General discussion and Summary

HPV infections have been associated with different malignancies, including cancers of the cervix, vulva, vagina, anus, penis, and some head and neck cancers. Although the incidence of cervical cancer has been decreased by population based cytology screening in the western world, new cases of cervical cancer still occur. The success of treatment by surgery, radiotherapy, chemotherapy or a combination thereof is often high in lower stages of the disease but decreases at higher stages. Therefore new treatment modalities are needed. In this context, immunotherapeutic strategies may hold promise.

Adoptive transfer of cytotoxic T cells has been demonstrated to be effective in clinical trials for the treatment of chronic myelogenous leukemia, cytomegalovirus mediated diseases, and Epstein Barr Virus positive B cell lymphomas. Ideally, the target antigens for adoptive transfer should be (over)expressed by malignant cells and not by normal cells. However, even low level expression of the target gene in normal tissues can lead to deleterious effects upon adoptive transfer of TCR transgenic T cells, as has been documented in two recent publications. Patients suffering from renal cell carcinoma who were treated with carboxy-anhydrase-IX specific transgenic T cells showed liver toxicity. This was most likely due to the reactivity of transduced T cells against normal tissue, that is, the epithelial cells lining the bile ducts. In addition, in an attempt to treat patients with ERBB2 overexpressing tumors, a chimeric antigen receptor (CAR) was used to transduce PBLs. Soon after cell infusion one patient experienced severe side effects and five days later died. It was shown that the gene modified cells localized in the lung and released cytokines upon recognition of low levels of ERBB2 on lung epithelium cells. Therefore it should be kept in mind that adoptive transfer of TCR transgenic T-cells should be monitored carefully for this type of serious side-effects.

The antigens to target for cervical cancer are antigens derived from the human papilloma virus. These virus antigens are only expressed in tumor cells and not in healthy tissues. Bonafide HPV specific T cells, which are able to detect HPV antigens in the context of MHC class I or II on tumor cells, are required for adoptive transfer purposes. Different strategies can be applied to isolate HPV specific T cells. One approach is to isolate HPV specific T cells directly from the blood of cervical cancer patients using an interferon-γ catch assay, resulting in a bulk population of T cells. Another approach is to isolate HPV specific T cell after several rounds of in vitro stimulation and expansion for adoptive transfer purposes.
As shown in Chapter 2 of this thesis we were successful in the isolation of HPV16E7 specific CTL clones from healthy donors, which were able to lyse cervical cancer cell lines. Such CTL clones hold promise for adoptive transfer in vivo to contribute to the eradication of CxCa tumors in patients, provided that sufficient CTL expansion can be reached in vitro. For adoptive transfer purposes approximately $10^{10}$-$10^{11}$ CTLs are required. Replicative senescence, a decrease in proliferative potential resulting from erosion of telomeric ends of chromosomes, imposes severe restrictions on T cell expansion in vitro and in vivo. Ectopic expression of human telomerase (hTERT) in human T cells can effectively compensate for this, mediating extension of the lifespan of both CD4+ and CD8+ T cells. We have been able to significantly extend the lifespan of multiple HPV16E711-20-specific CTL clones by retroviral hTERT transduction allowing continued expansion of such transduced CTLs. However, chromosomal aberrations were detected in hTERT-transduced HPV16 E7-specific CTL clones. Therefore, it is believed that these cells cannot be used safely in a clinical setting.

Equally important to the capacity of specific T cells to expand, is the affinity of the transferred TCR. The avidity of a T cell, which is greatly dependent on the TCR affinity, has been shown to correlate directly with its function. All T cell clones from which the HPV specific TCRs are isolated, were obtained after several rounds of in vitro stimulation using relatively high concentrations of peptide. These T cells often have low to intermediate affinity for its ligand, the peptide/MHC complex. This may hamper the efficacy of tumor/virus specific T cells in a clinical setting. Therefore, the isolation of HPV specific T cells from the tumor or the tumor draining lymph nodes might result in T cells with a higher affinity. Such TIL derived T cells may well be more effective in eradicating cervical cancers as compared to those T cells isolated after peptide stimulation.

