Summary

According to the theory of immune surveillance the immune system is able to detect and eliminate cells that are in the process of malignant transformation. Although the actual extent of immune surveillance in daily practice is unknown, it is clear that some tumours are immunogenic in their host in the sense that they present antigens that can be recognised by T and B cell receptors, resulting in immunological responses. The widespread success of vaccines for the prevention of viral diseases has provided a considerable base of immunologic information as well as a theoretical framework for immunisation against cancer antigens. Tumour cell vaccination is appealing since vaccines are easily administered on an outpatient basis and generally do not cause severe side effects.

In this thesis studies are described in which strategies are explored that try to maximise the immunological reaction of the tumour bearing host towards his/her own cancer cells by means of vaccinations with irradiated autologous tumour cells.

Chapter 1 provides an overview of the relationship between the immune system and cancer. Various immunotherapeutic anti-cancer approaches are discussed that have been explored in human clinical trials.

Chapter 2 describes a clinical study performed with 81 stage III and stage IV metastatic melanoma patients. Forty-nine of these patients had no sign of residual disease after metastasectomy, although the chances of tumour recurrence were considered high. After metastasectomy tumour cell vaccines were produced of the resected tumour tissue. In aid of vaccine production the tissue was cut into small pieces, after which a single cell suspension was produced by means of the addition of various enzymes. Cells were aliquotted to $10^7$ cells per vaccine and lastly 200 Gy of irradiation was administered leaving the tumour cells alive yet unable to proliferate. One month after surgery the first of three weekly administered vaccines was given intracutaneously in one of the lower extremities. To the first two vaccines $10^7$ live BCG organisms were added as an immunostimulatory adjuvant. At three monthly intervals booster vaccinations were given in the upper extremities, provided vaccines were available. Side effects of these vaccinations consisted of ulcer formation after BCG-containing injections in 65% of patients. In some patients mild systemic side effects were observed consisting of low grade fever and chills. In patients
with evidence of disease during the vaccination period (N = 38) no clinical responses occurred and survival was not better than what could have been expected in case no vaccinations would have been given. In patients without evidence of disease (stage III, N = 35 and stage IV, N = 14) 5-year overall survival was around 45%, which is superior to historical controls. However, since this was a non-randomized trial no definitive conclusions can be drawn on clinical efficacy.

Following the non-BCG-containing injections, i.e. vaccinations three and further, a local non-ulcerating inflammatory skin reaction could be discerned consisting of erythema and skin induration. Importantly, the size of this Delayed Type Hypersensitivity (DTH) response was found to correspond positively with overall survival, possibly indicating that patients with strong DTH-reactions had developed anti-tumour immunity due to the vaccinations. However, since melanoma cells often secrete immune suppressive factors like TGF-β, IL-10 and VEGF an other possible explanation could be that a small DTH response is merely a reflection of a suppressed immune status due to a large tumour load. Finally, in this study it was observed that the size of DTH-reactions weaned after consecutive non-BCG-containing vaccinations, even in patients that after a follow up period of over five years proved to be free of melanoma. This may indicate that booster vaccinations can only be effective in case of co-administration of an immunostimulating adjuvant.

Chapter 3 presents a subgroup of 25 melanoma patients included in the study described in chapter 2 which were analysed in more detail. Patients could be included in this analysis in case blood and/or tumour tissue was available and patients were macroscopically free of disease at the time of tumour cell vaccinations. Primary purpose of these analyses was to investigate whether the autologous melanoma cell vaccinations resulted in increased numbers of circulating tumour-associated-antigen-(TAA) specific cytotoxic T lymphocytes (CTL). To this end HLA-tetramers were used for the following peptides: MART-126-35, tyrosinase368-376, gp100280-288 and gp100154-162 for HLA-A2 and MAGE-A196-104 for HLA-A3. In blood taken before start of the vaccination procedure low numbers of TAA-specific CTL were found in 5 of 17 patients of whom blood samples were available. After subsequent tumour cell vaccination no changes at all were observed in all 17 patients of which blood samples were available for research purposes. In none of the 12 patients without TAA-specific-CTL preceding vaccination such a population became apparent after any of the vaccinations during the immunization process. In the 5 patients
in which TAA-specific-CTL were found preceding vaccination, these cell populations remained unchanged during the vaccination period. Subsequently it was hypothesized that effective TAA-specific CTL might not be found in the circulation, but might rather reside inside the tumour tissue. To further study this, we analyzed tumour cell vaccines that had been left over after the vaccination procedure. In 12 of 16 patients of which tumour vials were available for research purposes, TAA-specific CTL were found. Generally numbers of these cells were much higher than in circulating blood. Interestingly, a strong correlation was found between the presence of CTL in the tumour tissue and overall survival of the melanoma patients, whereas no such correlation was found for circulating TAA-specific CTL and survival. Possibly even more important, we found an inverse relation between the presence of TAA-specific CTL in blood and in tumour tissue, implying that measuring TAA-specific CTL in peripheral blood does not provide information on the immunological processes taking place inside tumour tissue. Unfortunately we could not study TAA-specific CTL in recurrent metastases since no such material was available for research purposes. In future studies it may be interesting to take serial biopsies from a single metastasis in melanoma patients receiving vaccination therapy.

