observed in active MS lesions,[160] SLE disease,[161] Behcet's disease and uveitis.[162] The presence of Th17 cells thus represents a poorly controlled immune response causing tissue damage in autoimmune diseases. Therapies directed at targeting the Th17 cell immune response using antibodies against IL-17 and its receptor are already used in clinical trials to treat autoimmune diseases like psoriasis, asthma, RA, MS and Crohn's disease.[163,164] This type of therapy might be extended to be used as an anti-cancer treatment, if the role of Th17 cells in cancer is further elucidated.

3.4 Th17 cells and IL-17 in cancer

A low frequency of circulating Th17 cells is generally present in cancer patients, which is usually increased in tumors compared with healthy tissues.[165,166] The role of Th17 cells in cancer is unclear. Both tumor suppressing and tumor promoting functions have been described. Tumor growth control has mainly been correlated with IFNγ production and a CTL response.[85,167] Th17 cells have also been described to display a stem-cell like phenotype, with the ability to differentiate to Th1 cells in vivo.[125] The tumor targeting immune response observed after Th17 cell adoptive transfer may thus partly be due to the conversion of Th17 to Th1 cells.[112] Th17 cells have a memory phenotype and express CC chemokine receptor 4 (CCR4) and CCR6.[112,118] The ligand of CCR6 is CCL20, which is also expressed by Th17 cells and might thus facilitate their continued recruitment.[115] Tumor promoting functions are the recruitment of myeloid cells and

other functions ascribed to the pro-inflammatory cytokines produced, as described before.

The role of IL-17 in cancer is also poorly understood. Although IL-17, as described before for Th17 cells, has been correlated with the production of IFNγ and tumor suppression, the induction of angiogenesis and neutrophil recruitment have been correlated with poor survival in cancer patients.[53,54,151] IL-17 has also been shown to directly induce survival and proliferation of cancer cells in mice,[168] and to induce human cervical cancer cell lines to secrete pro-inflammatory cytokines and obtain increased tumor size when transplanted.[169,170] IL-17 signaling has furthermore been shown to directly promote tumorigenesis in mouse transformed intestinal epithelial cells.[171] Since the functions of IL-17 and Th17 cells in cancer are controversial, their role in cervical cancer is investigated in this thesis.

4. Thesis outline

The IL-17 cytokine and Th17 cell type have recently been identified. The aim of the present thesis was to elucidate the role of the IL-17 and Th17 cell immune response in cervical cancer. Different aspects of the IL-17 and Th17 pathway have been investigated. In **chapter 2** the roles of the cytokines IL-1β, IL-6, IL-23 and IL-12 are described. While IL-12 stimulates a tumor targeting Th1/CTL response, the IL-12 family member IL-23 as well as the unrelated cytokines IL-1β and IL-6 can induce a Th17 cell immune response.[97] Expression of the cytokine subunits *IL12p35*, *IL12p40* and *IL23p19* was analyzed by mRNA *in situ* hybridization. Protein expression of IL-1β and IL-6 was studied by immunohistochemistry. In this study, the correlations between cytokine expression levels and patient survival were determined.

After studying the roles of IL-1β, IL-6 and IL-23 in cervical cancer, we studied the frequency and localization of Th17 cells using immunohistochemistry (**chapter 3**). The frequency of the two most important cell types expressing IL-17, granulocytes and Th17 cells, was also determined in other common cancer types. The correlation between the frequency of neutrophils, mast cells and Th17 cells, which expressed IL-17 in the tumor microenvironment to varying degrees, and clinical outcome was analyzed in squamous cervical cancer. The effect of IL-17 on cervical cancer cell lines was studied in a real-time cell analysis device to explain part of its function.

Many aspects of the immune response have so far been found to differ in cervical adenocarcinoma when compared to cervical squamous cell cancer. To study whether the role of IL-17 and Th17 cells was similar in cervical squamous cell cancer and cervical adenocarcinoma, the correlations between the number of total T cells, Tregs, Th17 cells and other IL-17 expressing cells and survival were studied in cervical adenocarcinoma in **chapter 4**.

The tumor microenvironment comprises a complex network of immune response and vascularization factors. The correlations between different immune cell pathways including IL-17/Th17 cells, vessel formation pathways and clinical outcome were studied in **chapter 5**. The inverse correlation suggested to exist between the formation of new vessels, termed angiogenesis, and vessel adhesiveness or maturation, was also studied. The expression levels of markers characterizing the different pathways were determined by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis.

Distinguishing between tumor and immune cell processes is often hampered by the mixed cellular composition of the tumor. To identify novel biomarkers for patient survival specifically derived from tumor epithelial cells versus infiltrating immune cells, we performed whole transcriptome analysis on both the tumor cells and the immune cells in **chapter 6**. Fluorescence-activated cell sorting of cervical cancer cell suspensions was used to separate the tumor infiltrating immune cells from the tumor epithelial cells. Total mRNA was sequenced and genes that were significantly differentially expressed in either the tumor cells or the immune cells based on clinical outcome were analyzed. Additionally, we studied the differential expression based on the presence of a Th17 cell immune response, which is described in the discussion of this thesis (chapter 8).

A small number of studies have investigated the correlation between IL-17 or Th17 cells and cancer patient survival to date. A systematic search of the literature was performed to study this correlation (**chapter 7**). We hypothesized that IL-17 is correlated with poor survival and Th17 cells are correlated with improved survival in cancer patients.

The results described in chapters 2 to 7 and the implications of our findings for future research are discussed in **chapter 8**.

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II

Role of IL-12p40 in cervical carcinoma

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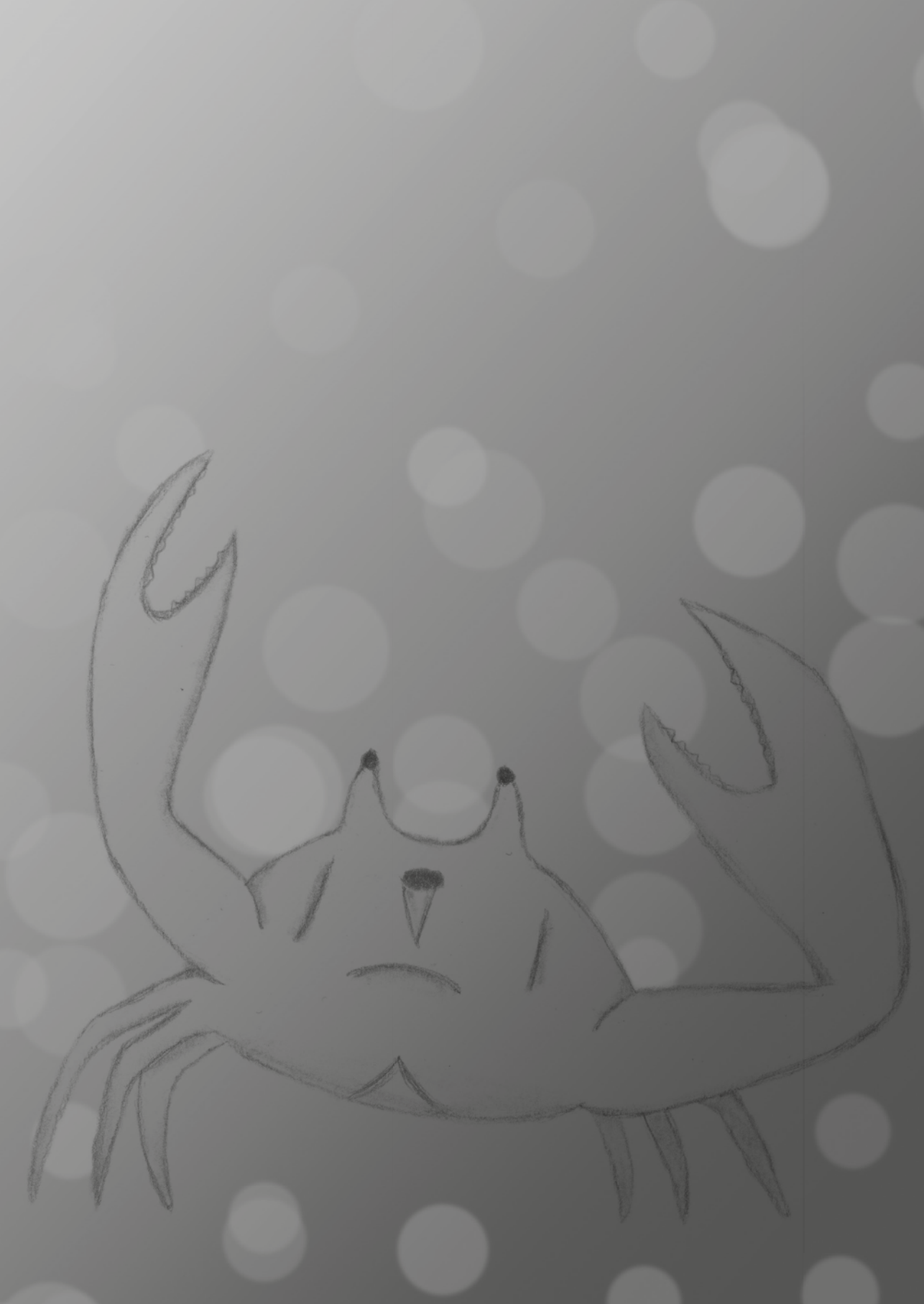

Abstract

Previously, we have shown that low *IL12p40* mRNA expression by cervical cancer cells is associated with poor survival of cervical cancer patients. As IL-12p40 is both a subcomponent of IL-12 and IL-23, the aim of this study was to elucidate the role of IL-12p40 in cervical cancer. We have measured the expression of *IL23p19*, *IL12p35* and *IL12p40* mRNA using mRNA *in situ* hybridization. As IL-23 is a component of the IL-17/IL-23 pathway, a pathway induced by IL-6 in humans, we have studied IL-1β and IL-6 expression. IL-1β and IL-6 were measured by immunohistochemistry. Only a high number of stromal IL-6^+ cells was correlated with poor disease-specific survival. The worst disease-specific survival was associated with a subgroup of patients that displayed a high number of IL-6^+ cells and low *IL12p40* expression ($p<0.001$). Both a high number of IL-6^+ cells and a high number of IL-6^+ cells plus low *IL12p40* expression were shown to be clinico-pathological parameters independent of lymph node metastasis, parametrial involvement and Sedlis score ($p=0.009$ and $p=0.007$, respectively). These results are in accordance with the hypothesis that the IL-17/IL-23 pathway has a tumor promoting role in cervical cancer.

Introduction

Cervical cancer is a leading cause of morbidity and mortality among women worldwide, especially in the developing countries.[1,2] Infection with oncogenic types of human papillomavirus (HPV) is an important factor in the development of cervical cancer.[3,4] The persistent HPV infection induces an inflammatory response. Inflammation is an important component in the majority of tumor types. The outcome of this inflammatory response surrounding the cancer cells is dependent on the composition of the inflammatory infiltrate and locally produced signaling molecules.[5] Although inflammatory cells within the neoplastic lesion are capable of generating an anti-tumor response this does not efficiently occur.[6,7]

Inflammatory cells are attracted to the tumor site by locally produced cytokines and chemokines.[8,9] Cervical cancer cells are known to produce an extensive range of cytokines and chemokines, such as CCL2, GM-CSF, TNFα and IL-12.[10-12] In addition to attracting inflammatory cells, these cytokines and chemokines influence the activation status and function of infiltrating antigen presenting cells and stromal cells, thus influencing the course of the disease.[9,13]

In a previous study, we have shown that high expression levels or undetectable levels of *IL12p40* mRNA in cervical carcinoma are associated with improved overall survival, as compared to low *IL12p40* levels that were associated with poor survival.[14] As IL-12 is known to stimulate effector cell populations such as cytotoxic T cells and natural killer cells,[15,16] our results suggest a dual role for IL-12p40.

The IL-12 cytokine family includes IL-12, IL-23, IL-27 and IL-35.[17] From this family IL-12 and IL-23 share the IL-12p40 subchain. IL-12 is composed of IL-12p40 and IL-12p35, whereas IL-23 is composed of IL-12p40 and IL-23p19. IL-23 plays, amongst others, an important a role in the IL-17/IL-23 pathway resulting in the maintenance and expansion of Th17 cells.[18] In addition to IL-23, IL-1β and IL-6 are thought to play an important role in the induction of Th17 cells in humans.[19] The effect of IL-23 on cancer progression or cancer eradication is still not clear.[20,21]

To further delineate the role of IL-12p40 in cervical carcinoma, we have quantified the mRNA expression levels of *IL23p19* and compared its expression level with *IL12p35* and *IL12p40* to investigate the relative importance of IL-12 and IL-23 in the tumor microenvironment. In addition, we have investigated the roles of IL-1β and IL-6 in the tumor microenvironment by determining the number of IL-1β^+ and IL-6$^+$ cells. Finally, we have assessed the correlations between *IL23p19, IL12p35, IL12p40,* IL-12 and IL-23 expression (low or high), a high number of IL-1β^+ and IL-6$^+$ cells and clinico-pathological parameters.

Materials and Methods

Patient material

Between 1985 and 1995, 254 untreated patients suffering from primary cervical carcinoma with stage IB and IIA underwent a radical hysterectomy type III with lymphadenectomy. From the tissue obtained, based on the availability of the material, 90 tissue samples were accessible for research. Tissues were routinely embedded in paraffin after 10% formalin fixation. The tissue samples of each patient were examined by a pathologist for the presence of tumor. Tumor percentage varied between 20% and 90%, median 60%. The characteristics of the patients are depicted in Table 1. Forty-seven patients received postoperative radiotherapy because of either tumor positive lymph nodes or the presence of positive risk factors described by the Sedlis criteria[22] (a combination of two of the following unfavorable prognostic parameters: depth of infiltration ≥ 15 mm (deep stromal invasion; middle or deep third), tumor size ≥ 40 mm and presence of vasoinvasion). Human tissue samples were used according to the guidelines of the Ethical Committee of the Leiden University Medical Center.

Table 1. Patient and tumor clinico-pathological characteristics

Clinico-pathological parameter	Category	N
Age	45 (mean)	90
	29 – 76 (range)	
FIGO stage[1]	IB	68
	IIA	21
Lymph node metastasis	No	66
	Yes	22
Tumor size[1]	< 40 mm	37
	≥ 40 mm	26
Depth of infiltration[1]	< 15 mm	58
	≥ 15 mm	26
Vascular space involvement[1]	No	39
	Yes	47
Sedlis criteria[2]	Positive	28
	Negative	52
Parametrial invasion[1]	No	74
	Yes	14
HPV status[3]	16, 18	66
	Other	15
Histology	Squamous	58
	Adenosquamous	18
	Adeno	7
	Other	6

[1]Data were not available for all patients. [2]Sedlis criteria (Sedlis et al, 1999): a combination of two of the following unfavorable prognostic parameters: depth of infiltration ≥ 15 mm (deep stromal invasion; middle or deep third), tumor size ≥ 40 mm and presence of vasoinvasion. [3]Only data for cervical carcinoma samples with a determined HPV type were included. HPV subtypes other than 16 and 18 included: HPV31 (n=2), HPV33 (n=6), HPV35 (n=1), HPV45 (n=3), HPV58 (n=1), HPV59 (n=2), HPV68 (n=1). Abbreviations: FIGO=Fédération Internationale de Gynécologie et d'Obstétrique

Preparation of IL12p35, IL12p40 and IL23p19 probes

RNA was isolated from frozen human spleen using TRIzol® (Invitrogen, Breda, The Netherlands) and first-strand cDNA was synthesized with oligoDT primers and Reverse Transcriptase AMV (both Roche Diagnostics GmbH, Mannheim, Germany), both according to manufacturer's instructions. Oligonucleotide primers for *IL23p19*, *IL12p35*

and *IL12p40* were chosen on the basis of known sequences (see Table 2) and cDNA encoding for the different cytokines was amplified. A pGEM®-3Zf(+) Vector (Promega, Madison, WI) was linearized with SmaI and the PCR products were cloned into the vector. After transferring the vector to E. coli strain Top 10 (Invitrogen Corp., San Diego, CA), the plasmids were isolated by using the QIAfilter Maxi KITS protocol (QIAGEN GmbH, Hilden, Germany). The sequence of the PCR product was confirmed by DNA sequencing. Plasmids were linearized with BamH1 and EcoR1 (both Boehringer, Mannheim, Germany) in case of *IL12p40,* BamH1 and SacI in case of *IL12p35* and SacII, SalI and SpeI (Boehringer, Mannheim, Germany) in case of *IL23p19* using One-Phor-All Buffer Plus (Amersham Biosciences, Roosendaal, The Netherlands). Both strands were translated in a digoxigenin (DIG) labeled RNA probe according to manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). The concentration of the DIG-labeled sense and antisense RNA probes were determined on a 1% agarose gel stained with ethidium bromide (Sigma, St. Louis, MO). Probes were stored at -20°C until further use.

Table 2. RNA probes used and RNA *in situ* hybridization conditions

Target	Primer	Sequence (5'-3')	Product (bp)	Accession nr	Position	Hybr. temp. (ºC)
IL23p19	Fw	AGAGCCAGCCAGATTTGAGA	487	NM_016584.2	134-620	55
	Rev	GCAGATTCCAAGCCTCAGTC				
IL12p35	Fw	TGCTCCAGAAGGCCAGACAAAC	465	XM_003121	320-784	42
	Rev	CCCGAATTCTGAAAGCATGAAG				
IL12p40	Fw	GGACCAGAGCAGTGAGGTCTT	373	XM_004011	189-561	42
	Rev	CTCCTTGTTGTCCCCTCTGA				

Abbreviations: Fw=forward; Rev=reverse Hybr. temp.=hybridization temperature

RNA in situ hybridization

RNA *in situ* hybridization (RISH) was performed as previously described.[23,24] In short, 3 µm thick paraffin sections were pre-treated and hybridized with 100 ng/ml DIG labeled RNA probe diluted in hybridization mixture containing NaCl and saline-sodium citrate (SSC; Table 2). Hybridization was allowed for 16 hrs at either 55°C (*IL23p19*) or 42°C (*IL12p35* and *IL12p40*), in a humidified chamber. Slides were washed in 2x SSC for 30 min followed by 0.1x SSC with 20 mM β-mercaptoethanol for 45 min (Merck, Darmstadt, Germany), both at used hybridization temperature (see Table 2). Subsequently the slides were incubated with 2 U/ml ribonuclease (RNase) T1 (Roche Diagnostics GmbH, Mannheim, Germany) in 2x SSC, 1 mM EDTA at 37°C for 30 min.

RNA hybrids were detected using subsequently mouse anti-digoxigenin (1:2000, Sigma-Aldrich Chemie GmbH, Steinham, Germany), rabbit anti-mouse Ig (1:50, DAKO, Glostrup, Denmark) and mouse alkaline phosphatase anti-alkaline phosphatase (APAAP, DAKO, Glostrup, Denmark).[10]

Immunohistochemistry

Serial sections of formalin-fixed and paraffin-embedded tissue, 3 µm thick, were mounted on aminopropylethoxysilane-coated slides. Sections were deparaffinized, rehydrated and treated with 0.3% H_2O_2 in methanol for 20 min to block endogenous peroxidase activity.

Antigen retrieval was performed (0.01 M citrate, pH 6.0) and sections were rinsed in phosphate-buffered saline (PBS). Subsequently, sections were stained overnight using either a 1:100 dilution of an affinity-purified polyclonal goat anti-human IL-1β antibody (AF-201-NA; R&D Systems, Minneapolis, MN, USA) or a 1:300 dilution of anti-human polyclonal rabbit anti-IL-6 antibody (Abcam, Cambridge, UK). For anti-IL-1β, the slides were incubated with a goat HRP-polymer kit (Biocare Medical, Concord, CA, USA) according to the manufacturer's instructions. For anti-IL-6, the slides were incubated with a biotinylated swine anti-rabbit antibody (1:200; DAKO, Glostrup, Denmark) and subsequently with a biotinylated horseradish peroxidase-streptavidin complex (1:100, DAKO). Immune complexes were visualized with diaminobenzidine as previously described.[24]

Evaluation of RISH and immunohistochemistry

RISH was scored as previously described.[25] Intensity was scored as none (0), mild (1), moderate (2) or intense (3) at low magnification (100x). Furthermore, the percentage of positive tumor cells was determined and divided in six groups: 0% (0, absent), 1-5% (1, sporadic), 6-25% (2, local), 26-50% (3, occasional), 51-75% (4, majority) and 76-100% (5, large majority). The sum of both the percentage and the staining intensity of the positive cells resulted in an overall score (0 or 2 to 8). The scores were combined into three groups: category 0 (score 0, no expression), category 1 (scores 2, 3, 4 and 5, low expression) and category 2 (scores 6, 7 and 8, high expression). mRNA expression was scored by two independent researchers without knowing the identity and clinical outcome of patients. IL-1β^+ and IL-6$^+$ cells were quantified in the tumor by counting the number of stained cells per 6, randomly selected, high-power fields of view (HPF, 400x).

Statistical analysis

Data from immunohistochemistry as well as RISH are given as the mean $\pm$ the standard deviation. Statistical analysis was performed using SPSS 17.0 (SPSS Inc., Chicago, IL).

Data were processed using the Chi-square test. Kaplan-Meier survival curves were generated to assess differences in disease-free period (defined as the observation time in months from surgery to relapse of the disease (disease-free survival)) or cumulative disease-specific survival (defined as time in months from surgery to death due to cervical cancer). A Cox regression was used for multivariate survival analysis. P values below 0.05 were considered statistically significant.

Results

Patients

Of the group of 90 patients, 68 patients were diagnosed with FIGO stage IB and 21 with FIGO stage IIA and all underwent a radical hysterectomy combined with pelvic lymph adenectomy (Table 1). Fortyseven patients received postoperative radiotherapy because of either tumor positive lymph nodes or meeting the terms of the Sedlis criteria[22] (a combination of two of the following unfavorable prognostic parameters: depth of infiltration ≥ 15 mm, tumor size ≥ 40 mm and presence of vasoinvasion). Twentyfive patients suffered from recurrent disease. At the end of the study 70 patients were alive, 7 suffered from a recurrence and 18 patients had died of disease.

Expression of IL23p19, IL12p35 and IL12p40 in cervical cancer

As IL-12p40 is a subunit of both IL-12 and IL-23, we have determined the expression pattern of *IL23p19*. Both *IL23p19* and *IL12p40* were expressed by cervical cancer cells (Figure 1A, C). The expression of *IL12p40* was stronger than the expression of *IL23p19*. *IL23p19* was expressed in 63% of the samples (n=54), *IL12p40* was expressed in 54% of the samples (n=90) and *IL12p35* was expressed in 84% of the samples (n=90; Table 3). All samples that expressed either *IL23p19* or *IL12p40* also expressed *IL12p35*. In contrast, 13 out of 44 samples that expressed *IL12p40* did not express *IL23p19*.
A slightly positive correlation between *IL23p19* and *IL12p40* was found (n=54, r^2 = 0.117, p=0.011; data not shown). No statistically significant correlation between *IL23p19* and *IL12p35* was found (n=54, r^2 = 0.061, p=0.072; data not shown).

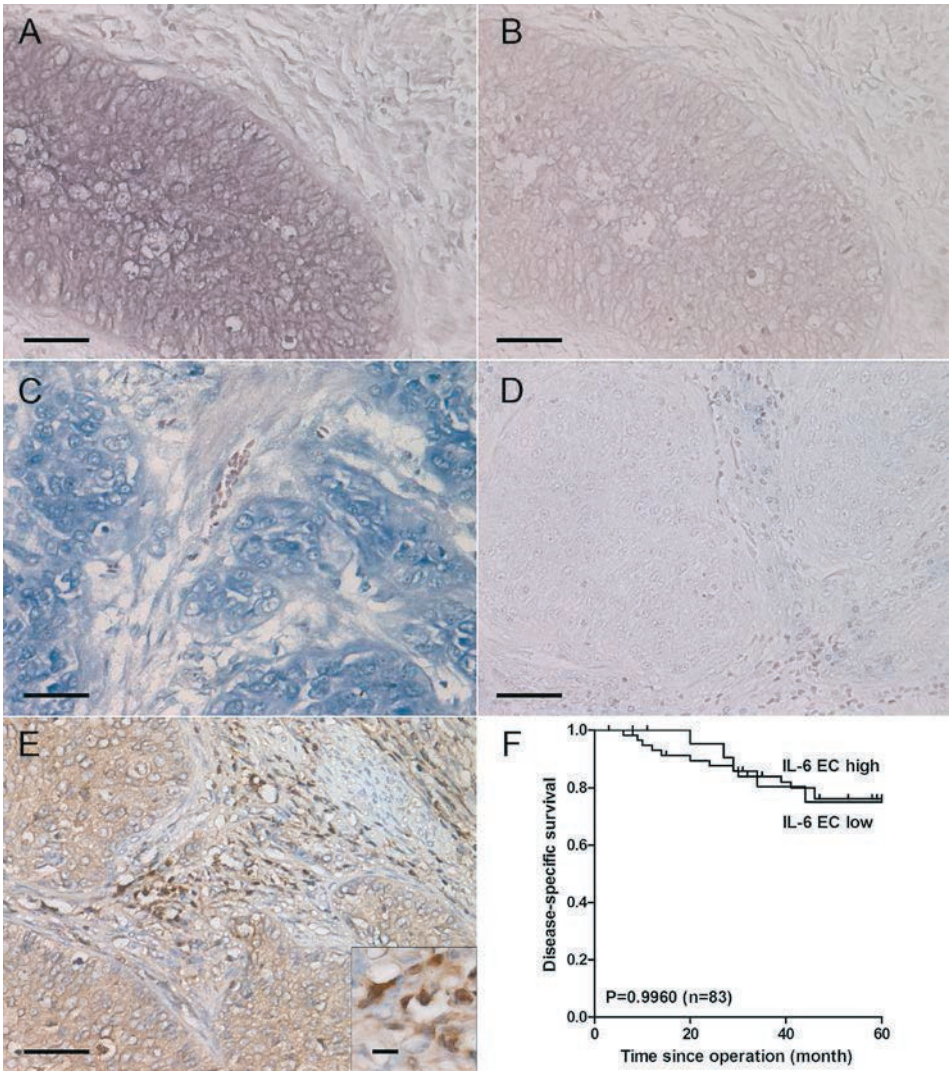

Figure 1. The expression of *IL23p19*, *IL12p40* and IL-6

Representative images of a cervical tumor expressing *IL23p19* (A) and *IL12p40* (C) determined using RISH (x250 magnification). Tumor cells stained positive (moderate) for *IL23p19* and positive (strong) for *IL12p40*. The negative (sense) controls of *IL23p19* and *IL12p40* RISH are shown in figures B and D, respectively. A representative image of cervical tumor expressing IL-6 determined by immunohistochemistry is shown in E. Both cells in the epithelial compartment (EC) as well as cells in the stroma expressed IL-6. Arrow indicates stromal IL-6^{+} cells, shown enlarged in the inset (x400 magnification). The association between cells in the epithelial compartment with low (IL-6 EC low) and high IL-6 (IL-6 EC high) expression and disease-specific survival is shown in F. Bars correspond to 50 mm in A–E and to 10 mm in the inset of E.

Table 3. Correlation between *IL23p19*, *IL12p35* and *IL12p40* expression in cervical cancer

Cytokine		***IL12p35***			
	Expression level	Absent	Low	High	Total n (%)
IL12p40	Absent	14	21	6	41 (46)
	Low	0	14	14	28 (31)
	High	0	3	18	21 (23)
Total	n (%)	14 (16)	38 (42)	38 (42)	90 (100)
p value					**<0.001**
		IL23p19			
		Absent	Low	High	Total n (%)
IL12p40	Absent	7	1	2	10 (19)
	Low	8	5	11	24 (44)
	High	5	4	11	20 (37)
Total	n (%)	20 (37)	10 (19)	24 (44)	54 (100)
p value					0.188
		IL23p19			
		Absent	Low	High	Total n (%)
IL12p35	Absent	1	0	0	1 (2)
	Low	8	5	8	21 (39)
	High	11	5	16	32 (59)
Total	n (%)	20 (37)	10 (19)	24 (44)	54 (100)
p value					0.619

The scores were combined into three groups: absent expression, low expression and high expression as described in the Materials and Methods. Statistically significant p values are shown in bold.

Association between IL-12 and IL-23 and disease-specific survival in cervical cancer

To investigate the relationship between the expression of *IL23p19, IL12p35* and *IL12p40* and disease-specific survival, Kaplan Meier plots were created. A log rank test was used to determine statistical differences in disease-specific survival. As the absence of *IL12p40* will result in neither IL-12 nor IL-23, we first confirmed that low expression of *IL12p40* was correlated with poor disease-specific survival (Figure 2A; n=48, log rank test 5.753, p=0.017) in this cohort. The expression of *IL12p35* (Figure 2B; n=74, log rank test 0.2019, p=0.653) and the expression of *IL23p19* (Figure 2C; n=33, log rank test 1.930, p=0.165) were both not significantly correlated with disease-specific

survival. The expression of *IL23p19, IL12p35* or *IL12p40* showed no significant difference in disease-free survival (data not shown).

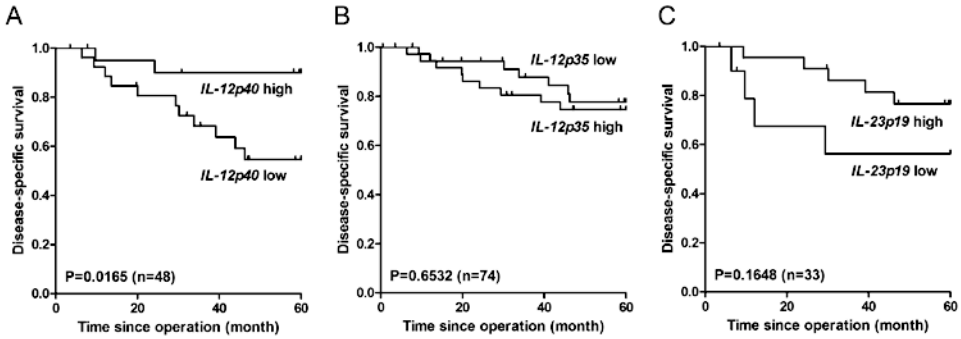

Figure 2. Correlation between IL-12 and IL-23 cytokine subunits and survival

The correlation between *IL12p40* (A), *IL12p35* (B) and *IL23p19* (C) expression and disease-specific survival in cervical cancer.

Presence of IL-6$^+$ cells and association with disease-specific survival in cervical cancer

The presence of *IL23p19* suggests that IL-23 may sustain a Th17 cell population in cervical cancer. As differentiation toward the IL-17/IL-23 pathway is thought to occur in the presence of IL-1β and IL-6 in humans,[19] we have determined the presence of IL-1β$^+$ and IL-6$^+$ cells using immunohistochemistry. IL-1β was predominantly expressed by cells in the stromal compartment. Occasionally, tumor cells also showed weak IL-1β expression. No statistically significant association between a low or high number of IL-1β-expressing cells and disease-specific survival was observed (Figure 3A). IL-6 was expressed by both cells in the epithelial (tumor cell) compartment as well as cells in the stromal compartment (Figure 1E). No significant association was observed between a low or high number of IL-6$^+$ cells in the epithelial compartment and disease-specific survival (Figure 1F). Subsequently, we quantified the number of IL-6$^+$ cells in the stroma. The presence of a high number of stromal IL-6$^+$ cells (median 17 IL-6$^+$ cells/HPF) was significantly correlated with disease-specific survival (Figure 3B; n=83, log rank test 12.57, $p<0.001$). No statistically significant difference was observed for disease-free survival (data not shown). We also determined whether the presence of both a high number of stromal IL-6$^+$ cells and low *IL12p40* expression was correlated with disease-specific survival. In this latter case an even stronger decrease in disease-specific survival was observed (Figure 3C; n=47, log rank test 20.38, $p<0.001$).

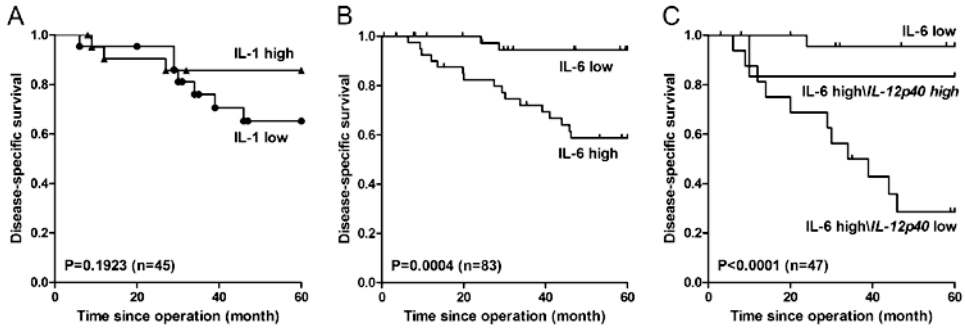

Figure 3. Correlations between IL-1β and IL-6 and survival

The correlation between a high number of IL-1β$^+$ cells (A), stromal IL-6$^+$ stromal cells (B) and *IL12p40* expression in combination with a high number of stromal IL-6$^+$ cells (C) and disease-specific survival.

Association between low IL12p40 expression, high number of stromal IL-6$^+$ cells and clinico-pathological parameters

To determine the relevance of our findings, we studied the correlations between our immunological findings and clinico-pathological parameters. First a univariate Cox analysis was performed, using the clinical parameters Sedlis criteria (two out of three of the following criteria positive: tumor size ≥ 40 mm, vasoinvasion and deep stromal invasion), lymph node metastasis and parametrial involvement and the immunological parameters low *IL12p40* expression, a high number of IL-6$^+$ cells and a high number of IL-6$^+$ cells plus low *IL12p40* expression. In the univariate Cox analyses, all the included parameters showed a significantly increased hazard ratio (HR; Table 4). Subsequently, a multivariate Cox analysis with the three clinico-pathological parameters and each of the statistically significant immunological parameters was performed. In this case two of the three immunological parameters were shown to be independent predictors of poor disease-specific survival: a high number of stromal IL-6$^+$ cells (HR 7.447, p=0.009) and a high number of stromal IL-6$^+$ cells and low *IL12p40* expression (HR 20.123, p=0.007).

Table 4. Cox regression of clinico-pathological variables and *IL12p40* and the number of IL-6-expressing cells in cervical cancer

Variable	Univariate hazard ratio (95% CI)	p value	Multivariate hazard ratio (95% CI)	p value	Multivariate hazard ratio (95% CI)	p value
Sedlis positive	4.354 (1.608-11.789)	**0.004**	3.848 (1.270-11.663)	**0.017**	0.706 (0.150-3.310)	0.659
Lymph node metastasis	3.266 (1.286-8.296)	**0.013**	1.333 (0.439-4.052)	0.612	2.887 (0.836-9.965)	0.094
Parametrial involvement	3.645 (1.411-9.411)	**0.008**	1.526 (0.542-4.444)	0.438	1.168 (0.283-4.822)	0.830
Low *IL12p40*	5.231 (1.156-23.661)	**0.032**				
Low number of IL-6$^+$ cells			Reference			
High number of IL-6$^+$ cells	8.975 (2.063-39.051)	**0.003**	7.447 (1.659-33.432)	**0.009**		
High number of IL-6$^+$ cells/low *IL12p40*	21.832 (2.805-169.915)	**0.003**			20.123 (2.248-180.147)	**0.007**

The hazard ratios and p values of compared expression levels are shown. Statistically significant p values are shown in bold.

Discussion

In a previous study, we found an association between low expression of *IL12p40* and poor disease-specific survival, whereas high expression of *IL12p40* or lack of expression of *IL12p40* were associated with a favorable disease-specific survival.[14] As IL-12p40 combines with both IL-12p35 and IL-23p19, to form IL-12 and IL-23, respectively, in the present study, we have further investigated the role of IL-12p40 in cervical cancer.

Both *IL23p19* and *IL12p35* were expressed in the majority of the samples. Of the 44 samples that expressed *IL12p40,* 13 samples did not express *IL23p19*. As *IL12p35* expression seems to be ubiquitous in cervical cancer,[14] the level of *IL12p40* or *IL23p19* expression most probably determines whether IL-12, IL-23 or both are expressed. In our study, we observed a trend between *IL23p19* and *IL12p35* expression, whereas in the study of Wolf *et al*. in ovarian cancer (n=112), a significant correlation between the expression of *IL23p19* and *IL12p35* was found.[26] The discrepancy between our results and the results of Wolf *et al*. may be due to the smaller size of our study group. Very few studies have investigated the association between local expression of IL-12 or IL-23

and prognosis. Using immmunohistochemistry, IL-12 has been associated with improved survival in patients with (advanced) gastric carcinoma.[27,28] In the study of Wolf *et al*., using RT PCR, both *IL12p35* and *IL23p19* were associated with a superior outcome.[26] In a multivariate analysis, *IL12p35* was found to be an independent factor for overall survival of ovarian carcinoma. As stated previously, we have observed a statistically significant correlation between low expression of *IL12p40* and poor disease-specific survival in cervical carcinoma.[14] In the present study and our previous study,[14] we did not find a significant correlation between either *IL23p19* or *IL12p35* expression and disease-specific survival. As *IL23p19* and *IL12p35* are both expressed, it is important to determine which cytokine, IL-23 or IL-12, has a dominant effect on the tumor microenvironment.

The molecular interaction between IL-23p19 and IL-12p40 has been studied by Beyer et al.[29] These authors reported that the interface region of IL-23p19 and IL-12p35 on IL-12p40 overlap. Due to different interresidue interactions of IL-12p35 and IL-23p19 with IL-12p40, these molecules interact with a different affinity with IL-12p40. Therefore, the availability of IL-12p40 in combination with the affinity for IL-12p35 and IL-23-p19 may result in skewing of the IL12/IL23 response. This is supported by experiments performed by Zwiers *et al*. These authors showed that in an experimental animal model, polymorphic variants of IL-12p40 can skew IL-12/IL-23 synthesis.[30] Thus, both differences in protein interactions between IL-23p19 and IL-12p35 on the one hand and IL-12p40 on the other hand and genetic polymorphisms in the protein chains, such as IL-12p40, contribute to the amounts of IL-12 and IL-23 formed. Our results support a previously suggested immunosuppressive role for IL-23.[20] This is further supported by a study in ovarian carcinoma were genetic differences in the IL-23 receptor have been reported to influence prognosis.[31]

In contrast, it has also been shown that overexpression of IL-23 reduces tumor growth and metastasis formation and that IL-23 is able to elicit a strong cytotoxic T-cell memory response,[21,32] underscoring our view that the level of expression of the different cytokines and chemokines plays an import role in the final outcome.

Our data suggest that in the presence of a limited amount of *IL12p40*, the biological effect of IL-23 dominate, whereas in the presence of a high amount of *IL12p40*, the biological effect of IL-12 prevails. As IL-12 polarizes the immune response toward an anti-viral response,[33] the favorable cumulative overall survival of patients with a high IL-12^{+} cell density,[27,28] high expression level of *IL12p35*[26] and *IL12p40*[14] can be explained by the capacity of this cytokine to increase the lytic activity and the production of interferon-γ of natural killer cells and cytotoxic T-lymphocytes.[33] Interestingly, we previously observed an association between high expression of *IL12p40* and high expression of *TGF-β* (p=0.024),[14] suggesting that the tumor cells are selected to counteract the effect of IL-12 or skew the response to the IL-17/IL-23 pathway.

In humans, in addition to TGF-β, IL-1β and IL-6 have been implicated to play a role in the IL-17/IL-23 pathway.[19,34] Previously, we have shown that it is likely that activated TGF-β is present in the tumor microenvironment, as PAI-1 expression, a target gene of TGF-β, is correlated with survival.[35] Furthermore, both the integrin avβ6 and active matrix metalloproteinase-2, known to activate TGF-β, are associated with poor disease-specific survival.[36,37] Even though the role of TGF-β in inducing Th17 in humans has been questioned, TGF-β may suppress Th1 and Th2 development thus favoring Th17 development.[38,39]

In our study, low or high numbers of IL-1β-positive cells were not correlated with disease-specific survival. The presence of a high number of stromal IL-6^+ cells was significantly associated with poor disease-specific survival. Previously, IL-6 has been implicated as an autocrine or paracrine growth factor for cervical cancer.[40,41] IL-6 has been shown to induce VEGF transcription via the STAT3 signaling pathway, thus promoting an angiogenic switch.[42] Indeed, blockade of the IL-6 receptor on cervical cancer cell lines was shown to interfere with cell survival signals and blocked expression of VEGF.[43]

As HPV vaccines will become available for the treatment of metastasized cervical carcinoma, the local cytokine/chemokine profile may be important to discriminate patients with a beneficial immune response from non-responding patients.

