Molecular approaches to identify cancer T cell antigens and improve immunogenicity
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Chapter 1

General introduction

En route to cancer vaccines

“...the person who has been thus affected [by the Cow-pox] is for ever after secure from the infection of the Small Pox...” By these words, Edward Jenner bridged a gap of more than 22 centuries between him and Thucydides, who observed in 430 BC that Athenians cured from the plague could take care of the sick thanks to their resistance to the illness. Edward Jenner’s work caused a major shift in understanding that the risk of exposure to a lethal virus can be curtailed by inoculation with an attenuated virus. Eighty years later, it was Louis Pasteur, Émile Roux, and Charles Chamberland who made the first vaccine of a deliberately attenuated pathogen. And last year, humankind has witnessed another major revolutionary step with the widespread use of a completely synthetic and defined vaccine against SARS-CoV-2. This last development has been possible due to ongoing research for harnessing the immune system against cancer.

In the early 20th century the first link for the role of the immune system fighting cancer was shown by Paul Ehrlich. His observation was based on the reduced outgrowth of tumors in mice who have received an “impfbrei” (vaccine broth, made from tumor material) and were resistant to later inoculations with the tumor of the same origin. Subsequently, the work by James Murphy between 1911 till 1926 described the central role of cells from the lymph node (LN) in the rejection of cancer- and normal tissue grafts. He observed a reduced “resistance” after x-ray induced lymphopenia and linked “round cell infiltrate” from the LN into the (tumor) grafts to the rejection. However, it took several decades to fully recognize the role of lymphocytes, thanks to the recognition of the lymphocyte function, and coincided with a resurfaced interest in cellular immunity after the description of the clonal selection theory. Thereafter, the machination underlying acquired immunity was rapidly disclosed: the division in B and T cell compartment, thymic development and selection, antigen rec-
Oginition, major histocompatibility complex (MHC), and the role of antigen presentation for lymphocyte activation. Unfortunately, one aspect remained enigmatic; how to get the acquired immunity to recognize tumor cells?

"Wenn es gelingt, die kleinen Versuchstiere in einfacher und sicherer Weise gegen die Infektion mit Tumormaterial von kolossalster Virulenz zu schützen, so besteht doch sicher die Möglichkeit, solches in gleicher Weise auch beim Menschen zu erzielen." As Paul Ehrlich stated here, he was optimistic based on the results he found in his experiments with vaccines made from cancerous cells. Ever since the first observation that antibodies were formed against HLA, MHC heterogeneity was identified as the immunological origin for the rejection of transplanted tissues and gives a clear reason for Paul Ehrlich's optimistic results, through differences between the murine origin of the cancer and the host mice due to genetic heterogeneity or immunological differences, but not cancer-intrinsic factors. These confounding situations were already considered, but it took till the 50's to confirm these suspicions when it was shown that tumor-specific antigens (TSAs) were needed for clearance of tumors in the mice of origin. Since then, it has been recognized that the acquired immunity can be specific for TSAs and could take a central place in the suppression of cancer and cure of patients.

Engagers of the corrupted self

When cells are corrupted by mutations and escape from their boundaries set by homeostasis in favor of their own survival and expansion, a confrontation with one's personal immune system ensues. The immune system within each individual is specialized in the continuous defense and elimination of infiltrating forces like intracellular pathogens and is therefore the right instrument to fight cancer cells. One of the most effective method of the immune system, and the intention of prophylactic vaccines, is the production of antibodies by B cells to neutralize their target antigen. Unfortunately, B cell antigens on cancer cells are (near-) indistinguishable from healthy cells, thus the elimination of cancer cells has to depend on immune cells sensitive for minor differences between cancer cells and healthy cells. There are two types of immune cells that are especially suited for this: CD8+ cytotoxic T cells and CD4+ helper T cells. Respectively, these T cells function through direct cell killing and the supervision of responses from other immune cells.

