Cancer vaccine strategies to improve immunotherapy: many roads lead to Rome

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1 INTRODUCTION

Immunotherapy of cancer

Immunotherapy of cancer is a spectacularly progressing field which comprises a growing number of treatments aimed at modulating the immune system to eradicate malignancies. It is a direct result of our understanding, which developed over the past 50 years, that the immune system is an active player in cancer pathophysiology and that it can determine both regression and progression of the disease.

The primary reason for which the immune system has evolved is to recognize abnormal situations jeopardizing our health and survival, such as the invasion of unwanted pathogens, the wounding of a tissue and, in the case of cancer, the presence of abnormally growing mutated cells. The immune system can spontaneously react against cancerous cells and exert some control over the progression of the disease [1]. In fact, it has been postulated and to a certain extent demonstrated that the immune system mediates the clearance of most of premalignant cells which start to accumulate mutations. However, premalignant and malignant cells possess the ability to evolve and adapt rapidly, eventually subverting immune surveillance [2, 3]. The aim of immunotherapy is to reawake these spontaneous mechanisms as well as to mobilize other immune pathways to generate a powerful immune response, strong enough to overturn tumor suppression.

Even though several cellular targets of immunotherapeutic approaches have been described, therapeutic efficacy of many immunotherapies is dependent on the activation of one specific type of immunity, namely T cell immunity. In fact, from all immune cell subsets, T cells have the potential to directly recognize cancerous cells and mediate tumor specific eradication. T cells can, by virtue of highly specific receptors, discriminate malignant cells form healthy cells based on molecular changes as small as single point mutations [1]. Importantly, the mobilization of an optimal T cell response strongly depends on a favorable environment, both at the time when the T cell response is initiated and during the effector phase [4]. For these reasons, different immunotherapeutic strategies act by intervening at different stages of the T cell response.

There are various immunotherapeutic strategies which directly or indirectly aim at enhancing anti-tumor T cell responses, or the effects of which converge to T cells for therapeutic efficacy. Based on their mode of action, they can be grouped in distinct categories: therapeutic vaccination, T cell transfer, immune-modulatory antibodies, bispecific antibodies, cytokine-based therapies

or immune-stimulating molecules (see **Figure 1**).

To understand T cell-based immunotherapies, it is fundamental to know how T cell immunity is initiated, how it acts and how it is regulated.

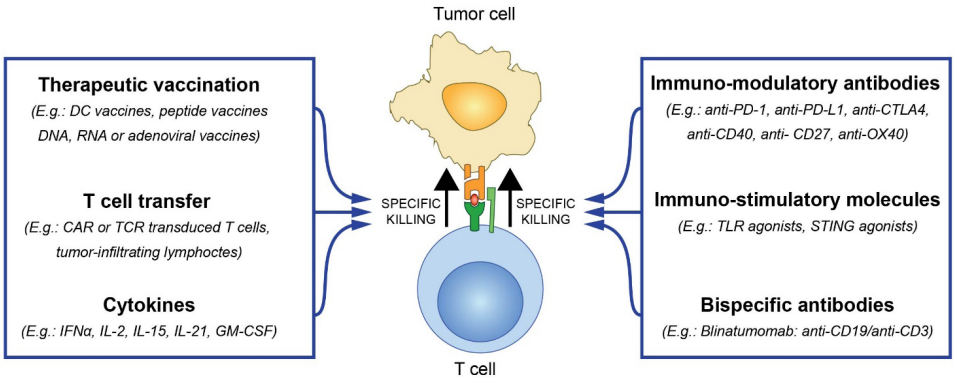

Figure 1: Immunotherapies converging to T cell immunity for therapeutic efficacy

T cell immunity

T lymphocytes derive from hematopoietic lymphoid precursors in the bone marrow and develop in the thymus. Every T cell possess a unique T cell receptor (TCR) generated by genetic recombination that can recognize antigenic peptide fragments bound to MHC molecules. During development in the thymus, self-antigen reactive clones are deleted through the central tolerance process, while clones positively selected for MHC recognition differentiate into naïve lymphocytes and start circulating in the body, ready to differentiate into effector cells at the encounter with their antigen [5].

