Mechanisms underlying the resistance of human papillomavirus-infected or -transformed cells to Th1 immunity

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Chapter 6

Discussion

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Human papillomavirus is one of the most common sexually transmitted pathogens in the world [1]. A persistent HPV infection can lead to the development of malignancies. The immune system plays a crucial role in controlling the progression of the disease and about 90% of the infections are cleared within three years, while 10% persist and less than 1% develop into cervical cancer [2]. A type 1 T-cell response is important for the control of HPV infections and individuals with a suppressed T-cell response display more infections [3, 4]. Furthermore, an HPV-specific Th1 immune response is frequently detected in healthy donors [5, 6] and the induction of strong HPV-specific type 1 T-cell responses by therapeutic vaccination is associated with regression of CIN or VIN lesions [7-12]. Finally, also in cancer, a type 1 immune contexture is associated with a better response to standard therapy and immunotherapy [13], including CxCa and OPSCC [13, 14]. In this thesis, we study the mechanisms allowing HPV-infected and -transformed cells to resist the attack of a type 1 T-cell response.

1. Human papillomavirus-infected cells are less sensitive to the antiproliferative effects of IFNγ

Human papillomavirus has developed multiple direct and indirect mechanisms to influence cell proliferation. The HPV E6 and E7 oncoproteins act to increase the proliferation of HPV-infected cells in the epithelium. Human papillomavirus interferes with the normal terminal differentiation process, thereby increasing the number of HPV-infected cells, which eventually produce more infectious virions [15-17]. Under certain conditions, the E2 protein can induce growth arrest, cell senescence and apoptotic cell death [18-20]. The receptors for IFNγ and TNFα are widely distributed among all nucleated cells, and activation of these receptors may have antiproliferative and proapoptotic effects. Regarding apoptosis, IFNγ induces IRF1 which reduces BCL2 and BAK, leading to the release of cytochrome C from the mitochondria and caspases, resulting in apoptosis [21]. Furthermore, IFNγ may trigger tumor cells to produce high concentrations of RNI and ROS, with apoptosis of the cell as a

result [22]. IFNγ is also reported to induce autophagy in HCC [23] and has an inhibitory effect on proliferation. Binding of IFNγ to the IFNγ receptor (IFNγR) leads to JAK1/2-mediated STAT1 phosphorylation, dimerization and nuclear translocation, which results in interferon-stimulated gene (ISG) expression [24]. IFNγ has been shown to induce growth arrest and differentiation of KCs [25, 26], as well as the arrest of cancer growth by IFNγ downregulating cyclins E and A, thereby inhibiting tumor growth [27]. Furthermore, activated STAT1 interacts with cyclins D1/CDK4, resulting in cell-cycle arrest [28]. Moreover, IFNγ has been shown to upregulate the cell-cycle inhibitors p27 and p21, which suppress the activity of E2F transcription factor and inhibit the activation of genes involved in cell proliferation [29]. A previous study shows that STAT1 is selectively suppressed by HPV to allow for HPV genome amplification and maintenance of episomes [30]. In chapter 2, we confirm that HPV downregulated STAT1 expression but also show that the inhibition of STAT1 was not complete, as IFNγ was still able to induce phosphorylation of STAT1. Importantly, we found that HPV resisted the antiproliferative effects of IFNγ through downregulation of the STAT1 downstream targets *IFITM1* and *RARRES1* (chapter 2). IFNγ-mediated activation of IFITM1 results in the inhibition of ERK phosphorylation, thereby suppressing MAPK signaling. IFITM1 also increases the stability of p53 and arrests the cell cycle at G1 phase [31]. Indeed, in our experiments, IFNγ treatment reduced about 50% of the cells in the S phase in normal KCs, though this was not observed in the HPV-positive KCs, indicating that HPV resisted the anti-proliferation effects of IFNγ by downregulating the expression of *IFITM1*. Furthermore, we found that the expression of *RARRES1* was significantly decreased in the HPV+ KCs. *RARRES1* is considered as a putative tumor-suppressor gene, largely based on the hyper-methylation of its promoter in many tumor types and ageing normal tissues [32-35]. Expression of RARRES1 inhibits cell growth in prostate and endometrial cancer cells [36, 37]. Moreover, we found that HPV significantly increased the expression of the proliferating cell nuclear antigen (PCNA), which is essential for the DNA replication of small DNA tumor viruses associated with HPV infection and the

progression of cervical intraepithelial neoplasia (CIN) [38], and is considered to be a marker for cell proliferation in various cancers [39].

