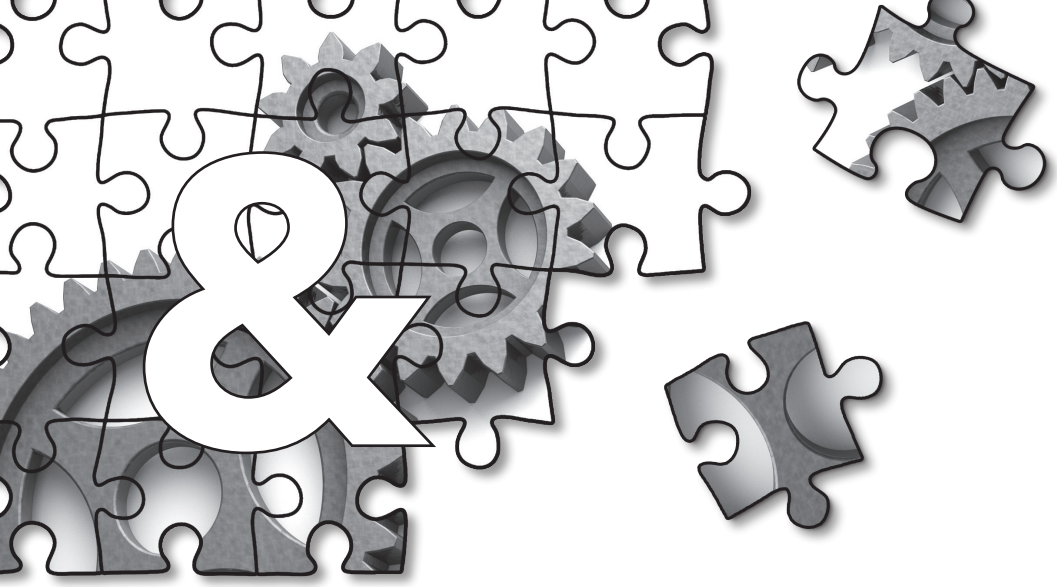

# ENGLISH SUMMARY

The immune system protects the body against pathogens such as parasites, bacteria and viruses but also against tumor cells. The immune system contains many different types of white blood cells that collaborate to keep the body healthy. It is divided in an innate (non-specific) and an adaptive (specific) part.

Characteristic for cells of the adaptive immune system is that they recognize small, but specific parts of pathogens or tumor cells (antigens), leading to a response which typically takes one to two weeks to develop from low numbers of precursor immune cells with unique receptors and results in the development of "memory" for these antigens. This memory enables the immune system to respond faster and better when the same pathogen or tumor is encountered. The adaptive immune system consists of two main types of lymphoid cells: T cells and B cells. T cells are further divided in two kinds: the helper T cells and the cytotoxic T cells. Helper T cells are characterized by the expression of "cluster of differentiation" (CD)4 molecules on their cell surface and are important for the regulation of an immune reaction. The cytotoxic T cells are characterized by the expression of CD8 molecules on their cell surface and the production of compounds that can mediate death of virus infected cells and tumor cells.

Examples of cells of the innate immune system are the macrophages and dendritic cells, both belonging to the myeloid lineage. Macrophages can kill pathogens and tumor cells upon activation and produce components (cytokines and chemokines) that attract other immune cells to the site of inflammation. Dendritic cells function as "professional" antigen presenting cells (APCs) and are crucial for activation of T cells. They can take up and digest proteins from their environment. Small pieces of these proteins (peptides) create the antigens that bind to so called "major histocompatibility complex" (MHC) surface molecules that are presented to T cells that have unique T cell receptors to recognize the peptide-MHC complex. For optimal T cell activation, the T cell needs to receive three signals. The first is the recognition by the T cell receptor of the appropriate peptide-MHC molecule complex. The second consists of interactions between various molecules on the cell surface of the T cell and the APC (co-stimulation) while the third signal consists of cytokines that are produced by APCs. For a strong T cell activation it is crucial that all three signals are present in sufficient amounts, as accomplished by matured APCs. Maturation of APCs takes place when the APC receives danger signals, through the recognition of for instance foreign structures and proteins by one of their various pattern recognition receptors. T cells that are activated by matured APCs will replicate (divide) and develop the capacity to produce effector molecules. These molecules are important for the T cell to kill virus-infected cells and also to kill tumor cells. A T cell response must be powerful but should not go on for too long leading to immunopathology: an immune response that is too long or too strong can result in unwanted tissue damage of healthy tissue. To avoid this, a T cell response usually wanes after the initial expansion. T cells that recognize tumor antigens are regularly detected in the blood and tumors of cancer patients. Examples of the antigens that are recognized by T cells are mutated proteins or proteins that are overexpressed on tumor cells. Another example of tumor antigens is viral proteins derived from viruses that cause cancer. Tumor specific T cells are often not effective or abundant enough to kill all the tumor cells.