Chapter 4 describes a clinical trial with 56 stage III (Dukes C) colon cancer patients that received four autologous tumour cell vaccinations as well as adjuvant chemotherapy consisting of 5-fluorouracil (5-FU) and leucovorin. Aims of this trial were to investigate feasibility, toxicity and the effects of chemotherapy on vaccination induced anti-tumour immunity. Five weeks after surgery the first of three weekly vaccinations was given, of which the first two contained BCG as an immunostimulatory adjuvant. Two weeks after the third vaccination the first of six four-weekly cycles of chemotherapy was given. A few weeks after the last cycle of chemotherapy the patients received a fourth and final booster vaccination. The toxicity of this chemotherapy regimen was severe, with grade III or grade IV toxicity occurring in 30% of patients and even 1 toxic death. Toxicity from chemotherapy in this study was similar to toxicity as reported in previous adjuvant colon cancer studies. Apart from ulcer formation after BCG containing injections no serious side effects of vaccination were observed. Median time for the ulcers to heal was 2.6 months (range 0.8-7.1 months), being comparable to what had previously been seen in colon cancer patients that had not received chemotherapy.
Anti-tumour immunity was measured before and after chemotherapy by means of delayed type hypersensitivity (DTH) reactions, taken 48 hours after the third and fourth vaccination. DTH-reactions before chemotherapy had a median size (induration) of 20.3 mm, while after chemotherapy DTH-size was 18.4 mm (p = 0.01), suggesting that chemotherapy hardly affected anti-tumour immunity.

Regular booster immunizations may maintain or even augment vaccination-induced anti-cancer immunity. For booster immunizations to be successful vaccines should not only contain the antigens of interest, but also an immunostimulatory adjuvant. Instead of augmenting immunity, booster vaccinations without an adjuvant may even lead to immunological tolerance. In the clinical studies described in this work the adjuvant which was used was live Bacillus Calmette Guerin (BCG), a potent and well known immune stimulus. However, a major side effect of intracutaneous injections of BCG is the almost inevitable formation of slowly healing ulcers at the site of inoculation. For this reason the use of BCG as an adjuvant for frequent booster vaccinations seems far from optimal.

For a long time it had been thought that cell wall constituents (i.e. endotoxins) of bacteria cause its immunogenicity. However, Tokunaga et al found the DNA-component of the BCG bacillus to be able to induce an immunological response as well. Further research led to the discovery that this immunogenicity resulted from unmethylated Cytosine-phosphate-Guanosine (CpG) motifs in the bacterial DNA after binding with the Toll Like Receptor 9 (TLR9) on immune cells. In animal models the immunogenicity of intradermally injected synthetically prepared unmethylated CpG motifs equals the effects of BCG while in humans the only cells thus far known to directly respond to CpG are the plasmacytoid DC and B cells. Subcutaneously administered CpG has been reported to be an excellent immunological adjuvant in humans too. However, the expression of TLR9 on human skin DC and the reaction of human skin DC to CpG were unknown.

Chapter 5 describes a search for a less toxic but at least equally effective adjuvant compared to BCG. To this end the immunostimulatory properties of BCG and several bacterially derived Toll Like Receptor ligands on human skin DC were studied using a human skin explant model. Human skin samples from healthy donors, obtained through the department of plastic surgery, were injected with BCG, CpG 7909 (now called PF-3512676) and LPS, respectively. CpG was used for its ability to act through the TLR9 receptor, while LPS was
used for its known ability to act through the TLR4 receptor. Comparisons were made with the immune-stimulating cytokines GM-CSF and IL-4. Immediately after the injections of the stimulus of interest, skin biopsies were taken at the injection site, and placed in culture medium enabling migration of DC into the culture fluid. After two days migrated DC were harvested and tested for maturation characteristics using flowcytometric (FACS) analysis. T cell stimulatory capacities were investigated by means of mixed leukocyte reactivity (MLR) assays. The same investigations were done with DC harvested seven days after the skin injections.

Injections of BCG, LPS and GM-CSF/IL-4 all led to a long-lasting up-regulation of the maturation markers CD40, CD80, CD83 and CD86, whereas injections of CpG did not result in any significant changes. Comparable results were seen investigating the T cell stimulatory capacity of the DC in MLRs. On human skin DC no expression of TLR9 was found and no plasmacytoid DC were encountered in the skin samples. We conclude that the immune potentiating properties of BCG and LPS on human skin DC are comparable to GM-CSF&IL-4, and that CpG does not have a direct effect on these cells. The reported adjuvanticity of CpG 7909 in humans most likely results from PDC activation in draining lymph nodes.