The IL-17 and Th17 cell immune response in cervical cancer : angels or demons : it depends on the context

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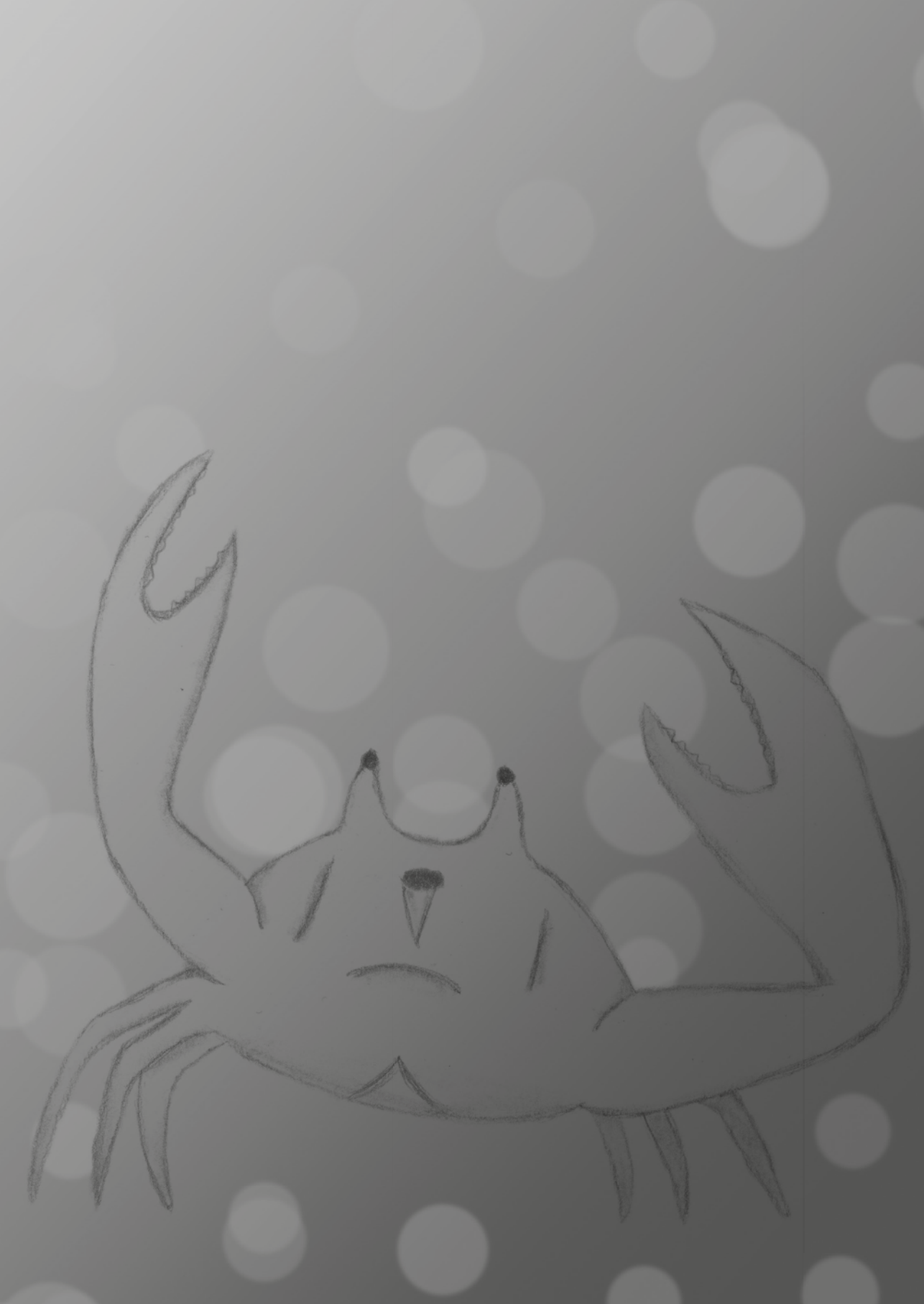
General introduction

1. Cervical cancer
   1.1 Etiology
   1.2 Staging and treatment

2. The immune response to cancer
   2.1 The tumor microenvironment
   2.2 The innate immune response
   2.3 Cytokines and chemokines
   2.4 Antigen presentation
   2.5 The adaptive immune response

3. The Th17 cell immune response
   3.1 Phenotype and function of Th17 cells
   3.2 Interleukin-17
   3.3 Th17 cells in autoimmunity
   3.4 Th17 cells and IL-17 in cancer

4. Thesis outline



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]

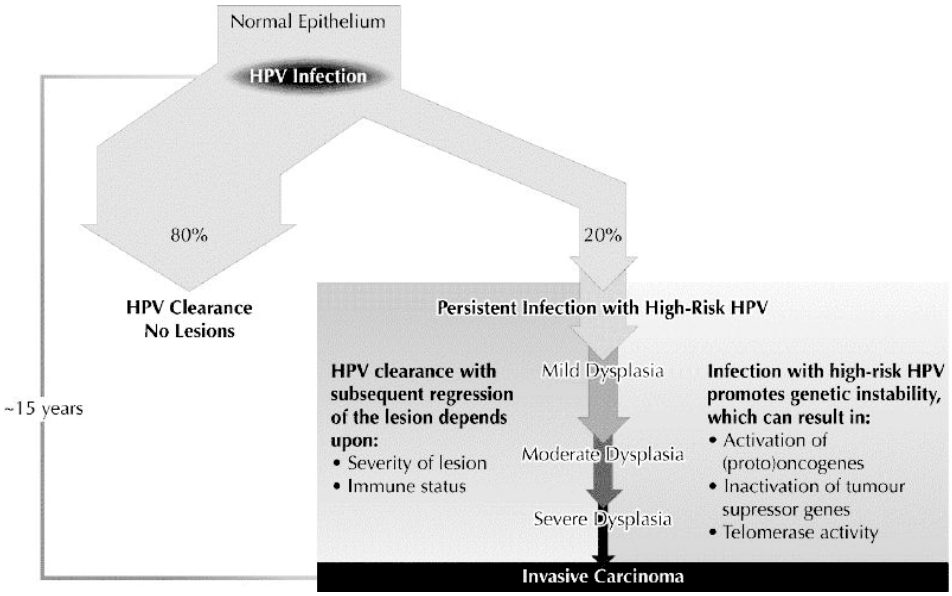


Figure 1. Etiology of cervical cancer

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.