The activation of T cells against cancer and pathogens requires the binding of a specific T cell receptor (TCR) to a peptide (epitope) presented by MHC class I (CD8+ T cells) or class II (CD4+ T cells) molecules. MHC class I is presented on all nucleated cells in the body and is loaded with endogenous peptides, an internal defense mechanism to alert the presence of intracellular pathogens like viruses. MHC class II is mostly expressed within the immune system as a mechanism to present peptides from exogenous origin (i.e. extracellular pathogens like bacteria). The TCR is a unique molecule for each T cell that undergoes strict selection during T cell development. During the first stages of development of T cells, the gene encoding the TCR undergoes V(D)J-recombination event whereby a combination is made from V, D, and J subregions to assemble a complete TCR molecule. Additional nucleotide insertions are possible at the hypervariable CDR3-region to increase diversification. The estimated diversity of a TCR is in the order of magnitude of 10^18 per individual, therefore strict selection of this huge repertoire is required to avoid autoreactivity. The expressed TCRs of the developing T cell are selected in the thymus for recognition of MHC class I or class II (positive selection), and non-specificity for proteins from the 'self' (negative selection). Thanks to the large number of possibilities for TCR recombination, the T cell compartment can recognize nearly all antigens from a 'foreign' origin. Cancer cells originate from somatic mutations in the DNA and carry diverse bystander mutations, these mutations are
de facto non-self or ‘foreign’ due to their absence during TCR selection. Therefore, T cells expressing a unique TCR with fine-specificity can distinguish malignant cells from healthy cells by the presentation of mutated peptides in MHC class I or class II thus playing a role in immune control of cancer.

After development, naïve T cells which have not yet recognized antigen circulate the blood and home into secondary lymph structures to inspect our body for foreign intruders until their TCR strongly binds to a peptide-MHC complex. This first encounter (priming) happens in a highly controlled setting in the lymphoid structures and is facilitated through dendritic cells (DCs) from the innate immunity, which acts as a licensee for the T cells to activate and engage the intruder. The antigens needed for DC-mediated T cell priming arrive inside the LN through active transportation by migrating DCs, by passive transport through the lymph (and intranodal conduit) and screening thereof by subcapsular sinus lining macrophages and DCs, and exchange of antigen from (apoptotic) lymphoid endothelial cells to migratory DCs.13-19 Subsequent activation of T cells by DCs is tightly orchestrated by multiple cell types and in several steps.

Licensees of T cell activation

DCs effective in the activation of T cells come in two subsets similar to the CD4+ and CD8+ division: the conventional type 1 (cDC1) and type 2 DCs (cDC2). cDC1s are well-capable to ‘cross-present’ antigens from exogenous origin as if the antigen comes from an endogenous origin into MHC class I and are thus the primary activators of CD8+ T cells. cDC2s are primarily presenting exogenous antigens into MHC class II and target the activation of CD4+ T cells. Both subsets exist in migratory and LN-resident versions, but the LN-resident cDC1 are located deep within the T cell zone while LN-resident cDC2s are located below the lymphoid sinus and LN conduits.14,20 This increases the likelihood that CD4+ T cells are activated first and in greater number of clusters14, and CD8+ T cells later, which is essential in the two-step priming method of CD8+ T cells. Another key-player in the activation of, primarily, CD8+ T cells is the LN-resident plasmacytoid DC (pDC), but this DC appears to function without the (direct) presentation of antigen.22 In the murine setting these DC subsets can be identified by the expression of the markers CD11c+CD103+ (migratory cDC1), CD8αα+XCR1+ (LN-resident cDC1), CD11b+SIRPa+ (cDC2, CD11c+ when migratory), Clec4C+ (pDC). Other types and origins of DCs exist but fall outside the scope of this thesis.23 In the human setting, the existence and functionality of these DC-subsets are comparable with few changes to extracellular markers.