To make sure that naïve lymphocytes properly react during danger situations but at the same time to avoid inappropriate activation, the immune system has evolved a process to control the initiation of T cell activation, called T cell priming [6]. T cell priming is a necessary step to activate T lymphocytes and to allow them to optimally differentiate into effector cells. It requires the integration of three separate signals. The first signal consists in the recognition by the TCR of an antigenic peptide presented by another cell on MHC molecules. The second signal is given through the co-stimulatory molecules that are present on the T cell surface. These receptors are proteins that are present on all naïve cells (CD27, CD28) and that can be further upregulated upon TCR triggering (OX40, ICOS, 4-1BB). When stimulated, these molecules activate an intracellular signaling cascade that promotes the expression of T cell differentiation and effector genes.

The third signal is given by cytokines. Similarly to co-stimulation, cytokine receptors stimulate critical T cell functions, such as cell division and differentiation. Cytokines known to be important during priming are IL-12, GM-CSF and type I interferons. Once these three signals are delivered to a naïve T lymphocyte, the T cell clone will start proliferating and differentiating, as well as producing proteins for the effector functions. These three signals are indispensable and irreplaceable for the generation of a functional T cell response. Sub-priming can occur when lymphocytes do not receive all three signals. Sub-primed T cells become dysfunctional or anergic, which results in tolerance for the antigen.

The key mediator for T cell priming, which uniquely ensures proper T cell activation, is a class of cells called antigen-presenting cells (APCs) [7]. Dendritic cells (DCs) are the most specialized type of APCs known for priming T cells. In fact, DCs are equipped with a series of sensing and uptake receptors [8]. The sensing receptors can recognize either PAMPs (pathogen-associated molecular patterns) or DAMPs (danger-associated molecular patterns) and are vital to alert the immune system about a possible abnormal situation. Among pattern recognition receptors (PPR) is the Toll-like receptor (TLR) family, a family of structurally related receptors that has evolved in superior organisms. Their primary function is to alert the immune system by activating innate immunity and starting an inflammatory response, which is propaedeutic for the initiation of adaptive immunity. For this reason, TLR stimulation is also exploited for vaccination. Other PRRs are the C-type lectin receptor family, the NOD-like receptors and RIG-I-like receptors. Once the ligands bind to the sensing receptors of DCs, they start a signaling cascade which induces the upregulation of co-stimulatory molecules (such as CD80, CD86 and CD70) and cytokines (i.e. IL-12, IL-15, type I interferons), a process known as DC maturation [9]. Moreover, they promote antigen uptake and proteasomal processing for loading of peptide on MHC molecules. Combined, these features create the perfect conditions for T cell priming and provide all three signals. Within the DC population, different subsets have been distinguished based on their localization and subspecialization [10]. Lymph node-resident DCs reside uniquely in lymphoid organs and participate to the initiation of the T cell responses. Within this subset, conventional DCs (cDCs) 1 and 2 and plasmocytoid DCs (pDCs) are found. cDCs1 are specialized in CD8 T cell stimulation, while cDC2 play an important role in the induction of CD4 T cell responses. pDCs are strong producers of type I interferons and are key mediators during antiviral immunity. Migratory DCs are found in tissues and peripheral organs,

and only travel to the lymph nodes upon antigen encounter. They comprise different types, which are alike to lymph-node resident cDCs and similarly show segregated abilities to stimulate either CD8 or CD4 T cells. In the skin, dermal CD103+/XCR1+ cDC1s are the responsible subset for antigen transport to the lymph nodes. During inflammation, another type of DC arises from monocytes (mo-DCs) which also participate to the promotion of an immune response.

