Molecular approaches to identify cancer T cell antigens and improve immunogenicity

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Chapter 8

General discussion
Large strides forward

In the last decade immunotherapy of cancer has become an established therapeutic strategy of clinical oncologists. For some cancer types, like melanoma, immunotherapy is now the standard of care in the clinic. Crucial scientific advancements preceded this development, including high resolution DNA/RNA sequencing of tumor samples and the development of clinically approved antagonistic antibodies to immunologic brakes like PD-1- and CTLA4-, so-called immune checkpoint inhibitors (ICIs). With these tools the analysis of the tumor mutanome became possible and established our understanding of neoantigen-specific T cell responses in cancer control. Subsequently, it was understood that tumors with high mutational burden (i.e. melanoma, lung cancer) are more sensitive to ICIs due to an increased chance of expressing immunogenic neoepitopes. Patients treated with ICIs are unfortunately susceptible to serious side effects and the application of vaccination with defined cancer antigens can act more specific with less side effects. Several clinical trials have been carried out to determine the feasibility of mutanome-based personalized vaccination in patients with cancer. Objective regression of tumors, correlating with vaccine-induced immune responses against the identified mutations, was observed in these patients, often through the combined treatment of vaccination with PD-(L)1 antagonists. Furthermore, the establishment of the novel mRNA-based modality for delivery of neoantigens has been successfully applied. Using this mRNA vaccine platform also improves the efficacy of shared antigen vaccines in patients with recurrent tumors after ICI treatment. These developments show the field is maturing, but improved understandings of the epitope selection process and vaccine platforms are still required. These subjects were the focus of this thesis and were analyzed at molecular and immunological level.

Sifting through the exome

Computational analysis of sequence databases is an essential step in the design of personalized vaccines of patients to optimally target a selection of relevant (neo)epitopes from the exome or mutanome. \textit{In silico} tools offer potential for a streamlined and time-effective discovery of antigens. Current methods acquire their predictive power from the determination of anchor residues in the MHC binding groove (e.g. SYFPEITHI) or through deep-learning of high-throughput MHC-binding assays with a large peptide database (e.g. netMHCpan). Despite their usefulness, algorithms carry inherent drawbacks for clinical application. The largest limitation is the necessity of established MHC-allele specific databases, and needs to accommodate for the heterodimerization of MHC class II alpha and beta chains. Due to the large diversity of HLA haplotypes, (current) databases are inevitably incomplete and have the risk to omit relevant epitopes. The open nature of the MHC class II binding groove also decreases the confidence of adequate CD4 epitope prediction. Algorithms also do not take processing into account, meaning that predicted epitopes never may be produced during antigen presentation/cross-presentation. Finally, algorithms are inherently probabilistic and limited in the required variables to fully determine the chance of an antigen being expressed, processed, and presented. This became obvious in the work leading to \textbf{chapter 2} when we looked for MHC class I epitopes purely based on ranked binding. The identified epitope in mutated RPL18 was located on the 126th location of the ranked list of predicted peptides, and one of the last peptides manually selected prior to mass spectrometric analysis. Manual selection was based on mutations which constituted an alteration in physicochemical property of the amino acid compared to the wild type sequence, thus resulting in a physicochemical change which has a higher likelihood to escape systemic tolerance. Moreover, the testing of immunogenicity of selected mutations by peptide vaccination in naïve mice, resulted in broad CD4 and CD8 responses (Figure 1A). But when tested on tumor-fed T cell bulk from MC38 cured mice, specific responses were limited to CD8 responses against the previously identified mutation in Adpgk and the novel mutation in RPL18 (Figure 1B); thereby illustrating the discrepancy between peptide immunogenicity and relevance in tumor-protective responses. No apparent reason prior to mass-spectrometric analysis would have selected
Rpl18 as a logical choice if a limited set of peptides (i.e. top ten candidates) had to be chosen for a vaccination. Of note, this newly identified mutated RPL18 neoepitope was strikingly missed in a key Nature publication by Yadav et al (2014) using a similar identification approach based on exome sequencing, MHC I prediction algorithms and MHC I peptide elution. Such an uncertainty of in silico selection supported the reasoning in this work for the incorporation of a ‘wet’ approach to identify potential vaccine candidates with a higher frequency.

