Discovering Drugable Immune Targets in Gastrointestinal & Hepatic Disease

Ontdekken van nieuwe behandelbare immunologische aangrijppunten in gastro-intestinale en leveraandoeningen

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CHAPTER 1

General introduction

1.1 Introduction

Pancreatic cancer (PC) is one of the world's most deadly form of cancers [1, 2]. One of the main reasons this type of cancer is so deadly, is because it is often not recognized and diagnosed too late. Although new studies argue the impact of a diagnostic delay on the survival [3]. Precursor lesions are hard to detect by medical imaging and the symptoms of patients are too aspecific to be attributed to PC, which complicates diagnosis. This results in patients having developed tumors that are often already metastasized at the time of diagnosis. This causes them overall to have a very short survival of <6 months [4, 5]. The only potentially curative treatment up till now is surgery. However, only a small fraction of patients are eligible for surgery and even if they receive a resection of the tumor, most of the time patients suffer from recurrence or metastatic spread [6]. This, as in other tumor types, poses a great problem and unlike other tumors, no other therapies really seem to work in PC [7, 8]. Unfortunately, life expectancy following a diagnosis of PC has not significantly improved over the past few decades. The best therapeutic approach used to be surgery, followed up by adjuvant treatment with Gemcitabine, resulting in a median survival of 26 months with a 5-year survival of 30% [9]. Currently, Gemcitabine has been largely replaced by FOLFIRINOX, which is a combination of the following drugs: **FOL**inic acid, **F**luorouracil (5FU), **IRIN**otecan and **OX**aliplatin. FOLFIRINOX compared to gemcitabine monotherapy increased disease free survival and median overall survival after surgery even further. However, gemcitabine remains a treatment option for patients with contraindications to FOLFIRINOX [10-12]. Hence, defining improved diagnosis and treatment of PC represents a major challenge in contemporary biomedical research.

To understand the hypotheses that I formulate in this thesis, it is important to introduce various aspects of established PC and some key-features during its development. Some patients have a hereditary form of PC, which is linked to mutations in certain genes like kRAS [13]. However, these will not be discussed in this thesis, for readers interested in this aspect of the cancer process I can refer to a review by Grover et al [14]. Inflammation has a key-role in the development of cancer and often precedes the oncological transformation of cells in that niche [15-20]. As a consequence of chronic inflammation, genetic alterations might occur. If these occur in one of the so-called driver genes of PC (e.g.: kRAS, CDKN2A, TP53 and SMAD4), this increases the risk on the malignant transformation of healthy cells into tumor cells [21]. Historically Rudolf Virchow was one of the first to notice leukocytes in neoplastic tissue and postulated that this observation was a reflection of the origin of cancer in chronically inflamed tissue [22]. Now, decades later, the scientific field is still puzzled by the exact mechanisms via which chronic inflammation consequently links to cancer. In various (gastrointestinal)

cancer types there is a strong link between inflammation and cancer (e.g.: Inflammatory bowel disease and Colorectal cancer, Chronic viral hepatitis and Hepatocellular carcinoma or Helicobacter Pylori infection and Gastric cancer [23-25]). Comparably, also patients or individuals with inflammation in their pancreas (Pancreatitis) are at increased risk to progress into PC [26]. Increased understanding of the inflammatory component in the progression of PC is likely to provide important new insight into the pathophysiology of this disease and provide novel avenues for designing rational treatment.

Not only chronic inflammation (chronic pancreatitis) but also acute pancreatitis has been designated as a risk factor for developing PC. Inflammation has a broad effect on the (tumor) microenvironment and tissue structure/architecture. The end product is a complex network of cancer cells and other stromal cells (cancer associated fibroblasts (CAFs), vascular cells and inflammatory immune cells (e.g. tumor associated macrophages (TAMs)). The stroma, which is nowadays recognized for its significant influence on tumor characteristics and development, is a major responder to inflammation and can actually also maintain chronic inflammation. Inflammation in general is a key feature of the innate immune response and plays a huge role in wound healing, but the adaptive immune response caught the main interest over the past few years. Hence my interest to include not only the spatial analysis of adaptive immune cells in the tumor in this thesis, but also my interest in stromal factors (e.g. CAFs) that might explain the phenotype of a tumor [27-32].

Major efforts have been executed by the field to understand and evaluate drugable targets in cancer. It is known that cancer, as an abnormal growth, also can be recognized as foreign by the human immune system. Over the recent years, drugs and vaccines have been developed to reactivate or initiate adaptive immune responses to cancer. Unfortunately, most of these approaches have failed in PC and my perspective on why this may be and how it can be overcome is further discussed in chapter 1.2 and chapter 2. However, the foreignness of PC can now finally properly be characterized and this thesis will describe a relatively new biochemical methodology/tool and its further development that will contribute to the discovery of novel therapeutic targets in various diseases, including PC and other gastrointestinal cancers.

1.2 Immunotherapy and vaccines and application in pancreatic cancer

The most prominent rationale in the field is the tumor immunity cycle as has been first proposed by Chen and Mellman in 2013 [33]. They proposed the model of a closed tumor immunity cycle in cancer (which has been adapted and put into context of the most recent literature in chapter 2) where, just as in a normal resolving immune response in viral infections, the immune system is presented with antigens by professional antigen presenting cells (APCs), antigens are trafficked to the lymph node for presentation to T cells, after which the T cells home back to the site where they need to exert their function killing cancer cells resulting in antigen release and starting the cycle anew.

As discussed in literature and in chapter 2, various factors might compromise the anti-tumor immune response. This has been extensively discussed by Chen and Mellman in 2017 in their work on the cancer-immune set point [34]. To summarize, mechanisms might be at play that completely prevent the uptake by or release of antigens to APCs, the sentinels of the immune system, preventing initiation of an immune response resulting in an immune deserted tumor phenotype. Other conditions might allow for the elicitation of an immune response, resulting in T cells homing to the tumor site, but because of lack of immune supportive mechanisms [35, 36] may prevent the T cells to infiltrate and exert their function in the tumor, leading to immune excluded tumors. Lastly, it could be that the immune system mounts an effective response against the tumor, T cells are sufficiently able to infiltrate the tumor, but fail to efficiently kill tumor cells because of mechanisms like immune checkpoint inhibition (extensively discussed in chapter 2). These would be the inflamed tumors. To which extent each of these mechanisms are operative in PC largely remains an open question. I share my thoughts on how these mechanisms that prevent a complete tumor immunity cycle could be tackled in chapter 2. Furthermore, in chapter 3 I will question what the proportion of PC tumors is that can be categorized in either a neglected, excluded or inflamed phenotype. I will also study the dynamics of T cells and Fibroblast activation protein (FAP, as a marker of CAFs) in the tumors from the PC patients. This work was done in response to Ogawa and colleagues who suggested that they found a link between FAP expression in PC patients and exclusion of T cells. By qualifying the work of Ogawa [37], I hope to direct investigators active in the field to other potentially more productive research directions.

1.3 Vaccine design

To aid and elicit immune responses against either infectious diseases (as now is urgently needed with COVID-19) or the oncological setting like in PC, vaccines can be used [38]. The most simple description of a vaccine is it being a sample of to be encountered (potentially lethal) disease causing pathogen. It is a taste of the pathogen to which the immune system is exposed to generate immunological memory and prepare itself for a real infection. Traditionally vaccines are used in a prophylactic setting (historically the Pox virus would be the best example [39]). Vaccines can, however, also be used therapeutically.

Various methods can be used to develop vaccines, for example sequencing and subsequently computational predictions of protein translation. These identified regions that present itself as suitable vaccine targets are subsequently selected and synthesized, commonly combined with an adjuvant and (after extensive safety assessment) tested *in vitro* or in animal models [40]. But I focused on a biochemically evident method that is partially not dependent on predictions and might favor discovery of the unexpected (that which currently could not have been modelled). The partially needed piece of predictions that is still missing as preparation in this method, is the study of the antigen itself (selecting an interesting region on the various proteins of the pathogen). Before biochemically determining antigen presentation, the selected region of a potential vaccine (like a synthetic long peptide (SLP) as used in my studies) needs to be validated for various aspects (e.g.: the ability to synthesize/produce the vaccine, conservation of selected sequences in the pathogen, predictions for T-cell responses). This was not the goal of my research and I have utilized data from de Beijer et al. [41] to establish our biochemical methods, however, aspects like conservation of regions within an antigen of a pathogen and the amount of described epitopes in that region contribute to the selection of a region for the eventual vaccine.

The biochemical method in question is human leukocyte antigen (HLA) immunopeptidomics. This is the study of all the HLA-peptide ligands presented on cells. In general every nucleated cell in the body inherently has the mechanism to present everything that is going on within that cell to the immune system. Presentation occurs via HLA class I (also called major histocompatibility complex (MHC), but from here on called HLA) to the surveyors of the human body, the T cells. Whenever anything foreign might occur in a cell, by default it becomes a potential target for T cells. Foreign is defined here as everything non-self. This could be pathogenic material like a virus, but also mutations that lead to slight changes in amino acid composition of proteins are foreign to the body.

By taking a sufficient amount of a specific cell type and subjugating it to a capture of the HLA-class I complexes, it becomes possible to reveal the HLA-peptidome of that cell [42-44]. Mass spectrometry (MS) is involved in the step of translating the biological sample containing HLA-class I molecules with bound peptides to a list of annotated peptides which can be used for further studies. Through immunoprecipitation HLA complexes containing the peptide (potential epitope) of interest are isolated. The peptides are eluted from the HLA-complexes with an acid wash and separated through high-performance liquid chromatography (HPLC) and injected into the MS for characterization of the peptides. The characterization results in raw files that are used for the generation of the aforementioned list of annotated peptides through alignment to reference databases. In this thesis I first set out to determine how the HLA-peptidomics based epitope discovery pipeline should be handled. In chapter 4 I make an empirical evaluation of the use of computational HLA binding as an early filter to the mass spectrometry-based epitope discovery workflow. I evaluated the use of statistical thresholds, that were taken over from the generic trypsin-based proteomics field and applied in the HLA immunopeptidomics field, and assessed whether a less strict threshold can be applied for HLA-peptidomics. With the results from chapter 4 implemented in my own pipeline, I continue to study the processing and presentation of SLP vaccines in chapter 5 and the identification of soluble HLA (sHLA) peptides in chapter 6.

In Chapter 5 I study antigen processing by dendritic cells (DCs) using HLA-peptidomics. Chronic viral infections and malignancies like PC share many traits with regard to the immune system and its exhaustion or dysfunction. Consequently also therapeutic approaches are very similar and include therapeutic vaccination. This thesis mostly deals with PC, but for the aim of developing the tools to generate potent (peptide) vaccines, hepatitis B virus (HBV) derived SLPs/ antigens have been taken as a model system because these were more readily available. Nonetheless, key findings from this work may contribute to therapeutic vaccine design for the treatment of PC or other gastrointestinal cancers and the understanding of antigen processing and presentation by APCs.
1.4 sHLA origin of tissue peptidomics study

A challenge in the field of PC is the diagnosis of the disease. The late diagnosis of disease leads to the fact that often PC is already progressed into a late stage and has metastasized throughout the body [45]. Treatment in late stage PC is significantly more difficult and an earlier diagnosis would contribute to the treatability of PC patients and extend life-expectancy [46-48].

Although, as discussed earlier in this introduction, inflammatory disease (Pancreatitis) predisposes PC patients to the development to the actual tumor, diagnosis is still largely dependent on screening of the supposedly healthy

population or the presentation of a patient with symptoms. The ideal method would be a non-invasive screening method that would be able to dissect healthy individuals from patients that already have established yet still remain in early stages of disease without symptoms. Inspired by the findings in chapter 5 and earlier HLA-peptidome work from the group (hepatocyte work from de Beijer et al, unpublished), in chapter 6 I set out to investigate sHLA and its immunopeptidome in the plasma of confirmed PC patients and try to use it as a biomarkers providing evidence of malignancy in liquid biopsies.

1.5 Aims of this thesis

To summarize the introduction and to put this thesis in the greater perspective of modern science and medicine, the following has been the motivation to initiate this research. As has been introduced earlier, PC patients currently still have a poor perspective on survival. Huge developments have taken place in the recent years, with the leading development immunotherapy. James Allison and Tasuku Honjo even received the Nobel prize for physiology or medicine in 2018 for their contribution to cancer immunotherapy [49]. However, although major efforts are being invested now in earlier diagnosis of PC patients and immunotherapy in PC, we still do not have a sufficient fundamental understanding of PC to make rational clinical considerations.

Although patients are currently enrolling in trials with immunotherapy [50-52] which are partially focused and depending on T cell activation or reinvigoration, we still did not know if T cells were even present. In PC tumors, like introduced earlier, from the three large immune phenotypes in cancer, inflamed would be the best situation as these are the best responders to immunotherapy. However, in **chapter 3** I questioned if and where T cells are present in the tissue of PC patients. This to design better treatment regimens/approaches as I have extensively discussed in **chapter 2**. This chapter goes back to the fundamental side of the science, to redirect research to features or mechanisms of tumors that can be inhibited to improve effects of currently used immunotherapy.

Even if existing T cell responses can be reinvigorated, this does not ensure that these responses are specific for the tumor. As also outlined in **chapter 2**, tumors are extremely versatile and adapt to their environment and environmental pressure. Tumors are a good small-scale example of evolution and continuously demonstrate the “survival of the fittest” concept, weaker tumors cells are selected out, while stronger tumor cells stay and keep dividing and evolving. This happens also in coordination with the host immune system, hence the cancer immunoediting theory as has been proposed by Robert Schreiber [53]. So, for immunotherapy to work, introduction of novel specific T cell responses might

be needed in PC and other tumor types. This can be achieved by therapeutic vaccination. But how can we develop these vaccines? This thesis contributes to the development of a novel vaccine discovery platform with the empirical evaluation of the Mass Spectrometry-Based Epitope Discovery Workflow in **chapter 4** and subsequently in **chapter 5** applying the most optimal workflow to SLP loaded DCs to study HLA presentation from SLPs, which, as a first in the scientific field, generates unbiased biochemical evidence of SLP derived peptide presentation on HLA. As has been introduced earlier, I utilize MS to characterize epitopes in the binding cleft of HLA. Biochemical evidence of SLP derived antigen presentation is needed by the field because of several reasons. The main reason is that with this technique it can be validated in an unbiased fashion if the designed SLP vaccine components lead to presentation of a peptide in HLA and thus are likely to elicit the desired T cell response. On the other hand, in light of other work from the scientific field on immune dominance of epitopes, one would also like to exclude the presentation of epitopes that lead to elicitation of exhausted T cell responses. In short, one would like to exclude the situation where the developed vaccine leads to the presentation of epitopes that lead to nothing.

As a common occurrence in scientific research, there is never enough time. The chapters leading to this thesis were forced to have this structure and order due to a large time-investment in the development of the technology used in chapters 4, 5 and 6 and the time it cost to retrieve patient samples and organize the experiments in chapter 3. Altogether these lead to the establishment of this thesis where I aid the scientific field on the clinical side providing insight into the immune phenotype of pancreatic cancer. On the fundamental side I provide the field with novel methods to biochemically study antigen presentation of desired or yet to be discovered epitopes.

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CHAPTER 2

Opportunities for conventional and in situ cancer vaccine strategies and combination with immunotherapy for gastrointestinal cancers, a review

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Abstract: Survival of gastrointestinal cancer remains dismal, especially for metastasized disease. For various cancers, especially melanoma and lung cancer, immunotherapy has been proven to confer survival benefit, but results for gastrointestinal cancer have been disappointing. Hence, there is substantial interest in exploring the usefulness of adaptive immune system education with respect to anti-cancer responses though vaccination. Encouragingly, even fairly non-specific approaches to vaccination and immune system stimulation, involving for instance influenza vaccines, have shown promising results, eliciting hopes that selection of specific antigens for vaccination may prove useful for at least a subset of gastrointestinal cancers. It is widely recognized that immune recognition and initiation of responses are hampered by a lack of T cell help, or by suppressive cancer-associated factors. In this review we will discuss the hurdles that limit efficacy of conventional cancer therapeutic vaccination methods (e.g. peptide vaccines, dendritic cell vaccination). In addition we will outline other forms of treatment (e.g. radiotherapy, chemotherapy, oncolytic viruses) that also cause the release of antigens through immunogenic tumor cell death and can thus be considered unconventional vaccination methods (i.e. *in situ* vaccination). Finally, we focus on the potential additive value that vaccination strategies may have for improving the effect immunotherapy. Overall, a picture will emerge that although the field has made substantial progress, successful immunotherapy through the combination with cancer antigen vaccination, including that for gastrointestinal cancers, is still in its infancy, prompting further intensification of the research effort in this respect.

Keywords: cancer vaccines; *in situ* vaccination; immunotherapy

1. Introduction

Clinical management of oncological disease of the gastrointestinal tract remains very challenging especially when surgical options have been exhausted. The problem gastrointestinal cancer pose for medicine and society at large is compounded by the nosidynamics of this group of diseases, for many gastrointestinal cancer showing a trend to higher incidence [1]. For advanced disease combinatory chemotherapy remains the mainstay of clinical management but outcomes are disappointing and prompt pursuit of alternative treatment modalities. Generally speaking, immunotherapy and especially immune checkpoint-directed therapy is now revolutionizing the management of oncological disease, an endeavor even awarded the Nobel prize [2]. Cancers are antigenic and evoke immunological responses, but can escape the resulting tumor destruction through a variety of mechanisms including upregulation of so-called checkpoints: inhibitory elements to limit self-damaging autoimmunity. By counteracting these inhibitory signals the cancer can be combatted. Such strategies have proven successful for treatment options in a range of solid tumors, including melanoma [3-5] and cancer of the lung [6-8]. Unfortunately, results for immune checkpoint inhibitors for treating gastrointestinal cancers have proven disappointing, urging exploration of strategies that might augment the potential of such drugs that are depending on the a priori presence of immune responses, as they do not initiate but enhance these [9].

An obvious strategy to improve anti-cancer immunity apart from checkpoint inhibition is vaccination. Vaccinating is the act of injecting a pathogen or foreign protein with the goal to induce antigen specific immune responses and immunological memory. Vaccination relies on the action of professional antigen presenting cells (APCs) such as dendritic cells (DCs) that via presentation of antigens on MHC class I and MHC class II initiate CD8+ cytotoxic T cell (CTL) and CD4+ T helper (Th) responses, respectively. The latter are required to obtain long-lived and effective CTL responses [10, 11].

Because of lack of efficacy by immune checkpoint inhibitors in gastrointestinal cancers, vaccination is of high interest to be explored to initiate responses which can then be later on enhanced by add-on treatment with immune checkpoint inhibition. Design of vaccination strategies is complicated by the complex tumor microenvironment (TME) and other characteristics like mutational load and expression of tumor antigens, which are largely unique to various types of tumors and may vary even within tumors. This is not different for gastrointestinal cancers.

As a consequence of the expression of embryonic or germline antigens, or because of genomic alterations leading to neoantigens, cancers can become immunogenic.

Neoantigen load shows substantial variation between different forms of cancer and correlates to a certain extent with the success of checkpoint-directed immunotherapy [12]. Concordantly, mismatch repair deficient gastrointestinal (e.g. colorectal and pancreatic) cancers that bear many mutations are more responsive to checkpoint-directed therapies [13]. However, high neoantigen levels do not correlate with survival for pancreatic- and liver cancer per se [14-16]. Yet also for these cancers it is rational to assume that stimulating cancer-specific immune responses will be associated with better outcomes. However, in these situations optimal exploitation of the available antigenic targets and combination therapies that overcome tumor specific suppressive mechanism are likely required.

Nowadays we discriminate between two types of vaccination. Prophylactic (preventive) vaccines and therapeutic vaccines. A few examples can be given of prophylactic vaccines that are very effective in preventing cancer, the human papilloma virus (HPV) vaccine, preventing cervical cancer and the hepatitis B virus (HBV) vaccine, preventing liver cancer [17-19]. For established disease however these vaccines are not effective also because they typically induce effective humoral rather than cellular responses. In the present manuscript we shall overview most important therapeutic cancer vaccine forms, elude on non-immune related cancer therapies that may trigger systemic immunity as a side effect, and will discuss how these therapies mechanistically offer potential for combination with other forms of immunotherapy to find opportunities for treatment of gastrointestinal cancers.

2. The ideal anti-tumor immune response and the limitation of vaccination

A long-established cancer immune-editing theory describes the interplay between a cancer and the immune system, encompassing 3 phases: Elimination, Equilibrium and Escape (the 3 E's) [20]. According to this view, initially the immune system can control cancer cells (Elimination), a process also termed immune surveillance. However, certain clones of malignant cells missed by the immune system (e.g. due to a non-immunogenic phenotype), escape the elimination phase (Equilibrium). The clones that survive are then subject to immune pressure driven (epi)genetic editing, which ultimately leads to Escape of the tumor from immune control [21, 22]. In cancers these three phases can occur simultaneously in patients. Immune checkpoint directed therapy has the potential to shift the balance to elimination and equilibrium. Importantly, low-fitness neoantigens may be leveraged by vaccination, i.e. marginal antigens in the immunosuppressive environment of a cancer that do not provoke effective immunity, when triggered by vaccination may confer effective anti-cancer responses [23].

Suppressive mechanisms however may limit the effect of vaccination. Tumors actively keep the immune system at bay by shielding themselves from the outside with a thick stroma or fibrotic shell [24], an anti-inflammatory microenvironment containing immune suppressive cells like M2-macrohpages [25], regulatory T cells [26], myeloid derived suppressor cells (MDSCs) [27], or by utilizing immune pathways like the PD1-PDL1 axis to suppress responses [28-30]. For gastrointestinal cancers these anti-cancer immune suppressing mechanisms show substantial redundancy as in situ approaches to enhance immune system activity through local application of non-relevant vaccines (e.g. anti-rotaviral vaccines or anti-yellow fever vaccines) only generate local immune responses to cancer when combined with immune checkpoint blockade (ICB) [31, 32]. Hence, overcoming the resistance to immune response development in gastrointestinal cancer, requires targeting multiple pathways.

How this may be achieved is outlined in the canonical tumor immunity cycle of Chen and Mellman. Here, the cancer immune response is described as an ongoing cycle of tumor cell killing and subsequent initiation of new responses which may combat the adaptation of tumors [33]. To prevent tumor escape continuous killing of tumor cells is required to trigger responses also against novel antigens expressed by escaping tumor cells. Vaccination may trigger an initial “therapy-induced hit”, further releasing antigens and danger signals kick-starting the cycle. Ideally this therapy-induced hit should also alter the anti-inflammatory environment in the tumor to a favorable pro-inflammatory environment, and facilitate the influx of novel T cell clones recognizing antigens beyond those starting the response and thereby create a snowball effect leading to a broad T cell repertoire. [34, 35]

To obtain an effective immune response in cancer patients 3 steps are generally thought to be required (figure 1): **(1) Creation of the response:** under certain circumstances a tumor specific CTL response might already exist, but in many cases there is either no response or the response is ineffective. Absence of a response is likely present in immune desert tumors that encompass a minor but significant part of gastric, colorectal and pancreatic cancers [36]. Although for some tumors antigenic targets may have been largely absent (restricting vaccination opportunity), for others responses may have lacked because tumor specific antigens did not (yet) reach APCs/DCs or the APC triggered response was subsequently not properly shaped. The treatment modalities outlined in table 1 and 2 mostly can support this very first step, the initiation of CTL and Th responses. Initiation can be achieved through conventional vaccination, with manually selected target antigens, or through *in situ* vaccination, releasing antigen via immunogenic cell death (ICD) to initiate the response. The latter option has the benefit that this is not limited to a set of patients expressing a specific selected

antigen. **(2) Shaping of the response,** during T cell priming by APCs in the lymph node (LN), the costimulatory signals received by the T cells are detrimental for the efficacy of the eventual response. These signals are provided by DCs activated and maturated by danger signals and/ or by contact with activated MHC class II primed Th cells. It is pivotal for their efficacy that CTLs receive the correct 'help' signals during priming in the lymph node. The most prominent example is the CD28-CD80/86 axis, but other pathways like the Th supported CD40-CD40 Ligand or CD27-CD70 axes have also been proven essential for the ability of CTLs to migrate towards, infiltrate in and ultimately to kill tumors [10, 11]. As such lack of help may contribute to the immune exclusion phenotype which mark a large fraction of gastric, colorectal and pancreatic cancers [36]. Furthermore, the absence of appropriate costimulatory and help signals can contribute to the exhausted or dysfunctional T cell phenotype often observed in cancer [10, 11, 37]. These signals are also a point of intervention for immunotherapy. Examples are blocking antibodies for CTLA-4 (Ipilimumab; a competitive inhibitor of CD28) or agonists for CD40 that each may enhance or direct the shape of the response [38, 39]. Combination of such drugs with vaccination could thus enhance the potency of the vaccine-induced response. **(3) Executing the response**, after the adaptive response has been established, fully primed and armed, T cells need to infiltrate the tumor and kill the tumor cells. Only then T cells will start a new cycle, tailoring immunity to the evolving cancer until the tumor is eradicated and memory is established, thus preventing also future growth of the tumor. Execution of CTL responses, however, are in many cancers in including gastrointestinal cancers locally suppressed by an array of suppressive molecules and cells such as PD1-PD-L1 or MDSCs respectively [40, 41]. In addition cancer specific suppressive mechanism may prevent immune effector function and thus limit the effect of vaccination. Pancreatic ductal adenocarcinoma for example is notorious for its fibrotic immune suppressive TME that may need to be tackled (e.g. by focal adhesion kinase inhibitors or for immune responses to take effect) [42-44]. In colorectal cancer aberrant WNT/β-catenin signaling shapes the TME and can render these tumors unresponsive to checkpoint inhibitors and may therefore require specific attention when combined with vaccination or immunotherapy [45]. A suppressive TME may especially impair the effect of conventional vaccines that start the response outside the tumor and do not much to improve the local environment. On the other hand, this might be an extra opportunity for *in situ* vaccines, that by definition also affect the local environment and, by disruption of the tissue or the release of chemotactic factors, might enable infiltration of immune cells [46]. How do presently employed strategies relate to the above-described idealized scenario?

