Introduction
Cancer therapy

Tumors are divided in benign and malignant. Benign implies that the tumors gross and microscopic characteristic are mostly considered innocent; the tumor will remain localised and will not metastasize. Malignant tumors, also referred to as cancers, share invasive (crab-like) growth characteristics, destroy adjacent structures and metastasize to lymphnodes and other organs. Cancer is one of the main causes of death in the modern world. Cancers can be divided according to the cell type from which they were derived. Carcinomas are derived from epithelial cells, melanomas from melanin pigment bearing cells, sarcomas from mesenchymal cells and haematological malignancies (leukemias and lymphomas) from different cell types of haematopoiesis. Clinically, a distinction is often made between solid tumors and non-solid tumors. Solid tumors refer to carcinomas, melanomas and sarcomas which often feel as a solid nodule, whereas leukemias consist of circulating individual neoplastic haematologic cells. Tumors share a profound growth dysregulation. They are the result of uncontrolled cell growth. In normal cells, cell growth is limited by means of checkpoints during cell cycle replication. When these checkpoints are compromised by genetic alterations, cells can keep on growing [1]. Other mechanisms to control cell growth, such as apoptosis, immune surveillance and energy metabolism, are also affected during oncogenesis [2].

Treating patients with cancer can be done in several ways, depending on tumor type and characteristics. Common forms of treatment are surgery, chemotherapy and radiotherapy or a combination of these. Despite further refinement and new developments of these therapies the mortality rate for some types of cancer is still high, necessitating the development of new types of treatment. Cancer immunotherapy is a promising form of treatment of which various strategies have been implemented into clinical practice; for example antibodies aimed at slowing down tumor growth or enhancing immune responses against the tumor. A number of approaches of immune therapy will be discussed further in this thesis.

Role of the immune system

(Tumor) immune surveillance

Our immune system protects us from pathogens like bacteria. In the last decades numerous publications have described a protective role of the immune system against tumor cells as well [3-5]. The immune system consists of the innate and adaptive immune system. The innate immune system forms the first line of defense. Macrophages, NK cells and neutrophils belong to the innate immune system and play an important role in clearing various types of pathogens [6]. Furthermore, the innate immune system activates the adaptive immune system, which is the second line of defense and targets pathogens more specifically [7]. This second line of defense is characterised by the generation of antibodies by B cells which can specifically target and neutralize pathogens [8] and the production of antigen specific T cells which can kill the pathogen [9]. Moreover, it builds up memory against these pathogens: renewed contact with the pathogen induces a faster response resulting in better protection against the pathogen. Vaccination uses this memory
response to pathogens of the adaptive immune system. Exposure to a small amount of inactive pathogens can induce long lasting immunological memory through the generation of memory B cells and memory T cells. Especially a swift B cell response (generation of antibodies) against the pathogens, enables the body to effectively eliminate the pathogen during a subsequent encounter.

In addition to eliminating foreign pathogens, the immune system is also responsible for preventing the outgrowth of cells that can potentially form malignant tumors. Antibodies produced by B cells are capable of recognizing membrane bound proteins on the surface of the cell. T cells on the other hand can probe tumor cells for intracellular proteins of which parts are presented on the cell. Tumor associated antigens (TAA) are antigens that are presented on tumor cells, but not or in a lesser extend on normal cells. The presence of TAA on tumor cells enable the immune system to identify and kill tumor cells. TAA derived from proteins that are important to maintain a malignant phenotype of the cell form important targets in T cell anti-tumor immunity. However, the immune system is not always capable of adequately eliminating potentially cancerous cells. When the immune system is compromised, the probability of tumors arising spontaneously, increases. Patients with a compromised immune system, such as patients with immunodeficiency, autoimmune diseases, AIDS or transplant patients have a higher chance of developing tumors [10,11]. These observations underline the importance of a well-functioning immune system in the prevention of the development of large clinically detectable tumors.

**Immune cells involved in tumor surveillance**

As described earlier, the adaptive immune system makes use of T cells and antibodies to kill their targets. Membrane bound proteins can form good targets for antibodies. *Ex vivo* produced antibodies specific for proteins expressed on tumor cells or T cells can be used for immunotherapeutic strategies. Antibodies directed against CD20 (B cell lymphoma) and Her2/neu (breast cancer) have been one of the first antibodies clinically used to treat cancer [12,13]. Some other well known examples of monoclonal antibodies are cetuximab (anti-EGFR) [14] for the treatment of head and neck cancer and colorectal cancer and bevacizumab (anti-VEGF) [15] for the treatment of colorectal cancer. The activation of T cells is facilitated by antigen presenting cells like dendritic cells (DC) [16], which can take up (parts of) pathogens and present peptides to T cells, which subsequently will proliferate and kill the pathogens [17]. MHC class I is present on all nucleated cells in the body, whereas MHC class II is primarily present on immune cells such as DC, B cells and T cells. In humans, the MHC complexes are called human leukocytes antigens (HLA). HLA class I and II complexes vary per person, resulting in an HLA restricted presentation of different sets of peptides [18].