Once primed, T cells can differentiate into effector and memory cells [11]. T lymphocytes are divided into two subsets based on the expression of the TCR co-receptor CD8 or CD4. The two co-receptors determine their affinity for peptide-bound MHC class I or class II. CD8 T cells recognize peptides form intracellular proteins presented on MHC class I molecules, while CD4 T cells recognize peptides on MHC class II, which are derived from antigens acquired extracellularly. Their ability to distinguish intracellularly- or extracellularly- derived peptides also determines their different effector functions. CD8 T cells differentiate into cytotoxic T cells (CTL) which can directly attack and kill cells that are presenting the epitope. Effector functions of CTLs consist in transferring granules containing enzymatic proteins that will damage the target cells, production of cytokines such as IFNγ or TNFα and upregulation of death-inducing ligands such as FasL or TRAIL [12].

CD4 T cells differentiate into helper cells (Th). There are distinct Th phenotypes that can develop, which are determined both by the stimuli received during priming and by the type of threat [13]. All these responses differ in cytokine production and influence the recruitment of immune cell subsets as well as their effector functions on target cells. Th1 responses support the clearance of intracellular infections by enhancing both CTL priming and function as well as promoting IgG antibody production by B cells and activating macrophages. Th2 responses are instead more skewed to resolving extracellular infections, by inducing the recruitment and activation of mast cells and eosinophils and supporting the production of IgE antibodies. Several other Th types have been discovered throughout the years. A particular type of differentiated CD4 T cells are regulatory T cells (Treg), a specialized type of immune suppressing cells functional for the induction of peripheral tolerance.

Finally, after a pathogenic invasion has been controlled, T cell necessitates of negative regulation to end the response and avoid overreactions. Mechanisms that negatively regulate T cell activation are the upregulation on APCs of CTLA4, a protein that competes with CD28 for binding of the co-stimulatory molecules

CD80 and CD86, stimulation of inhibitory receptors (PD-1, TIM-3, LAG3, NKG2A) or cytokine-driven suppression (IL-10, TGF-beta). Most effector cells will eventually die, however, a pool of memory cells can survive and readily activate upon antigen re-encounter [14].

Anti-tumor T cell immunity

The appreciation of the role of T lymphocytes in anti-tumor immunity was a gradual process that occurred between the 1970s and the 1990s, right after the discovery of CTLs, mainly through mouse studies. Starting from the late 1980s, these discoveries were also extended to humans, opening the way for additional mechanistic, therapeutic and diagnostic studies that are still advancing nowadays [15]. This early research unraveled both the nature of the antigens recognized as well as the concept of tumor immunoediting and escape.
Three main sources of tumor expressed antigens have been discovered:

1. **Viral antigens**: tumors that have been induced by oncogenic viruses (for example human papilloma virus, Epstein-Barr virus or T cell leukemia virus) can present epitopes derived from viral proteins. These epitopes can be recognized by T cells as they derive from foreign, non-self proteins.
2. **Mutation-derived antigens**: mutations that occur in expressed genes can give rise to new sequences and potential epitopes. Non-synonymous point mutations that result in one amino acid alterations give rise to so-called neoantigens. Other type of immunogenic mutations are frameshift mutations, which result in completely novel reading frames during protein translation or fusion genes. All these alterations are tumor-specific and are not present in normal cells, so no central tolerance is induced against them.
3. **Cancer germline antigens**: this class includes non-mutated genes that are found to be expressed or overexpressed by a large range of tumors. This group can be further divided into three subclasses:
    a. Some tumors can express genes that are normally expressed only during development or by germline cells. Examples of these genes are the melanoma antigen family (MAGE), the cancer/testis antigen (CTAG) and the antigen G (GAGE) families. Interestingly, many of these genes are found on the X chromosome but for many of them the function is still unknown. For these proteins no central tolerance is generally induced.
    b. Certain tumors express tissue-specific differentiation antigens such as

Melan-A or GP100, which are found on melanocytes and melanomas.

c. Lastly, yet other tumor types can overexpress certain genes compared to healthy tissue and lower the threshold for immunogenicity. Known overexpressed shared antigens are HER2, which is found in many epithelial tumors, and MUC-1 in adenocarcinomas.

