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Chapter 1

Introduction
1 Human papilloma viruses

Human papilloma viruses (HPV) are non-enveloped double stranded DNA viruses (7-8 kb) that infect human skin and mucosa and are the causative agents of mostly benign proliferative lesions such as common (genital) warts. However, persistent infection with ‘high-risk’ HPV subtypes is associated with the development of anogenital malignancies such as cervical, vulvar, penile and anal cancer, and also a growing subset of oropharyngeal cancers. The association is the strongest for cervical cancer as illustrated by the finding that HPV DNA can be detected in over 99% of cervical cancers. Notably, cervical cancer is the second most common cancer in women worldwide, with an estimated death toll of almost 300,000 women annually, mostly in developing countries.

More than 180 types of HPV are known and 15 are thought to be high-risk and tumorigenic. HPV16 is the most common high-risk type, and responsible for about half of all tumors; HPV18 accounts for another 10-15%; and HPV types 31, 33, 45, 52, and 58 account for an estimated 2-5% each.

1.1 Squamous epithelia

The surface of the human body that is exposed to the outside milieu is largely covered by stratified squamous epithelia, which are built up of multiple layers of keratinocytes (KCs) to create a robust physical barrier. Examples of such epithelia are the skin and the lining of the oropharyngeal and the lower female reproductive tracts. Tightly regulated division of the undifferentiated KCs in the basal layers results in the continuous production of new cells that gradually differentiate as they move to the upper layers in the epithelium, where they can serve as replacement for the cells that are lost from the epithelial surface due to wear and tear. The apical layers are not only exposed to physical stress, but also to a great variety of infectious pathogens, including bacteria and viruses. Similarly, the cells in the basal layers can be targeted by pathogens through wounds and micro-abrasions.

1.2 The viral life cycle

HPV infects the keratinocytes in the basal layer of the epidermis and mucosal epithelium, and the viral life cycle is tightly regulated through the differentiation program of keratinocytes. Following infection and uncoating, the virus genome is maintained as episomes at a low copy number in the basal cells of the epithelium, where proliferation-inducing early genes (including E6 and E7) are expressed, resulting in lateral expansion of the infected cells. Later, the suprabasal layers of epithelium support viral replication where hundreds to thousands HPV genomes are present within a single cell. The L1 and
L2 capsid proteins are expressed in the most superficial layers of the epithelium where viral assembly takes place, and finally new infectious viral particles are released.\(^1,3\)

### 1.3 Malignant transformation

For high-risk HPV infections, E6 and E7 effectively block the negative regulators of the cell cycle, whereby the cells remain active in cell cycle progression with the cessation of differentiation and apoptosis. As such, the infected cells acquire genomic instability and genetic alterations, ultimately driving malignant transformation of an infected cell into an invasive cancer cell.

E6 and E7 start oncogenesis by inactivating tumor suppressors. The tumor suppressor protein E6 targets TP53 for degradation via the ubiquitin proteasome pathway, preventing apoptosis and enabling potentially transformed cells to replicate.\(^8\) The tumor suppressor protein E7 contributes to oncogenesis through its interaction with the retinoblastoma tumor suppressor family members RB1, RBL1 and RBL2 and targets them for degradation.\(^9\)

### 2 Innate Immunity

The mammalian innate immune system provides a first line of defense against microbial attack through antimicrobial factors, phagocytosis and the induction of inflammation. Mucus covers the internal surface of the anogenital tracts and functions to trap infectious microorganisms and pollutants. Mucus contains mucins and various other microbicidal molecules, including antimicrobial peptides (calprotectin, lysozyme, lactoferrin), secretory leukoprotease inhibitor, and human β-defensins), immunoglobulins, and complement factors that directly bind to and kill microorganisms before they reach the host epithelial cell layer.

Invading viruses and microbes contain pathogen-associated molecular patterns (PAMPs) that are recognized by the host's pattern recognition receptors (PRRs). Two main classes of PRRs have been described in mammalian cells: 1) membrane-bound receptors, such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), and 2) cytoplasmic sensors, including NOD-like receptors (NLRs), pyrin and HIN domain-containing (PYHIN) family members, RIG-I-like receptors (RLRs) and an increasing range of cytosolic nucleic acid sensors.\(^10\) All of these receptors activate conserved signaling cascades that lead to activation of NF-κB via the canonical route, while RLRs and some TLRs activate interferon regulatory factors (IRFs) that together with NF-κB induce the production of type I interferons (IFN) and other effector molecules. Other PRRs initiate the assembly of cytoplasmic signaling complexes, termed inflammasomes,
which activate inflammatory caspases and cause maturation and secretion of IL-1β and IL-8. Each member of the PRR family recognizes distinct PAMPs. TLRs recognize a diverse array of PAMPs including bacterial lipoproteins, lipopolysaccharide (LPS), flagellin, peptidoglycan, nucleic acids as well as viral glycoproteins and nucleic acids, such as double-stranded RNA (dsRNA), uncapped single-stranded RNA (ssRNA) and viral DNA. NLRs recognize peptidoglycan fragments and RNA while RLRs comprising retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA5) and LGP2 (also known as DHX58) detect several different ssRNA and dsRNA viruses.