Thus, under normal physiological conditions, Th1 cells may migrate to HPV-infected lesions and secrete IFNγ to control viral replication by inhibiting cell proliferation of HPV-infected cells via the increased expression of *IFITM1* and *RARRES1*. According to our results, HPV may escape these effects of immune surveillance by downregulating the expression of these anti-proliferation genes and upregulating the proliferation marker *PCNA*. As *RARRES1* and *PCNA* also play a role in oncogenesis, the alteration of their expression by HPV may also contribute to the malignant transformation of the infected KCs. Hence, if the cytokines produced by type 1 T cells cannot interfere sufficiently with cell growth to prevent virus production or division of transformed cells, there is a need to kill the infected or transformed cells by induction of cell death.

2. Human papillomavirus impairs TNFα/IFNγ-induced necroptosis

Necrosis is an inflammatory type of cell death characterized by cell swelling, loss of plasma membrane integrity and release of cytosolic contents into the extracellular space [40], and plays a role as a host defense strategy to prevent viral infections [41]. The murine cytomegalovirus [42, 43] and influenza A virus (IAV) [44-46] activate DAI-dependent necroptosis via RIPK3. Reovirus induces caspase-independent cell death [47], which forms part of the mechanism that leads to immune control of these viral infections. In an attempt to prevent the attraction of the immune system, many viruses have developed mechanisms to suppress necroptosis. Herpes simplex virus 1 (HSV-1) ICP6 and herpes simplex virus 2 (HSV-2) ICP10 proteins prevent necroptosis in human cells by inhibiting the interaction between the receptor-interacting protein kinases RIP1 and RIPK3 [48]. Human cytomegalovirus suppresses RIPK3-dependent necroptosis [49].

In chapter 2, we examine IFNγ+TNFα-mediated apoptosis in both KCs and HPV+ KCs. Our data suggest that IFNγ+TNFα alone did not cause substantial apoptotic cell death in either of these cells. Necroptosis can be induced by IFNγ and TNFα when cIAPs and caspase-8 are

inhibited by BV6 and zVAD-fmk, respectively. We examined the expression of cIAPs and caspase-8 in the normal KCs and HPV+ KCs, and found that both of these molecules were still present in HPV+ KCs. In order to prime KCs and HPV+ KCs for necroptosis, the cells were treated with BV6 and zVAD-fmk. However, IFNγ+TNFα-induced necroptosis was significantly higher in KCs than in HPV+ KCs. We show that downregulation of RIPK3, which is the key component of the necrosome, was the underlying mechanism (chapter 2). As necroptosis is key in initiating the adaptive immune response for the control of viral infections, HPV evolved to remain stealthy and evade necroptosis induced by the Th1 cytokines IFNγ and TNFα. Moreover, Fas, granzymes and perforins are important mediators of cell death used by type 1 T cells. Others found that RIPK3 knockout endothelial cells resisted necroptosis induced via these molecules [50]. As RIPK3 is downregulated by HPV, HPV+ KCs may also partly resist the cytotoxicity effects of T cells' Fas, granzymes and perforins.

We found that RIPK3 was downregulated at the transcription level by HPV, indicating that methylation may be involved. We found that treatment of the cells with DZNep, which is a global inhibitor of histone methyltransferases that depletes EZH2, restored the expression of RIPK3 in HPV+ KCs. As a result, DZNep also restored the sensitivity of IFNγ- and TNFα-induced necroptosis in HPV+ KCs. However, catalytic EZH2-inhibitor GSK503 did not restore RIPK3 expression, indicating that EZH2 indirectly suppresses RIKP3 expression or that other histone methyltransferases are also involved. We tested about 40 methyltransferases in the KCs and HPV+ KCs, and found that HPV altered about eight methyltransferases in KCs. Therefore, the downregulation of RIKP3 by HPV may be a complex effect due to HPV's alteration of several methyltransferases (chapter 2).