In conclusion, IL-12p40 plays at least a dual role in cervical carcinoma by associating with both IL-23p19 and IL-12p35. We have shown that low *IL12p40* expression was significantly correlated with poor disease-specific survival. Also, a high number of stromal IL-6^+ cells was shown to associate with poor disease-specific survival. The worst disease-specific survival was observed in a subgroup of patients that displayed a high number of stromal IL-6 expressing cells and low *IL12p40* expression. Furthermore, both a high number of stromal IL-6 expressing cells and a high number of stromal IL-6 plus low *IL12p40* expression were shown to be independent clinico-pathological parameters as compared to lymph node metastasis, parametrial involvement and Sedlis score. Our results support the hypothesis that IL-23 plays a tumor promoting role in cervical cancer.

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III

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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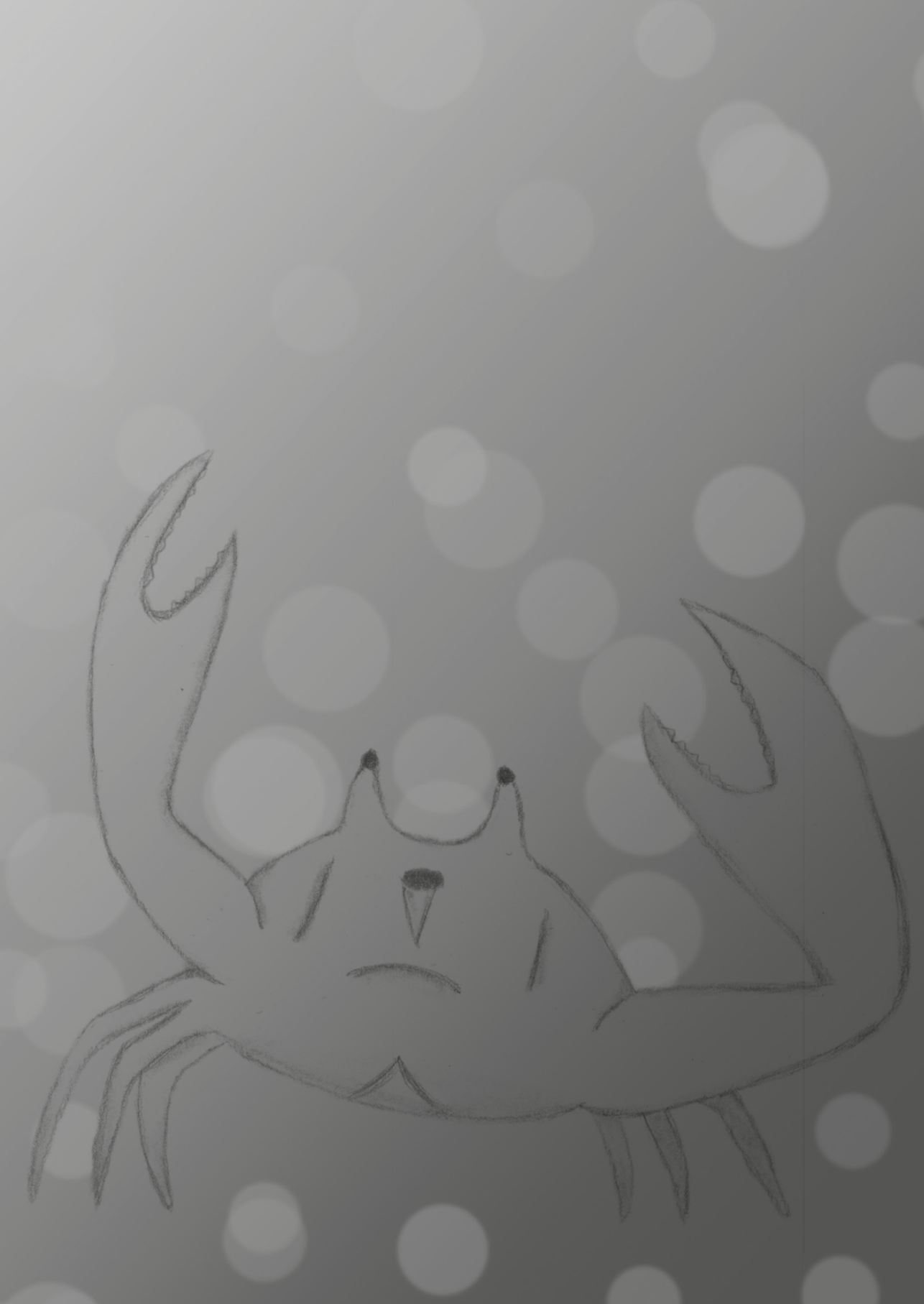


Abstract

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.

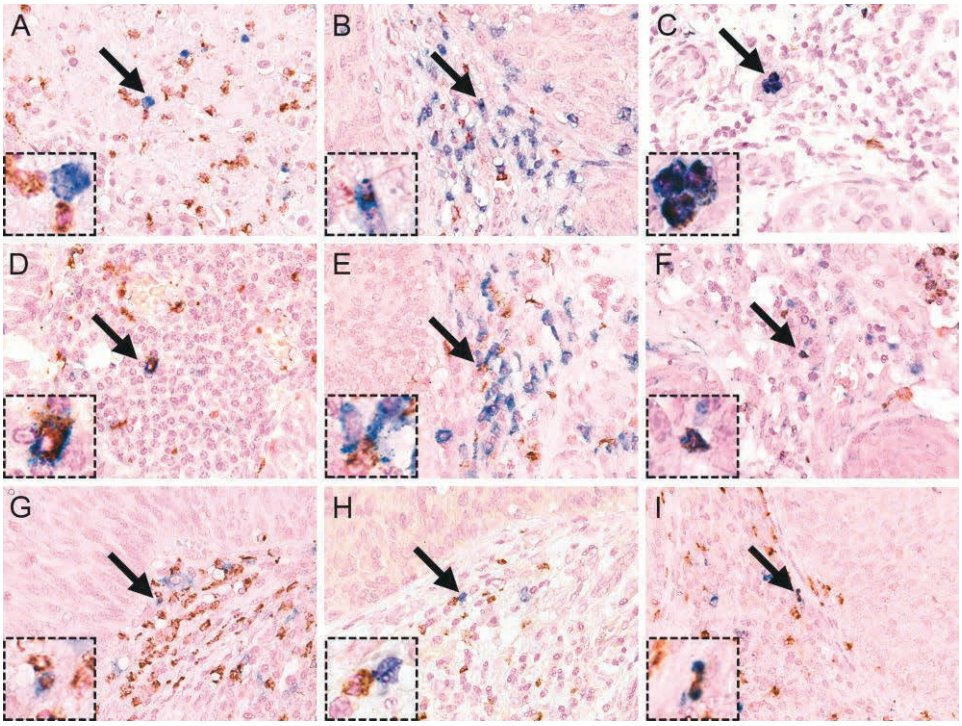
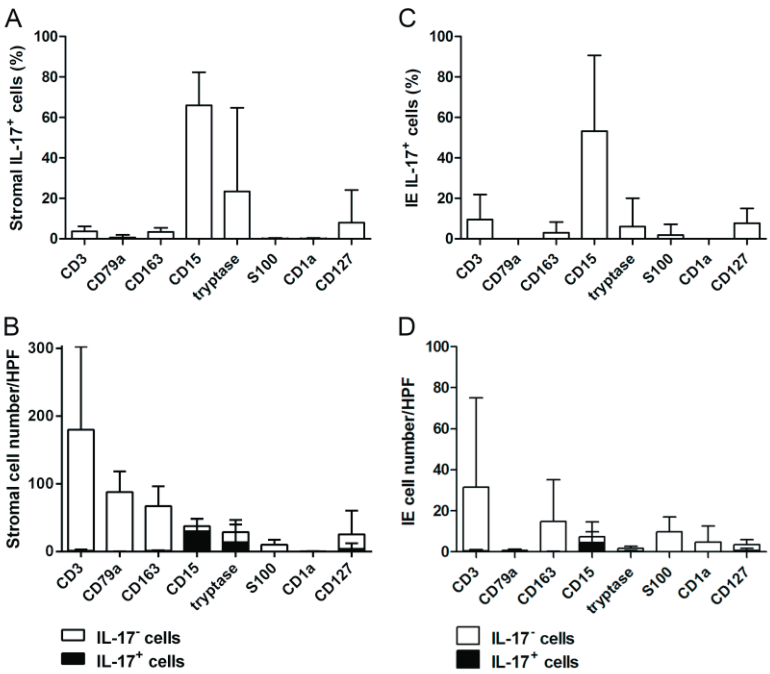


Figure 1. Immunohistochemical double staining of IL-17 and different phenotype markers
Representative images of double stainings for IL-17 (DAB) and CD1a (A), CD3 (B), CD15 (C), CD33 (D), CD79a (E), CD127 (F), CD163 (G), S100 (H) and tryptase (I) (all PermaBlue) at a x630 magnification are shown. Arrows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



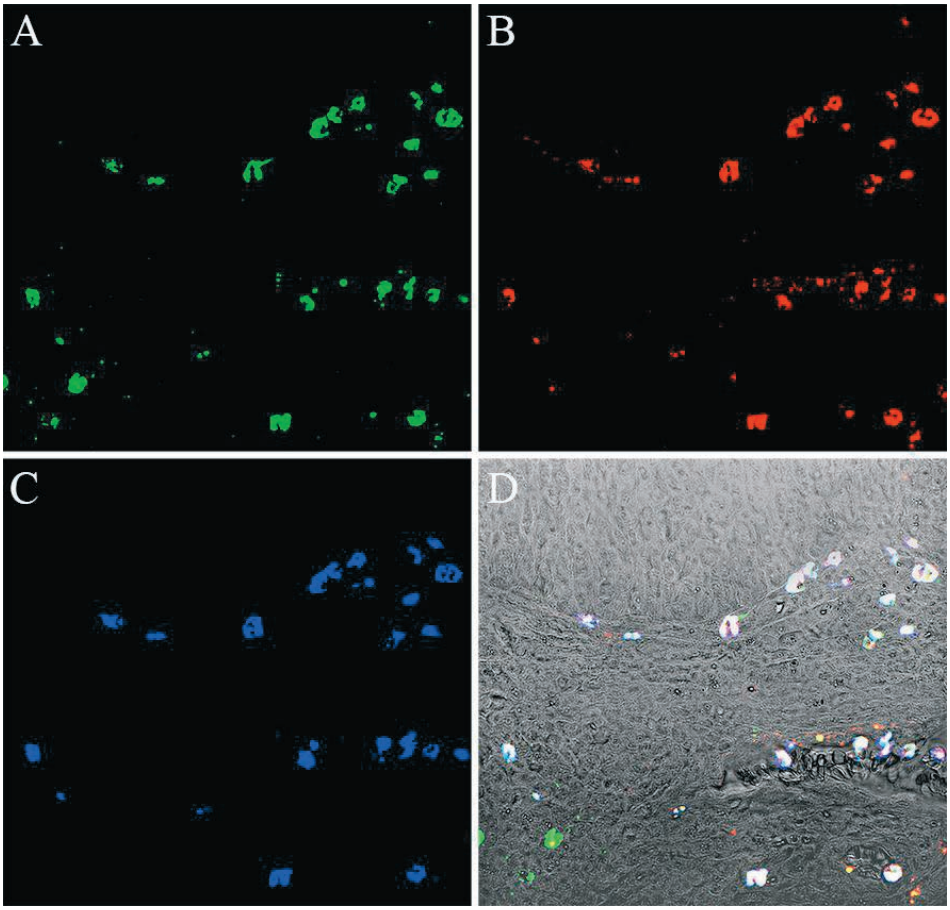


Figure 3. Triple immunofluorescent staining of IL-17$^+$ granulocytes

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.

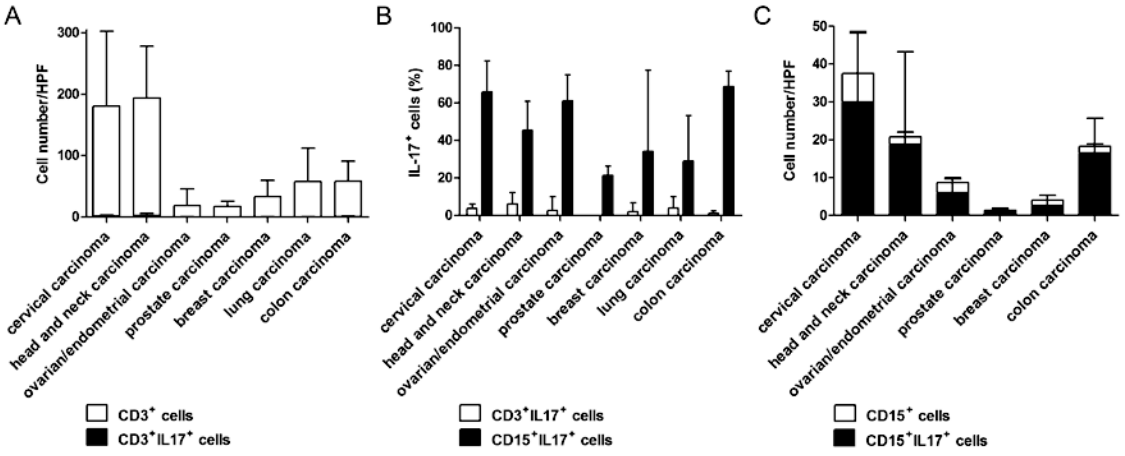


Figure 4. Phenotype of IL-17$^+$ cells in other carcinoma types

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).

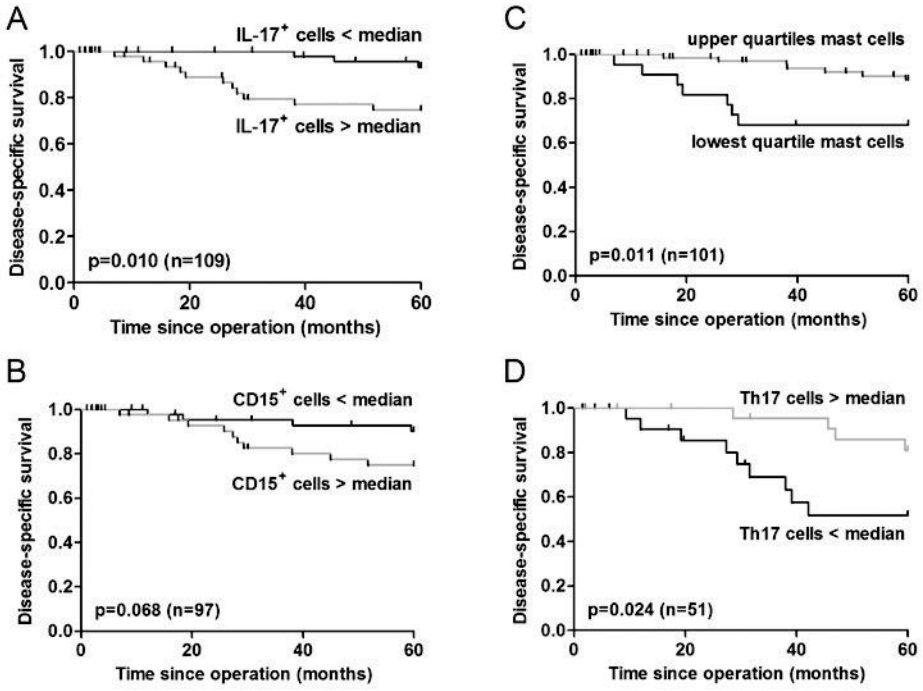


Figure 5. Correlation between IL-17, CD15, tryptase, Th17 and survival

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.

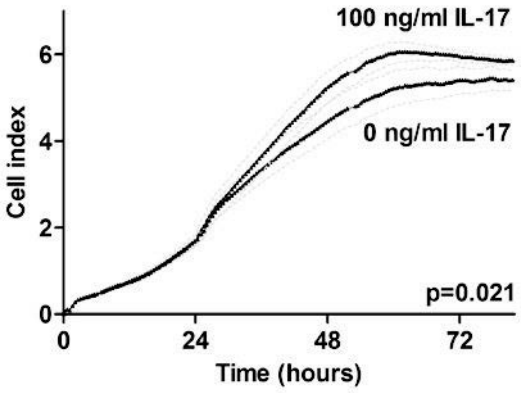


Figure 6. Effect of IL-17 on cervical cancer cells *in vitro*

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.

Acknowledgements

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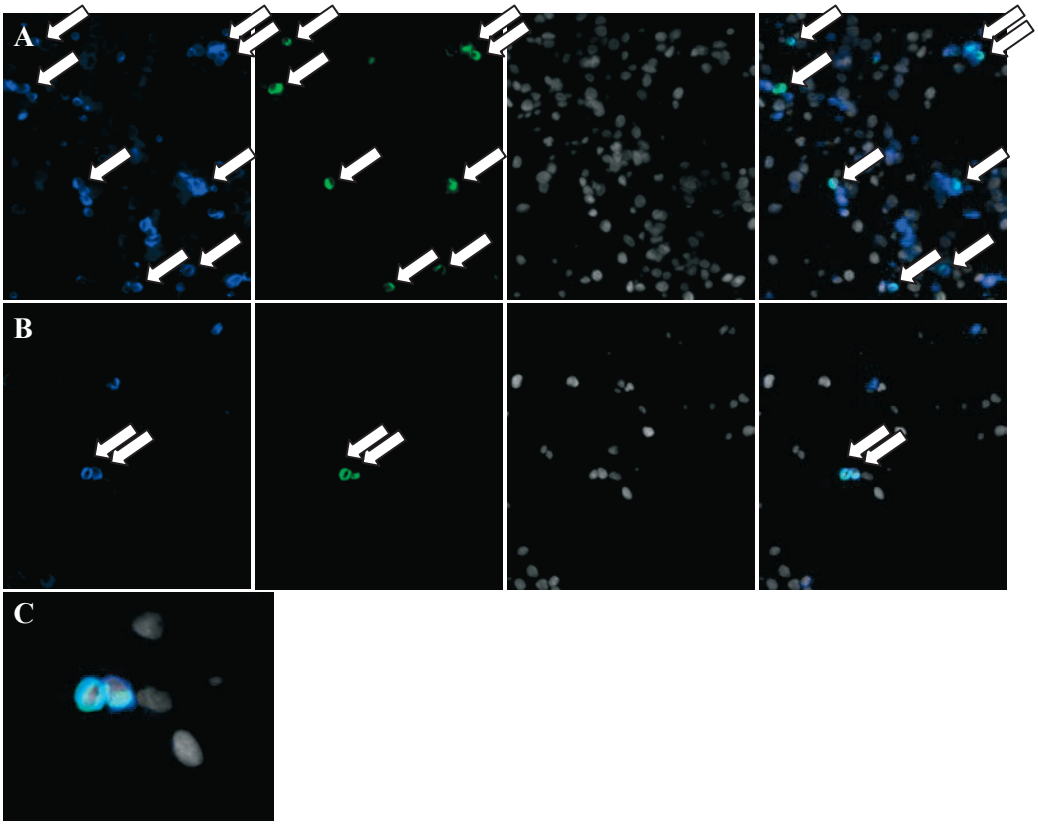
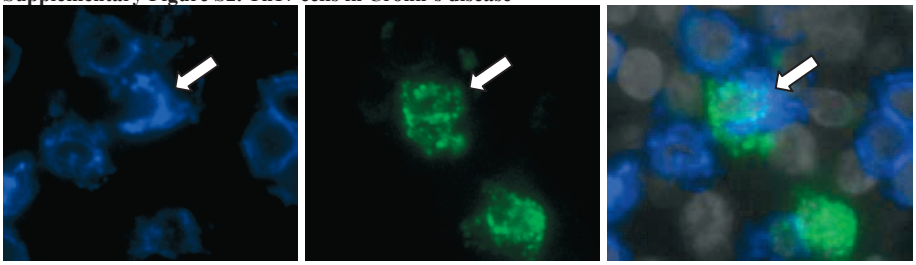
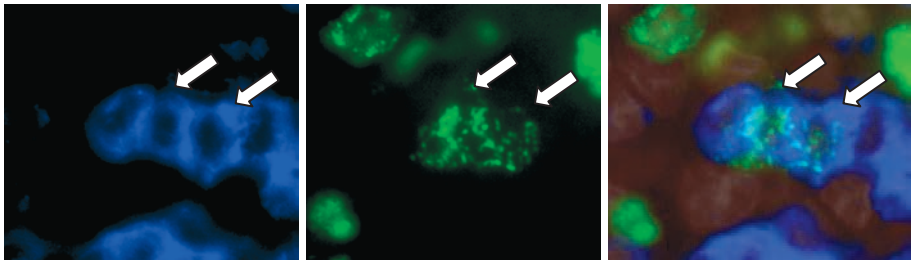
Supplementary Figure S1. Confocal analysis of activated memory T cells including Th17 cells



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.

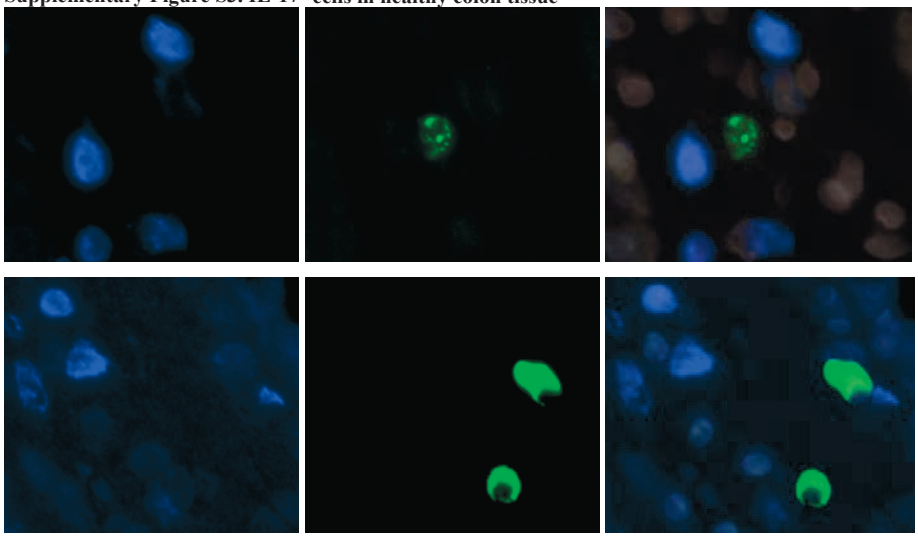
Supplementary Figure S2. Th17 cells in Crohn's disease





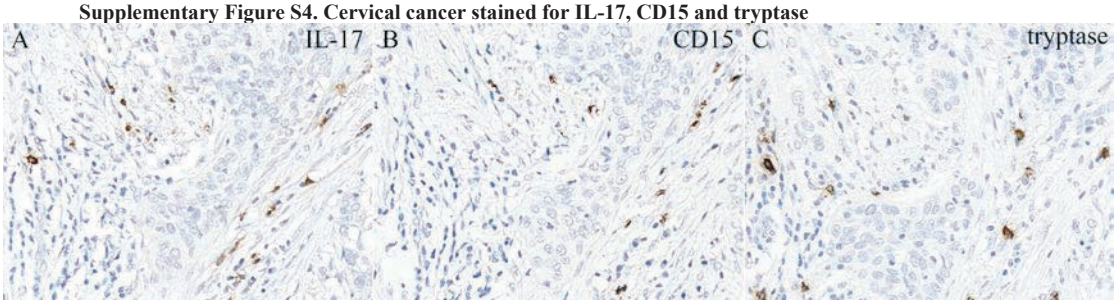
Cells double positive for CD3 (blue) and IL-17 (green) were observed in FFPE tissue from colon biopsies from Crohn's disease patients. Nuclei are stained by DAPI, shown in grey in the merged image on the right. Arrows indicate double positive cells. Representative pictures from two random samples are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

IV

FoxP3^{+} and IL-17^{+} cells are correlated with improved prognosis in cervical adenocarcinoma

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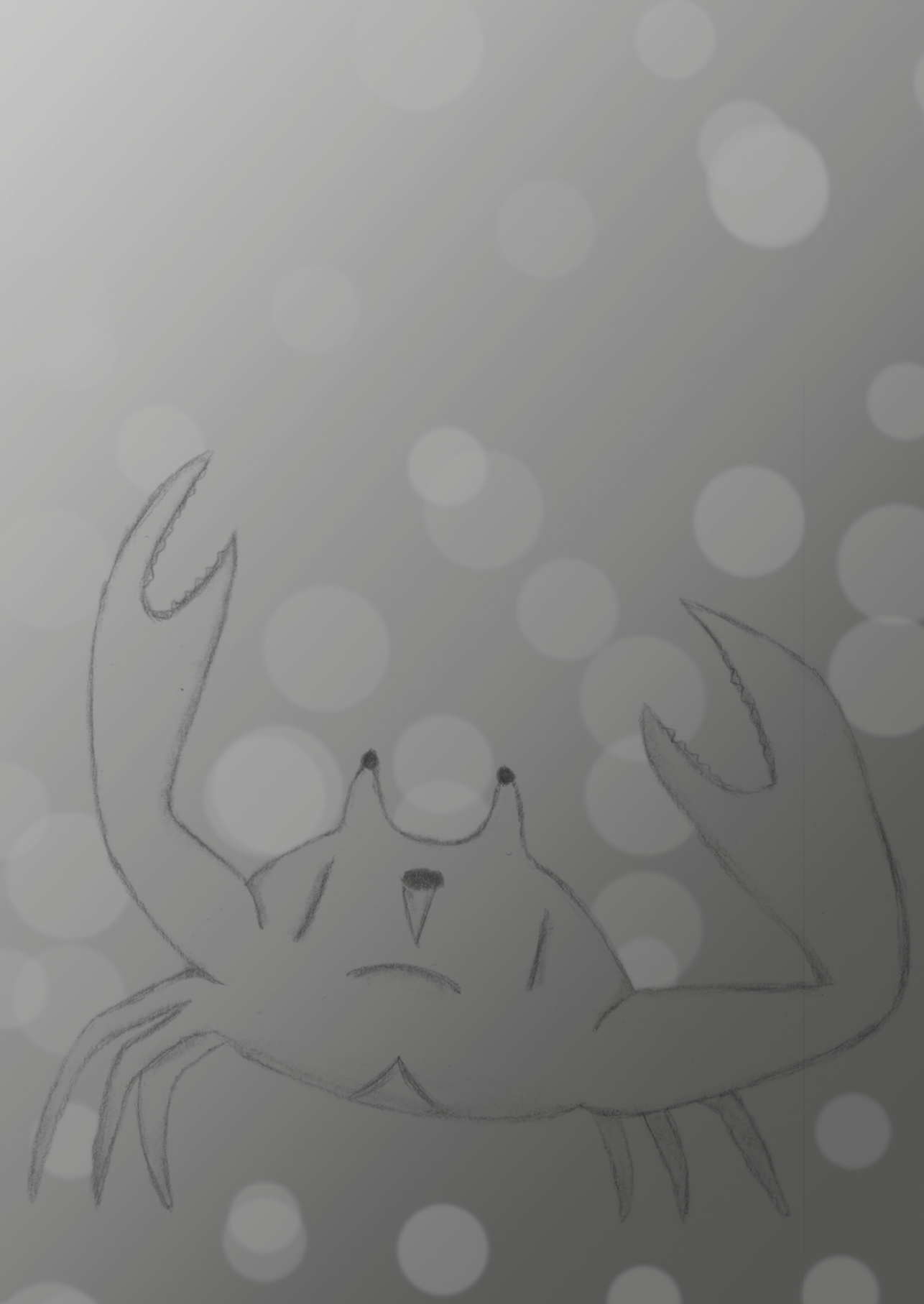

Abstract

Cervical adenocarcinoma comprises approximately 15% of cervical cancer cases. This histological subtype has different characteristics than cervical squamous cell carcinoma, which may influence disease progression. To study whether the infiltration of T cell subpopulations was correlated with cervical adenocarcinoma patient survival, similar to squamous cell carcinoma, the tumor infiltrating T cells, Tregs, Th17 cells and IL-17$^+$ cell frequencies were analyzed in a cohort of cervical adenocarcinoma patients (n=67). Intraepithelial, stromal and total cell frequencies were scored using triple immunofluorescence. The majority of Tregs was present in the tumor stroma, while other T cells and IL-17$^+$ cells infiltrated the tumor epithelium three times more frequently. A high total number of Tregs was significantly correlated with improved disease-specific and disease-free survival (p=0.010, p=0.007). Within the tumor epithelium, a high T cell frequency was significantly correlated with improved disease-free survival (p=0.034). Especially a low number of both Tregs and IL-17$^+$ cells was correlated with poor disease-specific survival (p=0.007). A low number of Tregs combined with Th17 cells present was also correlated with poor survival (p=0.018). An increased number of IL-17$^+$ cells was significantly correlated with the absence of vaso-invasion (p=0.001), smaller tumor size (p=0.030) and less infiltration depth (p=0.021). These results suggest that Tregs and IL-17$^+$ cells represent a beneficial immune response, whereas Th17 cells might represent a poor response in cervical adenocarcinoma. This contrasts with the correlations described in squamous cell carcinoma, suggesting that the local immune response in cervical adenocarcinoma contributes differently to tumor growth than in squamous cell carcinoma.

Introduction

Cervical cancer is the second leading cause of cancer death in young women.[1] The majority of cervical cancer cases can histologically be divided in squamous cell carcinoma (~75% of cases), adenocarcinoma and adenosquamous carcinoma (together 20-25% of cases).[2] Several studies have reported that the prognosis for patients with cervical adenocarcinoma is worse than for squamous cell carcinoma,[3-8] although this is still controversial.[9,10] Additionally, although the overall incidence of cervical cancer has declined in developed countries as a result of cytological screening programs, the incidence of adenocarcinoma has remained stable or even increased, predominantly in young women.[11,12] Cervical adenocarcinoma differs from squamous cell carcinoma in growth pattern, molecular background and sensitivity to radio- and chemotherapy.[13-15] However, because of the lower incidence, extensive analyses have been lacking.

Since practically all cases of cervical cancer are caused by a persistent infection with high-risk human papillomavirus (HPV),[16] immunosurveillance plays a critical role.

Most cervical HPV infections are cleared in over 90% of cases within two years.[17] In case of tumor progression, the immune response is thought to contribute to tumor development rather than eradication.[18] The type and number of immune cells present in the tumor microenvironment are both crucial for clinical outcome. T helper 1 (Th1) cells are required to overcome intracellular pathogens and can induce or stimulate a tumor targeting immune response. Th2 cells protect against extracellular pathogens, and have been shown to support cervical cancer progression,[19] but their role in cancer has not been fully elucidated. Th17 cells are essential to protect against extracellular pathogens, and play a dominant role in autoimmune diseases.[20,21] Their role in cancer is unclear, since they have been shown to both be able to promote as well as to counteract tumor growth.[22] Tregs suppress the activity of other T cells,[23] which may dampen either a tumor suppressing or tumor promoting immune response. Especially the potentially different role of the immune response in squamous versus adenocarcinoma has not been thoroughly studied, although there are indications for differences between the subtypes.[24]

We have shown before that Tregs are more frequently present in cervical squamous cell carcinoma than adenocarcinoma and that these cells, relative to cytotoxic T cells, were correlated with poor survival in a representative cohort of cervical cancer patients, i.e. predominantly squamous cell carcinoma cases (77%).[25] In addition, we have recently shown that Th17 cells were correlated with improved survival in a cohort of cervical squamous cell carcinoma patients.[26] Strikingly, in the same cohort, interleukin-17 (IL-17) was predominantly expressed by neutrophils and correlated with poor survival.[26] The aim of this study was to determine the number of intraepithelial, stromal and total T cells, Tregs, Th17 and other IL-17$^+$ cells. The correlations between the different cell frequencies and patient survival in cervical adenocarcinoma were analyzed. The contrasts with the correlations described in cervical squamous cell carcinoma and other cancer types are discussed.

Materials and Methods

Patient material

Formalin-fixed, paraffin-embedded (FFPE) cervical adenocarcinoma specimens obtained from all patients who underwent primary surgical treatment for cervical cancer between 1985 and 2005 with sufficient material available for analysis, were obtained from the archives of the Department of Pathology, Leiden University Medical Center (n=67). Cervical adenocarcinoma was defined as an invasive epithelial tumor showing glandular differentiation (moderate to highly differentiated) or staining Periodic Acid Schiff Plus and Alcian Blue positive and lacking squamous elements (undifferentiated).[27,28] None of the patients had received preoperative anticancer therapy and follow-up data were obtained from patient medical records. Patient and tumor

characteristics are listed in Supplementary Table S1. 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.

Immunofluorescent stainings

Triple immunofluorescent staining was performed on 4 μm thick FFPE sections. After antigen retrieval using Tris-ethylenediaminetetraacetic acid (EDTA) buffer (10 mM Tris plus 1 mM EDTA pH 9.0), rabbit anti-CD3 (ab828, Abcam, Cambride, UK), mouse IgG1 anti-FoxP3 (ab20034, Abcam) and goat anti-IL-17 (AF-317-NA, R&D Systems, Abingdon, UK) diluted in 1% w/v bovine serum albumin (BSA) in phosphate buffered saline (PBS) were incubated at room temperature overnight. Alexa Fluor labelled donkey anti-rabbit-A546 (A10040), donkey anti-mouse-A647 (A31570) and donkey anti-goat-A488 (A11055; all from Invitrogen, Life Technologies, Carlsbad, USA) were incubated at room temperature for one hour. Slides were mounted using VectaShield mounting medium containing DAPI (Vector Laboratories, Burlingame, USA). Omitting the primary antibodies and substituting them for antibodies of the same isotype class with an unknown specificity were used as negative controls.

Microscopic analyses

Immunofluorescent images were acquired with an LSM700 confocal laser scanning microscope equipped with an LCI Plan-Neofluar 25x/0.8 Imm Korr DIC M27 objective (Zeiss, Göttingen, Germany). In the majority of cases, four random images were obtained at a 250x magnification, sampling a total tumor area of generally 1.0 mm^2, comprising vital areas of both tumor epithelium and stroma. Double or triple positivity of cells as well as the tumor epithelium and stroma area were determined using the overlay tool in the LSM Image Browser software (version 4.2.0.121, Zeiss). The numbers of single, double and triple positive cells were scored in the tumor epithelium and tumor stroma separately using the open source image processing program ImageJ version 1.47 (http://rsb.info.nih.gov/ij). Cells within blood vessels or lumina were not counted.

Statistical analysis

Statistical analyses were performed using SPSS version 20.0 (IBM Corp., Armonk, USA). Correlations between the number of positive cells and clinico-pathological variables were tested using the Spearman's rank correlation rho (r) and Wilcoxon Mann-Whitney tests. Correlations between the number of positive cells and disease-specific or disease-free survival were tested using the Kaplan-Meier and Cox proportional hazards models. For survival analyses, patients were divided in two groups

based on the median numbers of positive cells (high and low). All tests were two sided and p-values below 0.05 were considered statistically significant.

Results

Infiltrating immune cells

The cell density for all cell types analyzed was higher in the tumor stroma than the epithelium (Figure 1). Although the IL-17$^+$CD3$^+$ cells might comprise both αβ and γδ T cells, we designated the presumed predominantly αβ T cell population expressing IL-17 and CD3 as Th17 cells. Tumor infiltration by CD3$^+$ T cells was observed in all samples analyzed, while only a minor population of Th17 cells was observed (Supplementary Figure S1, Supplementary Table S2). FoxP3$^+$ cells were always positive for CD3. Since a single FoxP3$^+$IL-17$^+$ cell was only observed in two tumor samples (0.02% of FoxP3+ cells), these cells were not analyzed. Although approximately three times more CD3$^+$ T cells and four times more (non-Th17) IL-17$^+$ cells were present in the tumor stroma compared with the epithelium, especially Tregs were more strongly represented in the tumor stroma with on average over ten times higher cell counts compared with the epithelium.

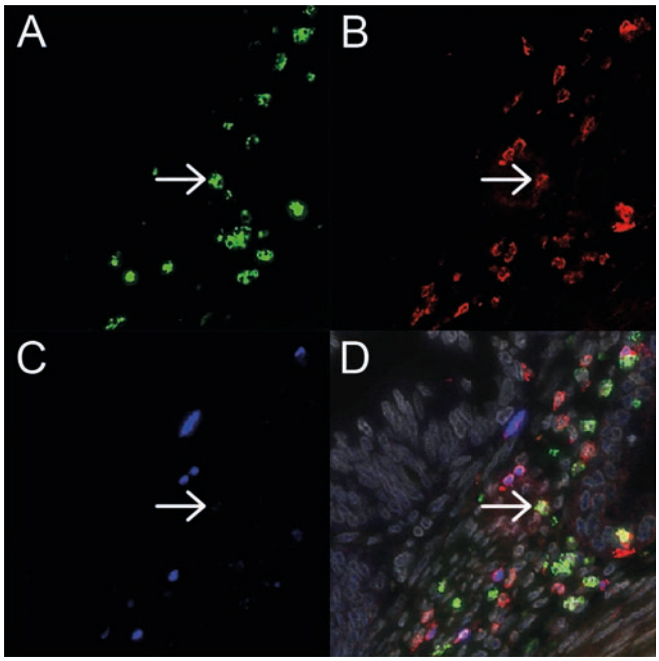

Figure 1. T cells, Th17 cells, Tregs and IL-17$^+$ cells in cervical adenocarcinoma

Representative image of a cervical adenocarcinoma stained by triple immunofluorescence for IL-17 (A), CD3 (B) and FoxP3 (C), with the combined stainings together with DAPI counterstain (grey) shown in D. The arrow indicates a cell double positive for IL-17 and CD3. Multiple CD3/FoxP3 double positive cells are present.

Correlation between infiltrating immune cells and survival

A high total number of T cells was significantly correlated with improved disease-specific (p=0.010, Figure 2A) and disease-free survival (p=0.001, Figure 2D). This was specifically due to a high number of $CD3^+FoxP3^+$ Tregs, since a high number of $CD3^+FoxP3^-$ T cells was less strongly correlated with disease-free survival (p=0.032, Figure 2F) than a high number of Tregs (p=0.007, Figure 2E). More importantly, there was no significant correlation between a high number of $CD3^+FoxP3^-$ T cells and disease-specific survival (p=0.254, Figure 2C), but high Tregs were significantly correlated with improved disease-specific survival (p=0.010, Figure 2B). Additionally, a high number of total $CD3^+$ T cells within the tumor epithelium was significantly correlated with improved disease-free (p=0.034, Figure 3D) but not disease-specific survival (p=0.248, Figure 3A). These correlations were practically similar for the number of $CD3^+FoxP3^-$ T cells (Figure 3C,F), because the number of FoxP3+ cells infiltrating in the tumor epithelium was relatively low. The number of Tregs in the tumor epithelium was not significantly correlated with disease-free or disease-specific survival (Figure 3B,E). The separate analyses of the correlation between the other cell types present in the tumor epithelium or the tumor stroma compartment and survival were not significant.

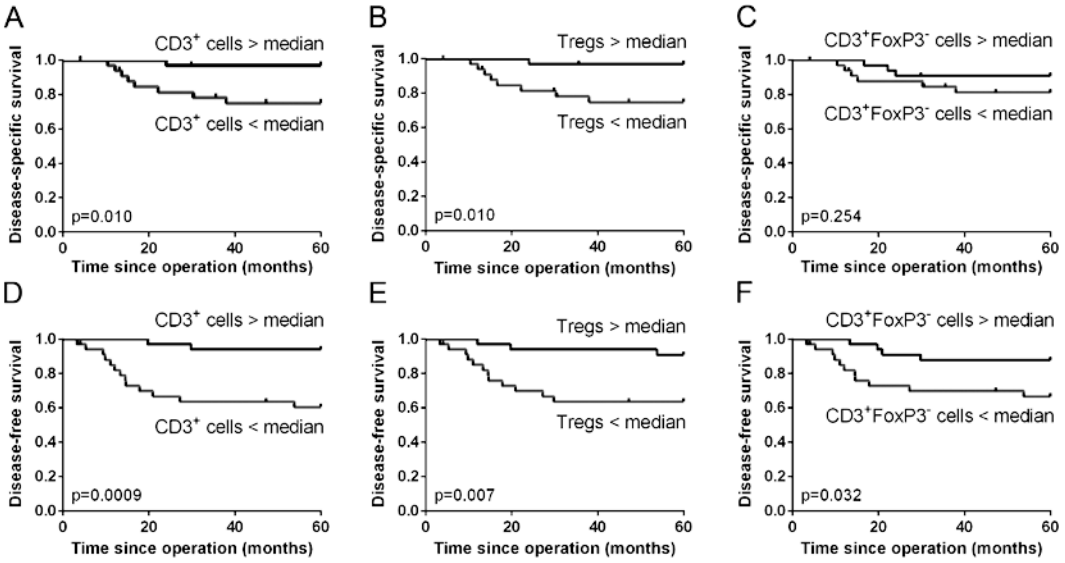

Figure 2. Correlation between total T cells, Tregs and survival

Kaplan-Meier survival curves for a high versus low number of total $CD3^+$ T cells (A,D), $CD3^+FoxP3^+$ Tregs (B,E) and $CD3^+FoxP3^-$ T cells (C,F) are shown for disease-specific (A-C) and disease-free survival (D-F).