2.1 Expression of pattern recognition receptors in keratinocytes

KCs are equipped with sensors for pathogens, including TLRs, protein kinase R (EIF2AK2), and the RNA helicases RIG-I (DDX58) and MDA5 (IFIH1) which enable them to generate pro-inflammatory and anti-viral signals in response to PAMPs. Of these receptors, TLR1, 2, 4, 5, 6 and 10 are expressed at the cell surface and specialized in the detection of macromolecules that constitute the building blocks of pathogenic micro-organisms, while TLR3, 7, 8 and 9 protrude into the lumen of intracellular vesicles and detect nucleic acids of foreign origin. RIG-I and MDA5 are cytosolic sensors. The vast majority of published studies have shown that KCs express TLR 1, 2, 3, 5, 6, but lack TLR 7 and 8, while different conclusions were reached with respect to the expression of TLR4 and 9 in these cells. In KCs, activation of these PRRs leads to direct NF-kappa-B activation and results in the upregulation of pro-inflammatory cytokines including IL-8, CCL2, CCL20, CCL27, and/or activation of type I interferon (IFN) response genes including transcription factors IRF3 and IRF7 regulating the production of antiviral cytokines.

2.2 Ubiquitins regulate cell signaling

Post-translational modification of proteins by ubiquitination regulates many cellular processes including the generation of innate and adaptive immune responses to pathogens. Ubiquitin is a highly-conserved 76-amino-acid polypeptide that can be covalently attached to cellular proteins through an enzymatic cascade involving three classes of enzyme termed E1, E2 and E3. E1 enzyme activates ubiquitin. Activated ubiquitin is transferred to an E2 ubiquitin-conjugating enzyme. The E2 enzyme-ubiquitin complex interacts with an E3 ubiquitin ligase that facilitates transfer of the ubiquitin to a lysine (K) residue on substrate protein. Ubiquitination can be reversed by deubiquitinating enzymes (DUBs). In humans, there are two E1 enzymes, about 50 E2 enzymes and 600 E3 enzymes and about 100 DUBs.
A protein can be modified on one lysine residue with a single ubiquitin (monoubiquitination) or with a chain of ubiquitin (polyubiquitination). Lysine 48 (K48)-linked polyubiquitination usually targets protein for proteosomal degradation, whereas K63-linked polyubiquitination is involved in activating proteins in signal transduction cascades (Figure 1).

**Figure 1 | The Ubiquitin-Proteasome System (UPS).** First, ubiquitin activating enzyme, E1, forms a thioester linkage with the C-terminal glycine residue of ubiquitin (Ub) in an ATP-dependent manner. Ub is then transferred to ubiquitin conjugating enzyme, E2. Finally, E2 enzyme binds to ubiquitin ligase enzyme, E3, and the complex mediates isopeptide linkage formation between carboxy terminal glycine residue of Ub and lysine ε-amino group of the substrate. Repetition of this catalytic cycle leads to polyubiquitination of the substrate. Ub primarily binds to the substrate either through its N-terminal or other internal lysine residues (K6, K11, K27, K29, K33, K48 and K63). While K48 polyubiquitination marks the protein for proteosomal degradation, K63 polyubiquitination activates the protein leading to the activation of signaling pathways. Deubiquitinating enzymes (DUBs) remove Ub from polyubiquitinated proteins and recycle Ub during protein degradation (Adapted from S.H. van der Burg, unpublished).

However, pathogens have evolved many ways to exploit the ubiquitination system of the hosts. A common evasion strategy for viruses is to target key immune proteins for degradation. By degrading host’s adaptor and signaling molecules, viruses disable many immune response pathways including the production of interferons and other innate host defense mechanisms. Additionally, viruses inhibit the ligation of ubiquitin or remove ubiquitin from host cell proteins to favor propagation and pathogenesis.
of viruses. For instance, the NS5 proteins of dengue virus inhibits IFN signaling by selective ligation of K48-linked polyubiquitin chains in STAT2, thus promoting the degradation of STAT2, an essential component of ISGF3 complex required for ISG induction. Moreover, Epstein-Barr virus encoded BPLF1 protein acts as a deubiquitinase and removes ubiquitin from TRAF6 to inhibit NF-κB signaling during lytic infection resulting in enhanced lytic replication of the virus.

2.3 Regulation of innate immunity by ubiquitination

TLR pathways use two main adaptor proteins: myeloid differentiation primary response protein 88 (MYD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF). MYD88-dependent pathways are used by all TLRs except TLR3 while TRIF-dependent pathways transmit signals from TLR3 and TLR4. Downstream of both MYD88 and TRIF-dependent pathways, ubiquitination plays critical roles in the activation of NF-κB and the mitogen-activated protein kinase (MAPK) signaling cascades. Following activation of MYD88-dependent pathways, MYD88 recruits kinases of the IL-1 receptor-associated kinase (IRAK) family, which then recruit TNF-receptor-associated factor 6 (TRAF6), a ubiquitin E3 ligase. Together with a ubiquitin E2 complex containing UBC13 and UEV1A, TRAF6 catalyze the synthesis of K63-linked polyubiquitin chains (Figure 3). These polyubiquitin chains bind to TAK1-binding protein 2 (TAB2) and TAB3 leading to the activation of the TGF-β-activated kinase 1 (TAK1) and the downstream MAPK cascade. K63-linked polyubiquitin also bind to the NF-κB essential modulator (NEMO), a regulatory subunit of the IκB kinase (IKK) which contains the IKKa and IKKβ catalytic subunits. Binding of K63-linked polyubiquitin chains to both the NEMO and TAK1 complexes facilitates the phosphorylation of IKKβ by TAK1, leading to the activation of IKK. IKK phosphorylates NF-κB inhibitor (IκB) proteins which are then recognized by the SCF-βTrCP ubiquitin E3 ligase complex targeting the IκB for K48-linked polyubiquitination and subsequent degradation by the proteasome. This allows NF-κB to enter the nucleus to turn on the target genes (Figure 2).