In addition to T cells, other sorts of immune cells are present in tumors. The balance between the immune cells that support tumor development and those that do not is important for the prognosis of cancer patients. Myeloid cells, such as macrophages, are abundant in tumors. Some subpopulations can stimulate the development of blood vessels and the survival of tumor cells, while other myeloid cells can aid in the cleanup of tumor cells.

Cancer therapy has focused long on the direct killing of cancer cells, for example by chemotherapy or radiotherapy. These therapies can result in a rapid killing of tumor cells and tumor regression. However, they frequently have severe side effects and the tumor often loses its sensitivity for these types of treatments allowing it to reappear.

A relatively new type of cancer treatment is immunotherapy. This therapy is intended to stimulate the immune system to kill the tumor cells. Although the clinical response to immunotherapy is often slower than to traditional treatments such as chemotherapy, it regularly results in a complete and sustained recovery of the patient. Immunotherapy can be used to induce or improve a tumor specific T cell response. An example of a method to do this is the infusion of large quantities of tumor-specific T cells that have been expanded outside the body. Another possibility is the injection of antibodies that relieve the inhibition of the T cell response. A third possibility is therapeutic vaccination, a method by which tumor antigens are injected into the body of the patient whereby the primary aim is to stimulate a tumor-specific T cell reaction.

An example of such a therapeutic vaccine is a synthetic long peptide (SLP) vaccine. The patient is injected with small fragments of protein (peptides), so that dendritic cells can then take up these long peptides and present a shorter version (epitopes) to T cells, resulting in a tumor-specific T cell response. Our research group had previously used SLP vaccination to treat tumors that are caused by the human papillomavirus (HPV). The success of this treatment lies in the fact that only dendritic cells and not other cell types are able to efficiently process the SLP for presentation as epitopes at their surface by MHC class I and II molecules. This type of vaccination has shown efficacy in patient with premalignant stages of HPV related tumors. However, in patients with advanced stages of HPV-induced cancers, it was shown that vaccination was not sufficient to induce a strong immunological or clinical response.

Insights in the mechanisms underlying vaccine failure can help to improve the development of immune therapy and provide insights in the possibilities to combine vaccines with other therapies in order to improve clinical outcome. During my PhD research I have used laboratory animals to study what the effect is of combinations of various types of cancer therapies. Identification of the underlying immune system-related mechanisms has led to the insight that it is not always necessary to blindly accept the side effects of some therapies and has also led to knowledge about which therapies cooperate well and which therapies do not in the treatment of cancer.

In **chapter 2** a mouse model for melanoma was used to study the role of myeloid cells in these tumors. At first it was observed that the percentage of a specific kind of myeloid cells (macrophages that express the "CSF-1 receptor") increased in the blood of tumor bearing mice. To circumvent this enhancement we treated mice with "PLX3397", a compound that can block the CSF-1 receptor. This resulted in reduction to normal levels of the myeloid cells in the blood

of mice and in a decrease of the macrophages in the tumor. In addition to this, it also caused a small delay of tumor growth of the mice treated with PLX3397, resulting in a slightly enhanced survival of these mice. To improve the effect of this therapy PLX3397 was then combined with injection of tumor-specific T cells and vaccination. This treatment combination resulted in an even stronger delay of tumor growth. From this we concluded that PLX3397 can be safely combined with infusion of tumor specific T cells and vaccination. In addition we concluded that the combination of these three cancer therapies is better than only one or two of the three.