The analysis of MHC presented epitopes by mass spectrometry as a wet approach requires significant time and material compared to in silico analysis, but offers the benefit of higher certainty of finding a relevant epitope. Lam et al. reported the full mutanome screen for immunogenicity with autologous PBMCs as a method to identify as many immunogenic mutations as possible.9 They describe an elaborate method of transfecting plasmids encoding each mutated peptide into E. coli, ex vivo culturing of monocyte derived DCs (moDCs) and (nonspecific) expansion of T cells from autologous PBMCs, for the discovery of mutation-specific T cell responses through presentation of the mutations by E. coli-fed moDCs. Additionally, potential neoantigens can be screened for stimulatory and inhibitory responses. Just as our approach of manually selecting potential epitopes, it failed to limit the selec-
tion of epitopes to a select few. The explored use of mass spectrometry in chapters 2 and 4 for the identification of MHC binding epitopes on tumor cells did, in fact, limit the list to a handful of identified mutations. Moreover, two MHC class I presented neopeptides and three MHC class II presented neopeptides were shown to be relevant in immunological protection against MC38, a drastic increase in the hit-rate compared to a full immunogenicity scan and an added benefit of being algorithm independent.

In chapter 4 we have reported a novel approach to identify MHC class II presented (neo) peptides. The independence from MHC-binding prediction algorithms was the primary advantage of mass-spectrometric identification of MHC class II eluted neopeptides. Initially, we aimed to obtain MHC class II presented peptides by the feeding of tumor cells to DCs followed by elution-MS techniques. Obtaining sufficient tumor material and APCs for a reliable quantity of eluate was a major drawback in this setting. Transfection of tumor cells with a single gene encoding CIITA offered a great shortcut to limit this dependency, as Armstrong et al. showed that CIITA transfection results in the efficient loading of endogenous and exogenous antigen in MHC class II, while preventing loading of erroneous peptides in the ER when transfected with MHC class II alone.10,11 This seems to happen in the MC38 model as well, as injection of CIITA-transfected MC38 in mice conferred adequate CD4+ T cell protection against non-transfected MC38.12 Moreover, the observations with the MuLV-specific T cell helper line in chapter 4 indicates that CIITA transfection-induced presented peptides are highly similar to tumor-specific antigens presented by APCs. Most likely the introduction of the master regulator CIITA regulates expression of all molecules involved in MHC class II presentation like CD74 and DM, in a controlled and balanced fashion leading to natural presentation of an immunological relevant peptide ligand repertoire.

Clinical application of MHC class I and II elution analysis is limited by the quantity of tumor material needed for mass spectrometric analysis of peptide-MHC complexes. Developments seen in the organoid platform offer a solution to alleviate the limitation on tumor material, as some tumor-types manage to return 90% success rates in establishing patient-derived organoid cultures.13-16 Importantly, these cultures maintain high similarity to the tumors of origin in clonality of mutations.17 Another promising development is the improvement of mass spectrometric sensitivity which will reduce the amounts of tumor material needed for adequate mass-spectrometric analysis.18,19 Analysis of presented peptides from small biopsies or shortening the timeline for expansion of an organoid culture may be a feasible future perspective. On the other end, the creation of MHC-allele specific elution databases based on MHC class II eluted peptides of tumor cells, offers the possibility to provide sequence information for deep learning algorithms, as recently sought after by Abelin et al.20 Thus improved in silico approaches can be applied or combined with wet approaches to accelerated the identification of relevant shared- and neoantigens.

Mind the gap: genetic discrepancies in tumor models

The MC38 tumor model was established in 1975 by chemical mutagenesis and has recently become a well-known tool for immunotherapeutic research around the world. Since the line is present in the field for more than 40 years, its lineage has become ill-defined with consequences for reproducibility between research groups. Experiments with MC38 prior to the start of the work presented in this thesis, showed little protection by vaccination with immunogenic epitopes described by Yadav et al.21 and suggested genetic drift or technological differences. Additionally, ICI-protected mice induced low levels of specific T cells against the described epitopes. T cell lines raised from these cleared mice on in-house MC38 cells also failed to recognize MC38 cells from Kerafast (chapters 2 and 3), a publicly available source of MC38 with the national cancer institute/NIH as origin. To delve into the origin of these immunological differences, the full genomic landscape was compared in chapter 3 between the in-house and Kerafast cell lines. The observed differences in the mutational burden between the two cell lines also included the lack of the CD8+ specific mutations in the Adpgk and
Rpl18 proteins and provided an explanation for the observed lack of immune recognition of the Kerafast MC38.

As a consequence, genetic differences create a problem for the interpretation of immunotherapeutic treatments, since other unknown neoepitopes are likely relevant for the immunological protection against Kerafast tumors. Interpretation of different immunotherapy studies is therefore challenging since individual neoantigens are commonly different in expression and may differ in sensitivity to immunological escape. The observed effects of PD-1 antagonistic antibodies on MC38 regression in published work is a distinct example for dissimilarity issues between these two MC38 cell lines. The MC38 tumors used in this thesis appears relatively insensitive to PD-1 blockade22, comparable to other reports23-25, while other groups have observed strong regressions after treatment.26-28 Ancestry or genomic analysis of the used MC38, or any tumor cell line, in publications is therefore needed for correct interpretation of the reported observations. The observed genetic drifts of the MC38 cell line also offers interesting opportunities to interrogate subtleties in immunogenicity of neoantigens and the sensitivity of cancer for cancer vaccines and immunotherapeutics. Such differences also occur between patients and even in separate tumor metastases within an individual patient.