Unlike apoptosis, necroptosis is a highly inflammatory process. It mediates the release of intracellular DAMPs, including interleukin 1a, HMGB1, uric acid, ATP and DNA, resulting in the recruitment of proinflammatory cell types to sites of infection [51]. In this process, RIPK3 also drives the production of IL-1β [52], which is an important

factor for the initiation of inflammation and the activation of immune cells such as macrophages and T cells [53]. In HPV16-immortalized human KCs, IL-1β secretion is impaired because the pro-IL-1β is degraded in a proteasome-dependent manner, mediated via the ubiquitin ligase E6-AP and p53 [54]. Biopsies from different progression states (cervical intraepithelial neoplasia, CIN I-III) and cervical cancer show a decrease of pro-IL-1β protein expression with an increased progression stage [54]. Thus, HPV prevents IFNγ and TNFα-mediated necroptosis, which may be one of the mechanisms contributing to the escape of HPV from immune surveillance and could explain why HPV behaves as a stealthy virus.

3. HPV-positive head and neck cancer is not sensitive to IFNγ- and TNFα-induced necroptosis, is there a need for chemotherapy co-treatment?

Subsequently seeking to understand whether a similar mechanism plays a role in cancer and whether this is HPV specific, we studied oropharyngeal cancers, as half of them are induced by HPV. Moreover, HPV-positive OPSCC displays a far better prognosis than HPV-negative tumors after (chemo)radiation therapy [55, 56], which is associated with a strong adaptive immune response at the tumor site [56-58]. In chapter 4, we show that the majority of HPV-positive OPSCCs is infiltrated with HPV16-specific T cells, producing high concentrations of IFNγ, TNFα and IL17A. By contrast, the tumor-infiltrating T cells from the group of patients who lacked an HPV-specific immune response displayed a low production of IFNγ and IL17A, while the production of IL-5 was increased, suggesting a shift towards a type 2 cytokine profile. The presence of HPV16-specific Th1/Th17 cells was strongly associated with better survival, suggesting that a Th1/Th17 immune response mediated the control of cancer cells.

To understand whether Th1/Th17 cells may contribute to necroptosis, we examined several proteins related to the apoptosis and necroptosis in the TNFR pathway in HPV- and HPV+ OPSCC cell lines in vitro. The different cell lines displayed some variance in the expression of these proteins. TRAF2 is considered as an antiapoptosis

protein that recruits cIAP1/2 to promote NF-κB signaling [59]. TRAF2 is an NF-κB-activating oncogene in epithelial cancers which is amplified and rearranged in 15% of human epithelial cancers [60]. TRAF2 was downregulated by IFNγ and TNFα treatment in UM-SCC19 (HPV-), UM-SCC47 (HPV+) and UM-SCC104 (HPV+), while TRAF2 expression was already relatively low in two other HPV cell lines (UM-SCC6 and UM-SCC4). cIAP1 and cIAP2 (cIAP1/2) are cellular inhibitors of apoptosis proteins, the amplification or genetic mutation of which has been associated with cancers and may promote tumor cell survival [61]. cIAP1/2 expression was at a low level in UM-SCC4 and UM-SCC104. Fas-associated death domain protein (FADD) is a classical adaptor protein mediating apoptotic stimuli-induced cell death. In cancer, however, FADD protects pancreatic cancer cells from drug-induced apoptosis [62]. Fas-associated death domain protein also plays a role in necroptosis. When caspase-8 is inhibited by inhibitors or the depletion of FADD by shRNA, cells undergo necroptosis via RIP1-RIPK3-complex formation and the activation of downstream pathways [63-66]. We found that FADD expression in UM-SCC6, UM-SCC47 and UM-SCC104 was relatively low and that RIPK3 is absent in the HPV-positive HNC cell lines. Furthermore, we examined whether treatment with the Th1 cytokines IFNγ and TNFα induced apoptosis and necroptosis in these cell lines. Similar to our results with KCs, no significant apoptosis was induced and the HPV-positive head and neck cancer cells were less sensitive to IFNγ- and TNFα-induced necroptosis. RIPK3 expression is absent in various cancer cell lines, including the HPV-positive cell line Hela, due to the genomic methylation at the site of the RIPK3. The loss of RIPK3 expression in many cancer cell lines is due to hypermethylation in the promoter region, which was high for RIPK3 [67]. The hypomethylating agent 5-AAD can restore the expression of RIPK3 and consequently increase the sensitivity to chemotherapeutics in a RIPK3-dependent manner [68]. It needs to be tested whether the absence of RIPK3 in the HPV-positive HNC cell line is due to DNA methylation or, similar to HPV-positive KCs, is the result of histone methylation.