In TRIF-dependent pathways downstream of TLR3, receptor-interacting protein 1 (RIP1) undergoes K63-linked polyubiquitination by ubiquitin E3 ligases such as TRAF6. RIP1 polyubiquitination recruits TAK1 and NEMO, leading to the activation of NF-κB. TRIF also recruits another ubiquitin E3 ligase, TRAF3, which activates the kinases TBK1 and IKKe, leading to interferon responsive factor 3 (IRF3) phosphorylation and type I IFN production. Additionally in IFN-induction pathway, TRAF3 and TRAF6 are recruited, TRAF3 undergoes K63-linked polyubiquitination leading to IFN induction by unknown mechanisms.
Ubiquitination plays crucial roles too in the RIG-I and melanoma differentiation-associated gene 5 (MDA5)-mediated pathways that sense viral RNA. Viral RNA binds RIG-I and induces a conformational changes that exposes the N-terminal CARD domains of RIG-I which binds the unanchored K63 polyubiquitin chains synthesized by TRIM25 and Riplet ubiquitin E3 ligases. RIG-I then interacts with and activates the mitochondrial membrane protein MAVS, further recruiting K63-linked polyubiquitinated TRAF6, TRAF3, TRAF2/5, and cIAP1/2 ultimately activating IKK and TAK1 complexes similar to TLR signaling as described above.
2.4 Regulation of innate immunity by deubiquitination

DUBs are proteases that cleave ubiquitin from target proteins and therefore oppose the function of ubiquitin E3 ligase. Consistent with a key role of ubiquitination in activating immune signaling cascades, several DUBs have been shown to negatively regulate immune responses. A20 and CYLD are two best known DUBs that inhibit PRR pathways.

Overexpression of A20 inhibits NF-κB activation in response to TNF or IL-1 stimulation. Mice lacking A20 die shortly after birth due to multi-organ tissue inflammation and cachexia due to uncontrolled activation of NF-κB. Mechanistically, A20 inhibits NF-κB via its DUB domain by removing or inhibiting the K63-linked polyubiquitination on key NF-κB signaling molecules such as TRAF6, RIP1 and RIP2, thereby, inhibiting proinflammatory outcomes of PRR pathways. Additionally, A20 contains one of the C-terminal zinc finger domains (ZnF4) that harbor intrinsic E3 ligase activity selectively conjugating K48-linked polyubiquitin chains onto target molecules and cause their degradation. Therefore, A20 is a novel ubiquitin-editing enzyme with both DUB and E3 ubiquitin ligase activity.

Additionally, CYLD is a tumor suppressor, and loss of CYLD function has been linked to several types of skin tumor. CYLD contains a ubiquitin protease domain which specifically cleaves K63-linked polyubiquitin chains, thus functioning as a suppressor of NF-κB signaling. CYLD is a NEMO-interacting protein that inhibits IKK and NF-κB by removing K63-linked polyubiquitin chains from TRAF2, TRAF6, and NEMO. Other CYLD substrates important for NF-κB regulation include TAK1, Bcl3, and RIP1. HPV E6 has been shown to degrade CYLD in cervical cancer cell lines possibly contributing to the development of cancer.

2.5 HPV and innate immunity

Anogenital HPV infections are very common and the cumulative lifetime incidence of infections is estimated to be as high as 80-85%. However, most of the lesions are cleared and low-grade CIN lesion often regress spontaneously indicating that in the majority of individuals the immune system succeeds in controlling the viral infection before malignant disease develops. The prevalence of persistent HPV infections and HPV-positive lesions is greatly increased in immunosuppressed subjects, such as transplant recipients and HIV-positive patients, indirectly indicating that the immune systems plays a major role in controlling the HPV infections.
In healthy individuals, the duration of a transient anogenital detectable HPV infection, before it is controlled and viral DNA becomes undetectable, ranges from 7-14 months \(^{34, 35}\). This indicates that active viral infections are capable of persisting in the host for quite some time. This lag time suggests that HPV is able to evade and/or interfere with the innate and adaptive immune defenses \(^{36}\). Effective avoidance of immunity might be related to the characteristic infectious cycle of HPV. HPV life cycle is non-lytic and therefore does not elicit a strong proinflammatory signals to attract and activate the APCs as would be generated by dying cells of the host. Additionally, there is no blood-borne or viremic phase of the life cycle. This suggests that HPV-infected KCs need to sense the infection in order to activate an immune response. \(^{37}\). Additionally, HPV does not infect DCs, nor does it express its protein in DCs, therefore, DC need to cross-present the HPV early antigens derived from HPV-infected KCs in order to mount immunity against HPV \(^{38}\). Thus priming of antiviral immunity depends on highly specialized APCs, such as the Langerhans cells (LCs) that can access HPV proteins in the epidermis. Clinical observations show that the number of LCs is significantly reduced at sites of HPV infected premalignant lesions \(^{39, 40}\). Adhesion molecules, such as E-cadherins, are necessary to mediate contact between LCs and keratinocytes but HPV reduce the expression of E-cadherins on keratinocytes cell surface \(^{41}\), suggesting that innate immune signaling, required for the recruitment of APC, is altered by HPV.

HPV oncoproteins have been shown to downregulate the expression of type I interferons and pro-inflammatory cytokines of keratinocytes \(^{42}\). For instance, retrovirally transfected HPV16 E6/E7 in keratinocytes show reduced production of MIP-3α, the most potent chemotactic agent for LC precursors, which results in reduced migration of LCs to the site of HPV infection \(^{t}\) and helps HPV to persist \(^{43}\).