Attention on CD4 T cells

The usefulness in targeting CD4 responses against cancer has become an important but not trivial part of selecting candidate antigens for personalized vaccines. Strong evidence has been found for the need of CD4 immunity in the tumor microenvironment and draining lymph nodes to activate the cytotoxic functions and memory formation of CD8+ T cells, respectively.29-31 Despite this appreciation of CD4 immunity, the identification of MHC class II presented tumor-specific antigens has remained a difficult process and limits the incorporation of such epitopes in personalized treatment. Previous studies have used CD8-epitope predicting algorithms and observed a high portion of mutation-specific CD4 responses within these antigens.2-5,32 The relevance of these ‘accidental’ CD4 responses compared to directly identified CD4 epitopes (e.g. as identified by the CIITA method) remains to be determined.

Thanks to the identification of our novel MHC class II presented peptides, fundamental questions on CD4 immunity in cancer can be addressed. First, it is unknown to what extent inclusion of neoantigen-specific help differs from non-specific activation in vaccination approaches. Recently, it has been reported that two-step activation of cDC1-CD8 T cells includes positive feedback from CD4+ T cells and results in the optimal programming of CD8 T cells when potent CD4-specific epitopes are included in the vaccine.29,33,34 The inclusion of CD4-specific neoantigens in a vaccine as shown in chapter 4 supports the added benefit in prophylactic and therapeutic settings. It would be of interest if distinction could be made between lymph node- or TME-spatial functions of CD4 immunity. Thus far the functionality of CD4 responses against neoantigens has been investigated in a limited set of tumor models, but thanks to the efficiency of our CIITA method this can be easily expanded to other cancer cell lines. Secondly, the presentation of CD4 neoepitopes in the TME appears important for tumor rejection30,31, but due to a general lack of MHC class II expression by tumor cells or direct CD4-cytotoxicity, this property of CD4 responses is most likely attributed to the interaction with local myeloid cells. The details of such mechanisms remain elusive, but the characterization of the involved antigens will help to investigate these local interactions. Furthermore, with the role of CD4 regulatory functionality often observed in cancer patients, the question arises whether shared- or neoantigens included in personalized vaccines induce tolerizing CD4 responses. In this thesis, however, no inhibitory effect were observed after vaccination in tumor-challenged mice, although this could be the result from agonistic OX-40 antibodies. It is compelling to hypothesize about the possibility of reprogrammed regulatory responses, when given the right targeted immunotherapeutic conditions.
Besides identifying MHC class II binding neoepitopes independent of algorithms and MHC alleles, the CIITA transfection method was also successful for the identification MHC class II binding shared antigens. Analysis of the eluate from B16F10 melanoma cells identified several presented peptides from well-established TAAs of melanoma. When tested in naïve mice, most epitopes were strongly immunogenic. Moreover, the method identified many MHC class II derived peptides from known TAAs in murine and human colorectal cancer. This is in concordance with recent studies that identified novel epitopes in TAAs from glioblastoma and hepatocellular carcinoma using a similar CIITA approach.35,36 The addition of tumor specific analysis of TAA expression would benefit the development of personalized vaccines when low mutational burden decreases the chance of expressed immunogenic neoepitopes. In addition, building such a database of shared MHC II epitopes will increase the options for design of generic vaccines suited for HLA selected cancer patient groups.

Delivering antigens

The formulation of vaccines is very important for therapeutic efficacy of cancer vaccines as briefly discussed in chapter 5. Chapters 2 and 4 applied basic vaccination approaches for the identification of immunogenic neopeptides with synthetized “long” peptides (i.e. peptides containing a suspected epitope with a centrally located mutation and flanking amino acid additions for processing-dependent presentation). This defined synthetic peptide sequence is effective for determination of immunogenicity, but therapeutic efficacy in tumor-bearing mice can be more consistent and stronger after conjugation to a potent TLR ligand e.g. Upam, a derivative of the Pam3, CysSK4 TLR-ligand with improved affinity for TLR-1/2 heterodimer37, as exemplified by the conjugation of an RPL18 synthetic peptide. Conjugation to TLR-ligands effectively increases maturation of antigen-presenting DCs and improves T cell activation.38,39 In general, the effectiveness of TLR-conjugation is attributed to the dual effect of efficient uptake and the simultaneous activation signals delivered to the antigen presenting cell, which can then upregulate co-stimulatory ligands, migrate and activate specific T cells.