Isolation and expansion of HPV specific T cells on a per patient basis is very laborious, time consuming and not always feasible. An alternative method to generate HPV specific T cells is by means of TCR gene transfer, which is described here in Chapter 3. TCRα and TCRβ open reading frames were isolated from different HPV16E711-20 specific CTL clones. Using HPV16 specific TCR transduction we were able to confer antigen specific, HLA-A2 restricted, functional activity to recipient CD8+ T cells. Several HPV16E7 specific TCR transgenic CTL clones were obtained, which all showed specific lysis of an HPV16 positive, HLA-A2 positive cervical carcinoma cell line. Expression levels of the introduced TCR at the cell surface of recipient T cells are often lower compared to the parental T cell
clone, necessitating limiting dilution cloning to obtain TCR-high T cell clones. There are several explanations for this reduced level of expression. First, the introduced TCR has to compete for cell surface expression with the endogenous TCR and mixed TCR dimers, which are composed of the α-chain from one TCR and the β-chain from the other TCR. The TCR complex with the best intrinsic pairing properties will be in favor of cell surface expression. Therefore, the concept of weak and strong TCRs was introduced. TCR transfer into T cell clones showed that a strong TCR was able to replace a weak TCR on the cell surface. Furthermore, two strong TCRs could be co-expressed on the cell surface of the same cell.

Secondly, TCRαβ dimers can only be expressed at the cell surface when properly assembled with components of the CD3 complex. TCRs with a higher intrinsic affinity for binding to the CD3 complex will be more favorable for expression at the cell surface. Therefore, selecting TCRs with high intrinsic capacities for binding to CD3 might be crucial for high TCR expression levels and target recognition. This requires further isolation of multiple HPV specific T cells to enable comparative studies.

Another explanation for low expression levels of the introduced TCR at the cell surface is suboptimal translation of the encoding mRNA. In Chapter 4 we showed that the TCR expression levels were greatly enhanced after codon-modification, in combination with the omission of mRNA instability motifs, of an HPV16E7 specific TCR. Furthermore functional activity in vivo is also improved after codon-modification. Regrettably, codon-modification of TCRα and TCRβ chains does not always lead to increased TCR expression, as has been documented in Chapter 5. This can be explained by the promiscuity of newly introduced TCRβ chains, which are able to pair with the endogenously present TCRα chains. The formation of such mixed TCR dimers can result in autoreactive TCR transgenic T cells. Previous reports on preclinical and clinical trials did not show signs of autoimmunity induced by the transfer of genetically engineered T cells. However, van Loenen et al showed potentially harmful reactivity of human TCR transgenic T cells in an in vitro model system. Moreover, in mice, the occurrence of lethal autoimmune pathology was observed as a consequence of adoptive transfer of TCR transgenic T cells resulting in the fatal destruction of the hematopoietic compartment. Therefore, it is of utmost importance to further develop expression platforms which prevent the formation of mixed TCR dimers. Thus far, different molecular engineering approaches have been developed which can contribute to the prevention of cross-pairing between the endogenous and introduced TCR chains. These include the inclusion of murine constant domains, the introduction of an extra cysteine in the
constant domains\textsuperscript{15;16}, referred to as cysteinization\textsuperscript{17}, and the “hole-into-knob” configuration\textsuperscript{18}. In Chapter 5, we introduced an extra cysteine into the constant domains of the TCR and observed increased TCR expression levels. However, cross-pairing between the endogenously present and introduced TCR chains was not prevented.

Another approach to prevent the formation of mixed TCR dimers is to use small interfering RNAs\textsuperscript{19}, which specifically inhibit the expression of endogenous TCR\(\alpha\) and/or TCR\(\beta\) chains. Okamoto et al showed that siRNAs can reduce pairing of endogenous and exogenous TCR chains\textsuperscript{20}. Since the TCR sequences differ between codon-modified and wild-type TCRs, siRNA was designed to knock down the endogenous TCR specifically. Careful comparative analysis of different engineering approaches should be performed to determine which is best applicable for a specific TCR. As an alternative approach one may choose to use effector cells devoid of endogenous TCR\(\alpha\) and TCR\(\beta\) chains like \(\gamma\delta\) T cells\textsuperscript{21} or NK cells\textsuperscript{22}.