A high number of (non-Th17) IL-17^+ cells per se was not significantly correlated with survival. However, when combining the Treg and IL-17 scores, patients could be better categorized in groups with poor or improved survival. Compared with a high number of

Tregs and a low Treg number but high IL-17$^+$ cells, the combination of a low number of both Tregs and IL-17$^+$ cells was correlated with worse disease-specific survival (p=0.007, Figure 4A). Since a high number of Tregs was correlated with favorable prognosis, the number of IL-17$^+$ cells did not discriminate between patients with poor or improved survival in this patient group. Having a low number of Tregs and Th17 cells present was correlated with worse survival than high Tregs or low numbers of Tregs and absence of Th17 cells (p=0.018, Figure 4B). So despite the generally low numbers of Th17 cells present, their presence still contributed to the effect of the number of Tregs present. Both correlations were also significant for disease-free survival (data not shown).

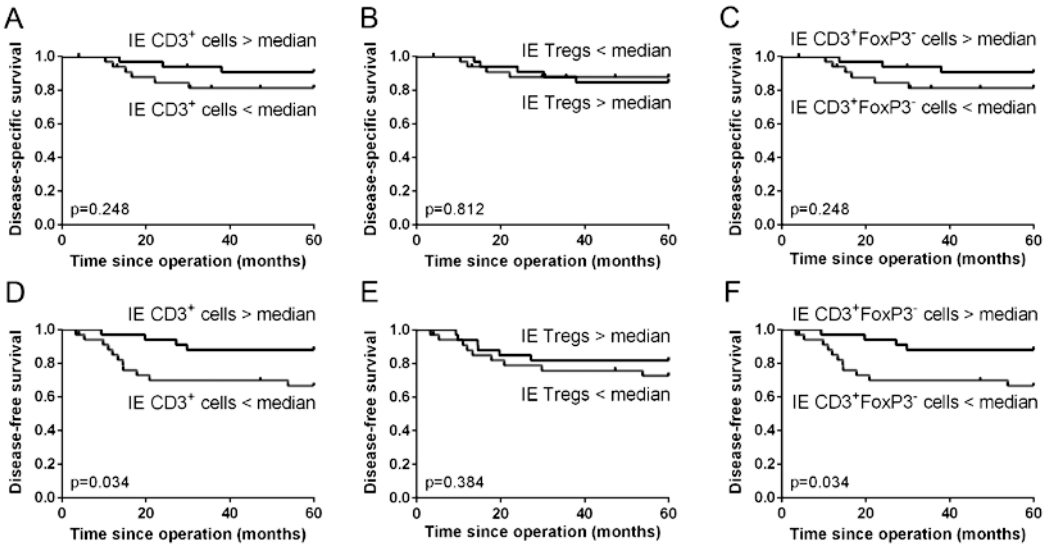

Figure 3. Correlation between tumor epithelium infiltrating T cells, Tregs and survival

Kaplan-Meier survival curves for a high versus low number of CD3$^+$ cells (A,D), CD3$^+$FoxP3$^+$ Tregs (B,E) and CD3$^+$FoxP3$^-$ T cells (C,F) infiltrating in the tumor epithelium are shown for disease-specific (A-C) and disease-free survival (D-F).

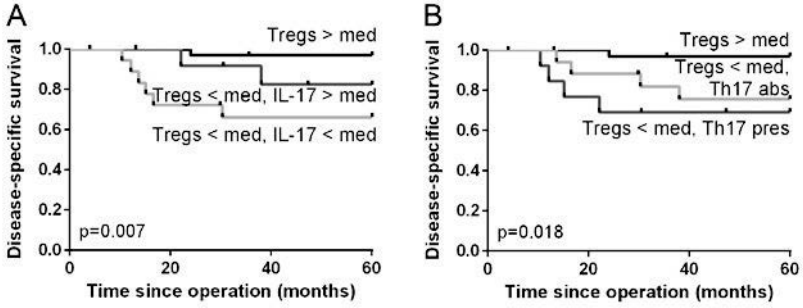

Figure 4. Correlation between Tregs combined with IL-17, Th17 and survival

Kaplan-Meier survival curves for disease-specific survival based on a high (above median, abbreviated as med) number of Tregs compared with a low number of Tregs combined with high or low IL-17$^+$ cells (A) or absence (abs) or presence (pres) of Th17 cells (B).

Hazard ratio for low Tregs and IL-17$^+$ cells infiltration

The hazard ratio for disease-specific survival in case of a low number of Tregs was 9.38 (95% CI: 1.17-75.09, p=0.035, Table 1). This remained significant when correcting for tumor lymph node metastasis (TNM) stage. The hazard ratio for disease-specific survival for tumors with a low number of both Tregs and IL-17$^+$ cells was 13.91 (95% CI: 1.67-115.73, p=0.015, Supplementary Table S3) when compared with a high number of Tregs, which also remained significant after correcting for TNM stage. A low number of Tregs combined with the presence of Th17 cells gave a hazard ratio of 12.83 (95% CI: 1.43-114.93, p=0.023, Supplementary Table S4) when compared with a high number of Tregs, which also remained significant after correcting for TNM stage.

Table 1. Hazard ratio for a low Tregs frequency

Variable	Univariate hazard ratio (95% CI)	p value	Multivariate hazard ratio (95% CI)	p value
TNM stage	1.606 (1.133-2.276)	0.008	1.643 (1.144-2.361)	0.007
Tregs low	9.384 (1.173-75.093)	0.035	10.131 (1.256-81.719)	0.030

Univariate and multivariate Cox regression analyses for the TNM stage and a low number of Tregs on disease-specific survival are shown. The 95% confidence interval (95% CI) of the hazard ratio is indicated between brackets.

Correlation between IL-17$^+$ cells and clinico-pathological parameters

Finally, we investigated whether the different cell populations were associated with prognostic clinico-pathological parameters (lymph node metastasis, tumor size, vaso-invasion and infiltration depth). A high number of IL-17$^+$ cells was significantly correlated with the absence of vaso-invasion (p=0.001, Figure 5A), decreased tumor infiltration depth (r = -0.29, p=0.021, Figure 5B) and decreased tumor size (r = -0.28, p=0.030, Figure 5C). No other significant correlations were found.

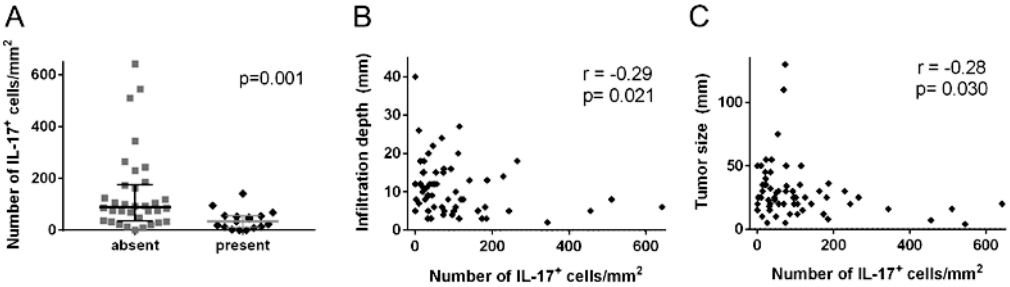

Figure 5. Correlation between IL-17 and clinico-pathological parameters

The p value for the correlation between non-Th17 IL-17$^+$ cells and vaso-invasion (A) was calculated using the Wilcoxon Mann-Whitney test. The correlations between non-Th17 IL-17$^+$ cells and tumor infiltration depth (B) and tumor size (C) were calculated using the Spearman's rank correlation rho test.

Discussion

The current study showed that a high total number of Tregs was significantly correlated with improved disease-free and disease-specific survival in cervical adenocarcinoma patients. Although tumor infiltrating immune cells are more frequently present in the tumor stroma than the tumor epithelium, especially Tregs were about three times less frequently present in the tumor epithelium than T cells and IL-17$^+$ cells. Within the tumor epithelium, a high T cell frequency was significantly correlated with improved disease-free survival. Strikingly, specifically a low total number of both Tregs and IL-17$^+$ cells was strongly correlated with poor survival. The IL-17$^+$ cells were inversely correlated with vaso-invasion, tumor size and infiltration depth. The number of IL-17$^+$ cells could thus further discriminate between patient prognoses after Treg determination. In addition, a low number of Tregs combined with the presence of Th17 cells was correlated with worse prognosis.

The current data suggest that, of the immunological parameters studied, the total number of Tregs is the most important determinant correlated with survival for cervical adenocarcinoma patients. Tregs thus seem to represent a beneficial immune response in cervical adenocarcinoma, which contrasts with Tregs correlating with poor survival in cervical squamous cell carcinoma.[25,29] This corresponds with studies that indicate that cervical adenocarcinoma differs substantially from squamous cell carcinoma,[13-15,24] and suggests that the composition and effect of the tumor infiltrating immune cells differ per histological tumor subtype. However, a direct correlation between total Tregs and survival in cervical squamous cell carcinoma has not been shown: the significant correlations were specifically found within the tumor epithelium and especially when compared with the number of cytotoxic T cells present. A specific correlation between a high ratio of total T cells or CTL over Tregs and improved survival has recently also been shown in glioblastoma.[30] When we studied the tumor epithelium separately, total T cell frequency was correlated with improved disease-free survival. The latter correlation was irrespective of Tregs, because the intraepithelial T cell frequency predominantly comprised FoxP3$^-$ cells. Thus, intraepithelial T cell infiltrate seems to be a general marker for improved survival. These intraepithelial T cells might predominantly be cytotoxic T lymphocytes (CTL). Another partial explanation for the differences found between the histological subtypes might be that the tumors of this cervical adenocarcinoma cohort were generally smaller in size than the squamous cell carcinomas, as was described before.[27] Supporting our data, other studies have also reported correlations between Tregs and poor survival,[31-33] indicative of the dampening of an anti-tumor immune response. However, Tregs have also been found to be correlated with improved prognosis in different types of cancer,[34-37] suggesting they may also dampen a tumor promoting immune response. Indeed, the role of Tregs in cancer is controversial and seems to be context and tumor type dependent.[38] The current

data support a predominant role in suppressing tumor growth favoring inflammation in cervical adenocarcinoma.

IL-17 has, in general, been shown to correlate with poor survival, and Th17 cells with improved survival in cancer.[39] The pro-inflammatory cytokines IL-6 and IL-23, which are implicated in the induction of IL-17 expression,[40] have also been shown to be correlated with poor survival in cervical cancer.[41] Since IL-17 has been shown to be generally expressed by granulocytes,[26] this suggests that a pro-inflammatory environment may attract granulocytes and other innate myeloid cells favoring tumor growth in cervical squamous cell carcinoma. The correlation between increased IL-17$^+$ cells and improved survival especially in case of low Treg frequencies in cervical adenocarcinoma suggests that these cells might rather counteract tumor growth in cervical adenocarcinoma. We have previously shown that IL-17 producing cells represent a heterogenous cell population,[26] and we propose that IL-17$^+$ cells may predominantly represent tumor targeting myeloid cells in cervical adenocarcinoma, potentially mast cells and type 1 neutrophils and macrophages. Correspondingly, Chen et al. showed that a high number of infiltrating IL-17$^+$ cells was significantly correlated with improved survival in a large cohort of gastric adenocarcinoma patients.[42]

Our results showed that the presence of Th17 cells, specifically when a low number of Tregs was present, was correlated with poor survival. A pro-inflammatory Th17 response, despite the low frequencies, might thus rather be correlated with a tumor promoting immune response. This does correspond with a study by Yan et al., showing that an increased frequency of circulating Th17 cells is correlated with poor survival in hepatocellular cancer.[43] Also this correlation is in contrast with its role in a beneficial immune response in cervical squamous cell carcinoma[26] as well as other cancer types.[39]

These different correlations suggest that the local immune response in cervical adenocarcinoma differs substantially from the immune response in squamous cell carcinoma. This might be related to differences in the molecular constitution of the two cancer types. Cervical adenocarcinoma has recently been shown to contain more frequent TP53[44] and KRAS mutations and less frequent PIK3CA and PTEN mutations compared with cervical squamous cell carcinoma.[45] In our patient cohort, we found significantly more frequent somatic KRAS mutations in cervical adenocarcinoma, whereas PIK3CA mutations were more frequently found in squamous cell carcinoma (manuscript submitted). We have furthermore shown that CXC chemokine receptor 4 (CXCR4), CXCR7 and epidermal growth factor receptor (EGFR) were more frequently expressed in cervical squamous cell carcinoma than adenocarcinoma.[46] Additionally, we showed that HLA-E was overexpressed more frequently in cervical adenocarcinoma than in cervical squamous cell carcinoma.[27] High HLA-E expression was significantly correlated with improved disease-free and disease-specific survival in cervical adenocarcinoma, while no correlation was found in squamous cell carcinoma. Cervical adenocarcinoma samples have also been shown to produce higher levels of transforming growth factor-β (TGF-β) than squamous cell carcinoma samples.[24] Since we showed in

the present study that Tregs, Th17 cells and other IL-17$^+$ cells also show opposed correlations in cervical adenocarcinoma compared with cervical squamous cell carcinoma, this suggests that the molecular differences are correlated with a different type of immune response. We speculate that the increased HLA-E and TGF-β expression might cause an effector T cell response to have limited efficacy. Under these circumstances, classically activated myeloid cells such as type 1 neutrophils might be more effective in cervical adenocarcinoma. Infiltration of T cells into the tumor epithelium was correlated with improved survival in both cervical cancer types.

To conclude, our data show that the role of T cells, including Tregs, Th17 cells and other IL-17$^+$ cells is context and tumor type dependent. Tregs and IL-17$^+$ cells represented a beneficial immune response correlated with improved survival, while Th17 cells might contribute to tumor progression and poor prognosis in cervical adenocarcinoma. Future research should determine how these cell types are correlated with improved prognosis, what other immune cell types are involved and how this might be used to guide patient prognosis and treatment.

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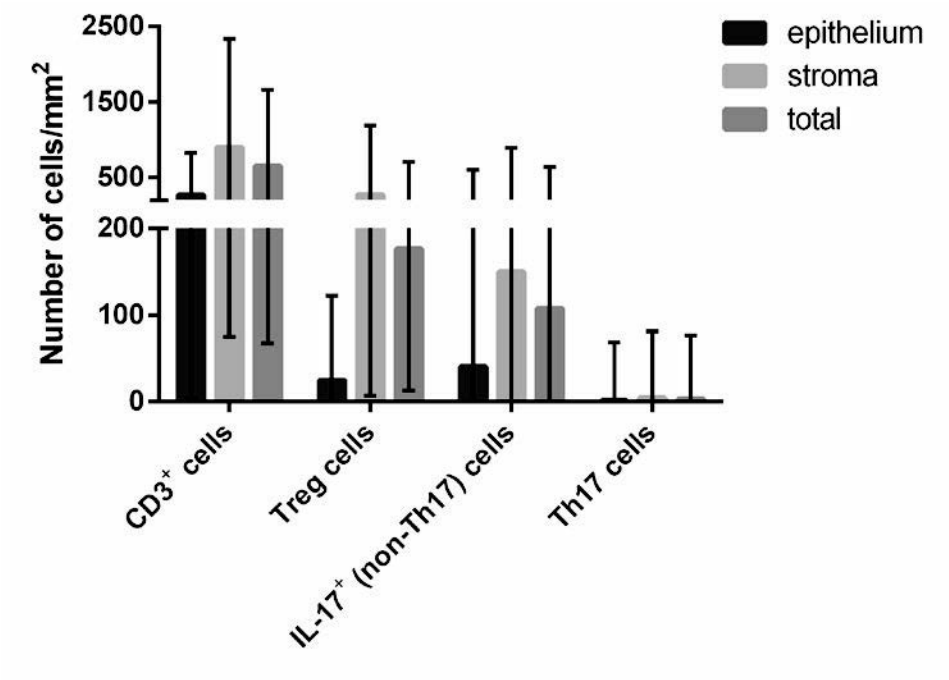
Supplementary Figure S1. Quantification of tumor infiltrating cells



The mean and range of the number of total $CD3^+$ T cells, $FoxP3^+CD3^+$ Tregs, $CD3^-IL\text{-}17^+$ cells and $CD3^+IL\text{-}17^+$ Th17 cells observed infiltrating in the tumor epithelium, tumor stroma and combined total area per mm^2 is shown (n=67).

Supplementary Table S1. Patient and tumor clinico-pathological characteristics

Clinico-pathological parameter	Category	N = 67 (%)
Age	Median	40
	Range	26-80
FIGO stage[1]	IA	2 (3)
	IB	59 (88)
	IIA	6 (9)
TNM stage	IAII	4 (6)
	IB1	40 (60)
	IB	4 (6)
	IB2	8 (12)
	IIA	7 (10)
	IIB	1 (1)
	IIIA	1 (1)
	IIIB	2 (3)
Lymph nodes	negative	54 (81)
	positive	13 (19)
Tumor size (mm)[2]	<40	52 (78)
	≥40	12 (18)
Vaso-invasion[2]	Absent	36 (54)
	Present	17 (25)
Infiltration depth (mm)[2]	<15	46 (69)
	≥15	17 (25)
HPV type	16	26 (39)
	18	24 (36)
	other	17 (25)

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

Supplementary Table S2. Number of T cells, Tregs and IL-17$^+$ cells present in cervical adenocarcinoma

	CD3$^+$ cells		Treg cells		IL-17$^+$ (non-Th17) cells		Th17 cells	
	mean (range)	median	mean (range)	median	mean (range)	median	mean (range)	median
Epithelium	268 (5-827)	187	25 (0-123)	17	41 (0-601)	7	2 (0-69)	0
Stroma	898 (75-2338)	762	271 (7-1193)	175	150 (0-889)	95	4 (0-82)	0
Total	649 (68-1664)	601	176 (13-705)	119	107 (0-642)	69	3 (0-76)	0

The mean, minimum, maximum and median number of total CD3$^+$ cells, FoxP3$^+$CD3$^+$ Tregs, CD3$^-$IL-17$^+$ cells and CD3$^+$IL-17$^+$ Th17 cells observed infiltrating in the tumor epithelium, tumor stroma and combined total area per mm^2 is indicated.

Supplementary Table S3. Effect of Tregs in combination with IL-17$^+$ cells on survival

Variable	Univariate hazard ratio (95% CI)	p-value	Multivariate hazard ratio (95% CI)	p-value
TNM stage	1.606 (1.133-2.276)	0.008	1.545 (1.051-2.273)	0.027
Tregs high	reference		reference	
Tregs low, IL-17 high	5.587 (0.506-61.635)	0.160	7.561 (0.659-86.742)	0.579
Tregs low, IL-17 low	13.906 (1.671-115.730)	0.015	12.261 (1.454-103.383)	0.021

Univariate and multivariate Cox regression analyses for the TNM stage and a low number of Tregs combined with a high or low number of (non-Th17) IL-17$^+$ cells versus a high number of Tregs on disease-specific survival are shown.

Supplementary Table S4. Effect of Tregs in combination with Th17 cells on survival

Variable	Univariate hazard ratio (95% CI)	p value	Multivariate hazard ratio (95% CI)	p value
TNM stage	1.606 (1.133-2.276)	0.008	1.619 (1.131-2.317)	0.008
Tregs high	reference		reference	
Tregs low, Th17 absent	8.361 (0.934-74.863)	0.058	8.460 (0.942-75.983)	0.057
Tregs low, Th17 present	12.825 (1.431-114.930)	0.023	14.668 (1.603-134.179)	0.017

Univariate and multivariate Cox regression analyses for the TNM stage and a low Tregs number combined with a high or low number of Th17 cells versus a high number of Tregs on disease-specific survival are shown.

V

Correlations between immune response and vascularization qRT-PCR gene expression clusters in squamous cervical cancer

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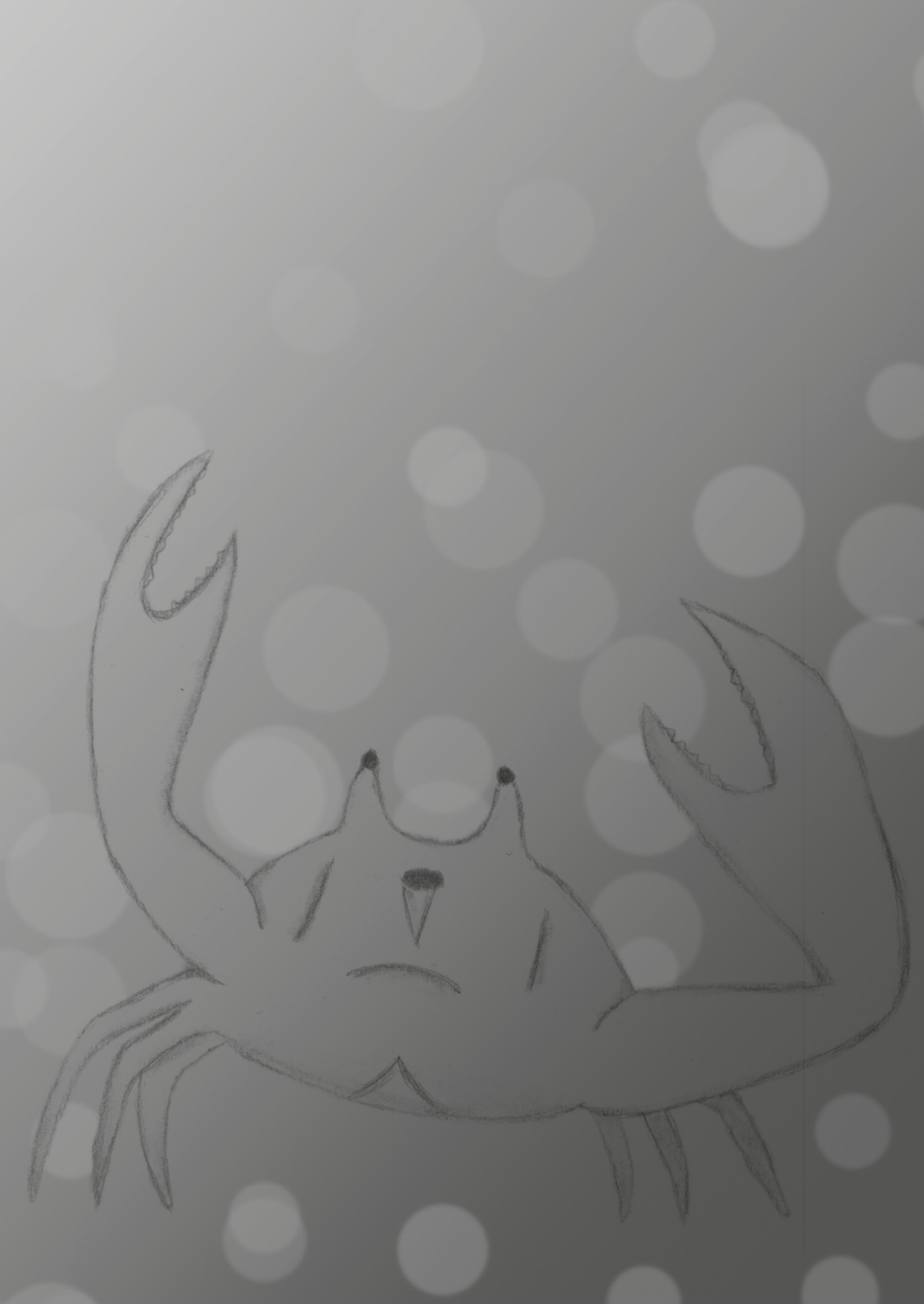

Abstract

The tumor microenvironment comprises a network of immune response and vascularization factors. From this network, we identified immunological and vascularization gene expression clusters and the correlations between the clusters by determining the expression of 42 genes in 52 fresh frozen squamous cervical cancer samples using qRT-PCR. Weighted gene co-expression network analysis and mixed-model analyses were performed to identify gene expression clusters. We subsequently determined which factors were correlated with each other and with patient survival. We identified four immune response clusters: 'T cells' (*CD3E* / *CD8A* / *TBX21* / *IFNG* / *FOXP3* / *IDO1*), 'Macrophages' (*CD4* / *CD14* / *CD163*), 'Th2' (*IL4* / *IL5* / *IL13* / *IL12*) and 'Inflammation' (*IL6* / *IL1B* / *IL8* / *IL23* / *IL10* / *ARG1*) and two vascularization clusters: 'Angiogenesis' (*VEGFA* / *FLT1* / *ANGPT2* / *PGF* / *ICAM1*) and 'Vessel maturation' (*PECAM1* / *VCAM1* / *ANGPT1* / *SELE* / *KDR* / *LGALS9*). The 'T cells' module was correlated with all modules except for 'Inflammation', while 'Inflammation' was most significantly correlated with 'Angiogenesis' ($p<0.001$). High expression of the 'T cells' cluster was correlated with earlier TNM stage ($p=0.007$). High *CD3E* expression was correlated with improved disease-specific survival ($p=0.022$), while high *VEGFA* expression was correlated with poor disease-specific survival ($p=0.032$). Independent predictors of poor disease-specific survival were *IL6* (hazard ratio=2.3, $p=0.011$) and a high *IL6*/*IL17* ratio combined with low *IL5* expression (hazard ratio=4.2, $p=0.010$). To conclude, 'Inflammation' marker *IL6*, especially in combination with low levels of *IL5* and *IL17*, was correlated with poor survival. This suggests that *IL6* promotes tumor growth, which may be suppressed by a Th17 and Th2 response. Measuring *IL6, IL5* and *IL17* expression may improve the accuracy of predicting prognosis in cervical cancer.

Introduction

Cervical cancer is caused by a persistent infection with human papillomavirus (HPV) and represents the second leading cause of cancer-associated deaths among young women.[1] Infection with HPV initiates an immune response that can generally clear the infection. In some cases the infection can lead to chronic inflammation, which may provide growth signals and support carcinogenesis.[2] Once a tumor has been established, the type of immune response present in the microenvironment is thought to be important for clinical outcome.

Tumor infiltrating T lymphocytes have been shown to be an independent predictor for survival in ovarian and colorectal cancer.[3,4] T lymphocytes can be subdivided in different populations, including cytotoxic $CD8^+$ T lymphocytes (CTL) and $CD4^+$ T

helper 1 (Th1), Th2, Th17 and regulatory T cells (Tregs). CTL and Th1 cells are generally appreciated for their potential to induce or stimulate a specific tumor suppressing immune response. Synthetic long-peptide vaccination in women with $HPV16^+$ high-grade vulvar intraepithelial neoplasia has been shown to induce $CD4^+$ T helper and $CD8^+$ CTL responses, which were correlated with tumor regression.[5] In cervical cancer, we have previously shown that a low number of CTL combined with a high number of Tregs is an independent predictor for poor survival.[6] Since Tregs can control the activity of other T cells, these cells may dampen both a tumor suppressing and a tumor promoting immune response. Indeed, Tregs have been found to be correlated with less invasion in thyroid cancer and improved recurrence-free survival in head and neck cancer.[7,8] A Th2-induced immune response has also been shown to support cervical cancer progression.[9] The role of Th17 cells in cancer is still unclear, as they are capable of inducing both tumor growth and tumor regression.[10]

The innate immune system also plays an important role in cervical cancer progression. Our group has shown that mature $CD14^+CD163^-$ M1 type macrophages are an independent predictor for improved survival.[11] $CD163^+$ M2 type macrophages have been correlated with poor survival, although the results of different studies are not consistent.[12,13] Tumor associated neutrophils are a heterogeneous cell population associated with poor outcome in different types of cancer.[14]

Another important factor for an adequate immune response is the vascular system, which delivers nutrients, but also enables immune cells to enter the tumor site. There appears to be an inverse relationship between new vessel formation (angiogenesis), which supports tumor progression, and vessel adhesiveness (maturation), supporting infiltration of immune cells in the tumor tissue.[15] Angiogenesis is induced by growth factors or cytokines such as vascular endothelial growth factor A (VEGFA), angiopoietin-2 (ANGPT2), fibroblast growth factors (FGFs) and interleukin-8 (IL-8).[15,16] Angiogenesis, as represented by a high number of blood vessels, has been associated with poor survival in cervical cancer.[17] Vessel maturation, on the other hand, is characterized by signaling and adhesion proteins including vascular cell adhesion molecule-1 (VCAM1), intercellular adhesion molecule-1 (ICAM1), E-selectin (SELE) and ANGPT1.[15,16]

In the present study, we identified combinations of immunological and vascular factors (expression clusters) in cervical carcinoma and determined the associations between the different clusters, prominent genes and their correlations with clinico-pathological parameters and patient survival.

Materials and methods

Patient material

Fresh frozen squamous cervical cancer specimens was obtained from the biobank of the departments of Pathology and Gynaecology from the Leiden University Medical Center.

Table 1. Patient and tumor clinico-pathological characteristics

Clinico-pathological parameter	Category	N = 52 (%)
Age	Median	43
	Range	24-77
FIGO stage[1,2]	IB	41 (79)
	IIA	10 (19)
TNM stage	IB1	16 (31)
	IB	2 (4)
	IB2	19 (37)
	IIA	8 (15)
	IIB	5 (10)
	IIIB	1 (2)
	IV	1 (2)
Lymph nodes	Negative	33 (63)
	Positive	19 (37)
Tumor size (mm)[2]	<40	20 (38)
	≥40	29 (56)
Vaso-invasion[2]	Absent	22 (42)
	Present	29 (56)
Infiltration depth (mm)[2]	<15	26 (50)
	≥15	22 (42)
HPV type	16	31 (60)
	18	11 (21)
	Other	10 (19)

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

Patients had underwent primary surgical treatment for cervical cancer between 1989 and 2005 and had not received preoperative therapy. Follow-up data were obtained from patient medical records. Samples with sufficient material available for analysis, containing 50-90% tumor epithelial cells and no uninvolved normal tissue were selected by staining a 4 μm section with haematoxylin and eosin (n=56). Median follow-up time was 6.2 years. Patient and tumor characteristics are listed in Table 1. 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.

RNA isolation and quality control

RNA was isolated from four 20 μm slides using Trizol (Life Technologies, Carlsbad, USA) and DNase treated and purified using RNeasy Mini columns (Qiagen, Hilden, Germany). The RNA integrity and quantity were analyzed using RNA 6000 Nano chips in a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). Samples with RIN<5 were excluded from further analysis (n=4), because the Cq values of the reference genes were disproportionally low. The median RIN value was 7.9.

qRT-PCR array immune response markers

Genomic DNA removal, cDNA synthesis, pre-amplification and qRT-PCR were performed using RT^2 Profiler PCR Arrays (Qiagen) according to the manufacturers' instructions. In brief, 300 ng RNA was treated with DNA elimination mix and cDNA was subsequently synthesized. The intended PCR products were preamplified, followed by Side Reaction Reducer and heat inactivation. A Sybr Green-based qRT-PCR reaction was performed in duplicate on a CFX384 system (Bio-Rad, Hercules, USA). A custom combination of primer sets was used to analyze different T cell and macrophage markers: *ARG1*, *CD14*, *CD163*, *CD3E*, *CD4*, *CD8A*, *FOXP3*, *GATA3*, *IDO1*, *IFNG*, *IL1B*, *IL10*, *IL12A*, *IL13*, *IL17A*, *IL17F*, *IL2*, *IL23A*, *IL4*, *IL5*, *IL6*, *RORC*, T-bet encoding *TBX21*, *TGFB1* and *TGFB3*. From the four reference genes included based on reported stability in cervical cancer tissue, the most stably expressed genes *EEF1A1* and *RPLP0* were used for normalization. Averaged duplicate measurements were scaled by standard deviation. Negative genomic DNA contamination and positive reverse transcriptase and PCR controls were included for each sample.

cDNA synthesis and qRT-PCR IL-17A and neutrophil markers

Since *IL17A* expression measured by the RT^2 Profiler PCR Array was low for all samples and not detected in eight samples, an additional qRT-PCR with Primer-BLAST[18] designed primers for *IL17A* was performed on all samples. To further complement the assay, qRT-PCRs were performed for the neutrophil markers

fucosyltransferase 9 (*FUT9*) and neutrophil elastase (*NE*). cDNA was synthesized as described previously.[19] Sybr Green-based qRT-PCR was performed in duplicate using 1:125 diluted cDNA and 3 pmol primers. Primers and annealing temperatures used were for *IL17A*: forward 5'-CCCCCGGACTGTGATGGTCAAC-3' and reverse GCGGCACTTTGCCTCCCAGAT at 56.7°C, *FUT9*: forward 5'-AGGCCACCCTTCCAGAAATG-3' and reverse 5'- TGCTTGGCACTTCAAACACG-3' at 64.5°C and *NE*: forward 5'- ATTCTCCAGCTCAACGGGTC-3' and reverse 5'-GATTAGCCCGTTGCAGACCA-3' at 63.8°C. The PCR products were validated by sequencing. Reference genes *EEF1A1* and *RPLP0* were quantified using 4 pmol RT^2 qPCR primer assays (Qiagen) at an annealing temperature of 60°C as per the manufacturers' instructions. Replacing the cDNA template by milliQ was used as negative control. The qRT-PCR, normalization and scaling were performed as described for the RT^2 Profiler PCR array. Since RNA expression was detected in all samples by the Primer-BLAST primers, all *IL17A* analyses were performed with the *IL17A* expression measured by the Primer-BLAST primers.

cDNA synthesis and qRT-PCR vascularization markers

Vascularization markers *ANGPT1*, *ANGPT2*, basic fibroblast growth factor (*bFGF*), platelet endothelial cell adhesion molecule (*PECAM1*), placental growth factor (*PGF*), *IL8*, Galectin-1 (*LGALS1*), Galectin-3 (*LGALS3*), Galectin-9 (*LGALS9*), *VEGFA*, VEGFR1 encoding fms-related tyrosine kinase 1 (*FLT1*), VEGFR2 encoding kinase insert domain receptor (*KDR*), *ICAM1*, *VCAM1* and *SELE* were measured on all samples, except for samples containing less than 100 ng RNA per µl (n=2). cDNA synthesis, primers and qPCR conditions were as described before.[20,21] Reference genes, normalization and scaling were performed as described for the immune response markers.

Immunohistochemistry

In previous studies, immunohistochemistry (IHC) has been performed on at least ten formalin-fixed, paraffin-embedded (FFPE) samples corresponding to the fresh frozen samples used in this study. Briefly, $CD3^+CD8^+$ CTL were stained by a mixture of rabbit anti-CD3 (Abcam, Cambridge, UK), mouse IgG2b anti-CD8 (Novocastra, Newcastle, UK) and mouse IgM anti-CD57 (developed in-house), followed by goat anti-rabbit IgG-A546, goat anti-mouse IgG2b-A647 and goat anti-mouse IgM-A488 (Invitrogen, Life Technologies, Carlsbad, USA).[6] Fifteen images per slide were obtained in 26 samples using an LSM510 confocal laser scanning microscope equipped with a PH2 Plan-NEOFluar 25x/0.80 oil objective (Zeiss, Göttingen, Germany) in a multitrack setting. The number of $IL\text{-}6^+$ cells was scored in 14 samples stained with rabbit anti-IL-6 (Abcam) followed by biotinylated swine anti-rabbit (Dako, Glostrup, Denmark) and biotinylated HRP-streptavidin (Dako).[22] The percentage of positive cells was counted in

six random high-power fields. The number of IL-1β$^+$ cells was scored in 12 overlapping samples stained with goat anti-IL-1β (R&D Systems) and the goat HRP-polymer kit.[22] Positive cells were counted in the tumor stroma of six random high-power fields. IL-17 was stained on all 52 samples by goat anti-IL-17 (R&D Systems, Abingdon, UK) followed by goat HRP-polymer (Biocare Medical, Concord, USA).[23] Cells were digitally scored in 4-6 random images at a 200x magnification. CD3$^+$IL-17$^+$ cells were stained by a mixture of mouse IgG1 anti-CD3 (Dako) and goat anti-IL-17 followed by rat anti-mouse IgG1-AP (Southern Biotech, Birmingham, USA) and donkey anti-goat-HRP (Abcam). Six random images were taken in 18 samples using a DM4000B spectral microscope equipped with a HC Plan APO 20x objective (Leica Microscopy CMS GmbH, Wetzlar, Germany).

Statistical analysis

Weighted gene co-expression network analysis (WGCNA) was performed using R version 3.0.2.[24] Weights were computed using a power of 3 to obtain the best combination of power, scale-free topology and connectivity values. Average linkage hierarchical clustering was performed. The distance between subclusters was determined by using the average distances between all potential gene pairs. Small clusters were only assigned to clusters belonging to the same branch. Gene expression clusters were summarized using the first principal components.

To test for correlations between gene expression clusters and genes, mixed model analyses were performed using SPSS version 20.0 (IBM Corp., Armonk, USA). All combinations between the six expression clusters were tested for correlations with a Bonferroni corrected significance level of $p<0.003$. A cutoff for single gene correlations (r) of 0.3 and significance level of $p<0.05$ were used. Correlations between RNA expression and IHC data was tested using the Spearman's rank correlation rho test. A p value<0.05 was considered statistically significant.

Correlations between gene expression clusters or separate genes and clinico-pathological variables were tested using the independent samples t-test and Wilcoxon Mann-Whitney U test for categorical variables and the Pearson and Spearman's rank correlation rho for continuous variables. Six gene expression clusters were tested for each parameter with a Bonferroni corrected significance level of $p<0.008$.

Normalized Cq values were converted to expression values to obtain correlations corresponding with increased presence of the gene product for Kaplan-Meier and Cox proportional hazards survival models. For Kaplan-Meier curve generation and log rank analyses, gene expression levels were divided in four equal quartiles and the lowest quartile (low expression) or highest quartile (high expression) was compared with the other quartiles.

Results

Gene clustering

We investigated the expression of 27 immune response and 15 vascularization marker genes in 52 squamous cervical cancer samples by WGCNA, a method developed for network analysis of gene expression data.[25] A gene expression cluster is composed of genes with similar expression patterns. Genes that were not included in a cluster due to lack of correlation with other genes were removed from cluster analysis, including *bFGF*, *FUT9*, *LGALS1*, *LGALS3*, *GATA3*, *IL17A*, *IL17F*, *IL2*, *NE*, *RORC*, *TGFB1* and *TGFB3*, resulting in the dendrogram shown in Figure 1A. Of note, the neutrophil markers *NE* and *FUT9* were expressed at very low levels and were not detected in 26 and 34 out of 52 samples, respectively. *IL17A* was also generally expressed at levels lower than the other genes measured.

Small gene expression distances represent strong correlations. The strongest correlations were found between the T cell markers, for instance between *TBX21* and *CD3E* ($r = 0.939$, $p<0.0001$). Additional clustering was found for the T cell (*CD3E*), CTL (*CD8A*), Th1 (*TBX21*, *IFNG*) and Treg (*FOXP3*, *IDO1*) markers. This gene cluster is referred to as the 'T cells' cluster. A 'Th2' cluster was represented by the expression of *IL4*, *IL5*, *IL13* (Th2 markers) and *IL12* (Th1 marker). As the expression distance between *IL12* and the other markers was high, *IL12* only weakly correlated with the Th2 markers. The expression of *CD4*, *CD14* and *CD163* represented a 'Macrophage' cluster. The last immune response cluster consisted of genes representative of an 'Inflammation' response, including *IL1B*, *IL6*, *IL8*, *IL10, IL23* and *ARG1*. In this cluster, *IL10* and *ARG1* were weakly correlated. The 'Angiogenesis' cluster included the genes *ANGPT2*, *FLT1* (encoding VEGFR1*)*, *VEGFA*, *PGF* and *ICAM1*, while the 'Vessel maturation' cluster included the genes *PECAM1*, *KDR* (encoding VEGFR2*)*, *ANGPT1*, *VCAM1*, *LGALS9* and *SELE* (encoding E-Selectin).

To analyze the correlations between the different gene clusters, we performed mixed model analyses, summarized in Figure 1B. All gene clusters significantly correlated with 'Vessel maturation', while the 'T cells' cluster significantly correlated with all other clusters except for 'Inflammation' (all $p<0.001$). The 'Inflammation' cluster significantly correlated with the 'Vessel maturation', 'Angiogenesis' and 'Macrophages' clusters (all $p<0.0001$), although the individual 'Inflammation' genes did not significantly correlate with any of the individual 'Macrophages' genes.