T cell activation is first of all dependent on the presentation of epitopes in MHC (primary signal), but requires co-stimulatory signals from DCs during their priming event for full activation. The co-stimulatory signals are acquired by the DCs through their own activation from environmental cues, termed maturation. DCs express certain pattern recognition sensors (PRRs) that can recognize pathogen- or danger-associated molecular patterns (PAMPs/DAMPs) for a switch to a mature, T cell activating state.24 A major group of PRRs are the Toll-like receptors (TLRs), which are capable at recognizing unique pathogen-specific molecules like lipopolysaccharide, lipoproteins, single-/double-stranded RNA, and unmethylated CpG. Another major group of PRRs is known for its capacity to recognize polysaccharides chains, the c-type lectin receptors (CLRs). They can bind pathogenic or aberrant sugar-chains on pathogens and cancer cells and promote phagocytosis or adjust downstream signals from TLRs.25 More recently, the cytoplasmic DNA sensing STING-pathway has been identified as an important inducer of type-I interferons and was identified as an endogenous inducer of anti-cancer immunity.26,27 Other PRRs are present in the cytoplasm of DCs that recognize cellular stress (NOD-like receptors) and viral RNA (RIG-I-like receptors), but their function
for the activation of DCs against cancer is limitedly defined.

The two-step priming model of naïve CD4+ and CD8+ T cells by antigen-presenting cDCs involves several molecular cues and starts at separate sites. Maturation of DCs causes a rapid expression of maturation markers CD80/86. When these bind their receptor CD28 expressed by the T cells, it sends a strong (secondary) signal to the TCR binding and the priming of the T cell is initiated. It appears that this T cell ‘checkpoint’ can be tightly regulated by the DCs through PD-L1 expression, and is resolved by the heterodimerization with CD80 or the selective immunotherapeutic targeting of PD-L1 interaction with its receptor PD-1. CD4+ T cells are the most probable to encounter presented antigen and co-stimulation first due to their (co-)location close to the lymphatic sinus and LN conduits, where migrating DCs enter the LN or LN-resident DCs acquire antigen from the afferent lymph. The activation of CD4+ T cells when receiving signals through their TCR and CD28 can be strengthened by expression of OX40-ligand (and to a lesser extent 4-1BB-ligand) on the DC and the OX40 receptor on the T cell. This signal promotes CD4+ T cell survival, memory, clonal expansion, and strongly enhances IL-2 cytokine production and its receptor CD25, further augmenting its activation and proliferation. In a later stage, antigen presenting DCs reach T cell follicles where CD8+ T cells reside, where several antigen-presenting cell-types are described to be necessary for full activation of CD8+ T cells. After the initial interaction with a cDC1 through the TCR and CD28 receptor, the cDC1 and CD8+ cells attract pDCs and LN-resident XCR1+cDC1 through the chemokine expression of CCL3, CCL4, and XCL1. The attracted XCR1+cDC1 receive antigen from the migratory DC and enhance the presentation of the antigen in duration or quantity. The pDCs perform an essential role by their production of type-I interferons to assist XCR1+ DC cross-presentation. Importantly, the XCR1+ DCs are accessible to ‘helper’ signals from CD4+ T cells. When pre-activated CD4+ T cells bind the antigen presented on MHC class II, it sends an enforcing signal through the expression of CD40-ligand, which binds CD40 on the DC and strengthens the expression of CD80/86. CD40-signaling also induces the expression of CD70 on DCs, which binds CD27 on the CD8+ T cell for enhanced activation, proliferation, and memory recall. The proximity of several cell types at the CD8+ priming site facilitates the paracrine activation of CD8+ T cells with cytokines (tertiary signals) like IL-2 from CD4+ T cells, IL-15 from DCs, and IFN-I from the previously mentioned pDC, respectively. The fulfillment of these signals results in activated CD8+ T cell with optimal cytotoxicity, tumor-infiltration, and memory cell formation.

Acquisition of the primary signal

Upon encountering activation of PRRs in the tissues, DCs start a program of enhanced engulfment via endocytosis or phagocytosis, express maturation and chemotactic markers, and increase the presentation of antigens in MHC. The antigens follow several, independent, intracellular routes that determines their presentation in MHC class I or MHC class II.