**Tumor associated antigens**

T cells can roughly be divided into CD8+ cytotoxic T cells (CTL) and CD4+ T helper cells. Both are necessary for an effective anti-tumor immunity. CD8+ T cells recognize TAA presented in HLA class I present on DCs. The T cell receptor (TCR) of CD8+ T cells recognize peptides consisting 8 to 12 amino acids in the contexts of the HLA class I complex [19]. Whereas CD4+ T cells recognize peptides of around 20 amino acids in the context of the HLA class II complex. TAA are derived from proteins that are utilized
to promote transformation and tumorigenesis to ascertain a malignant phenotype. There are multiple types of TAAs, they can be either overexpressed by the tumor compared to normal tissue, neoantigens, mutated self antigens or viral antigens. Overexpressed antigens like Survivin [20] and hTert [21] are also presented on normal cells, however, only at low levels or for short periods of time. TAA can also be specific for tumors cells, like oncogene products. Oncogene products can be derived from altered proteins such as BCR/ABL, mutations in TP53 [22] or frame shift mutations leading to altered peptides. Other important targets for T cells are antigens derived from viral proteins. Viruses known to induce tumor growth are human papilloma virus (HPV), Epstein Barr virus (EBV), Human T-Lymphotropic virus-1 (HTLV-1) and Hepatitis B virus (HBV) [23]. For example the HPV-derived viral proteins E6 and E7 induce transformation of cells infected with HPV (human papillomavirus) [24], which can cause carcinomas of the cervix, penis, vulva [25] and head and neck [26,27]. Antigens derived from viral proteins, e.g., E6 and E7 of HPV are considered ideal candidates for immunotherapy because they are non-self antigens and essential for tumor formation.

**T cell functions**

T cells start of as naive T cells; they have not yet been activated or encountered their antigen. Naive T cells have a relatively long lifespan (at least several months). After T cells have been activated they can become effector T cells, who have a relatively short lifespan (several weeks). A percentage of these cells become (central or effector) memory T cells; they have a long lifespan to ensure long lasting immunological memory [28,29]. In what stage (naive, effector or memory) a T cell is in, can be determined by phenotypical markers. For example CD27 and CD28 are present on naive and central memory T cells and are associated with a ‘young’ phenotype [30].

CD4+ T cells or T helper cells can activate CTLs, and recruit cells from the innate immune system such as macrophages and mast cells [31]. CD8+ T cells or CTLs main functions are to kill (tumor) cells with Granzyme and perforin [32] and produce IFN-γ. Naïve CD4+ T cells can differentiate into several subtypes; Th1, Th2, Th17 and T regulatory (T-regs) cells. Each T cell subtype produces a different range of cytokines. Cytokine receptor signalling in turn plays an important role in T cell skewing towards a certain subtype. Th1 CD4+ T cells produce IL-2 and IFN-γ, Th2 CD4+ T cells mainly produce IL-4 and IL-5, Th17 CD4+ T cells mainly produce IL-17 and T-regs mainly produce IL-10 and TGFβ. IL-10 can inhibit pro-inflammatory cytokines such as INF-γ and IL-2 by Th1 cells and inhibit antigen presentation capabilities of antigen presenting cells (APC). TGFβ can cause immunesuppression and angiogenesis, and has been found to suppress T cell proliferation[33]. IL-2 produced by Th1 cells can stimulate T cell proliferation and activation, whereas IL-10 is an inhibiting factor. These cytokines influence T cell behaviour and thereby anti-tumor immunity.
**Immunotherapy**

Various immunotherapeutic strategies have been designed to shift the balance towards an anti-tumorenvironment. For some strategies the immune system of the patient is being stimulated to eradicate tumor cells (active immunotherapy). Other strategies do not require the patients own immune system to play an active role (passive immunotherapy).