In order to study the relationships of more distant genes, we also studied their correlation with separate genes. Extending on the ´Inflammation´ associations, expression of ´Inflammation´ marker *IL6* was inversely correlated with 'Vessel maturation' marker *VCAM1* ($r = -0.340$, $p=0.021$). The 'Inflammation' and 'T cells' clusters were not significantly correlated, but the 'Inflammation' marker gene *IL1B*

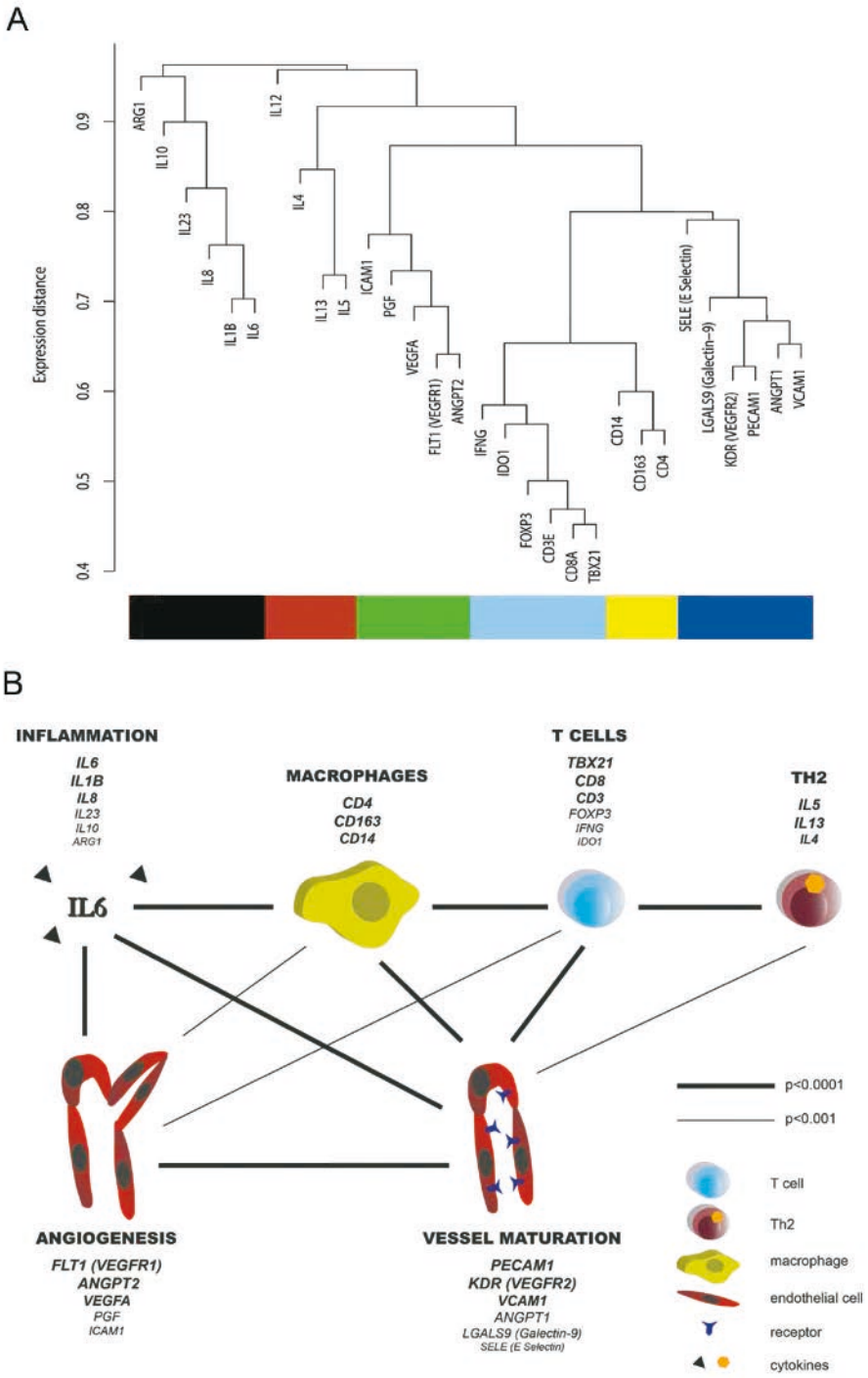

Figure 1. Gene expression clusters

WGCNA was performed to detect gene expression clusters, as shown in Figure 1A. Expression clusters are indicated by color bars below the genes that are included in the different clusters. The smallest gene expression distances are present between the genes at the bottom of the figure, representing strong associations. Frequently used gene synonyms are shown between brackets. The correlations between the clusters were tested by mixed model analyses, represented in Figure 1B. Only correlations with $p<0.003$ are shown, based on the Bonferroni correction for multiple testing.

negatively correlated with *CD3E* (r = -0.384, p=0.009), *CD8* (r = -0.384, p=0.009), *FOXP3* (r=-0.307, p=0.031) and *TBX21* expression (r=-0.307, p=0.032). Expression of the 'Th2' cluster gene *IL12* was distant from other 'Th2' genes, and only significantly correlated with *IL4* expression (r = 0.506, p=0.001). Similarly, *ARG1* expression only weakly correlated with *IL10* (r = 0.423, p=0.004) and *IL23* expression (r = 0.400, p=0.007), but not with the other genes in the cluster.

Correlations between gene expression and clinico-pathological parameters

Correlations between the gene cluster first principal components and the individual genes and clinico-pathological parameters were investigated. Increased expression of the 'T cells' cluster was significantly correlated with early tumor node metastasis (TNM) staging (r = 0.37, p=0.007; see Table 2). This could mainly be attributed to the expression of *FOXP3* (r=0.39, p=0.004) and *CD3E* (r=0.37, p=0.006).

Table 2. Correlations between clusters and clinico-pathological parameters

Clinico-pathological parameter	Cluster	Correlation (r)	p value	Gene name	Correlation	Cq mean (SEM)	p value
TNM	T cells	0.37	0.007	*FOXP3*	0.388		0.004
				CD3E	0.374		0.006
Vaso-invasion	Inflammation	0.38	0.005	*IL6*			0.002
					absent	-0.486 (0.193)	
					present	0.348 (0.173)	
Lymph node metastasis				*LGALS9*			0.008
					negative	0.277 (0.166)	
					positive	-0.492 (0.220)	
				IL2			0.003
					negative	0.306 (0.176)	
					positive	-0.518 (0.171)	

Correlations between normalized Cq values of cluster first principal components or separate genes and clinico-pathological parameters with p<0.008 are shown (for the correlation between *LGALS9* and lymph nodes p=0.0077). The correlations between single gene expression and TNM stage were tested by the Pearson correlation test and since increased Cq values represent decreased expression, an inverse correlation was found. The correlations for separate genes and vaso-invasion or lymph nodes were tested using independent samples t-tests. Mean normalized Cq values of the gene expression within a category are given to indicate the direction of correlation (inverse for vaso-invasion).

Low expression of *CD3E* was significantly correlated with poor disease-specific survival (p=0.022; Figure 2A). For disease-free survival, in addition to low *CD3E* expression, low expression of both *FOXP3* and *CD8* were correlated with poor outcome (p=0.014, p=0.008 and p=0.034, respectively; Figures 2B-D).

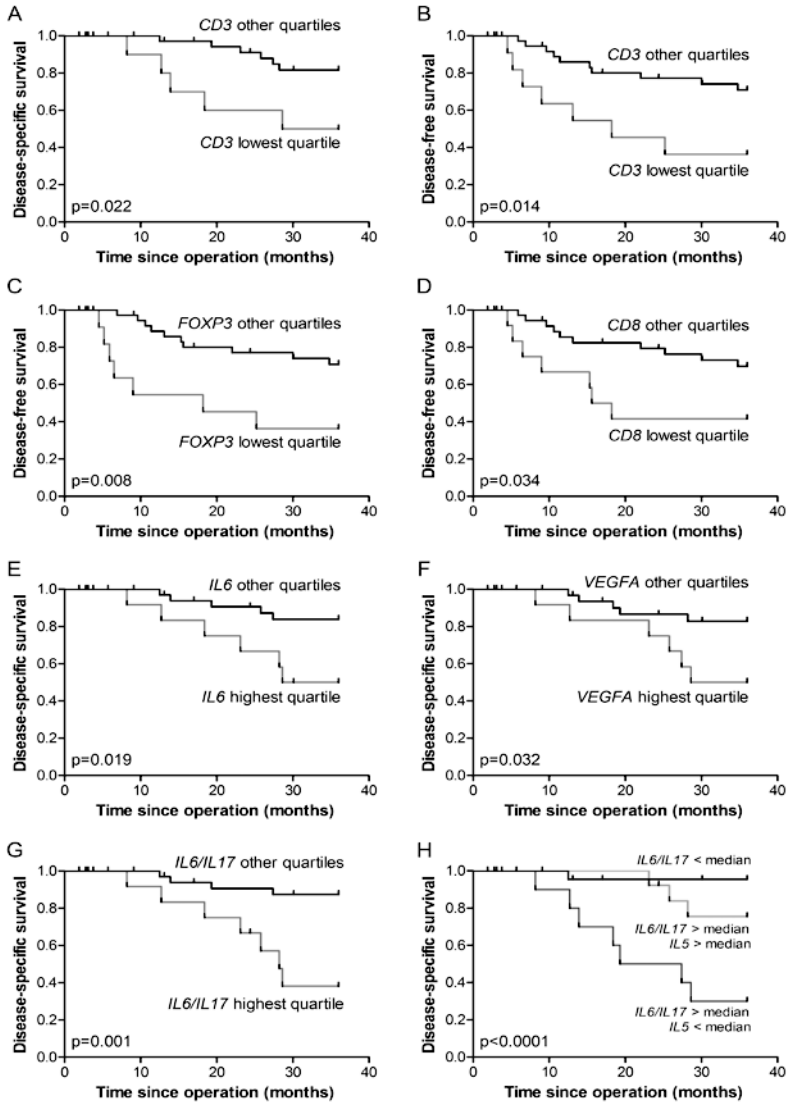

Figure 2. Correlations between immune response and vascularization markers and survival

Kaplan-Meier survival curves for gene expression divided in quartiles. Disease-specific survival for the quartile group with lowest CD3E expression compared with the other groups is shown in A. Disease-free survival with the same division for CD3E, FOXP3 and CD8 expression is shown in B, C and D. Disease-specific survival for the quartile groups with highest IL6 and VEGFA expression compared with the rest are shown in E and F. Disease-specific survival for the quartile group with the highest ratio IL6/IL17 expression compared with the rest is shown in G. The combination of an above median IL6/IL17 ratio combined with a below median level of IL5 compared with the presence of above median expression of IL5 or relatively low IL6 expression is shown in H.

High expression of the 'Inflammation' gene cluster was significantly correlated with the absence of vaso-invasion (r = 0.38, p=0.005), mainly as a result of the contribution of *IL6* (p=0.002). High expression of *IL6* was significantly correlated with poor disease-specific survival (p=0.019; Figure 2E).
Expression of the 'Vessel maturation' marker *LGALS9* was correlated with lymph node metastasis (p=0.008). This association was also observed for the expression of *IL2* (p=0.003).
Within the 'Angiogenesis' cluster, high expression of *VEGFA* (highest quartile) was significantly correlated with poor disease-specific survival (p=0.032; Figure 2F).

Hazard ratios for independent prognostic factors

We investigated which genes were independent prognostic factors for survival using a multivariate analysis with one representative gene per cluster (Table 3). High *IL6* expression was the best predictor of poor disease-specific survival with a hazard ratio of 2.2 (p=0.002). After correction for clinico-pathological parameters, the hazard ratio of *IL6* expression was 2.3 (p=0.011). IL-6 was a signature cytokine of the 'Inflammation' gene cluster, which included cytokines that are often associated with the induction of an IL-17 response: IL-1β, IL-6, IL-8 and IL-23.[26] Studying the correlation between IL-17 and survival, we found that high expression of *IL17* showed a trend toward an association with improved survival (p=0.087). Since *IL6* and *IL17* expression were not correlated in our study, we studied whether the ratio of *IL6* and *IL17* has an association with survival. Indeed, a high ratio of *IL6* over *IL17* expression was significantly correlated with poor survival (p=0.001; Figure 2G). Since the intratumoral immune response in cervical cancer is dominated by Th1, Th2, Th17 and Treg cells, we further analyzed the contribution of the Th1 marker *TBX21* and Th2 marker *IL5*. In order to maintain sufficient group sizes for this analysis, groups were divided based on the median expression level. An above median *IL6* over *IL17* ratio remained significantly correlated with poor survival (p=0.004). We did not observe a significant effect for the addition of *TBX21*, which was significantly correlated with Th1, Treg and CTL markers. In contrast, the combination of above median *IL6* relative to *IL17* expression combined with a below median level of *IL5* was significantly correlated with a worse prognosis (p<0.0001; Figure 2H). This combination was shown to be an independent predictor for poor prognosis corrected for lymph node status, tumor size and infiltration depth with a hazard ratio of 4.2 (p=0.010; Table 3). For both multivariate Cox regression analyses, the results remained similar and significant upon adding postoperative therapy as a parameter.

Table 3. Cox regression analyses

Variable	Univariate hazard ratio (95% CI)	p value	Multivariate hazard ratio (95% CI)	p value	Multivariate hazard ratio (95% CI)	p value
Lymph node status	1.20 (0.35-4.11)	0.769	1.56 (0.30-8.07)	0.599	1.35 (0.27-6.77)	0.718
Tumor size	1.05 (1.02-1.09)	0.006	1.05 (0.99-1.11)	0.121	1.06 (1.00-1.12)	0.073
Vaso-invasion	1.22 (0.36-4.15)	0.756				
Infiltration depth	1.06 (0.99-1.13)	0.096	1.01 (0.92-1.11)	0.785	0.97 (0.89-1.06)	0.556
IL6	2.21 (1.34-3.66)	0.002	2.29 (1.21-4.34)	0.011		
IL5	0.96 (0.61-1.51)	0.853				
ANGPT2	1.12 (0.56-2.24)	0.746				
TBX21	0.997 (0.53-1.87)	0.993				
CD14	0.45 (0.17-1.18)	0.104				
PECAM1	0.90 (0.46-1.76)	0.754				
IL6/IL17 + IL5	4.66 (1.90-11.41)	0.001			4.17 (1.41-12.40)	0.010

Univariate Cox regression hazard ratios are shown for the critical prognostic categorical clinico-pathological parameter lymph node tumor positivity and the continuous variables tumor size (per mm) and infiltration depth (per mm), as well as for the expression of genes representative for the different clusters. Normalized Cq values were converted to expression values to obtain hazard ratio's corresponding with increased presence of the gene product. Multivariate Cox regression analyses are shown for the genes significant in the univariate analysis combined with the most critical clinico-pathological parameters (restricted by the number of patients). The combination of the *IL6*/*IL17* ratio and *IL5* is divided in three categories: a low ratio, a high ratio and high *IL5* levels and a high ratio combined with low *IL5* levels.

Correlation between RNA and protein expression

We studied whether the expression levels of some of the most relevant genes determined by qRT-PCR on fresh frozen tissue correlated with the number of cells expressing the corresponding proteins determined by IHC in FFPE tissue. The RNA

expression level of CD8 was significantly correlated with the number of $CD3^+CD8^+$ cells (r = -0.640, p=0.0004; Figure 3A). The expression of *IL6* and the percentage of IL-6^+ cells were also significantly correlated (r = 0.574; p=0.032; Figure 3B). The number of cells expressing IL-1β was significantly correlated with *IL1B* RNA expression (r=0.628; p=0.029; Figure 3C).

We further studied whether *IL17* RNA expression was correlated with the number of IL-17^+ cells, mainly comprising neutrophils, or specifically with Th17 cells. There was no significant correlation between *IL17A* RNA expression and the total number of IL-17^+ cells (r = -0.048, p=0.737; Figure 3D), but there was a trend toward a positive correlation with the number of IL-17^+CD3^+ Th17 cells (r=-0.438, p=0.069; Figure 3E).

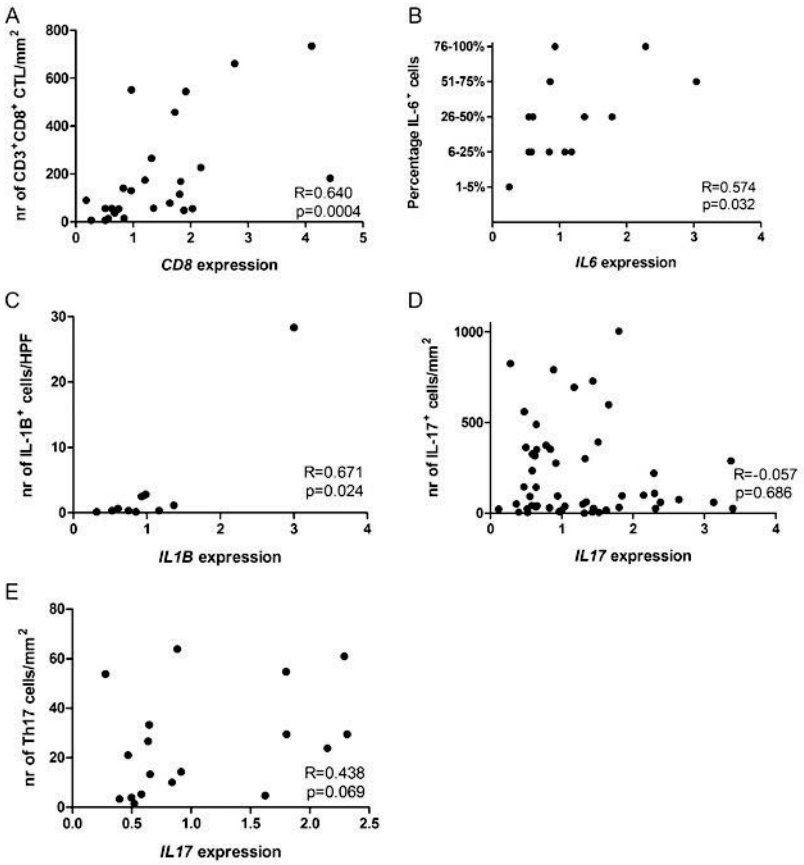

Figure 3. Correlations between RNA and protein expression

The RNA expression levels determined by qRT-PCR in fresh frozen tissue were compared with the number of cells expressing the corresponding protein as determined by IHC. Correlations were determined for CD8 expression and the number of CD3+CD8+ cells (A), IL6 and the percentage of IL-6+ cells (B), IL1B and the number of IL-1β expressing cells (C), IL17 and the total number of IL-17+ cells (D) and Th17 cells (E).

Discussion

In the present study, we used WGCNA to identify gene expression clusters associated with immune response and vessel formation processes in cervical carcinoma. Six gene expression clusters were identified: 'T cells', 'Macrophages', 'Th2', 'Inflammation', 'Vessel maturation' and 'Angiogenesis'. The clusters were named according to the pathways the genes are involved in, but may be expressed and induced by tumor epithelial or infiltrating immune cells, or both. High expression of the 'T cells' cluster was associated with early TNM staging, and low expression of *CD3E*, *CD8* and *FOXP3* were correlated with poor disease-specific and disease-free survival. This supports earlier observations where the absence of a lymphocytic infiltrate was shown to be a predictor of poor survival.[27] The 'T cells' cluster also showed strong correlations between the expression of CTL, Th1 and Treg marker genes. In agreement with these observations, we and others have previously shown that the number of $FoxP3^+$ Tregs strongly correlates with the number of tumor infiltrating T cells.[6,8] In contrast, we have shown before that a high number of $FoxP3^+$ Tregs scored specifically within the tumor epithelium, especially relative to the number of $CD8^+$ CTL, was correlated with poor survival, an observation also made in other tumor types.[27-29] Collectively, these data indicate that T cell infiltration is correlated with improved survival, whereas a relatively high number of Tregs, specifically within the tumor epithelium, counteracts the tumor suppressing immune response.

The 'Th2' gene expression cluster was characterized by *IL4*, *IL5*, *IL13* and *IL12* expression. Surprisingly, expression of the transcription factor *GATA3* was not correlated with the expression of any of these genes, suggesting that *GATA3* RNA expression may not be suitable marker for the Th2 response. Although a Th2 response is regarded as immunosuppressive in cervical cancer,[9] HPV-specific T cells associated with regression of high-grade VIN lesions, have been shown to produce high levels of both IFNy and IL-5.[5] In agreement with the latter observation, low *IL5* levels were an indicator of poor prognosis in combination with high *IL6* relative to *IL17* levels.

The 'Inflammation' gene expression cluster was not significantly correlated with the 'Th2' and 'T cells' clusters, indicating that these clusters represent distinct pathways. High expression of *IL6* represented a dominant 'Inflammation' response and was significantly correlated with poor disease-specific survival. A high number of stromal $IL\text{-}6^+$ cells was previously shown to be correlated with poor disease-specific survival in an overlapping patient cohort[22] and other types of cancer.[30] IL-6 might drive STAT3 expression in tumor cells,[31] induce angiogenesis and epithelial-mesenchymal transition[32,33] and induce differentiation of dendritic cells and macrophages toward tumor promoting cells.[34,35] Additionally, *IL6* was correlated with the absence of vaso-invasion. We have observed a significant correlation between IL-17 and IL-1β and the absence of vaso-invasion by IHC as well (reference 23 and unpublished data). *IL1β*, also a member of the 'Inflammation' gene cluster, showed a trend toward a correlation

with the absence of vaso-invasion. These results suggest that this type of inflammatory response may prevent metastatic spread of the tumor cells via the blood or lymphatic vasculature.

The 'Inflammation' gene cluster was characterized by cytokines that are often associated with the induction of an IL-17 response: IL-1β, IL-6, IL-8 and IL-23.[26] However, *IL17* expression was not significantly correlated with the 'Inflammation' cluster, T-cell or neutrophil related genes. Mature neutrophils have been shown to express no or very low mRNA levels for granule proteins.[36] In our study, both *IL17* and neutrophil markers *NE* and *FUT9* were expressed at very low levels. Since we have shown by IHC that IL-17 is mainly expressed by neutrophils in cervical cancer,[23] this suggests that *IL17A* RNA expression is primarily derived from Th17 cells. The absence of a correlation with T cell markers and the 'Inflammation' gene cluster is likely due to the small size of the Th17 population.

While a qRT-PCR analysis by Tosolini *et al*. showed that a Th1 cluster and high *FOXP3* expression were correlated with improved disease-free survival in colon cancer, corresponding with our results, this group also found a correlation between a high Th1/Th17 gene cluster ratio and improved disease-free survival.[37] In the current work, the 'Inflammation' gene cluster was more important for patient survival. To study whether *IL17* might have an opposite effect on survival compared to *IL6*, the ratio of *IL6* and *IL17* was analyzed. Indeed, this ratio was significantly correlated with poor survival, corresponding with previous observations that a high number of Th17 cells is correlated with improved disease-specific survival in cervical carcinoma.[23] Since the immune response in cervical cancer is predominantly characterized by Th1, Th2, Th17 and Treg cells, the contributions of the 'T cells' signature marker *TBX21* and the 'Th2' signature marker *IL5* were studied. We did not observe a significant association with the Th1 marker *TBX21*, which is supposed to be critical for an tumor suppressing immune response.[5] Strikingly, the most prognostic independent risk factor was a high *IL6* over *IL17* ratio combined with a low expression level of Th2 marker *IL5*, with a hazard ratio of 4.2 (p=0.010). Since we did not find a correlation between *IL5* and *IL6* expression in cervical cancer, this suggests that the effect of a high amount of *IL6* is dampened by a Th2 response.

'Vessel maturation' adhesion markers were correlated with *KDR* encoded VEGFR2 expression, which has been described before[38] and suggests that VEGFR2 might primarily be involved in vessel maturation in cervical cancer. Expression of the 'Vessel maturation' marker *LGALS9* was correlated with tumor positive lymph nodes, suggesting it might play a role in metastasis to the lymph nodes.

The 'Inflammation' cluster was most significantly correlated with the 'Angiogenesis' cluster. Although the 'Angiogenesis' cluster marker ICAM1 is an adhesion protein, VEGFA has been described to first induce ICAM1 expression to prepare endothelial cells for migration, after which ICAM1 is downregulated.[39] The 'Angiogenesis' marker *VEGFA* was significantly correlated with poor disease-specific survival (p=0.032),

which is in agreement with our previous study where we showed that *VEGFA* expression correlates with the number of blood vessels in cervical cancer, in its turn correlated with poor disease-free survival.[40] Correspondingly, Yuan et al. showed that both RNA and protein expression of *VEGFA* were correlated with poor survival in non-small-cell lung cancer.[41]

Although IL-17 has been reported to induce vascularization via VEGF-dependent and -independent mechanisms in cancer,[42,43] we did not find a significant association between *IL17* and angiogenesis or vessel maturation. Correspondingly, we did not find a correlation between the number of IL-17^{+} cells and the number of CD105^{+} vessels in a series of 151 squamous cervical carcinoma samples (data not shown).

To conclude, by using a qRT-PCR array, we identified *CD3E*, *IL6*, *VEGFA* and a high *IL6*/*IL17* ratio combined with low *IL5* expression as the most prognostic factors in squamous cervical cancer. While high expression of T cell markers was correlated with improved prognosis, and high expression of angiogenesis marker *VEGFA* with poor prognosis, *IL17* expressed by Th17 cells could counteract the tumor promoting effects of *IL6*, even more so combined with a Th2 response characterized by *IL5*. A proposed model of the factors most relevant for disease outcome is shown in Figure 4.

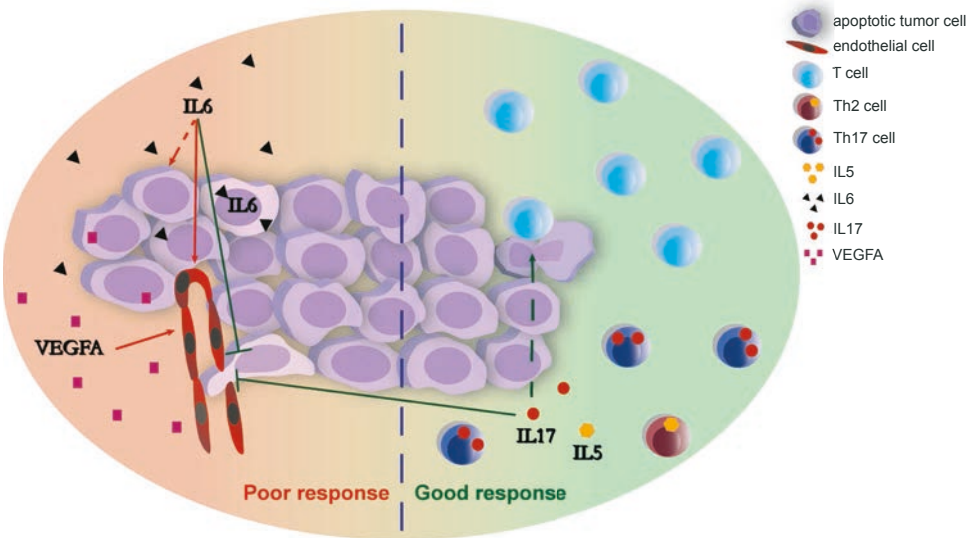

Figure 4. Th17 counteracts the effect of *IL6*

Of the 27 immunological and 15 vascularization markers analyzed, *CD3*, *IL5*, *IL6*, *IL17A* and *VEGFA* were most significantly correlated with the clinical outcome of cervical cancer patients. A 'T cells' response, indicating the presence of Th1, CTL and Treg cells and represented by blue cells in the image, was correlated with improved prognosis. *IL6* and *VEGFA*, the latter indicative of angiogenesis, were both correlated with poor prognosis. *IL6*, expressed by both tumor cells and infiltrating immune cells, may directly promote tumor growth, indicated by the interrupted arrow. High *IL17* (Th17) and *IL5* (Th2) expression were correlated with improved prognosis, specifically relative to *IL6* expression. *IL6* expression was also correlated with the absence of vaso-invasion, indicated by the blunted arrow on the tumor cell entering the vessel. We have also shown this correlation before for IL-17 protein expression,[23] suggesting that the IL-6/IL-17 pathway may prevent tumor spread via the blood or lymphatic vasculature.

Measuring *IL6,* especially in combination with *IL5* and *IL17* expression may improve the accuracy of predicting prognosis. Moreover, our data support the development of combined anti-IL-6 and anti-VEGF therapies. Since we have found correlations between 'Inflammation' markers and the absence of vaso-invasion, blocking IL-6 might increase the risk of tumor cell invasion. Since VEGFA expression has been correlated with tumor invasiveness,[44,45] and the presence of vaso-invasion negatively affects clinical outcome, blocking both IL-6 and VEGFA has the potential to counteract both tumor growth and invasion.

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VI

Whole-transcriptome analysis of flow-sorted cervical cancer samples reveals that B cell expressed TCL1A is correlated with improved survival

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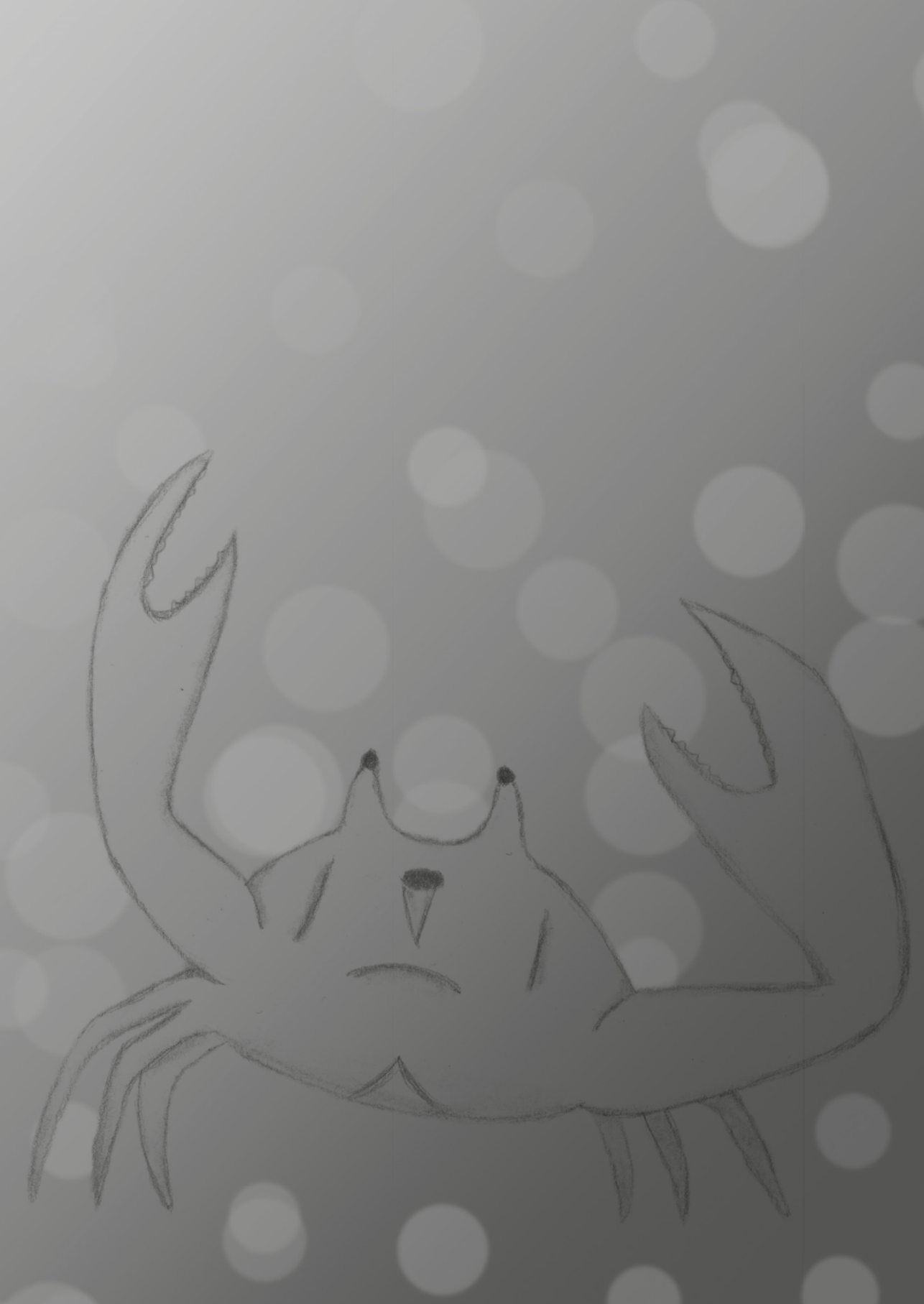

Abstract

Cervical cancer is typically well infiltrated by immune cells. Because of the intricate relationship between cancer cells and immune cells, we aimed to identify both cancer cell and immune cell expressed biomarkers for survival. Using a novel approach, we isolated RNA from flow-sorted viable $EpCAM^+$ tumor epithelial cells and $CD45^+$ tumor-infiltrating immune cells obtained from squamous cell cervical cancer samples (n=24). Total RNA was sequenced and differential gene expression analysis of the $CD45^+$ immune cell fractions identified *TCL1A* as a novel marker for predicting improved survival (p=0.007). This finding was validated using qRT-PCR (p=0.005) and partially validated using immunohistochemistry (p=0.083). Importantly, TCL1A was found to be expressed in a subpopulation of B cells ($CD3^-/CD19^+/CD10^+/CD34^-$). A high *TCL1A/CD20* (B cell) ratio, determined in total tumor samples from a separate patient cohort using qRT-PCR (n=52), was also correlated with improved survival (p=0.027). These results suggest that intratumoral $TCL1A^+$ B cells are important for controlling cervical cancer development.

Introduction

Cervical cancer is caused by persistent infection with human papillomavirus (HPV) and is currently the second-leading cause of cancer-related death in young women.[1] The prognosis for patients with cervical cancer relapse is poor. Although the host's immune system can usually clear an HPV infection, chronic inflammation can contribute to tumorigenesis.[2] Cervical cancer is characterized by a large number of infiltrating immune cells, and observational and functional studies have revealed that several immune cell types can play a crucial role in the prognosis of patients with cervical cancer. A high number of cytotoxic T cells and T helper type 1 cells is correlated with improved survival in several cancer types,[3,4] including cervical cancer.[5] Cytokines derived from immune cells determine the type of immune response, but they can also directly promote tumor growth.[6] For example, our group and others have shown that a high level of IL-6 expression is an independent predictor of poor survival.[7-9] However, because tumor cells can also produce cytokines and suppress a tumor-targeting immune response,[10] the complex interaction between tumor cells and the local microenvironment makes it challenging to discriminate between effects arising from the tumor cells and effects arising from immune cells.

Because of the intricate relationship between cervical cancer cells and the infiltrating immune cells, the infiltrating immune cells represent an opportunity to identify novel cell-specific biomarkers for improved patient survival. Immunohistochemistry (IHC) is a commonly used method for investigating separate cell types, but is suitable for studying only a select number of strongly expressed antigens. To detect global

differences in gene expression, total RNA is usually studied. However, studying gene expression in tumor samples usually fails to provide information regarding the origin of the cells expressing a particular RNA. Therefore, to distinguish between genes expressed by tumor epithelial cells and genes expressed by tumor-infiltrating immune cells, these two cell subpopulations must first be separated. Although fluorescence-activated cell sorting is a commonly used strategy for selectively identifying cancer stem cells[11-13] and for studying the molecular genetics of tumor cell subpopulations,[14,15] to the best of our knowledge flow cytometry has not been used for transcriptome analysis of matched tumor cell and immune cell subpopulations.

The aim of this study was to discover potential biomarkers for survival expressed by tumor cells or infiltrating immune cells using an unbiased approach. Tumor epithelial cells were separated from immune cells using flow cytometry. Subsequently, we performed total RNA sequencing (RNA-seq) and differential gene expression analysis to examine which genes may play a role in clinical outcome. Our analysis revealed that T-cell leukemia/lymphoma 1A (TCL1A) is a novel immune cell expressed marker for predicting improved survival in patients with cervical cancer. This finding was confirmed using both qRT-PCR and IHC analyses. In addition, we used multicolor immunofluorescence to demonstrate that TCL1A is expressed by a subpopulation of B cells in cervical cancer. This novel approach highlights the potential relevance of tumor infiltrating B cells.

Materials and Methods

Patient material

Tumor samples were obtained from patients who underwent primary surgical treatment for squamous cell cervical cancer from 1985 through 2005. Samples with sufficient material to dissociate and freeze cells available in the archives of the Department of Pathology (Leiden University Medical Center, Leiden, the Netherlands) were selected for this study (n= 36). None of the patients received preoperative therapy, and clinical and follow-up data were obtained from patient medical records. Samples were handled in accordance with the medical ethical guidelines described in the Code of Conduct for the Proper Secondary Use of Human Tissue established by the Dutch Federation of Biomedical Scientific Societies.

Tumor tissue dissociation and sample preparation

Fresh tumor samples were dissociated as described previously.[16-18] In brief, the tumor tissue was rinsed in RPMI medium (Gibco Life Technologies, Bleiswijk, the Netherlands) and subsequently dissected into 1-2 mm^3 pieces, which were incubated overnight in 10 ml DMEM (Gibco) containing 0.5 mg/ml collagenase type II and

0.002% DNase I type II (both from Sigma-Aldrich, St. Louis, MO). The suspension was then incubated in 0.25% trypsin (Gibco) for up to one hour at 37°C until a single-cell suspension was obtained. The cells were then filtered through a 100-µm pore size nylon sieve (Verseidag-Industrietextilien GmbH, Kempen, Germany), and 10% (v/v) fetal calf serum (FCS) was added. The cells were frozen in 90% FCS/10% DMSO at -180°C until further use.

The protocol for preparing the samples for cell sorting was adapted from Tighe and Matthew.[19] Cell suspensions were thawed rapidly in a 37°C water bath, added to a total volume of 10 ml RPMI medium and centrifuged at 1000x*g* at 4°C for 3 minutes. The cell pellet was washed in 100 U RNasin Ribonuclease Inhibitor (Promega, Madison, WI) in 1 ml phosphate-buffered saline (PBS), filtered through a 50-µm filter (BD Biosciences, San Jose, CA), stained with haematoxylin and counted. Depending on the total number of cells in each sample, 1-6 million cells were transferred to RNase-free tubes and centrifuged at 800x*g* at 4°C for 5 minutes. The cell pellets were resuspended in RNasin/PBS, RNasin/PBS containing 1:10 FITC-labelled mouse anti-CD45 antibody (clone T29/33; Dako, Glostrup, Denmark), 1:10 PE-labelled mouse anti-CD326 antibody (i.e., anti-EpCAM; clone EBA-1; BD Biosciences), or RNasin/PBS containing both antibodies. The cells were then incubated in the dark at 4°C for one hour. The samples were washed three times in 1 ml RNasin/PBS by centrifuging at 800x*g* at 4°C for 5 minutes. The pellets were then resuspended in RNasin/PBS containing 1:4000 TO-PRO-3 (Life Technologies, Carlsbad, CA) and transferred to RNase-free flow cytometry compatible tubes. Identical samples were pooled.

Flow cytometry

A FACSARIA IIu (BD Biosciences) was used for cell sorting. Prior to each sorting run, all parts of the sort chamber were cleaned with RNaseZap (Life Technologies), and the inlet tube and flow cell were cleaned with 10% Contrad70 followed by RNasin/PBS. Dead cells were excluded based on TO-PRO-3 staining.[20] Blue and red lasers were used for excitation. A live gate was set using a FSC-A versus TO-PRO-3 fluorescence dot plot. A cut-off of 3% living TO-PRO-3$^-$ cells was used to exclude samples from further analysis (7 samples exceeded this cut-off and were excluded). Cell doublets were excluded using FSC-H versus FSC-W and SSC-H versus SSC-W pulse-processing. Single-stained samples were used to correct for spectral overlap. EpCAM$^+$ and CD45$^+$ single-positive cells in the double-stained samples were sorted using a 16,16 purity mode, a 100-µm nozzle at 20 psi and a drop frequency of 30.8 kHz. A representative example of the restricted gating strategy for sorting pure EpCAM$^+$ tumor epithelial cells and CD45$^+$ immune cells is shown in Figure 1. Cells were collected in 2-ml tubes containing 1 ml ice-cold RNAprotect Cell Reagent (Qiagen, Hilden, Germany), mixed and kept on ice until RNA isolation.