The type I interferons (IFN-α, and IFN-β) have antiviral, anti-proliferative, anti-angiogenic, and immunostimulatory properties and act as a bridge between innate and adaptive immunity \(^{44}\). Both in vitro and in vivo data suggest that HPV has evolved mechanisms to avoid the effects of type I IFN. IFN-α does not effectively inhibit transcription of E6/E7 RNA in several cervical epithelial cell lines immortalized by recombinant HPV16, HPV18, and HPV33 DNA \(^{45}\). Furthermore, pre-malignant lesions from patients non-responsive to treatment with IFN-α have higher levels of E7 mRNA compared to patients that respond to treatment, suggesting that HPV E7 may inhibit IFN signaling \(^{46}\). E7 binds to IRF-9 and prevents the translocation of IRF-9 to the nucleus, thus inhibiting IFN-α-mediated signal transduction by preventing the formation of the ISGF-3 transcription complex that functions by binding to the interferon-specific response element (ISRE) in the nucleus \(^{47, 48}\). Moreover, E7 binds to IRF-1 and inhibits
the activation of IFN-β through recruitment of histone deacetylase to the promoter, thereby preventing transcriptional activation. Additionally, HPV18 E7 inhibits the transactivating function of IRF-1 resulting in reduced expression of IRF-1 target genes, such as the TAP1, IFN-β, and MCP-1 genes. The HPV E6 protein also targets the interferon pathway. By binding to IRF-3 and preventing its transcriptional activation, E6 prevents transcription of IFN-α mRNA. E6 also binds to TYK2 which prevents its binding to the cytoplasmic region of IFN-α receptor 1, and inhibits phosphorylation of TYK2, STAT1, and STAT2, thereby impairing the JAK-STAT signaling pathway resulting in reduced secretion of IFNs. More recently, microarray analysis showed that HPV16 E6 and HPV31 downregulates multiple IFN-responsive genes. E6 decreased expression of IFN-α and IFN-β, downregulates nuclear STAT-1 protein, and decreased binding of STAT-1 to the ISRE. In addition, HPV16 E6 has been shown to degrade pro-IL-1β in a proteasome dependent manner which is mediated via ubiquitin ligase E6-AP and p53.

2.6 Our questions

So far studies addressing HPV-mediated immune evasion have used retrovirally transduced HPV E6 and/or E7, mimicking the situation of KC transformation by HPV or have even used HPV-transformed KC or cervical cancer cell lines. While these studies are of interest with respect to the effects of HPV proteins on the immune response during malignant transformation, they are less likely to reflect how HPV escapes during infection because during cellular transformation the HPV E6 and E7 genes are integrated into the cellular genome and the production of infectious viruses is stopped. Furthermore, the expression of the different HPV early proteins (E1, E2, E4, E5, E6 and E7) is different in early infected and HPV-transformed cells. During the productive phase with infectious virions when the viral genomes are maintained as episomes in keratinocytes, HPVs need to avoid and/or suppress host immunity in order to establish a persistent infection. Thus, although the immunomodulating roles of HPV E6 and E7 in the setting of cell transformation, or quasi-infection have been studied, there is a complete gap in the literature when it comes to the effects of HPV on the responses of KC when the episomal HPV genome is present.

To circumvent this problem we made use of an unique experimental set up in which primary keratinocytes expressing episomal HPV genes were used. When these cells are grown in an organotypic raft culture system, they show differentiation-dependent production of infectious viruses mimicking the situation like early natural infection of HPV. In addition, we also infected primary keratinocytes with native HPV virions. Thus, in contrast to the published studies focused on the effect of HPV proteins on the
immune response during malignant transformation, our set up specifically allowed us to focus on the immune evasive effects of HPV infection at the early phase of infection.

The questions we wanted to answer were:

1) Do KCs express functional PRRs, how is this altered during KC differentiation, and is this altered upon infection of KCs with HPV?

2) Which (groups of) immune genes are deregulated in KCs upon HPV infection and if so what are the molecular mechanisms underlying the HPV effect?

We investigated this in uninfected and hrHPV-infected KCs using genome-wide analyses and biochemical approaches in Chapters 2 + 3.

3 Adaptive immunity against HPV infection

Adaptive immunity consists of T cells and B cells crucial for the formation of immunological memory enabling the immune system to respond rapidly and effectively to a specific pathogen that has been encountered previously. APCs capture HPV proteins and digest them into peptides. The APCs then migrate to the lymph nodes where HPV peptides are presented to the T cells. Simultaneously, DCs or macrophages expressing TLRs, NLRs, RLRs, C-type lectins are activated by binding to the viral PAMPs through innate immune receptors present on cell surface. Moreover, CD4+ T cells recognizing their cognate peptides may activate DCs via CD40-CD40L interaction. The activated immune cells release inflammatory cytokines including IL-1, IL-6, TNF-α, and IL-12, which induce local inflammation and function as chemoattractants to other immune cells or to polarize the immune response for instance to a TH1 (by IL-1, IL-12) type, important for the induction of adaptive immune responses.