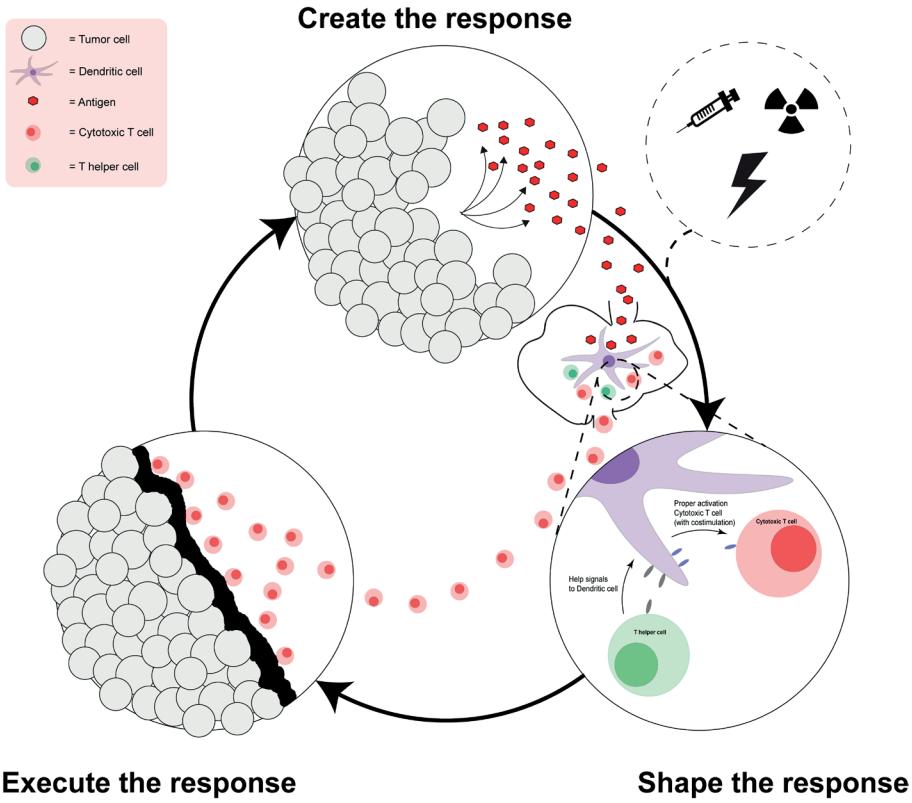

Figure 1. A simple representation of an anti-tumor immune response with integration of (in situ) vaccination. In case of naturally arising anti-cancer immunity, antigens are released from the tumor, **creating the response**. Antigens end up in the lymph node and are presented on dendritic cells, to T helper cells and cytotoxic T cells. T helper cells give help signals to dendritic cells resulting in enhanced costimulation for cytotoxic T cells, **shaping the response**. Activated T cells will migrate to the tumor and kill the tumor cells, **executing the response.** However, T cells at the tumor site may encounter a harsh microenvironment which often starts with a physical barrier. By killing the tumor cells new antigens are released and the cycle can continue. In of the absence of naturally arising immunity, (in situ) vaccines can be used to kick start the response.

Table 1. overview of conventional cancer vaccines with pros and cons.

Therapy	Pros	Cons	References
Peptide vaccines	· Cheap, easy to produce · Long peptides: Th and CTL epitopes, not HLA-restricted · Personalized (neo-antigens) and semi-personalized (peptide "warehouse" for prevalent tumor antigens) · High epitope concentration	· Short peptides: no or less Th epitopes cells · Restricted to selected epitopes/antigens · HLA-restricted · Poor immunogenicity (need adjuvants)	[47-51]
Genetic vaccines	· Native structure of protein · Induce humoral and cellular response · Personalized possible · Th and CTL epitopes · Cheap, easy to produce	· Poor immunogenicity (needs adjuvants)	[52-57]
Tumor cell vaccines	· Contains characterized and uncharacterized tumor antigens · Th and CTL epitopes · Allogeneic vaccine can be given, broader target population	· Poor clinical efficacy · Self/ normal proteins in the vaccine pose toxicity risk · Possibility of release immunosuppressive cytokines · Rejection of vaccine because of allogeneic HLA	[58-63]
Dendritic cell vaccines	· Measurable antigen presentation efficiency and DC maturation · Th and CTL epitopes	· Not fully matured DCs/ tumor impaired DCs may induce tolerance · Logistically challenging · Costly, labor intensive	[64-70]

Table 2. overview of *In Situ* cancer vaccines with pros and cons.

Therapy	Pros	Cons	References
Radiotherapy	· Depending on dose, can induce immunogenic cell death · Can release uncharacterized/ personal tumor antigens · Easy to combine with immune checkpoint inhibitors	· Will cause 'late effects' · Elevated risk of cancer due to treatment · Destruction of healthy tissue	[46, 71-77]
Chemotherapy	· Can cause immunogenic cell death depending on the compound · Can suppress specific types of immune suppressive cell populations · Easy to combine with immune checkpoint inhibitors · Will release uncharacterized/ personal tumor antigens	· Overall toxicity · Not all chemotherapeutic compounds have the favored immunogenic effect · Destruction of healthy cells	[78-84]
Oncolytic virus	· (Engineered to) Specifically target tumor cell · Cause immunogenic cell death Will release uncharacterized/ personal tumor antigens · Easy to combine with immune checkpoint inhibitors · Can be engineered to express a tumor antigen or cytokines to modify the tumor micro environment	· Anti-viral response can neutralizing the therapy, shortening the window of opportunity, · Specialized facilities to monitor patients due to safety concerns	[32, 85-92]

3. Conventional vaccines

The first cancer vaccine exploiting the immune system for cancer treatment, named 'Provenge' or "Sipuleucel-T", was an infusion of DCs, isolated from the patient and loaded with a specific antigen *ex-vivo* [93]. Over the years many more vaccine forms/platforms have been developed aiming to bypass the first step in the cycle (figure 1), to create an immunological response by offering the antigen in various forms, processed or unprocessed, to the patient. Various vaccine platforms deliver antigens in many forms and complexities ranging from tumor lysates to whole proteins, protein encoding mRNA, protein fragments or synthetic long peptides (SLPs) and to finally 9-11 AA short peptides of the minimal MHC class I binding epitope (table 1). Although vaccines thus far have yielded immunological and some clinical effects, their clinical efficacy is still disappointing [65, 94-96]. The use of suboptimal vaccine platforms and of low immunogenic vaccine target antigens (e.g. overexpressed self-antigens) together with a suppressive tumor microenvironment is held responsible, as has recently been extensively reviewed elsewhere [97].

We will first briefly go over the main vaccine platforms and discuss their ability to create or shape response and to what extent they may need additional support. Because danger signals are crucial for the shaping of a response, conventional vaccines are often combined with adjuvants. Especially when low immunogenic self-antigens are targeted, such as overexpressed tumor antigens for which central tolerance exists, adjuvants are likely very important. The need for adjuvants and the type of adjuvants used may also differs per vaccine platform as will be touched upon below. We will, however, not discuss the various types of adjuvants in detail as there are some recent excellent reviews on this matter [98, 99].

Peptide vaccine

Peptide vaccines exist in a short or long format, are generally stable, safe and can be used of the shelf for common tumor (specific) antigens or in a personalized fashion. Furthermore peptide vaccines are cheap and easy to produce (table 1). However, for personalization, genetic analysis of the tumor is required which may delay treatment and is not always possible to perform (for instance in inoperable pancreatic cancer). Short peptides (<15 Amino Acids (AA)) are convenient because of their ability to directly bind MHC, but short peptides are MHC subtype restricted and may also induce tolerance or on-target off- tumor toxicity by binding to MHC on non-professional APCs [38, 51, 100]. Synthetic long peptides (SLPs; ≈15-40 AA) in contrast, need to be processed by professional APCs rendering these safer and less tolerogenic and non-MHC restricted. For peptide vaccines obtaining sufficient MHC-epitope complexes for the creation of a response is easier than for whole protein based vaccines [68]. Furthermore, SLPs can also provide MHC class II epitopes facilitating activation of CD4 T helper cells and have a high epitope concentration. Peptide vaccines may benefit from Th-skewing adjuvants, which can also be conjugated to the peptide and can further help shaping the response [50, 101]. Combinations of peptide vaccines with forms of immune therapy that aid in the later stages of the response are obvious and good options, as long as sufficient T cells are induced and able to not only migrate to, but also infiltrate the tumor.

In clinical practice peptide vaccines, have been and are used treat premalignant advanced or recurrent HPV16-induced gynecological carcinoma but also a multitude of cancers targeting cancer (neo)antigens [48, 49]. Targeting HPV with SLPs may be of high interest also for the treatment of HPV related esophageal cancer [102]. Especially SLP vaccines have shown promising results with respect to the creation of both CTL and Th responses that also correlated with clinical effects. In premalignant HPV lesions more than 50% of patients showed a complete or partial response (i.e. regression of lesions) upon SLP vaccination [103]. In malignant disease responses were less overt. Although, immunological

responses induced by the vaccine were observed in a majority of tested patients, no regression of tumors nor prevention of progressive disease was observed likely because T cell were impaired in the execution phase by immune suppression [104]. To lift suppression, combination of SLPs vaccines, with low-dose chemotherapy to kill suppressive myeloid cells, was shown to improve T cell responses [105, 106]. Furthermore, It was found that the tumoricidal effects of PD-1 inhibition (with nivolumab) may be enhanced by combining it with an SLP vaccine. These encouraging results were, however, obtained in a phase II single-arm study and need to be confirmed through a randomized control trial before changes in clinical practice are indicated [107]. Recently, also a personalized neoantigen-based SLP vaccine showed highly promising immunological (i.e. Th and CTL) and clinical responses with and without additional ICB therapy in metastatic melanoma [108]. In an alternative approach, recently a Phase I immunotherapy trial with two chimeric HER-2 (commonly over-activated in gastrointestinal cancer) B-Cell long peptide vaccines were tested in solid tumors including gastrointestinal tumors and showed anti-tumor activity with a very acceptable side effect profile. This study indicates that long peptides may be even more versatile, triggering not only tumor directed cellular but also humoral immune responses [48]. It should prove very interesting to combine such approaches with immune checkpoint-directed therapy and assess the potential to control gastrointestinal cancer refractory to immune checkpoint-directed monotherapy.

Genetic vaccine

RNA and DNA vaccines are genetic vaccines. Genetic vaccines rely on the concept that DNA or RNA encoding for antigens are transfected into cells and serve as a template for proteins synthesis, maintaining the native structure of the protein. Material from transfected cells may engage the MHC class I and II pathways in DCs/ APCs or DCs can be directly transfected themselves and present peptides on MHC I via the endogenous route of antigen presentation. Genetic vaccines may thus theoretically induce humoral and both CD8 cytotoxic T cell responses and CD4 T helper cell responses although the extent of each may vary depending on the dominant target cell of a specific genetic vaccine. [55, 109] Genetic vaccines are relatively cheap and simple to synthesize. They are safe and highly flexible and a broad range of antigenic targets can be selected with this technique. However, genetic vaccines may be limited in immunogenicity and the antigen levels obtained are more variable and harder to control than for peptide vaccines. Yet, the genetic vaccine has come a long way with many optimizations in e.g.: codon optimization, novel plasmid vectors, vector boosting regimens and more. [56] Although genetic vaccines have intrinsic adjuvant properties by binding to pattern recognition molecules recognizing nucleic acids, this may not necessarily aid their effect as it induces and antiviral state, abolishing antigen translation

[109]. Rather, for optimal efficacy an adjuvant effect may need to be pursued after genetic vaccine induced antigen production. This can be achieved for example by co-expression of immune activating proteins (e.g. CD40L, CD70) or cytokines (e.g. IL12) [109]. Alternatively, potential for combination with other forms of immune therapy might also lie in the priming and shaping phase. For example therapeutic compounds targeting the APCs for enhanced immunogenicity like CD40 agonists or other T cell activators in clinical development to aid in the shaping of the response.

DNA vaccination has been clinically tested in HPV related neoplasia and a multitude of cancers. On precancerous HPV lesions the vaccine had beneficial effects causing histopathological regression in a significant amount of patients. [110] However, clinical trials with DNA vaccines in more established diseases like melanoma, prostate-, colorectal- or breast cancer disappointed in terms of therapeutic outcome, despite the immunological responses induced. [53, 54, 57, 111-117] Yet, these results pave the way for combinations with therapies to lift the suppressive mechanisms of the tumor. Also mRNA vaccines have been applied to many different cancers and have shown immunogenicity and some clinical responses [109]. Of special interest is a recent clinical study on the vaccination of 13 late stage melanoma patients with mRNA encoding mutated parts of proteins (27AA with the mutation in the middle; 10 potential immunogenic mutations per patient) that resulted in T cell responses against multiple neo-epitopes in all patients (mostly Th but also CTL). Despite low patients numbers this study also showed promising clinical effects including a complete response in one patient receiving the vaccine combined with PD-1 blockade [118].

For many approaches antigen selection remains a bottleneck. The most obvious way to address this is combining genetic analysis of the cancer and patient HLA phenotype with prediction tools that identify promising candidates. Now that many centers are building molecular precision medicine pipelines for drug selection in gastrointestinal cancer, it is also becoming feasible to use the infrastructure for selecting epitopes suitable for personalized genetic vaccines, which in combination with ICB therapy may prove exceedingly useful.

Tumor cell vaccine

Tumor cell vaccines are whole-cell vaccines consisting of inactivated allogeneic tumor cell lines or of autologous tumor cells. They contain characterized, but also uncharacterized, tumor antigens which lie at the basis of inducing the immune response. Examples are Canvaxin and GVAX [119]. GVAX is a tumor cell vaccine where the origin of tumor cells can be autologous or allogeneic (can be given to a broader target population). Because the vaccine consists of ‘whole protein’,

it will contain Th and CTL epitopes. In GVAX, the tumor cells are engineered to express granulocyte macrophage colony stimulating factor (GM-CSF). In mice genetically modified tumor cells engineered to express cytokines like interleukin 2 (IL-2), interferon gamma (IFN-y) or GM-CSF can be rejected and can induce systemic immunity. Subsequent characterizations of the induced immune response revealed a local influx of immature dividing monocytes, granulocytes and activated lymphocytes at the injection site. Moreover, paracortal hyperplasia was observed at the draining lymph node. Most of this preclinical work was done in mouse models of melanoma but was also extended to renal cell carcinoma, colon carcinoma and fibrosarcoma models. [119, 120]

Although these preclinical results were promising, the clinical efficacy of GVAX was thus far limited. Studies have mostly been performed in (but not limited to) prostate cancer, pancreatic cancer and colorectal cancer [58, 60, 61, 121]. Immunologically, Th cells have been demonstrated to be induced upon treatment with GVAX, however, these studies often included combination of GVAX with checkpoint inhibitors like ipilimumab [60]. This complicates our understanding of the sole effect of GVAX on the adaptive immune response. Also due to allogeneic HLA, the vaccine might be rejected and may not induce an effective anti-tumor immune response. GVAX-ICB combinations are currently pursued further in the clinic. [58] Interestingly, one of the biomarkers that was found associated with survival in pancreatic cancer following GVAX combined with ipilimumab was a diversification of the T cell receptor (TCR) repertoire [58, 121]. Although ipilimumab has this effect already by itself, diversification was most clear upon co-treatment with GVAX [58, 61, 121].

DC-vaccine

Dendritic cells are considered the most important professional APC crucial for the initiation of any adaptive response [122]. They are very efficient in the phagocytosis of antigens, and subsequently process these and load derived peptides on MHC class II. In addition DCs excel in the cross presentation of incoming antigens on MHC class I to T cells. DCs also provide the necessary costimulation to T cells for proper activation and function. Finally they can secrete cytokines that further shape T cell function. In vivo different DC subtypes can be discriminated (i.e. myeloid DC1, DC2, plasmacytoid DC and inflammatory monocyte derived DC) that differ in function [123]. Of particular interest are the rare subset of DC1 that are thought to excel in cross presentation and in mice have been demonstrated to be crucial to the activation of naïve T cells and are thought to transfer help signals to CD8 cytotoxic T cells through CD4 T helper cells [124-126]. DCs can be loaded with antigens and activated ex-vivo and be given to a patient as a therapy [15, 62, 63, 127-129]. For loading of vaccine DCs all the aforementioned forms of

antigens can be used (i.e. short and long peptides, DNA, RNA and tumor lysates). For DC vaccination monocyte derived DCs (moDCs) have been popular because they can be easily differentiated ex-vivo from monocytes that can be obtained in large numbers through leukapheresis. Current thought is, however, that moDCs are not the most optimal DC for vaccination [66, 130]. Primary DC subsets, which can only be harvested in lower numbers from patients, may be more effective and have recently also been used for vaccination with promising results. The DC type used, the antigen loaded and the activation method used together likely greatly determine the ability of the DC to create and shape a response. Efforts are currently directed at the exploitation of primary DC subsets including rare DC1 for vaccination and at optimizing DC loading and activation[130].

Although DC vaccination is time and resource consuming, antigen loading and DC activation can be well controlled and monitored which is less for other cancer vaccine platforms. DC therapy has been proven to be safe in the clinic and preliminary data deems it efficacious, triggering both Th and CTL responses and also yielding some clinical responses [66]. Currently DC vaccines are tested in several advanced phase II/III trials including gastrointestinal cancers [69]. Also for DC therapy, however, use as a stand-alone therapy has thus far been disappointing despite their proven ability to trigger T cells [67]. DC vaccines very likely require support of T cells in the execution phase for clinical effect. Concordantly, many trials with combinations of DC vaccines with checkpoint inhibitors like PD-1/ PD-L1 and CTLA-4 inhibitors are ongoing [64, 69, 131].

4. In situ vaccines

Besides these conventional vaccination strategies there are also several other therapies that can have an *in situ* vaccine effect which initial purpose was not to generate immunological memory or an immune response at all [74, 132, 133]. These treatment modalities can cause the release of antigen and thereby can have a vaccine-effect *in situ*, resulting in the induction of an immune response and the development of immunological memory [134]. Their strongest edge over most conventional vaccines is that screening of the patient for antigen-positivity is not needed [73, 88, 135-137]. For these *in situ* vaccines the effectiveness of the resulting immune response depends on the expression of immunogenic antigens in the tumor at the time of treatment. The response will by definition be ‘personalized’ AND The lack of need to screen for tumor antigen expression may save valuable time. Furthermore, in case of local treatment and induction of a systemic immune response also metastasis might be targeted indirectly due to the partial antigenic similarity of the main tumor and the metastasized tumors (i.e an abscopal effect) [72]. An overview of the most important therapies with a known *in situ* vaccination effect are summarized in table 2.

The concept of *in situ* vaccination comprises that the antigens causing the vaccine effect are already present in the tissue and are released upon therapy. Upon release these antigens are taken up by phagocytic cells and transported to the lymph node for the induction of specific, personalized adaptive immune responses [91]. In situ vaccination is thus an attractive form of personalized medicine as any tumor will have its own profile of tumor antigens and mutations that might form neoantigens (i.e. new antigens to be loaded on HLA-molecules). For treatments having an *in situ* vaccine effect, tumors do not necessarily have to be characterized before starting treatment, saving valuable time. Possible limitations of the *in situ* vaccination however, might be that antigens might not be present in such a concentration to allow effective antigen (cross)presentation and the creation of proper responses. Furthermore, as the antigens carrying the vaccine effect are not known it is difficult to monitor the response [77]. Lastly, antigen release following these treatments might not always be accompanied by sufficient danger signals to shape the response (and break tolerance in case of self-antigens). Especially in this scenario, responses following the *in situ* vaccination may benefit from immunotherapeutic agents that are designed to stimulate/initiate key mechanisms important to the shape and execution of an effective adaptive immune response [135, 137]. Although many conventional cancer therapies used to treat gastrointestinal cancers, may have an *in situ* vaccine effect we will restrict our discussion to those most widespread used.

Radiotherapy

Radiotherapy is still one of the most important treatment modalities for cancer and is also standard-of-care or at least a treatment option for many gastrointestinal cancers [138]. It causes radiation-induced cell death trough lethal DNA damage [139]. A secondary effect of radiotherapy is activation of the immune system as it leads to ICD of the tumor cells by ionizing radiation [81]. However, complications might arise due to destruction of not only cancerous tissue but also healthy tissue, might induce so called 'late-effects' and might even lead to an increased risk of getting cancer in a later stage of life due to the radiation.

Radiotherapy not only releases antigens for uptake by APCs but may also provide cell death-associated danger signals (e.g. cell surface calreticulin, ATP, nucleic acids, HMGB1) important for DC activation and immune cell recruitment [140]. Thus radiotherapy may be effective to create and shape the response. Apart from the activation of the immune system, effects of ionizing radiation are also seen in the tumor microenvironment for example on the vascular endothelium where factors involved in the recruitment of T cells were increased following radiotherapy [75, 76, 141-143]. It is important to note, however, that not every radiation dose has the same effect. In mice, the release of cell free DNA in the tumor was found to

be compromised by the expression of DNA exonuclease following a single high radiation dose. This was thought to prevent the activation of the cGas-STING pathway and therefore prohibited immune cell activation [144](70). In this same study combination of radiotherapy with a CTLA-4 inhibitor, provides not only a local immune stimulatory effect but also an abscopal effect by the generation of systemic immunity [144]. Furthermore, in a mouse model of pancreatic cancer the induction of tumor specific memory cells by radiotherapy was enhanced by combination with a CD40 agonist [145].

One of the concerns of combining radiotherapy, as the inducer of the immune response, and ICB, removing the brakes from the immune response, is safety especially as also self-antigens are released. Checkpoint inhibitors are known to have side effects, and when combined with each other, these might occur significantly more [146]. Combining radiotherapy with ICB, however, has thus far been reported to be safe and well tolerated [147-149].

In mice, anti-CTLA4 treatment predominantly inhibited regulatory T cells while radiation therapy enhanced the TCR repertoire of intratumoral T cells. When these therapies were combined, anti-CTLA4 promoted the expansion of T cells and radiation shaped the TCR repertoire of the expanded peripheral clones, thus these modalities synergized to create the response as well as to shape the response [149]. However, in patients this combination was less effective. Melanoma patients showing high PD-L1 expression in the tumor, when treated with ionizing radiation together with anti-CTLA4, developed T cells with an exhausted phenotype and the tumors progressed [149]. In lung cancer a case of clinical success of combination of RT with CTLA-4 was reported [150] and also combination with PD-1 blockade showed promising results [151]. In mice it was shown that in addition to ionizing radiation and anti-CTLA4, supplemented with PD-L1 blockade reversed T-cell exhaustion and aided in the execution of the response suggesting further combination of radiotherapy with multiple checkpoint inhibitors could be more effective [147-149] Although the combination with radiotherapy may be superior to just ICB, not all inhibitors have the same efficacy and are based on different mechanisms. More mechanistic insight is now required to make good combinations optimally covering all 3 requirements depicted in figure 1 [140].