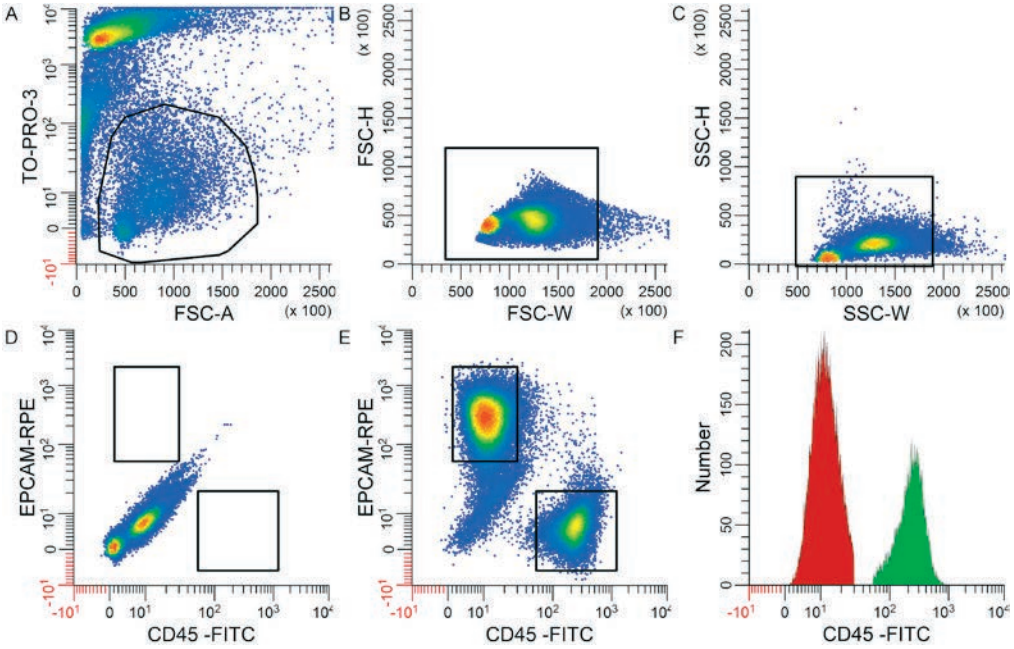

Figure 1. Example of EpCAM$^+$ and CD45$^+$ single-positive cells obtained from a cervical cancer cell suspension using flow cytometry

The negative control stained for TO-PRO-3 but not for EpCAM or CD45 is shown in A-D. Living cells were selected based on the absence of TO-PRO-3 staining (A), after which cell aggregates were excluded using forward scatter width (B) and side scatter height and width (C). Cell sorting was based on the expression of either EpCAM or CD45 in the double stained samples (E), but not in the unstained control (D). Restricted gates were used as indicated to obtain pure cell populations. Panel (F) shows the distribution of CD45 expression in the CD45$^+$ immune cells (in green) and the EpCAM$^+$ tumor cells (in red). Similar results were obtained with respect to EpCAM expression (data not shown).

Total RNA sequencing

RNA was isolated and DNA was removed using the RNeasy Plus Micro kit (Qiagen). The RNA was eluted from the column using two rounds of 14 µl RNase-free water. RNA concentration was measured using a Nanodrop device, and RNA integrity was analyzed using RNA 6000 Pico chips in a model 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). A cut-off of 100 ng RNA and an RNA integrity number (RIN) of 8 were used to exclude samples with an insufficient amount of RNA and/or insufficient quality RNA (5 samples were excluded based on these criteria). The mean RIN value of the included samples was 9.2 (range: 8.3-10.0). 20 U RNasin was added to each sample, and the samples were stored at -80°C until use.

External RNA Controls Consortium (ERCC) EXfold spike-in mix (Life Technologies) was added for quality control to 50 ng of total RNA. The RNA was then converted to cDNA and pre-amplified using the SMARTer Ultra Low RNA kit (Clontech Laboratories, Mountain View, CA). A sequencing library was generated from 1 ng

amplified cDNA using the Illumina Nextera XT kit (Illumina, San Diego, CA) as described previously.[21] Paired end 100-base pair sequencing was performed using a HiSeq 2000 v3 (Illumina). FastQ files were generated from each sample using CASAVA version 1.8.2 (Illumina).

RNA-seq data analysis

Sequencing adapters in the FastQ files were detected using FastQC version 0.10.1 and removed using the cutadapt tool (version 1.4.2). Base quality trimming was performed using the sickle tool (version 1.200). RNA was aligned to a custom index based on the hg19 human genome (GSNAP version 2014-05-15; novel splicing flag set to true), supplemented with the ERCC spike-in sequences and replacing the published mitochondrial chromosome with the Revised Cambridge Reference Sequence (GenBank acc. no. NC_012920_1). The resulting alignment file was compressed, indexed and name-sorted using the samtools tool (version 0.1.19-44428cd). Reads mapping to ribosomal RNA (obtained from the UCSC rmsk track on the hg19 genome) were removed using a custom-written script. The count table was generated using htseq-count (HTSeq suite version 0.6.1p1; --format flag: BAM; --stranded flag: no; --order flag: name; --mode flag: intersection-nonempty). The UCSC genePredToGtf generated RefSeq annotation (raw database dump of the refGene table) was used as a GTF reference. One of the immune cell fractions was excluded from differential expression analysis because the correlation between the external RNA controls was insufficient ($r<0.9$).

Differential expression analysis was performed using the R[22] package edgeR.[23] This package uses an overdispersed Poisson model to account for both biological and technical variability. Empirical Bayes methods were used to moderate the degree of overdispersion across transcripts, enhancing the reliability of inference.[23] The values were normalized using the trimmed mean of M (TMM) and the respective library sizes,[24] after which the expression differences between two groups were estimated. Reported p values are false discovery rate–corrected p values calculated using the Benjamini-Hochberg procedure. The Database for Annotation, Visualization, and Integrated Discovery (DAVID; david.abcc.ncifcrf.gov) was used to identify enriched GO terms.[25]

qRT-PCR analysis

RNA was obtained from 52 fresh-frozen squamous cell cervical cancer samples (obtained from 15 patients who were included in the RNA-seq analysis and an additional 37 samples) and is described in chapter 5. cDNA was synthesized as described previously.[26] cDNA was also synthesized from 150 ng of the RNA from the remaining immune cell fractions. The resulting cDNA from the immune cell fractions was purified using the QIAquick PCR Purification Kit (Qiagen). Sybr Green-based

qRT-PCR was performed in duplicate using 1:25 diluted cDNA and 3 pmol RT² qPCR Primer Assay for amplifying human *CD19*, *EEF1A1*, *MS4A1* (*CD20*), *RPLP0* and *TCL1A* (Qiagen) on a CFX384 system (Bio-Rad, Hercules, CA). Amplification of *ACTB* was performed using the forward 5'-GCCCTGAGGCACTCTTCCA-3' and reverse 5'-CGGATGTCCACGTCACACTTC-3' primers designed using Primer-BLAST.[27] As a negative control, the cDNA was replaced with milli-Q water. The most stably expressed genes (*EEF1A1* and *RPLP0*) were used for normalization. Expression was scaled by standard deviation. For statistical analysis, gene expression levels were divided into four quartiles, and the lowest quartile (i.e., low expression) was compared with the other three quartiles; in addition, the lower two quartiles were compared with the upper two quartiles.

Immunohistochemistry

Immunostaining was performed as described previously[7] on 4-µm thick formalin-fixed, paraffin-embedded (FFPE) sections obtained from the patients included in the RNA-seq analysis. In brief, 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 10 mM Tris and 1 mM EDTA (pH 9.0). The samples were incubated in monoclonal rabbit anti-TCL1A (EPR3949, Abcam, Cambridge, UK) diluted in 1% (w/v) bovine serum albumin (BSA) in PBS at room temperature overnight. The samples were then incubated in BrightVision poly-horseradish peroxidase (HRP) anti-mouse/rabbit/rat antibody (Immunologic, Duiven, the Netherlands) at room temperature for 30 minutes. HRP activity was visualized using 3,3'-diamino-benzidine-tetrahydrochloride (DAB+, Dako). The sections were counterstained with haematoxylin and mounted using CV Mount (Leica Biosystems, Newcastle, UK). The number of strong $TCL1A^+$ cells was counted in two to four hot-spots in the center of the tumor using an Axioskop-20 microscope equipped with a Plan-Neofluar 20x/0.5 objective (Zeiss, Göttingen, Germany), sampling a total tumor area of 1.6-3.1 mm^2 comprising vital tumor epithelium and stroma. For statistical analysis, the number of positive cells was divided into two groups based on the median of 56 cells/mm^2.

Double- and triple-fluorescent IHC was used to stain TCL1A in combination with mouse IgG1 anti-CD3 (clone F7.2.31, Dako), mouse IgG2b anti-CD8 (clone 4B11, NCL-L-CD8-4B11, Leica Biosystems), mouse IgG1 anti-CD10 (clone 56C6, Dako), mouse IgG1 anti-CD19 (clone 3B10, LifeSpan Biosciences, Seattle, WA), mouse IgG2a anti-CD20 (clone L26, Dako), mouse IgG1 anti-CD34 (clone QBEnd 10, Dako), mouse IgG1 anti-CD79a (clone JCB117, Dako), and/or mouse IgG2a anti-DNTT (clone 41C, Abcam); all incubated at room temperature overnight. The samples were then incubated in a combination of Alexa Fluor-labelled goat anti-rabbit-A488 (A11008), goat anti-mouse IgG1-A647 (A21240), goat anti-mouse IgG2a-A546 (A21133), and/or goat anti-mouse IgG2b-A546 (A21143; all from Life Technologies) at room temperature for one

hour. As negative controls, the primary antibodies were replaced with antibodies of the same isotype with unknown specificity. Images were acquired using an LSM700 confocal laser-scanning microscope equipped with an LCI Plan-Neofluar 25x/0.8 Imm Korr DIC M27 objective (Zeiss). Two to four random images were obtained, sampling a total tumor area of 0.5-1.0 mm^2 comprising vital tumor epithelium and stroma. Double and triple positivity of cells was determined using LSM Image Browser software (version 4.2.0.121, Zeiss). The numbers of $TCL1A^+$, $CD19^+$, and $CD20^+$ single-, double-, and triple-positive cells were scored manually in $TCL1A^+$ cell hot-spots at the center of the tumor using ImageJ version 1.47 (http://rsb.info.nih.gov/ij). For statistical analysis, the number of positive cells was divided into two groups based on the median of 43 $TCL1A^+$ cells/mm^2, 31 $TCL1A^+CD19^+$ cells/mm^2, 18 $TCL1A^+CD19^+CD20^-$ cells/mm^2 and 5 $TCL1A^+CD19^+CD20^+$ cells/mm^2.

Statistical analysis

Correlations between qRT-PCR and IHC data and both disease-specific survival and disease-free survival were analyzed using Kaplan-Meier curves in SPSS version 20.0 (IBM, Armonk, NY). Differences in expression between two groups were analyzed using the independent Student's *t*-test. All tests were performed two-sided, and differences with a p-value <0.05 were considered statistically significant.

Results

Cervical cancer samples

The patient and tumor characteristics are summarized in Table 1. First, we determined whether our patient cohort (n=24) was representative of the total squamous cervical cancer patient cohort followed from 1985 through 2005 (n=173). The only significant difference identified was increased tumor size in our patient cohort (p=0.008), which was due to the requirement for larger tumors to prepare cell suspensions. We found no difference between the study cohort and the other patient samples with respect to survival or postoperative radiotherapy treatment (data not shown).

Separation of tumor cell and immune cell fractions

Following the dissociation of fresh tumor samples, TO-PRO-$3^-$$EpCAM^+CD45^-$ viable tumor epithelial cells (hereafter referred to as the tumor cell fraction) and TO-PRO-3^- $EpCAM^-CD45^+$ viable immune cells (hereafter referred to as the immune cell fraction) were sorted using flow cytometry (Figure 1). RNA-seq data were obtained from total RNA isolated from both cell fractions. The gene expression pattern in the tumor cell fractions differed considerably from the expression pattern in the immune cell fractions,

Table 1. Patient and tumor clinico-pathological characteristics

Clinico-pathological parameter	Category	N = 24 (%)
Age	Median	43
	Range	26-63
FIGO stage[1]	IB	20 (83)
	IIA	4 (17)
TNM stage	IB1	3 (13)
	IB	1 (4)
	IB2	13 (54)
	IIA	5 (21)
	IIB	1 (4)
	IIIA	0 (0)
	IIIB	1 (4)
Lymph nodes	Negative	15 (63)
	Positive	9 (38)
Tumor size (mm)[2]	<40	4 (17)
	≥40	19 (79)
Vaso-invasion	Absent	11 (46)
	Present	13 (54)
Infiltration depth (mm)[2]	<15	8 (33)
	≥15	15 (63)
HPV type	16	13 (54)
	18	5 (21)
	Other	6 (25)

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

as shown by two separate clusters of all tumor cell and immune cell fractions upon performing a principal component analysis of all fractions (Figure 2).

Based on p value ranking, the most upregulated gene in the immune cell fractions was protein tyrosine phosphatase receptor-type C (*PTPRC*, p=6.54E-146; Figure 3, Supplementary Table S1), which is also known as *CD45*, the marker used to flow-sort the immune cells. The most significantly enriched biological process Gene Ontology (GO) terms in the immune cell fractions were immune response, leukocyte and lymphocyte terms.

In the tumor cell fractions, the most significantly enriched biological process GO terms were cell cycle, tissue development and cellular process, confirming the identity of this

fraction as well. The most upregulated gene in the tumor cell fractions (i.e., with the lowest p value) was epithelial membrane protein 2 (*EMP2*, p=5.49E-36; Figure 3). In addition, another highly significantly upregulated gene was *CDKN2A* (also known as *p16*; p=7.08E-25), which is upregulated specifically in cervical cancer cells.
The most strongly differentially expressed genes were upregulated in the immune cell fractions compared with the tumor cell fractions, as shown by the ranking of the 100 most differentially expressed genes (Figure 3).

Prognostic factors identified in the tumor cell fractions

Within the tumor cell fractions, no genes were significantly differentially expressed based on patient survival status five years after surgery. With respect to lymph node status, membrane metallo-endopeptidase (*MME*; log fold change: 9.1, p=0.02) and olfactomedin-like 2A (*OLFML2A*; log fold change: 3.0, p=0.02) were expressed at higher levels in patients without lymph node metastases compared with patients with lymph node metastases.

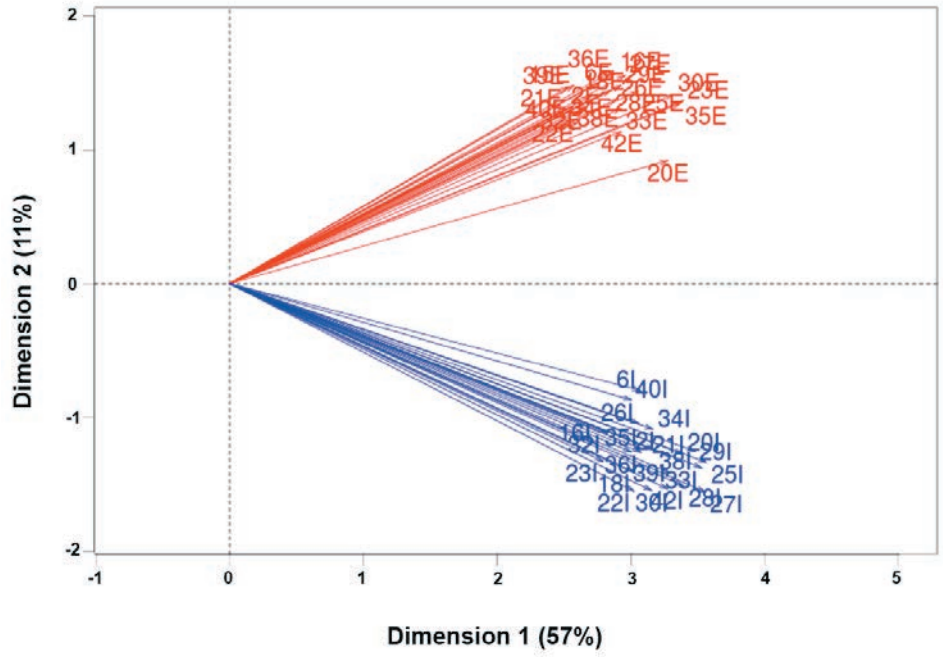

Figure 2. Principal component analysis of all fractions

Differential gene expression of the tumor cell fractions and immune cell fractions (n=46) obtained from 23 patients (one sample was excluded based on the external RNA controls) was compared using principal component analysis. The numbers indicate the individual patient-matched fractions; "E" indicates a tumor epithelial cell fraction, and "I" indicates an infiltrating immune cell fraction. The percentages in each dimension indicate the amount of variation explained by each dimension. Note that all tumor cell fractions clustered, and all immune cell fractions clustered.

Figure 3. The most differentially expressed genes in the tumor versus the infiltrate fractions

The 100 most significantly differentially expressed genes between the tumor cell (red dots) and infiltrate (blue dots) fractions are shown. The gene with the lowest p value (*PTPRC*) is listed at the top.

Prognostic factors identified in the immune cell fractions

Among the immune cell fractions, 17 genes were significantly differentially expressed based on patient survival status (Supplementary Table S2 and Supplementary Figure S1). The most prominent gene was *TCL1A*, which encodes a lymphocyte-specific protein. Specifically, *TCL1A* was expressed in the majority of surviving patients, but it was not expressed in any of the patients who died within five years of surgery. Comparing patients with lymph node metastases and patients without lymph node metastases revealed that the expression level of protein phosphatase 3 regulatory subunit 1 (*PPP3R1*) was significantly higher in patients with metastases (log fold change: 5.6, $p<0.001$), and endothelin 2 (*EDN2*) was expressed only in patients without lymph node metastases (log fold change: 7.0, $p=0.03$). Because we could best discriminate between patients who survived versus patients who had died based on *TCL1A* expression, we continued to validate the correlation between *TCL1A* expression and patient survival.

Validation of the correlation between TCL1A expression and improved survival

Based on our RNA-seq data, the expression of *TCL1A* was significantly correlated with improved disease-free survival ($p=0.047$) and disease-specific survival ($p=0.007$; Figure 4A). To validate the technique used, we performed a qRT-PCR analysis of *TCL1A* expression using the same 24 immune cell fraction RNA samples that were used for generating the RNA-seq data. This analysis confirmed that *TCL1A* was expressed at significantly increased levels in patients who survived compared with patients who died within five years ($p=0.0003$; Figure 4B). Our qRT-PCR analysis confirmed that *TCL1A* expression was significantly correlated with improved disease-free survival ($p=0.033$) and improved disease-specific survival ($p=0.005$), with Kaplan-Meier survival curves that were qualitatively similar to Figure 4A (data not shown).

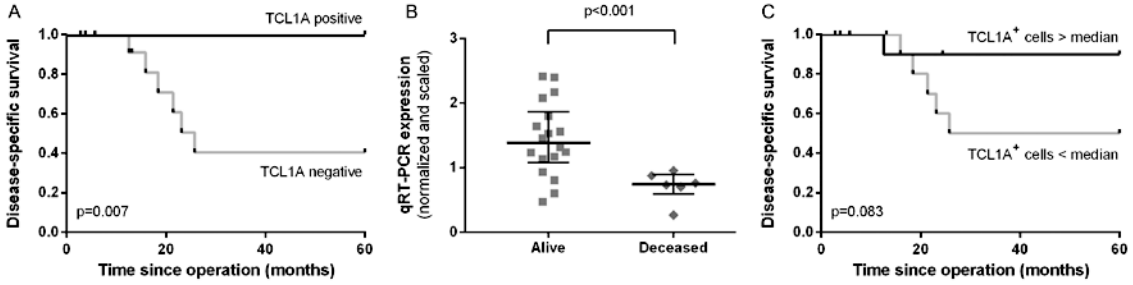

Figure 4. Correlation between TCL1A expression and survival

A Kaplan-Meier curve and log-rank survival analysis based on the presence or absence of *TCL1A* sequence reads is shown in A. The *TCL1A* qRT-PCR expression values were compared between patients who survived and patients who were deceased five years after surgery in B. The median and interquartile range are depicted. A survival analysis for a high number of $TCL1A^+$ cells versus a low number of $TCL1A^+$ cells based on immunohistochemistry is shown in C.

To validate this correlation at protein expression level, the corresponding FFPE samples were stained for TCL1A using IHC. A survival analysis was performed by comparing patients with a high (i.e., above the median of 56 cells/mm^2) versus a low (i.e., below the median) number of strong TCL1A$^+$ cells (Figure 4C). One patient died despite having a high number of TCL1A$^+$ cells; nevertheless, the data revealed a trend toward improved disease-specific survival (p=0.083).

Phenotypic characterization of TCL1A$^+$ cells

Immunofluorescence staining was used to determine the phenotype of the TCL1A$^+$ cells in FFPE samples from four patients included in the RNA-seq analysis. Strikingly, the TCL1A$^+$ cells did not express CD3 or CD8 (Figure 5A), but the majority of TCL1A$^+$ cells expressed the pan B cell marker CD19 (Figure 5B). Upon further investigation of the B cell phenotype, we found that the majority of TCL1A$^+$ cells also expressed CD10. A smaller population of cells expressed CD20 (Figure 5B,C); relatively few cells expressed CD79a (Figure 5D) or DNA nucleotidylexotransferase (DNTT; Figure 5E), and none of the cells expressed the pro B cell marker CD34 (Figure 5F).

To quantify the relative frequencies of the two predominant TCL1A$^+$ B cell subpopulations (i.e., TCL1A$^+$CD19$^+$CD20$^-$ and TCL1A$^+$CD19$^+$CD20$^+$ cells), the numbers of these cell subpopulations were scored in the FFPE samples of all 24 patients who were included in the RNA-seq analysis. On average, 73% of the TCL1A$^+$ cells expressed CD19 (range: 39-95%), and an average of 23% of these TCL1A$^+$CD19$^+$ cells also expressed CD20 (range: 0-84%).

Taken together, the majority of TCL1A$^+$ cells expressed CD19 and CD10; in contrast, a minority of cells expressed CD20, and relatively few cells expressed CD79a or DNTT. Most of the DNTT$^+$ cells did not express CD10. Interestingly, the CD19$^+$ B cells were distributed throughout the stroma, although some were organized in B cell structures adjacent to tumor epithelial fields.

Correlation between B cells, TCL1A expression and survival

We examined the correlations between patient survival and TCL1A$^+$CD19$^+$ cells, TCL1A$^+$CD19$^+$CD20$^-$ cells and TCL1A$^+$CD19$^+$CD20$^+$ cells on the FFPE samples (n=24). A high (i.e., above the median of 31 cells/mm^2) number of TCL1A$^+$CD19$^+$ cells showed a trend toward improved survival, similar to the correlation for the total number of TCL1A$^+$ cells (see Figure 4C). We found no significant correlation between TCL1A$^+$CD19$^+$CD20$^+$ cells or TCL1A$^+$CD19$^-$CD20$^-$ cells and patient survival (data not shown), suggesting that TCL1A$^+$CD19$^+$ cells play the most clinically relevant role.

Next, we used qRT-PCR analysis to validate the correlation between TCL1A and CD19$^+$ B cells and improved survival in cervical cancer patients by analyzing the expression levels of *CD19*, *CD20* and *TCL1A* in fresh-frozen cervical cancer samples obtained from 15 patients in the same cohort and an additional 37 samples. We found

no significant correlation between *CD19* expression and disease-specific survival (p=0.125; Figure 6A); in contrast, high *CD19* expression was significantly correlated with improved disease-free survival (p=0.036; Figure 6B). High expression of *CD3E*, *CD20* or *TCL1A* was not significantly correlated with survival (data not shown), although a high ratio of *TCL1A*/*CD20* expression showed a trend toward improved disease-specific survival (p=0.053).

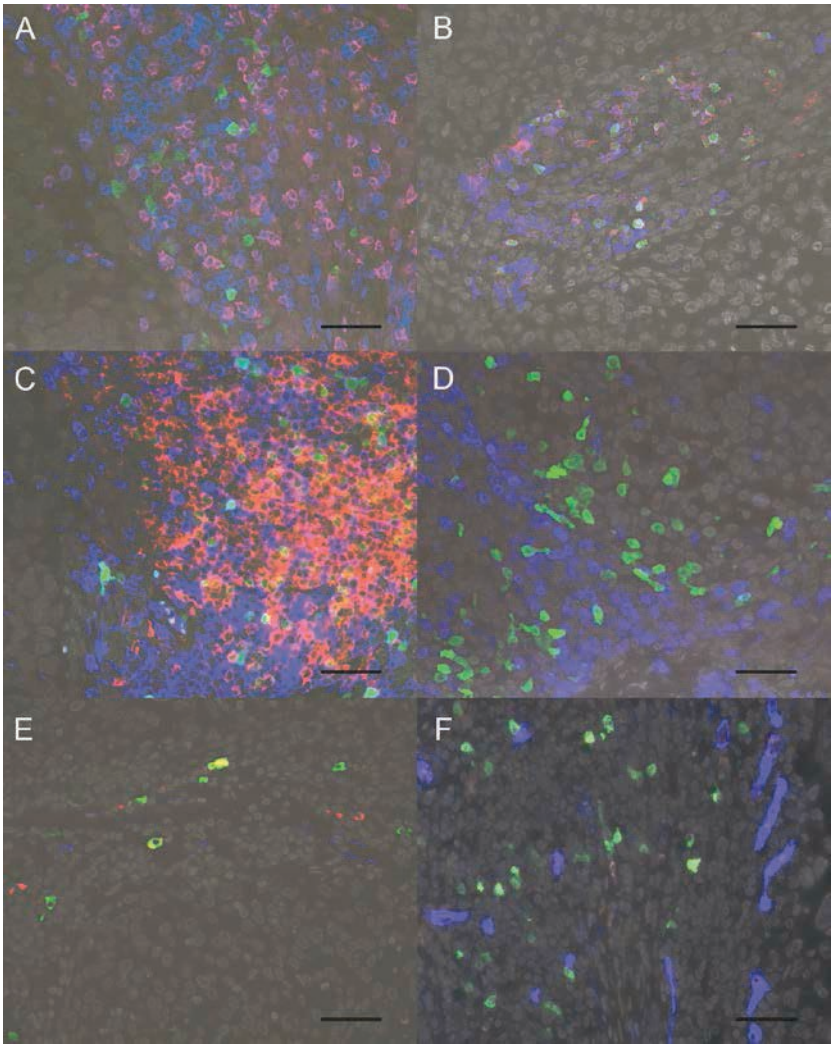

Figure 5. Phenotype TCL1A$^+$ cells

A representative image of a squamous cervical cancer FFPE specimen stained for TCL1A (green), CD8 (red) and CD3 (blue) shows that all TCL1A$^+$ cells were negative for T cell markers (A). A staining for TCL1A, CD20 (red) and CD19 (blue) indicates that TCL1A$^+$ cells predominantly expressed CD19, and part of these cells also expressed CD20 (purple membrane, B). Similarly, a staining for TCL1A, CD20 and CD10 (blue) shows that the majority of the TCL1A$^+$ cells expressed CD10 and part expressed CD20 (C). Few TCL1A$^+$ cells were found to express CD79a (blue, D). An example of infrequent TCL1A$^+$ cells expressing DNTT (red) is shown in E (CD10 is shown in blue). TCL1A$^+$ cells did not express CD34 (blue, F). In all panels, the nuclei were counterstained with DAPI (shown in gray), and the scale bar represents 50 µm.

Based on a quartile division, a low ratio (i.e., the lowest quartile) of *TCL1A*/*CD20* expression was significantly correlated with poor disease-specific survival (p=0.027; Figure 6C) and showed a trend toward poor disease-free survival (p=0.051; Figure 6D). Lastly, the ratio of *TCL1A*/*CD19* expression was not significantly correlated with survival.

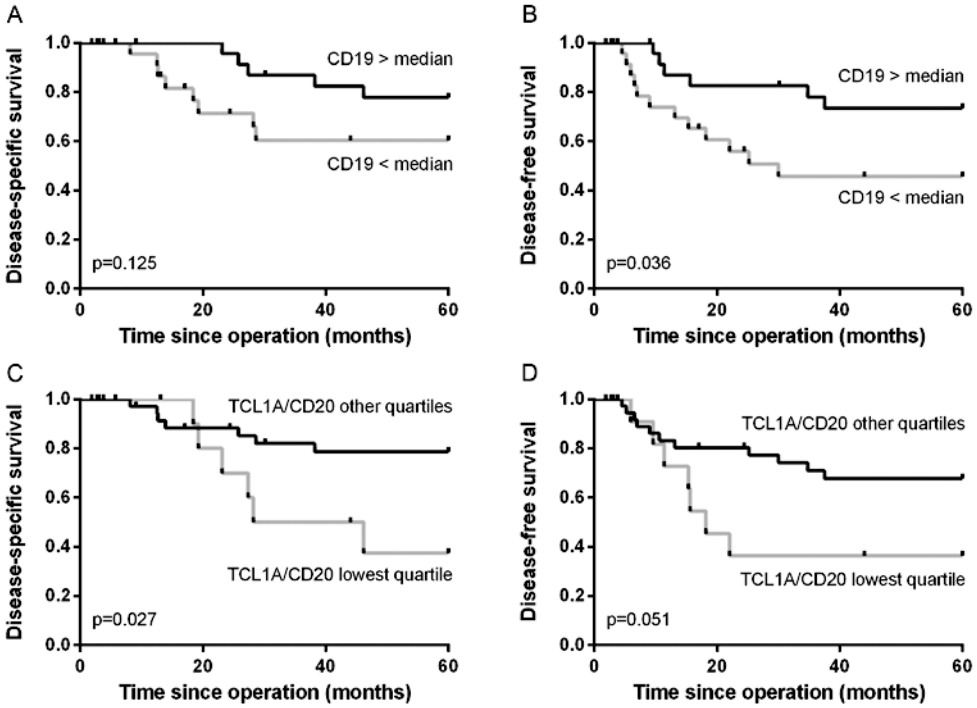

Figure 6. B cell and TCL1A correlations with survival

Kaplan-Meier analyses are shown based on qRT-PCR analyses of fresh-frozen tumor samples. The correlation between high versus low *CD19* expression and disease-specific (A) and disease-free survival (B) is shown. The ratio of *TCL1A/CD20* expression levels was divided in quartiles. The correlation between the lowest versus the other three quartiles and disease-specific (C) and disease-free (D) survival is shown.

Discussion

Tumors contain several cell types, including tumor cells, fibroblasts, endothelial cells and infiltrating immune cells. This heterogeneous nature of tumors can complicate the expression analysis of cell subpopulations in the tumor. Here, we report the first study in which RNA was isolated separately from viable tumor epithelial cells and tumor-infiltrating immune cells using flow-sorted fresh-dissociated squamous cell cervical carcinomas. Our aim was to use this novel approach in an attempt to identify factors that are correlated with the clinical outcome of cervical cancer patients.

Differential gene expression analysis of the RNA-seq data revealed that the expression profile in the tumor cell fractions differed from the expression profile in the immune cell fractions. In addition, the most differentially expressed genes matched the epithelial cell and leukocyte origins of the flow-sorted fractions, thus validating the study approach. Interestingly, the most significantly differentially expressed genes were upregulated in the immune cell fractions as compared with the tumor cell fractions. One possible explanation for this finding is that the immune cells in these tumors are a highly heterogeneous population of cells characterized by a higher number of function-specific proteins. The tumor epithelial cells on the other hand might represent a more homogeneous cell population characterized by a more uniform set of proteins.

Our novel approach identified *TCL1A* as a novel putative biomarker for predicting survival in patients with cervical cancer. Specifically, *TCL1A* was not expressed in the immune cell fractions of the deceased patients. We validated the correlation between high *TCL1A* expression and improved survival using both qRT-PCR and IHC analyses. Despite the relatively small number of patients in our study, *TCL1A* expression was robustly correlated with improved survival in the flow-sorted immune cell fractions. A clear trend toward improved prognosis was also found based on the number of $TCL1A^+$ cells in the FFPE samples obtained from the same patients that were included in our RNA-seq analysis. Noh *et al*. reported the presence of TCL1A protein in cervical cancer cells,[28] which we did not observe in our patient samples. The reason for this discrepancy might be the use of different antibodies. Since that study did not find a correlation between TCL1A expression and survival, strong lymphocyte-specific expression might be specifically correlated with patient survival.

TCL1A is expressed in immature $CD4^-CD8^-$ T cells and in several stages of developing B cells, including pre-B cells, virgin, mantle and germinal center B cells.[29-31] TCL1A is also expressed in activated peripheral lymphocytes, promoting cell proliferation and survival by activating protein kinase B (Akt).[32] In humans and mice, overexpression of TCL1A due to chromosomal rearrangement of the *TCL1A* gene to the T cell receptor locus causes mature T cell leukemia and lymphoma.[33,34] Indeed, TCL1A overexpression is a common finding in both leukemia and lymphoma,[34] although amplification of this gene was also reported in a pre-malignant cervical lesion.[35] In addition, Hoyer *et al*. showed that TCL1A expression induces proliferation by increasing T cell receptor signaling in mature T cells.[36] We found that TCL1A was not expressed by T cells in our patient samples. In contrast, TCL1A was expressed predominantly in $CD19^+$ and $CD10^+$, $CD34^-$ B cells. Some of these cells also expressed CD20 and—in rare cases—some cells expressed CD79a or the pro/pre B cell marker DNTT. Based on these markers, we conclude that the $TCL1A^+$ B cells seem to be predominantly germinal center or mature B cells, as well as a subpopulation of pre or immature B cells.

Specifically, the presence of $TCL1A^+CD19^+$ cells was correlated with a trend toward improved survival. Remarkably, high *CD19* expression was significantly correlated with improved disease-free survival (based on fresh-frozen tumor tissue samples

obtained from an additional cervical cancer patient cohort), whereas *CD3E*, *CD20* and *TCL1A* were not significantly correlated with survival. A low ratio of *TCL1A*/*CD20* expression was significantly correlated with poor disease-specific survival, indicating that high *TCL1A* expression relative to the expression of the B cell marker *CD20* in total tumor samples is correlated with improved survival. These results suggest that $CD19^+$ B cells are an important determinant of clinical outcome, and they suggest that the $TCL1A^+$ and $CD20^+$ B cell populations play an essential role in this outcome. Further validation on an independent patient cohort is required for TCL1A to be used as a prognostic marker.

$TCL1A^+$ B cells were distributed throughout the stroma, although they were also organized in B cell structures located adjacent to tumor epithelial fields. We speculate that $TCL1A^+$ B cells might indicate lymphoid follicular processes that facilitate B cell maturation, somatic hypermutation and isotype switching. Recently, intratumoral lymphoid structures were reported in several types of cancer,[37,38] and the presence of these structures was correlated with improved survival.[39,40] Compared to T cells, the role of B cells in cancer has been studied less extensively and is controversial. In addition to producing antibodies, tumor-infiltrating B cells can also function as antigen-presenting cells.[41] In melanoma and breast cancer mouse models, $CD19^+CD20^+CD137L^+$ B cells were reported to activate cytotoxic T cells.[42,43] High numbers of tumor-infiltrating B cells have been correlated with improved patient survival in several cancer types.[44,45] Moreover, antibodies can undergo antigenic selection and affinity maturation in cervical cancer.[46] Our data indicate that intratumoral B cells may play an important role in controlling cervical cancer, and TCL1A is a potential marker for a beneficial B cell response.

Based on both positive lymph nodes and death by disease, *PPP3R1* was the most upregulated gene in the immune cell fractions. *PPP3R1* encodes a regulatory subunit of calcineurin, which activates the transcription factor NFAT (nuclear factor of activated T cells).[47] In addition, the vasoconstrictor endothelin 2 was expressed only in the immune cell fractions of patients without lymph node metastases. Because the expression levels of these two genes were inadequate for discriminating between patients and did not differ significantly within the tumor cell fractions, we did not pursue these proteins as potential targets.

With respect to the tumor cell fractions, the only significantly increased expression was observed in the genes *MME* and *OLFML2A* in the patients without lymph node metastases. This low number of differentially expressed genes suggests that the tumor cell fractions were highly homogeneous. We could thus not identify a differential expression pattern based on positive lymph nodes or death by disease. Because of this relative homogeneity, we believe that this technique is a promising tool for identifying pathways that are specific to different cancer types.

In summary, we report that RNA-seq data can be analyzed reliably using tumor cells and immune cells isolated from cancer samples using flow cytometry. The most

distinguishing gene identified by differential expression analysis was *TCL1A*. Specifically, *TCL1A* was not expressed in the immune cells of patients who died from cervical cancer. This correlation between *TCL1A* expression and improved survival was validated using qRT-PCR and IHC analyses. We also found that TCL1A was predominantly expressed in B cells, possibly reflecting intratumoral lymphoid follicular processes. Based on these findings, we conclude that TCL1A may be a prognostic biomarker for predicting improved survival in patients with cervical cancer. This is the first study demonstrating the prognostic value of separating tumor epithelial cells from tumor-infiltrating immune cells and determining their RNA expression profile for identifying putative cancer biomarkers. Because B cell expressed *TCL1A* was the most prominent marker correlated with patient survival, perhaps we should broaden our T cells biased view to include B cells in cancer immunology. The emerging interest in cancer immunotherapy supports studying the use of B cells in anticancer treatments.

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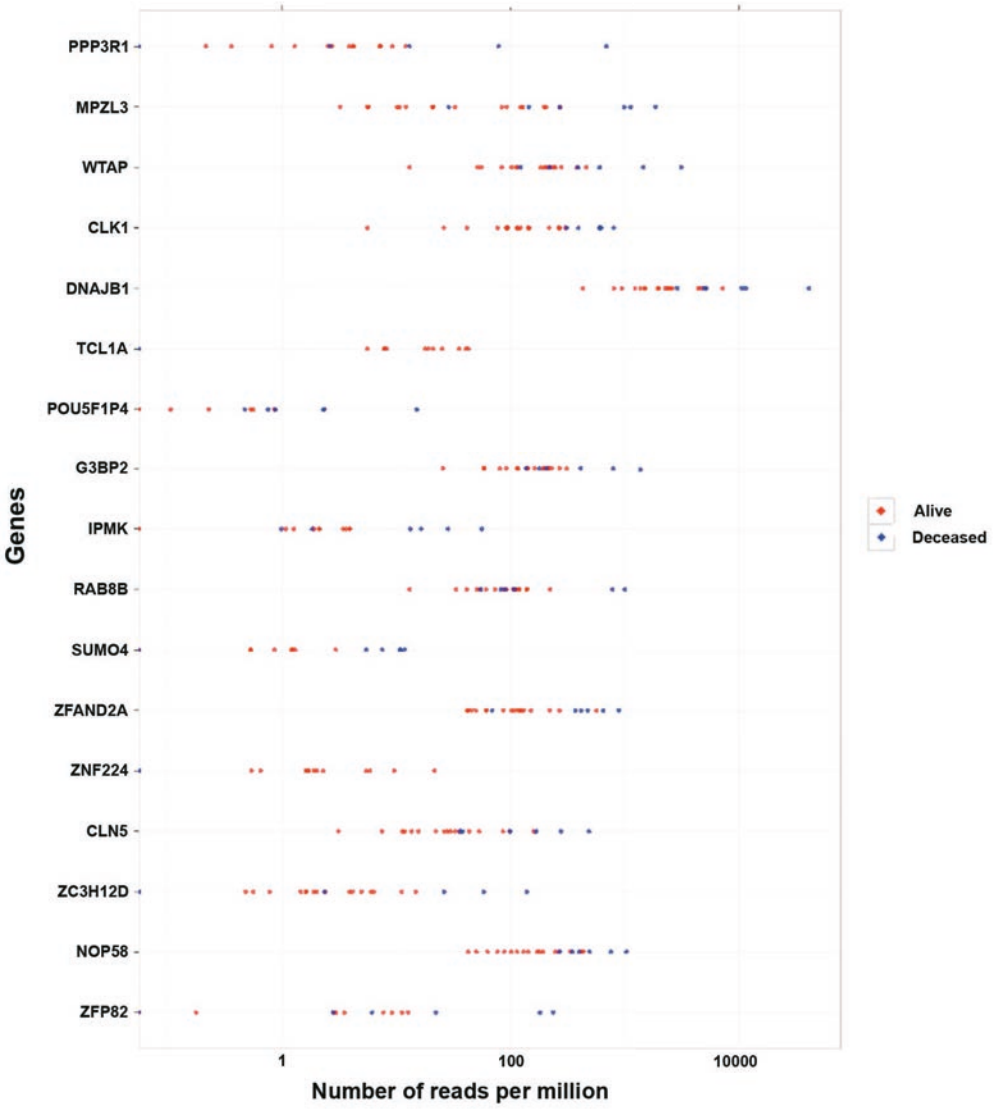
Supplementary Figure S1. Differentially expressed genes in infiltrating immune cell fractions based on patient survival status



The significantly differentially expressed genes in the tumor-infiltrating immune cell fractions between patients who were alive (red dots) and patients who were deceased (blue dots) is shown. The gene with the lowest p value (*PPP3R1*) is shown at the top.

Supplementary Tables S1 and S2 were too extended to include in this thesis, and can be obtained from the author.

