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Harnessing neoantigens for targeted cancer treatment

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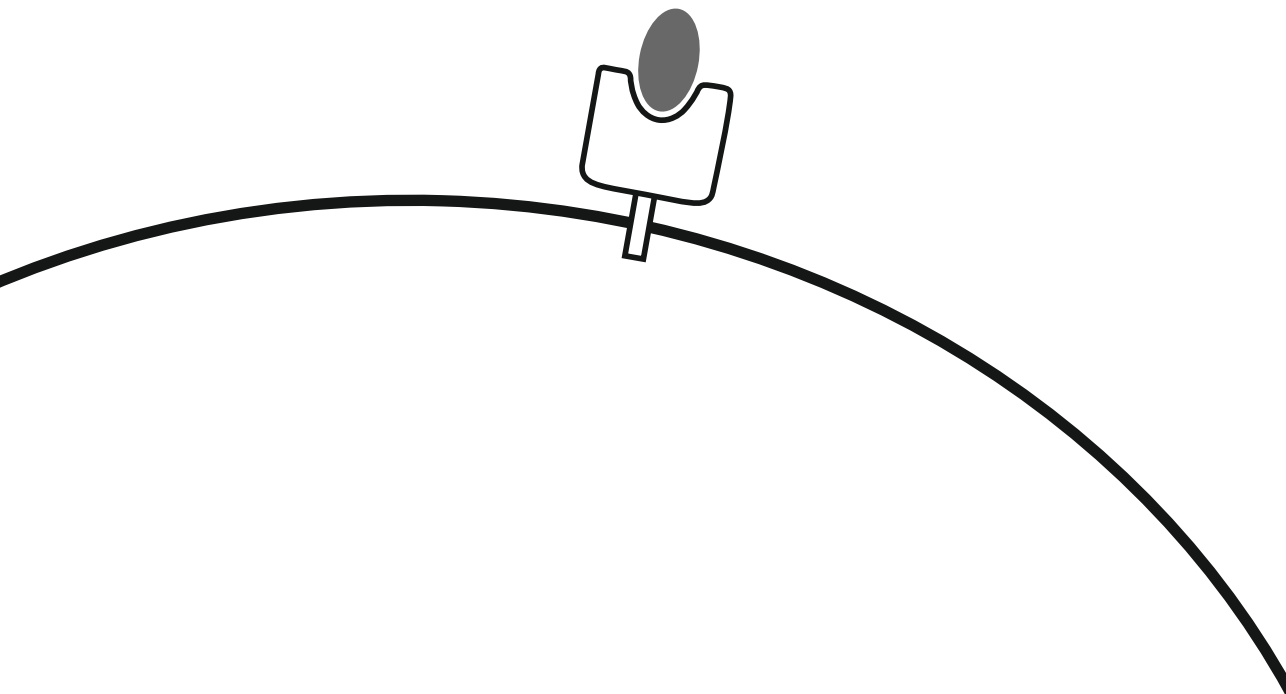
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CHAPTER 1

General introduction and thesis
outline

CANCER INITIATION

Cells in the human body proliferate in a regulated manner in order for tissues to grow or to replace damaged or dead cells. With each cellular division mistakes can occur during DNA replication, and the exposure to endogenous or exogenous DNA-damaging agents can also induce alterations in the DNA. In healthy cells, several DNA damage response and repair pathways ensure that such DNA lesions are detected and, either repaired or signalled so that an aberrant cell can be eliminated^{1,2}. Nevertheless, during a lifetime, multiple mutations go unrepaired and are stably integrated in the DNA of cells. When oncogenes and tumour-suppressor genes are mutated in parallel this may lead to uncontrolled cell proliferation, evasion of growth suppressors, replicative immortality and resistance to cell death³. The disruption of cellular homeostasis results in tumours which can be classified as either benign or malignant. Benign tumours remain localized in their tissue layer (e.g. polyps), while malignant tumours can invade surrounding tissues and eventually spread (metastasize) throughout the body. Consequently, the latter is also referred to as cancer and can lead to severe illness and death.