Chemotherapy

Chemotherapy is extremely versatile and apart from stopping tumor proliferation directly it may also aid the generation of anti-tumor immunity. In general it is used as a therapy to manage disease and treat lower grade cancers. [152] However, chemotherapy also holds potential to enable other therapies

to become more efficacious in late stage cancer. Immunological effects of chemotherapy can be induction of ICD, releasing both danger signals and tumor antigens facilitating antigen presentation, induction of a cellular senescence program in tumor cells that alert the immune system by activation of natural killer (NK) cells and finally the inhibition of immune suppressive cell populations like regulatory T cells or myeloid derived suppressor cells (MDSCs). [78, 82, 83] Most important chemotherapeutics that lead to ICD are idarubicin, epirubicin, doxorubicin, mitoxantrone, oxaliplatin, bortezomib and cyclophosphamide [153]. These individual chemotherapeutic agents have been extensively discussed for their specific immune modulatory properties elsewhere [153-155]. It is important to note that the immunological effects of chemotherapeutic drugs might vary greatly. This is illustrated by differential ICD in response to the related chemotherapeutics cisplatin (no ICD) and oxaliplatin (ICD), both commonly used to treat gastrointestinal cancers [153, 156]. Thus, not all chemotherapeutics may benefit similarly from a combination with immunotherapy.

In general, combination of chemotherapy with checkpoint inhibitors (anti-CTLA4 or anti-PD-(L)1) is well tolerated. In various types of cancer like lung cancer and biliary tract cancer, the combination also seems to be superior compared to single immunotherapy or chemotherapy alone. [157-160]. Also for pancreatic cancer combination of gemcitabine chemotherapy with PD1 was well tolerated and holds promise [161].

Although combination of ICB with chemotherapy occurs very often in clinical trials, this is most often because it was a standard of care treatment for these patients rather than a rational choice based on the immunological effects of specific chemotherapeutic agents. However, there are several exceptions that are explicitly meant to exploit the immune stimulating actions of ICD-inducing chemotherapeutics [153, 162]. One of these is a recent multi-arm phase II study comparing the combination of various ICD and non-ICD inducing chemotherapeutics with PD-1 inhibitor nivolumab [163]. Results confirmed the superiority of combining ICB with ICD-inducing chemotherapy (in this case doxorubicin).

Oncolytic viruses

Oncolytic viruses (OV) have been discovered by accident in patients from cases that experienced tumor reduction after contracting a natural viral infection [90, 92]. OV based on naturally replicating viruses are selective for tumor cells in particular. These viruses exploit the fact that tumor cells, in order to attain features beneficial for uncontrolled growth, trade in some basic biological processes, one if which is the innate response mechanism to viral infection. Because this

is lacking in tumor cells, oncolytic viruses can specifically infect the tumor and cause cell death. Although various viruses have been tested for this purpose, of particular interest are two recent studies in mice, demonstrating oncolytic effects after intratumoral vaccination with common prophylactic vaccines based on attenuated viruses (i.e. yellow fever and rotavirus) [31, 32]. Another class of oncolytic viruses is formed by viruses genetically modified to target and kill a tumor [92]. Although it was thought that direct cell killing by the virus was responsible for tumor control/regression, evidence is accumulating that systemic immunity that can originate from this killing (an *in situ* vaccine affect) is also very important. Like radiotherapy and chemotherapy, OVs can cause ICD, releasing antigen and promote a local pro-inflammatory environment, leading to an adaptive immune response [87]. Additionally recombinant OVs are being tested in the clinic carrying various tumor antigens, using the OV simultaneously as a viral vector [85]. In comparison to radiotherapy and chemotherapy, the experience with the combination of oncolytic viruses and ICB is still in its infancy [31, 92]. Clinical trials combining several different form of OVs (including recombinant OVs carrying various types of antigen) with PD1/PD-L1-, CTLA-4 inhibitors or other forms of immunotherapy are currently ongoing [164]. Pioneering clinical results have been obtained in melanoma where response rates with a genetically modified GM-CSF expressing herpes simplex based OV (T-VEC) in the presence of CTLA-4 or PD-1 blockade were promising and even improved was better in patients treated with OV combination therapy compared to anti-CTLA4 alone [89, 165, 166].

5. Future perspective

To obtain the best therapy combination of immune stimulatory approaches that create and shape an effective adaptive anti-tumor response and also support this response optimally in the execution phase, it is important to characterize the immune status of a patient (-population) [167]. In case of evidence of an ongoing active immune response, ICB can be considered as a stand-alone treatment. However, in most cases there is no proper adaptive immune response against the tumor. Vaccination offers the possibility to create a response, inducing T cells, but especially for gastrointestinal cancers additional support of the response through checkpoint inhibitors may prove essential. There are many forms of vaccination and therapies with an in situ vaccine effect, as were discussed in this review. Therapies with in situ vaccination effects provide considerable opportunities, as they do not depend on the characterization of tumor antigens or vaccine design/manufacturing and may also disrupt the TME which greatly limits immune resolution of many gastrointestinal cancers. Novel therapies like OVs are of high interest but also more common therapies like radiotherapy and chemotherapy that are already part of routine clinical practice may prove exceedingly useful in

this respect. To find more effective therapies for ICB resistant gastrointestinal cancers, it seems worthwhile to evaluate and investigate immunological effects of non-immune standard-of-care treatments. A direction might be to identify and investigate intrinsic features of gastrointestinal cancers like composition of the TME. A tumor devoid of T cells, or populated with mainly exhausted terminally differentiated non-responding T cells might be in need of a new immune response cycle. Such tumors are ideal candidates for (in situ) vaccination. For tumors with a low mutational load targeted therapies like peptide vaccines could be utilized to induce or enhance CTL responses. However, with a higher mutational load and/or higher immunogenic antigen presentation radiotherapy or chemotherapy may be the preferred way to get that initial release of antigens.

However, immune regulatory mechanisms may still be in place that will prevent antitumor immunity. Identifying these mechanisms in a personalized manner can aid in the selection of immune checkpoint inhibitors (or alternative therapies) to combine with vaccines to give that last push to shift the equilibrium to tumor cell killing and promote remission in patients. For example, a patient harboring only little or dysfunctional/helpless CTLs could be treated with a vaccine and subsequently with PD(L)1 inhibition to rescue effector function at the tumor site. To make effective combinations we now need more detailed insight into the power and mechanism of each (in situ) vaccine form to create and shape the response and also more knowledge on the timing of the created response. Furthermore, we need to know what essential properties induced T cells may lack, so specific signals or blockages can be provided to fix these shortcomings. [168] Only then can we provide the best combination immunotherapy at the right moment. In the near future the repertoire of checkpoint inhibitor options will expand. Many new forms of such therapy are currently in clinical development including blockade of LAG3, TIGIT, IDO, CD47, or TIM3, especially the latter may be of particular interest to combine with treatments causing ICD as it is a receptor for HMGB1 keeping this compound and associated DNA from triggering TLRs [169, 170]. Also several immune stimulators are currently clinically evaluated and may be used to enhance the shaping of adaptive responses following (in situ) vaccination (e.g. activators of OX40, GITR) [169]. Also for combination of (in situ) vaccine forms with these compounds we need more insight into the level at which vaccine-induced responses require support. Especially for *in situ* vaccines this may be challenging, as the nature of the antigens driving the vaccine effect is not known. State of the art analysis techniques may give answers. For example immune responses in these patients could be followed by non-invasive tests like a screening for the TCR repertoire diversity in blood before and after initial therapy and by subsequently tracing back the phenotype of cells carrying prevalent TCR using single cell sequencing. [171] By verifying induction of an immune response after a first 'therapeutic hit', as described in figure 1 (create the response), this can

be followed-up by treatment modalities that support the effector cells and aid in executing the response, potentially leading to a superior treatment strategy against cancers in general and gastrointestinal cancers in particular.

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CHAPTER 3

Three Distinct Stroma Types in Human Pancreatic Cancer Identified by Image Analysis of Fibroblast Subpopulations and Collagen – Letter

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Ogawa and colleagues reported the presence of three distinct stromal subtypes in pancreatic ductal adenocarcinoma (PDAC), associated with alternative disease characteristics [1]. These data are important as stromal heterogeneity defining PDAC subtypes supports developing anti-stromal therapy [1]. Intriguingly, the authors report the presence of a fibroblast activating protein (FAP)-dominant stroma (F-stroma), that compared to other stromal types was low in CD8 T cells and associated with poor survival. T cell exclusion is well-recognized as a disease progression-promoting factor and appears to be the dominant immune phenotype in PDAC (Figure 1A). The authors suggest that F-stroma may contribute to T cell exclusion but do not test this notion directly. Thus prompted, we performed a morphometric analysis of the spatial distribution of both T cells and FAP on 31 treatment-naïve resected PDACs. We found that T cells indeed were excluded from the tumor *per se* (Figure 1A & B), while FAP expression did not show a specific centromarginal gradient. Importantly, no association between local FAP expression and T cell presence was found (Figure 1C). The most straightforward interpretation of our results, in conjunction with those of Ogawa and colleagues, is that the appearance of F-stroma is a manifestation of a T-cell-excluding phenotype but not a major contributor to exclusion itself. In this sense the relation with Hedgehog signaling, as also pointed out of Ogawa *et al.* is interesting, as it has been shown in experimental rodents that Hedgehog can both suppress immune responses through regulating CXCL12 expression, and concomitantly enlarge the size of specific stromal compartments [2], but obviously further work is necessary to substantiate this notion. The observation that T cells are generally excluded in PDAC (Figure 1D) has major implications in the field and will direct research towards stromal factors. Counteracting T cell exclusion with immune checkpoint inhibition (ICI) by targeting PD-(L)1 might not be optimal as mainly inflamed cancer types (melanoma or microsatellite instability-high gastrointestinal cancers) benefit from this type of therapy, but unfortunately not PDAC [3, 4]. The goal would certainly be to convert excluded (cold) tumors into inflamed (hot) tumors and subsequently consider combinations with treatments that further potentiates such T cell responses [5]. The key stromal target in PDAC, however, that will allow restoration of the tumor immunity cycle remains, unfortunately, obscure at best.

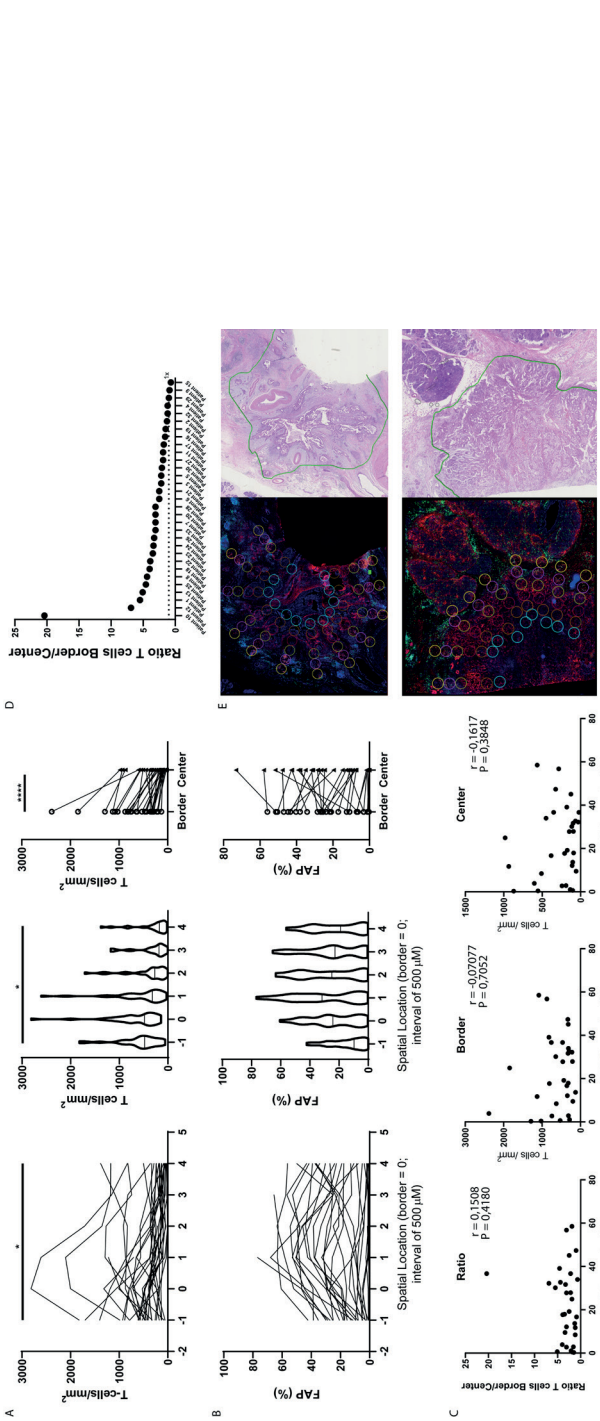
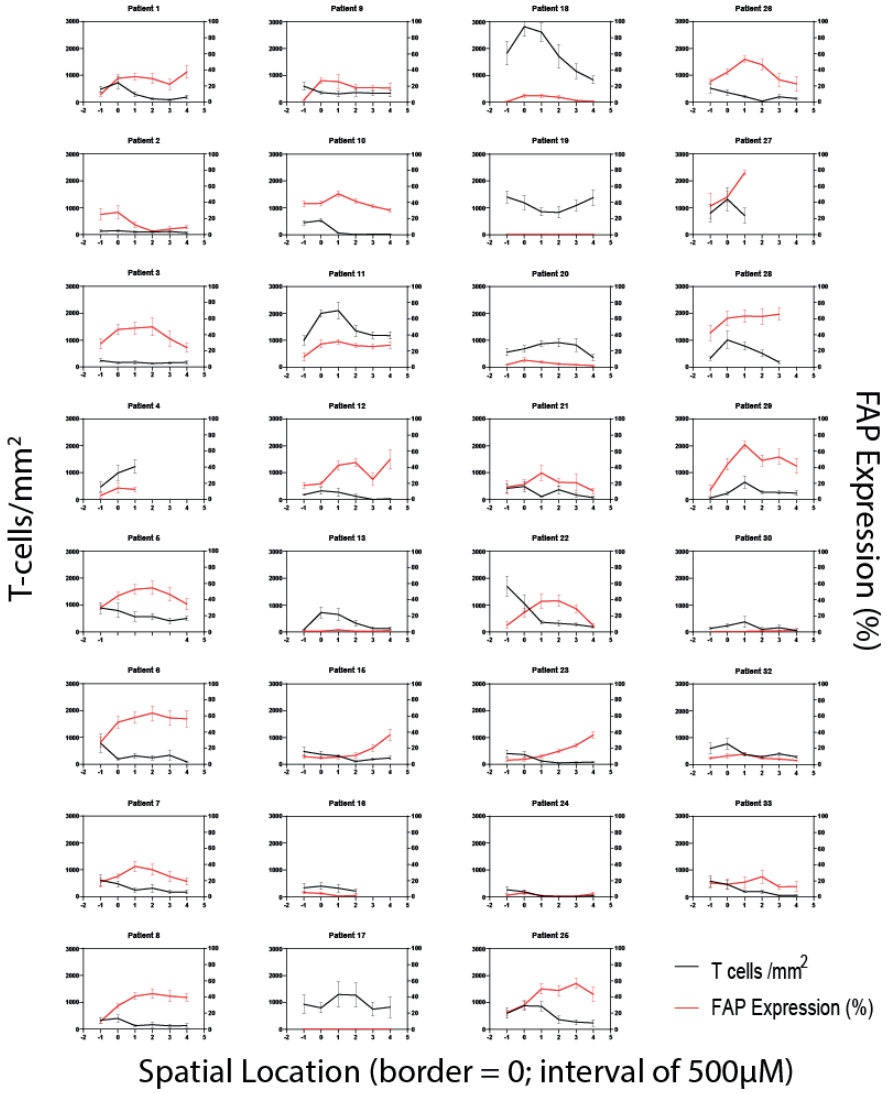


Figure 1: (A,B) The measurements of T cells **(A)** and FAP **(B)**, with in the left panel the individual measurements at the spatial location indicated on the X-axis (a representation from the 2 patients shown in the left panel in E), in the middle panel the data summarized in a violin-plot and in the right panel a direct comparison between the border and the center region from the tumor. Grouped analysis was performed with a one-way ANOVA with a Kruskal-Wallis multiple comparisons test and the direct comparison between border and center with a Wilcoxon test, P<0.05 = *, P<0.005 = **, P<0.0005 = ***, P<0.0001 = ****. **(C)** Correlation between FAP expression and various T cell parameters (as indicated on the Y-axis) were plotted and Spearman correlation test was applied, statistical results are indicated in every individual plot. **(D)** Ratios of T cells calculated from the border and the center measurements from 31 patients were ranked and plotted with a dotted line to indicate the 1x threshold. Examples of immune fluorescence staining and selection of tumor regions by the pathologist with a H&E staining are given in **(E)**.Immunofluorescence (IF) and H&E pictures have a 40x zoom. The IF pictures contain DAPI (blue), FAP (red) and CD8 (green). Areas used for quantification of CD8 content and FAP expression are circled. Circle color indicates spatial location ranging from blue (center) to yellow (2mm from border) The pathologist (M.D.) determined the tumor margins based on H&E staining (green lines) by assessing cell density, morphology and stroma atypical to normal pancreatic tissue.

Supplementary figure



Supplementary figure 1: an overview of the spatial distributions from the individual patients that are summarized in figure 1. Distinctive patterns can be identified in the individual patients with regard to the spatial distribution of T-cells when studying areas deeper into the tumor. T-cells/mm^2 are displayed in black and the relative FAP expression in the same area of analysis are quantified in red.

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CHAPTER 4

Empirical evaluation of the use of computational HLA binding as an early filter to the Mass spectrometry-based epitope discovery workflow

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Simple Summary: Many different human leukocyte antigen (HLA)-types exist across the population that each bind a specific motif of amino acids. HLA-peptide complexes are the driving force behind recognition of cancers and infected cells by cytotoxic T cells. HLA-immunopeptidomics aims to identify peptides derived from (cancer)antigens in the HLA-binding cleft with mass spectrometry (MS). Peptides eluted from HLA are analyzed by MS and translated to a protein derived amino acid sequence by specialized software. These software packages use statistical thresholds to limit false discoveries and return only the most confidently identified peptides. However, we and others believe that many useful peptides can still be found in the excluded pool of peptides. This idea drove the development of specialized algorithms that utilize HLA specific motifs to retrieve additional relevant peptides. It is unknown however how many peptides could potentially be found in this pool. By adjusting the statistical threshold, we empirically demonstrate the vastness of valuable data beyond the traditional thresholds that awaits to be discovered.

Abstract: Immunopeptidomics is used to identify novel epitopes for (therapeutic) vaccination strategies in cancer and infectious disease. Various false discovery rates (FDRs) are applied in the field when converting liquid chromatography-tandem mass spectrometry (LC-MS/MS) spectra to peptides. Subsequently, large efforts have recently been made to rescue peptides of lower confidence. However, it remains unclear what the overall relation is between the FDR threshold and the percentage of obtained HLA-binders. We here directly evaluated the effect of varying FDR thresholds on the resulting immunopeptidomes of HLA eluates from human cancer cell lines and primary hepatocyte isolates using HLA-binding algorithms. Additional peptides obtained using less stringent FDR-thresholds, although generally derived from poorer spectra, still contained a high amount of HLA-binders and confirm recently developed tools that tap into this pool of otherwise ignored peptides. Most of these peptides were identified with improved confidence when cell input was increased, supporting the validity and potential of these identifications. Altogether, our data suggests that increasing the FDR threshold for peptide identification in conjunction with data filtering by HLA-binding prediction, is a valid and highly potent method to more efficient exhaustion of immunopeptidome datasets for epitope discovery and reveals the extent of peptides to be rescued by recently developed algorithms.

Keywords: Cancer, Immunopeptidomics, Antigen presentation

1. Introduction

The action specificity of the adaptive immune system critically depends on the repertoire of peptides presented on human leukocyte antigen (HLA) molecules to T cells [1, 2]. As a consequence, rational development of therapy to exploit the adaptive immune system to combat cancer, infection and autoimmune disease, requires insight into which epitopes of which disease-related antigens are presented on HLA. With this purpose, the recent decade has seen an advent of so-called immunopeptidomics, a novel discipline that aims to comprehensively characterize the full complement of peptides presented by HLA complexes to T cells in specific clinical or experimental settings. In immunopeptidomics, cell lines or patient material of interest are typically detergent-lysed and subjected to HLA immunoprecipitation (IP) [3, 4]. Peptides are then eluted from HLA at low pH and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). This generates MS/MS spectra, which allows the identification of peptides (peptide spectrum matches; PSM) presented by HLA in the original sample. The correct identification of HLA-binding peptides from the obtained MS/MS spectra is currently considered one of the most challenging steps in immunopeptidomics.

Identification of potential HLA-binding peptides from MS/MS spectra can be done in two ways: 1) by matching MS/MS spectra to an experimental and/or in silico generated spectral database (e.g.: Mascot [5], Maxquant [6] or Peaks DB [7] database searching-algorithms) or 2) by performing “de novo” sequencing, i.e. reconstructing the amino acid sequence independent of any database, based on the peptide fragmentation pattern (e.g.: PEAKS [7], pNovo [8] or Novor [9] de novo algorithms). Identified peptides can subsequently be cross-referenced to existing biomedical literature or subjected to specialized search algorithms that allow the determination of the protein origin of these fragments, even though these peptide sequences may not occur in the reference proteomes [10-12].

In the first peptide identification approach where raw MS/MS spectra are matched to in silico generated fragmentation spectra, the false discovery rate (FDR) is used as a filter to control the expected proportion of discoveries that are false. The FDR reflects the rate of type I errors expected when testing the null hypothesis in a large dataset. In a typical bottom-up LC-MS/MS-based proteomics workflow, peptides are generated by digestion of proteins with trypsin or another protease. By convention, an FDR of 1% is set by comparing the PSM scores obtained from a database alignment of the experimentally obtained MS/MS spectra to the PSM scores obtained by alignment to a decoy database [13]. However, application of this ‘standard’ FDR threshold may not necessarily be most efficient for immunopeptidomics for several reasons. First, the databases

and PSM score used to derive the FDR threshold were optimized for, and may favor, tryptic peptide identification. While trypsinization of proteins leads to either an arginine or lysine at the peptide C- terminus, HLA peptides are rather generated by endogenous proteolytic cell processing mechanisms, yielding a wide variety of amino acids at the peptide C-terminus [14, 15]. Additionally, since HLA peptides binding to different HLA-types also differ in their binding properties at the so-called anchoring regions, each immunopeptidome may have its own specific bias towards a certain amino acid composition [16]. Lastly and importantly, in the immunopeptidomics discovery pipeline, LC-MS/MS analysis is followed-up by the selection and further validation of only those peptides that derive from a specific tumor- or pathogen-associated antigen or mutated protein sequence. This selection already greatly reduces the number of hits to investigate and allows for a somewhat less stringent screening approach in the initial stages of the pipeline. In fact, especially for tumor (neo)antigens, immunogenic peptides are rare and validating a few more may sometimes be favored over missing out on potentially curative epitopes. Currently, a range of FDR thresholds has been reported in different immunopeptidomics studies, mostly varying from 1 - 5% [17-24]. Efforts have been made to develop algorithms that utilize for example the binding motifs of HLA peptides to rescue relevant peptides in the discarded dataset [25-27]. These algorithms demonstrated that there are valuable peptides beyond the used statistical thresholds. However, it remains unknown to what extent in general potentially interesting peptides remain below the conventionally used thresholds or how the application of a less stringent FDR affects the resulting peptide set.

A useful feature of HLA peptides is that the ligandome of each different HLA-type has preferred (and non-preferred) amino acids at the anchor residues that enable the peptide to bind to that particular HLA-type. This feature lies at the basis for in silico HLA-binding prediction algorithms (e.g. NetMHCcons [28], MHCFlurry [29] or Pickpocket [30]). An LC-MS/MS-derived immunopeptidome would therefore be expected to display a good match between the HLA-type expressed on the cell of origin and the sequence motifs present in the identified peptides [11]. This same principle also underlies the rescue algorithms that utilize HLA-peptide sequence motifs to retrieve motif-containing peptides from discarded datasets [25, 27].

Here, using a multitude of HLA-eluates of various origins, we systematically evaluated the influence of varying the FDR threshold during peptide identification on the size of the resulting immunopeptidome and on its content of predicted HLA binders for the HLA-types expressed on the cells of origin. Our results underscore that common stringent FDR thresholds, although surely yielding most confident peptide identifications, may leave a significant number of potential HLA-peptides undiscovered. In general, our data show that

filtering on specific HLA sequence motifs justifies looking for valuable peptides in datasets beyond statistical confidence which could yield additional epitopes of therapeutic value.