Many types of cancer cell lines can undergo necroptosis by classic necroptosis inducers and existing chemotherapeutic agents, including colorectal cancer, leukemia, multiple myeloma, lung cancer, ovarian cancer, breast cancer, hepatocellular carcinoma, bladder carcinoma, head and neck carcinoma, glioblastoma, cervical cancer and neuroblastoma [69]. Necroptosis is activated in response to many chemotherapeutic agents and contributes to chemotherapy-induced cell death [68]. Among the abovementioned cancer cells, colorectal cancer cells and hematopoietic neoplasms (e.g., leukemia and multiple myeloma) appear to be more sensitive and responsive to necroptosis inducers [69]. Thus, triggering necroptosis may be an alternative way to eradicate apoptosis-resistant cancer cells. However, numerous cancer cell lines develop mechanisms to evade necroptosis. Similar to the HPV-positive head and neck cancer cell lines UM-SCC47 and UM-SCC104, the cervical cancer cell line Hela, which is also HPV positive, is resistant to necroptosis due to the low level of RIPK3. In primary colon cancer tissues, RIPK1 and RIPK3 are downregulated [70], similarly to the OPSCC cell line UM-SCC6. Stimulation of cervical cancer cells can occur with poly I:C-induced necroptotic cell death but relies on the expression of RIPK3 [71], which is known to gradually decrease during cervical carcinogenesis [54]. In acute myeloid leukemia samples, RIPK3 is decreased without a significant decrease of RIPK1 [72]. In addition, RIPK3 and CYLD are markedly downregulated in chronic lymphocytic leukemia, which is resistant to TNFα and zVAD-induced necroptosis [73]. RIPK3-mediated phosphorylation of mixed lineage kinase domain-like (MLKL) protein triggers necroptosis and leads to plasma-membrane disruption [74]. Reduced MLKL is found in pancreatic adenocarcinoma and is associated with decreased overall survival [75]. However, MLKL expression was high and not altered in our OPSCC cell lines (chapter 4). Resistance to cell death is one of the hallmarks of a cancer cell and tumor formation often selects against the expression of cell death proteins [76]. While RIPK3 expression is lost in HPV-positive OPSCC cell lines and many other cancer cell lines [68], RIPK3 is present in normal tissue and primary cells [68], which suggests that RIPK3

expression is negatively selected during initial tumor development or growth.

To improve necroptotic cell death, several drugs can be used. Breast cancer cells MCF-7 overexpress Bcl-2 and are resistant to proapoptosis drugs. Shikonin, a naturally occurring naphthoquinone, induces necroptotic cell death in MCF-7 [77]. Obatoclax, a putative antagonist of Bcl-2 family members, triggers autophagy-dependent necroptosis to reverse glucocorticoids resistance in acute lymphoblastic leukemia [78]. IAP antagonist with caspase-inhibitor zVAD treatment induces TNF-dependent necroptotic death in cisplatin and IAP-antagonist-resistant ovarian carcinoma cell lines [79]. However, the caspase inhibitors also inhibit T-cell proliferation, thus making it inadvisable to combine zVAD with immunotherapy [80, 81]. RIPK3 expression may be restored in most cells by the use of simple hypomethylating agents such as 5-AD, which is far more effective when combined with other chemotherapeutic drugs [68]. Hence, the RIPK3 expression status in cancer cells may critically influence the outcome of immunotherapeutic approaches and should therefore be assessed prior to immunotherapy.

To test the potential effect of the OPSCC-infiltrating Th1/Th17 cell-produced cytokines IFNγ and TNFα on tumor cell proliferation, we used the supernatant from antigen-stimulated HPV-specific Th1 or Th17 cells. This revealed a reduction in cell proliferation and an increase in the expression of the antiproliferative genes *IFITM1* and *RARRES1*, both of which have antiproliferative effects, suggesting that these cytokines hamper the proliferation not only of HPV-infected KCs but also of OPSCC (chapter 4). However, there was quite some variability between the different OPSCC cell lines that were tested. UM-SCC4 (HPV-), UM-SCC19 (HPV-) and UM-SCC47 (HPV+) were more sensitive to cytokine treatment compared to UM-SCC6 (HPV-) and UM-SCC104 (HPV+). It is probable that multiple mechanisms regulate the proliferation of and contribute to cell death induced by IFNγ and TNFα are involved. We found that IRF1 expression, which may lead to apoptosis [21], was significantly upregulated by IFNγ and TNFα in our OPSCC cell lines. STAT1 expression, which via the cyclins D1/CDK4