The course of disease depends on a variety of elements, including the tumour type, the genetic make-up and how cancer cells interact with the remaining cells of the body and, in particular, with immune cells. All these features determine the persistence and progression of a cancer and, therefore, their characterization may reveal important mechanisms that can be manipulated in a therapeutic manner. This thesis focusses on the interaction between cancer cells and cancer-specific T cells and how cancer-specific DNA mutations give rise to anti-tumour immune responses. The combination of these analyses provides clues for additional strategies to target cancers by means of immunotherapy.

THE IMMUNE SYSTEM

The immune system comprises a broad variety of cells and molecules, which together form a defence barrier against, primarily, infectious agents. Our immune system can be grossly divided into two components which cooperatively prevent and suppress infections by eliminating organisms that are detected as being “non-self”⁴. The innate immune system forms the first layer of defence and responds quickly but in a non-specific manner to insults⁵. The adaptive immune system on the other hand, is characterized by a slower response, directed to specific targets and has the capacity to establish memory enabling a quick response at re-infection.

Throughout evolution, the immune system developed to battle pathogens which formed a major threat for survival of species⁶. Importantly, the same immune defence mechanisms have also been found to possess the capacity to recognize and eliminate cancer cells via e.g. cancer-specific antigens which are detected as non-self. Moreover, the fact that increased infiltration of CD4⁺ (T helper 1) and CD8⁺ T cells is generally associated with a

better patient prognosis as well as the clinical efficacy of immunotherapeutic approaches aiming at their activation, strongly point at the role of these immune cells on tumour control⁷⁻⁹. Consequently, cancer cells must adopt immune evasive mechanisms to avoid immune destruction via e.g. limiting the expression and/or presentation of cancer antigens to the immune system^{10,11}. This anti-cancer potential of the immune system, however, is thought to be mainly an accessory attribute derived from its ability to recognize the “non-self” rather than a consequence of natural selection since cancer most often arises after reproductive age. On the other hand, the capacity of our immune system to eliminate cancers is potentially masked by the recognition and elimination of developing cancers before clinical manifestation and diagnosis.

NEOANTIGENS

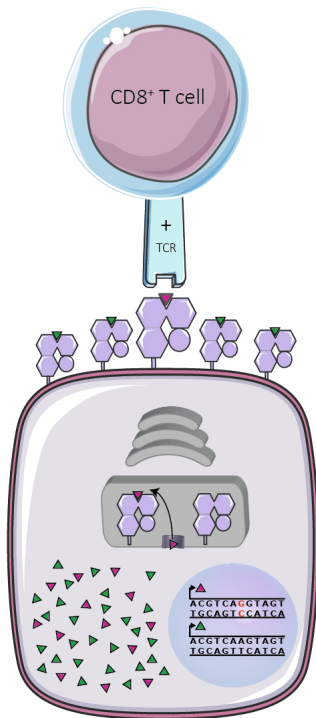


Figure 1 | Neoantigen presentation to T cells. Small peptides derived from normal (green) or mutant (pink) DNA sequences are loaded on HLA molecules and presented on the cell surface. Subsequently, T cells can recognize the peptides with their T cell receptor (TCR).

In theory, antigens recognized by T cells can be derived from all internally available proteins (including internalized external proteins) which are broken down to small peptides by the (immune) proteasome¹². T cells can recognize specific peptides in complex with HLA molecules on the surface of target cells using their T cell receptor (TCR; Figure 1)¹³. Anti-tumour immune responses can be directed to so-called tumour-associated antigens, cancer testis antigens, viral antigens and/or cancer-mutated antigens (neoantigens)¹⁴. Tumour-associated and cancer testis antigens are “self” antigens that are differentially expressed in cancer tissue compared to healthy tissue, which allows immune recognition.