2. Materials and Methods

Cell culture

All cell lines were cultured in RPMI1640, supplemented with glutamine, penicillin/streptomycin and 10% fetal calf serum (FCS; Sigma-Aldrich). Cell lines JY, HepG2, PanC1, MiaPaCa2 and BxPC3 were cultured in T75 or T175 flasks up to ~80% confluency for adherent cells or up to 1-2*10^6 cells/ml as counted by trypan blue exclusion for suspension cultures. Adherent cells were detached with trypsin-EDTA. After harvest, all cells were washed 2-3 times by centrifugation (5 minutes 450xg) with PBS. Primary hepatocytes were isolated from non-tumor tissue obtained from a liver resection. Briefly, the liver tissue was cut into small pieces, treated with collagenase and DNase and subjected to ficoll density centrifugation to collect a hepatocyte faction that was then washed with PBS, counted and stored on -80°C in a dry pellet. Usage of this patient material for research purposes was approved by the local ethics committee (MEC2014-060) and the patient provided informed consent.

Sample preparation, immunoprecipitations and HLA-typing

Frozen dry pellets were resuspended with cold (4°C) cell suspension buffer (CSB; 50 mM Tris-Cl pH 8 + 150 mM NaCl + 5 mM EDTA) in presence of one protease inhibitor tablet per 50 mL (complete tablets mini easypack, Roche) to 2*10^8 cells/ml and diluted 1 on 1 with CSB + 1% zwittergent 3-12 detergent (N-Dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate; Sigma). For cell lysis the suspension was incubated for 1 hour on ice and vortexed every 15 minutes. Subsequently, cell nuclei and large membrane fragments were removed by centrifugation at 17.000xg for 10 minutes at 4°C to obtain a post nuclear supernatant (PNS). 100 µl nprotein A fast flow sepharose beads (GE Healthcare), empty (as a pre-clear) and coated with anti-HLA-I beads (in-house produced W6/32 antibody and cross-linked; 3,2 mg antibody/ml packed beads) were used to IP HLA class I from PNS of 10^8 cells. An exception was made for primary hepatocytes where the number of cells varied as indicated in Figure 1 and only 25 µL beads were used per 10^8 cells. Both during the pre-clear and subsequent IP, PNS to which beads had been added, was incubated on a roller bench for 1 hr at 4°C. After IP the beads were washed (2 mL per 100 µL packed beads) several times with Tris-NaCl and in the following order with: 20 mM Tris-Cl pH 8,0 + 120 mM NaCl (2x), 20 mM Tris-Cl pH 8,0 + 1 M NaCl (1x), 20 mM Tris-Cl pH 8,0 + 120 mM NaCl (2x), PBS + 20 mM Tris-Cl pH 8,0 (1x) and PBS (1x) prior to peptide elution (described below). For HLA typing

purposes, DNA was isolated with a DNA isolation kit (the QIAamp DNA Mini kit; Qiagen 51304) and sent to the Institute for immunology and Infectious Diseases (Murdoch, Australia) making use of their sequencing based HLA-typing service (NGS illumina-based).

LC-MS/MS data acquisition

HLA-I peptides were eluted from the beads with 500 µL 0,15% Trifluoroacetic acid (TFA) at room temperature (RT). This elution was repeated three times and eluates per sample were combined. The eluted HLA peptides were lyophilized and stored at -20°C until mass spectrometry analysis. In order to separate HLA peptides from contaminating proteins, lyophilized peptides were first dissolved in 400 µL 0,1% TFA and then filtered using a 10 kD MWCO spin column (Amicon 42407). The filtered peptide fraction was desalted using a 1 mL Sep-Pak column containing 10 mg C18 and 10 mg HLB resin that was prepared in-house. Peptides were eluted with 28% acetonitrile containing 0,1% TFA and the solvent was removed by vacuum centrifugation.

Nanoflow liquid chromatography tandem mass spectrometry (nLC-MS/MS) was performed on an EASY-nLC 1200 coupled to an Orbitrap Lumos Tribrid mass spectrometer (ThermoFisher Scientific) operating in positive mode. Peptide mixtures were trapped on a 2 cm x 100 µm Pepmap C18 column (ThermoFisher Scientific 164564) and then separated on an in-house packed 50 cm x 75 µm capillary column with 1,9 µm Reprosil-Pur C18 beads (Dr. Maisch) at a flowrate of 250 nL/min, using a linear gradient of 0–32% acetonitrile (in 0,1% formic acid) during 2 hr. Mass spectra were acquired from 375 to 1200 m/z in the Orbitrap at 120,000 resolution. Upon selection peptides were fragmented by higher-energy collisional dissociation (HCD) with a collision energy of 30% and MS/MS spectra were recorded in the Orbitrap at 30,000 resolution.

Bioinformatics analysis

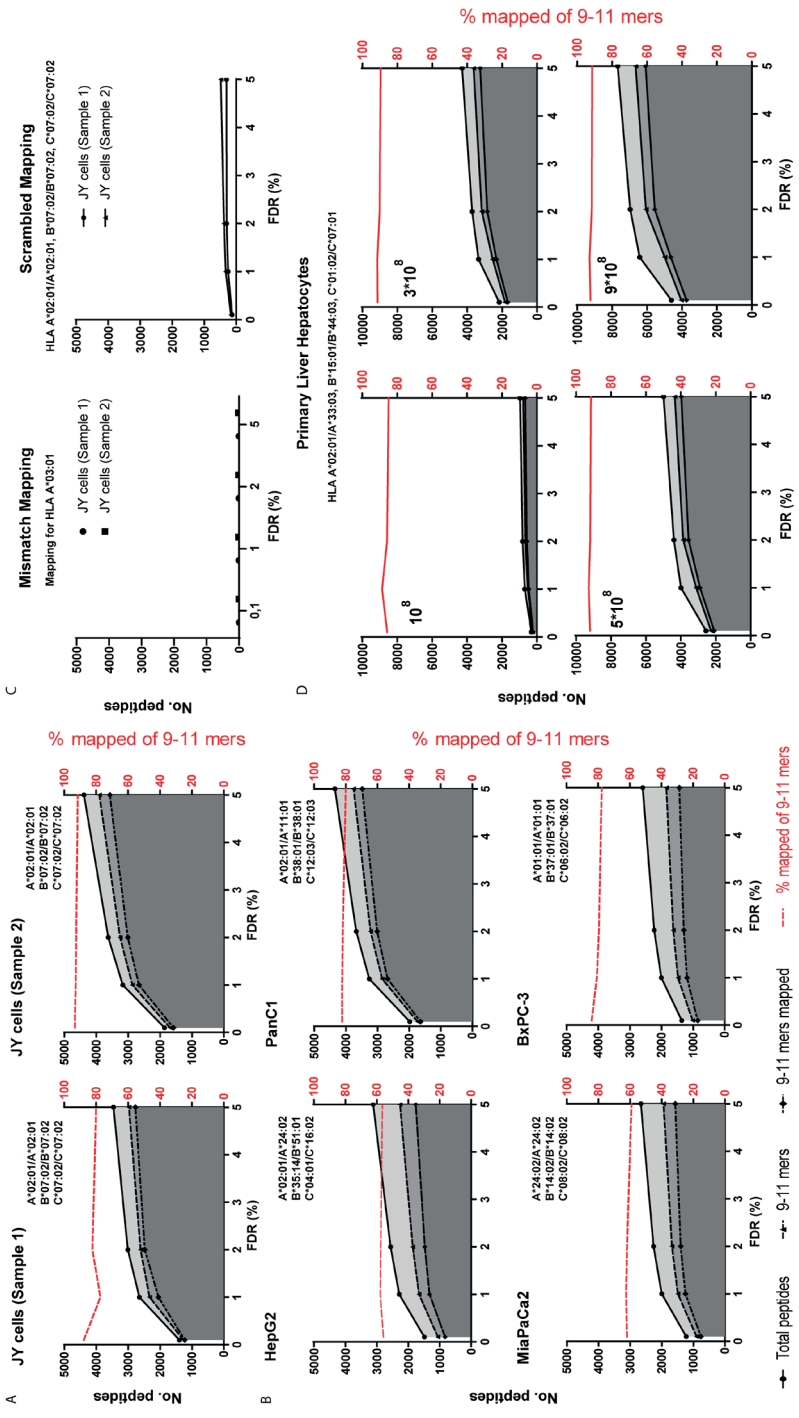
Mass spectrometry data were analyzed with PEAKS Studio v 10.5 (bioinformatics Solutions Inc.). MS/MS spectra were searched against a database containing sequences downloaded from Uniprot for H. sapiens (version August 2019). The digest mode was set to ‘unspecific’ (no enzyme), error tolerances for parent mass and fragment masses were 10,0 ppm and 0,02 Da, respectively. The peptide FDR was varied from 0,1 to 5%. NetMHCcons v1.1 (DTU Bioinformatics [28]) was used to predict HLA-binding properties of peptides to HLA-types of interest. A peptide was called an HLA binder at a predicted IC50 ≤500 nM or rankscore ≤2%. HepG2 proteome data was downloaded from a mass-spec characterization study [31]. Extracted proteins were ranked based on quantification from high

to low expression based on the average of their 3 measurements for HepG2. A complimentary list was generated by extracting all source proteins (obtained via PEAKS, by accession numbers) from our immunopeptidome data. The protein list from the Geiger et al. was taken as the leading list and every time a protein was present in our immunopeptidome list, it received a score of 1. Prism GraphPad was used to generate plots and barcode figures.

3. Results

Experimental data set

To test the effect of various FDR values on the size of the immunopeptidome and number of bona fide HLA binders, we performed an extensive immunopeptidome analysis on HLA eluates of various cell lines and primary cell samples (Figure 1). We included five different cell line models in this study: in casu JY cells, a professional antigen presenting leukemic B cell line often used for immunopeptidomics studies; HepG2 cells, a model liver hepatoma cell line that represents liver cancer which is often considered an attractive target for therapeutic vaccination; and three different pancreatic cancer cell lines (PanC1, MiaPaCa2, and BxPC-3), representing an oncological disease that is usually considered to be very challenging with regard to immunotherapy. All five experimental models were expanded to a final experimental size of 10^8 cells, after which cells were lysed and HLA was immunoprecipitated (see methodology). Typically, 50-70% of all HLA complexes were retrieved in this procedure (data not shown; determined by western blot analysis as the relative HLA signal retrieved by IP compared to input material).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B*07:02/B*07:02
C*07:02/C*07:02
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FDR (%)
% mapped of 9-11 mers
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HepG2
A*02:01/A*24:02
B*35:14/B*51:01
C*04:01/C*16:02
MiaPaCa2
A*24:02/A*24:02
B*14:02/B*14:02
C*08:02/C*08:02
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BxPC-3
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B*37:01/B*37:01
C*06:02/C*06:02
Total peptides
9-11 mers
9-11 mers mapped
% mapped of 9-11 mers
C
Mismatch Mapping
Mapping for HLA A*03:01
JY cells (Sample 1)
JY cells (Sample 2)
Scrambled Mapping
HLA A*02:01/A*02:01, B*07:02/B*07:02, C*07:02/C*07:02
D
Primary Liver Hepatocytes
HLA A*02:01/A*33:03, B*15:01/B*44:03, C*01:02/C*07:01
10^8
5*10^8
3*10^8
9*10^8

Figure 1. FDR score analysis for various cell lines and primary samples. (A-B) Obtained immunopeptidomes with the database search of (A) JY cells in duplicate and (B) various pancreatic and hepatic cancer cell lines. (A, B, D) Shades of grey (top-down) represent the total number of identified peptides, total number of 9-11mers identified and the total number of 9-11mers predicted to bind cell- expressed HLA at the indicated FDR (all left y-axis). The percentage of predicted HLA-binders of identified 9-11mer peptides is indicated in red (% mapped on right y-axis). (C) The left graph shows predicted binding of HLA-derived 9-11mers to the indicated irrelevant HLA types (mismatch binders) of two independent JY datasets. The right graph depicts the predicted binding of a scrambled peptide dataset containing peptides that are matched in number, length and amino acid composition to peptides derived from two independent JY HLA datasets across indicated FDR thresholds. (D) Immunopeptidome of various cell numbers of isolated primary hepatocytes ranked on cellular input from low to high (input number indicated in graph) from left to right and top to bottom. (A-D) The HLA types used for *in silico* prediction of HLA-binding are indicated above each graph.

More permissive FDR settings improve coverage of the immunopeptidome

We next assessed the effect of different FDR thresholds in a mass spectrometry proteomics based database search using the PEAKS database (DB) search algorithm. Figure 1 shows the resulting identified peptide sets for different cell lines and different FDR value thresholds. As HLA-I-bound peptides typically contain nine to eleven amino acids (9-11mers), we subsequently selected only 9-11mers that were identified from fragmentation spectra (the m/z detection window of the mass spectrometer was also limited to this range, see methodology). To gain further insight into the probability that an identified peptide would have been retained in the peptide-binding groove of the HLA molecules expressed on the cell of origin, we predicted HLA binding strengths for each peptide sequence. For this, we used the MHCcons 1.1 software tool [28] utilizing most commonly applied binding criteria (i.e. IC50 ≤500nM or rank score ≤2%; see methods). For all five cell lines that express widely divergent HLA-types (indicated in Figure 1), we observed that increasing the FDR threshold increased putative peptide identifications and that the majority of these additional identified HLA peptides were invariably also predicted to bind the HLA-types expressed on the cell of origin (stable red lines in Figure 1). Thus, the application of a less stringent FDR threshold results in an overall increased yield of potential HLA-peptides. This effect was tested and observed for FDR values of up to 5% (Figure 1A & B).

The identification of the immunopeptidome from JY cells was performed in duplicate. Duplicates yielded very similar results underscoring the reproducibility of our analysis (Figure 1A). To further test the specificity of the *in silico* HLA-binding prediction tool, we also predicted binding to an irrelevant HLA-type for all cell lines (Figure 1C for JY and Supplementary Figure 1 for the other cell lines). For the JY sample, for example, identified peptides were mapped to HLA A*03:01, which is a mismatch for A*02:01 (full HLA-type of JY cells depicted in Figure 1A). The

prediction to irrelevant HLA-types yielded only a low number of predicted binders (<3% of 9-11mers), even at higher FDR thresholds, indicating that the identified peptides were indeed specific binders exclusively for HLA-types expressed on the source material. Then, to also rule out any aspecific prediction results based on the amino acid content of our dataset, we randomly generated a database of 9-11mer peptide sequences with an identical total number of peptides and identical length and amino acid distributions to each of the datasets obtained with the different FDR thresholds (i.e. scrambled). HLA-binding prediction for these scrambled sequences using the netMHCcons 1.1 tool resulted in only very few predicted HLA-binding sequences (Figure 1C).

To subsequently assess the sensitivity of HLA binding prediction to peptide misidentifications we evaluated how the *in silico* prediction of HLA-binders would perform on a peptidome generated by *de novo* sequencing. In PEAKS, the probability that a peptide is correctly identified using the *de novo* sequencing algorithm is indicated by the average local confidence (ALC) score. Peptides identified with higher ALC scores are more likely to be identified correctly and decreasing the permitted ALC score is expected to result in more falsely identified peptides which in turn can be expected to affect predicted HLA-binding. Indeed, we found a direct inverse relationship between the ALC score and the number of correctly predicted HLA binders (Supplementary Figure 2). This pattern contrasted with the stable high percentages of correctly predicted HLA binders that were observed at less stringent FDR values, suggesting that the latter represent bona fide HLA binders (based on the prediction algorithm). Together, these additional controls support the idea that releasing the FDR threshold for HLA peptide discovery combined with HLA binding prediction is a valid approach.

Identification of more targets from existing immunopeptidome datasets could benefit target discovery and subsequent vaccine design, which are of large interest in the field of Oncology. Our results imply that there may be false negative identifications of peptides when stringent FDR values are used in peptide database searching algorithms. To illustrate this we therefore searched for peptides from tumor associated cancer/testis antigens (CTA) in the cell line-derived immunopeptidomes obtained using variable FDR cutoffs. A higher number of CTAs was identified using increasing FDR cutoffs (Supplementary Table 1), although the highest gain was observed when increasing the FDR threshold from 0.1% to 1%, yielding 6 and 17 CTA-derived HLA peptides respectively. One additional CTA-derived peptide was added when further releasing the FDR threshold to 5%.

Immunopeptidomic analysis of variable amounts of primary cells yielded similar results

Our results so far were obtained in transformed cell line models displaying uncontrolled growth. It is widely recognized that antigen presentation on HLA molecules may be markedly different in such model systems as compared to untransformed primary cell types. Hence, it is of interest to validate our findings also on primary cells. Thus, we extended our analysis to primary hepatocytes and also included a titration of cell input to explore the dynamics across peptide abundancy levels. In line with our expectation, it was observed that the amount of cells highly affected the overall number of uniquely identified peptides. Importantly, for all samples irrespective of cellular amounts, increases in peptide yield were observed as a consequence of releasing the FDR threshold and again the relative number of predicted HLA-binders remained stable (red line Figure 1D). Our results thus suggest that the potential to discover additional HLA peptides at higher FDR thresholds is a general property of antigen presenting systems.

Next, we reasoned that less abundant peptides can be expected to have a lower quality spectrum and therefore may be less likely to be identified when applying relatively low FDR thresholds. To test this, we investigated the effect of increasing cellular input on the identification of low quality peptides. We first isolated the predicted HLA-binders from the 10^8 cell-sample that were identified in the FDR range of 1 – 5% (174 peptides). Subsequently, we looked for these specific peptides in the sample with a higher input of $9*10^8$ cells. Strikingly, 150 of the 174 HLA-binders (86,2%) were identified in this high input sample when applying a more stringent FDR value of 1%. Moreover, when we extended our search to an FDR of 1 – 5% we found an additional 13 peptides back. Altogether, the majority of the predicted HLA-binders with poorer spectra in the low input sample could be found back at a stricter FDR in the high input sample, likely due to more robust peptide spectra as a result of higher peptide abundance.

Comparing immunopeptidomic results to full cellular proteomes.

Previously, others have demonstrated that peptides derived from more abundant proteins are also more frequently identified in immunopeptidomes[32]. If more abundant proteins are indeed more frequently presented on HLA, these may yield better PSM scores upon MS/MS analysis of HLA eluates favoring their identification at a more restrictive FDR as exemplified by our primary hepatocyte titration result. However, peptide loading on HLA is a complex process, which also involves competition between peptides depending on their binding affinity and half-life, as well as other factors including peptide generation and degradation kinetics. This means that theoretically the HLA molecule may not necessarily favor only the peptides from the highest expressed proteins. To test the relation

between cellular protein abundance and the number of HLA peptides identified from a protein, we mapped our immunopeptidome of HepG2 cells to a publicly available quantitative proteome dataset from this same cell line[31]. We then ranked the relative cellular abundances of HepG2 proteins from high to low and marked those proteins for which one or more peptides were identified in our HLA peptidome (x-axis in all panels in Figure 2). This yielded a binary barcode graph visualizing the relation between the presentation of a protein in HLA and its reported cellular abundance (Figure 2A). Indeed, most lines representing peptide identifications in our immunopeptidome clustered on the left side of the bar code, indicating that they originated from highly abundant cellular proteins. We generated similar plots across FDR thresholds to visualize the effect of applying different FDR values on the abundance distribution of HLA peptide source proteins, finding additional hits in the lower abundant proteins (on the right side) in case of more lenient FDR values (Figure 2A). The effect of varying the FDR thresholds, however, was hard to discern visually. To obtain a more quantitative assessment of enriched peptides from highly abundant source proteins in our HLA peptidome, a cumulative score was calculated by walking from highest abundant protein to lowest abundant protein and adding a score of 1 every time a HepG2 protein was encountered in our converted (from peptide to protein) immunopeptidome dataset. This cumulative score was then plotted at each position of the abundance ranked protein list as a proportion of the HepG2 cell proteome covered in our immunopeptidome (Figure 2B). If HLA peptides would derive equally frequent from all proteins along the abundance spectrum, an exact diagonal line would be expected (Figure 2B; broken line). Preference for peptides to derive from more abundant proteins would deviate the graph upwards. We observed that the immunopeptidomics data set for all FDR values favored higher abundant proteins (Figure 2B). Only small differences were observed between the application of an FDR of 1% or 5%. At an FDR of 1% half of the presumed HLA peptides in the dataset derived from the top 35,94% of most abundant proteins (figure 2B; left arrow). Using an FDR value of 5%, however, half of the detected peptidome derived from the top 37,93% of most abundant proteins (Figure 2B; right arrow). At an FDR threshold of 5% hundreds of additional source proteins were detected in the immunopeptidome including some more moderately expressed in the cell. The total coverage of the HepG2 proteome in the immunopeptidome dataset was 18,86% at an FDR of 1% vs: 22,40% at an FDR 5% (Figure 2C). Taken together, our results confirm previous findings that most detected HLA peptides are derived from more abundantly expressed cellular proteins irrespective of the FDR threshold used but indicate a slight deviation towards less abundant proteins at more permissive FDR thresholds.

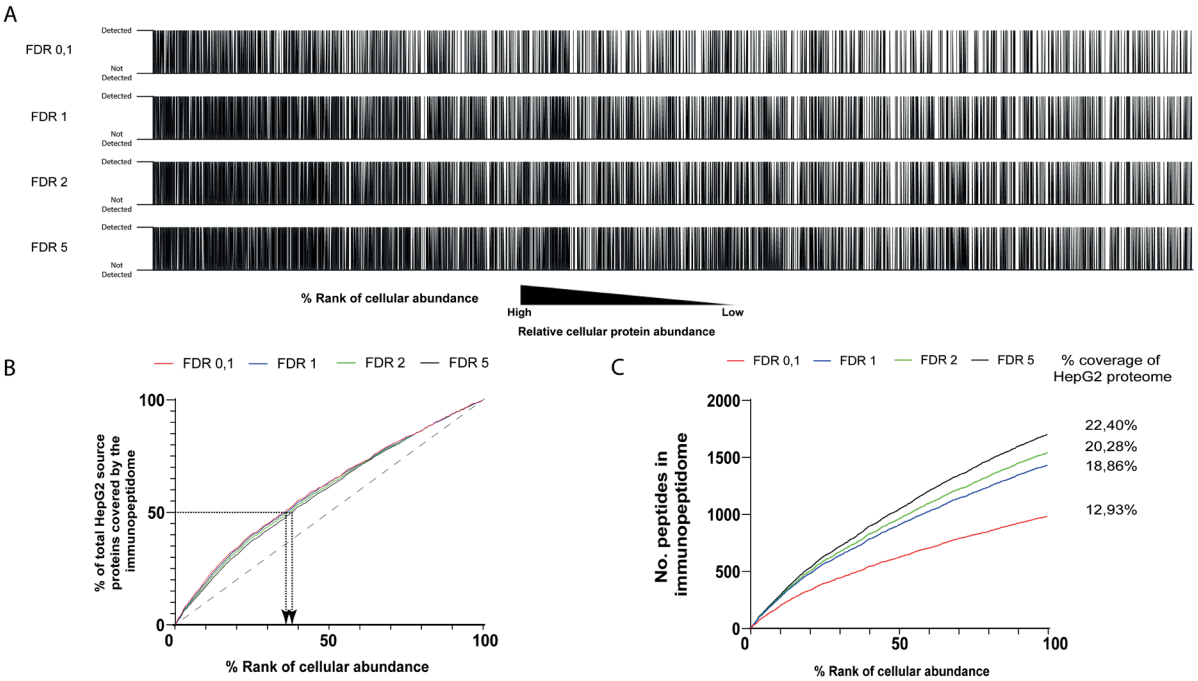

Figure 2 . Comparison of our HepG2 immunopeptidome to the quantitative HepG2 cellular proteome: (A) proteins from the HepG2 proteome were sorted on cellular expression from high (left) to low (right). Then source proteins in this list for which one or more 9-11mer peptides were identified in the HepG2 immunopeptidome using indicated FDR cutoffs were marked by a vertical line to yield barcodes. (B) While "walking" from left to right over these barcodes a cumulative score was calculated by adding a 1 for each protein hit in the immunopeptidome. At each position in the abundance ranked protein list (X-axis) this cumulative score was then plotted as a percentage of the total of proteins covered by the immunopeptidome and also by the full HepG2 proteome (Y-axis). Arrows indicate the % of top ranking source proteins that produced 50% of HLA peptides. (C) As in B but representing the cumulative absolute number of proteins covered by the immunopeptidome at each cellular abundance rank. Indicated on the right is the percentage of proteins derived from the immunopeptidome that could be found back in the HepG2 proteome.