VII

The correlations between IL-17 versus Th17 cells and cancer patient survival: a systematic review

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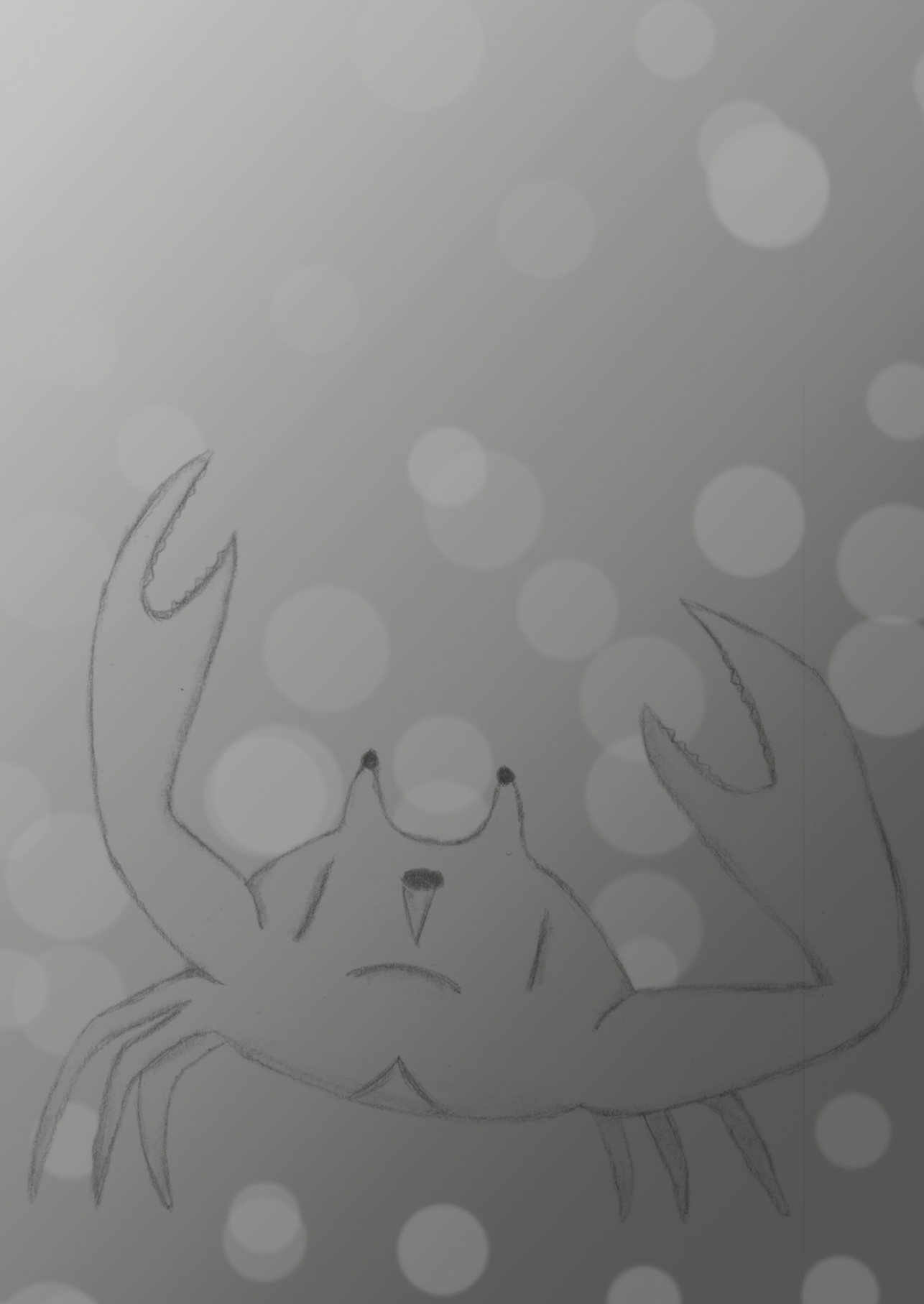


Abstract

Both IL-17 and Th17 cells have been ascribed tumor promoting as well as tumor suppressing functions. We reviewed the literature on correlations between IL-17 versus Th17 and survival in human cancer, following the PRISMA guidelines. Serum, formalin fixed paraffin embedded tissue and peripheral blood samples were most frequently studied. High IL-17 quantities were correlated with poor prognosis, whereas high Th17 cell frequencies were correlated with improved prognosis. Since Th17 cells are a subpopulation of IL-17$^+$ cells and had a different correlation with prognosis than total IL-17, we substantiate that a distinction should be made between Th17 and other IL-17$^+$ cells.

Introduction

Interleukin-17 (IL-17) was discovered in 1993 and originally named cytotoxic T lymphocyte-associated-8 (CTLA-8).[1] IL-17 was more recently renamed IL-17A and has five family members: IL-17B-F.[2] Only IL-17F shows some homology and overlapping functions with IL-17A. The main functions of IL-17 are the attraction of neutrophils and stimulation of inflammation.[3] The T helper 17 (Th17) cell, one of the predominant producers of IL-17 that was characterized in 2005,[4] is essential to protect the host against pathogens that are not handled well by Th1 and Th2 cells.[5] This pro-inflammatory cell type plays a dominant role in a variety of autoimmune diseases.[3] Antibodies targeting IL-17 and its receptor are now used in clinical trials to treat autoimmune diseases like psoriasis, rheumatoid arthritis and Crohn's disease.[6] Since IL-17 can also be produced by innate immune cell types including both lymphoid derived (e.g. γδT cells, invariant natural killer T cells and innate lymphoid cells)[7,8] and myeloid derived cells (e.g. neutrophils, macrophages and mast cells),[9] it may bridge the activities of the innate and adaptive immune system.[10]

Much less studied is the role of IL-17 in cancer. Both tumor suppressing and tumor promoting functions have been ascribed to the IL-17 protein and Th17 cells.[11] This ambiguity about the function of IL-17 and Th17 cells in cancer has limited the potential for targeting the molecule or using cell-based immunotherapy. Part of the ambiguity may have arisen because different aspects of the IL-17 response are studied. Total protein amount or cells expressing IL-17 protein have been measured in serum and tumor associated fluids by ELISA and in formalin-fixed, paraffin-embedded (FFPE) tissue by immunohistochemistry, respectively. The effect of Th17 cells has been analyzed mainly in peripheral blood, but also in tumor associated fluids, FFPE and fresh frozen tissue by flow cytometry, immunohistochemistry or RT-PCR. A review on Th17 cells in cancer by Wilke *et al*. in 2011 already noted that correlations between the IL-17 protein and survival may be different from correlations with the Th17 cell population.[12]

To systematically study the correlations between the IL-17 protein and Th17 cells and survival in human cancer, we investigated all publications in NCBI PubMed, Ovid Embase and Web of Science addressing this subject. The aim of our study was to identify the correlations between both IL-17 protein and Th17 cells and prognosis in cancer. The studies were classified by the sample type used to study IL-17 or Th17 cells: serum, FFPE tissue, peripheral blood, tumor associated fluids and fresh frozen tissue. Subsequently, the effect on survival was analyzed for each of the sample types studied. The implications for further research of IL-17 and Th17 cells are discussed.

Results

Study design and selection criteria

Of the 2643 publications identified through database searching on IL-17 or Th17 and cancer, 56 studies met the inclusion criteria (Figure 1). The main reasons for a publication to be excluded were: being a conference abstract (23%), an animal study (24%), no study on cancer (27%) or not reporting on survival data (19%). Two articles were excluded due to lack of other references on the same method and survival analysis. One article reported on an IL-17 SNP analysis,[13] while the other studied RNA levels of Th17 cell expressed retinoic acid receptor-related orphan receptor gamma (RORγt).[14] Neither of these studies found a correlation with survival. An overview of the included studies sorted by sample type and clinical outcome is shown in Table 1.

Studies reporting on survival analysis or risk of recurrence were included regardless of the outcome of the study. A potential publication bias was caused by excluding articles that reported on correlations with other clinico-pathological parameters but not survival. This bias was minimized by screening all articles that reported on correlations with clinico-pathological parameters for having performed a survival analysis. The survival criterion enabled us to focus on studies that are relevant for the potential targeting of IL-17 or Th17 cells in a clinical setting.

Generally, a random or consecutive group of patients was analyzed for relatively objective measures (see supplementary Tables S1-4). Although most studies did not provide details on the sample selection method, the majority of the studies used as a continuous variable or categorized IL-17 or Th17 cell numbers in groups based on the presence, mean or median to analyze the effect by Kaplan-Meier and Cox regression analyses. Potential risks of bias identified in categorizing IL-17 or Th17 expression were optimal cut-off values chosen arbitrarily[15,16] or using a minimum p value,[17-23] ROC curve[24-27] or regression tree analysis.[28] Furthermore, one study compared the six long (>3 years) versus short (<1.5 years) surviving patients.[29] Another study reported that post-chemotherapy samples were used when no pretreatment samples were available for immunohistochemistry.[30] A final potential risk factor was observed in a study of leukemia patients treated with allogeneic stem cell transplantation after myeloablative

conditioning, which included donors that varied from related to unrelated and different prophylaxis regimens to prevent graft-versus-host disease.[31] Additional study details and concerns are listed per sample type in supplementary Tables S1-4. Clinico-pathological characteristics of the different studies per measurement method are provided in supplementary Tables S5-8.

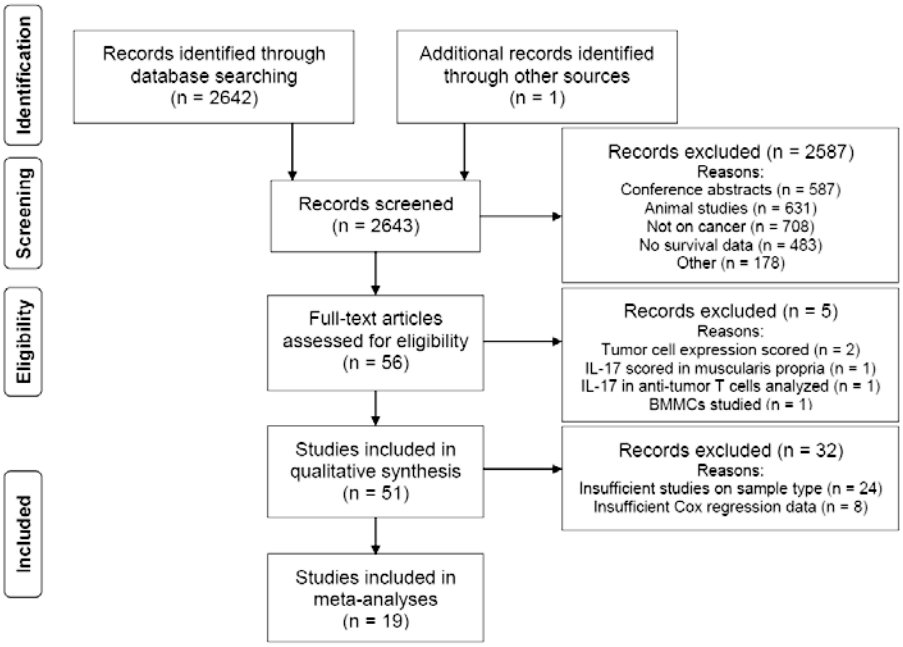


Figure 1. PRISMA Flow diagram

Database searching identified 2642 publications on IL-17 or Th17 and cancer. One publication on this topic of our group published in this issue of OncoImmunology was added manually. Using the search term 'tumor' caused studies on tumor-necrosis factor to be selected regardless of whether the study was on cancer. Although tumor necrosis factor was excluded as a major topic, many publications that were not on cancer had to be excluded manually. All inclusion criteria were met by 56 articles. Five studies were excluded from analysis for using different sample types or methods than all other articles. Another 32 studies were excluded from meta-analysis because not enough studies were available on sample types other than tissue analyzed for IL-17 expression or insufficient data were provided. Figure adapted from Moher et al.[80]

High IL-17 serum levels are correlated with poor survival

Serum, paraffin tissue, peripheral blood mononuclear cells (PBMCs) and occasionally tumor associated fluids or fresh frozen tissue were used to measure IL-17 protein or RNA and Th17 cells. Since the cell source and related activity measured may differ in different sample types, we sorted and analyzed the studies by sample type. The amount of IL-17 protein in serum was measured by ELISA (Table 1.1). Since total protein quantity was measured, the IL-17 could have been derived from Th17 cells but also from innate immune cell types. Five studies out of ten reported that a high amount of serum IL-17 protein was correlated with poor survival.[17,24-26,31] One study showed a

correlation between a high IL-17 level and improved survival in leukemia.[32] Four studies did not observe a significant correlation between high serum IL-17 levels and survival,[33-36] although one group did find a trend toward poor prognosis (p=0.05).[36] Overall, a high amount of IL-17 protein in serum has predominantly been correlated with poor survival (Table 2).

Table 1.1. Correlation between IL-17 in serum and survival

Cancer type	N	Outcome	Correlation	Multivariate Cox p<0.05	Notes	Ref #
NSCLC	128	OS	Poor	Yes		25
HBV-related HCC	105	OS, DFS	Poor	DFS: Yes OS: NA		26
Leukemia treated with myelo-ablative conditioning and SCT	95	DFS	Poor	Yes		31
Gastric carcinoma	85	OS	Poor	Yes		17
CRC	80	DFS	Poor	NA		24
Acute leukemia	93	OS	Improved	No		32
CLL	294	OS	No correlation		sample type: plasma	33
CLL	84	OS	No correlation			34
Pancreatic AC	62	OS	Trend toward poor			36
Multiple myeloma	50	OS	No correlation		peripheral blood, bone marrow	35

Table 1.2. Correlation between IL-17$^+$ cells in tissue and survival

Cancer type	N	Outcome	Correlation	Multivariate Cox p<0.05	Notes	Ref #
HCC	323	OS, DFS	Poor	No		40
HCC	300	OS, DFS	Poor (intratumoral)	NA		18
HCC	150	OS, DFS	Poor (intratumoral)	No	peritumoral IL-17$^+$ cells correlated with improved survival	41

HCC	108	OS, DFS	Poor (intratumoral)	Yes	hot-spot areas scored	19
HCC	56	OS, DFS	Poor DFS	NA	both intra- and peritumoral cells scored	42
HCC	43	OS, DFS	Poor	Yes		43
Intrahepatic cholangiocarcinoma	123	OS	Poor (intratumoral)	Yes		44
CRC	104	DFS	Poor	NA	both tumor center and invasive margin scored	20
CRC	102	OS	Poor	NA		45
CRC	52	OS	Poor	Yes		16
NSCLC	102	OS	Poor	NA		46
NSCLC	52	OS, DFS	Poor	Yes	same research group as ref 16	15
Breast carcinoma	207	OS, DFS	Poor DFS	Yes	scores in tumor center and front hot-spots averaged	30
Gastric carcinoma	112	OS	Poor	Yes	mainly mast cells were IL-17^{+}	50
Cervical SCC	109	OS	Poor (TNM stage I)	Yes	NS in all TNM stages	37
Gallbladder carcinoma	104	OS, DFS	Poor OS	No		47
Laryngeal SCC	71	DFS	Poor	No		49
Pancreatic AC	46	OS	Poor	NA	condensed expression areas scored	48
Gastric AC	192	OS	Improved	Yes		51
Esophageal SCC	181	OS	Improved	No		52
Cervical carcinoma	153	DFS	Improved	Yes	densest lymphocytic infiltrates scored	21
Recurrent ovarian carcinoma	47	OS, DFS	Improved DFS	No		28

Pancreatic ductal AC treated with vaccine, CT, RT	12	OS	Improved	NA	lymphoid aggregates scored	29
HCC	132	OS, DFS	No correlation		densest lymphocytic infiltrates scored	22
Nasopharyngeal carcinoma	106	OS	No correlation			53
Epithelial ovarian carcinoma	104	OS	No correlation		consecutive hot-spot areas scored	23
Giant cell tumors of bone	74	DFS	No correlation			54
Esophageal SCC	215	OS	High IL-17$^+$ cells in tumor muscul. propria correlated with improved OS	Yes	correlation between IL-17$^+$ cells in tumor nests and survival not studied	39*
CRC	78	OS, DFS	Improved	Yes	mainly tumor cells positive	55*
Stage IV glioblastoma	41	OS	Improved	Yes	mainly tumor cells positive	56*

Table 1.3. Correlation between Th17 cells and survival

Cancer type	Sample	N	Measurement	Outcome	Correlation	Multivariate Cox p<0.05	Notes	Ref #
Cervical SCC	FFPE	51	IHC CD3$^+$ IL-17$^+$ cells	OS	Improved	Yes		37
Diff. thyroid carcinoma	FFPE	266	IHC CD4$^+$ IL-17$^+$ cells	DFS	Trend toward improved			59
HCC	PBMC	150	FC CD4$^+$ IL-17$^+$	DFS, OS	Poor	Yes		41
Gastric carcinoma	PBMC + PMA/iono/ mon 4u	32	FC CD4$^+$ IL-17$^+$IFNG$^-$	OS	Poor	NA		62

Acute leukemia	PBMC + PMA/iono/ mon 5h	93	FC $CD3^+CD4^+$ $IL\text{-}17^+$ cells	OS	Improved	Yes		32
CLL	PBMC + 5h PMA/iono/ mon	66	FC $CD3^+CD4^+$ $IL\text{-}17^+$ cells	OS	Improved	No	Same group: ref 34	38
End-stage melanoma treated with aCTLA4	PBMC + 2.5h PMA/iono/ 2h mon	47	FC $CD4^+CD8^-$ $IL\text{-}17^+$ increase 6m after/1wk before therapy	DFS	Improved	NA		61
HCC treated with transarterial chemoem-bolization	PBMC + activation mix at -5h and +30d	30	FC $CD4^+$ $IL\text{-}17^+$ cells	OS	Improved (+30d measurement)	Yes	NS for -5h mea-sure-ment	60
CLL	PBMC + PMA/iono/ mon 5h	150	FC $CD3^+CD4^+$ $IL\text{-}17^+$ cells	OS	No correlation			33
Hematologic melagnancy treated with allogeneic HSCT	PBMC	30	FC $CD3^+CD4^+$ $CD8^-IL\text{-}17^+$ cells	DFS	No correlation	NA	60% of patient got GVHD	64
HCC	PBMC	26	FC $CD4^+$ $IL\text{-}17^+$ cells	DFS	No correlation		IL-17 ELISA <detect limit	63
Stage IV melanoma with anti-tumor antigen T cell response	Melan-A reactive $CD3^+CD4^+$ PBMC + 12d peptide mix + 12h antigen/mon	38	FC IL-17 present/absent	OS	Poor	Yes	IL-17 present in n=3; CD4 related with poor OS	65*
Acute myeloid leukemia	BMMC + 5h PMA/iono /bref	98	FC $CD3^+CD8\text{-}$ $IL17^+$	OS	Poor	NA	no corr. for PBMC (n=30)	66*
CRC	normal biopsies ~10cm from tumor center	19	qRT-PCR IL-17	OS	No correlation			57

Ovarian carcinoma	fresh frozen tumor tissue	17	agarose gel RT-PCR IL-17 present	OS	No correlation			58

Table 1.4. Correlation between IL-17 and Th17 cells in tumor associated fluids and survival

Cancer type	Sample	N	Measurement	Outcome	Correlation	Multivariate Cox p<0.05	Notes	Ref #
Lung carcinoma	MPE supernatant	78	ELISA IL-17	OS	Poor	Yes		27
Ovarian carcinoma	tumor ascites	85	ELISA IL-17	OS	Improved	Yes		69
Lung carcinoma	MPE	30	FC $CD4^+$ $IL\text{-}17^+$ cells	OS	Improved	Yes		67
Lung AC with pleura metastasis	MPE +2h PMA/iono +4h Bref/mon	24	FC $CD3^+CD4^+$ $IL\text{-}17^+$ cells	OS	Improved	NA		68
Gastric carcinoma	peritoneal lavage	114	qRT-PCR IL-17	OS	Improved (curative resections; n=79)	Yes	NS for all patients (n=114)	70

Table 1. Correlation between IL-17 or Th17 and survival

All studies describing a correlation between a measurement of IL-17 or Th17 cells and overall or disease-free survival are shown. The analyses were sorted by clinical outcome, cancer type and study size. N indicates the number of patients on which the correlation between the IL-17 measurement and survival was reported. The column 'Correlation' indicates whether a high IL-17 or Th17 cell measurement was correlated with poor or improved survival. If this correlation was significant under a certain condition (e.g. for OS or DFS only, or for a scoring location), this is also indicated. A dark grey row indicates a correlation with poor survival, a white row a correlation with improved survival and a light grey row no significant correlation. Whether or not the correlation found was independently correlated with survival when corrected for clinico-pathological parameters in a multivariate Cox regression analysis is also indicated. A multivariate analysis including both the IL-17 measurement as well as another variable also containing this IL-17 measurement (e.g. a ratio) was not included in our analysis since the potential effect might be lost by correcting for it. Measurement deviances are indicated under 'Notes'. Only if a study on a certain cancer type was performed by the same research group as another included study, a note is included because of potential sample overlap. An asterisk behind a reference number indicates that the study was not included in the quantitative analyses in Table 2 and Figure 2. Table 1.1 shows analyses on IL-17 quantifications in serum by ELISA. Table 1.2 is a representation of studies on tumor infiltrated IL-17+ cells quantified by immunohistochemistry on FFPE tissue slides or tissue microarrays. If 'intratumoral' is indicated, peritumoral cells were scored as well. Table 1.3 shows analyses of Th17 quantification on FFPE tissue, peripheral blood PBMCs and fresh frozen samples. Table 1.4 represents the analyses on tumor associated fluids sorted by measurement type. Abbreviations: AC=adenocarcinoma; Bref=brefeldin A; CLL=chronic lymphocytic leukemia; CRC=colorectal carcinoma; CT=chemotherapy; FC=flow cytometry; GVHD=graft-versus-host disease; HCC=hepatocellular carcinoma; Iono=ionomycin; Mon=monensin; MPE=malignant pleural effusion; NA=not applicable or not mentioned in the article; NS=not significant; NSCLC=non-small cell lung carcinoma; PMA=phorbol 12-myristate 13-acetate; RT=radiotherapy; SCC=squamous cell carcinoma

Table 2. Correlations per measurement type

Target	Sample type	Measurement method	#analyses improved prognosis	#analyses poor prognosis	#analyses no effect	Total # analyses	Factor difference
IL-17	Serum	ELISA	1	5	4	10	0.2
	FFPE tissue	IHC	5	18	4	27	0.3
	Tumor associated fluid	ELISA	1	1	0	2	1.0
		Total	**7**	**24**	**8**	**39**	**0.3**
Th17	FFPE tissue	IHC Th17	1	0	1	2	NA
	Peripheral blood	Flow cytometry	4	2	3	9	2.0
	Tumor associated fluid	Flow cytometry	2	0	0	2	NA
		RT-PCR	1	0	0	1	NA
	Fresh frozen tissue	RT-PCR	0	0	2	2	NA
		Total	**8**	**2**	**6**	**16**	**4.0**

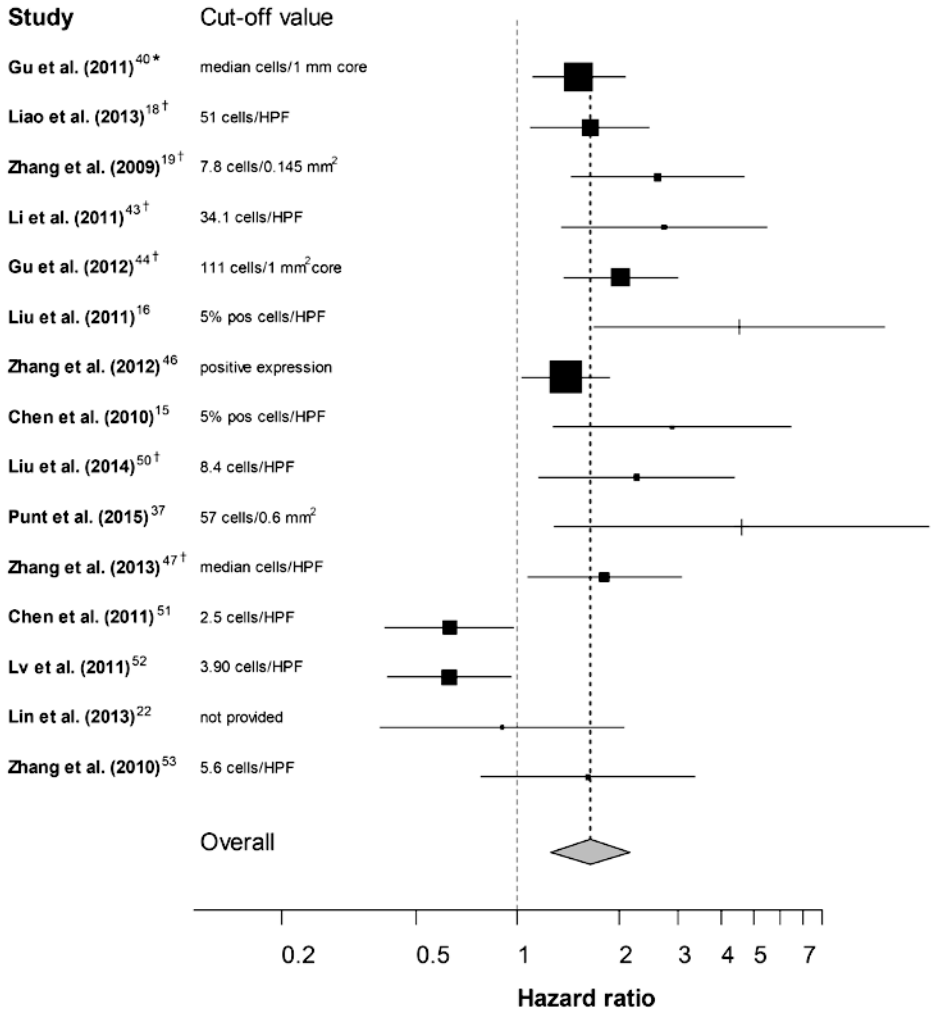
The number of analyses per sample and measurement type of IL-17 protein or Th17 cells showing a correlation with improved or poor prognosis or no effect is indicated. The final column denotes the ratio of the number of analyses showing a correlation with improved prognosis over the number of analyses showing a correlation with poor prognosis, as an indication of the factor difference. A white box indicates a correlation with improved survival, a dark grey box a correlation with poor survival and a light grey box no clear correlation.

A high number of IL-17$^+$ cells in tissue is correlated with poor survival

The total number of IL-17$^+$ cells was quantified on cancer tissue FFPE whole slides or tissue microarrays using immunohistochemistry. This type of analysis allows for quantification of the total number of IL-17$^+$ cells within the tumor microenvironment. IL-17 is expressed by different types of tumor infiltrating immune cells in cancer, predominantly neutrophils and mast cells.[37-39] The total number of IL-17$^+$ cells was correlated with poor prognosis in 18 out of 27 studies (Table 1.2).[15,16,18-20,30,37,40-50] Five studies reported on a correlation between a high number of IL-17$^+$ cells and improved survival.[21,28,29,51,52] It is important to note that in two of these five studies, the IL-17$^+$ cells were scored in areas with the densest lymphocytic infiltrate, one of which was on pancreatic ductal adenocarcinoma patients who had received immunotherapy (the correlation between IL-17 and survival was based on 12 patients).[21,29] Four studies did not observe a significant correlation between total IL-17$^+$ cells in the tumor and survival.[22,23,53,54] Again the scoring in two of these four studies had been performed in hot-spot or dense lymphocytic infiltrate areas, while only three of the 18 studies reporting on a negative correlation had focussed on hot-spots. Three more studies did

not focus on IL-17^{+} tumor infiltrating immune cells and are included with their reported correlations in Table 1 for completeness, but not in the quantitative analyses.[39,55,56] Collectively, 18 studies reported on a significant correlation between high IL-17 and poor prognosis, over 3.5 times more than the studies showing a correlation with improved prognosis (n=5, Table 2). To visualize the overall correlation, forests plots are shown for the hazard ratio of a high number of IL-17^{+} cells on overall (Figure 2A) and disease-free survival (Figure 2B). Of the 22 studies reporting on overall survival, 7 were excluded from the meta-analysis due to insufficient Cox regression data. Of the 16 studies reporting on disease-free survival, 4 were excluded due to insufficient Cox regression data.

A



B

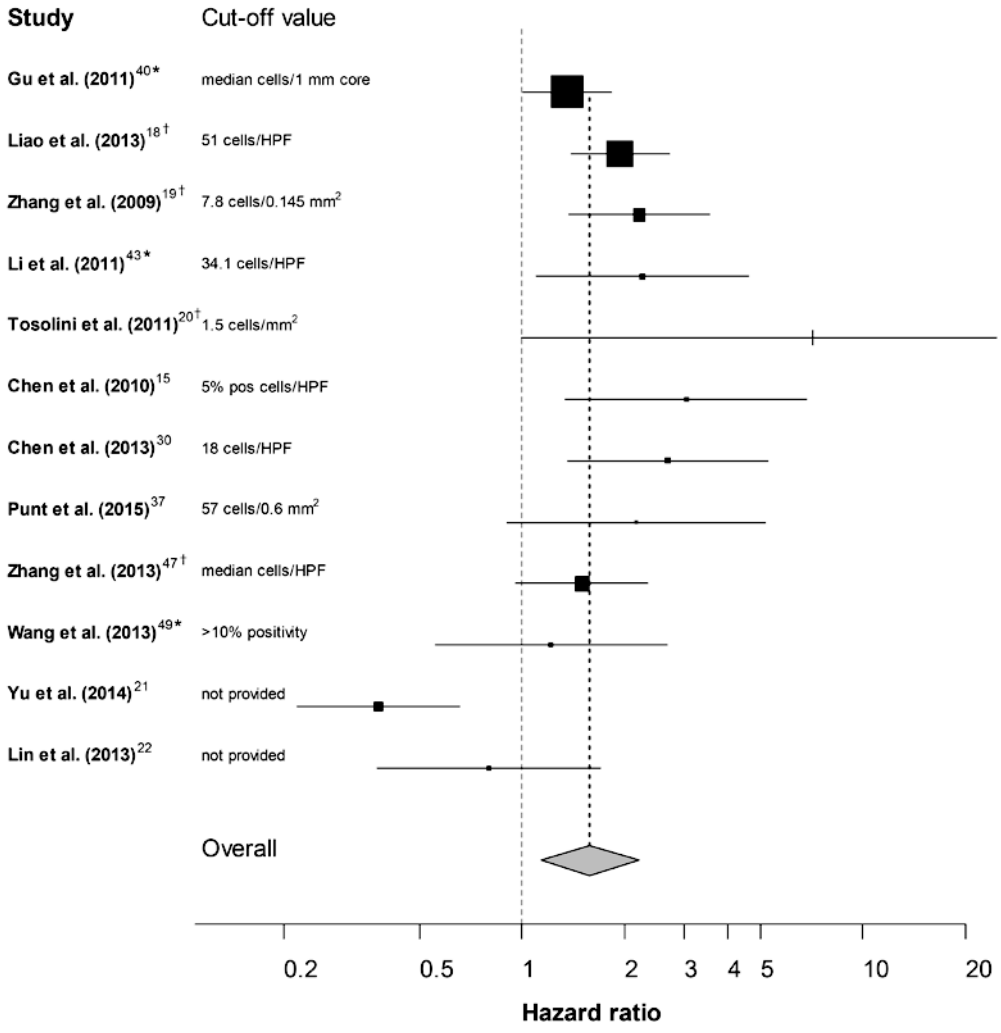


Figure 2. Forest plots for IL-17$^+$ cells in tissue

Schematic quantitative analyses of the studies on the number of IL-17$^+$ cells in FFPE tissue is shown by forest plots. Cox regression hazard ratios and 95% confidence intervals for the correlation between a high number of IL-17$^+$ cells and overall survival (A) and disease-free survival (B) were obtained from the articles or via personal communication with the authors. An asterisk (*) indicates that a multivariate Cox regression analysis was used because a univariate analysis was not provided. A dagger (†) indicates that part of the data were obtained via e-mail. The cut-off value used to divide the IL-17$^+$ cell frequency in a high and low group is indicated for comparison. The center of the random effects model represents the pooled hazard ratio, while the 95% confidence interval is represented by the diamond horizontal borders.

Correlation between IL-17 RNA expression in fresh frozen tissue and survival inconclusive

Two studies have analyzed IL-17 RNA expression in fresh frozen samples using RT-PCR (Table 1.3). Both studies, on small study populations, did not find associations with survival. One study analyzed IL-17 expression in tumor adjacent normal appearing biopsies (~10 cm from the tumor center) from 19 colorectal cancer patients.[57] The other study in 17 ovarian cancer patients only measured the presence of PCR products on agarose gel.[58] Insufficient data were available to conclude on an association between IL-17 RNA expression in fresh frozen tissue and survival.

A high number of Th17 cells in tissue is correlated with improved survival

The total number of Th17 cells can be quantified using a combination of a T cell marker and IL-17 in FFPE slides. Using immunohistochemistry, our group has shown that a high number of Th17 cells was correlated with improved prognosis in squamous cervical cancer,[37] while another study found a trend toward improved disease-free survival (p=0.06) in differentiated thyroid cancer (Table 1.3).[59] We did not include analyses on the ratio of the number of IL-17$^+$ cells over the number of CD3$^+$ or CD4$^+$ T cells, because we do not regard this as a measure for Th17 cells since IL-17 is also produced by other cell types.

A high number of Th17 cells in peripheral blood is correlated with improved survival

Flow cytometry was used to quantify the Th17 cell frequency among PBMCs, usually defined as CD4$^+$IL-17$^+$ cells (see Table 1.3 for details). A high number of Th17 cells was correlated with improved survival in four studies.[32,38,60,61] Two studies found a correlation with poor survival,[41,62] while three studies did not find a significant correlation.[33,63,64]

Two studies focused on a different aspect of the Th17 response and are included in the overview in Table 1 for completeness, but not in the quantitative analyses.[65,66] Notably, while two studies reported on a correlation between a high number of Th17 cells and poor prognosis, twice as many studies (n=4) reported on a correlation with improved prognosis (see Table 2).

A high number of Th17 cells in tumor associated fluids is correlated with improved survival

Tumor associated fluids have infrequently been studied for the correlation between IL-17 or Th17 numbers and survival (Table 1.4). Two studies have analyzed the number

of Th17 cells in lung cancer malignant pleural effusion by flow cytometry.[67,68] Both found a significant correlation between a high number of Th17 cells and improved overall survival.

One group has studied the correlation between high IL-17 protein levels in lung cancer malignant pleural effusion measured by ELISA and described a correlation with poor survival.[27] Another study found a correlation between high IL-17 protein levels in ovarian carcinoma ascites and improved survival.[69] Finally, a study in gastric cancer patients showed a significant correlation between high IL-17 RNA expression measured by qRT-PCR and improved survival in patients treated with curative resection.[70]

Collectively, of the five studies on tumor associated fluids, the studies quantifying Th17 cells using flow cytometry (n=2) and qRT-PCR (n=1) found a correlation with improved prognosis. Of the two studies quantifying IL-17 using ELISA, one found a correlation with improved, and one with poor prognosis.

Differences between cancer types

While functional differences between IL-17 and Th17 cells may be due to the cellular source of IL-17 and the accompanying immune response, this might also depend on the cancer type. In studies on liver cancer (n=13), a negative (n=9) or no significant (n=3) correlation was found between high IL-17 or Th17 cells and prognosis, except for the study of hepatocellular cancer treated with transarterial chemoembolization. All studies on colorectal cancer (n=6) also found a correlation between high IL-17 and poor prognosis (n=4) or no significant correlation (n=1), except for one study that reported on IL-17 being expressed mainly by tumor rather than tumor infiltrating immune cells. The studies on non-small cell lung cancer (n=3) reported a significant correlation between IL-17 and poor prognosis as well.

In contrast, all analyses described in six leukemia studies (n=8) showed a significant correlation between PBMC Th17 cells or serum IL-17 and improved prognosis (n=3) or no effect (n=4), except for one study of serum IL-17 in patients that received stem cell transplantation after myeloablative conditioning. This might indicate that the immune response in haematological malignancies may differ from solid tumors. Of the studies on ovarian cancer (n=4), two described a correlation between high IL-17 and improved survival. The other two groups did not find a significant correlation with disease-specific survival, but one of the studies described a correlation between high IL-17 and improved progression-free survival.

These findings indicate that there may be context specific effects on the IL-17 or Th17 cell immune response, although the number of studies per cancer type was too limited to determine whether the cancer type or sample type is more important for the effect on survival.

Discussion

The clinical impact of Th17 cells has remained unresolved in cancer.[71] The aim of this review was to identify the correlations between a high amount of IL-17 protein or high number of Th17 cells in human cancer and patient survival. Following an extensive electronic database search, publications were manually selected without format or language restrictions. Survival analyses were studied in the full article if any analysis regarding prognosis was mentioned in the abstract, minimizing the risk of publication bias. Although the risk of bias in included studies was limited, all studies used different cut-off levels to divide IL-17 or Th17 expression in a high and low expression group due to a lack of established cut-off levels. This study limitation makes it difficult to compare different studies directly.

The sample type studied proved to be crucial for the correlation with clinical outcome. This may partly be explained by a difference in cell source. Some tumor microenvironments may be more favorable for Th17 cells, while others may be more readily infiltrated by IL-17 producing neutrophils. Additionally, the method used determines whether Th17 cells, IL-17 protein or all IL-17 producing cells are measured. A high amount of IL-17 protein, predominantly produced by neutrophils and mast cells in cancer[37-39] and measured in serum, FFPE tissue and tumor associated fluids, was over three times more frequently correlated with poor than with improved prognosis. A meta-analysis could only be performed for IL-17 in FFPE tissue due to the limited number of studies on the other sample types. The forest plots clearly showed that a high number of $IL\text{-}17^+$ cells was correlated with an increased hazard ratio, despite the use of a range of cut-off values, which might depend on the type of cancer and analysis. In contrast, a high number of Th17 cells measured in FFPE tissue, peripheral blood or tumor associated fluids was four times more often correlated with improved than with poor prognosis. Since IL-17 RNA can generally not be quantified in neutrophils[37,72] the data obtained by RT-PCR analyses most likely represent IL-17 produced by Th17 cells. The PCR measurements in tumor associated fluids and fresh frozen tissue are thus regarded as an indicator of the Th17 cell frequency. Because of limited data available, we could not conclude on an association between IL-17 RNA expression and survival.

Th17 cells seemed to primarily have a tumor suppressing effect, whereas IL-17 was generally associated with poor outcome. IL-17 has been shown to be produced by only a small Th17 cell population.[37-39] The tumor promoting function can be explained by the role of IL-17 in inducing angiogenesis[73] and recruiting neutrophils.[74] Neutrophils have been reported to convert to a tumor promoting phenotype and to induce angiogenesis.[75] The immune cells capable of producing IL-17 include neutrophils as well as other cell types,[7-9] which may determine an important part of the clinical outcome. The tumor suppression by Th17 cells is probably due to different properties than the secretion of IL-17. Th17 cells might stimulate the Th1 and cytotoxic T cell tumor targeting immune responses.[76] Additionally, Th17 cells have been shown to have memory stem-cell like

properties and the ability to differentiate to Th1/Th17 cells that produce interferon-gamma.[77] Th17 cells may thus either directly or indirectly suppress tumorigenesis.

The type of IL-17 response is thus likely to be context dependent. Liver cancer, colorectal cancer and non-small cell lung cancer seem to be correlated with an unfavorable IL-17 response.[15,16,18-20,24-26,40-46] Leukemia on the other hand might provide an environment favorable for Th17 cells to suppress tumor growth.[32,38] Similarly, ovarian cancer might attract a tumor suppressing IL-17 response, as the majority of studies found a correlation with a favorable outcome.[28,69] A possible explanation might be that different microenvironments favor infiltration of or differentiation toward more or less tumor promoting immune cell phenotypes. Not only Th17 cells, but also innate cell types capable of producing IL-17 may be correlated with improved prognosis, as we and others have shown for mast cells.[37,39] Although we cannot discriminate whether the cancer type or method used is more important for the correlations found, it is likely that both are important for the cell source studied and thus clinical outcome.

Based on the findings described in the current review, cancer patients with high total IL-17 protein levels might benefit from anti-IL-17 treatment, blocking the tumor promoting response. Adoptive transfer of Th17 cells might be another promising treatment. The feasibility of both approaches needs to be investigated further. Human Th17 cells can be induced by a combination of IL-1β, IL-6 and IL-23, although the exact conditions required are still under debate.[78] The functions of differentially obtained Th17 cell populations should be determined by functional studies. Animal models may be helpful to clarify this, as the induction of Th17 cells by IL-6 and TGF-β is clearer in mice than in humans, although the described effects on survival are still contradictory.[12,79]

We conclude that while IL-17 primarily promotes tumorigenesis, the subpopulation of IL-17 producing Th17 cells seems to have a tumor suppressing effect. Future research should use methodology that makes a distinction between soluble IL-17 protein, Th17 cells and other IL-17$^+$ cells. This will help to determine whether IL-17 and Th17 cells should be targeted or used in a clinical setting.

Materials and Methods

Study design

A systematic search in the NCBI PubMed, Ovid Embase and Web of Science bibliographic databases was conducted without language restriction in collaboration with information specialist JML following the PRISMA guidelines.[80] Since IL-17 is frequently studied in autoimmune diseases and together with tumor necrosis factor, these terms were excluded as major topic. The full search terms are provided in Supplementary Table S9.

Selection criteria

All original research studies reporting on an IL-17A or Th17 cell measurement and overall (OS) or disease-free survival (DFS) in human cancer published until 10 September 2014 were included. Articles that did not meet all inclusion criteria (e.g. conference abstracts; studies on IL-17 producing γδTCR or $CD8^+$ cytotoxic T cells or only describing remission or progression-free survival) were excluded. All relevant references in reviews were manually checked for presence in the systematic search.