After the recognition of an antigen, CD4 T cells may differentiate into for instance Th1, Th2 cells or T regulatory cells, largely determined by the cytokine milieu in the local microenvironment and the activation status of the DCs. T regulatory cells will suppress adaptive immune responses, Th1 cells promote cell-mediated immune responses, and Th2 cells sustain humoral effector responses. B cells are responsible for producing antibodies which function to neutralize and opsonize viral antigens. The growth, maturation, and production of antibodies by B cells are dependent on interaction with APCs and the cytokines profile secreted by CD4 helper T cells. HPV has many targets (E2, E6, E7, L1 and L2) against which antibodies can be generated during natural infection. While antibodies directed against E2, E6, E7 are weak and unable to mediate protective immunity against HPV infection, L1 and L2 capsid proteins are targets
of neutralizing antibodies and may prevent viral infection. The majority of these antibodies are of the IgG1 class, a frequent response against viral antigens. Eight to nine months after natural infection sero-conversion and neutralizing antibodies can be detected, but their levels are low, not apparent in all women, and not likely to prevent against subsequent infections.

The control of HPV infection requires an effective T cell response comprising both virus-specific CD8+ CTLs and CD4+ IL-2/IFN-γ-producing Th1 cells. In healthy individuals, circulating HPV16-specific CD4+ Th1 and Th2 cells and CD8+ CTLs reactive to a broad array of epitopes in the viral early (E2, E6, E7) and late (L1) antigens can be detected that are able to migrate to areas where viral antigens are presented. Furthermore, spontaneous regression of HPV-induced lesions is associated with the presence of circulating CD4+ and CD8+ T cells specific for HPV early antigens and coincident with the infiltration of the lesions by CD8+ CTLs and CD4+ T cells in numbers that surpass those of CD25+ Tregs.

By contrast, most individuals with HPV-induced progressive disease show an undetectable or a weak circulating T cell response to the HPV early antigens. Additionally, progressive disease is associated with a loss of locally present IFN-γ and an increase in immunosuppressive IL-10. In addition, T cells expressing TGF-β have been detected in HPV-induced lesions. IL-10 and TGF-β may directly suppress HPV-specific immunity since IL-10 can strongly inhibit the production of pro-inflammatory cytokines and TGF-β has a potent negative effect on the proliferation and Th1-differentiation of T cells. Moreover, there is a steady increase in the number of tumor-infiltrating Foxp3+ Tregs, IDO+ cells, and macrophages, further suppressing the anti-tumor immunity. Intratumoral CD4+ and CD8+ T cells in this suppressive milieu generally lack the expression of granzyme B and thus are functionally impaired.

3.1 Mechanisms of escape of HPV from adaptive immunity

In hrHPV-induced cancer, HPV E7 downregulates the expression of the transporter associated with antigen protein (TAP1) which is essential in mounting MHC Class I presentation of HPV peptides by transformed cells resulting in suppression of HPV’s antigen presentation thereby impeding the recognition of transformed cells by effector CTLs. Additionally, HPV16 E5 downregulates MHC/HLA class I.

The function of regulatory T cells (Tregs) is the induction of tolerance, but they also suppress anti-tumor responses. The number of Tregs is increased in HPV-induced tumors probably attracted by tumor-produced CXCL12. Tregs inhibit the
proliferation and cytokine (IFN-γ and IL-2) secretion of activated naïve CD4 T cells and Th1 cells. As for APCs, Tregs alter their protein expression necessary for efficient antigen presentation and evoke the production of indoleamine 2,3-dioxygenase (IDO) by dendritic cells, which is an enzyme toxic to T cell populations. Treg derived products like TGF-β, carbon monoxide, galectins, and IL-10 are considered to be immunosuppressive. Notably, IL-10 producing HPV-specific Tregs highly capable of inhibiting the proliferation and cytokine (IFN-γ and IL-2) production of recently activated naïve CD4+ T cells, Th1 cells, and CD8+ CTLs have been isolated from premalignant lesions and cancer, indicating that local immune suppression milieu may be a result of erroneously skewed HPV-specific T cell response. Importantly, high numbers of intratumoral Tregs are associated with poor prognosis of cervical cancer implying role of Tregs in suppression of anti-tumor immunity.

3.2 T cell co-inhibitory molecules in cancer

Effective activation of T cells requires two signals: the first is mediated by the recognition of an antigen presented via the major histocompatibility complex (MHC) on antigen presenting cells (APC) by a corresponding antigen-specific T cell receptor (TCR). Second, co-signaling occurs via T cell co-signaling receptor molecules binding to ligand molecules expressed on the APCs, which can further enhance or dampen primary signaling pathways. Co-signaling is involved in all phases of T cell function including priming, activation, expansion, effector function and contraction.

The best characterized co-signaling molecules includes member of the CD28 and B7 superfamily, which are involved in both co-stimulatory and co-inhibitory processes. The interaction between CD28 and B7 family molecules are critical for immune response for infection and diseases. For example, T cell activation depends on binding of CD28 to B7-1 (CD80) and B7-2 (CD86) on APCs while cytotoxic T-lymphocyte antigen-4 (CTLA-4, CD152), another member of CD28 family downregulates T cell activity by binding B7-1 and B7-2. Molecules of the B7-H1/PD-1 pathway are also critical modulators of the immune responses. Programmed Death-1 (PD-1, CD279) is a member of the CD28 family expressed on activated T cells, B cells, dendritic cells and macrophages. PD-1 has two ligands B7-H1 (CD274, PD-L1) and B7-DC (CD273, PD-L2) of the B7 family. While B7-H1 expression is inducible on a variety of cell types in lymphoid and peripheral tissues, B7-DC is more restricted in myeloid cells including dendritic cells. The major role of B7-H1/PD-1 pathway is to tune down inflammatory immune responses in order to protect tissues and organs from collateral damage.
Co-inhibitory molecules associated with inactivation of T cells include PD1, TIM3, CTLA4, CD160, LAG3 and 2B4. Initial studies indicated that PD-L1- PD1 was a crucial pathway in the regulation of CD8+ T cell exhaustion, as blockade of PD-L1-PD1 interactions in chronic infection or tumor microenvironment restored CD8+ effector function, whereas blockade of other individual co-inhibitory pathways alone (TIM3, CTLA4, and LAG3) showed less effective in rescuing T cells. However, combined blockade of PD-L1-PD1 with these co-inhibitors, notably has a synergistic effect in reversing T cell exhaustion.