Viral antigens can be specifically presented in virus-induced cancers and form non-self tumour-specific targets for immune cells. At last, neoantigens form a particularly interesting target due to their cancer-specific origin¹⁵. For example, DNA mutations in cancer cells can give rise to amino acid changes in the resulting proteins and thus aberrant, cancer-specific peptide presentation. Single nucleotide variants (SNV) occur frequently and are rarely shared among patients. Other types of genomic mutations include: (1) insertions and deletions that can result in frameshifts, (2) gene fusion and (3) chromosomal rearrangements. These genomic variants are in general more immunogenic than SNVs, because they differ more from the wildtype sequences¹⁶. Besides neoantigens derived from genomic mutations – which will be the focus of this thesis – can neoantigens also be derived from transcriptomic aberrations or post-translational modifications^{17,18}.

The fact that neoantigens form a basic characteristic of cancer and can be recognized by the immune system opens a window of opportunity for leveraging adaptive immune responses.

T CELL-MEDIATED IMMUNITY

T cells are a type of white blood cell. The ‘T’ refers to the thymus, a lymphoid organ in which developing T cells undergo TCR gene arrangement, yielding a broad TCR repertoire that is able to recognize an almost unlimited variety of targets¹⁹. The developing T cells are selected in the thymus for their ability to functionally respond to peptide-HLA complexes while lacking strong responses to self-antigens in order to avoid auto-immunity²⁰. This already points out the challenges for the induction of strong T cell responses to the above mentioned “self” antigens as negative selection in the thymus particularly ensures elimination of T cells that strongly respond to “self” antigens²¹. T cells can be differentiated into major subsets based on the expression of either CD4 or CD8 molecules as co-receptor of the TCR complex, i.e. CD4⁺ T cells and CD8⁺ T cells, respectively. CD8⁺ T cells often have the capacity to kill cells and are also known as cytotoxic T cells, while CD4⁺ T cells include, amongst others, helper T cells and regulatory T cells (Tregs)¹⁹. The latter dampen ongoing immune responses and are often characterized by the nuclear expression of the transcription factor FoxP3²².

In order to enable naïve T cells to proliferate and exert their effector function, they need to be properly activated in lymph nodes by antigen presenting cells, particularly dendritic cells^{4,23}. Dendritic cells can engulf exogenous material, including tumour cell material, and present antigens derived thereof on HLA class II molecules to CD4⁺ T cells, as well as cross-present these on HLA class I molecules to CD8⁺ T cells²⁴ (Figure 2). Besides recognition of these antigens by the TCR, T cells require co-stimulatory signals to become fully activated. These co-stimulations are provided by antigen presenting cells which upregulate molecules like CD80, CD86 and CD40 on their cellular surface, which bind

respectively CD28 and CD40L on T cells, and additionally secrete cytokines upon exposure to danger signals (e.g. infection)²⁵.

