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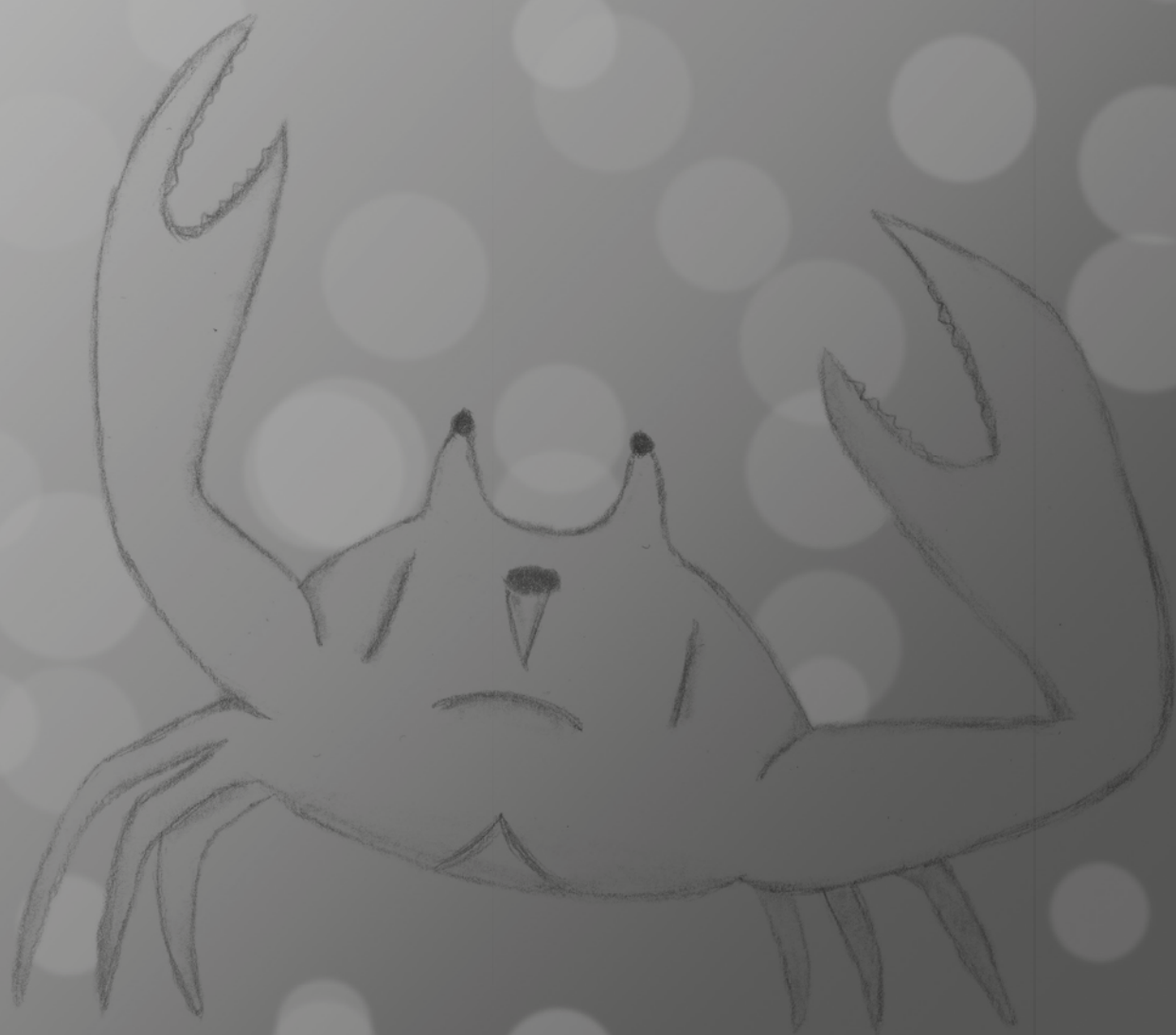
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VIII

Conclusion and Discussion



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

In **chapter 2**, the roles of IL-12, IL-23, IL-6 and IL-1 β were described. We showed by RNA in situ hybridization that *IL12p35*, *IL12p40* and *IL23p19* were predominantly expressed by tumor cells, while the immunohistochemical stainings showed that IL-1 β was mainly expressed by stromal cells and IL-6 was expressed by both tumor cells and infiltrating immune cells. A high level of *IL12p40* expression represented a beneficial IL-12 related immune response. *IL12p35* was never a limiting factor, and the only significant correlation was found between *IL12p35* and *IL12p40* levels. Since the affinity of *IL12p40* for *IL12p35* differs from the affinity for *IL23p19*, our data suggested that IL-12 is produced if high levels of *IL12p40* are present, while IL-23 is produced when *IL23p19* is present in combination with only low levels of *IL12p40*.

While a high frequency of stromal IL-6⁺ cells was strongly correlated with poor survival, especially a high number of IL-6⁺ cells combined with IL-23 was correlated with worse prognosis. Interestingly, the absence of the shared *IL12p40* subunit was also correlated with improved survival as compared with low *IL12p40* levels.⁵ Since the absence of *IL-12p40* implicates the absence of both IL-12 and IL-23, this indicates that the absence of IL-23 was more favorable than the presence of IL-12. Specifically IL-23 secretion rather than the absence of IL-12 was thus correlated with poor survival, especially when combined with a high number of IL-6⁺ cells.