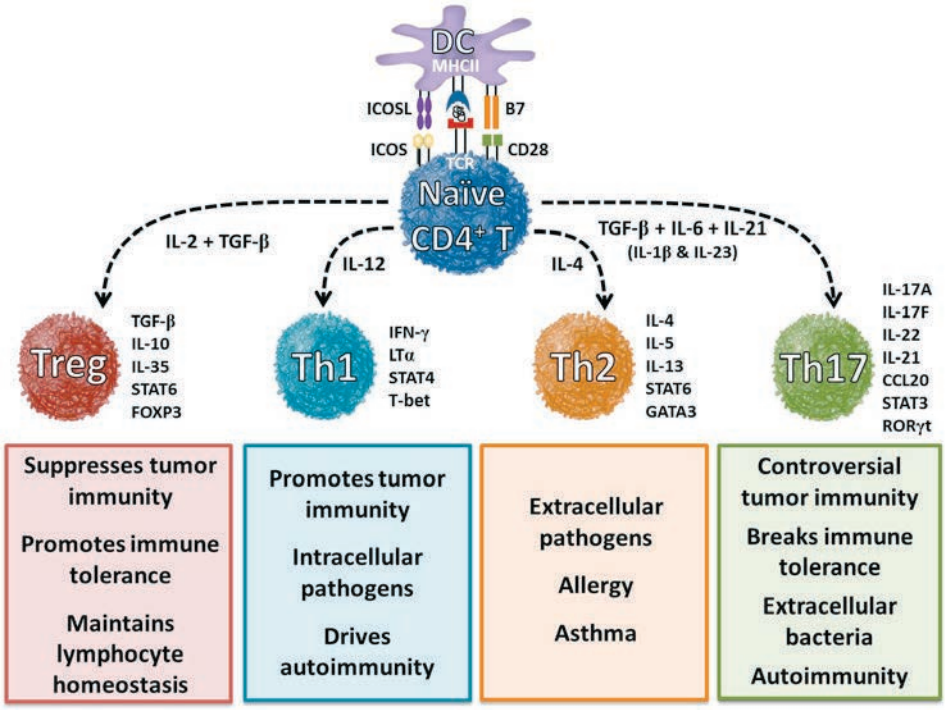


Figure 2. T helper cell subtypes

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]

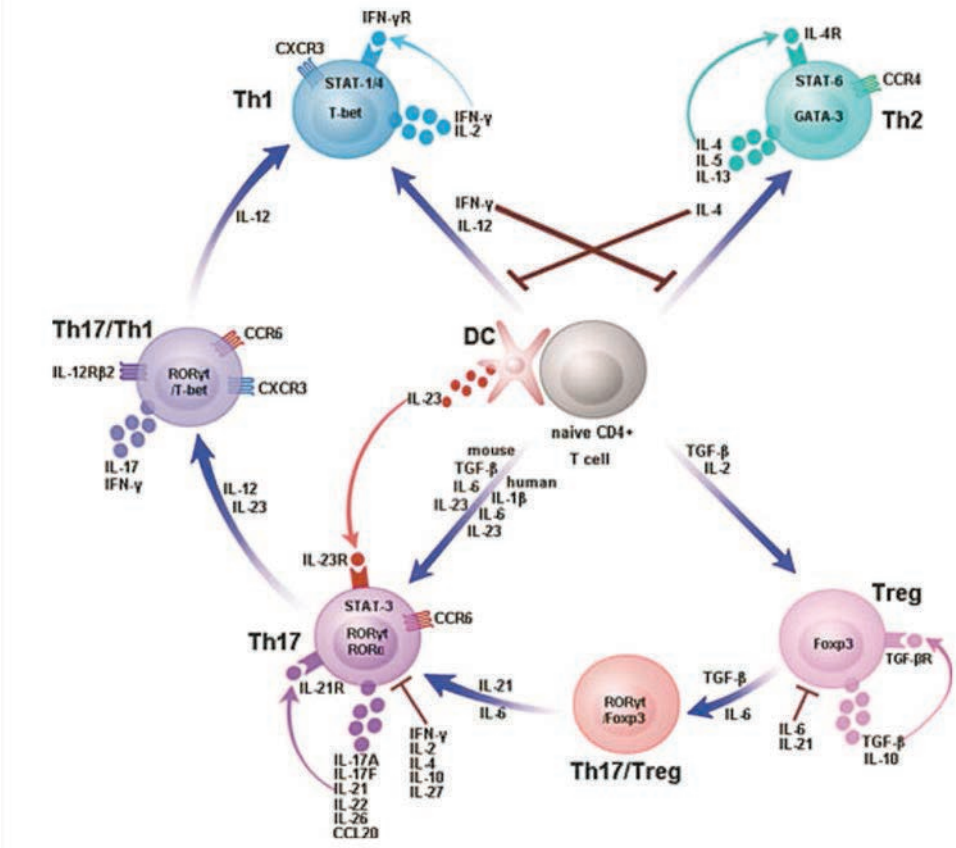


Figure 3. Th17 cell phenotype

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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