may arrest the cell cycle [28], was also significantly increased by IFNγ and TNFα in all OPSCC cancer cell lines. TNFα has multiple effects on the cancer cells. We found that TNFα alone did not cause a significant increase of apoptosis in OPSCC cell lines, but experiments in mice indicate that together with cisplatin it could synergize to induce apoptosis [82]. Cisplatin is the chemotherapeutic drug for the treatment of OPSCC. The combination of TNFα and cisplatin resulted in an increased percentage of apoptotic tumor cells and especially in the HPV-positive cell lines, as no synergistic effect was observed the HPV-negative cell lines, probably because cisplatin alone efficiently caused cell death in most HPV-negative cells. TNFα was also shown to enhance the anti-cancer effects of doxorubicin through suppressing the antiapoptotic activity of p21- and p53-deficient cancers [83].

4. The EGFR pathway suppresses the amplification of T-cell infiltration.

We show that head and neck cancers could resist the attack of type 1 T cells by interfering with mechanisms of cell proliferation and cell death. From our studies in KC, we obtained evidence supporting that the EGFR pathway is upregulated by HPV and interferes with the IFNγ and TNFα-induced expression of cytokines and chemokines, which may attract T cells [84]. The EGFR is frequently overexpressed in the cancers of patients with poor prognosis and is found to be overexpressed in 80–90% of HNSCC [85]. Therefore, as discussed in chapter 5, we examined the phosphorylation of proteins downstream of EGFR after treatment with cetuximab and found that cetuximab blocked most of the downstream pathways of EGFR, including the RAF-MEK-ERK, AKT-mTOR and MAPK pathways. We also found that inhibition of EGFR by cetuximab combined with IFNγ and TNFα led to increased cytokine production, including CCL5, CXCL9 and CXCL10, which function as T-cell-attracting chemokines to tumor sites. Chemotaxis assays in vitro confirmed that more lymphocytes migrated after the treatment of tumor cells with cetuximab and IFNγ and TNFα. This is coherent with the observation that the presence of an activating EGFR mutation is related to a lower T-cell infiltration of

human tumors [86]. Moreover, previous studies reveal that EGFR has important immune-regulatory effects. Activation of EGFR repressed the expression of MHC class I and II [87]. Overexpressed EGFR significantly correlated with JAK2 and PD-L1 expression in a large cohort of HNC specimens and PD-L1 expression was induced in an EGFR- and JAK2/STAT1-dependent manner [88]. In lung tumors, the expression of mutant EGFR in bronchial epithelial cells induced the expression of PD-L1, which was reduced by EGFR inhibitors in non-small cell lung cancer cell lines. Furthermore, the blockade of PD1 improved survival of mice in EGFR-driven murine lung tumors [89]. Together, these data suggest that EGFR has negative effects on the recruitment and effector function of T-cell immunity. Although not formerly proven in humans, data in mice suggest that the clinical effect of effective EGFR blockade indeed depends on T-cell immunity. Depletion of either $CD8^+$ or $CD4^+$ T cells was reported to abrogate the beneficial effects of EGFR inhibitor treatment in mice [90].

Importantly, we found that cetuximab alone did not significantly alter chemokine expression. Only when combined with the Th1 cytokines IFNγ and TNFα did EGFR blocking by cetuximab increase cytokine expression (chapter 5). As patients whose OPSCCs are infiltrated with type 1 T cells display far better survival, the presence of a type 1 T-cell response may improve the anti-tumor effects of EGFR inhibition. Indeed, TNFα was shown to enhance the tumor-regression effects of monoclonal antibodies against EGFR to cancer cell xenotransplants, as well as spontaneously occurring tumors from the larynx, pharynx, mammary gland, uterine cervix and vulva [91]. Moreover, TNF-α treatment sensitized tumors that initially did not respond to antibody treatment [91].