IL-6 and IL-23 are part of a response correlated with poor survival in cervical cancer, which is schematically indicated in a model of the IL-17 and Th17 cell immune response axes in cervical cancer in Figure 1 (page 184). Both IL-6 and IL-23 have been correlated with poor survival in different cancer types as well. This is thought to be due to the attraction of macrophages and neutrophils in the tumor microenvironment,⁶ or direct stimulation of tumor growth.⁷ Because both IL-6 and IL-23 also play an important role in the stimulation of a Th17 cell immune response,⁸ we hypothesized that Th17 cells may have an immunosuppressive function in cervical cancer.

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

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

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

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

activation.^{19,20} This may further amplify the positive feedback loop between IL-17 production and neutrophil attraction. IL-6 and IL-23 may thus primarily induce neutrophils to produce IL-17, rather than T cells (indicated in Figure 1).

The role that we have shown for innate IL-17 producing cells in squamous cervical cancer is probably tissue and context dependent. Indeed, we found different distributions of IL-17 producing cells in the different cancer types studied. However, in all cancer types studied, the frequency of granulocytes producing IL-17 exceeded the Th17 cell frequency. The obvious next question was whether the role of Th17 cells and other IL-17⁺ cells is similar in cervical adenocarcinoma.

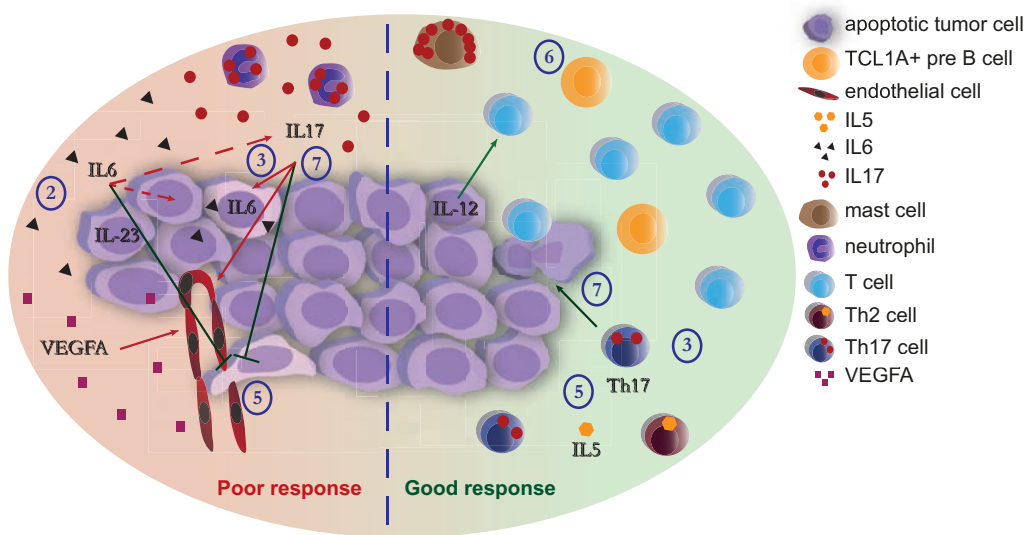


Figure 1. The IL-17 and Th17 cell immune response axes in cervical cancer

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

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

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

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

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

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

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

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

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

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

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

Future prospects

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

provide insight in the type of immune response that induces IL-17 production and its associated effects.

The phenotypical differences between classically activated tumor targeting and alternatively activated tumor promoting neutrophils are still largely undetermined. Studying these different subpopulations could aid in identifying whether it is indeed alternatively activated cells that produce IL-17 and play a detrimental role in cervical cancer.³⁸

There may also be substantial differences between the sources of IL-17 in autoimmune diseases, which would be interesting to investigate. Several studies have already indicated that IL-17 is expressed by mast cells, macrophages, eosinophils and neutrophils in different autoimmune diseases.³⁹⁻⁴⁴ Because the dependency of the function of secreted IL-17 on its cellular source is unclear, studying the composition of the microenvironment and the other stimulatory factors involved is important.

Although IL-17 was shown to predominantly correlate with poor survival and Th17 cells with improved survival, there was substantial variance in the magnitude of the effect. This might be attributable to the tumor type, which might even affect the direction of the effect (described in chapter 7). In squamous cervical cancer, we showed that Th17 cells were significantly and independently correlated with improved survival, and could counteract the tumor promoting effects of *IL6*. In contrast, Th17 cells showed a trend toward poor survival in cervical adenocarcinoma. IL-17 was predominantly expressed by neutrophils in squamous cervical cancer, and was significantly and independently correlated with poor survival in early stage cancer. In cervical adenocarcinoma, non-Th17 IL-17⁺ cells showed a trend toward improved survival. The trends in adenocarcinoma thus contradict the general correlations described in this thesis. Whether this is true for adenocarcinomas in general needs to be studied further. Overall, the cancer types in which Th17 or other IL-17⁺ cells play an important role should be determined. It would also be interesting to determine the cell types that produce IL-17 per cancer type or perhaps even per patient. To study the significance of IL-17 produced by Th17 cells, the other immune cell types or factors correlated with the IL-17 and Th17 cell immune response should be studied. Functional assays should also be performed to study whether Th17 cell derived IL-17 is crucial to stimulate a Th1/CTL immune response, or rather reflects plasticity of stem-cell like cells that can differentiate toward Th1 cells.

Similarly, the mechanisms behind the correlations between Tregs and intraepithelial T cells and improved prognosis in cervical adenocarcinoma should be investigated. Studying the immune cell types correlated with the Treg and intraepithelial T cell responses will provide insight into how these cell types might be used to guide patient prognosis and treatment.

Based on the findings described in chapter 7, cancer patients with high IL-17 levels might benefit from anti-IL-17 treatment. Neutralizing IL-17 has been shown to inhibit

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

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

Conclusion

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

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

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