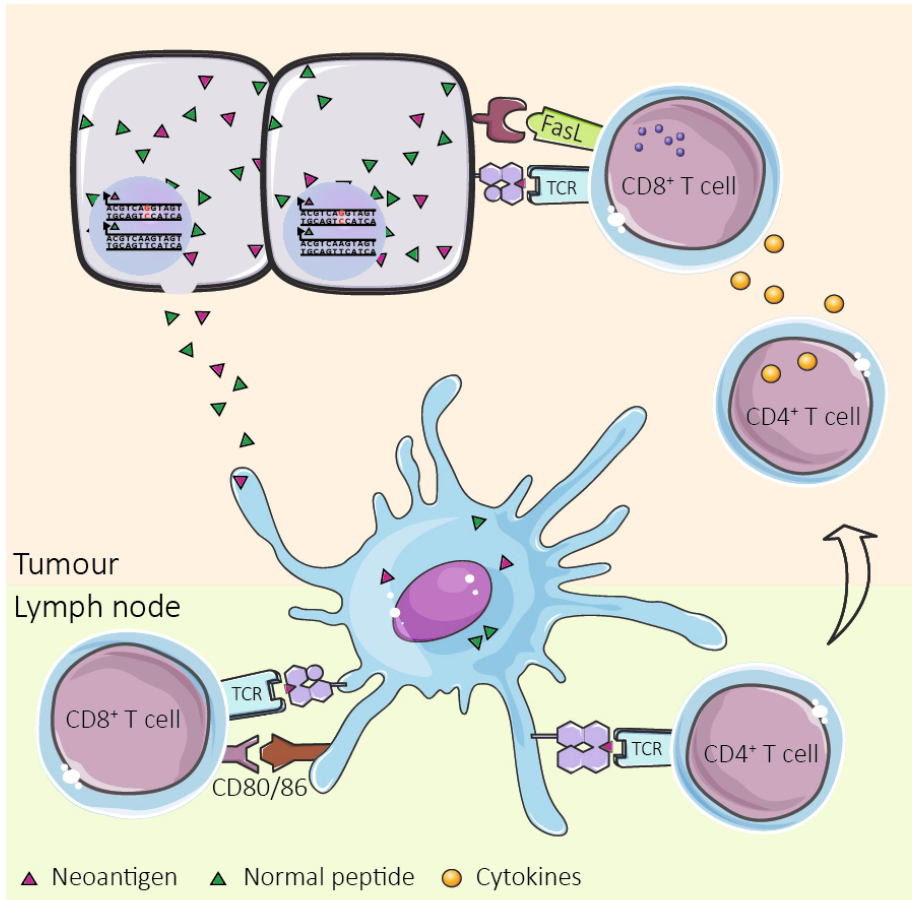


Figure 2 | T cell activation by tumour-antigens. Dendritic cells engulf tumour material at the tumour site and migrate to lymph nodes where they (cross-) present neoantigens to naïve CD4⁺ and CD8⁺ T cells, so T cell receptors (TCR) can bind the HLA-peptide complex accompanied by binding of co-stimulatory molecules (CD80/86). The activated T cells migrate to the tumour where they exert their effector function.

After activation in the lymph nodes, the T cells can migrate to the tumour site where they execute their effector function upon binding to the same antigen-HLA complex presented at target cells. CD4⁺ T cells can relay support signals towards CD8⁺ T cells to improve their effector and memory functions²⁶⁻²⁸. Additionally, CD4⁺ T cells contribute directly to the tumour control by secretion of cytokines (e.g. IFN- γ and TNF- α), and indirectly via re-organization of the tumour microenvironment via attraction of other cells and the activation of innate effector cells²⁹⁻³¹. CD8⁺ T cells release lytic granules with perforin, to create pores in the cellular membrane of target cells, and granzymes which can subsequently induce

programmed cell death with their protease capacity^{32,33}. An immune synapse is formed between the T cell and its target cell, to regulate the direction in which the granules are released. Additionally, by expressing FasL, CD8⁺ T cells can bind Fas if expressed by tumour cells and, thereby, induce another cell death pathway³²⁻³⁴.

ANTI-CANCER THERAPIES

The most commonly applied anti-cancer strategies include surgery, radiotherapy, chemotherapy, targeted therapy and hormonal therapy or a combination of these. Over the years, many cancer patients were cured or benefitted from prolonged survival based on these therapies, but each therapy comes with certain limitations and more importantly, not all patients are or remain susceptible to these treatments. It is for example not feasible to perform surgery on all anatomical locations and (so far) undetected metastases that might have spread throughout the body will not be tackled. Chemotherapy and radiotherapy are not specifically targeting tumour cells but all, particularly rapidly proliferating, cells to induce cell death through induction of more DNA damage. Targeted therapy and hormonal therapy are directed to a cancer-specific protein or hormone (receptor), respectively, but development of resistance mechanisms to these approaches by tumours is often observed.

The relatively unspecific mechanisms of action of these approaches also come with short and/or long-term side-effects for patients. Consequences of surgery highly depend on the tissue that was removed and varies greatly. For chemotherapy treatments, side-effects are often observed as especially rapidly dividing cells like e.g. the intestines, hair and blood cells are heavily affected. And even though stereotactic ionizing radiation beams can be directed to specific locations in the body aiming to provide a high dose to the tumour and limit the exposure to healthy tissue, it can still induce skin irritation and the exposure to the DNA-damaging beam is traceable in the long-term on surrounding healthy tissues. Hormonal therapy likely deregulates the balance of the hormone system causing related symptoms.