We aimed to find the underlying pathway responsible for the regulation of cytokines. We blocked the downstream pathway of EGFR by several inhibitors with or without the stimulation of IFNγ and TNFα and found that the inhibition of MEK1 and JNK significantly increased the cytokines at the gene level. A previous study showed

that activation of cells via IFNγR/TNFR results in cytokine mRNA production, but this mRNA is destabilized via EGFR-mediated overexpressed MEK/ERK1/2. Inhibition of ERK1/2 induces an even more severe inflammatory response in the skin [92], showing that EGFR and its downstream pathway suppresses the local immune response. Others have demonstrated that the MEK pathway selectively downregulates the human rhinovirus-16-induced epithelial production of CXCL10. Furthermore, PD98059 and U0126, two inhibitors of the MEK1/2-ERK MAPK pathway, significantly enhanced HRV-16-induced CXCL10 [93]. Our data presented in chapter 5 show that MEK1-inhibitor PD98059 alone did not alter *CCL5, CXCL9* and *CXCL10*, but when combined with IFNγ and TNFα significantly enhanced the gene expression of *CXCL9* and *CXCL10*, as well as *CCL5* in SCC4. Similar results were also observed after JNK inhibition by SP600125 when combined with IFNγ and TNFα stimulation. The JNK pathway plays a complex role in innate and adaptive immune systems. When MKP1 is knocked out, JNK signaling is activated, resulting in enhanced cytokine production of CCL2, CXCL10, TNF, IL6 and IL10, which leads to massive neutrophil infiltration to the lung and liver [94]. In Mkp5 knockout mice, activated JNK signaling increases TNF, IL6, IFNβ, IFNγ and TGFβ production by innate immune cells, and decreases Th1 and Th2 cytokines production by adaptive immune cells [95, 96]. Our data show that JNK inhibition combined with IFNγ and TNFα significantly increased *CCL5*, *CXCL9* and *CXCL10* gene expression, especially in the HPV+ OPSCC cell lines, while EGFR blocking enhanced *CCL5, CXCL9* and *CXCL10* production mainly via the downstream JNK and MEK1 pathways. Interestingly, EGFR blocking by cetuximab plus IFNγ and TNFα increased the T-cell-attracting chemokines but decreased IL1β expression. Tumor-derived IL1β secreted into the tumor microenvironment has been shown to induce the accumulation of MDSC possessing an enhanced capacity to suppress T cells [97]. Blocking of the downstream molecule c-RAF by GW5074 blunted the IFNγ and TNFα-induced expression of the T-cell-attracting chemokines while enhancing the expression of IL-1β, thereby confirming earlier reports that RAF can be activated by both IFNγ [98] and TNFα [99], and revealing an important role for c-RAF in

relaying the signals induced by IFNγ and TNFα that lead to cytokine production. Thus, EGFR blockade may stimulate the attraction of T cells while suppressing that of MDSC.

Besides the EGFR signaling pathway, many other oncogenic signaling pathways may also have an impact on immune signaling [100]. β-catenin-positive tumors had minimal T-cell infiltration due to the reduced production of CC-chemokine ligand 4 (CCL4) by tumor cells, resulting in a failure to recruit basic leucine zipper transcriptional factor ATF-like 3 lineage dendritic cells (BATF3 DCs) into the tumor microenvironment. Owing to a lack of CXCL10 production by BATF3 DCs, effector T cells are not recruited into the tumor [100, 101]. In addition, activation of MYC signaling enhances the expression of leukocyte surface antigen CD47 and PD-L1 on the tumor, thus interfering with antigen uptake by antigen-presenting cells (APCs) via engagement with signal-regulator protein-α (SIRPα) and inhibiting T-cell function via PD1 engagement, respectively [102]. Furthermore, loss of liver kinase B1 (LKB1) signaling within tumor cells results in increased expression of various cytokines, contributing to reduced T-cell infiltration and promotion of T-cell dysfunction [103]. Loss of PTEN protein function activates PI3K, thereby inhibiting autophagy in tumor cells [104, 105], which diminishes T-cell priming and also mediates resistance to T-cell-mediated apoptosis [106-108]. Finally, TP53-mutated tumor cells lack production of key chemokines required for the recruitment of NK cells to the tumor microenvironment [109, 110]. Moreover, by using the pharmacological p53 activator nutlin-3a, local p53 activation reversed immunosuppression in the tumor microenvironment and induced tumor immunogenic cell death, leading to activation and expansion of polyfunctional $CD8^+$ CTLs and tumor regression. P53 activation only enhanced the antitumor response when the tumor microenvironment already comprised tumor-infiltrating leukocytes [111], suggesting that, similar to our findings with respect to EGFR blockade, p53 activation can amplify the local immune response.