Data collection and analysis

Survival analyses, generally Kaplan-Meier survival curves, a log rank test and Cox regression analyses, but in some cases a Spearman's rank correlation,[33,68] Wilcoxon signed-rank test[29] or Satterwaithe t-test[61] were obtained. All included articles were reviewed by both SP and ESJ. If extracted data did not match, the data were discussed until a consensus was reached. Cox regression hazard ratios and confidence intervals were used to perform a meta-analysis. The authors of all articles that did not provide sufficient information on study details, clinico-pathological data or Cox regression analyses were contacted via e-mail. Due to the number of studies with sufficient data per sample type studied, only the analyses of the number of $IL\text{-}17^+$ cells in FFPE tissue were suitable for meta-analysis. To keep the data as comparable as possible, scores in the tumor center were used in the cases where data were reported on different scores (e.g. invasive margin, peritumor).

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Supplementary Table S1. Details of the studies on IL-17 in serum

Ref #	Inclusion criteria	Risk of bias (study level)	Risk of bias (outcome level)
25	absence of inflammatory disease, chronic liver disease, allergies or other concomitant diseases capable of interfering with the IL-17 assay		potential: cutoff value based on ROC curve
26	primary HCC without extrahepatic metastasis or prior therapy; Child-Pugh class A; no thrombus in main veins		potential: cutoff value based on ROC curve
31	not receiving multiple allogeneic SCTs	potential: consecutive patients, but relatedness of donor to patient and GVHD prophylaxis differed among patients	negligible: correlation determined for continuous variables
17	no prior RT/CT or other medical interventions		potential: cutoff value based on most significant difference in prognosis
24			potential: cutoff value based on ROC curve
32			
33		negligible: consecutive patients	negligible: measurement divided by median
34			negligible: measurements divided by median
36			negligible: correlation determined for continuous variables
35			

Inclusion criteria and risks of bias at study and outcome level provided for the studies on IL-17 measured by ELISA reported in Table 1. An empty cell means no data were reported on this topic. A shaded cell indicates a potential risk of bias. Abbreviations: CT=chemotherapy; GVHD=graft-versus-host disease; HCC=hepatocellular carcinoma; RT=radiotherapy

Supplementary Table S2. Details of the studies on IL-17 in tissue

Ref #	Inclusion criteria	Risk of bias (study level)	Risk of bias (outcome level)
40	no distant metastasis or receiving prior anticancer therapy	negligible: consecutive patients	negligible: measurements divided by median
18		negligible: consecutive patients	potential: cutoff value determined by minimum p value approach
41	no prior anticancer therapy, metastasis or concurrent autoimmune disease		
19	no prior anticancer therapy or concurrent autoimmune disease, HIV or syphilis		potential: cutoff value determined by minimum p value approach
42	no concurrent HCV/HIV infection, autoimmune disease or alcoholic liver disease		negligible: measurement divided by median
43			negligible: measurements divided by mean
44	no metastasis to lymph nodes beyond the hepatoduodenal ligament		negligible: measurements divided by median
20			potential: cutoff value determined by minimum p value approach
45			negligible: measurements divided by mean
16			potential: cutoff value arbitrarily chosen
46	no prior RT/CT treatment		negligible: measurement determined as positive/negative
15	no prior anticancer therapy		potential: cutoff value arbitrarily chosen
30	TNM stage I-III and available clinical follow-up data	potential: if no prechemotherapy biopsy was available, a post-chemotherapy sample was used to score IL-17	negligible: measurements divided by mean

50	effective resection, no prior anticancer therapy and without distant metastasis, autoimmune disease, HIV or syphilis		negligible: measurements divided by median
37	primary surgical treatment, no prior anticancer therapy and sufficient material available for analysis	negligible: consecutive patients	negligible: measurements divided by median
47	no concurrent autoimmune diseases or incomplete clinico-pathological data		negligible: measurements divided by median
49	no prior anti-cancer radio-, chemo-, and bio-therapy		negligible: measurement divided by more/less than 10% positive
48	no prior RT, CT or immune therapy or autoimmune or infectious diseases		negligible: measurements divided by median
51	no prior anticancer treatment or autoimmune disease		negligible: measurements divided by median
52	no autoimmune diseases, other esophageal cancers or prior anticancer treatment		negligible: measurements divided by median
21	FIGO stage II undergoing primary radical hysterectomy and pelvic lymphadenectomy without preoperative CT/RT		potential: cutoff value determined by minimum p value approach
28			potential: cutoff value determined by regression tree analysis
29	no liver metastases, grossly residual tumors, immediate recurrence, ampullary/neuro-endocrine/ undifferentiated cancer or autoimmune pancreatitis		potential: correlation determined for continuous variables, but extremely long surviving patients (n=6) were compared with poor surviving patients (n=6)
22			potential: cutoff value determined by minimum p value approach
53			negligible: measurements divided by median

23	stage III–IV with no prior anti-cancer or anti-inflammatory treatment and sufficient histological sections for immunohistochemical staining	potential: cutoff value determined by minimum p value approach
54		negligible: measurement divided by more/less than 25% positive
39*	no concurrent autoimmune diseases, distant metastasis or neoadjuvant therapy	negligible: measurements divided by median
55*	no prior CT/RT/anti-inflammatory treatment	negligible: measurement determined as positive/negative
56*	no prior anticancer therapy or patients that were immunocompromised	negligible: measurements divided by median

Inclusion criteria and risks of bias at study and outcome level provided for the studies on IL-17 measured by immunohistochemistry reported in Table 1. An empty cell means no data were reported on this topic. A shaded cell indicates a potential risk of bias. An asterisk behind a reference number indicates that the study was not included in the quantitative analyses. Abbreviations: CT=chemotherapy; FIGO=Fédération Internationale de Gynécologie et d'Obstétrique; RT=radiotherapy

Supplementary Table S3. Details of the studies on Th17 cells

Ref #	Inclusion criteria	Risk of bias (study level)	Risk of bias (outcome level)
37	primary surgical treatment, no prior anticancer therapy and sufficient material available for analysis	negligible: consecutive patients	negligible: measurements divided by median
59		negligible: consecutive patients	negligible: measurements divided in groups (absent, 1-10, > 10 pos cells/TMA spot)
41	no prior anticancer therapy, metastasis or concurrent autoimmune disease		
62	no prior RT/CT treatment		negligible: measurement divided by median
32			negligible: measurement divided by median

38		negligible: samples obtained before (n=55) and after treatment (n=11), effect remained significant	negligible: measurements divided by median
61	MART-1/Melan-A/HMB-45/tyrosinase/HLA-A*0201 expression; no autoimmune disease, steroid dependence or prior ipilimumab treatment		negligible: measurements divided by median
60	patients aged 18 - 75 years with the largest tumor diameter ≥ 5 cm, no previous treatment or concurrent diseases (see reference for details)	negligible: consecutive patients	negligible: measurements divided by median
33		negligible: consecutive patients	negligible: measurement divided by median
64			negligible: means compared
63			negligible: measurements divided by integer value near the mean/median value
65*	unresectable distant metastases at the time of blood draw, T cell reactivity against Melan-A and available survival follow-up data		negligible: measurement determined as positive/negative
66*			negligible: measurements divided by median
57			
58		potential: sample size possibly too small	potential: not very sensitive method (presence of RT-PCR product bands)

Inclusion criteria and risks of bias at study and outcome level provided for the studies on Th17 cells measured by immunohistochemistry, flow cytometry or PCR analysis reported in Table 1. An empty cell means no data were reported on this topic. A shaded cell indicates a potential risk of bias. An asterisk behind a reference number indicates that the study was not included in the quantitative analyses. Abbreviations: CT=chemotherapy; RT=radiotherapy

Supplementary Table S4. Details of studies on IL-17 or Th17 cells in tumor associated fluid

Ref #	Inclusion criteria	Risk of bias (study level)	Risk of bias (outcome level)
27		negligible: consecutive patients	potential: cutoff value based on ROC curve
69		potential: 2 different chemotherapy regimens were given	negligible: measurements divided by median
67	no anticancer therapy, corticosteroids, nonsteroid anti-inflammatory drugs, invasive procedures in the pleural cavity or suffering chest trauma within 3 months prior to hospitalization		negligible: measurements divided by median
68	not receiving disease-modifying therapy; no autoimmune disease, empyema, chest trauma, pregnancy or lactation before recruitment		negligible: correlation determined for continuous variables
70	not receiving prior anticancer therapy or having autoimmune disease, inflammatory bowel disease or viral infections		negligible: measurement divided by median

Inclusion criteria and risks of bias at study and outcome level provided for the studies on IL-17 and Th17 measured on tumor associated fluids reported in Table 1. An empty cell means no data were reported on this topic. A shaded cell indicates a potential risk of bias.

Supplementary Table S5. Clinico-pathological data of the studies on IL-17 in serum

Ref #	Cancer type	Histology	Stage	Female (%)	Median age (range)	Median follow-up (months) (range)
25	NSCLC	46,9% SCC, 53,1% AC		40%	54 (mean 59 ± 11)	24 (3 - 81) for living patients
26	HBV-related HCC	71% Edmondson-Steiner grade I-II, 30% III-IV	4% BCLC 0, 79% A, 17% B	13%	53 (46 - 60)	20
31†	Leukemia treated with allogeneic SCT after myeloablative conditioning	55% AML, 40% ALL, 5% CML	87% standard, 13% advanced	42%	32 (15 – 57)	17 (9 - 26) for surviving transplants
17	Gastric carcinoma	52% differentiated, 48% undifferentiated	69% TNM I-II, 31% stage III-IV			
24†	CRC	4% well, 89% moderate, 6% poorly differentiated	18% TNM I, 37% II, 28% III, 17% IV	45%	67 (30 - 91)	mean 44 (32-60)
32	Acute leukemia	68% AML, 32% ALL		45%	19 - 63	
33	CLL		31% Rai 0, 21% I, 30% II, 6% III, 12% IV	55%	64 (38 - 87)	9 (1 - 124)
34	CLL		64% Rai 0-1, 25% 2-4		65 (34 - 91)	
36†	Pancreatic AC		11% TNM II, 24% III, 65% IV	37%	65 (31 - 80)	7 (1 - 66)
35	Multiple myeloma		12% ISS I, 36% II, 52% III	38%	mean 60 ± 7	23 (8 - 35)

Tumor histology and stage, female/male distribution and median age and follow-up are provided for the studies on IL-17 measured by ELISA reported in Table 1. Most studies provided the median and range of the age and follow-up distributions. Indications are given in cases where other units were provided, mainly the mean and standard deviation. Data of the studies by Tseng et al.[24], Yamada et al.[17] and Hus et al.[33] are provided for the total study cohort rather than the subpopulation IL-17 was measured in. An empty cell means no data were reported on this topic. A dagger behind a reference number indicates that data have (partially) been obtained via e-mail. Abbreviations: AC=adenocarcinoma; ALL=acute lymphoid leukemia; AML=acute myeloid leukemia; BCLC=Barcelona Clinic Liver Cancer Classification; CLL=chronic lymphocytic leukemia; CML=chronic myeloid leukemia; CRC=colorectal carcinoma; HCC=hepatocellular carcinoma; ISS=International Staging System; NSCLC=non-small cell lung carcinoma; SCC=squamous cell carcinoma

Supplementary Table S6. Clinico-pathological data of the studies on IL-17 in tissue

Ref #	Cancer type	Histology	Stage	Female (%)	Median age (range)	Median follow-up (months) (range)
40	HCC	79% Edmonson grade I-II, 21% III-IV	48% TNM I, 52% II-III	14%	51% ≤ 50	60 (2 - 74)
18†	HCC	73% grade I-II, 27% III-IV	67% TNM I, 33% II-III	16%	52% ≤ 53	53 (18-79)
41	HCC					
19†	HCC	56% Edmonson grade I-II, 44% III-IV	76% TNM I-II, 24% III	12%	46 (17 - 71)	44 (2-80)
42	HCC	16% well, 70% moderate, 14% poorly differentiated	61% TNM I-II, 39% III-IV	11%	mean 54 ± 10	36 (2 - 73)
43	HCC		58% TNM I-II, 42% III-IV	15%	80% ≤ 60	
44†	Intrahep. cholangio-carcinoma	massforming type; 70% Edmonson grade I-II, 30% III-IV	51% TNM I, 23% II, 26% III	50%	55 (18 - 78)	13 (4 – 111)
20†	CRC	22% mucinous colloid type; 63% well, 35% moderate, 2% poorly differentiated	12% TNM I, 33% II, 29% III, 26% IV	84%	26% < 65	36 (29-55)
45	CRC	15% well, 56% moderate, 30% poorly differentiated	33% TNM I-II, 67% III-IV	44%		
16	Colorectal AC	83% tubular, 17% mucinous type; 65% well-moderate, 35% poorly differentiated	TNM III	40%	62% < 60	
46	NSCLC	43% SCC, 52% AC, 5% ASC	41% TNM I, 26% II, 32% III	35%	mean 65 (40 - 73)	30
15	NSCLC	31% AC, 44% SCC, 25% other; 19% well-moderate, 81% poorly differentiated	63% TNM I-II, 37% III	21%	52 (29 - 77)	
30	Breast carcinoma	82% ductal, 4% lobular, 4% mixed, 10% mucinous, 1% metaplastic; 30% grade I, 40% II, 30% III	20% TNM I, 46% II, 34% III	100%	51 (23 - 78)	mean 67 (7 - 144)

50†	Gastric carcinoma	24% well-moderate, 75% poorly differentiated	44% TNM is/I/II, 56% III	30%	60 (33 - 89)	51 (39 - 57)
37	Cervical carcinoma	100% SCC	77% FIGO IB, 22% IIA, 1% IIB; 69% TNM I, 28% II, 3% III, 1% IV	100%	45 (22 - 87)	100 (1 - 296)
47†	Gall-bladder carcinoma	29% well, 38% moderate, 33% poorly differentiated	7% TNM I, 36% II, 46% III, 12% IV	61%	mean 66 ± 12	mean 39 (2 - 76)
49	Laryngeal carcinoma	100% SCC	24% TNM I, 23% II, 25% III, 28% IV	3%	59	58 (38-85)
48	Pancreatic AC	26% well, 33% moderate, 41% poorly differentiated	15% TNM I, 33% II, 39% III, 13% IV	35%	mean 61 (43 - 75)	5 - 48
51	Gastric AC	3% well, 21% moderate, 76% poorly differentiated	4% TNM I, 37% II, 49% III, 10% IV	33%	58 (17 - 85)	61 (0 - 82)
52	Esophag. carcinoma	100% SCC	65% TNM I-II, 35% III-IV	22%	56 (33 - 79)	44 (1 - 87)
21	Cervical carcinoma	86% SC, 12% AC, 2% ASC	57% FIGO IIA1, 41% IIA2, 2% IIB	100%	17% < 40	
28	Ovarian carcinoma with recurrence	94% high-grade serous papillary carcinoma	6% FIGO II, 84% III, 4% IV	100%	34 - 77	
29	Pancreatic ductal AC					
22	HCC	48% grade I-II, 52% III-IV	62% TNM I-II, 38% III	8%	51 (20 - 71)	
53	Nasopha-ryngeal carcinoma	1% SCC, 12% NKC, 87% UC	6% TNM I, 30% II, 37% III, 27% IV	21%	49 (22 - 73)	
23	Epithelial ovarian cancer	74% serous, 6% mucinous, 10% endometrioid, 1% clear cell, 10% AC; 12% grade I, 89% II-III	90% FIGO III, 10% IV	100%	53 (27 - 81)	
54	Giant cell tumors of bone	54% Campanacci grade I-II, 46% III	61% Enneking T1, 39% T2	55%	22% > 30	

39*	Esophageal SCC	19% well, 57% moderate, 24% poorly differentiated	7% TNM I, 46% IIA, 2% IIB, 34% III, 2% IV	26%	56 (23 - 82)	29 (2 - 157)
55*†	CRC	42% well, 29% moderate, 10% poorly differentiated	5% Duke's stage A, 32% B, 33% C, 29% D	41%	41% < 60	
56*	Glio-blastoma		TNM IV	56%	47 (14 - 65)	13 (4 - 24)

Tumor histology and stage, female/male distribution and median age and follow-up are provided for the studies on IL-17 measured by immunohistochemistry reported in Table 1. Most studies provided the median and range of the age and follow-up distributions. Indications are given in cases where other units were provided, mainly the mean and standard deviation. Data of the study by Lin et al.[22] are provided for the total study cohort rather than the subpopulation IL-17 was measured in. An empty cell means no data were reported on this topic. An asterisk behind a reference number indicates that the study was not included in the quantitative analyses. A dagger behind a reference number indicates that data have (partially) been obtained via e-mail. Abbreviations: AC=adenocarcinoma; CRC=colorectal carcinoma; FIGO=Fédération Internationale de Gynécologie et d'Obstétrique; HCC=hepatocellular carcinoma; NSCLC=non-small cell lung carcinoma; SCC=squamous cell carcinoma

Supplementary Table S7. Clinico-pathological data of the studies on Th17 cells

Ref #	**Cancer type**	**Histology**	**Stage**	**Female** (%)	**Median age** (range)	**Median follow-up** (months) (range)
37	Cervical carcinoma	100% SCC	67% FIGO IB, 31% IIA; 63% TNM I, 33% II, 2% III, 2% IV	100%	40 (22 - 75)	85 (2 - 296)
59	Differentiated thyroid carcinoma	95% papillary, 5% follicular	59% TNM I, 11% II, 15% III, 15% IV	82%	54% < 45	
41	HCC					
62	Gastric carcinoma	25% moderate, 75% poorly differentiated	28% TNM I-II, 72% III-IV	34%	54 (31 - 69)	
32	Acute leukemia	68% AML, 32% ALL		45%	19 - 63	
38	CLL		30% Rai 0, 35% I, 15% II, 12% III, 8% IV			

61†	Melanoma treated with ipilimumab (a-CTLA4)		39% TNM IIIc, 61% IV	41%	49% < 56	26 (14-68)
60†	HCC treated with transarterial chemoem-bolization		TNM III	20%	55 (26 - 71)	11 (1 - 35)
33	CLL		31% Rai 0, 21% I, 30% II, 6% III, 12% IV	55%	64 (38 - 87)	9 (1 - 124)
64†	hematologic malignancy treated with allogeneic HSCT	13% MDS, 63% AML, 23% ALL		23%	30 (2-66)	7
63†	HCC		BCLC A or B	31%	62 (41-81)	14
65*†	Melanoma		TNM IV	33%	53 (19 - 90)	12 (1-135)
66*†	Acute myeloid leukemia	7% M1, 15% M2, 27% M3, 30% M4, 18% M5, 2% M6, 1% M7		44%	46 (16 - 79)	9
57	CRC					
58	Ovarian carcinoma	24% serous cyst AC, 6% mucinous cyst AC, 41% clear cell, 29% endometr. AC	41% FIGO I, 12% II, 35% III, 12% IV			

Tumor histology and stage, female/male distribution and median age and follow-up are provided for the studies on Th17 measured by immunohistochemistry, flow cytometry or PCR analysis reported in Table 1. Most studies provided the median and range of the age and follow-up distributions. Indications are given in cases where other units were provided, mainly the mean and standard deviation. Data of the studies of Sarnaik et al.[61] and Hus et al.[33] are provided for the total study cohort rather than the subpopulation Th17 cells were measured in. An empty cell means no data were reported on this topic. An asterisk behind a reference number indicates that the study was not included in the quantitative analyses. A dagger behind a reference number indicates that data have (partially) been obtained via e-mail. Abbreviations: AC=adenocarcinoma; ALL=acute lymphoid leukemia; AML=acute myeloid leukemia; BCLC=Barcelona Clinic Liver Cancer Classification; CLL=chronic lymphocytic leukemia; CRC=colorectal carcinoma; FIGO=Fédération Internationale de Gynécologie et d'Obstétrique; HCC=hepatocellular carcinoma; SCC=squamous cell carcinoma

Supplementary Table S8. Clinico-pathological data of the studies on IL-17 or Th17 cells in tumor associated fluid

Ref #	Cancer type	Histology	Stage	Female (%)	Median age (range)	Median follow-up (months) (range)
27	Lung cancer with MPE	86% AC, 6% SCC, 8% small cell ca		54%	mean 56 ± 13	
69	Ovarian carcinoma	64% serous/ mucinous/endometroid, 36% clear cell/ undifferentiated; 13% grade G1,15% G2,69% G3,2% G4	6% FIGO II, 61% III, 33% IV		mean 61 (25 - 80)	
67	Lung carcinoma with MPE	37% SCC, 63% AC		60%	64 (32 - 81)	
68	Lung AC with pleura metastasis			33%	mean 50 ± 9	
70	Gastric carcinoma	52% differentiated, 48% undifferentiated	51% TNM I, 23% II, 27% III	27%	44% ≤65	61 (1 - 99)

Tumor histology and stage, female/male distribution and median age and follow-up are provided for the studies on IL-17 and Th17 measured in tumor associated fluids reported in Table 1. Most studies provided the median and range of the age and follow-up distributions. Indications are given in cases where other units were provided, mainly the mean and standard deviation. An empty cell means no data were reported on this topic. Abbreviations: AC=adenocarcinoma; FIGO=Fédération Internationale de Gynécologie et d'Obstétrique; MPE=malignant pleural effusion; SCC=squamous cell carcinoma

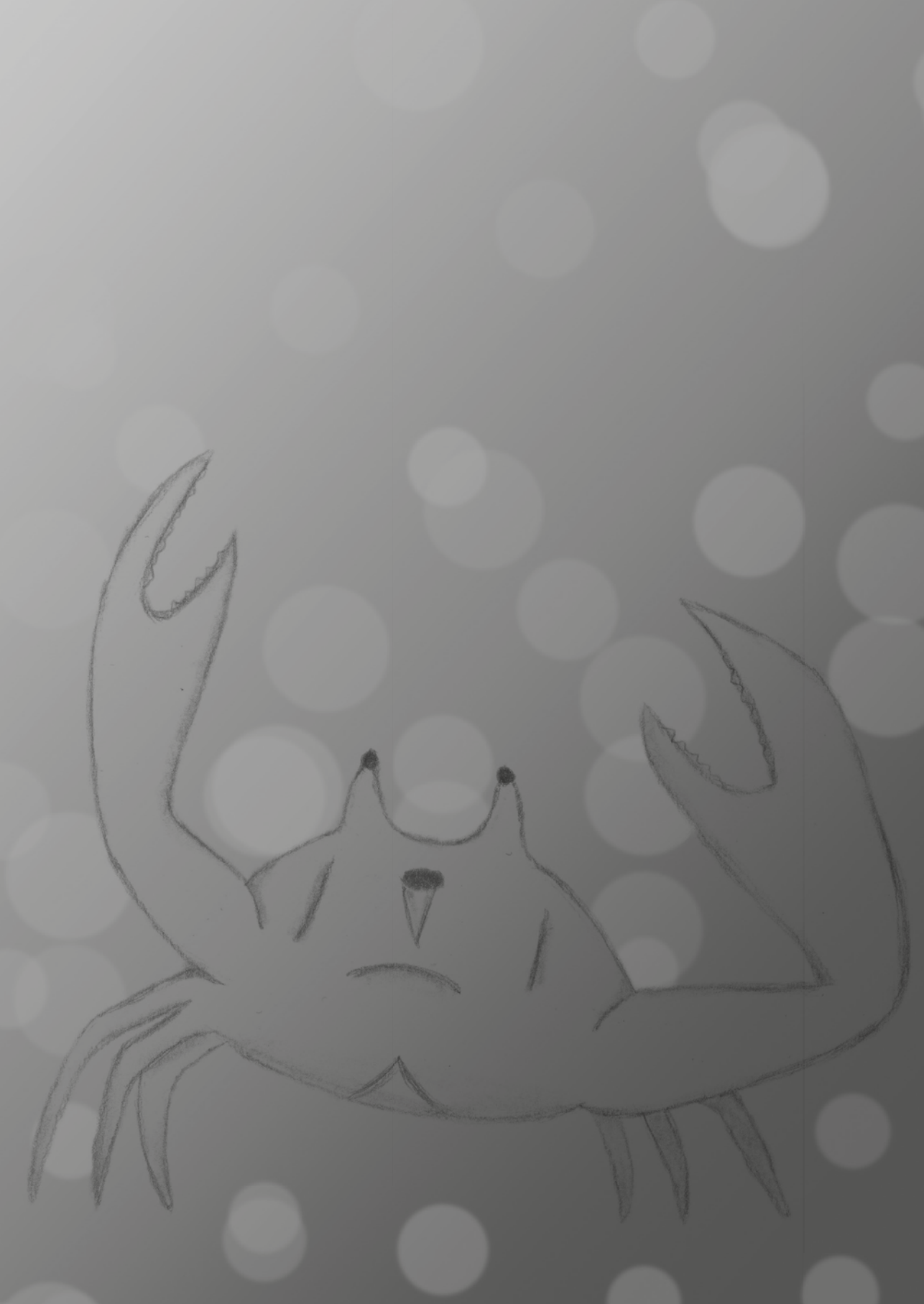
Supplementary Table S9. Database search strategies

Database	Search Strategy
PubMed	(("Interleukin-17"[majr] OR "Interleukin-17"[tiab] OR "Interleukin 17"[tiab] OR "Interleukin17"[tiab] OR "IL17"[tiab] OR "IL-17"[tiab] OR "Th17 Cells"[Majr] OR "Th17"[tiab] OR "Th-17"[tiab]) AND ("Neoplasms"[majr] OR "Neoplasms"[tiab] OR "Neoplasm"[tiab] OR "cancer"[tiab] OR "carcinoma"[tiab] OR "malignancy"[tiab] OR "malignancies"[tiab] OR "tumor"[tiab] OR "tumour"[tiab] OR "tumors"[tiab] OR "tumours"[tiab])) NOT ("Tumor Necrosis Factors"[Majr] OR "Tumor necrosis factor"[ti] OR "Tumour necrosis factor"[ti] OR "Tumor necrosis factors"[ti] OR "Tumour necrosis factors"[ti] OR "TNF"[ti] OR "Autoimmune Diseases"[Majr] OR “autoimmune”[ti] OR “auto-immune”[ti] OR “autoimmunity”[ti] OR “arthritis”[majr] OR “arthritis”[ti] OR “arthritic”[ti] OR “multiple sclerosis”[ti] OR “psoriasis”[majr] OR “psoriasis”[ti] OR “inflammatory bowel diseases”[majr] OR “inflammatory bowel disease”[ti] OR “inflammatory bowel diseases”[ti] OR “Crohn Disease”[majr] OR “crohn”[ti] OR “crohn’s”[ti] OR “lupus”[ti] OR “colitis”[majr] OR “colitis”[ti])
Embase	((*interleukin 17/ OR "Interleukin-17".ti,ab. OR "Interleukin 17".ti,ab. OR "Interleukin17".ti,ab. OR "IL17".ti,ab. OR "IL-17".ti,ab.) AND (*neoplasm/ OR "Neoplasms".ti,ab. OR "Neoplasm".ti,ab. OR "cancer".ti,ab. OR "carcinoma".ti,ab. OR "malignancy".ti,ab. OR "malignancies".ti,ab. OR "tumor".ti,ab. OR "tumour".ti,ab. OR "tumors".ti,ab. OR "tumours".ti,ab.)) NOT (*tumor necrosis factor/ OR "Tumor necrosis factor".ti. OR "Tumour necrosis factor".ti. OR "TNF".ti. OR exp *autoimmune disease/ OR "autoimmune".ti. OR "auto-immune".ti. OR "autoimmunity".ti. OR exp *arthritis/ OR "arthritis".ti. OR "arthritic".ti. OR *multiple sclerosis/ OR "multiple sclerosis".ti. OR exp *psoriasis/ OR "psoriasis".ti. OR *inflammatory bowel disease/ OR "inflammatory bowel disease".ti. OR "inflammatory bowel diseases".ti. OR *crohn disease/ OR *ulcerative colitis/ OR "crohn".ti. OR "crohn’s".ti. OR "lupus".ti. OR "colitis".ti.)
Web of Science	(TS=("Interleukin-17" OR "Interleukin 17" OR "Interleukin17" OR "IL17" OR "IL-17") AND TS=("Neoplasms" OR "Neoplasm" OR "cancer" OR "carcinoma" OR "malignancy" OR "malignancies" OR "tumor" OR "tumour" OR "tumors" OR "tumours")) NOT TI= ("tumor necrosis factor*" OR "tumour necrosis factor*" OR "TNF" OR "autoimmune" OR "auto-immune" OR "autoimmunity" OR "arthritis" OR "arthritic" OR "multiple sclerosis" OR "psoriasis" OR "inflammatory bowel disease" OR "inflammatory bowel diseases" OR "crohn" OR "crohn’s" OR "lupus" OR "colitis")

The specific search strategies per database are indicated.

VIII

Conclusion and Discussion



Cervical cancer is caused by a persistent HPV infection. Although this infection induces an immune response that can generally clear the infection,[1] chronic inflammation can promote tumor growth by causing DNA mutations, promoting tumor cell survival and suppressing the adaptive immune response.[2,3] Because the roles of IL-17 and Th17 cells in cancer have been controversial and seem to be tissue and context dependent,[4] the aim of this thesis was to elucidate the roles of IL-17 and Th17 cells in cervical cancer. **Chapter 1** provides an introduction on cervical cancer, the immune response in general and the IL-17 and Th17 cell immune response.

In **chapter 2**, the roles of IL-12, IL-23, IL-6 and IL-1β were described. We showed by RNA in situ hybridization that *IL12p35, IL12p40 and IL23p19* were predominantly expressed by tumor cells, while the immunohistochemical stainings showed that IL-1β was mainly expressed by stromal cells and IL-6 was expressed by both tumor cells and infiltrating immune cells. A high level of *IL12p40* expression represented a beneficial IL-12 related immune response. *IL12p35* was never a limiting factor, and the only significant correlation was found between *IL12p35* and *IL12p40* levels. Since the affinity of *IL12p40* for *IL12p35* differs from the affinity for *IL23p19*, our data suggested that IL-12 is produced if high levels of *IL12p40* are present, while IL-23 is produced when *IL23p19* is present in combination with only low levels of *IL12p40*.
While a high frequency of stromal IL-6$^+$ cells was strongly correlated with poor survival, especially a high number of IL-6$^+$ cells combined with IL-23 was correlated with worse prognosis. Interestingly, the absence of the shared *IL12p40* subunit was also correlated with improved survival as compared with low *IL12p40* levels.[5] Since the absence of *IL-12p40* implicates the absence of both IL-12 and IL-23, this indicates that the absence of IL-23 was more favorable than the presence of IL-12. Specifically IL-23 secretion rather than the absence of IL-12 was thus correlated with poor survival, especially when combined with a high number of IL-6$^+$ cells.
IL-6 and IL-23 are part of a response correlated with poor survival in cervical cancer, which is schematically indicated in a model of the IL-17 and Th17 cell immune response axes in cervical cancer in Figure 1 (page 184). Both IL-6 and IL-23 have been correlated with poor survival in different cancer types as well. This is thought to be due to the attraction of macrophages and neutrophils in the tumor microenvironment,[6] or direct stimulation of tumor growth.[7] Because both IL-6 and IL-23 also play an important role in the stimulation of a Th17 cell immune response,[8] we hypothesized that Th17 cells may have an immunosuppressive function in cervical cancer.

To study the function of Th17 cells in cervical cancer, we analyzed the frequency and localization of Th17 cells using immunohistochemistry (**chapter 3**). We did not find tumor cells expressing IL-17. Only a minority of tumor infiltrating IL-17$^+$ cells were CD3$^+$IL-17$^+$ Th17 cells. This was further confirmed by using different antibodies against both CD3 and IL-17, as well as an antibody against CD4 (data not shown), and

only approximately 5% of all IL-17 expressing cells were found to be T cells. By performing double and triple immunostainings for a panel of immune cell phenotype markers, we found that 66% of the IL-17$^+$ cells were neutrophils and 23% were mast cells. In a variety of different common types of cancer, we also found that IL-17 was mainly expressed by granulocytes, while Th17 cells were always a minor cell population. This indicates that the distribution of cell populations expressing IL-17 was not specific to cervical cancer. Interestingly, while IL-17 was found to be correlated with poor survival in early stage squamous cervical cancer, Th17 cells were correlated with improved disease-specific survival. As a potential mechanism for tumor growth in early stage disease, IL-17 was shown to increase the growth or intercellular adhesion of cervical cancer cell lines. These data suggest that IL-17 has a tumor promoting role in squamous cervical cancer, while Th17 cells are likely to stimulate a tumor targeting immune response (indicated in Figure 1).

While the role for Th17 cells in a tumor targeting immune response can be understood from their Th1 cell and CTL stimulating functions, the roles of both IL-17 and neutrophils in cancer are less clear. Neutrophil infiltration has been correlated with angiogenesis and poor survival in cancer,[9,10] but extensive research has been lacking. Immature myeloid cells, which also comprise neutrophils, have recently been shown to be correlated with IL-17 expression, CCL4 dependent T helper cell infiltration and tumor growth in a melanoma mouse model, indicating that the myeloid cells may create a pro-inflammatory environment that induces tumor progression.[11] A high total number of neutrophils only showed a trend toward poor survival in our study on squamous cervical cancer. This may be due to the heterogeneity of this cell population, suggesting that IL-17 may particularly be expressed by alternatively activated neutrophils. Several of the pro-inflammatory cytokines that induce IL-17 production are also involved in the generation of myeloid derived suppressor cells (MDSCs),[12] suggesting that the IL-17 response may also be correlated with concurrent infiltration of MDSCs, which is generally correlated with poor prognosis.[13] Although granulocytic MDSCs have been described to resemble neutrophils, and there is no consensus on the markers to identify MDSCs yet,[14] we did not find substantial overlap between the expression of granulocyte marker CD15 and MDSC marker CD33. We thus concluded that IL-17 was mainly expressed by CD15$^+$MPO$^+$ neutrophils, but we have not studied their correlation with (CD33$^+$) MDSCs. Another mechanism for IL-17 to promote tumor growth is their potential to induce macrophage programmed death-ligand 1 (PD-L1) expression, which suppresses CTL activity.[15]

Since IL-17 signaling can induce IL-6 and IL-8 production,[16] and IL-8 induces neutrophil recruitment,[17] IL-17 production by neutrophils may induce a positive feedback loop to attract more neutrophils, leading to more IL-17 production. The transcription factor RORγt induces IL-17 production and has been described to be expressed by both innate and adaptive IL-17-producing cells.[18] IL-23 and RORγt have been shown to induce GM-CSF expression, which induces neutrophil infiltration and

activation.[19,20] This may further amplify the positive feedback loop between IL-17 production and neutrophil attraction. IL-6 and IL-23 may thus primarily induce neutrophils to produce IL-17, rather than T cells (indicated in Figure 1).
The role that we have shown for innate IL-17 producing cells in squamous cervical cancer is probably tissue and context dependent. Indeed, we found different distributions of IL-17 producing cells in the different cancer types studied. However, in all cancer types studied, the frequency of granulocytes producing IL-17 exceeded the Th17 cell frequency. The obvious next question was whether the role of Th17 cells and other IL-17^{+} cells is similar in cervical adenocarcinoma.

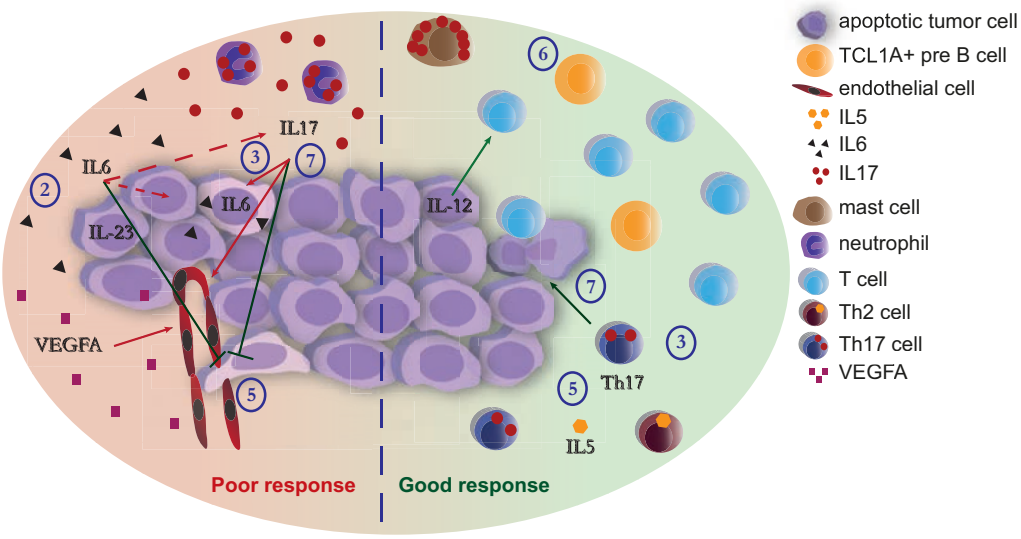


Figure 1. The IL-17 and Th17 cell immune response axes in cervical cancer

Schematic representation of the results obtained from the studies described in this thesis. The encircled numbers indicate the chapters in which the indicated correlations have been described. IL-12 was described to stimulate a tumor targeting Th1/CTL response (discussed in chapter 2), while T cells were correlated with improved survival (chapter 5). Angiogenesis induced by VEGFA has been shown to characterize a detrimental tumor microenvironment, as was the case for IL-6 (chapter 5). IL-6 is produced by both tumor and infiltrating immune cells (chapter 2), and may directly promote tumor growth (interrupted arrow). IL-6 and IL-23 may also stimulate IL-17 production (interrupted arrow), which characterizes a poor immune response when produced by non-Th17 cells, predominantly neutrophils (chapter 3). IL-17 may directly stimulate tumor growth, angiogenesis and neutrophil infiltration. The IL-6 and IL-17 response may prevent metastatic spread via the vasculature (blunted arrows; chapter 3, 5). Th17 cells may stimulate the tumor targeting T cell response, and might counteract the tumor promoting effects of IL-6, especially when combined with an IL-5 response (chapter 5). The functions of IL-17 and Th17 cells are probably tumor type and context dependent, as described in chapters 4 and 7.

We and others have observed that the immune response present in cervical adenocarcinoma differs substantially from the immune response in squamous cervical cancer (reference 21 and unpublished data). The correlations between the number of total T cells, Tregs, Th17 cells and other IL-17 expressing cells and survival in cervical adenocarcinoma were described in **chapter 4**. The most significant correlation was

found between a high number of total intratumoral (intraepithelial and stromal) Tregs and improved disease-specific survival. IL-17 could further discriminate between patients with a low Treg frequency and poor prognosis, which was worse in case of a low frequency of IL-17$^+$ cells. Low Tregs and the presence of Th17 cells was also correlated with worse prognosis. This contradicts our results in squamous cervical cancer, and shows that the roles of Tregs, Th17 cells and other IL-17$^+$ cells are context and tumor (sub)type dependent. In cervical adenocarcinoma, a pro-inflammatory environment might attract classically activated innate immune cells that suppress tumor growth. Tregs might then suppress a harmful tumor promoting immune response of other immune cells, including Th17 cells and other T helper cells. A high number of intraepithelial T cells was correlated with improved prognosis, which was not due to Tregs or Th17 cells. The intraepithelial T cells might predominantly be cytotoxic T lymphocytes (CTL), but this cell population was not studied.

The correlations between different immune response and vessel formation pathways present in the tumor microenvironment were described in **chapter 5**. In fresh frozen squamous cervical cancer samples, we studied markers for immune cell subpopulations that had been shown to be correlated with cancer progression in the literature, using qRT-PCR analysis. The correlations between the different markers were analyzed by weighted gene co-expression network analysis followed by mixed-model analyses. To identify the genes that are most strongly related with patient survival, correlations with survival were studied at single gene expression level. While high expression of T cell markers (*CD3E*, *CD8A, FOXP3*) was correlated with improved prognosis, *IL6* and angiogenesis marker *VEGFA* were correlated with poor disease-specific survival in squamous cervical cancer. Especially a high *IL6*/*IL17* ratio combined with low *IL5* expression was strongly correlated with poor survival. Using qRT-PCR analysis, we found very low expression levels of *IL17* and the neutrophil markers fucosyltransferase 9 (*FUT9*) and neutrophil elastase (*NE*). Since mature neutrophils have been shown to express no or very low mRNA levels for granule proteins,[22,23] the *IL17A* RNA expression is probably mainly derived from Th17 cells. Th17 cell derived *IL17* could thus counteract the tumor promoting effects of *IL6*, even more so combined with a Th2 response characterized by *IL5* (indicated in Figure 1). We concluded that measuring *IL6,* especially in combination with *IL5* and *IL17* expression may improve the accuracy of predicting patient survival.