CANCER IMMUNOTHERAPY

On top of these older 'standard' treatment regimens, new immunotherapeutic strategies that employ autologous anti-tumour immune responses, have been introduced via clinical studies. Some of these have been approved as new standards of care. This provides additional treatment options with the possibility to overcome some of the treatment limitations discussed above (e.g. inability to reach a tumour and substantial off-target effects). Currently, a series of clinical trials is ongoing in order to investigate and validate new or combination treatment strategies implementing immunotherapy.

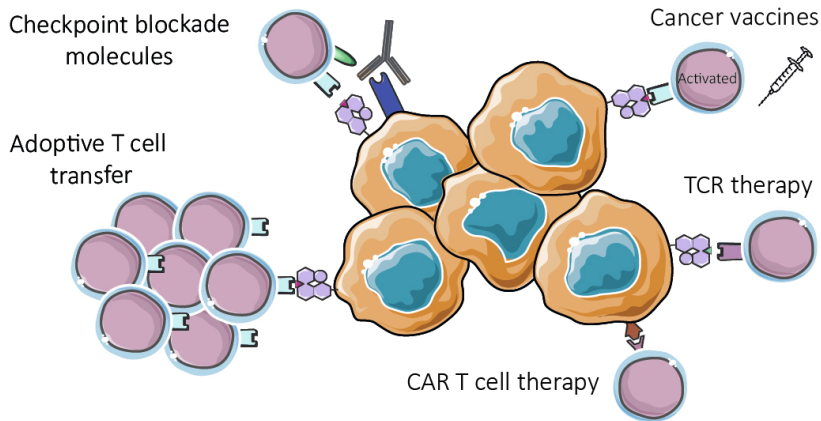


Figure 3 | Schematic representation of T cell-mediated immunotherapeutic strategies that can be exploited in cancer patients.

Immunotherapeutic strategies regularly employ neoantigens, which can evoke natural T cell responses and potentially induce cancer cell elimination. This mechanism is well recognized as it has been observed that especially highly mutated tumours like melanoma and non-small cell lung cancer (induced by UV and tobacco exposure, respectively) are highly infiltrated by immune cells and autologous tumour-specific T cell responses have been identified^{35,36}. The tumour mutation burden (TMB) positively correlates to e.g. colorectal cancer patient outcome and, again, T cell infiltration³⁷. Furthermore, the TMB correlates to the objective response rate of checkpoint blockade therapy in pan-cancer analyses³⁸⁻⁴⁰. However, the immune system of patients that present in the clinic with cancer has not been able to eliminate the tumour yet. Apparently, the tumours employ diverse immune evasive mechanisms, including antigen presentation defects, suppression of T cell functionality by expression of inhibitory molecules and modulation of the tumour microenvironment through secretion of certain molecules^{10,41,42}. Strikingly, there are indications that (neo)antigen-specific T cell responses shape cancer immunogenicity, a mechanism known as immunoediting, driving immune evasion⁴²⁻⁴⁴ and underscoring the important role of (neo)antigen-specific T cells in the anti-cancer immune response. Current T cell-mediated immunotherapeutic strategies (Figure 3) aim to tip the balance by inducing and/or enhancing immune responses and, thereby, enable tumour cell eradication.

Targeting checkpoint blockade molecules formed a major breakthrough in the field of immunotherapy and has been recognized as such with a Nobel prize in 2018. The checkpoint blocking antibodies (anti-PD-(L)1 and CTLA-4) were designed to prevent inhibitory signalling between the T cell and cancer cells or myeloid cells and thereby re-invigorate the pre-existing anti-tumour immune response or enhance T cell priming, respectively^{38,39,45}. Clinical responses were mainly observed in tumours with a high

mutation burden, and checkpoint blocking therapies thus have been approved for use in patients with e.g. melanoma and lung cancer, but also in a tumour-agnostic manner to all mismatch-repair deficient tumours as these accumulate many mutations over time^{40,46}. The clinical success of blocking checkpoint molecules on T cells in a variety of cancer types emphasizes the significant role of (neo)antigen-specific T cells for anti-tumour responses^{38,39,45,47}.