4. Discussion

Understanding the nature of the antigen repertoire presented to the adaptive immune system is essential for better treatment of cancer and autoimmune disease, but is technically challenging. Although important progress in the field of HLA-immunopeptidomics has been made [4], many questions remain. An important realization has been that the standardized approaches to analyze the cellular proteomes, i.e. bottom-up tryptic proteomics, can only be partially transposed to the analysis of the immunopeptidome. The present study adds to this notion by evaluating whether commonly used FDR thresholds in bottom-up proteomics are optimal for immunopeptidome analysis using the theoretical property of predicted HLA-binding for quality assessment. For bottom-up tryptic proteomics an FDR of 1% is the widely accepted standard [13]. However, we show that a less stringent FDR threshold yields a larger collection of PEAKS peptide identifications. This finding is in line with results of reported rescue algorithms exploiting the concept that MS identified peptides should contain a binding motif for one of the specific HLA-types expressed in the cells of origin [25]. Our data thus provides further rationale for such strategies to uncover additional peptides of potential interest for epitope discovery. However, overall quality of spectra of peptide identifications in the FDR range of 1 – 5% was found reduced (based on expert opinion), despite their predicted HLA-binding, but in agreement with their inherent lower PSM scores. This leaves us with the challenge of how to deal with putative peptide identifications, that contain a binding motif of the corresponding HLA-type, but harbor too poor spectra for manual validation. Although expert opinion is not to be neglected, HLA binding prediction may still render peptides with poorer unevaluable spectra of interest for epitope discovery. This is especially valuable when such peptide is derived from a specific protein of interest, for example in a study trying to acquire potential epitopes for a vaccine against a certain tumor associated- or pathogen-derived protein. Evidence for correct peptide identifications is obtained by using synthetic forms of discovered PSMs to validate their identification by MS identification, *in vitro* HLA-binding confirmation and immunogenicity assays (Figure 3). Such a workflow may grant an efficient trade-off between the ends of the sensitivity and specificity spectrum. At the end of maximal specificity, the application of an FDR of 1% without additional HLA binding algorithms, acquiring limited data filtered for only the peptides with highest technical quality, but possibly missing valuable data in a discovery setting. On the other end of the spectrum optimal sensitivity can be reached by not applying any statistical thresholds to control the size of the dataset, capturing all the potentially valuable data, but likely also many false hits. Our data supports a workflow that combines the best of both worlds by releasing the first FDR filter but adding a second filter specific to this field of research extracting only HLA-binding peptides to keep the amount

of data manageable and reliable (Figure 3). This could be done manually by utilizing HLA-binding algorithms and set binding parameters for known HLA-types expressed in the source material. However, great efforts have been made to develop algorithms that directly implement the binding motifs of HLA peptides identified at high confidence to distill peptides harboring this same motif from beyond the set FDR threshold [25]. Peptides derived from specific proteins of interest or from mutated protein sequences can be subsequently extracted from the dataset for further validation. In this scheme, we propose to restrict manual inspection of spectra to peptides of specific interest and possibly only to call certain misidentifications. Unevaluable spectra of peptides that are predicted to bind donor HLA, however, may still be considered to be followed-up.

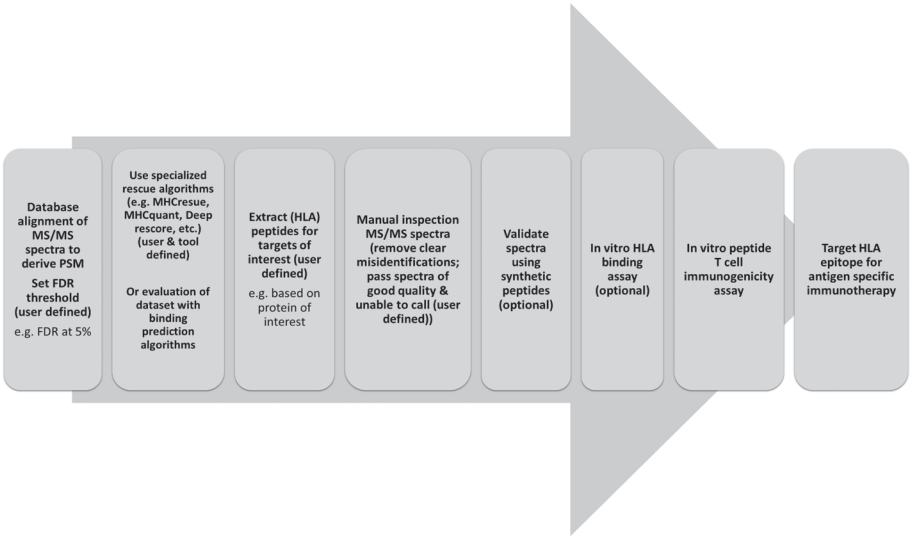

Figure 3. Proposed workflow regarding the use and handling of mass spectrometry data in the application and discovery of HLA-peptides to be used for antigen-specific immunotherapy.

In the present study, an FDR threshold of 5% was the most permissive FDR analyzed and this threshold still delivered peptides equally well predicted to bind to HLA as those obtained using lower FDR threshold cutoffs. Here, it should be noted that HLA binding was a theoretical assessment that for translation to immunological relevance remains to be validated *in vitro*. It is unclear whether even less strict FDR thresholds would still provide more opportunity. From primary hepatocyte HLA, peptides could be found back with a higher amount of cells at a more restrictive FDR, suggesting that more cells will support the discovery of additional peptides. On the other hand, often a limited amount of cells is available. Because we here show that the majority of identified peptides at more lenient FDR settings can still

be found back with a more strict FDR at a higher input, release of FDR in these situations certainly deserves consideration. Our findings demonstrate the ability and power of the peptide spectrum match algorithm to identify these peptides even at lower abundances. While rescue algorithms [25-27] can capture peptides beyond the set statistical threshold of confidence, experiments with primary hepatocytes argue that using more cells contributes to a better profile and a more complete dataset. The maximum amount of cells/HLA- input for complete data capture remains to be determined but is consequently more likely reached when also lower confidence peptides can be added to the equation.

Others have previously found that HLA peptides preferably derive from the most abundant cellular proteins and those with the highest turnover [32]. Interestingly, usage of a more restrictive FDR threshold seems to favor detection of HLA peptides derived from more abundantly expressed source proteins, possibly suggesting also a higher abundance of these peptides in our peptidome and associated better spectra. One could argue that the increased source protein coverage and the slightly more widespread distribution over the abundance spectrum of source proteins of peptides derived using a more permissive FDR, points to a higher level of false identifications. However, primary hepatocyte data shows that a majority of peptides discovered at permissive FDR settings can also be found with an increased input of cells with a stricter FDR threshold. Furthermore, these additional HLA peptides identified at more permissive FDR settings equally bound source cell HLA-types. For these peptides to still be false positive hits, they would need to contain the correct amino acid motif to pass the filter of the HLA mapping which we believe unlikely to occur at a high rate due to chance. This is supported by the results of our control experiments predicting binding of peptides to irrelevant HLA-types and using scrambled matched data sets as input for HLA mapping. In addition, considering the sensitivity of HLA-prediction to sequence uncertainty by *de novo* sequencing, we believe that the amount of false positive peptides after HLA-mapping is likely small.

Our observations strengthen our confidence in the validity of applying a workflow of combining (a more permissive) FDR filter with a HLA-binding filter like proposed (Figure 3). The decision to use stricter or more permissive FDR thresholds may need to be tailored to the situation taking into account tissue availability, the scarcity of target epitopes options, the manageable number of peptides to validate with downstream assays and lastly the goal of the study.

Taken together, our study supports that guided by *in silico* HLA-binding calculations, FDR thresholds used to identify peptides from HLA-eluates can be used in a more permissive manner to yield more potential HLA-binders for usage in antigen specific immunotherapeutic approaches such as vaccines or adoptive T cell transfer.

5. Conclusions

The empirical evaluation of computational HLA-binding in this study revealed that beyond the traditionally used statistical threshold, relevant and valuable data can still be distilled by applying a HLA-binding motif based filter. Altogether we conclude that the use of data beyond conventional statistical thresholds retrieved by specialized algorithms or *in silico* prediction tools is justified to enhance the coverage of the immunopeptidome.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Mismatch mapping analysis of cell lines used in this study, Figure S2: ALC score analysis of De Novo acquisition of cell lines used in this study, Table S1: Overview of identified cancer testis antigen peptides from cell lines.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy and ethical reasons.

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CHAPTER 5

Biochemical evaluation of Synthetic Long Peptide antigen presentation in human monocyte derived dendritic cells

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Manuscript in preparation

Abstract

The recent COVID-19 pandemic demonstrated that the design of good vaccines is still a big challenge. Antigen presentation is a complex process and algorithms are not perfect in their prediction of presented epitopes. We utilized monocyte derived dendritic cells (moDCs) in order to establish a model where we can load antigen presenting cells with our antigen of interest and assess antigen presentation. We demonstrate that our antigen presentation model is capable of being loaded with certain regions from pathogens, in this case synthetic long peptides (SLPs) from the hepatitis B virus, and that upon stimulation of SLP-loaded moDC with Toll like receptor ligands, various HLA-binding peptides can be retrieved by Mass spectrometry. The HLA-binding peptides identified in this manuscript exist out of both previously reported and verified epitopes or HLA-binders, and new potential epitopes. With this study we provide the field with a novel tool that can swiftly be used in times of urgent need (as with the recent pandemic) or in the discovery of novel epitopes like for example in the challenging field of immune oncology. Furthermore, as in our case we can utilize this method to aid in the selection of candidate SLPs for an eventual vaccine against chronic Hepatitis B.

Introduction

Fundamental research or design of vaccines in general have always been laborious [1-4]. The discovery or validation of epitopes that are presented to the human immune system are generally done by synthesizing peptides and performing *in vitro* assays to validate HLA-binding and the ability of a HLA-peptide complex to induce a T cell response. However, depending on the pathogen (or even cancer), the amount of peptides to test could rapidly reach numbers that are not convenient to test in a laboratory setting. All this without even taking into account the target population and their most prevalent HLA-types with further complicates off-the-shelf vaccine design. To tackle this problem, human leukocyte antigen (HLA)-immunopeptidomics can be utilized [5].

HLA immunopeptidomics is the study of peptides presented on HLA to the immune system. Generally cells of interest are isolated, lysed and processed in order to capture the HLA-complexes that harbor the presented peptides. Peptides are then eluted from the HLA-molecules and measured in a mass spectrometer. Data retrieved from this methodology can be used in various ways. A dataset of everything presented on a cell type or tissue can be obtained and can be used in a descriptive way for that respective sample to gain fundamental insights [6, 7]. However, datasets can also be used to mine for peptides and thereby for potential epitopes from a particular pathogen or cancer that could serve as candidates for antigen-targeted forms of immunotherapy (e.g. therapeutic vaccination or adoptive T cell therapy). In this study we utilized synthetic long peptide (SLP) technology to develop our antigen presentation model for vaccine-candidate discovery, establish a method independent of T cells as a read-out of potential epitope presentation and we report various new hepatitis B virus (HBV) peptides/epitopes discovered in our model system.

SLPs are a convenient approach to vaccination [8]. They can contain multiple epitopes as they cover a sequence from a region of a pathogen or tumor associated protein. Depending on the multitude of epitopes that bind to various HLA-types each SLP can be used in a smaller or broader range of patients with differing HLA-type backgrounds. Furthermore, SLPs need to be processed by dendritic cells (DCs) in order to be presented to the immune system. This restricted processing of SLPs by DCs prevent induction of tolerance by antigen presentation without proper co-stimulation as has been described for short peptide vaccines [9]. In addition, SLPs hold the advantage over comparable approaches with whole-protein (lysates) due to a better processing of SLPs and higher potential to eventually activate T-cells [10]. SLPs have also been shown to be a safe therapeutic modality in the setting of human papilloma virus (HPV) and are currently tested in various clinical trials [11-13]. Although SLPs seem to

be promising and have been demonstrated to elicit immune responses for both cytotoxic (CD8+) and helper (CD4+) T-cell responses, it is often unknown what the range of epitopes was that lead to the response. A dominant epitope is frequently used to validate a response with for example HLA-multimers, but a complete unbiased overview of potential epitopes as a consequence of antigen presentation of the SLP is more informative and might contribute to further development or the discovery of novel epitopes. Altogether, a more complete overview of potential epitopes from the SLPs might also aid in the selection of SLPs for an eventual vaccine.

DCs are pivotal for antigen presentation, multiple naturally occurring primary DC populations exist but unfortunately their numbers in human blood are low [14]. This limits the potential of primary DCs to be used as a model *ex vivo*. However, a DC-like cell type can be obtained by isolating monocytes from blood and differentiating these *ex vivo* into a monocyte-derived DC (moDC) [15]. MoDCs, because they originate from monocytes which are abundant in blood, can be generated in greater amounts than primary DC subsets can be obtained. Their greater numbers make them favorable to be used as a model system for antigen presentation in HLA-immunopeptidomics, where an increasing amount of cell input generates increasingly more data and therefore a higher chance of discovering novel epitopes. Although valid discussions remain about the differences in moDCs and primary DC populations, moDCs are able to cross present exogenous antigens to CD8 T-cells as has been demonstrated in the human setting [16-18].

HBV still remains a worldwide problem in its chronic form [19-21]. A prophylactic vaccine is available and effective, however, treatment options for those who have contracted chronic HBV (cHBV) are only marginally effective [22-25]. Various aspects of cHBV make it a challenge to develop a proper treatment for it. Especially mechanisms of disease that exhaust T cell responses complicate the development of therapeutics [26, 27], however, inducing novel T cell responses or enhance existing ones might be key to resolving chronic disease and restore immune surveillance. Therefore it is interesting to map which epitopes are effectively presented to the immune system beside the most dominant well known HBV epitopes in order to develop vaccines that may target more naive T cell populations [28].

Furthermore we made an attempt to study the differences in antigen presentation depending on the mode of activation of the moDC. Commonly used adjuvants target the Toll-like receptors (TLRs) on antigen presentation cells (APCs) and induce their antigen presentation program and pro-inflammatory signature [29-33]. We discriminated between TLRs that are dependent on the

MyD88 pathway (e.g. TLR2) and those independent of the MyD88 pathway (e.g. TLR3) in order to get a complete overview of presented potential epitopes from our SLPs. Furthermore we were interested to evaluate whether certain potential epitopes will only be presented depending on the mode of activation.

Altogether, in this study we aim to characterize novel HBV epitopes in our moDC antigen presentation model system by making use of HLA-immunopeptidomics. We aim to demonstrate novel and existing epitopes for HBV based on a set of developed SLPs that were based on among others data from a recent study into novel epitopes and their conservation [28]. This methodology can be used in context of various diseases and pathogens in order to select potential candidates in vaccine design. Furthermore we demonstrate the application for our model-system where exogenous antigens can be offered to APCs and in an unbiased fashion potential epitopes can be detected and used for further studies. In our hands this system may also help us to select between various SLP candidates for the eventual vaccine in terms of their overall capacity to yield potential epitopes, and therefore potential responses.

Methods

Cell isolation and monocyte derived dendritic cell culture

Monocytes were isolated from healthy donor buffy coats (BC) sourced by Sanquin blood bank (Amsterdam, the Netherlands) and were handled according to known practice and legal guidelines. All blood donors were informed of the use of this material for research and have granted their written consent. BC (typically 50 ml) were diluted 1/5 with PBS + 2 mM EDTA (AccuGENE, Lonza) and loaded on Ficoll-Paque (GE Healthcare) for density gradient centrifugation. Hereafter, peripheral blood mononuclear cells (PBMCs) were seeded in cell-adherent plastic flasks (T75, Corning) in RPMI1640 (Lonza) containing 2% human serum and monocytes were allowed to adhere for 1 hr at 37°C. Subsequently monocytes were differentiated into moDCs with IL-4 (300 U/ml, Peprotech) and GM-CSF (450 U/ml, Peprotech) (in RPMI-1640 supplemented with 8% fetal calf serum; cytokines added/refreshed on day 3 and 6 of 7-day protocol) based on existing protocols [34]. Cells were stimulated with a toll-like receptor (TLR) 2 agonist Pam3Cys (Amplivant ® provided by ISA pharmaceuticals, MyD88 dependent, 2 μM) or a TLR 3 ligand (Poly I:C, InvivoGen, 20 μg/ml, MyD88 independent). Synthetic long peptides (provided by ISA pharmaceuticals, Leiden, Table 1) were added simultaneously with stimulants in a concentration of 2 μM. After a total of 7 days cells were harvested with cold PBS and washed at least 3x with PBS prior to freezing in a dry pellet at -80°C until further use.

Table 1: Overview of used synthetic long peptides

SLP nr.	SLP sequence	Derived from HBV protein/region (amino acids)
1	HYFQTRHYLHTLWKAGILYKRETTR	Polymerase 140-164
2	TSFPWLLGCAANWILRGTSFVYVPS	Polymerase 758-782
3	SVVRRAFPHCLAFSYMDDVVLGAKS	Polymerase 535-559
4	LSAMSTTDLEAYFKDCLFKDWEELG	HBx protein 100-124
5	HLSLRGLPVCAFSSAGPCALRFTSA	HBx protein 52-76
6	HHIRIPRTPARVTGGVFLVDKNPHN	Polymerase 358-382
7	AARLCCQLDPARDVLCLRPVGAESR	HBx protein 2-26
8	RKLHLYSHPIILGFRKIPMGVGLSP	Polymerase 499-523
9	ARQRPGLCQVFADATPTGWGLAIGH	Polymerase 688-712
10	SPSVPSHLPDRVHFASPLHVAWRPP	Polymerase 819-843
11	ASSSSSCLHQSAVRKAAYSHLSTSK	Polymerase 276-297
12	GFAAPFTQCGYPALMPLYACIQAKQA	Polymerase 641-666

Sample preparation, immunoprecipitation, western blot and flow cytometry

Frozen dry pellets were re-suspended with cold (4°C) cell suspension buffer (CSB; 50 mM Tris-Cl pH 8 + 150 mM NaCl + 5 mM EDTA) in the presence of one protease inhibitor tablet per 50 mL (complete tablets mini easypack, Roche). A suspension was made of 2*10^8 cells/mL and diluted 1:1 with CSB + 1% Zwittergent 3–12 detergent (N-Dodecyl-N, N-dimethyl-3-ammonio-1-propanesulfonate; Sigma-Aldrich (St. Louis, MO, USA). For cell lysis the suspension was incubated for 1 h on ice and vortexed every 15 min. Subsequently, cell nuclei and large membrane fragments were removed by centrifugation at 17.000 xg for 10 min at 4°C to obtain a post-nuclear supernatant (PNS). For IP 100 µL nProtein A fast flow sepharosebeads (GE Healthcare) were used either empty (as a pre-clear) or coated with anti-HLA-I monoclonal antibody (mAb); clone W6/32; 3,2 mg antibody/mL packed beads were used to IP HLA class I from PNS. W6/32 mAb was produced in-house from a hybridoma cell line (ATCC). Hybridoma culture medium (without FCS) containing the secreted antibody was pumped over a column of the nProtein A beads. W6/32 was subsequently covalently conjugated to the beads using 20 mM DMP (dimethyl pimelimidate; Sigma-Aldrich, St. Louis, MO, USA) in 0.2 M sodium borate buffer pH 9.0 for 30 minutes on RT. Afterwards non-covalently attached antibodies were removed by washing beads in 0.1 M glycine (pH 2.5). For all samples 100 µL W6/32 coated beads were used. Beads were loaded on an in-house fabricated mini column using pipet tips, holding

the beads in place and allowing fluids to flow through. After IP the beads were washed (2 mL per 100 µL packed beads) several times with Tris-NaCl and in the following order with: 20 mM Tris-Cl pH 8.0 + 120 mM NaCl (2x), 20 mM Tris-Cl pH 8.0 + 1 M NaCl (1x), 20 mM Tris-Cl pH 8.0 + 120 mM NaCl (2x), PBS + 20 mM Tris-Cl pH 8.0 (1x) and PBS (1x) prior to peptide elution (described below).

Samples of captured HLA-complexes with these beads were assessed for efficiency with western blot on a 10% gel by comparing the post-IP supernatant to the PNS and calculating the efficiency based on a fluorescent signal. PNS of 100,000 JY cells was used as positive control, and membranes were stained with primary antibody anti-human HLA-A,B,C clone EMR8-5 (1:20,000, Abcam) in blocking buffer for 1 h at RT and secondary antibody goat anti-mouse IRDye® 800CW (1:10,000) in 1:1 suspension of blocking buffer and 0.05% Tween in 1x PBS for 1 h at RT.

LC-MS/MS Data Acquisition

HLA-I peptides were eluted from the beads with 500 µL 0.15% trifluoroacetic acid (TFA) at room temperature (RT). This elution was repeated three times and eluates per sample were combined. The eluted HLA-peptides were lyophilized and stored at -20 °C until mass spectrometry analysis. In order to separate HLA-peptides from contaminating proteins, lyophilized peptides were first dissolved in 400 µL 0.1% TFA and then filtered using a 10 kD MWCO spin column (Amicon 42407). The filtered peptide fraction was desalted using a 1 mL Sep-Pak column containing 10 mg C18 and 10 mg HLB resin that was prepared in-house. Peptides were eluted with 28% acetonitrile containing 0.1% TFA and the solvent was removed by vacuum centrifugation. Nanoflow liquid chromatography tandem mass spectrometry (nLC-MS/MS) was performed on an EASY-nLC 1200 coupled to an Orbitrap Lumos Tribrid mass spectrometer (ThermoFisher Scientific) operating in positive mode. Peptide mixtures were trapped on a 2 cm x 100 µm Pepmap C18 column (ThermoFisher Scientific 164564, Waltham, MA, USA) nand then separated on an in-house packed 50 cm x 75 µm capillary column with 1.9 µm Reprosil-Pur C18 beads (Dr. Maisch) at a flow rate of 250 nL/min, using a linear gradient of 0–32% acetonitrile (in 0.1% formic acid) over 2 h. Mass spectra were acquired from 375 to 1200 m/z in the Orbitrap at 120,000 resolution. Upon selection, peptides were fragmented by higher-energy collisional dissociation (HCD) with a collision energy of 30% and MS/MS spectra were recorded in the Orbitrap at 30,000 resolution.

Bioinformatics Analysis

Mass spectrometry data were analyzed with PEAKS Studio v 10.5 (Bioinformatics Solutions Inc. Waterloo, ON, Canada). MS/MS spectra were searched against a database containing sequences downloaded from UniProt for H. sapiens (version

August 2019) and the SLP sequences. The digest mode was set to “unspecific” (no enzyme), error tolerances for parent mass and fragment masses were 10.0 ppm and 0.02 Da, respectively. The peptide FDR was set at 5% [35]. NetMHCcons v1.1 (DTU Bioinformatics [36]) was used to predict HLAbinding properties of peptides to HLA-types of interest. A peptide was called an HLA-binder at a predicted IC50 ≤ 500 nM or rank score ≤ 2%.

Results

Molecular characteristics of the moDC model system

moDCs were generated from buffy coats from 7 healthy donors (selected on HLA A2 and A11) and were loaded with 12 SLPs (HLA*A2 epitopes in 4 SLPs and HLA*A11 epitopes in 4 SLPs, together covering 6 SLPs from the 12, Table 1). These moDCs were stimulated with Amplivant® or Poly I:C as described in the methodology. The immunoprecipitation (IP) efficiency of every experiment was calculated by comparing western blot signals before and after the IP. The average IP efficiency was 63.0±15.3% and did not significantly differ between the various conditions (Figure 1A). Furthermore, the yield of moDCs, and therefore the cellular input, was comparable between the various conditions (An average of $13.14\pm6.35*10^6$ moDCs were used in the IP per sample) (Figure 1B). Although cellular input and IP efficiency were comparable between the conditions, the overall peptide yield was slightly higher when maturated with TLR2 ligand Amplivant®(Figure 1C). However, numbers of identified peptides derived from the SLP-sequences did not differ in absolute amounts (Figure 1D). Total HLA-expression as determined by western blot, but not the IP efficiency or cellular input, had the best correlation with overall peptide yield (Figure 1E). Although it seemed like Amplivant® stimulated moDCs had a higher peptide yield, this increase was diminished after numerically correcting yield for HLA-expression, IP efficiency and the cellular input (Figure 1E). We performed the same analyses for only SLP-derived peptides, but, likely due to the overall lower numbers of peptides, we were not able to detect any relevant correlations (Figure 1F). We compiled the data and assessed the length distribution of peptides to confirm their HLA-origin. The majority of peptides consisted out of 9-mer long peptides, the maturation stimulus did not affect the length distribution of detected peptides (Figure 1G).