We also found a significant correlation between *IL6* expression and the absence of vaso-invasion, while *IL1β* expression showed a trend toward a significant correlation. Total IL-17$^+$ cells, IL-1β$^+$ cells and neutrophils were also significantly correlated with the absence of vaso-invasion (described in chapter 3). This suggests that this type of inflammatory response may prevent metastatic spread of the tumor cells via the blood or lymphatic vasculature (indicated in Figure 1). Together, these data support the development of combined anti-IL-6 and anti-VEGF therapies. Because of the

correlation with absence of vaso-invasion, blocking IL-6 might increase the risk of tumor cell invasion. Since VEGFA expression has been correlated with tumor invasiveness,[24,25] and the presence of vaso-invasion negatively affects clinical outcome, blocking both IL-6 and VEGFA has the potential to counteract both tumor growth and invasion. Blocking antibodies to neutralize IL-6 have been shown to lead to some clinical responses in different solid cancer types.[26-28] VEGFA neutralizing antibodies have led to clinical responses in cervical cancer.[29,30] These results warrant further investigation of the clinical effects of simultaneously blocking IL-6 and VEGFA in cervical cancer.

To further examine which factors are most important for patient survival, and determine whether these are derived from the tumor epithelial cells or infiltrating immune cells, we studied total mRNA sequence (RNA-seq) data from cervical cancer cell suspensions flow-sorted into a tumor cell and immune cell fraction (**chapter 6**). To test whether any pathways were differentially expressed, we performed weighted gene co-expression network analysis for the tumor and infiltrate samples.[31] The tumor cell samples as well as the infiltrating immune cell samples showed clear cell source dependent clustering. However, although we found a substantial number of gene clusters, none of the clusters was significantly correlated with clinical outcome or *IL17* expression after correcting for multiple testing. This indicates that the cell sorting technique worked well, and both the tumor cells and immune cells had similar expression patterns among patients when considering clinical outcome. The samples were thus relatively homogeneous, and the pathway toward tumorigenesis may be comparable between patients. This technique may therefore be useful to analyze and map differences between tumor types.

Differential gene expression analysis of the sequencing data revealed that *TCL1A* was not expressed in lymphocytes of patients that had died within five years after surgery. This was confirmed by qRT-PCR and immunohistochemical analyses. We subsequently found that TCL1A was expressed by a subpopulation of B lymphocytes. Furthermore, high expression of *CD19* and a high *TCL1A/CD20* ratio were significantly correlated with improved survival on another cervical cancer patient cohort. These results suggest that intratumoral B cells are crucial to control cervical cancer, and the $TCL1A^+$ and $CD20^+$ B cell populations play an important role (see Figure 1). This warrants further investigation for the potential of B cells in anticancer treatment.

We also studied the differential gene expression when comparing *IL17* RNA-seq positive with *IL17* RNA-seq negative immune cell samples. *IL17* was expressed at very low levels, corresponding with the small number of Th17 cells we have observed in cervical and other types of cancer (described in chapter 2). Additionally, the main cell source of IL-17 was the neutrophil, which has a short lifespan and is unlikely to have survived the sample preparation.[32] We could not further elucidate the function of IL-17 or Th17 cells in cervical cancer using this technique, because no genes were differentially expressed in the immune cell fractions. Only three genes were expressed

at increased levels in the tumor cell fractions when comparing samples with matched *IL17*$^{+}$ versus *IL17*$^{-}$ immune cell fractions. The most significantly differentially expressed gene, *OMA1* zinc metallopeptidase, is involved in mitochondrial quality control, acting in stress situations.[33] Since Th17 cells have been described to function well in hypoxic or other stress conditions, the tumor *OMA1* response and Th17 cell immune response might be linked.

IL-17 is usually regarded as a marker for Th17 cells. A limited number of studies has been published on the correlation between IL-17 or Th17 cells and cancer patient survival. The opposing correlations between IL-17 versus Th17 cells and survival described in cervical cancer in this thesis might explain the controversy about their role in cancer. To test this hypothesis, we specifically studied the correlations between IL-17 versus Th17 cells and survival in cancer patients in a systematic literature review described in **chapter 7**. We found that high IL-17 measurements were three times more frequently correlated with poor than with improved survival in cancer. A high Th17 cell frequency on the other hand was four times more frequently correlated with improved than with poor survival. So although the type of tumor microenvironment certainly determines for an important part whether IL-17 and Th17 cells have a tumor promoting or suppressing function, IL-17 seems to generally induce tumor promoting angiogenesis and neutrophil recruitment,[9,17,34] corresponding with our results in squamous cervical cancer (described in chapter 3 and illustrated in Figure 1). Since IL-17 can be produced by a variety of cell types, the cell types present will also determine the type of immune response. Th17 cells seem to be predominantly involved in tumor suppression, potentially by stimulating Th1 and CTL immune responses (indicated in Figure 1).[35,36] Since Th17 cells are a subpopulation of IL-17^{+} cells and had a different correlation with prognosis than total IL-17, it is important to distinguish between Th17 and other IL-17^{+} cells. The systematic review could thus substantiate our hypothesis on the opposing roles of IL-17 and Th17 cells in cancer.

Future prospects

The studies and insights described in this thesis have also led to new research questions. The most essential topics that require further investigation are described in this section of the discussion. For a start, the finding that IL-17 is predominantly expressed by neutrophils rather than Th17 cells in cancer, raises some interesting discussion points. For example, although the requirements for the differentiation of naïve T cells to Th17 cells are still under debate, the conditions required to induce IL-17 production by neutrophils are completely unknown. The expression of RORγt has been shown to induce IL-17 in mouse neutrophils,[37] but whether its expression is required for or supporting IL-17 production in human neutrophils is not known. Knowledge about the cytokines and transcription factors involved in IL-17 expression in neutrophils would

provide insight in the type of immune response that induces IL-17 production and its associated effects.
The phenotypical differences between classically activated tumor targeting and alternatively activated tumor promoting neutrophils are still largely undetermined. Studying these different subpopulations could aid in identifying whether it is indeed alternatively activated cells that produce IL-17 and play a detrimental role in cervical cancer.[38]
There may also be substantial differences between the sources of IL-17 in autoimmune diseases, which would be interesting to investigate. Several studies have already indicated that IL-17 is expressed by mast cells, macrophages, eosinophils and neutrophils in different autoimmune diseases.[39-44] Because the dependency of the function of secreted IL-17 on its cellular source is unclear, studying the composition of the microenvironment and the other stimulatory factors involved is important.

Although IL-17 was shown to predominantly correlate with poor survival and Th17 cells with improved survival, there was substantial variance in the magnitude of the effect. This might be attributable to the tumor type, which might even affect the direction of the effect (described in chapter 7). In squamous cervical cancer, we showed that Th17 cells were significantly and independently correlated with improved survival, and could counteract the tumor promoting effects of *IL6*. In contrast, Th17 cells showed a trend toward poor survival in cervical adenocarcinoma. IL-17 was predominantly expressed by neutrophils in squamous cervical cancer, and was significantly and independently correlated with poor survival in early stage cancer. In cervical adenocarcinoma, non-Th17 IL-17$^+$ cells showed a trend toward improved survival. The trends in adenocarcinoma thus contradict the general correlations described in this thesis. Whether this is true for adenocarcinomas in general needs to be studied further. Overall, the cancer types in which Th17 or other IL-17$^+$ cells play an important role should be determined. It would also be interesting to determine the cell types that produce IL-17 per cancer type or perhaps even per patient. To study the significance of IL-17 produced by Th17 cells, the other immune cell types or factors correlated with the IL-17 and Th17 cell immune response should be studied. Functional assays should also be performed to study whether Th17 cell derived IL-17 is crucial to stimulate a Th1/CTL immune response, or rather reflects plasticity of stem-cell like cells that can differentiate toward Th1 cells.
Similarly, the mechanisms behind the correlations between Tregs and intraepithelial T cells and improved prognosis in cervical adenocarcinoma should be investigated. Studying the immune cell types correlated with the Treg and intraepithelial T cell responses will provide insight into how these cell types might be used to guide patient prognosis and treatment.
Based on the findings described in chapter 7, cancer patients with high IL-17 levels might benefit from anti-IL-17 treatment. Neutralizing IL-17 has been shown to inhibit

tumor growth in a lymphoma mouse model.[45] Antibodies directed against human IL-17 and its receptor are already used in clinical trials to treat a variety of autoimmune diseases.[46] Adoptive transfer of Th17 cells might be another promising treatment, especially since adoptive transfer of Th17 cells in mice has been described as more efficient than Th1 cell transfer.[47-49] Specifically, IL-17 antibodies did not affect the tumor targeting effect of Th17 cells, while IFNγ antibodies abrogated their efficacy,[47] again indicating that the effects of Th17 cells and IL-17 are not identical in cancer. The feasibility of both approaches should be further investigated.

Finally, as mentioned previously, the efficacy of combination therapies should be investigated. Not only the combination of targeting IL-17 or IL-6 and VEGFA might be promising, but also combining immunotherapies and targeted therapies or radio- or chemotherapy. The potential efficacy of combination therapies lies beyond the scope of this thesis, but is a major interest of current investigations.

Conclusion

In conclusion, this thesis provides novel insights into the role of IL-17 and Th17 cells in cervical cancer. Figure 1 summarizes the results obtained from the studies described in this thesis. While IL-17 was shown to be predominantly produced by innate myeloid cells such as neutrophils and correlated with poor survival, Th17 cells were generally a small cell population correlated with improved survival. Since IL-6 and IL-23 were also found to be strongly correlated with poor survival, we hypothesize that these cytokines may induce IL-17 production by myeloid cells. Th17 cells may counteract this pro-inflammatory response characterized by IL-6 and IL-17. The IL-6/IL-17 response was correlated with the absence of vaso-invasion. The immune responses described are tissue and context dependent, as indicated by the predominant correlation between Tregs and improved survival in cervical adenocarcinoma. In addition to innate and T lymphocytes responses, B lymphocytes may also play an important role in cervical cancer. Few studies have investigated this cell type. We found that B cells expressing TCL1A are strongly correlated with improved survival in squamous cervical cancer.

These novel data will, together with the proposed future studies discussed in this chapter, provide a better understanding of the immune response in cervical cancer. This will advance our knowledge in cancer evolution, diagnostics, prognostics and therapeutics.

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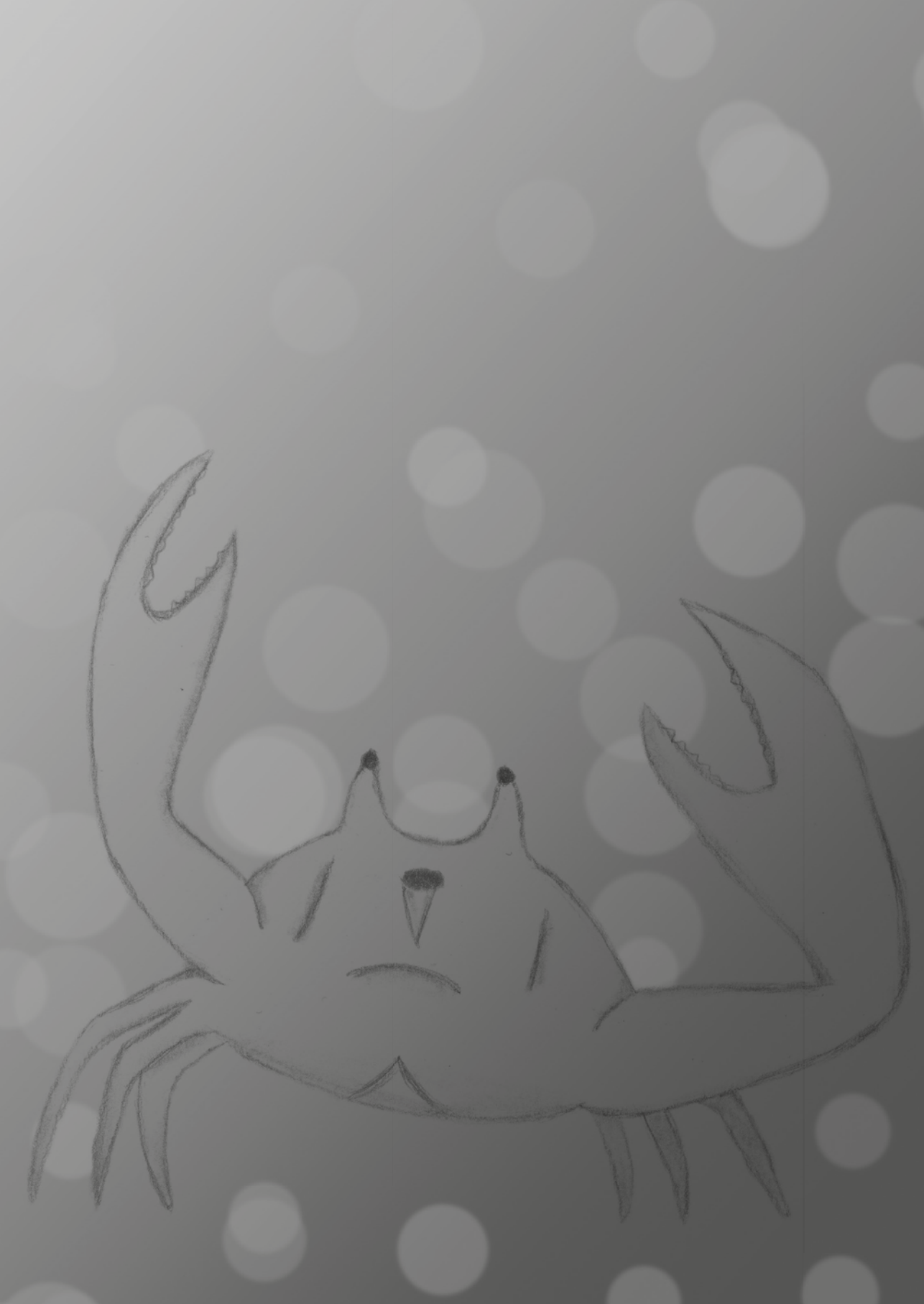
IX

Nederlandse samenvatting



De IL-17 en Th17 cel immuunrespons in baarmoederhalskanker

In **hoofdstuk 1** wordt een algemene introductie gegeven over baarmoederhalskanker en de immuunrespons gericht tegen kanker, waaronder de rol van IL-17 en Th17 cellen. Baarmoederhalskanker komt vooral voor bij jonge vrouwen, en is wereldwijd de vierde voornaamste doodsoorzaak door kanker bij vrouwen. Dit komt met name door de hoge incidentie van baarmoederhalskanker in ontwikkelingslanden. De oorzaak van baarmoederhalskanker is een infectie met hoog-risico humaan papillomavirus (HPV). Hoewel naar schatting zo'n 80% van de vrouwen ooit wordt besmet met HPV, wordt het virus in meer dan 80% van deze besmettingsgevallen geëlimineerd. Slechts in 10-20% van de infecties wordt het virale genoom ingebouwd in het humane genoom, wat expressie van de virale oncogenen E6 en E7 veroorzaakt en uiteindelijk kan leiden tot premaligne voorstadia en uiteindelijk kanker. Er zijn drie belangrijke histologische typen baarmoederhalskanker te onderscheiden: plaveiselcelcarcinoom, adenosquameus carcinoom en adenocarcinoom. Plaveiselcelcarcinoom heeft met 75% de hoogste incidentie.

De behandeling is afhankelijk van het FIGO stadium: een klinische beoordeling van de tumorgrootte, ingroei in het omliggende weefsel en uitzaaiingen op afstand. Tumoren die geclassificeerd worden met een laag FIGO stadium worden geopereerd, terwijl tumoren die geclassificeerd worden met hoge FIGO stadia doorgaans worden behandeld met (adjuvante) radiotherapie al dan niet in combinatie met chemotherapie. Klinische factoren die de prognose beïnvloeden zijn tumorgrootte, infiltratie in het omliggende weefsel en de bloedvaten en uitzaaiing naar de lymfeklieren. Na de behandeling wordt het TNM stadium bepaald door een patholoog. De TNM stadiëring omvat een classificatie op basis van tumorgrootte en uitzaaiingen naar de lymfeklieren alsook uitzaaiingen op afstand.

Het immuunsysteem beschermt organismen tegen pathogenen (ziekteverwekkers), middels een grote variëteit aan witte bloedcellen met elk een specifieke functie. Deze immuuncellen kunnen de pathogenen of geïnfecteerde cellen fagocyteren, of aanvallen met behulp van zuurstofradicalen en enzymen. Daarnaast kunnen ze cytokinen produceren: kleine signaalmoleculen die cellen kunnen activeren en het type immuunrespons kunnen reguleren. Zo kan het immuunsysteem op een effectieve manier reageren op verschillende soorten pathogenen. Inmiddels weten we dat het immuunsysteem het lichaam ook beschermt tegen de ontwikkeling van kanker. Echter, zodra de getransformeerde cellen weten te ontsnappen aan de immuunrespons, kunnen deze uiteindelijk uitgroeien tot een tumor. Gedurende dit selectieproces kunnen de tumorcellen bijvoorbeeld ook cytokinen produceren en hiermee de immuunrespons reguleren. Er kan dan een chronische ontsteking ontstaan, die juist de tumorgroei bevordert. Door de expressie van niet-lichaamseigen virale eiwitten, induceren HPV

geïnfecteerde en getransformeerde cellen een natuurlijke immuunrespons. Deze immunogeniciteit wordt al preventief toegepast door middel van vaccinatie, maar therapeutische behandeling van baarmoederhalskanker met immuuntherapie heeft nog weinig efficiënte behandelingen opgeleverd. Het is daarom van belang om de lokale immuunrespons in baarmoederhalskanker verder te onderzoeken.
Een specifiek type immuuncel is de T lymfocyt. De T lymfocyt ontwikkelt in de thymus en heeft een unieke receptor, waardoor elke T cel uniek is voor een bepaald antigen, afkomstig van een pathogeen of anderszins abnormaal eiwit. Zodra het unieke antigen wordt herkend, kunnen deze cellen direct een doelwit cel lyseren (cytotoxische T cellen). Daarnaast zijn er T helper cellen, die hulp bieden aan andere lymfocyten en andere cellen activeren middels cytokinen. Een voorbeeld van deze T helper cellen is de T helper 17 (Th17) cel, ontdekt in 2000. De naam van de Th17 cel berust op het feit dat dit celtype het cytokine interleukine 17 (IL-17) produceert. IL-17 induceert de groei van bloedvaatjes en indirect het aantrekken en de activatie van neutrofiele granulocyten. Neutrofiele granulocyten kunnen heel efficiënt pathogenen opruimen. Th17 cellen zijn met name van belang bij de eliminatie van bepaalde extracellulaire pathogenen, omdat ze verschillende ontstekingsbevorderende (pro-inflammatoire) cytokinen produceren waaronder IL-17. Aan de andere kant spelen Th17 cellen door het pro-inflammatoire karakter een dominante rol in verschillende auto-immuunziekten.
De rol van IL-17 en Th17 cellen in kanker is onduidelijk. De aanwezigheid van Th17 cellen en IL-17 kan gepaard gaan met de productie van interferon-γ (IFNγ) en activatie van cytotoxische T cellen, en leiden tot afname van de tumorgroei. Daarentegen kunnen IL-17 en Th17 cellen ook vaatgroei (angiogenese) induceren en myeloïde cellen zoals neutrofiele granulocyten aantrekken, welke de tumorgroei bevorderen. Omdat er geen consensus bestaat over de rol van IL-17 en Th17 cellen in kanker, werd in dit proefschrift de rol van IL-17 en Th17 cellen in baarmoederhalskanker onderzocht.

In **hoofdstuk 2** wordt de rol van de cytokinen IL-1β, IL-6, IL-23 en IL-12 beschreven. IL-12 stimuleert een immuunrespons gericht tegen de tumor, terwijl IL-1β, IL-6 en IL-23 een Th17 cel immuunrespons kunnen induceren. IL-12 en IL-23 bestaan uit twee ketens, een gedeelde component (IL-12p40) en een unieke component (IL-12p35 respectievelijk IL-23p19). De expressie van de cytokinen *IL12p35*, *IL12p40* en *IL23p19* werd onderzocht met RNA in situ hybridisatie. Alleen de expressie van *IL12p40* was significant gecorreleerd met de expressie van *IL12p35*. Omdat *IL12p35* altijd aanwezig was als *IL12p40* aanwezig was, en de affiniteit voor *IL12p35* verschilt van de affiniteit voor *IL23p19*, veronderstelden wij dat IL-12 wordt geproduceerd bij een hoge *IL12p40* expressie, terwijl IL-23 wordt geproduceerd als *IL23p19* aanwezig is in combinatie met lage *IL12p40* expressie. De eiwitexpressie van IL-1β en IL-6 werd bestudeerd met immuunhistochemie. Een hoog aantal IL-6 positieve cellen in het stroma (steunweefsel rondom de tumorcellen) was gecorreleerd met slechte overleving. Met name de combinatie van een hoog aantal IL-6 positieve cellen en IL-23 expressie door de tumor

was sterk gecorreleerd met slechte overleving. De afwezigheid van zowel IL-12 als IL-23, in het geval van *IL12p40* afwezigheid, was ook gecorreleerd met goede overleving, wat aangeeft dat de afwezigheid van IL-23 gunstiger is dan de aanwezigheid van IL-12 voor wat betreft de prognose. Met name IL-23 productie was dus gecorreleerd met slechte overleving, niet zozeer de afwezigheid van IL-12. Omdat IL-6 en IL-23 een belangrijke rol spelen bij de inductie van een Th17 immuunrespons, suggereert dit dat Th17 cel differentiatie een rol zou kunnen spelen bij suppressie van het immuunsysteem.

De sterke correlatie tussen IL-6 en IL-23 en slechte overleving leidde tot de hypothese dat de inductie van Th17 cel differentiatie gecorreleerd is met suppressie van het immuunsysteem in baarmoederhalskanker. Om deze reden werden de lokalisatie en het fenotype van IL-17 producerende cellen beschreven met behulp van immuun-histochemie in **hoofdstuk 3**. Hoewel IL-17 doorgaans wordt beschouwd als marker voor Th17 cellen, vonden we tegen onze verwachting in dat IL-17 met name tot expressie kwam in neutrofiele granulocyten en mestcellen, en slechts in een kleine populatie Th17 cellen in baarmoederhalskanker. Ook in andere typen kanker werd IL-17 vaker tot expressie gebracht in granulocyten dan in T cellen. Vervolgens werd de frequentie van IL-17 positieve cellen, granulocyten, mestcellen en Th17 cellen in de tumoren van een cohort patiënten met plaveiselcelcarcinoom van de baarmoederhals bestudeerd. Een hoog aantal IL-17 positieve cellen in de tumor was gecorreleerd met slechte overleving in patiënten geclassificeerd met een laag TNM stadium (stadium I). Een mogelijke gedeeltelijke verklaring hiervoor is de directe stimulatie van tumorcel proliferatie, gebaseerd op experimenten waarbij een toename in celindex van baarmoederhalskanker cellijnen werd waargenomen na toevoeging van IL-17 in een real-time cel analyse. Opzienbarend genoeg was een hoog aantal Th17 cellen juist gecorreleerd met goede overleving. Dit suggereert dat IL-17 de tumorgroei in baarmoederhalskanker stimuleert, terwijl Th17 cellen onderdeel zijn van een immuunrespons gericht tegen de tumor.
Aangezien IL-17 zorgt voor de infiltratie van neutrofiele granulocyten, zou de productie van IL-17 door de neutrofiele granulocyten zelf voor een positieve terugkoppeling kunnen zorgen die weer leidt tot meer infiltratie van neutrofiele granulocyten. Het lijkt er in ieder geval op dat IL-6 en IL-23 niet alleen zorgen voor de aantrekking van macrofagen en tumorgroei, maar ook neutrofiele granulocyten zouden kunnen stimuleren IL-17 te produceren, resulterend in slechtere overleving.

De rol van T cellen en IL-17 in adenocarcinoom van de baarmoederhals zijn het onderwerp van **hoofdstuk 4**. Adenocarcinoom verschilt van plaveiselcelcarcinoom van de baarmoederhals in groeipatroon, moleculaire achtergrond en gevoeligheid voor radio- en chemotherapie. Omdat de incidentie van adenocarcinoom een stuk lager ligt, zijn er niet veel uitgebreide studies naar de rol van het immuunsysteem in dit type tumor

gedaan. Omdat de rol van IL-17 en Th17 cellen context en weefsel afhankelijk lijkt te zijn, en het ontstekingsinfiltraat in adenocarcinoom een andere samenstelling lijkt te hebben dan in plaveiselcelcarcinoom, onderzochten wij of de rol van T cellen en IL-17 in deze twee histologische tumortypen vergelijkbaar is. Een laag aantal regulatoire T cellen gemeten op basis van immuunfluorescente score bleek sterk gecorreleerd te zijn met slechte overleving. Een laag aantal regulatoire T cellen gecombineerd met een laag aantal IL-17 positieve cellen was nog sterker gecorreleerd met slechte overleving. Zo was ook de combinatie van een laag aantal regulatoire T cellen en de aanwezigheid van Th17 cellen gecorreleerd met slechtere overleving. In tegenstelling tot onze bevindingen in plaveiselcelcarcinoom, was er een trend naar goede overleving bij aanwezigheid van veel IL-17 positieve cellen in de tumor, en een trend naar slechte overleving bij aanwezigheid van Th17 cellen. Ook de rol van regulatoire T cellen is controversieel en lijkt context en tumortype afhankelijk te zijn. Het zou kunnen dat een pro-inflammatoir milieu in adenocarcinoom immuuncellen aantrekt die de tumorgroei onderdrukken. De Th17 cellen zouden een tumorgroei stimulerende immuunrespons kunnen vertegenwoordigen, die geremd wordt door de regulatoire T cellen. Dit komt niet overeen met onze bevindingen bij plaveiselcelcarcinoom en dient nog verder te worden bevestigd. Ook de correlaties met andere immuuncel typen dienen verder onderzocht te worden. Deze resultaten ondersteunen de hypothese dat de rol van het immuunsysteem, waaronder IL-17 en Th17 cellen, context en tumortype afhankelijk is.

Een tumor bestaat niet alleen uit tumorcellen, maar bevat ook bloed- en lymfevaten, immuuncellen en een matrix van cellen, eiwitten en suikerstructuren. In **hoofdstuk 5** werden de correlaties tussen verschillende typen immuunrespons en factoren van invloed op vaatvorming beschreven. Op deze manier werd de rol van de IL-17 en Th17 cel immuunrespons in baarmoederhalskanker verder onderzocht. De factoren werden bestudeerd met behulp van kwantitatieve reverse transcriptase PCR (qRT-PCR) analyse op vers ingevroren monsters van plaveiselcelcarcinoom van de baarmoederhals. Hoge expressie van T cel markers (*CD3E*, *CD8A, FOXP3*) was gecorreleerd met goede prognose, terwijl *IL6* en de angiogenese marker *VEGFA* gecorreleerd waren met slechte overleving. Met name een hoge *IL6*/*IL17* ratio gecombineerd met lage *IL5* expressie was sterk gecorreleerd met slechte overleving. Opvallend genoeg bleken *IL17* en neutrofiele granulocyten markers fucosyltransferase 9 (*FUT9*) en neutrophil elastase (*NE*) zeer laag tot expressie te komen. Aangezien neutrofiele granulocyten in weefsels zeer weinig tot geen RNA tot expressie brengen dat codeert voor de eiwitten die ze in hun granules bevatten, is de *IL17* expressie die we hebben gemeten waarschijnlijk afkomstig van Th17 cellen. Omdat we eerder hebben beschreven dat de Th17 cel populatie gering is in kanker (hoofdstuk 3), bevestigt dit de lage gemeten concentraties *IL17* en kunnen we met deze studie de rol van neutrofiele granulocyten niet verder onderbouwen. Wel suggereren de data dat *IL17* afkomstig van Th17 cellen de tumorgroei bevorderende effecten van *IL6* kan remmen, in het bijzonder in

aanwezigheid van *IL5*. Gezien de sterke correlaties met overleving, concluderen wij dat het meten van *IL6*, met name in combinatie van *IL5* en *IL17*, zou kunnen bijdragen aan het meer nauwkeurig kunnen voorspellen van de prognose van de patiënt.
We vonden ook een significante correlatie tussen lage *IL6* expressie en vaso-invasie, de aanwezigheid van tumorcellen in vaatjes. Hoge *IL1β* expressie gaf ook een trend naar een correlatie met afwezigheid van vaso-invasie. Een hoog aantal IL-17 en IL-1β positieve cellen was ook significant gecorreleerd met de afwezigheid van vaso-invasie (hoofdstuk 3). Dit suggereert dat de IL-1β/IL-6/IL-17 respons uitzaaiing van tumorcellen via de bloed- of lymfevaten remt.

Hoofdstuk 6 handelt over het meten van genexpressie specifiek afkomstig van ofwel tumorcellen, ofwel immuuncellen. Bij moleculaire analyses van de tumor als geheel, zoals in hoofdstuk 5, zijn de celtypen waar de moleculen vandaan kwamen vaak niet te achterhalen. Daarom hebben wij de tumorcellen en immuuncellen geïsoleerd uit baarmoederhalskanker celsuspensies met behulp van flowcytometrie. Uit deze twee cel fracties werd RNA geïsoleerd en de totale expressie van het RNA werd vervolgens gemeten met RNA sequentiebepaling. Binnen beide fracties werden de genen die significant hoger of lager tot expressie kwamen op basis van overleving, lymfekliermetastasen of Th17 respons geanalyseerd. Deze nieuwe aanpak leidde tot een duidelijke scheiding in genexpressie die overeenkwam met de originele celtypen. Gebaseerd op klinische uitkomst bleek er weinig variatie tussen de verschillende tumoren te bestaan, wat zou kunnen betekenen dat de RNA expressie in de tumoren relatief homogeen is. Omdat de ontwikkeling van een tumor tumortype-specifiek zou kunnen zijn, en vergelijkbaar tussen patiënten, zou deze techniek ook geschikt kunnen zijn om de verschillen tussen tumortypen in kaart te brengen.
Binnen de immuuncel fracties bleek dat *TCL1A* expressie significant gecorreleerd was met overleving en dat *TCL1A* niet tot expressie kwam in deze cellen bij patiënten die binnen vijf jaar na de operatie overleden waren. Deze correlatie werd bevestigd met qRT-PCR en immuunhistochemische analyses. Vervolgens toonden we aan dat *TCL1A* tot expressie komt in B cellen. Tot slot waren hoge expressie van B cel marker *CD19* en een hoge *TCL1A/CD20* (B cel marker) ratio in de ingevroren tumoren van een aanvullend patiënt cohort gecorreleerd met goede overleving. Deze data geven aan dat B cellen in de tumor mogelijk een belangrijke rol spelen bij het verloop van baarmoederhalskanker, met voornamelijk een belangrijke rol voor TCL1A en CD20 positieve B cellen. Rijpe B cellen staan bekend om hun vermogen om antilichamen te produceren, maar kunnen ook antigenen presenteren. Recent onderzoek heeft aangetoond dat lymfoïde structuren, waar B en T cellen normaal geactiveerd worden, ook in tumoren aanwezig kunnen zijn. Deze lymfoïde structuren lijken gecorreleerd te zijn met goede overleving. Hoewel de rol van B cellen in kanker nog onduidelijk is, toont deze studie aan dat B cellen in baarmoederhalskanker een belangrijke rol kunnen spelen bij het onderdrukken van tumorgroei. Deze rol moet verder worden uitgezocht.

Ook in deze studie werd IL-17 erg laag tot expressie gebracht. Omdat slechts drie genen verschillend tot expressie kwamen tussen IL-17 positieve en negatieve tumoren, was het niet mogelijk om de rol van IL-17 of Th17 cellen verder te verklaren op basis van deze resultaten. In de tumor fracties met corresponderende *IL17* positieve immuuncel fracties kwam *OMA1* zinc metallopeptidase het meest significant verhoogd tot expressie vergeleken met de tumoren met *IL17* negatieve immuuncel fracties. OMA1 functioneert in situaties van stress, en omdat Th17 cellen hier ook om bekend staan, zou een OMA1 respons in de tumor gerelateerd kunnen zijn aan een Th17 cel immuunrespons.

Th17 cellen worden doorgaans gekarakteriseerd door IL-17 expressie. De tegengestelde correlaties tussen IL-17 versus Th17 cellen en overleving in baarmoederhalskanker, zouden een mogelijke verklaring kunnen zijn voor de controverse over de rol van Th17 cellen in kanker. Om deze hypothese te toetsen, werd in **hoofdstuk 7** een systematische literatuurstudie naar de correlaties tussen IL-17 versus Th17 cellen en overleving in patiënten met kanker beschreven. In overeenstemming met onze bevindingen in plaveiselcelcarcinoom van de baarmoederhals, beschreef het merendeel van de studies naar de rol van IL-17 in kanker een significante correlatie met slechte patiënt overleving. Ook werd meestal een significante correlatie tussen Th17 cellen en goede overleving beschreven. Wel was er variatie in de richting en grootte van het effect, zoals ook aangetoond in onze studie in adenocarcinoom (hoofdstuk 4), een indicatie dat de effecten van IL-17 en Th17 cellen in kanker context en tumortype afhankelijk zijn.
Omdat IL-17 door verschillende celtypen kan worden geproduceerd, kunnen deze celtypen ook het type immuunrespons bepalen. Over het geheel lijkt IL-17 echter tumorgroei te stimuleren, waarschijnlijk via de inductie van angiogenese en aantrekking van neutrofiele granulocyten. Th17 cellen, hoewel een kleine cel populatie, zijn over het algemeen juist betrokken bij het onderdrukken van tumorgroei, wellicht door een Th1 en cytotoxische T cel respons te stimuleren. We concluderen dat het belangrijk is om onderscheid te maken tussen Th17 en andere IL-17 positieve cellen.

Hoofdstuk 8 omvat een discussie van de resultaten beschreven in hoofdstuk 2 tot 7 en de implicaties voor verder onderzoek. Een samenvatting van de resultaten en discussie is hierboven beschreven. Een van de belangrijke vraagstukken die verder dient te worden bestudeerd betreft de factoren die IL-17 expressie bij neutrofiele granulocyten induceren. Alhoewel de factoren voor de inductie van Th17 cellen nog steeds niet geheel bekend zijn, zijn de factoren voor de inductie van IL-17 producerende neutrofiele granulocyten geheel onbekend. Deze kennis zou inzicht bieden in het type immuunrespons dat hierbij betrokken is, en de effecten ervan. Zo dient ook verder onderzocht te worden of IL-17 door een specifieke subpopulatie neutrofiele granulocyten wordt geproduceerd, de zogenaamde alternatief geactiveerde neutrofiele granulocyten.

In dit proefschrift is beschreven dat IL-17 voornamelijk gecorreleerd is met slechte patiënt overleving, en Th17 met goede overleving in kanker. Er was echter substantiële variatie in de mate van correlatie. Dit zou te maken kunnen hebben met het tumortype, zoals beschreven in het review (hoofdstuk 7). Dit werd ook belicht door de verschillen tussen plaveiselcelcarcinoom en adenocarcinoom van de baarmoederhals (hoofdstuk 3, 4). Het is daarom van belang om te bestuderen in welke tumortypen IL-17 en Th17 cellen welke rol spelen. Het is ook interessant om de celtypen te bestuderen die in de verschillende tumortypen IL-17 produceren, en welke andere celtypen of factoren hiermee gecorreleerd zijn.

Gebaseerd op de bevindingen die beschreven zijn in hoofdstuk 7, zouden kanker patiënten met veel IL-17 wellicht baat kunnen hebben bij het blokkeren van de werking van IL-17. Antilichamen tegen IL-17 worden momenteel al gebruikt in klinische trials voor de behandeling van verschillende auto-immuunziekten. Ook in een experimenteel diermodel werd de tumorgroei van een lymfoom geremd met antilichamen tegen IL-17. Aan de andere kant zou de toediening (‘adoptive transfer’) van Th17 cellen kunnen helpen bij het bestrijden van de tumor. Op grond van de resultaten verkregen uit diermodel studies is beschreven dat de toediening van Th17 cellen leidt tot meer tumorregressie dan de toediening van Th1 cellen. De haalbaarheid van beide benaderingen dient verder onderzocht te worden.

List of abbreviations

Act1	NF-κB activator protein 1
ANGPT	angiopoietin
AP	alkaline phosphatase
APAAP	alkaline phosphatase anti-alkaline phosphatase
BCR	B cell receptor
BSA	bovine serum albumin
CCL	CC motif chemokine ligand
CCR	CC motif chemokine receptor
CD	cluster of differentiation
CI	confidence interval
CIN	cervical intraepithelial neoplasia
CTL	cytotoxic T lymphocyte
CTLA-8	cytotoxic T lymphocyte-associated-8
CXCL	CXC motif chemokine ligand
CXCR	CXC motif chemokine receptor
DAB	3,3'-diamino-benzidine-tetrahydrochloride
DFS	disease-free survival
DIG	digoxigenin
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence-activated cell sorting
FFPE	formalin-fixed, paraffin-embedded
FGF	fibroblast growth factor
FIGO	Fédération Internationale de Gynécologie et d'Obstétrique
FoxP3	forkhead box P3
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPF	high-power field of view
HPV	human papillomavirus
HR	hazard ratio
HRP	horseradish peroxidase
ICAM	intercellular adhesion molecule
IFNγ	interferon-γ
IHC	immunohistochemistry
IL	interleukin
IL-17RA	IL-17 receptor A

LLETZ	large loop excision of the transformation zone
MDSC	myeloid derived suppressor cell
MMP	matrix metalloproteinase
MPO	myeloperoxidase
MS	multiple sclerosis
NF-κB	nuclear factor-κB
NK	natural killer cell
OS	overall survival
Pap smear	Papanicolaou cytology test
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PD-L1	programmed death-ligand 1
PECAM	platelet endothelial cell adhesion molecule (also known as CD31)
PGE_2	prostaglandin E2
PRR	pattern-recognition receptor
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RA	rheumatoid arthritis
RISH	RNA *in situ* hybridisation
RNA	ribonucleic acid
RNase	ribonuclease
RNA-seq	total mRNA sequence data
RORα	retinoic acid receptor-related orphan receptor α
RORγt	retinoic acid receptor-related orphan receptor γ t
SEM	standard error of the mean
SLE	systemic lupus erythematosus
SSC	saline-sodium citrate
STAT	signal transducer and activator of transcription
TBS	Tris-buffered saline
TCL1A	T-cell leukemia/lymphoma protein 1A
TCR	T cell receptor
TGF-β	transforming growth factor β
Th	T helper cell
TLR	Toll-like receptor
TNFα	tumor necrosis factor α
TNM	tumor, node, metastasis staging system
TRAF-6	tumor necrosis factor receptor-associated factor-6
Treg	regulatory T cell
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
WGCNA	weighted gene co-expression network analysis

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BMC Cell Biology, 2010, 11: 33

Curriculum Vitae

Birgitte Simone Punt was born on 1 February 1987 in Hoek van Holland, the Netherlands. She graduated at the Stedelijk Gymnasium Schiedam in 2005. She studied Biomedical Sciences at the University of Leiden between 2005 and 2010. For her Bachelor's thesis, she studied the effects of MiHa HA-1 on cell transformation under the supervision of Dr. Kam-Wing Ling and Prof. Dr. Els Goulmy, at the department of Immunohematology and Bloodtransfusion (IHB), Leiden University Medical Center (LUMC). During her first Master's internship, the cellular localisation of HCV target protein GLT25D1 was studied under the supervision of Dr. Hans van Leeuwen and Dr. J. Liefhebber at the department of Medical Microbioloy of the LUMC. For her Master's thesis, she studied collagen cross-link maturation under the supervision of Dr. Reinout Stoop at the department of Tissue Repair, TNO Quality of Life. After following the courses Science Based Business Fundamentals and Orientations on Technopreneurship, she also performed a market analysis for Quickzyme Biosciences under the supervision of Dr. Roeland Hanemaaijer at Quickzyme Biosciences. During her Master study, Simone was a member of the committee organizing the Biomedical Sciences Symposium. She received her Master of Science degree in October 2010. Following the last master internship, Simone worked on several projects at the TNO department of Tissue Repair under the supervision of Dr. Stoop for three more months. She acquired a PhD position at the department of Pathology, LUMC in January 2011. The PhD project was supervised by Prof. Dr. Gert Jan Fleuren, Dr. Arko Gorter and Dr. Ekaterina Jordanova. The results obtained during her PhD project are described in this thesis. During her PhD project, Simone obtained a SASC sequencing grant (January 2013) and a LUF travel grant (September 2014). She was also trained to become an SMBWO certified Immunologist. Simone was a board member of the LUMC Association for PhD candidates (LAP) from 2012 to 2015. She was also chairman of the PhD day committee for the PhD day organized by the LAP in October 2013. Simone obtained a position as a postdoctoral fellow in the laboratory of Prof. Dr. Patrick Hwu at the department of Melanoma, MD Anderson Cancer Center, University of Texas, Houston, USA (August 2015).

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Simone