In vitro expansion of (neo)antigen-reactive T cells, either from tumour-infiltrating lymphocytes or via induction of peripheral blood with tumour cells, forms a way to create tumour-reactive T cell products that can be infused into a patient in order to increase the number of cells that can exert anti-tumour activity *in vivo*. This strategy, known as adoptive T cell transfer, led to long-term (complete) responses in, amongst others, a subset of metastatic melanoma patients further emphasizing the critical role that neoantigen-reactive T cells could play^{30,43,48-51}. Currently, a large number of T cells is reinfused of which a substantial fraction consists of bystander T cells. Selective expansion of (neo)antigen-reactive T cell subsets could potentially increase the clinical efficacy and reduce the number of cells that have to be infused. For this, cell surface molecules should be identified that specifically pinpoint tumour-reactive cytotoxic T cells among the bulk in solid tumours. Alternatively, the autologous immune response against solid tumours can be enhanced using T cell engineering approaches. To this end (neo)antigen-specific TCRs need to be identified in a patient, after which these TCRs can be expressed on patient's T cells via genetic engineering. Infusion of these engineered (neo)antigen-specific T cells expands the patient's functional TCR repertoire. Another T cell engineering approach makes use of chimeric antigen receptors (CAR) which can recognize epitopes in an HLA-unrestricted manner⁵². CAR T cell therapy was shown to be particularly effective in haematological cancers as these cells provide tissue-restricted antigens⁵³. Solid tumours appeared challenging to target using this strategy due to the lack of suitable cancer-specific targets and the difficulty for CAR T cells to infiltrate the tumours.

Another, largely experimental, approach to boost the autologous anti-tumour response is the use of vaccination strategies. Cancer vaccines can consist of e.g. tumour material or defined tumour-specific derivatives thereof. In addition, adjuvants can be provided to stimulate the immune system to respond to the provided compounds and increase the chances that neoantigen-reactive T cells become activated, migrate to the tumour site and locally perform their effector function⁵⁴. Proof-of-principle has been provided in melanoma patients that received neoantigen-based vaccines which resulted in persistent T cell responses for years, with broadening of the TCR repertoire due to epitope spreading⁵⁵⁻⁵⁷.

OUTLINE OF THIS THESIS

Manipulating the immune system could potentially add to the standard treatment regimens of a larger group of cancer patients, while making use of an even broader reper-

toire of strategies than presented here. The human immune system is highly complex and, therefore, we do not yet thoroughly understand all the interactions that take place between cancer and immune cells. However, this complexity might at the same time form the crux in establishing durable treatment benefits, as observed in some patients after immunotherapy. In order to fill in some of these knowledge gaps, this thesis focusses on elucidating the role of neoantigen-directed T cell reactivity in cancer immunotherapy.

Chapter 2 comprises a review that provides an overview on cancer immunotherapy and discusses how to extend the benefit of immunotherapy to low mutation burden tumours. In **chapter 3**, we investigated whether neoantigen-specific T cell responses are present in mismatch-repair proficient colorectal cancer patients, a particular low mutation burden tumour type. Neoantigen-specific T cell responses were identified in more than half of our patient group, particularly in tumours of consensus molecular subtype 4, which are characterized by abundant TGF- β signalling. Hence, **chapter 4** reviews the role of TGF- β on immune cells and, consequently, its effect on and potential for the design of immunotherapeutic strategies. In **chapter 5**, we continue to further investigate the phenotype of neoantigen-specific T cells by studying the neoantigen-reactivity of cytotoxic T cells with specific phenotypic markers (i.e. CD39 and CD103) as a means to select and manufacture an enriched tumour-specific T cell product for ACT. **Chapter 6**, describes the stable neoantigen landscape and T cell infiltration of melanoma metastases during progression, suggesting potential for neoantigen-directed immunotherapy even in late-stage disease. Finally, a discussion on the collective results of this thesis will be provided in **chapter 7**, also addressing the future perspectives to exploit neoantigens for cancer immunotherapy.

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