To initiate an immune response against intracellular pathogens and cancer, cDC1s rely on a presentation route that presents exogenous antigens into MHC class I. This ‘cross-presentation’ is possible through two processing routes: the cytosolic and vacuolar. In the cytosolic route, the central understanding is defined by the transportation of antigen across intracellular membranes. Phagocytosed antigens end up into the cytosol where they are processed by the proteasome before being transported across membranes through the TAP-complex. This translocation of peptides by TAP can happen at the endoplasmic reticulum (ER), where newly synthesized MHC class I molecules reside, or at endosomal compartments where recycled MHC class I from the extracellular membrane are reloaded with peptides derived from endocytosed antigens. The vacuolar pathway incorporates this same MHC class I recycling mechanism, in which antigens are not transported into the cytosol, but are broken down by proteases (e.g. cathepsins) in the endolysosomal compartment and
bind MHC class I recycled from the membrane to the antigen-laden endolysosomes.\(^{38}\) This loading process is highly similar to MHC class II loading.

DCs which prefer the activation of CD4+ T cells primarily increase the expression and longevity of MHC class II on their extracellular membranes, and display a limited increase of MHC class I upon maturation.\(^{36,39}\) While MHC class I can be loaded through the TAP-complex in the ER, the activation of the MHC class II presentation machinery also incorporates the expression of invariant chain (\(\text{Ii, also known as CD74}\))\(^{40}\) This molecule binds tightly to the binding groove of MHC class II, thus preventing any endogenous peptide from binding. The MHC class II-\(\text{Ii}\) complex is subsequently transported towards an MHC class II-loading compartment, whereupon the \(\text{Ii}\) is cleaved and the residual peptide CLIP remains bound in the binding groove. Only when DM, another MHC class II machinery component, binds MHC class II does CLIP release and antigens can be loaded.\(^{41}\) DM itself is limited to interact with MHC class II by H-2O (HLA-DO in humans), which is tightly bound to DM until the pH of the endolysosomal compartment containing the MHC class II loading molecules is sufficiently low.\(^{36,39}\) All MHC class II presentation molecules involved in the peptide-loading of MHC class II are under expressive control by the master regulator class II transactivator (CIITA). Constitutive expression of CIITA is primarily found in APCs and is largely lacking in healthy tissue and cancer cells, although expression can be induced by IFN\(\gamma\).\(^{42-44}\)

Once upon reaching maturity, DCs remain capable of presenting antigen in MHC class II and I for several days. Cross-presented antigen in MHC class I, which was targeted via FcR or TLR routes, remained functionally presented from identified antigen storage compartments for

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**Figure 1.** Specific cytotoxic and helper T cell responses start with the translocation of antigen from tissue to draining lymph nodes by diffusion and lymph node sinusoid sensing or active migration of migratory DCs. Naïve (and memory) T cells in the lymph node are primed (and activated) through presentation of antigen by local or migratory DCs. In a 2-step process, CD4 responses can enhance CD8 activation through enhanced costimulatory expressions on local DC subpopulations. After the expanding phase, activated CD8 and CD4 T cells migrate to the tumor or infected tissue to fulfill their programmed roles of cytotoxicity of infected and cancerous cells or the inflammatory modulation of microenvironment. (designed with BioRender)
at least three days.\textsuperscript{45} Long term antigen cross-presenting capacity of splenic cDC1 and cDC2 has been shown after in vivo uptake of circulating antigen-antibody complexes for up to seven days.\textsuperscript{45} Recently it was shown that this is not dependent on mode of internalization, as antigen endocytosed via Fc receptors and C-type lectin receptors was routed to the same storage compartment and remained a viable source for antigen presentation and T cell activation for several days.\textsuperscript{45-47} This specialized storage function of DC may be of importance for migration of DC which have gathered antigenic intel from the tumor or site of infection to draining lymphoid organs, which may take several days, to exert their T cell priming function.

**T cell targets in cancer**

The research field investigating the origin and characteristics of epitopes is developing and changing since the day that cancer cells were seen as potential targets of the immune system. Several types of antigens have been proposed to provide epitopes for the adaptive immunity to lock onto: self-antigens, viral antigens, and neoantigens. Moreover, technical developments are improving the detection and identification of target epitopes.