Many of these antigens were identified by analyzing patients-derived T cells *ex vivo* after co-culture with autologous tumors [16-18]. However, despite the existence of detectable tumor-specific T cell responses, in many cases tumors eventually grow out, leading to the development of the disease. The reason why this occurs has also been the subject of extensive studies. Two main mechanisms were discovered. The first is the process of immunoediting [1]. This theory postulates that in the early stages of tumor growth, tumor-specific T cell responses shape the composition of the tumor by exerting an evolutionary pressure on the different tumor clones. Only tumor cells that will manage to evolve escaping strategies will survive. For example, mutations that modify the targeted antigen, or cause the loss of it, will give an evolutionary advantage to the clone, as it can be no longer recognized by the T cells. Another possible escape mechanism is the mutation of one of the components of the antigen presentation machinery that will affect the presentation of the epitope on the cell surface. The third mechanism involves the development of immune suppression mechanisms. The three main strategies of immune suppression consist in the upregulation on the tumor cells and in the tumor microenvironment of ligands of immune inhibitory receptors (PD-L1, PD-L2), inhibitory cytokines (IL-10, IL-6, TGF-b), and the recruitment of Tregs in the tumor [19].

Also on the T cell side, some factors can demote proper activity. It is not completely understood how these spontaneous responses are initiated in the first place, but it is highly likely that T cells are sub-primed and do not receive full co-stimulation, leading to low functioning T cells [20]. Because of their lower potency, T cells are subjected to chronic activation and antigen exposure, which culminates with an exhausted phenotype characterized by low killing capability, upregulation of inhibitory receptors (PD-1, LAG3, CTLA4, TIM-3) and low proliferative potential [21].

Altogether, these factors contribute to the weakening of anti-tumor T cell responses. At the time of tumor detection, patients often present dysfunctional T cell responses which may be rescued by immunotherapy.

Current status of therapeutic vaccination

Therapeutic vaccination aims to induce or re-activate T cell responses against tumor-specific antigens. The vaccine can be targeted to any of the antigen classes described above, depending on the tumor type and the antigens it expresses. Even though cancer vaccination is based on a straightforward logic, years of research have failed to bring this concept into clinical translation. The reasons for this are multiple: the challenge of properly activating T cells in an immune-suppressed environment, the different inhibitory and evading mechanisms taking place in tumor cells, and the realization that not all antigens are necessarily relevant in mediating tumor rejection [22]. However, the latest successes of other immunotherapies, together with advancements in immune profiling and neoantigen identification, provide a new rationale for improving previous attempts, since therapeutic vaccination represents a highly specific therapy against cancer cells, that could potentially leave healthy cells unharmed.

The field of cancer vaccines is currently focused on two fronts: the identification of relevant antigens and the refinement of vaccine formulations. The progress in different high throughput technologies such as DNA and RNA sequencing, has finally made it possible to identify the numerous patient-specific mutation-derived antigens [23-26]. Moreover, the advances in proteome and peptidome techniques are enriching our knowledge about the immune landscape of tumors and how frequently epitopes can be found to be presented on tumor cells [24, 27-29]. The challenge remains on how to make the best use of these potential antigens. Many studies reveal that vaccine formulation can influence the quality of the T cell response generated by the vaccine [22, 30]. The choice of the adjuvant also influences immune activation and can differentially skew the response induced.

Based on current knowledge, an optimal cancer vaccine should fulfill the following requirements:

- Preferential targeting of the vaccine to DCs, to create the optimal conditions for T cell priming
- Inclusion of an adjuvant that will skew a type 1 immunity (CTL and Th1 responses)
- Inclusion of multiple antigens to induce a broad specificity
- Flexibility of manufacture to produce not only off-the-shelf products but also personalized vaccines

Many different strategies are being investigated to tackle these challenges, among which peptide- and DNA-based vaccines.