Biochemical assessment of antigen presentation on dendritic cells

After analysis of Mass spectrometry results from our immunopeptidomics pipeline we identified 20 unique sequences that were potentially derived from the 12 SLPs that had been loaded onto the moDCs (peptides from 6 of the 12 SLPs could be detected). Peptide length ranged from 7 to 13 amino acids longs but the most common length of detected peptides were 9 amino acid long

(n=6). To investigate whether these were likely to have originated from HLA-A2 or A11, we performed an *in silico* HLA-binding prediction for these peptides and alleles and also for other alleles known to be expressed in the used donors. One peptide was shorter than 8 amino acids and therefore the binding affinity could not be determined. In total 7 peptides were predicted to be binders for either HLA-A2 or HLA-A11 (derived from SLP 3, 8 and 11). Of the 14 peptides, 6 peptides (respectively: KLHLYSHPI, TPARVTGGVF, HLSLRGLPV, YMDDVVLGA, SVVRRAFPH and HLYSHPIIL) have been already reported in the immune epitope database (IEDB) confirming the presentation of these epitopes by the dendritic cells. The remaining 8 peptides have not been reported so far in the IEDB. (Table 2)

The adjuvant might affect the presented peptide repertoire

Not all detected peptides were found in the same stimulatory condition. We stimulated moDCs with a synthetic TLR 1/2 ligand (MyD88 pathway dependent, Amplivant) or with a synthetic TLR 3 ligand (MyD88 independent, Poly I:C). 12 out of 14 detected SLP derived peptides were found in the MyD88 dependent condition and 12 out of 14 peptides were found in the MyD88 independent condition. Overall 10 out of 12 peptides were detected in both conditions. The peptides derived from moDCs subjected to either one of the stimuli did not have a preferred peptide length. Previously reported epitopes found in the IEDB did not necessarily correlate with one stimulus.

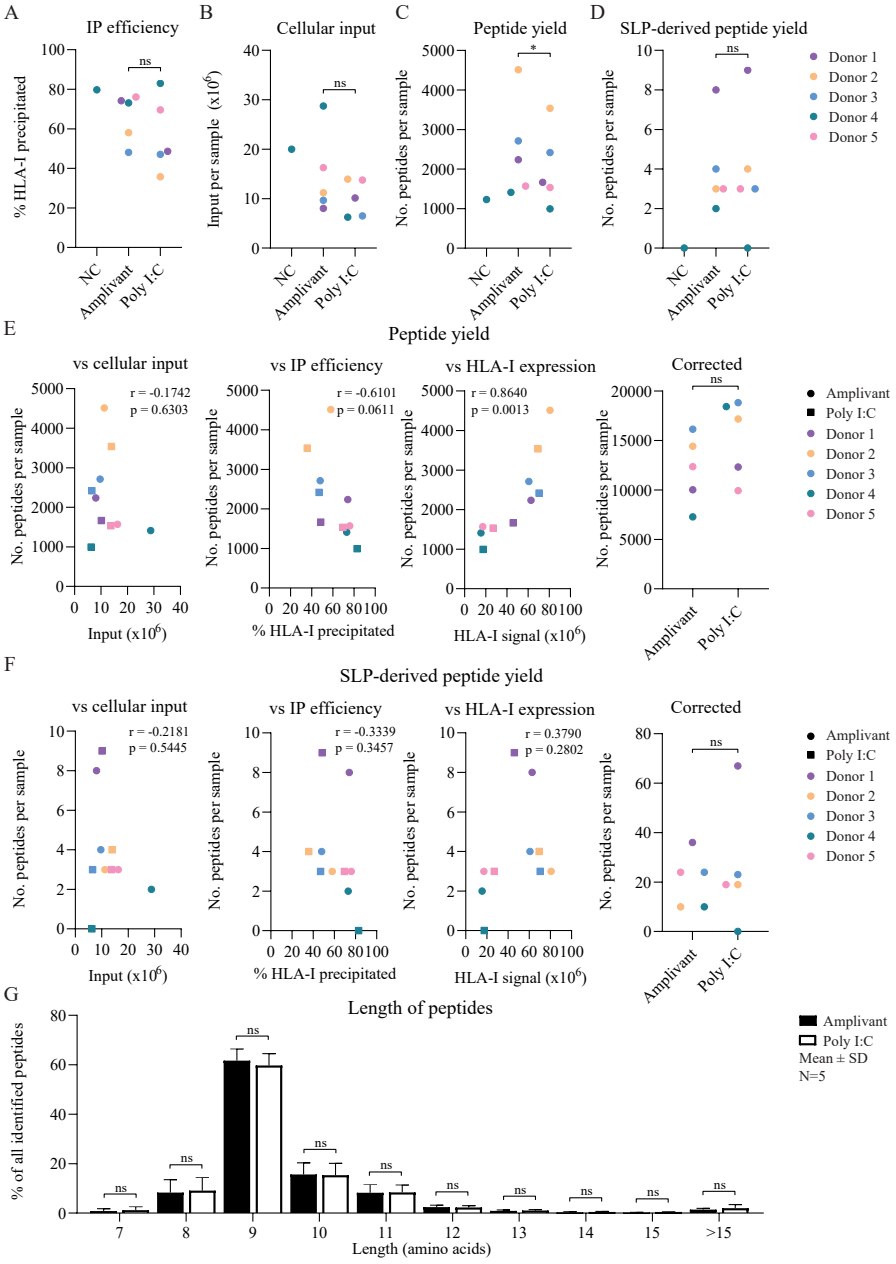

Figure 1. Mass spectrometry analysis of HLA-I IP from moDCs loaded with HBV-based SLPs (n=5). MoDCs were stimulated with Amplivant® or poly I:C, and loaded with 12 HBV-based SLPs. **A.** IP efficiency as calculated by comparing the western blot signal of HLA class I prior to and after IP. **B.** The cellular input of moDCs per IP. **C.** Number of peptides identified by MS per sample. **D.** The amount of peptides identified in IP samples that align to the HBV-based SLP sequences. **E-F.**

The cellular input (First panel), IP efficiency (Second panel), and HLA-I expression (Third panel) were plotted against the peptide yield for the overall identified peptides (E) and SLP-derived peptides (F). **G.** The length distribution of identified peptides for Amplivant® (*Black bars*) and poly I:C (*White bars*) in percentage of total peptides (n=5; Mean ± SD). Statistical test: paired t test (A-D, and G), Pearson correlation (E-F) (ns: $p>0.05$; *: $p\leq0.05$; **: $p\leq0.01$; ***: $p\leq0.001$).

Discussion

We here demonstrated that HLA-immunopeptidomics can be used in combination with the moDC antigen presentation model in order to study presentation of (exogenously loaded) antigen by DCs either to verify existing epitopes, discover novel presented peptides and aid in the selection of SLPs for an eventual vaccine against chronic Hepatitis B. This data could be used in for example the development of new vaccines. However, this model is somewhat limited by a few factors. Due to higher HLA expression on moDCs compared to other non- antigen presentation cells (APCs), and the large amount of peptide to which DCs can be exposed in vitro it is easier to detect peptides from exogenously loaded antigens. HLA immunopeptidomics demands high cell numbers in case of cell samples directly derived from patient's diseased cells or representative cell lines. Furthermore, not every peptide derived from these sources might be a good and relevant target as opposed to our model system where the antigen (in our case SLPs) is selected and validated to cover relevant regions of the pathogen of interest (or in case of cancer-vaccines, the regions of the tumor antigen or harboring the mutation to be targeted). Even when taking into account, the date generated in this study, the upper limit of this APC model system remains unknown. Furthermore, generation of moDCs still largely depends on the efficiency of differentiation and viability of the donor PBMCs and the amount of PBMCs investigators are able to obtain. We made use of buffy coats which are already enriched in the concentration of PBMCs, but to even further increase the yield of monocytes techniques like apheresis could be considered. However, this limitation could be solved by transforming moDCs into a cell line or creating an artificial DC cell line by for example activating various key genes [37], the latter has the advantage that it has high similarities to naturally occurring DC populations [38]. Furthermore, another limitation of moDCs compared to naturally occurring DCs is that their similarity in terms of antigen presentation remains to be determined [39-41]. As mentioned in the introduction of this manuscript, moDCs are at least able to cross-present exogenous antigens to, and activate cytotoxic T-cells [16]. However, it remains a challenge to isolate sufficient amounts of naturally occurring DC subsets in order to perform HLA immunopeptidomics. We attempted to isolate primary DC subsets but the numbers, as expected, were too low (data not shown). Even after performing HLA immunopeptidomics, the resulting data, due to a low sample input, was overcrowded by contaminating longer peptides and no SLP derived peptides could be detected.

Although we did find more peptides overall in the various donors per condition in context of stimulation with a synthetic TLR 1/2 ligand than a TLR 3 ligand, we did not assess the strength of activation of both ligands in comparison to each other in detail. By flow cytometry, however, we did not find any surprising changes in activation markers (data not shown). It might still be that the strength of activation by moDCs is different at the concentrations of the two stimulants used in this study. To resolve this, our study would need to be followed up by titration experiments of the used adjuvants and also an assessment of moDC activation by cytokine secretion and HLA class I upregulation. However, although in total less SLP derived peptides were found in moDC samples stimulated with the TLR 3 ligand (MyD88 independent) compared to those stimulated with Amplivant, for both stimuli there was still a unique peptide found not identified with the other stimulus. This suggests that the antigen presentation pathway might affect which peptides are eventually presented. However, studying the exact mechanism behind this lies beyond the scope of this study.

We showed that we are able to find novel potential epitopes with our antigen presentation model. However, it remains a valid discussion regarding the moDCs whether these truly represent primary DCs. Primary DCs, depending on their subset, have a different role and the probability remains that epitopes found with this model might not be found on primary DC subsets. We made various attempts to isolate primary DC subsets and succeeded, but numbers were too low to perform HLA immunopeptidomics analysis when loaded *ex vivo*. However, during clinical evaluation of such vaccines, extraction of primary DC subsets can be achieved through for example suction blisters applied to the skin [42, 43].

Altogether, we demonstrated our novel model and the potential of HLA immunopeptidomics. We report 8 new potential HBV derived epitopes that need to be verified for their immunogenicity. In conclusion we demonstrate how especially the vaccinology field might benefit from this approach in situations of rapid need for characterization of epitopes, as for example the recent COVID-19 pandemic.

Table 2: Overview of detected SLP-associated peptides/epitopes from moDCs

				Stimuli moDCs (Amplivant/Poly I:C)				
SLP	**Antigen**	**Peptide sequence**	**HLA type[a]**	**Donor 1**	**Donor 2**	**Donor 3**	**Donor 4**	**Donor 5**
2	Pol	SFVYVPS	Too short	-	-	-	Amp/ Poly	Amp/ Poly
3	Pol	SVVRRAFPH	A*11:01	Amp/ Poly	-	-	-	-
3	Pol	YMDDVVLGA	A*02:01	-	-	-	Amp[b]	Amp[c]/ Poly[c]
5	HBx	HLSLRGLPV	Known A*02:01 binder	-	-	Amp	-	-
6	Pol	HIRIPRTPA	B*07:02	Amp/ Poly	-	-	-	-
6	Pol	TPARVTGGVF	B*07:02	Amp/ Poly	Amp/ Poly	-	-	-
6	Pol	TPARVTGGVFL	B*07:02	Amp/ Poly	-	-	-	-
6	Pol	IPRTPARVTG-GVF	B*07:02	Poly	-	-	-	-
8	Pol	FRKIPMGVGL	B*25:05	-	-	-	Amp[b]	Amp[c]/ Poly[c]
8	Pol	HLYSHPIIL	A*02:01	Amp/ Poly	Amp/ Poly	Amp/ Poly	Amp/ Poly	Amp
8	Pol	HLYSHPIILG	Not predicted to bind donor HLA	Amp/ Poly	-	Amp	-	Amp
8	Pol	KLHLYSHPI	A*02:01	Amp	-	-	-	-
11	Pol	AAYSHLSTSK	A*11:01	Amp/ Poly	Amp/ Poly	Amp/ Poly	-	-
11	Pol	KAAYSHLSTSK	A*11:01	Poly	Poly	Poly	-	-

[a]= Binding of peptide was predicted with the MHC con 1.1 prediction tool; [b]= Also identied with oxidation of methionine (M);
[c]= Only identified with oxidation of M; Pol = Polymerase; HBx = Hepatitis B virus X protein; Amp = Amplivant; Poly = Poly I:C

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CHAPTER 6

Characterization of the soluble human leukocyte antigen content in pancreatic ductal adenocarcinoma and chronic pancreatitis

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Manuscript in preparation

Abstract

Human leukocyte antigen (HLA) molecules are pivotal for anti-tumor immunity, as they flag tumor cells for immune recognition by displaying non-self, tumor-derived peptides on the tumor cell surface. Until now, efforts to understand antigen presentation in cancer have focused on membrane-bound HLA complexes, yet the soluble form of HLA complexes (sHLA) has received much less attention. sHLA complexes exist in bodily fluids as a membrane-bound form on vesicles, or as free-floating entities. In cancer patients, sHLA complexes have not only been found to be significantly increased in blood, but they also carry a similar peptide repertoire compared to cell surface HLA. Pancreatic cancer is infamous for its late-stage diagnosis, resulting in a poor prognosis for the majority of patients. This raises the question whether sHLA complexes can be utilized as diagnostic tool in pancreatic ductal adenocarcinoma (PDAC). In this study we evaluate the levels of sHLA in crude and detergent-treated liquid biopsies from patients with both resectable and unresectable PDAC, patients with chronic pancreatitis (CP) serving as a non-malignant, inflammatory control cohort and healthy controls. No significantly elevated levels of sHLA were found in malignant or inflamed samples. However, we found that lysis of plasma samples enhances the detection of sHLA. Furthermore, when comparing the factor increase of the sHLA content, we found discrepancies between the diseased conditions and healthy controls. A trend towards more sHLA after lysis in PDAC and CP patients was observed, suggesting a higher contribution of the vesicular isoform of sHLA. Additional immunopeptidomics analysis on sHLA complexes from a PDAC patient revealed 19 tumor associated antigen (TAA)-derived peptides, demonstrating that a liquid biopsy from a malignant patient can be used in a versatile way. Altogether, this study demonstrates the diagnostic relevance and potential of sHLA complexes and the need for further sHLA research.

Introduction

The pancreas is a pivotal organ in the digestive system and has an important role in the human physiology. Worldwide, approximately 500,000 are diagnosed with pancreatic ductal adenocarcinoma (PDAC) annually of which 2500 people in the Netherlands. The poor prognosis of PDAC is a result of difficult early-stage diagnosis and a lack of effective therapy for advanced-stage disease. Curative-intent surgical resection of the tumor is only possible in early stages of disease. Clues for PC are, however, often lacking at early disease stages because symptoms are absent and/or non-specific. [1-3]

Local tissue-remodeling and genetic damage can be caused by chronic inflammation which might precede the formation of the tumor [4]. The 5-year survival rate for PDAC has stagnated around 10% for many years, partially due to generally poor treatment efficacy. Next to that, one of the biggest challenges in treating PDAC is arguably the lack of reliable methods to facilitate early diagnosis. The majority of patients are diagnosed with late-stage disease, and given that treatment is significantly more efficient when initiated as early as possible, it is clear that novel methods are needed for early-stage diagnosis and improvement of current treatment strategies. [1-3] In some cases of PDAC, but not all, chronic pancreatitis (CP) precedes the development of PDAC. [5] CP is a clinical condition during which the pancreas is subjected to persistent inflammation. Risk factors for PDAC are both environmental and inherited. Environmental factors include age and an unhealthy lifestyle. Inherited factors are a family history of PDAC, hereditary pancreatitis, Peutz-Jeghers syndrome, Lynch syndrome and germline mutations like BRCA1/2, CDKN2A, and p53. However, in spite of the overlap in risk factors and the link between PDAC and CP, a biomarker is needed to either diagnose an at-risk individual for PDAC or exclude an individual from progression from CP into PDAC. In this study CP patients will be included to characterize whether such biomarkers are truly unique to PDAC and do not occur in CP.

Human leukocyte antigen (HLA) molecules play a crucial role in the immune recognition of aberrancies such as malignancies. Their role in antigen presentation in the context of cancer has been researched extensively, leading to novel insights for the development of new generation immunotherapies. [6, 7] Yet, their function and importance have almost exclusively been assessed as membrane-bound complexes on the cell surface, despite the fact that it has been known since the 1970's that they also exist in soluble forms (sHLA). [8]

It has convincingly been shown that during inflammatory conditions (e.g.: auto-immune diseases and chronic viral infections) levels of sHLA are increased in bodily fluids. [9-18] This has proven to be specifically true in the context of cancer,

as reviewed previously and as has been shown also for PDAC in an early-day Japanese study [16, 19]. The exact role for sHLA is not completely understood although various subtypes of sHLA (classical HLA-ABC and non-classical HLA-EFG) have been investigated and have been implemented in immune-regulatory mechanisms. [6] Some of the immunological functions and effects of sHLA have been described in pregnancy [20] and transplantation [21, 22] but these are beyond the scope of this study where sHLA will be evaluated as biomarker.

sHLA secretion is thought to occur through three different mechanisms which involve active secretion by cells or cleavage from cell membranes through protease activity in the environment. Secreted forms of sHLA include those that are either still part of a lipid bilayer (exosomes or extracellular vesicles, depending on preferred nomenclature; sHLA size 44 kD), and those that result from alternative splicing, lacking the transmembrane domain (39 kD). The protease cleaved variant contains only the extracellular domain of HLA and is the smallest (35 kD) form. In general, studies that have assessed sHLA concentrations have not discriminated between these isoforms) [22, 23]. It is not unlikely though that sHLA derived from a cancer may be predominantly of one particular form. Based on this notion, we hypothesized that detection and quantification of sHLA could be affected by sample treatment prior to analysis. Depending on the proportion of vesicular sHLA in the sample, introducing a lysis step could affect the detectable levels of sHLA by sandwich ELISA because disruption of the membrane of vesicular sHLA prior to ELISA could increase sHLA availability (Figure 1B). Furthermore, we hypothesized that a part of the sHLA that enters the periphery might contain peptides derived from the inflamed or malignant tissue which can serve as diagnostic biomarkers [17]. Liquid biopsies are minimally invasive, and if such biomarkers could be detected in the periphery, this could have major implementations in the diagnostics and surveillance of patients with inflammatory disease or malignancies such as pancreatitis or PDAC.

Methodology

Plasma collection, processing and biobanking

Blood was collected from patients with clinically confirmed CP or PDAC (from n=10 included patients, 6 resectable, 3 unresectable and 1 unknown) in sodium heparin tubes and plasma was always isolated within 24 hours after blood withdrawal through an IV. Blood was centrifuged for 15 minutes at 120xG at room temperature (RT). Subsequently, plasma was transferred to a new tube and centrifuged at 3166xG for 15 minutes at 4°C to get rid of platelets and cellular debris. Cleared plasma was stored per mL on -80°C until further use.

Anti HLA class I ELISA

In-house produced W6/32 anti-HLA class I antibody (production described in Bouzid et al., 2021, Cancers [24]) was used to coat non-tissue culture treated ELISA plates (cat number: 260895, thermofisher scientific) at a concentration of 25 ug/ml. The W6/32 antibody recognizes both classical and non-classical sHLA-I complexes in native form [25]. Plates were coated twice on 4°C overnight prior to the day of the ELISA. A few wells were coated with isotype control IgG to verify aspecific capture by the ELISA, an mIgG2a mouse isotype antibody was used.

Plasma samples were thawed and kept on ice. Crude samples did not require processing hereafter. Plasma samples designated for detergent-mediated lysis were diluted 1:1 with PBS and centrifuged at 3166xG for 10 minutes at 4°C to exclude any protein precipitations from the freeze-thawing cycle. Samples were subsequently diluted 1:1 with lysis buffer (cell suspension buffer (CSB); 50mM Tris-Cl pH 8.0 + 150mM NaCl + 5mM EDTA + a protease inhibitor (complete tablets mini easypack, Roche) per 50 ml, supplemented with detergent to make it a lysis buffer) to obtain a final concentration of 0.5% zwittergent (n-Dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, Millipore, catalog nr. 693015). Lysis was performed by incubating for 1 hour on ice and briefly vortexing every 15 minutes. After lysis, samples were again centrifuged at 3166xG for 10 minutes at 4°C to guarantee no protein-aggregates were present in the sample.

After coating, plates were blocked with dilution buffer (0.1 M NaCl, 0.05M Tris, 0.1% BSA, 0.02% Tween 20, pH 8.0) for 30 minutes on a shaker at RT. Crude samples were diluted 1:16 and 1:32, lysed samples were incubated in the dilutions 1:8 and 1:16 (final dilutions 1:32 and 1:64, respectively) and incubated for an hour at 37°C. After capture of sHLA complexes on the plate, plates were washed 3 times with wash buffer (PBS with 0.05% TWEEN-20) and incubated with HRP anti-human β2-microglobulin detection antibody (0.3µg/mL; Biolegend, Cat# 280303) for 1 hour at 37°C. After 3 rounds of washing, substrate was added for 8 minutes in

the dark prior to the addition of stop solution, and absorbance was measured at 414nm within 15 minutes. Results from the dilution which resulted in a value within the calibration line were used for the analysis. Calibration line was done by including serial dilutions of recombinant HLA class I from biolegend (Flex-T™ ELISA Positive Control), starting at 506 ng/mL (Cat# 280301).

Preparation of beads and sHLA immunoprecipitation

Protein A Sepharose beads were coated with W6/32 anti-HLA class I capture antibody (hereafter called affinity beads), procedures were previously described in Bouzid et al., Cancers, 2021 [24]. Lysis was performed on the plasma sample as described above. In-house developed mini-precipitation columns were used to load the beads on and function as a matrix to pass the sample or washing buffers over. sHLA immunoprecipitation was performed as follows: 2 columns per sample were prepared (one column for a pre-clear step with empty protein A sepharose beads to capture aspecific binding proteins; the other column loaded with the affinity beads) and washed 3 times with lysis buffer (0.5% zwittergent). The sample was first passaged over the pre-clear beads once and the eluate was then transferred to the affinity-column where it was passaged 5 times. During passaging of the sample across the beads, a manual speed of one drop/second was defined as a controlled flow rate. Pressure was applied manually by applying a P1000 pipet on top of the tip. After 5 passages the affinity beads were then washed with lysis buffer and a low- and high-salt buffer various times (described in Bouzid et al., 2021 [24]). Beads were transferred to a lo-bind Eppendorf cup and centrifuged on maximum speed for 10 mins to pellet the beads. Supernatant was aspirated to just above the beads and sent to the department of Biochemistry, Proteomics core facility for further processing, elution and mass spectrometry acquisition of sHLA-derived peptides.

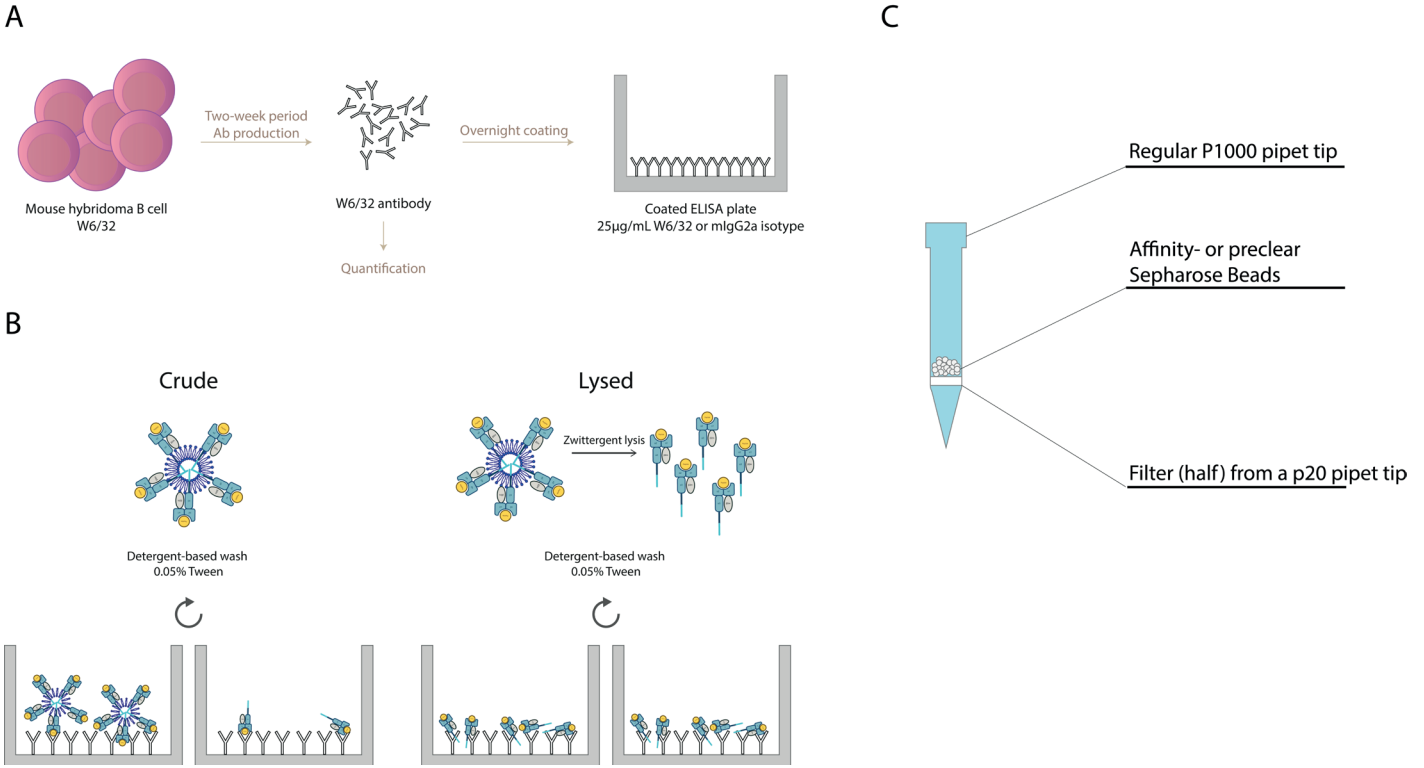

Figure 1: A schematic overview of the various methodological aspects used in this study. In (A) the in-house pipeline used to generate antibody and coat the ELISA plates twice overnight. In (B) a schematic overview of the hypothesis that lysis prior to ELISA could enhance the detection of sHLA. In (C) a schematic overview of an in-house constructed mini-column used for immunoprecipitations.