Major efforts have gone into inducing immune responses against self-antigens related to cancer. Although successful immunization would generally mean a breach of tolerance, several reasons made these attempts worthy to pursuit. A first subtype of self-antigens are so-called cancer/testis antigens (CTAs), proteins that are mostly expressed in immune-privileged sites like the eyes, testicles, or central nervous system. This also includes proteins specifically expressed during embryonic development. The benefit of targeting these antigens, is the supposition that continued peripheral tolerance against these antigens is lacking in adults. Thereby offering a window for T cells to by-pass peripheral tolerance with TCRs that escaped thymic selection. The selective expression profile of these antigens reduce the chance of targeting healthy tissue and is not harmful for immune-privileged sites. A second subtype of self-antigen are proteins selectively expressed in normal tissues. Selection of these tissue-specific antigens is based on differential or high expression on cancer cells and breaching tolerance against such antigens on healthy cells is deemed an acceptable risk of autoimmunity. Many immune responses to CTAs or cancer-associated self-antigens have been identified in patients and used as targets for a variety of active vaccination strategies, but a lack of clear objective responses was a sobering concern.\textsuperscript{48,49}

Alternatively, a class of non-self antigens is described as oncoviral antigen and derived from viral \textit{(onco)genes}, which are theoretically better candidates than self-antigens due to their absence during thymic selection of T cells. A major example is the human papilloma virus strain 16 or 18, which carry the E6 and E7 oncoproteins that functions by preventing cellular regulation and support cell growth.\textsuperscript{50} These viral antigens have oncogenic driver properties which makes targeting them highly valuable to prevent immunogenic escape. Studies targeting immunization against E6 and E7 oncoproteins in HPV-positive cervical cancer and head-and-neck cancer patients showed consistent induction of immune responses and benefit for early lesions in the vulva.\textsuperscript{51-53} However, improving the outcome for patients with late stage and established tumors remains challenging.\textsuperscript{54} The immunosuppressive environment in established tumors appears to limit effective tumor clearance, and combination with ICIs or optimally-timed platinum-based chemotherapy might prove beneficial in combination with strong vaccine modalities.\textsuperscript{55,56} Due to a limited set of described oncogenic viruses (e.g. Epstein-Barr virus, hepatitis B and C, Kaposi sarcoma herpesvirus, etc.), this class of antigens is restricted in the clinic to relatively few patients and most require another method of approaching non-self targeting.

Antigens derived from non-synonymous mutations in the DNA are currently receiving signific-
icant interest in the field as potential targets with non-self characteristics. Single nucleotide point mutations in the DNA can switch the codon of the amino acid and change the sequence of the translated peptide; which may influence hydrophobicity, charge, polarity, or even structure of the positions involved in TCR affinity. Small changes can also have significant effect on the processing and binding of the peptide to MHC. Additionally, non-synonymous mutations can lead to nucleotide deletion or insertion, thus resulting in frameshift mutations with high potential of non-self protein sequences. The strongest evidence supporting the role of mutation-derived neoantigens in immunological rejection of tumors came after the application of antagonistic antibodies specific for immunosuppressive signaling pathways PD-(L)1 and CTLA-4. Treatment of cancer patients with these immune checkpoint inhibitory (ICI) antibodies indicated a positive correlation between mutational load and immunotherapeutic response. Accordingly, genomic analysis of tumor cells from ICI antibody treated tumor-bearing mice suggest strong immunological pressure on mutations. Neoantigen specific T cell responses can be observed after immunotherapeutic treatments. Due to these understandings, the potential role of neoantigens have obtained a central position in immunological treatment strategies of cancer patients.

Prediction in epitope discovery

Identification of immunogenic (neo)antigens requires laborious analysis which is cost- and time-intensive and unavailable for most cancer patients. In 1991, a study in human melanoma identified for the first time the CTA family member MAGE as the target of autologous T cell clones. This process required the creation of a large cosmids library made from the patients’ melanoma cell lines and the in-depth screening of the cDNA-transfected feeder cells for cytolytic capacity by the autologous T cell lines. Alternatively, when an antigen is suspected or known, it can be explored for potential epitopes by synthesis of overlapping peptides and analyzed for MHC binding properties and T cell recognition, as was done for HPV. Both approaches, however, have several mostly technical limitations like the dependence of expression levels of the antigens by the cancer cells and the heterogeneity of MHC binding
properties between patients. Pertaining to these problems, the group of Rammensee described a molecular peptide elution approach in 2004. In short, this approach describes feasible discovery through analysis of expressed RNA in the tumor and compared to normal tissue expression, elution and mass-spectrometric analysis of MHC-presented peptides, followed by recognition of potential antigens by T cells. This approach proved to be suitable for the incorporation of neoantigen discovery by full genomic analysis combined with peptide elution.