**Adoptive cell therapy (passive immunotherapy)**

**Expansion of T cells**

Adoptive cell transfer (ACT) is a form of passive immunotherapy where a patients own cells are expanded *in vitro* and transferred to the patient [34]. ACT has been studied extensively in preclinical mouse models, and in human clinical trials mainly for metastatic melanoma patients [35]. Moreover, recent developments in methods to genetically engineer peripheral blood T cells to express anti-tumor T cell receptors ensure that ACT is now also applicable for patients with common epithelial (and not always easily resectable) tumors [36]. To generate sufficient numbers of effector T cells for ACT, extensive *ex vivo* expansion of T cells is required. In order for ACT treatment to work it is important that the T cells are capable of recognizing the tumor cells (tumor antigen specific) and subsequently killing the tumor cells (cytotoxic capacity). Another desirable quality of the generated T cells is that they can persist for a longer period of time *in vivo*. Effector T cells are the best type of T cell to kill tumor cells, however, due to their relatively short lifespan, it would be preferred to generate T cells that have the capacity to live longer, such as central memory T cells. T cells can be obtained from the patient’s peripheral blood or directly from the tumor; tumor infiltrating lymphocytes (TIL).

**Specificity of transferred T cells**

Only a very small percentage of peripheral blood T lymphocytes are TAA specific. A way to introduce tumor specificity is to transduce T cells with a tumor specific T cell receptor (TCR) [37]. A TCR consists of two chains (alpha and beta). The combination of the alpha and beta chain determines which specific epitope is recognized. The DNA sequence of the two chains of a TCR can be introduced into large quantities of T cells that can then be used for ACT. When this is applied in alpha/beta T cells it can result in mixed TCR dimers. These new TCRs can recognize an unforeseen epitope that is for example present on healthy cells, causing auto immune reactions. There are several methods to avoid this problem, for example using gamma/delta T cells [38].

**DC based immunotherapy (active immunotherapy)**

DC based immunotherapy is a form of active immunotherapy where dendritic cells loaded with TAA(s) are used to elicit a T cell response *in vivo*. The number of DCs found in peripheral blood is low. Abundantly present monocytes can also be used to generate DCs *in vitro*, thus providing an almost endless supply of professional antigen presenting cells [39,40]. Subsequently these moDCs can be loaded with TAAs and injected back into the patients to elicit a T cell response against the tumor.

*Ex vivo* loading of dendritic cells with antigens can be performed in several ways. Small
peptides of around nine amino acids can be used to induce T cell activation. These small peptides or epitopes are HLA restricted, therefore loading dendritic cells with known TAA epitopes is only applicable on a subset of patients. Furthermore, for effective anti-tumor immunity both cytotoxic and helper T cells have to be activated via MHC class I and II respectively [41]. By loading dendritic cells with long peptides, epitopes can be generated which can be recognised by CD4+ as well as CD8+ T cells [42]. To circumvent any HLA restriction, full length TAAs can also be used. Even complete tumor lysates can be used to load dendritic cells, in this way all tumor proteins can be presented to T cells. A safe and easy way to load dendritic cells is to transfect them with TAA DNA or mRNA [43-45]. This can be either DNA or mRNA derived from tumor cells or from a specific antigen. Additionally DNA or mRNA encoding cytokines can be co-transfected in the dendritic cell [46].

**Cytokines**

In a tumor environment the cytokine balance may be disturbed. Cytokines play an important role in maintaining the right balance of the immune system by skewing cells toward certain lymphocyte subtypes. IL-2 is probably the most widely explored cytokine in immune therapy, and is known for its T cell proliferation capacity [47]. However, it not only induces proliferation of T cells that can be of benefit in suppressing tumors, but also T regulatory cells. Another important cytokine is IL-12, which is mainly produced by mature dendritic cells and can promote NK and T cell activity [48]. A relatively new cytokine that is now being explored for the use in anti tumor immunity is IL-21 [49]. IL-21 has been shown to increase cytotoxic capacity of CD8+ T cells, while maintaining their ‘young’ phenotype [50]. Furthermore, unlike IL-2, IL-21 does not stimulate the growth of T regulatory cells [51]. Given in high doses systemically cytokines can cause severe toxicity, for example capillary leak syndrome after systemic administration of IL-2 [52].

**Tumor types and immunotherapy**

Immunotherapeutic strategies are becoming a more common form of treatment. Especially the use of monoclonal antibodies is becoming a more frequent form of adjuvant treatment, e.g. cetuximab for the treatment of head and neck cancer and colorectal cancer [53]. Cellular immunotherapeutic approaches are less commonly used as cancer treatment. The tumor microenvironment and cellular origin is different for each tumor, therefore different immunotherapeutic strategies should be considered per tumor type. In this thesis we focus on immunotherapeutic strategies for the treatment of head and neck cancer and of colorectal cancer.
Knowledge of the state of the patient’s immune system and the characteristics of the tumor are important factors in determining which kind of immunotherapeutic treatment would be most efficient. Although there have been many improvements over the last years, there are still many strategies to be investigated. Part 1 of this thesis explores immunotherapeutic options for the treatment of patients with head and neck cancer. Part 2 of this thesis explores the use of IL-21 for passive as well as active immunotherapy. Part 3 of this thesis explores active immunotherapy for colorectal patients, with an emphasis on the microsatellite status of the patients and on tumor infiltrating lymphocytes.