Mass spectrometry analysis and bioinformatics

Mass spectrometry analysis was performed as described in Bouzid et al., 2021, Cancers [24]. In brief, HLA-class I peptides were eluted from the beads with 500 µL 0,15% Trifluoroacetic acid (TFA) at room temperature (RT). This elution was repeated three times and eluates per sample were combined. The eluted HLA peptides were lyophilized and stored at -20°C until mass spectrometry analysis. In order to separate HLA peptides from contaminating proteins, lyophilized peptides were first dissolved in 400 µL 0,1% TFA and then filtered using a 10 kD MWCO spin column (Amicon 42407). The filtered peptide fraction was desalted using a 1 mL Sep-Pak column containing 10 mg C18 and 10 mg HLB resin that was prepared in-house. Peptides were eluted with 28% acetonitrile containing 0,1% TFA and the solvent was removed by vacuum centrifugation.

Nanoflow liquid chromatography tandem mass spectrometry (nLC-MS/MS) was per-formed on an EASY-nLC 1200 coupled to an Orbitrap Lumos Tribrid mass spectrometer (ThermoFisher Scientific) operating in positive mode on 50% of the sample eluate. Peptide mixtures were trapped on a 2 cm x 100 µm Pepmap C18 column (ThermoFisher Scientific 164564) and then separated on an in-house packed 50 cm x 75 µm capillary column with 1,9 µm Reprosil-Pur C18 beads (Dr. Maisch) at a flowrate of 250 nL/min, using a linear gradient of 0–32% acetoni-trile (in 0,1% formic acid) during 2 hour Mass spectra were acquired from 375 to 1200 m/z in the Orbitrap at 120,000 resolution. Upon selection peptides were fragmented by higher-energy collisional dissociation (HCD) with a collision energy of 30% and MS/MS spectra were recorded in the Orbitrap at 30,000 resolution.

Mass spectrometry data were analyzed with PEAKS Studio v 10.5 (bioinformatics Solutions Inc.). MS/MS spectra were searched against a database containing sequences downloaded from Uniprot for H. sapiens (version August 2019). The digest mode was set to ‘unspecific’ (no enzyme), error tolerances for parent mass and fragment masses were 10,0 ppm and 0,02 Da, respectively. The peptide FDR was set on 5%. Prism GraphPad was used to generate plots and figures.

Tumor antigen protein alignment and STRING analysis

Identified source proteins by PEAKS DB analysis were compared to 3 databases for the identification of tumor antigen associated (TAA)-derived peptides: Cancer Antigenic Peptide Database; TANTIGEN 2.0 [26] and the Cancer Tumor Database. Peptide sequences were also cross-referenced to the publically available dataset from Shraibman et al. [17]. A hit was defined as a 8-12 mer peptide that originated from one of the source proteins that had been annotated in one of these databases as a TAA.

STRING analysis was performed in the webtool [27]. Source proteins were included in the STRING analysis only if they yielded unique peptides (i.e. peptides that were mapped to solely one source protein) between 9-11 amino acids long. Source proteins that yielded peptides between 9-11 mers but also peptides of 13 or 13+ amino acids long were excluded from the analysis because in the absence of HLA-typing we were unable to check for HLA-binding and considered these likely contaminating of 9-11-meric non-HLA binders. Based on these criteria, the input for the STRING analysis software was 590 unique source proteins, of which eventually 565 were recognized by the software and used for STRING included KEGG and GO analysis. Further settings for the STRING, KEGG and GO pathway analysis were: usage of the full STRING network, highest confidence (0.900) and a medium FDR of 5% was applied.

Results

Detergent-based lysis of plasma enhances the proportion of detectable sHLA

To detect sHLA and test our hypothesis that sHLA might be elevated in malignant conditions, we performed an ELISA on crude plasma samples from PDAC patients, CP patients and healthy donors (HD) (patient characteristics have been described in Table 1). However, we could not find significant differences in sHLA levels between the various crude conditions (PDAC vs CP vs HD) (Figure 2A). Next we tested our hypothesis that lysis of plasma samples prior to the ELISA would enable us to detect a significantly better proportion of the present sHLA. lysis may contribute to enhanced availability of sHLA to the capture antibody thereby augment its ability to capture the total vesicular sHLA content (Figure 1B). Lysis indeed yielded a higher amount of detectable sHLA, but still no difference between the cohorts could be detected (Figure 2A). We did however notice a trend that PDAC and CP patients had a higher increase in sHLA levels after lysis compared to HD (Figure 2B). This might indicate a higher content of vesicular sHLA in plasma samples from PDAC and CP patients.

Table 1: Patient characteristics

Condition	PDAC (n = 10)	CP (n = 10)	HD (n = 10)
Mean age (±SD)	62.9 (7.9)	66.8 (7.9)	48.3 (7.4)
Gender (M/F)	(6/4)	(3/7)	(5/5)

When comparing samples crude versus lysed within each group, we could verify a statistically significant and consistent increase of the detection of sHLA in each sample. None of the conditions (PDAC, CP or HD) displayed a lysed sample which

did not increase in sHLA signal compared to the paired crude sample (Figure 2C). These results suggests that indeed the content of sHLA might be higher in plasma samples than previously thought and this underestimation is likely due to loss of vesicular sHLA during the ELISA detergent-based washing step.

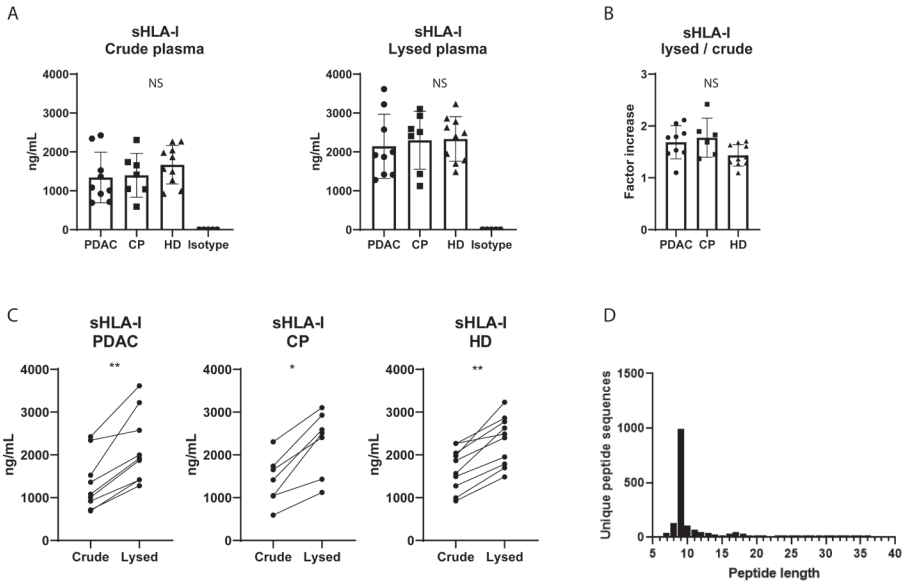

Figure 2 In panel (A) the pancreatic ductal adenocarcinoma (PDAC), chronic pancreatitis (CP) and healthy donor (HD) samples with an isotype control of the ELISA performed on multiple PDAC samples, a Kruskal-Wallis test was applied (excluding the isotype in this test) with a Dunn's multiple comparisons test. In panel (B) the factor increase calculated based on the results in (A) by dividing lysed by the paired crude sample, same statistical test as in (A) was applied. In panel (C) a paired analysis (Wilcoxon) of the samples per disease or control condition. NS = not significant, P<0.05 = *, P<0.005 = **, P<0.0005 = ***, P<0.0001 = ****. In panel (D) the length distribution of the mass spectrometry sample from a PDAC patient.

The sHLA-immunopeptidome of a PDAC patient

Next, we assessed whether sHLA peptide content from PDAC patients could hold additional information to facilitate cancer diagnosis. To this end we performed an HLA-immunopeptidomics analysis on sHLA from the plasma of a PDAC patient. From sHLA isolated from 5 ml PDAC patient plasma sample we identified a total of 1684 peptides (unique sequences) of which 1167 were 9-11 amino acid long peptides. The dominant length in the dataset derived from the sHLA from this patient was 9 amino acids which is in concordance with the expected length of HLA (class I) peptides, indicating we indeed succeeded in identifying HLA-peptides (Figure 2D). We then inspected peptide source proteins (see methodology for exclusion criteria) and we found peptides derived from 563

proteins. By cross-referencing these proteins to databases of tumor associated antigens (TAA) we determined 19 of these source proteins were TAAs (Table 2). Furthermore, several TAA peptides were identified in an earlier cancer-related sHLA peptidomes (Table 2; last column).

Table 2: overview of peptides that aligned to various TAA databases

	Sequence	Gene ID	Gene name	Length	Refs
1	IYHHTGNNTF	Q8N8S7	Protein enabled homolog	10	[17]
2	LPLVSSTY	Q99541	Perilipin-2	8	-
3	TLYEAVREV	P62906	60S ribosomal protein L10a	9	[28]
4	QMWISKQEY	Q6S8J3	POTE ankyrin domain family member E	9	-
5	EYQKVWNLF	Q9H0J9	Protein mono-ADP-ribosyltransferase PARP12	9	[29]
6	QQATPGPAY	P06731	Carcinoembryonic antigen-related cell adhesion molecule 5	9	-
7	TLKGPDSHY	Q9H0C5	BTB/POZ domain-containing protein 1	9	[30]
8	GLASFKSFL	O15539	Regulator of G-protein signaling 5	9	-
9	EVIPYTPAM	P09601	Heme oxygenase 1	9	[28]
10	ALKEATKEV	P09601	Heme oxygenase 1	9	-
11	FLGPWPAAS	P30533	Alpha-2-macroglobulin receptor-associated protein	9	[31, 32]
12	TVLELVTQY	Q9Y6K5	2'-5'-oligoadenylate synthase 3	9	[33]
13	IFIEVFSHF	P0C7T4	Minor histocompatibility protein HMSD variant form	9	-
14	RYFDPANGKF	P13639	Elongation factor 2	10	[34]
15	TYTYEKLLW	P35222	Catenin beta-1	9	-
16	FPMEIRQY	P42224	STAT1	8	[35]
17	AYMPHTFFI	O15294	UDP-N-acetylglucosamine--peptide N-acetylglucosaminyltransferase 110 kDa subunit	9	-
18	KFLEQVHQL	P42224	STAT1	9	-
19	YIKTELISV	P42224	STAT1	9	-

Unfortunately when performing a Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis or Gene Ontology (GO) analysis for overrepresentation of proteins related to specific functions, processes or cellular components in the sHLA peptidome, we were unable to find clear indicators of malignant disease in the reported terms (Table 3). The reported terms included those of metabolic pathways, lysosomes and phagosomes. The top 10 pathways for every analysis are shown in table 3 ranked on most significant hits as defined by the false discovery rate.

Table 3: Overview of KEGG and GO terms from PDAC-patient derived sHLA

#TERM ID	KEGG PATHWAYS
hsa03040	Spliceosome
hsa01100	Metabolic pathways
hsa01212	Fatty acid metabolism
hsa04120	Ubiquitin mediated proteolysis
hsa04142	Lysosome
hsa04145	Phagosome
hsa04510	Focal adhesion
hsa04610	Complement and coagulation cascades
hsa04611	Platelet activation
hsa04659	Th17 cell differentiation
GO	FUNCTIONS
GO:0003824	catalytic activity
GO:0005488	binding
GO:0005515	protein binding
GO:0036094	small molecule binding
GO:0043168	anion binding
GO:0044877	protein-containing complex binding
GO:0000166	nucleotide binding
GO:0003723	RNA binding
GO:0035639	purine ribonucleoside triphosphate binding
GO:0005524	ATP binding
GO	PROCESSES
GO:0044238	primary metabolic process
GO:0071704	organic substance metabolic process
GO:0008152	metabolic process
GO:0006950	response to stress
GO:0002376	immune system process
GO:0006807	nitrogen compound metabolic process
GO:0009987	cellular process
GO:0044237	cellular metabolic process
GO:0071840	cellular component organization or biogenesis
GO:1901564	organonitrogen compound metabolic process

GO	COMPONENTS
GO:0005622	intracellular
GO:0005737	cytoplasm
GO:0005829	cytosol
GO:0043229	intracellular organelle
GO:0043226	organelle
GO:0043227	membrane-bounded organelle
GO:0043231	intracellular membrane-bounded organelle
GO:0005623	cell
GO:0043232	intracellular non-membrane-bounded organelle
GO:0032991	protein-containing complex

Discussion

Early diagnosis in PDAC patients remains a major challenge, highlighting the need for novel non-invasive methods to start treatment as early as possible. Studies have shown that a potential biomarker for malignancies might be sHLA [16]. However, in our study we failed to confirm the elevation of crude sHLA in established PDAC. In the control condition, CP, we did also not observe a higher level of crude sHLA. However, our current results remain insufficient to conclude whether inflammation (which could be associated with malignancy) is not associated with elevated levels in total sHLA levels from lysed samples in pancreas-associated disease. A previously performed power analysis based on the Japanese study [16] (data not shown) indeed confirmed that we would need more specimens to demonstrate any statistical differences. Notably however, we also failed to observe any trend in increases of sHLA compared to healthy controls. Interestingly, when comparing the samples based on lysis prior to quantification, we found that there was a trend towards a larger increase in sHLA level as a result of lysis in PDAC and CP patients compared to healthy controls. Current results might be suggestive of a bigger proportion of vesicular sHLA in PDAC and/or CP patients. To verify this, more patients would be needed, but also further investigation could be done by utilizing methods like the Nanoparticle tracking analysis to quantify the amount of vesicular particles in plasma samples. This might confirm the part vesicular sHLA plays in the relative distribution of molecular forms of sHLA in PDAC or CP patients.

To get an insight into the sHLA-immunopeptidome of a PDAC patient and its potential usefulness for PDAC diagnosis, we performed an HLA-immunopeptidomics analysis. Immunopeptidomics methodology was tailored to be used with sHLA samples from liquid biopsies (plasma) by creating mini-columns from pipet tips and optimizing the process. We found a peptide length distribution compliant with what we would normally expect from an immunopeptidomics analysis performed on cell surface, membrane-bound HLA (predominantly 9-mer long peptides). We realize that the chances of finding clues of tumor growth in the pancreas might be slim because we utilized a liquid biopsy which was obtained via a venous blood sample form the arm of the patient. Others however have shown for various types of cancers that this is feasible. In our analysis we did manage to find 19 peptides that are derived from TAAs. Currently work is ongoing to establish profiles of pancreas enriched proteins and combine this with the TAA source proteins derived to assess the chance of sHLA complexes being derived from the pancreas. Whether the TAA peptides are derived from the tumor itself remains to be investigated by validating their absence in healthy donors. Optimally a biopsy/tissue from the tumor would be collected and subjected to the immunopeptidomics pipeline in tandem with the liquid biopsies. Earlier studies have reported a substantial overlap between relevant tumor derived peptides on the tumor and sHLA [17], but for that tumor tissue from the patient is required which was not the intent of the current study.

To aid in pinpointing pancreas-specific sHLA profiles, additional research on sHLA in liver disease could be performed. It is suggested that the liver secretes high levels of sHLA. Suspicions of PDAC often arise during clinical evaluation of the liver. It would be interesting to isolate samples from patients with liver disease, like hepatocellular carcinoma (HCC) or viral hepatitis, and generate an exclusion profile for PDAC to exclude any contaminating sHLA source proteins from the liver. However, the challenge remains that if a peptide derived from a TAA is found in both HCC and PDAC, it does not directly mean that this is not a valid target. In the same study, an extensive cohort of healthy controls would need to be included to verify such peptides are derived from a malignant source.

In general it seems like the sHLA peptidome would be better suited as a list in which relevant TAA peptides can be searched rather than a complete display of the disease state (considering the early stage of these analyses). The entire peptidome is of course usable for further analysis, however, only a fraction will be relevant to the disease of interest. Our KEGG and GO pathway analyses simply did not identify any processes that were indicative of malignant disease. This could be considered as not surprising since the sHLA peptidome is clouded with peptides that could be derived from any anatomical location in the body which makes

it hard to pinpoint malignant processes in the overall list of proteins. Possibly a liquid biopsy from for example pancreatic juice or via an EUS-FNA procedure, where blood is directly drawn from the portal vein to prevent a first-pass effect, could be candidates to generate a better representation of the pancreas in the sHLA-immunopeptidome. Furthermore, these type of analyses (KEGG and GO) will always remain only indicative of the processes going in due to their inherent nature that the source data is derived from (s)HLA. Meaning that the desired biological (or rather pathological) processes will always be clouded by the remaining 'normal' peptides in the immunopeptidome, unless only the relevant disease-associated peptides from the immunopeptidome can be isolated and used as input for these type of analyses.

HLA covers the presentation of almost all the proteins in the human body, but the efficient detection of all these peptides with mass spectrometry remains a challenge. Thus, the identification of proteins might be underestimated. Furthermore, we do not know whether there is a selection of peptides that end up on sHLA or if this is a completely random process, although some have been described to be more prevalent. [36] Altogether, the sHLA immunopeptidome might not be complete and sufficient enough to give a representation of what is going on in the pancreas, but it might definitely be sufficient to be used as a matrix where disease-associated peptides of interest can be searched upon. It might also be interesting to perform HLA-immunopeptidomics on pancreatitis patients and check for TAA peptides that can be found in PDAC patients as a biomarker and maybe even predictor of disease to reveal patients at risk. However, optimally the collection of longitudinal samples from patients that were once healthy, developed CP and subsequently PDAC would be advantageous to accurately verify the usage of TAA peptides as a biomarker for PDAC patients.

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CHAPTER 7

Discussion and future perspectives

To solve a problem it all starts with a scientific question, and through the design of a methodology and experimentation, one will arrive at an answer and thus the solution to that problem. But the answers obtained during experimentation will often not be satisfactory and only give rise to new questions. In this context it is impossible to give answer to the big question, 'How do we curatively treat Pancreatic cancer', in this thesis, or any individual thesis. It is the accumulation of scientific work from various disciplines over many years, if not decades, that together contribute to the solution of this problem. However, through the development of novel methodologies and the experimental work in tumors from pancreatic cancer patients, I have contributed to answering the smaller questions, which their answers will one day contribute to solving the main question of how to effectively treat those patients and lay the foundation for successive investigators and peers in the field.

Methodological contribution

Many methodologies exist to contribute to the identification of epitopes, small peptides that are not only able to bind to HLA, but also have a T cell clone that is reactive against this peptide-HLA complex. When I started the group of dr. Woltman, later taken over by dr. Buschow, I had the ambition of setting up a pipeline that would enable the discovery of novel epitopes as presented by cells to the immune system. At the time, the goal of developing this pipeline was to facilitate the design of a novel vaccine against chronic hepatitis B (cHBV). The envisioned method would enable the identification and verification of epitopes in the synthetic long peptide vaccine against cHBV. My predecessor (Monique de Beijer) as a Ph.D. student within this context attempted to adapt a protocol from the van Veelen group [1] that already specialized in immunopeptidomics, and was able to locally establish a working protocol (manuscript submitted). However, I embarked on a quest to not only make an improved user-friendly immunopeptidomics protocol but also overall improve the efficiency. This was needed to convert the relatively slow and user-unfriendly method with long elution times to a quick and high-throughput method which can easily be implemented and used even by those with little training in the laboratory. My goal was to not only limit ourselves to the understanding of the work performed on the wet lab, but also extend my vision to the dry lab. I posed the question whether the data analysis part downstream of Mass Spectrometry data acquisition could be directly transposed from traditional proteomics to HLA immunopeptidomics as many in the field were doing. However, I arrived to the conclusion that the biology behind the generation of presented epitopes on a cell, and the proteasomal processing in combination with the presence of certain anchoring residues in peptides depending on the HLA type, that we could not simply apply the same statistical thresholds to immunopeptidomics. The work in the above of this thesis is the result of my endeavors in attempting to realize this quest.

More specifically, in **chapter 4** I made an empirical evaluation of various threshold in *In Silico* pipelines in general HLA-immunopeptidomics. The idea that drove this research is that as immunologists we collaborate with biochemists that specialize in proteomics, but so far we had not been able to precisely translate all the challenges which could affect the results in effective protocols. The demands made on the analysis are manifold, precluding me to take a comprehensive approach and forcing me to tackle the issues involved in a serial fashion. Thus prompted, I started with the selection of the software that was used to translate the raw data obtained from our processed samples to lists of annotated peptides. I questioned what the best software package would be up to that point in time to get both qualitatively and quantitatively satisfactory data from our experiments. In general these software packages translate/align the amino acid mass peaks derived from the mass spectrometry analysis into annotated peptides [2]. Based on the developments in the field I recommended the use of the software package PEAKS [3, 4] which was designed taking in account immunopeptidomic applications. Subsequently I compared the performance of various software packages available to us during that time (Proteome Discoverer among others) on biological samples I generated. I found that PEAKS performed best in both qualitative as quantitative results. However, a huge part of discussion remains to not only my research, but all research in the field of immunopeptidomics. We collectively chose a software package and decided to continue with just this package as it performed best in our hands (meeting the demands of both immunologists and biochemists). It yielded extra results over other software and had a good overlap in the data it produced (data not shown). However, the remaining discrepant results in which data did not overlap with other software, gives rise to the discussion whether this software is truly comprehensive. Personally, I feel PEAKS is not likely to capture all information available in MS spectra, as the software was primarily designed to translate raw LC-MS data to interpretable peptide lists, comprehensiveness not being an overarching factor in the design of the software. Not everyone in the field also uses the same software which means that various research groups might uncover distinct elements in immunopeptidomics biology. The best case scenario would be a compilation of the various algorithms that would be able to calculate the data and subsequently would generate a list that contains interpreted peptides from the raw data with annotation through which algorithms this was obtained. The main problem with such an approach is calculation power, to be able to process vast amount of data, through various algorithms at the same time might be challenging to hardware currently available in medical universities and would require a more specialized informatics department with the appropriate equipment. Researchers have been trying to develop superior hardware based on quantum mechanics for decades [5], if developed in the future quantum computers might be the solution to the previously described lack of computational power.

Furthermore, most of the current software and/or operators have a basis in conventional proteomics, which focusses on the identification of whole-proteins, often through aligning them to tryptic fragments, rather as the correct annotation of small peptide fragments generated through the intracellular protease activity associated with the antigen-presenting machinery. Hence, as has been discussed in **chapter 4**, immunopeptidomics can be more challenging and complicated with respect to 'predicting' or pinpointing the mechanisms in play that are responsible for the cellular processing of a protein into eventually an HLA-binding peptide. I think that our understanding of antigen presentation is not sufficient yet to build models that can comprehensively predict which peptides will potentially become HLA-peptides. Currently still major progression is made in this field of science where new algorithms to predict HLA-binders are constantly being developed [6-14]. Furthermore we are not able to detect all the peptides from a biological sample as the detection of a peptide during the LC-MS procedure depends on its capacity to undergo ionization. The field has recognized this problem and is trying to obtain better ionization efficiency through various technological approaches for improving the comprehensiveness of the data [15-23]. Until this is achieved, we will not be able to capture the full spectrum of the immunopeptidome and although we know that we are missing some of the data, we remain unaware of the extent of the problem.