Several different safeguard systems have recently been developed for the elimination of potentially harmful TCR transgenic T cells. These include the Herpes Simplex Virus-thymidine kinase (HSV-TK) gene\textsuperscript{23} and a Fas chimeric molecules termed LV'VFas\textsuperscript{24;25}. However, T cells carrying HSV-TK rapidly disappeared due to induction of HSV-TK-specific immunity\textsuperscript{26}. Mechanisms based on human proteins, like CD20, might be more favourable for clinical use\textsuperscript{27}. We however, have observed loss of expression of CD20 on transduced T cells (unpublished results). Incorporation of a myc-tag between the signal peptide and the variable domain of the TCR\(\alpha\) chain guarantees continued expression of the marker-tag and allows for depletion of potentially harmful T cells. This has been shown in vitro and in an animal model\textsuperscript{28}. Similar approaches should be undertaken for the TCR\(\beta\) chain.

It should be kept in mind that in previous clinical trials of adoptive transfer of melanoma specific CD8+ T cells showed no objective clinical responses in these patients while the melanoma specific CD8+ T cells were highly reactive against tumor cells \textit{in vitro}\textsuperscript{29}. More recent clinical trials using both CD4+ and CD8+ T cells were more successful since 18 out of 35 patients showed a clinical response, including three complete responders\textsuperscript{12;30}. Thus, transfer of both CD4+ and CD8+ T cells needs to be taken into account in adoptive T cell transfer protocols. In Chapter 6, we redirected CD4+ T cells into HPV specific T cells using TCR gene transfer. TCR ORFs from an HLA-DP1 restricted T cell clone were introduced into CD4+ T cells. However, thus far very few MHC class II restricted CD4+ T cell clones have been isolated. To overcome this limitation, MHC class I restricted TCRs can be transferred to CD4+ T cells\textsuperscript{31;32}. Introduction of a MHC class I restricted HPV16 specific TCR in
combination with CD8 co-receptor resulted in functional activity of recipient CD4+ T cells. This allows for transfer of cytotoxic and helper T cell functions into recipient T cells.

**Future perspective**

TCR gene transfer has become a feasible treatment modality through technical advances made during the last couple of years. Although clinical trials have been conducted, TCR gene transfer needs further advancements to be both effective and safe.

A significant reduction in cervical cancer can be expected as a consequence of prophylactic vaccination protocols and population based screening programs. However, besides cervical cancer, HPV is also related to other types of cancer. Both men and women are still at risk of developing HPV induced malignancies such as anogenital cancer and oropharyngeal cancer. At present standard treatment are often surgery, radiotherapy or chemotherapy or a combination there-of. Patients for whom these standard treatment protocols are not curative might benefit from therapeutic vaccinations. A successful therapeutic vaccine would generate specific CD4+ and CD8+ T cells, in vivo or in vitro, which are able to induce cell death (apoptotic death) of HPV infected cells. TCR gene transfer is such strategy that is able to generate HPV specific CD4+ and CD8+ T cells in a relatively short period of time.

Currently used systems to deliver TCR genes into recipient T cells are electroporation of TCR mRNA and the use of retroviral or lentiviral vectors. Only transient expression of the TCR is observed after mRNA electroporation and therefore is not suitable for introduction of TCRs into T cells for adoptive transfer purposes. Retroviral or lentiviral vectors on the other site are stably expressed. However, the main safety concern of using retroviral or lentiviral vectors is related to the risk of malignant transformation following oncogene activation due to random genomic integration. Therefore, development and improvements of novel vectors to deliver TCRs to recipient T cells would be important to make adoptive transfer of TCR transgenic T cells safer for clinical purposes.

Also important for the safety and effectiveness of TCR gene transfer is the choice of TCR. Expression levels of the introduced TCRs are often very low necessitating antibiotic selection and in some cases even cloning for those cells that are functionally active. Therefore, introduction of TCRs into a polyclonal T cell population requires optimization of the TCRs to give them an advantage over endogenous TCR to be expressed on the cell surface. Furthermore, a major problem using TCR transfer is cross-pairing of the introduced
TCR chains with the endogenously present TCR chains. The currently described techniques to prevent cross-pairing are not 100% successful. The development of approaches like the knock down of the endogenously present TCR chains might be attractive to prevent cross-pairing.

Most tumor antigens to target are tumor associated antigens. These antigens are often also expressed on healthy tissue. As has been described targeting these antigens can result in severe side effects. Tumor specific antigens are only expressed at the cell surface of the tumor and not on healthy tissue. In case of HPV induced malignancies these include HPV antigen.

Finally, TCR gene transfer in combination with another type of treatment might be more effective in fighting cancer. Strategies to boost in vivo immune responses, to promote specific expansion and persistence of TCR transgenic T cells, and to allow tumor infiltration will be desirable.

Reference List