A decade after the publication of the Rammensee approach, several studies showed the practical feasibility of the approach with the murine melanoma and colorectal carcinoma models B16F10, MC38, and CT26. Each of these studies applied a similar methodology of whole-exome sequencing paired with algorithmic prediction of MHC class I binding properties, one study also combined this with mass-spectrometric analysis of MHC class I eluted peptides through immunoprecipitation. Since tumor material is often limited, major focus in development has been towards the accurate prediction based on whole-exome sequencing. This algorithmic approach is based on the computational learning on MHC allele-specific elutions to determine the likelihood of a given peptide to bind MHC. In the case of MHC class I, specific amino acids on certain locations of the binding groove can be the biggest influence on binding and are aptly named anchor residues. Potential binders can subsequently be calculated and ranked, of which the binding affinity is a major determinant. A binding affinity ($\text{IC}_{50} < 150 \text{ nmol/L}$) is considered an indispensable characteristic for selection criteria of a potential neoepitope and correlates to immunogenicity. Problematically, there are currently approximately 17,000 HLA alleles known which number grew rapidly in the years prior, while the most established and up-to-date prediction algorithm NetMHCpan is trained on 70+ HLA class I alleles. Obviously, this limits a reliable application of such prediction algorithms in the clinic. Even more so, the development of these MHC class I epitope prediction algorithms were mostly successful due to the closed nature of the MHC class I groove resulting in defined peptide lengths of binding epitopes. In contrast, the open-end nature of the MHC class II groove results in variable peptide length variants and has turned out to be challenging for algorithmic development. Discovery of novel MHC class II alleles is rapidly growing as well, the number currently stands at 6700, although their binding repertoire can be multiplied several times due to their αβ-chain heterodimerization. This MHC class II heterodimerization challenges the established mono-allelic approach used for the identification of MHC class I-bound peptides, and consequently the computational learning

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**Figure 3.** Clinical process of personalized development of cancer vaccines. Biopsies of patients are sequenced for expression of shared and neoantigens in cancer cells. Current prediction algorithms for MHC binding peptides can be supplemented with a wet approach to confirm predicted presentation, or establish novel (personalized) algorithms based on previously unestablished HLA-types. Selected candidate epitopes can be formulated in single or multiple vaccine modalities for optimal immunogenicity.
of binding rules for MHC class II molecules. This problem has recently been addressed with the development of a high-throughput method, which manages to isolate MHC class II alleles prior to elution and mass-spectrometric analysis and thereby defines binding properties per MHC allele.\textsuperscript{77}

**The next progression in personalized vaccines**

Several studies have brought the technological developments in personalized epitope identification to the clinic to test the feasibility of this approach in patients. The studies performed by Ott et al. and Sahin et al. used whole-exome sequencing and HLA I-binding prediction to select expressed somatic mutations for potential-ranked epitopes. All studies observed mutation-specific immunological responses and a correlation with reduced tumor outgrowth.\textsuperscript{78-80} The vaccine designs were well received and combined greatly with anti-PD-L1 antagonistic antibodies, and observed epitope spread after vaccination was correlated with increased progression-free survival.\textsuperscript{80} Interestingly, although both the murine and human studies based their epitope predictions on MHC class I, a more dominant immunogenicity was observed in the CD4\textsuperscript{+} T cell compartment. In preclinical studies, the inclusion of both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell-specific epitopes benefited the outcome of the treatment, with improved survival, increased tumoricidal activity, and tumor infiltration.\textsuperscript{81,82} The activation of CD4\textsuperscript{+} T cells by the vaccine directly helps the activation of CD8\textsuperscript{+} T cells in the LNs to confer greater responses against the tumor, and is irrespective of tumor-specificity.\textsuperscript{82} Tumor specificity of induced CD4\textsuperscript{+} T cells, and local expression of CD4\textsuperscript{+} specific antigen, is nevertheless beneficial for the local inflammation of the tumor microenvironment, and subsequently helps CD8\textsuperscript{+} T cell activation.\textsuperscript{75,81,83} Neoantigen-specific CD4\textsuperscript{+} T cells with antitumor activity are therefore considered good targets for induced responses, but it should be noted that neoantigen-specific regulatory (CD4\textsuperscript{+} and CD8\textsuperscript{+}) T cells have been described as well.\textsuperscript{84,85} What this means for vaccine induced responses in the LN remains to be determined.