More recently it was shown that the expression of these molecules are a sign of T-cell activation rather than exhaustion, only when the cognate ligands are expressed T-cell function may be downregulated. Notably, the expression of multiple co-inhibitory receptors by T cells is associated with a progressive loss in proliferation, production of proinflammatory cytokines (IL-2, TNF-α and IFN-γ), cytotoxicity and the ability to become memory cells.

3.3 PD-1 is a co-inhibitory receptor that can be expressed on activated T cells

PD-1 is a 50-55 kDa type I transmembrane glycoprotein composed of an extracellular Ig domain and a 20 amino acid stalk. Its cytoplasmic tail contains two conserved tyrosine-based signaling motifs, an immunoreceptor tyrosine-based inhibition motif (ITIM), followed by an immunoreceptor tyrosine-based switch motif (ITSM), both of which are phosphorylated upon PD-1 engagement and can have inhibitory function. PD-1 signaling interferes with the earliest tyrosine phosphorylation events in TCR signaling, thereby suppressed the activation of PI3K/Akt resulting in the inhibition of T cell expansion. Additionally, PD-1 engagement recruits both SHP-1 and SHP-2, SH-2-domain containing protein tyrosine phosphatases, which dephosphorylate and deactivate Ras-MEK-ERK and AKT pathways that ultimately result in cell cycle arrest of T cells.

The role of PD-1 in suppressing the antiviral response was first demonstrated by the rapid clearance of adenoviral infection in PD-1 knockout mice compared to wild type. In contrast, Barber et al. in the model of chronic LCMV infection showed that antigenic persistence resulted in high level of PD-1 expression on CD8 T cells which is associated with loss of effector function, and what they called an immune exhausted phenotype. A similar role for PD-1 has been reported in other chronic viral infection such as Hepatitis, SIV and HIV. T cells chronically exposed to antigen within tumor microenvironment may also develop such a functionally inactive phenotype.
3.4 PD-1 in anti-tumor immunity

There is accumulating evidence that tumors exploit PD-1-dependent immune suppression for immune evasion. The aberrant expression of B7-H1 and B7-DC has been found in many solid tumors and hematological malignancies. Additionally, PD-1 expression on tumor infiltrating lymphocytes (TILs) has been reported, suggesting that these T cells might be functionally suppressed. Importantly, a strong correlation between B7-H1 expression on tumor cells and unfavorable prognosis has been demonstrated for many cancers including kidney, ovarian, esophageal, bladder, gastric and pancreatic cancers and melanoma. In renal carcinoma, patients with high tumor and/or lymphocyte B7-H1 levels were 4.5 times more likely to die of their cancer than patients with low levels of B7-H1 expression. Ovarian cancer patients with tumors positive for both B7-H1 and B7-DC showed dramatically lower survival rate than patients with tumors negative for both of these ligands (46% versus 83% for 5-year survival). New studies focus on the role of other co-inhibitory markers and how they are exploited by cancers to evade host immunity.

3.5 PD-1 and regulatory T cells (Tregs)

Tregs play a critical role in the maintenance of immune tolerance. CD4+ Foxp3+ Tregs are the most studied suppressive T cell population. The Foxp3 knockout mice develop severe autoimmune conditions and the mutations of the human gene FOXP3 is associated with fatal human immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) indicating the crucial roles of Foxp3+ Tregs in peripheral tolerance. Foxp3+ Tregs can be divided into “natural” Tregs and “induced” Tregs. While “natural” Tregs develop as committed regulatory cells from the thymus, the “induced” Tregs arise in the periphery by polarization of naïve CD4+ T cells for instance when the microenvironment produces TGF-β and IL-2 or IL-10.

Tregs are often associated with solid tumors in both human and murine models. Increased number of Tregs is associated with a poorer prognosis in many human cancers. Foxp3+ Tregs highly express PD-1 and B7-H1. Surprisingly, B7-H1 binding to PD-1 on natural Tregs has been shown to inhibit Treg suppressive function, whereas PD-1 ligation on the conventional T cells has been shown to promote their differentiation into induced Tregs. The ability of PD-1 to deliver signal through B7-H1 on Tregs remain unclear. Moreover, the exact function of co-stimulatory and co-inhibitory molecules on both natural Tregs and induced Tregs needs further clarification.
3.6 Cancer immunotherapy targeting PD-1 and B7-H1

Monoclonal antibody-mediated immune checkpoint blockade of the inhibitory immune receptors CTLA-4, PD-1, and PD-L1 has shown to be successful in treating patients with advanced cancer. Treatment with CTLA-4 blocking monoclonal antibody ipilimumab improved the overall survival of untreatable metastatic melanoma patients. Strikingly, the durability of objective responses by CTLA-4 blockade leading to a possible cure for some patients has fuelled new enthusiasm on cancer immunotherapy in general.