Nevertheless, even if we recognize that lists of peptides obtained are not comprehensive (as discussed above), the incomplete results available to us can function already as the basis for the next step in the pipeline, as was also discussed in **chapter 4**, and serve as rich waters for fishing-expeditions for relevant data. I suggested the use of an HLA-binding algorithm in adjunct to raw identification of peptides. Others had developed algorithms based on this principle [24-26] but had not systematically evaluated their performance. However, certain aspects of the (immune-)proteasome remain unknown and these unknown aspects can obviously not be implemented in models that predict peptides. The most accurate method to date would be the implementation of anchoring residues for every HLA type (which has been done in some methods [24, 26]) employing HLA-binding algorithms. The best validation for using anchoring residues as a guide to score peptides for their likeliness to bind a certain HLA type, and therefore to exist in datasets obtained, would be to find peptides that slightly deviate from what would be optimal binding and that should end up near the detection threshold. Because such methods always rely on assumptions, results will be biased and are fundamentally incompatible with a truly unbiased discovery pipeline. In concordance with the notion that we are not able to detect all peptides yet, I feel it is beneficial to first invest in novel methods that make it possible to characterize the entire immunopeptidome. Hereafter, if the technology has advanced and would be available to Academia, artificial intelligence (AI) might

present a solution. If all data that can be acquired is available, it could be run through AI where the AI itself would find patterns and associations in the data independent of any bias. The problem here might be that initially AI will not accurately be able to tell which peptides from such dataset would be interesting. But in an oncological setting, various reference lists can be used, for example through exclusion of peptides that occur in the normal human proteome, and specific inclusion of overexpressed/cancer testis antigen (CTA) peptides. CTAs being predominantly interesting as these are a subset of tumor antigens with normal expression restricted to germ cells in the testis but not in developed somatic tissues making them a relative safe target in cancer patients. This group of antigens is unknown to the human immune system and therefore can be a safe but also effective target for immunotherapy [27-29].

In **chapter 5** I demonstrated in an antigen presentation model utilizing monocyte-derived dendritic cells, that in the context of an SLP-vaccine based on HBV a sufficient amount of peptides presented on HLA can be identified. The source for potential epitopes is more restricted due to the source of antigen being selected regions from the HBV genome. This however, allows for an efficient identification of potentially clinically relevant presented peptides. Overall, data processing remains a challenge and mainly because due to the size of data, the field will need to continue looking into automated controlled processes to process data and distill relevant results.

Scientific contribution

The field of immune oncology (IO) underwent a major development over the past few decades. With the introduction of IO drugs, like ipilimumab (blockade of CTLA-4) and nivolumab/pembrolizumab (blockade of PD-1), the treatment of solid tumors has seen a tremendous rise in response rates [30, 31]. With the use of these type of drugs researchers were able to further dissect the dysfunctional immune responses ongoing (or in some cases lacking) in malignancies. As has been described in **chapter 2** and with a reference to the tumor immunity cycle and cancer-immune set point, patients with various malignancies have a different level of involvement of the immune system with respect to combating the oncological process. Notably the IO drugs currently registered have a higher efficacy in patients with some form of pre-existing immunity and an immunogenic tumor [32-37]. However, in PDAC, it was hypothesized in the field that the involvement of the immune system was limited in this type of malignancy explaining the lack of responses to IO drugs like PD-1 inhibitors [38]. Recently, various studies have published data that demonstrated that certain subsets of the immune system, in this case T-cells, are present in tumors of PDAC patients [39, 40]. I have performed a spatial analysis on T-cells in the tumors

of PDAC patients and have confirmed that T-cells are indeed present in the margin of PDAC tumors, but tumor infiltration is limited to just a few patients. In concordance to other studies I arrived at the conclusion that PDAC has in general an immune excluded phenotype.

I have proposed alternative treatment approaches with respect to the different immune phenotypes in **chapter 2**. I agree with the field that cold tumors should be first converted to hot tumors prior to consideration of treating with immune checkpoint blockade. In case of PDAC, I would propose to further investigate the microenvironment [41] and identify targets that mediate the infiltration of T-cells [42]. I have argued whether cancer associated fibroblasts have a direct role in the exclusion of T-cells in **chapter 3**, seeing little apparent role for these cells in this respect. However, indirectly there might still be a role for the fibrotic component in PDAC tumors as these might present to be a physical barrier to T cell infiltration. Drugs that target the tumor microenvironment (TME) or cells that mediate the formation of a harsh TME might present an opportunity for T-cell infiltration. These T-cells might subsequently be stimulated with IO drugs that promote their cytotoxic activity. It remains to be studied whether the diversity of pre-existing T cell clones is sufficient to cover the heterogeneity expected to be present in these tumors. However, such studies should also focus on the potential of such an immune response for the release of new antigens and the capacity to prime new T cell populations. In case studies would fail to confirm a T-cell diversity potentially compatible with clinical responses, therapeutic options like (in situ) vaccination could present the solution as these bypass the release of antigens by the tumors depending on the endogenous immunity cycle.

Although previous discussion has mainly focused on the micro environment and my studies have focused mainly on the spatial distribution of T-cells, it remains a big question what factors intrinsic to T-cells might still affect the observed defect to infiltrate PDAC tumors. The presence of T-cells that lack their full functional potential (as evident from the observation that they are not able to infiltrate the tumor), could derive from various underlying mechanisms, on which there is much discussion but little consensus in the field [43-46]. Nowadays a distinction is made between exhausted and dysfunctional T-cells. In summary an exhausted T-cell is antigen-experienced, but has undergone many cell divisions and approaches the state of senescence. Such T-cells might be reinvigorated via the induction of a DNA damage response resulting in elongation of its telomeres, resulting in rejuvenation [47]. However, often in practice this approach is not successful and the T-cell remains exhausted, even in preclinical settings. In contrast, dysfunctional T-cells might pose a higher potency to be reinvigorated as they still have their polyfunctional potency, but are suppressed by for example signaling through immune checkpoints. These mechanisms might occur naturally as it

is a physiological negative feedback loop to keep immune responses in check and prevent the occurrence of auto-immune responses. However, in the setting of cancer, due to prolonged exposure to suppressive factors and their cognate antigen, T-cells accumulate the expression of immune checkpoints and are sensitive to general dysfunction [45]. Dysfunction can be initiated as early as the priming stage of T-cells if this happens without appropriate costimulation. Such T-cells develop without sufficient polyfunctional capacity and are less prone to infiltrate tumors due to a lack of expression of certain matrix metalloproteases [48-50]. The latter group constitutes the most promising target for therapy to improve T cell-mediated anti-cancer immunity.

Altogether, I have demonstrated in concordance with existing literature that PDAC tumors mostly have an immune excluded phenotype with regard to T-cells. As discussed it still remains unknown what the exact underlying mechanism is and whether this can be attributed to the tumor, intrinsic defects in the immune system or a combination thereof. Further research should thus focus on both aspects of failing anti-cancer immunity. However, from a therapeutical point of view the focus on improved function of the immune system *per se* should constitute the fast track to better clinical results. Patients are relatively easily stratified based on their immune status when compared to tumor immuno-evasive subtype. Many drugs altering immunity have already been identified. Thus immune systems might be a more favorable target for developing new drugs in comparison to the daunting wide range of tumors that have a higher intra- and inter-patient heterogeneity. But also this should be studied further in the future to aid patients in their fight against cancer. Hence our effort in **chapter 6** where we tried to find immune targets in both the levels of sHLA and the presentation of various potentially tumor associated peptides on sHLA in liquid biopsies from PDAC patients. In any case, with this thesis I hope to have performed the groundwork that should make such future studies possible.

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7

CHAPTER 8

Wetenschappelijke samenvatting

In deze thesis worden verschillende onderwerpen uiteengezet en behandeld. Deze onderwerpen vallen allen onder de noemer immunologie en het verrichte onderzoek richt zich op het beter begrijpen van kanker (specifiek alvleesklierkanker) en de behandeling hiervan en het beter begrijpen van de betrokkenheid van het immuun component in alvleesklierkanker. Het fundamentele gedeelte van deze thesis richt zich op de ontwikkeling en integratie van de discipline 'Immunopeptidomics' (deze discipline richt zich op de studie van peptiden die gepresenteerd worden door cellen op hun celmembraan) in het laboratorium ten behoeve van de ontwikkeling en het genereren van data voor vaccines.

De alvleesklier heeft een cruciale rol in het spijsverteringsstelsel. Wereldwijd worden er echter 500.000 mensen gediagnostiseerd met alvleesklierkanker. Hiervan zijn er 2500 diagnoses op jaarbasis in Nederland. Het vooruitzicht voor een patiënt met alvleesklierkanker is zeer slecht en komt mede door de complexiteit van het vaststellen van de diagnose in een vroeg stadium van de ziekte (door een gebrek aan symptomen of presentatie met veelvoorkomende algemene symptomen) en het gebrek aan effectieve geneesmiddelen in vergevorderde stadia. Chirurgische resectie is één van de beste middelen voor genezing, maar is alleen toepasbaar in vroegere stadia van alvleesklierkanker.

In deze tijd van de moderne wetenschap vinden er doorslaggevende ontwikkelingen plaats op het gebied van immuuntherapie ten behoeve van de behandeling van kanker. Sinds enige tijd was al bekend dat het immuunsysteem over het algemeen niet alleen maar een rol heeft in de afweer tegen pathogenen (virussen, bacteriën, schimmels, etc.), maar ook een rol speelt in zowel het voorkomen als bestrijden van tumoren. Tumorcellen kunnen ontstaan door verschillende mechanismen waarbij er over het algemeen een accumulatie van mutaties plaatsvindt in het genetisch materiaal van een gezonde cel. Het immuunsysteem is in staat om deze transformerende cellen te detecteren en te elimineren, echter is er de kans dat er een transformerende cel over het hoofd wordt gezien door het immuunsysteem en in staat is om uit te groeien tot een tumor. Zelfs in het geval van een ontwikkelde tumor speelt het immuunsysteem nog steeds een rol, een dubbele rol om exact te zijn. Het immuunsysteem kan aan de ene kant nog steeds tumorcellen herkennen en deze elimineren en de groei van de tumor op deze manier onder controle houden, aan de andere kant speelt het immuunsysteem ook een belangrijke rol (in het voordeel van de tumor) in het stromale gedeelte van de tumor. Het immuunsysteem kan hier de tumor helpen in het creëren of onderhouden van de meest gunstige omgeving voor tumorcellen door chronische ontsteking te bevorderen of juist door het uitscheiden van ontstekingsremmende signalen. Deze maken het andere componenten van het immuunsysteem moeilijk om de tumorcellen te elimineren en de tumormassa onder controle te houden.

In **hoofdstuk 2** heb ik verschillende aspecten uiteengezet die de behandeling van gastro-intestinale tumoren, maar alvleeskliertumoren in het bijzonder, verder zouden kunnen bemiddelen. In dit hoofdstuk staat beschreven dat een immuunreactie tegen de tumor in 3 fases verdeeld zou kunnen worden, en hoe deze met behulp van therapieën bemiddeld kunnen worden. Deze fases bestaan opeenvolgend uit; 1) initiatie van de immuunreactie, 2) het vormen van de immuunreactie en 3) de functie uitvoeren van de immuunreactie, namelijk het elimineren van tumorcellen. Dit laatste brengt het vrijlaten van tumor-eiwitten teweeg welke door het immuunsysteem kunnen worden opgepikt en kunnen dienen als nieuw doelwit voor een opvolgende response. Middels deze weg kan het immuunsysteem de tumor, en de continue plaatsvindende tumor evolutie, onderdrukken. Echter kan het in elke fase van deze anti-tumor immuniteitscyclus misgaan waardoor de tumor vrij spel krijgt en verder kan uitgroeien. In **hoofdstuk 2** staat ook beschreven welke middelen ingezet kunnen worden per fase waarin het immuunsysteem zich bevindt om op deze manier de anti-tumor immuniteitscyclus te herstellen.

Een beter begrip van het immuun component in alvleesklierkanker

Uit mijn onderzoek op biopten van tumoren, alsmede uit recente literatuur, is gebleken dat het immuunsysteem welke verantwoordelijk kan zijn voor het elimineren van tumorcellen (namelijk de cytotoxische T cellen), grotendeels wordt buitengesloten uit de tumor. Het exacte mechanisme wat hier verantwoordelijk voor is, is tot op heden onbekend. Er bestaat het vermoeden dat een bepaalde kanker-geassocieerde fibroblast een grote rol speelt in de geobserveerde exclusie van cytotoxische T cellen. Echter, na evaluatie van dit idee door monsters van de biopten te kleuren voor eiwitten die deze specifieke kanker-geassocieerde fibroblasten identificeren, kwamen wij tot de conclusie dat er geen direct verband bestaat tussen de locatie van deze kanker-geassocieerde fibroblasten en de exclusie van cytotoxische T cellen. Dit sluit echter niet uit dat deze fibroblasten geen indirecte of ondersteunende rol zouden kunnen hebben welke kan bijdragen aan de geobserveerde exclusie van cytotoxische T cellen. Dit gedeelte van het onderzoek staat beschreven in **hoofdstuk 3**. Deze observaties versterken wel het rationeel om te focussen op medicatie die ofwel potente cytotoxische T cel reacties kunnen induceren, ofwel zich richten tegen factoren in de stroma van de tumor en deze toegankelijk maken voor infiltratie van cytotoxische T cellen.

Tumorcellen kunnen door een accumulatie van mutaties eiwitten hebben die afwijken van gezonde cellen. Over het algemeen presenteren cellen stukjes van eiwitten op het celmembraan op humaan leukocytenantigeen (HLA),

tumorcellen doen dit in de meeste gevallen ook. De presentatie van afwijkende eiwitten is één van de aangrijppunten voor het immuunsysteem om een tumorcel te herkennen en vervolgens te elimineren. In **hoofdstuk 6** buig ik mij echter over het component van deze presentatie eiwitten ten behoeve van de diagnostiek van alvleesklierkanker, welke in cel-vrije vorm in het bloed te vinden zijn, ook wel 'soluble HLA' genoemd. Hier kwantificeren wij cel-vrij HLA in de plasma monsters van alvleesklierkanker patiënten, patiënten met een ontsteking in de alvleesklier (pancreatitis) en gezonde controles. Wij vinden niet direct aanwijzingen dat cel-vrij HLA in het plasma van alvleesklierkanker patiënten verhoogd zou zijn. Echter, na het oplossen van de van cellen afkomstige membranen in de plasma samples observeerden wij een toename in het detecteerbare cel-vrij HLA. Een verklaring hiervoor is dat Cel-vrij HLA kan voorkomen in verschillende vormen, waarvan één van de vormen een membraanblaasje kan zijn waar zich meerdere cel-vrije HLA moleculen op kunnen bevinden. Het oplossen van membranen maakt al deze moleculen beschikbaar voor detectie en verhoogd zo de hoeveelheid gemeten cel-vrij HLA. Alhoewel er geen statistisch significante verschillen te vinden waren tussen de patiëntengroepen en controles, kon er echter wel een trend onderscheiden worden waarbij het leek dat er mogelijk meer totale cel-vrije HLA terug te vinden is in alvleesklierkanker- en pancreatitis-patiënten. Echter is verder onderzoek nodig om deze observaties op te volgen en applicaties voor de gemaakte bevindingen te ontwikkelen.

Het ontwikkelen van immunopeptidomics ten behoeve van vaccine ontwikkeling

Zoals eerder beschreven kunnen cellen stukjes van alle aanwezige eiwitten in een cel presenteren op HLA. De discipline ‘immunopeptidomics’ richt zich op het bestuderen van de peptiden die afkomstig zijn van eiwitten die gepresenteerd worden op HLA. Over het algemeen wordt er een protocol gevolgd waarbij er een cel/monster van interesse is welke direct afkomstig zou kunnen zijn van een biopt van een patiënt, of in grote getalen op het laboratorium gekweekt kan worden. Dit biologisch materiaal wordt vervolgens gedigesteerd en gelyseerd om alle HLA eiwitten die de peptiden van interesse bevatten in een cel-vrije vorm te krijgen. Deze kunnen vervolgens geïsoleerd worden door gebruik te maken van een antilichaam welke aan een minuscule latex-kraal vastzit die het HLA molecuul herkent en kan wegvangen. Deze latex-kralen waar het HLA molecuul vervolgens aan vast zit wordt uitvoerig gewassen met verschillende buffers om een zuiver monster te genereren waarna de peptiden uit het HLA molecuul geëlueerd worden met een zuur alvorens het peptide monster wordt gemeten in massa spectrometer. De massa spectrometer schiet de peptiden vervolgens kapot en genereert een digitale piekenpatroon aan de hand van de massa en lading van de verschillende fragmenten.

In **hoofdstuk 4** bestudeer ik het proces van het omzetten van een digitaal piekenpatroon naar een geannoteerd peptide. Over het algemeen bestaan er verschillende softwarepakketten die commercieel aangeboden worden om piekenpatronen uit de massa spectrometer te kunnen vertalen naar peptidesequenties. Wij maken gebruik van het softwarepakket 'PEAKS' in deze studie, welke in onze handen het beste presteerde op het vlak van identificeren van peptiden die afkomstig zijn van HLA. Op basis van de onderliggende biologie van antigeenpresentatie in cellen stelden wij de hypothese dat de traditionele parameters (de 'False discovery rate', FDR, een statistische maat om binnen het totale resultaat de best scorende subjecten te identificeren) die gebruikt worden in de vertaalslag van piekenpatronen naar peptidesequenties te stringent zouden zijn. Door deze te versoepelen observeerden wij een toename in de totale hoeveelheid geïdentificeerde peptiden, een toename in de diversiteit van de peptiden en een trend tot identificatie van peptiden die afkomstig zijn van eiwitten met een lagere cellulaire expressie. Ondanks de toename in peptiden leverden we niet in op de kwaliteit van de geïdentificeerde peptiden, dit werd bevestigd door een analyse waarbij we gebruik maakten van een algoritme welke in kaart brengt tot welke HLA-type de peptiden te herleiden waren, en hier werd relatief geen verlies van kwaliteit geobserveerd. Op basis van onze observaties stellen wij een model voor, waarbij afhankelijk van het onderzoek soepelere parameters gebruikt kunnen worden in combinatie met algoritmen die de peptiden in kaart brengen en herleiden naar het HLA-type van de donor en op deze manier de efficiëntie en opbrengst van deze immunopeptidomics techniek verhoogt.

Met deze data vervolgde ik mijn onderzoek in **hoofdstuk 5** waar ik biochemisch bewijs van antigeen presentatie van een ontwikkeld 'lang-peptide' vaccine (synthetisch lang peptide (SLP)) probeer te ontdekken. De onderzoeksgroep van dr. Buschow heeft in samenwerking met ISA pharmaceuticals verschillende SLP's ontwikkeld afgeleid van het Hepatitis B virus. Door gebruik te maken van artificieel induceerbare antigeen presenterende cellen, namelijk van monocyt-afgeleide-dendritische cellen (moDC's), is er een modelsysteem tot stand gebracht waarbij moDC's beladen kunnen worden met SLP's en de antigeenpresentatie vervolgens bestudeerd kan worden met behulp van immunopeptidomics en de parameters vastgesteld in **hoofdstuk 4**. Ik rapporteer een verzameling aan HLA-bindende peptiden afkomstig van het vaccine welke gedeeltelijk eerder gerapporteerd zijn en het andere gedeelte nieuw gevonden zijn en verder onderzocht dienen te worden of deze ook te herkennen zijn door cytotoxische T cellen. Verder rapporteer ik dat afhankelijk van de manier waarop moDC's geactiveerd worden met verschillende adjuvantia een afwijkend patroon kunnen opleveren in peptiden die gepresenteerd worden. Echter is verder onderzoek nodig om deze observaties op te volgen en te integreren in de selectie van mogelijke kandidaten voor het samenstellen van een vaccin ter klinische evaluatie.

CHAPTER 9

Appendices
PhD Portfolio
List of publications
About the author
Acknowledgments

Portfolio

Name PhD student:	Rachid Bouzid
Promotors:	Prof. dr. M.P. Peppelenbosch, Prof. dr. M.J. Bruno
Co-promotor:	Dr. S.I. Buschow
Affiliation:	Department of Gastroenterology and Hepatology, Erasmus University Medical Center Rotterdam
PhD period:	2017-2022

Courses

Weekly MDL seminar program in experimental gastroenterology and hepatology (attending & presenting) (2017-2021)
Tumor Immunology Platform (TIP) meeting (attending & presenting) (2017-2021)
Erasmus MC - Gene expression data analysis using R: How to make sense out of your RNA-Seq/microarray data (2019)
Erasmus MC - Basic Course on 'R' (2019)
Erasmus MC - Scientific Integrity (2021)

Other conferences

Dutch society for Immunology (NVVI) (2017,2019-2020)
European Congress of Immunology (ECI) (2018)
Dutch Tumor Immunology meeting (DTIM) (2018-2021)

Supervision theses

HLO student, Robbie Luijten (2018)
Master student, Marija Jokic (2020)
Master student, Kim Klarenaar (2021)

List of publications

1. **Bouzid R**, Peppelenbosch M, Buschow SI. Opportunities for Conventional and in Situ Cancer Vaccine Strategies and Combination with Immunotherapy for Gastrointestinal Cancers, A Review. Cancers (Basel). 2020 Apr 30;12(5):1121. doi: 10.3390/cancers12051121. PMID: 32365838; PMCID: PMC7281593.
2. **Bouzid R**, de Beijer MTA, Luijten RJ, Bezstarosti K, Kessler AL, Bruno MJ, Peppelenbosch MP, Demmers JAA, Buschow SI. Empirical Evaluation of the Use of Computational HLA Binding as an Early Filter to the Mass Spectrometry-Based Epitope Discovery Workflow. Cancers (Basel). 2021 May 12;13(10):2307. doi: 10.3390/cancers13102307. PMID: 34065814; PMCID: PMC8150281.
3. **Bouzid R**, Kessler AL, Levink IJM, van den Bosch TPP, Buschow SI, Fuhler GM, Peppelenbosch MP, Cros J, Lévy P, Bruno MJ, Doukas M. Three Distinct Stroma Types in Human Pancreatic Cancer Identified by Image Analysis of Fibroblast Subpopulations and Collagen-Letter. Clin Cancer Res. 2022 Jan 15;28(2):425-426. doi: 10.1158/1078-0432. CCR-21-2257. PMID: 35045959.
4. Gonçalves FDC, Luk F, Korevaar SS, **Bouzid R**, Paz AH, López-Iglesias C, Baan CC, Merino A, Hoogduijn MJ. Membrane particles generated from mesenchymal stromal cells modulate immune responses by selective targeting of pro-inflammatory monocytes. Sci Rep. 2017 Sep 21;7(1):12100. doi: 10.1038/s41598-017-12121-z. PMID: 28935974; PMCID: PMC5608915.

About the author

Rachid Bouzid was born on September 4th 1994 in Rotterdam, the Netherlands. He attended the Hogeschool Rotterdam Biology and medical laboratory research bachelor. During this bachelor he performed two internships at the Erasmus Medical Center, in his third year studying the composition of the immune system of young children by the cytomegalovirus and/or the eppstein barr virus. In his graduation year he studied a relatively novel approach of gene-engineering working on the development of T cell receptors that could be engineered into T cells that ultimately would be used for adoptive cell transfer into cancer patients. After having received his Bachelor's degree, Rachid Bouzid pursued his scientific training by applying to the prestigious research master Infection & Immunity. During the research master he performed two internships. Although his interest was mainly reserved for the field of immune oncology, Rachid wished to broaden his immunological scope and performed an internship in the field of transplantation immunology. Rachid studied the generation of extracellular particles from mesenchymal stromal cells. The ultimate goal of this study was to generate and characterize particles that would be able to dampen and modulate immune responses. During this time, Rachid also took on a job on the side at Janssen Vaccines & Prevention (formerly Crucell) aiding in animal experimentation in infectious disease models. In his final internship Rachid studied the role of Bruton's tyrosine kinase (BTK) in the tumor micro environment in Mesothelioma models. After being approached by Sonja Buschow to explore a PhD-position in the group of Andrea Woltman (which later transitioned to the Buschow group), Rachid started his work as a PhD-candidate. Rachid worked on the development of the HLA-immunopeptidomics pipeline and initiated the work on the research on pancreatic cancer with the workgroup. After his PhD Rachid started to work as a translational scientist at Merus, a prestigious company developing novel therapies and drugs for cancer patients.

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