In summary, the field of personalized vaccines has advanced greatly in recent years with the first applications of TSA-incorporated vaccines resulting in positive outcomes. However, gaps remain in reliable prediction tools and methods for translation to the clinic, with emphasis on MHC class II binding epitope prediction. Recent technological developments makes the study of this field more accessible and is rightfully receiving major interest.

**Scope of this thesis**

Recent advances in our understanding of immunotherapeutic strategies against cancer and the development of improved analysis tools and computing power have led to a highly anticipated progression of the cancer-vaccine field. The possibilities of personalized approaches to vaccinate patients against tumor-specific antigens by the prediction of MHC-binding peptides are already in clinical studies. Currently, the field is lacking behind in antigen identification tools and optimal delivery of vaccines. The studies in this thesis are divided in two major parts which address these topics. The first part reports a novel approach for the identification of relevant MHC class I and II binding epitopes. The second part shows the development of a molecular approach for the tracking of ligands and peptides to improve our understanding of vaccine delivery.

In chapter 2, the identification of a novel and relevant neoantigen is described for a CD8\textsuperscript{+} T cell specific response in the murine MC38 tumor model. Through the use of an updated Rammensee-approach, a previously undescribed mutation in the Ribosome protein L18 has been confirmed as a relevant target for therapeutic vaccination.

At least several branched origins exist of the MC38 murine model due to its use in oncolog-
ical studies for half a century. Chapter 3 investigates the genomic differences between two independent MC38 cell lines and subsequent implications for previously reported immunological responses specific for neoantigens. Significant changes in mutational landscape have been identified, and relevant antigens for therapeutic vaccines are absent in one cell line, and consequently fails to be recognized by neoantigen-specific CD8+ T cells.

The incorporation of CD4+ T cell epitopes in vaccine formulations greatly benefit the induced immunological response against cancer. To establish a novel method for the identification MHC class II binding neoepitopes, chapter 4 explores the possibility of transfecting tumor cells with a class-II transactivator-encoding vector. Both human and murine cell lines were susceptible to transfection and capable of stably expressing the MHC class II presentation machinery. Depending on the origin of the tumor cell line, this method resulted in the identification of relevant viral-, self/cancer-testis-, and neoantigens.

Chapter 5 reviews molecular approaches to improve cancer vaccines and functions as an introduction into the second subject of this thesis. This chapter discusses the dependency of vaccine efficacy on the modus of delivery. The use of molecular analysis tools has resulted in a comprehensive understanding of the inter- and intra-cellular pathways of antigen processing and presentation. However, several subcellular interrogations remain elusive due to technological shortcomings of current tools. This review proposes the use of bioorthogonal labelling techniques to improve the resolution and quantitative power of established methods.

Efficacy of vaccine formulation can be inferred from the quantification of MHC-presented antigen by APCs. Chapter 6 discusses the optimization of bioorthogonal chemistry for in situ conjugation of a fluorophore to MHC-presented epitopes as a model-independent method to quantify MHC-presented peptides. The study explored the most favorable locations in the epitope for bioorthogonal conjugation of a quenched fluorophore, and reproduced the results in several well-known model epitopes.

Chapter 7 describes the exploration of a copper-catalyst independent bioorthogonal reaction to conjugate MHC-presented epitopes in live cells. Strained-alkenes and tetrazines were chosen and readily modified for reactivity in vitro through optimizations of chemical structure. On-cell bioorthogonal reactions of MHC-presented epitope and fluorophores resulted in low signal above background, but showed potential for further optimization.
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