Consequently, a number of clinical trials are examining the blockade of PD-1 by monoclonal antibody therapy (anti-PD-1 mAb; Bristol-Myers Squibb [New York, NY, USA], CureTech/Teva [Yavne, Israel] and Merck [Boston, MA, USA]) and B7-H1 (anti-B7-H1 mAb; Bristol-Myers Squibb, Genentech [San Francisco, CA, USA]) in the treatment of refractory solid tumors. Two large Phase I trials blocking PD-1/B7-H1 interactions in patients with advanced cancers including melanoma and non-small cell lung cancer (NSCLC) have reported highly promising results. Topalian et al. used an IgG4 monoclonal antibody (BMS-936558) that targets PD-1 while Brahmer et al. used a monoclonal antibody that targeted B7-H1. Tumor response rates were between 18-28% for patients treated with anti-PD-1 antibody and 6-17% for the anti-B7-H1 antibody. Moreover, combined blockade of CTLA-4 (with ipilimumab) and PD1 (with nivolumab) in advanced melanoma was associated with rates of objective response that exceeded the previously reported results with either ipilimumab or nivolumab alone, albeit that in another study with the anti-PD-1 antibody lambrolizumab a similar response rate was observed.

3.7 Our questions

The transition from normal epithelium, via low grade and high grade lesions to cervical carcinoma is associated with locally present influx of CD4+ and CD8+ T cells, nevertheless, these T cells are not always able to mount an effective anti-tumor response. Several mechanisms could affect the efficacy of the T-cell response, in particular the presence of regulatory T cells, the presence of immune suppressive myeloid cells and/or the expression of co-inhibitory receptors and their ligands. Earlier our lab showed a role for intratumoral regulatory T cells in hampering T-cell reactivity and later the role of myeloid cells was addressed. Therefore, in Chapter 4 of this thesis, we focused our studies on the expression of B7-H1/PD-L1 and B7-DC/PD-L2 by the tumor cells and the expression of PD1 by CD4, CD8 and regulatory T cells.
Chemokines in cancer related inflammation

Chemokines are immune-cell attracting cytokines (about 8-17 kDa) that binds to and activate a family of chemokine receptor. So far, over 50 chemokines have been described. On the basis of the position of four conserved cysteine residues, the chemokines can be divided into four families (CXC, CX3C, CC and C). Functionally, chemokines can be divided into inducible or inflammatory chemokines and constitutively expressed or homeostatic chemokines. Inflammatory chemokines are critical for attracting a diverse set of effector leukocytes to inflammatory sites and as such they play a key role in the innate immune response by recruiting neutrophils, monocytes/macrophages, dendritic cells (DC), and natural killer (NK) cells. Homeostatic chemokines play major roles in migration of antigen-presenting cells (APC) and lymphocytes into the lymph node, as well as in migration of effector T cells to reach tissues, thus play critical roles for an effective adaptive immune response. Recently, chemokines and their receptors have been identified as mediators of chronic inflammation which play a key role in the tumor growth, angiogenesis, and metastasis.

4.1 Chemokines, tumor-associated leukocytes and tumor microenvironment

Cancer cells create a favorable microenvironment by interacting with the stromal cells (including cancer associated fibroblast, CAFs, mesenchymal stem cells, MSCs) and triggering the homing of leukocytes (including tumor-associated macrophages, TAMs, and tumor-associated neutrophils, TANs). Intratumoral CAFs secrete altered types of chemokines including CXCL12 which promotes cancer cell proliferation and angiogenesis by recruiting endothelial cells into carcinomas. Moreover, CAFs secrete TGFβ which in turn increase the levels of CXCR4 in cancer cells, thus activating CXCR4/CXCL12 axis resulting in increased proliferation of cancer cells by activating Akt. Recent work has also shown that MSCs produce chemokines like CCL5, when they come in contact with cancer cells. Secreted CCL5 acts on CCR5 present at the surface of breast cancer cells promoting their metastasis to the lung. Cancer cells can also recruit circulating cells including monocytes and macrophages to the tumor. CC chemokines, especially CCL2 and CCL5, are major attractants of monocytes and macrophages to the tumor microenvironment and their levels correlate with the number of the infiltrating myeloid cells. Macrophages in tumors (TAMs) are usually of the M2-type, promote tumorigenesis and are associated with poor prognosis. Lymphocytes particularly TH2 lymphocytes are the other major leukocytes found in cancers. Their recruitment is controlled by CC and CXC chemokines. Infiltrating TH2 lymphocytes are tumor promoting and are associated with poor prognosis. In human cervical cancer, TAM secreted VEGF-C was proposed to be involved in peritumoral lymphoangiogenesis, and ultimately to lymphatic metastasis.
4.2 Tumor cell survival, and proliferation, tumor growth and progression.

The expression of chemokine receptors by tumor cells affects their own proliferation and survival \(^{136, 137}\). Early studies show that antibodies against CXCR2 inhibited melanoma cell growth in vitro implying the role of CXCR2 in tumor growth \(^{138}\). Similarly, CXCR4/CXCL12 chemokine/chemokine receptor pairs have been shown to be very efficient in enhancing tumor cell growth \(^{139-141}\). Additionally, the stable expression of CXCR7 was shown to increase the survival of breast cancer cells in vitro without affecting their in vitro proliferation \(^{142}\). Knockout of CXCR7 in cancer cells and use of the CXCR7 antagonist CCX754 reduces tumor growth in vivo. Altogether this suggests critical role for CXCR7 in growth and survival of cancer \(^{142, 143}\). Moreover, it has been reported that chemokines and growth factors can influence each other in some tumors. Estrogens increases the expression of CXC12 which activates CXCR4/CXCL12 signaling pathway that in turn promotes estrogen receptor transcriptional activity \(^{144}\).