In **chapter 3** the interaction between vaccination-induced T cells and myeloid cells was studied further. Two tumor models that both express HPV related tumor antigens were used. The mice were vaccinated with an SLP that was based on HPV antigens. Interestingly it was observed that the same vaccination protocol resulted in one of the tumor models (TC-1) in a strong, but temporally, regression while in the other model (C3) it had no effect at all. Analysis of the tumors of these mice revealed that TC-1 tumors were highly infiltrated with vaccination-induced, functional, tumor specific T cells. It was observed that T cell infiltration coincided with the influx of many types of myeloid cells into the tumor. Via the removal of different T cell subsets it was shown that CD8 T cells and especially the T cell produced cytokines Interferon-gamma (IFN-γ) and Tumor Necrosis Factor (TNFα), were responsible for the influx of myeloid cells. In the C3 model the vaccine-induced T cells failed to infiltrate the tumors, resulting in unaltered numbers of myeloid cells. We hypothesized that the effect of vaccination could be improved by the removal of macrophages by use of PLX3397, as done in **chapter 2.** However, the removal of a large proportion of the myeloid cells by PLX3397 resulted in a poorer survival of the mice after vaccination. Further research into the exact mechanisms is needed, but we think that vaccine-induced T cells have changed the function of the myeloid cells and caused them to contribute positively to the anti-tumor effect of vaccination. The removal of these cells by PLX3397 undermines the effect of the therapy. These data clearly show that the combination of differently acting immunotherapies is not always better than only one of the two, and that this is largely dependent on the mechanisms involved in the therapies chosen.

In **chapter 4** we used the same two tumor models to test the effect of cisplatin, a frequently used chemotherapeutic. An increasing dose of cisplatin for the treatment of tumor bearing mice resulted in increased side effects but also in an improved survival. The maximum tolerated dose cisplatin led to complete regression of tumors in half of the mice. Subsequent studies indicated that the long-term effect of cisplatin was dependent on the CD8 T cells. Analysis of the tumors of the treated mice showed that cisplatin treatment resulted in the infiltration of myeloid cells with a high expression of the costimulatory molecules CD70, CD80 and CD86. The expression of these molecules was relevant, indicated by the observation that tumor bearing mice lacking these costimulatory molecules displayed a decreased response to cisplatin treatment. Addition of SLP vaccination resulted to a strongly improved clinical effect.

Previously we have shown that SLP vaccination, intended to induce a T cell reaction against HPV-antigens, hardly resulted in a clinical response in patients with late-stage HPV-related tumors. Patients with HPV-induced cervical cancer are often treated with a combination of the two chemotherapeutics carboplatin and paclitaxel, together termed cabotaxol. In **chapter 5** the effects of combined treatment of carbotaxol and SLP vaccination have been tested. In TC-1

tumor bearing mice this combined treatment resulted in a strong regression of the tumor and in many cases in complete cure. Carbotaxol appeared to have no negative effect on the vaccine-induced T cell response. Comparable to the data presented in **chapter 2** we showed that also the mice with a TC-1 tumor displayed an elevated percentage of myeloid cells in the blood and in the tumor. The same observation was made in the blood of patients with advanced stages of cervical cancer, and also here treatment with carbotaxol resulted in a decrease in the abnormally high levels of myeloid cells in the blood, while the numbers of T cells were not affected. This decrease corresponded with an improved T cell reactivity, an observation that was most clear around two weeks after the second cycle of chemotherapy that these patients received. With this knowledge an new cohort of end-stadium cervical cancer patients was treated with standard carbotaxol and a single dose of the SLP vaccine two weeks after the second cycle of carbotaxol. It appeared that the vaccine-induced T cell reaction was much stronger than in previous clinical studies. This research has shown that carbotaxol normalizes the tumor related increase in myeloid cells which benefits the (tumor specific-) T cell response. However, larger studies with the appropriate control groups are now needed to study how this combination therapy can improve clinical responses compared to single treatment with vaccination or chemotherapy alone.

In **chapter 6a** we tested 7 different chemotherapeutics to investigate whether they work well in combination with SLP vaccination. The study showed that none of the seven clinically relevant chemotherapeutics had a negative effect on vaccination: three of the chemotherapeutics had no influence on the effect of SLP vaccination while the four others showed synergy with SLP vaccination in tumor eradication. Especially cisplatin was active in this regard and the mice receiving vaccination with twice 40% of the maximum tolerated dose of cisplatin had a comparable survival and decreased side effects compared with the mice that received SLP vaccination in combination with a single maximum tolerated dose of cisplatin. Analysis of the blood of these mice also showed that cisplatin had no effect on the vaccine-induced T cell response. Vaccination alone, but not cisplatin treatment alone resulted in the infiltration of the tumor with IFN-γ and TNFα producing T cells. Surprisingly, cisplatin had no effect on the number or function of the tumor-infiltrating T cells. An important observation was that the dividing capacity of the tumor cells in mice that were treated with cisplatin and SLP vaccination was lower than that of tumors of untreated, cisplatin or vaccination-treated mice. A second observation was the presence of more dead cells in the tumors of mice that were treated with the combined therapy. To understand this, various types of tumor cells were cultured in the laboratory and treated with cisplatin and the two major cytokines produced by T cells: IFN-γ and TNFα. The combination of TNFα and cisplatin resulted in a much higher percentage of dead tumor cells. The role of TNFα appeared to be vital as neutralization of this cytokine, by use if injection of antibodies, resulted in a decreased clinical response of tumor bearing mice that were treated with cisplatin and SLP vaccination. This data is further discussed in **chapter 6b**.