**Part 1 Immunotherapeutic approaches for head and neck cancer patients**

Active immunotherapy depends largely on the patient’s own immune system, therefore it is important to know the general status of the patient’s immune system. Head and Neck Squamous Cell Carcinoma (HNSCC) patients are thought to have a relatively weak immune status. The immune status of a patient is associated with the activation level of T cells and the presence of memory T cells. Part of the HNSCCs are HPV associated and thus have a clearly defined TAA. In Chapter 2 of this thesis we report on whether the immune status of HNSCC patients correlate with the stage of disease and whether the immune status of patients with HPV positive tumors can be distinguished from patients with HPV negative tumors. We monitored the numbers and types of lymphocytes present in the peripheral blood of HPV (+) and (-) HNSCC patients and compared them to age-matched healthy donors.

Most tumors overexpress survivin compared to normal tissue, survivin is therefore a likely candidate to target in active immune therapy. In Chapter 3 we investigated whether an active immune therapeutic strategy can be designed aimed at the tumor associated antigen survivin. We investigated the use of survivin for DC based vaccines in vitro for HNSCC patients.

HPV infection plays an important role in part of the HNSCC tumors especially Oro-Pharyngeal tumors. Thus HPV can be used as target in active as well as passive immunotherapy. In Chapter 4 we report on our investigations on whether we could improve passive immunotherapy for patients with HPV positive tumors and describe the possibilities to use HPV specific T cell receptor for the use of adoptive T cell transfer.

Immunotherapeutic strategies that have been or currently are being explored for HNSCC patients are reviewed and discussed in Chapter 5.

**Part 2 The role of IL-21 in immunotherapy**

In active as well as passive immunotherapy cytokines can be used to stimulate T cells to proliferate and increase their cytotoxic potential. In Chapter 6 we investigated whether optimization of T cell anti-tumor activity with the use of the relatively new cytokine IL-21 was possible and describe the use of IL-21 in DC based vaccines.

In Chapter 7 we compare IL-21 to IL-2 and IL-15 in the in vitro expansion phase of adoptive cell transfer using artificial APC.

The use of IL-21 in immunotherapeutic strategies is discussed in Chapter 8.
Part 3 Immunotherapy for Colorectal cancer patients

Immunotherapeutic trials have mixed outcomes, resulting in improved prognosis for some patients while unsuccessful in others. Tumor samples from colorectal cancer patients who participated in a trial investigating the effect of active specific immunotherapy (ASI) were analysed for parameters like microsatellite status and the numbers of tumor infiltrating lymphocytes. In Chapter 9 we evaluate the influence of the tumor’s microsatellite status on the clinical course of CRC patients after surgery with or without ASI treatment. In Chapter 10 we evaluated the presence of tumor infiltrating lymphocytes and the correlation to survival in these patients.

Discussion Part 3

In Chapter 11 we discuss our findings in colorectal cancer in a more general background.

Chapter 12 Future perspectives of immunotherapeutic strategies

Finally, we briefly discuss several new developments in cancer immunotherapy.
References

CHAPTER 1


CHAPTER 1

Thesis Chapters

Part 1: Immunotherapeutic approaches for head and neck cancer patients


Discussion part 1


Part 2: The role of IL-21 in immunotherapy


Discussion part 2: 
The role of IL-21 in immunotherapy

Chapter 8  Santegoets SJAM, Turksma AW, Powell Jr. DJ, Hooijberg E, de Grujil TD. IL-21 in cancer immunotherapy: at the right place at the right time. OncoImmunology in press.

Part 3: Immunotherapy for Colorectal cancer patients


Chapter 10  Turksma AW, Shamier MC, Lam KLH, Coupe VMH, de Weger VA, Belien JAM, van den Eertwegh AJ, Meijer GA, Meijer CJLM, Hooijberg E. Extent and location of tumor infiltrating lymphocytes in micro-satellite stable colorectal cancer predicts outcome to adjuvant active specific immunotherapy. Submitted to CCR.

Discussion part 3: 
Immunotherapy for Colorectal cancer patients

Chapter 11  ASI trial for colorectal cancer patients

Chapter 12  Future perspectives