5. Overall summary

In summary, we focused on the resistance of HPV-infected cells and HPV-related cancers to Th1 immunity. In HPV+ KCs, human papillomavirus impaired necroptosis by downregulating the key component of necroptosis RIPK3 through histone methylation. The global histone methyltransferase inhibitor DZNeP restored the expression of RIPK3 and thus enhanced Th1-cytokine-induced necroptosis. Human papillomavirus also made KC resistant to the antiproliferative effects of IFNγ by downregulating *IFITM1* and *RARRES1*, which are the antiproliferation genes. With respect to the Th1 immune response itself and the resistance to it in HPV-related cancer, we found that HPV-positive OPSCC was infiltrated with type 1 T cells, and if so, these patients displayed a far better survival when compared to the HPV-negative OPSCC. We found the presence of Th1 and Th17 cytokines, mainly IFNγ and TNFα, in the culture of TILs from HPV-positive OPSCC. IFNγ and TNFα can induce cell-growth arrest in OPSCC cell lines by upregulating the antiproliferation genes *IFITM1* and *RARRES1*. However, OPSCC cancer cell lines also display other mechanisms by which to escape the immune control of type 1 cytokines. Similar to HPV-positive KCs, the HPV-positive OPSCC cell lines lacked the expression of RIPK3 and were resistant to necroptosis induced by IFNγ and TNFα. In addition, our previous study showed that HPV+ KCs expressed high levels of EGFR and when this receptor was blocked by cetuximab it led to a decreased expression of IFRD1, resulting in increased NFkB/RelA K310 acetylation, and as a consequence enhanced expression and production of proinflammatory cytokines and chemokines [84]. We now showed that the EGFR is also overexpressed at the cell surface of OPSCC cell lines and that EFGR-signaling impaired the production of the T-cell-attracting cytokines CCL5, CXCL9 and CXCL10 when these cells are stimulated with the Th1 cytokines IFNγ and TNFα. We propose that this may prevent the start of an amplification cycle for the migration of T cells to the tumor environment. In contrast to our observations in KCs, the inhibition of cytokines by EGFR signaling resulted mostly from the activation of the downstream JNK and MEK pathways, albeit

that in one cell line a role for IFRD1 was found. Others found that overexpression of EGFR also induced the expression of PD-L1 while lowering that of MHC classes I and II [112-114]. This suggests that EGFR overexpression impairs both the attraction and function of T cells. Our previous study shows that the presence of intratumoral HPV16-specific T cells is important in controlling the disease progression and clinical outcomes [12-14], which makes it important to boost the HPV-specific type 1 T-cell response by vaccines [7-12]. However, due to the resistant mechanisms to immune control of HPV related OPSCC, a combination with other therapies is required. We show that cisplatin combined with TNFα was most effective in inducing apoptosis in OPSCC cell lines in vitro. Based on these results, showing that RIPK3 was absent in HPV-positive OPSCC because of DNA methylation, co-treatment of methylation inhibitor 5-AAD and caspase-8 inhibitor may have therapeutic effects on HPV related cancer. 5-AAD leads to increased T-cell recognition of tumor cells without influencing the proliferation and function of $CD4^+$ and $CD8^+$ T cells [115], and may increase the expression of RIPK3, followed by the inhibition of caspase-8 priming for necroptosis, whereby type 1 cytokines IFNγ and TNFα may consequently increase the necroptosis of OPSCC. However, both the pan-caspase inhibitor zVAD-FMK and the caspase-8 inhibitor z-IETD-FMK suppress human T-cell proliferation [81]. Ways of inhibiting caspase-8 without influencing T-cell proliferation are worthwhile to explore and a combination with adoptive T-cell therapy could potentially be tested. We also found that the EGFR-inhibitor cetuximab combined with IFNγ and TNFα increased the production of the T-cell-attracting cytokines CCL5, CXCL9 and CXCL10, which resulted in the increased migration of $CD4^+$ and $CD8^+$ T cells in vitro. The downstream of the EGFR, JNK and MEK1 pathways are mainly responsible for suppressing the production of *CCL5, CXCL9* and *CXCL10*. In vivo, EGFR-signaling blockade increased CCL2, CCL5 and CXCL10 in KCs, and in a mouse model, the use of a selective EGFR kinase blocker resulted in a markedly enhanced immune response with increased chemokine expression and a more dense inflammatory cell infiltrate in the skin [116]. This provides evidence that the blockade of EGFR may also increase tumor-

infiltrated immune cells *in vivo*. In all, this thesis presents the mechanisms of Th1 immune regulation in HPV and HPV-related head and neck cancer.

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