For the treatment of cancer, cisplatin is often combined with another chemotherapeutic, topotecan. This combination therapy results in a better survival compared to treatment with only cisplatin. In **chapter 7** it was studied whether this chemotherapeutic combination works

well with SLP vaccination. In nearly all mice with a TC-1 tumor, the tumor was cleared upon treatment with the combination of SLP vaccination and the two chemotherapeutics. This was also dependent on the presence of CD8 T cells, however it appeared that topotecan had an effect on the CD8 T cell reaction. The division and contraction of the vaccine-specific CD8 T cell response was clearly altered. While SLP vaccination typically results in a fast division of tumor-specific T cells it appeared that this was not the case when it was combined with topotecan. The proliferation of the T cells returned when topotecan treatment was stopped. Together these data show that although topotecan delays the expansion of vaccine-specific CD8 T cells, the combination of vaccination and cisplatin with topotecan can result in a strong anti-tumor response. These results are important for the clinic when chemotherapy is combined with immunotherapy. The insight in how chemotherapy alters the kinetics of immune therapy-induced T cell responses is crucial for determining the treatment schedule.

In **chapter 8** we studied the side-effects of another cancer immunotherapy; the blocking antibody against CTLA-4. The CTLA-4 molecule is expressed on activated T cells and functions as a brake on T cells. Systemic administration (via the blood) of this antibody can improve the tumor-specific T cell reaction in patients and in the recent years many patients have been successfully treated with this antibody. However, blockade of CTLA-4 is also associated with autoimmune and inflammatory reactions that appear at different locations of the body. To circumvent these side effects, in this chapter a lower dose of the antibody was injected in the drainage area of the tumor-draining lymph node. The antibody was administrated in an oil-water emulsion that results in a slow release of the antibody. In this way an eight time lower dose than the systemic dose could be used to eradicate tumors. A major advantage of this local administration is that a much lower dose of the antibody ended up in the blood, corresponding with a reduced risk for autoimmune reactions. Important was also that the local treatment not only resulted in the eradication of the tumor that was near the injection side, but also in the eradication of tumors at distant sites of the body of the mouse. Recently the first patients have been treated with locally administered CTLA-4 blocking antibodies and the first results seem promising.

In **chapter 9** I have discussed the most important scientific articles of the past few years about vaccination against HPV induced tumors. From these data it clearly appeared that a T cell reaction with a good therapeutic effect can only achieve its full potential when the immunosuppression is reduced. The best combinations of therapies can only be chosen when the greatest possible knowledge is acquired concerning the mechanisms of action of each individual component as well as their joint action.

In **chapter 10** I have discussed other aspects of this thesis. It appeared from the data presented in the first chapters of this thesis that myeloid cells in different types of tumors and after different types of treatment can become modulated and thereby mediate another effect on the outcome of the treatment. In addition to this I have added an **appendix** to the discussion describing research about checkpoint blockade molecules. It describes data obtained so far with anti PD-1 and anti PD-L1 monoclonal antibodies. Blockade of these molecules can improve a T cell response against tumors, a treatment that has proven effective in patients. SLP vaccination leads to the expression of PD-1 on intratumoral T cells and to the expression of

PD-L1 on myeloid cells and tumor cells. However, we showed that antibodies to PD-1 or PD-L1 did not greatly enhance the vaccine-induced tumor specific T cell response or improve tumor eradication. These data show once more that not all existing cancer therapies amplify each other.

Collectively, the data presented in this thesis have shown that it is important to gain insight in the interactions between different cell types and treatment modalities. Together this should lead to a reduction of the toxicity of the treatment and simultaneously an improvement in the clinical response of cancer patients.