The diversity of physicochemical properties across identified shared and neopeptides could influence and cause fluctuation in immunogenicity, which makes managed particulation interesting for clinical application of peptide-based vaccines.40 Nanoparticles and liposomes are, besides conjugates, synthetic methodologies capable of forcing particulation irrespective of physicochemical properties and offers higher control on characteristics like size, charge, and improves certainty of injected quantities.41-43 Nanoparticulates are reliable for the managed encapsulation of peptides of most physicochemical properties and can be easily mixed for reliable immunogenicity against multiple neoepitopes. The standardized encapsulation with cationic liposomes with identified neoepitopes of MC38 improved reliability of the vaccines and protection in vivo (Heuts et al. in submission). Alternatively, antigenic release might be extended for enhanced antigenic exposure through incorporation of peptides into polymeric (nano-)structures, that can similarly function as an artificial APC through incorporation of stimulatory molecules to enhance DC-selective uptake and activation.44 Large improvements in immunogenicity of tumor antigens has been made with the development of these technological platforms and currently the first successes are seen in the translation to the clinic.45

Progress in antigen visualization

The discovery of highly specialized functions of antigen presenting cells gave rise to educated attempts to design molecularly defined cancer vaccines for improved targeting of antigen to subsets of DCs, optimizing their processing and including optimal activation signals. These attempts have resulted in improved understanding of the mechanisms underlying antigen
uptake, processing, and presentation. Direct tracking of antigens by conjugation of probes and model-specific analysis tools for peptide-MHC complexes are important to acquire qualitative and quantitative data on the mechanisms of induced immune responses. Such model systems make use of well-defined antigens with, often, high immunogenic capacity. Previous tracking tools were specific for the model antigens, qualitative in nature and had drawbacks on reliability in later stages in antigen processing. Thus detailed interrogation of the antigen processing mechanisms in DCs would benefit from the development of novel analysis methods. Chapters 6 and 7 reported the potential of bioorthogonal chemistry for in situ ligation of probes, irrespective of the antigens of choice.

Current methods for linking distinct stages of processed antigen with antigen presentation and induction of T cells make use of fluorescent tags. The conjugation of probes or fluorescent proteins to antigens has the risk that physicochemical modification of peptides may interfere with intracellular localization or influence degradation of fluorescence. Incorporation of bioorthogonal groups by copper-catalyzed Huisgen cycloaddition in chapter 6, or Diels-Alder strained alkyn cycloaddition in chapter 7, are suitable solutions. These additions of bioorthogonal groups have a minimal effect on physicochemical properties on peptides and survival of endolysosomal processing and presentation. Copper-catalyzed ligation of fluorophores has already proven a useful tool with correlative-light-electron microscopy (CLEM) to achieve high spatial information from fluorophores and these studies were successful in the probing of pathogen-derived proteins. Fluorophore-conjugated peptides, however, are present in much lower quantities and require super-resolution technologies. These technologies offer the potential to detect single fluorophores, and thus single peptides, through lowering the ‘classical’ threshold of used wavelengths in light microscopy. For instance, higher resolutions are achievable with fluorophores through direct stochastic optical reconstruction microscopy (dSTORM) and could be used for the analysis of ‘rare’ molecules like presented peptides and offer direct quantification. Several other techniques are currently under development to detect single fluorophores, but in situ bioorthogonal ligation of individual intracellular antigenic peptides with fluorophores remain to be tested in live cell settings.

Paving the next stage

The successful application of ICIs in the clinic allowed the release of immunosuppressive blockades raised by cancer and opened avenues to other immunotherapeutic modalities. Defined vaccines with shared- and neoantigens derived from patient material offers a promising solution of a tumor-specific therapy without the serious side effects induced by non-specific ICI therapy. Vaccinations directed against tumor-specific antigens can be made more potent with the exploitation of other immunostimulatory adjuvants (e.g. OX40, 4-1BB, ICOS), whether or not in combination with ICIs. An important development is the inclusion of cancer-specific CD4 antigens which, in addition to helping the priming and activation of CD8 T cells, can contribute to stimulating immunogenic responses in suppressive areas in tumors and tumor-draining lymph nodes, and modulation of the TME. Optimal vaccine formulations will fulfil an important role in this, since it improves immunogenicity and provides consistency in the mixed physicochemical properties of antigenic peptides. The developments in novel analytical methods is necessary to gain knowledge about processing and presentation of such antigens in relation to these formulations. In this thesis, novel trails have been explored that shape the basis of these approaches. Future clinical studies are within reach to transform these trails into paved routes.
References