4.3 Tumor cell invasion and metastasis

The expression of chemokines and their receptors have been implicated in the distinct tropism of metastatic sites or cancer cells. The binding pairs CXCR4/CXCL12, CCL19-CCL21/CCR7 and CCL27/CCR10 are involved in metastasis in bone, lymph node and skin respectively \(^{145}\) due to high concentrations of chemokines produced by the site of metastasis that attract cancer cells to these locations or through generating a gradient of chemokines by the tumor cells creating autologous chemotaxis and a continuous cycle of recruitment of the cancer cells actively promoting their own metastasis and tropism \(^{146}\). Pancreatic ductal adenocarcinoma cells have been shown to express high levels of CX3CR1 and migrate towards a gradient of its ligand, CXC3CL1, produced by neurons and nerve fibers causing the cancer cells to metastasize in brain \(^{147}\). Clinical studies showed that CCR9-expressing human melanomas metastasize to the small intestine which expresses high level of CCL25, the ligand for CCR9 \(^{148}\). T cell acute lymphocytic leukemia (T-ALL) shows high risk of metastasis in the central nervous system (CNS). Silencing of CCR7 or its ligand CCL19 is sufficient to inhibit CNS metastasis in T-ALL \(^{149}\). Additionally, cancer stem cells expressing CXCR4 have been identified at the invasive front of the tumor which determines that metastatic phenotype of individual tumors \(^{150}\). CXCR4 and CCR7 expression are associated with lymph node metastasis as well as poor prognosis in patients with cervical cancer \(^{151-153}\). The distribution and the intensity of expression of CXCL12, CXCR4, CXCL16, and CXCR6 increases as neoplastic lesions progress through CIN1, CIN2, and CIN3 to invasive cervical cancer. Moreover, among those molecules only CXCR6 is associated with long-term outcomes in that the patients with high CXCR6 expression had significantly shorter overall survival than those with
low CXCR6 expression and the expression of CXCR6 is associated significantly with lymph node metastasis \(^{154}\).

### 4.4 Oncogene pathways involved in chemokine production and chemokine receptor signaling

Chemokines bind to G-protein-coupled receptors (GPCRs) and activate a series of downstream effectors and signal transduction pathways including PI3K, Jak-STAT \(^{155}\). In cancers, inactivating mutations of tumor-suppressors or activating mutations of oncogenes have been associated with deregulated production of chemokines in cancer cells which in turn cause tumor initiation and progression. Ras is frequently mutated in human cancers, which activates EGFR-ras-raf signaling pathways leading to the production of tumor-promoting chemokines including CXCL1 and CXCL8 \(^{156, 157}\). Myc is over-expressed in many human tumors and myc-activated tumor cells produce chemokines that can recruit mast cells to induce new vessel formation and tumor growth \(^{158, 159}\). Moreover, wild type p53 but not the cancer-specific mutants (R175H or R280K) represses CXCR4 expression resulting in reduced invasion of cancer cell lines through matrigel suggesting that mutation of tumor suppressor p53 increases the production of tumor-promoting CXCR4 \(^{160}\). Oncogenic changes not only produce tumor-promoting chemokines, but may also suppress homeostatic chemokines production. EGFR-Ras signaling in cutaneous tumor cells reduces their ability to express CCL27, resulting in impaired recruitment of anti-tumor T-lymphocytes to the site of tumors \(^{161}\). Transgenic mouse models, expressing the early genes from HPV 16 under the control of the human keratin 14 promoter, have shown that HPV-induced lesions release the chemokine CCL2 which enhances macrophage recruitment into tumors via CCR2 \(^{162}\). In human neoplastic cervical epithelial cells, HPV 16 E5, E6 and E7 oncogenes have been shown to induce the inflammatory cyclo-oxygenase (COX)-prostaglandin axis, by elevating the expression of COX-2 which is involved in oncogenesis \(^{163}\). These studies directly link HPV oncogenes with the activation of inflammatory cascades in promoting cervical cancer. Thus, HPV is not associated with inflammation at the initial phase of infection, however, it is likely that following HPV DNA integration and transformation, hrHPV-transformed cells drive dysregulated inflammatory cascades, such as the COX-prostaglandin pathway in transformed epithelial cells promoting immune cell infiltration, chronic inflammation and ultimately to tumor progression \(^{164}\).

### 4.5 Our questions

Chemokine receptors have been studied in the context of outcome of cervical cancer patients \(^{151}\) (and discussed above). Our genome-wide mRNA expression profiling data...
showed that CXCR7 expression is upregulated in hrHPV-infected KCs. Hence, we wondered if CXCR7 was also expressed in HPV+ tumors and whether its expression would be associated with disease outcome. Therefore, we studied a series of cervical cancers for the expression of CXCR7 and the co-dependency of expression of its putative receptors EGFR and CXCR4, the expression of its predominant ligand CXCL12 as well as associations with clinical outcome in cervical cancer patients in Chapter 5.
References


93. Fourcade, J. et al. CD8(+) T cells specific for tumor antigens can be rendered dysfunctional by the tumor microenvironment through upregulation of the inhibitory receptors BTLA and PD-1. *Cancer research* 72, 887-896 (2012).


103. Petrovas, C. et al. SIV-specific CD8+ T cells express high levels of PDI and cytokines but have impaired proliferative capacity in acute and chronic SIVmac251 infection. *Blood* 110, 928-936 (2007).


111. Gregori, S. *et al.* Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway. *Blood* 116, 935-944 (2010).