Peptide-based vaccines

Peptide vaccines rely on delivering the selected epitopes (which is on average 9 to 20 amino acids long) within an extended amino acid sequence (usually around 25 to 35 amino acids). This approach is more effective compared to administration of the exact (minimal) epitopes because it circumvents undesired binding of the peptide epitopes on the MHC molecules of non-professional APCs, which could trigger T cells in absence of proper co-stimulation [31, 32]. At the same time, peptides are better endocytosed and processed by DCs compared to full length proteins [33, 34]. Peptide vaccines can simply be delivered as a mixture together with the adjuvants. Even though this is sufficient to induce T cell responses, it is reportedly not enough to achieve tumor control in the clinic [22, 35-38]. Possible reasons behind this lack of success are inefficient peptide delivery causing poor antigen uptake by DCs in vivo, which are a rare subset compared to other cellular types, and their sub-optimal activation due to dispersal of adjuvant and antigen after injection. These factors may determine sub-optimal T cell priming. Many approaches are currently under investigation to address these issues, which will expectedly improve optimal T cell activation by optimized cancer vaccines.

TLR-ligand conjugated vaccines

The Toll-like receptor family (TLRs) is a family of receptors that recognizes pathogen-associated structures and plays a fundamental role in early immune activation and the initiation of an immune response. Because of their sensing role, TLRs can be found on various cellular subsets, but the highest diversity of expression is found on DCs . What makes TLRs interesting in the vaccination field is that many of the ligands and chemical structures responsible for TLR activation have been identified over the years, leading the way for their use as adjuvants both in their native form or as synthetically reproduced molecules (**Table 1**).

Synthetic TLR ligands can be manipulated for conjugation to protein antigens or peptides. Conjugation of peptide and TLR ligands represents one strategy to enhance DC targeting and at the same time to co-deliver antigen and maturation signals in the same cells, thereby potentiating T cell priming [39] (**Figure 2**).

Table 1: Overview of TLR ligands and agonists

TLR	Localization	Recognized structure	Ligands and agonists	Commercialized adjuvants
1/2	Surface	Bacterial lipoproteins	Pam_3CysSK_4, FSL-1, MALP-2 Zymosan,	Amplivant™
2/6	Surface			
3	Endosomes	Viral dsRNA	Poly-I:C, Poly A:U	Poly-ICLC, Ampligen
4	Surface	Bacterial glycolipids	LPS, MPA, GLA, AGPs	MPL™, CRX-527
5	Surface	Bacterial flagellin	Flagellin	-
7	Endosome	Viral ssRNA	Imidazoquinolines, Hydroxyadenines	Aldara, Imiquimod, Resiquimod
8	Endosomes			
9	Endosomes	Unmethylated DNA	CpG-ODNs	PF-3512676 (CpG 7909)
10	Surface	Unknown	-	-

Many pre-clinical studies have described the benefit of using TLR ligand-peptide conjugates to target different TLRs such as TLR2 [40-42], TLR4 [40], TLR7 [43, 44], TLR7/8 [45, 46] and TLR9 [42, 47]. All these studies report methodologies to successfully conjugate ligands to antigen without disrupting the immunological properties of the ligand or the antigen but, most importantly, they highlight how this strategy improves vaccine potency. Conjugated vaccines to a TLR2 ligand were shown to favor enhanced uptake and antigen presentation by DCs *in vitro* and *in vivo* compared to the soluble, separate components [41, 42].

The attachment of the TLR2 agonist was also described to increase DC targeting *in vivo*, via undetermined endocytic receptors, and to affect intracellular trafficking towards antigen presentation or storage compartments [34, 42, 48]. Overall, this was associated to enhanced T cell induction by the conjugated vaccine which translated into improved tumor control [49]. These studies winded up to the clinical evaluation of a peptide conjugate vaccine bearing the TLR2 ligand UPam (Amplivant) [50] for the treatment of malignancies positive for Human Papilloma Virus (HPV) (Trial identification number: NCT02821494). This vaccine contains two peptides covering immunodominant region of the E6 protein of HPV16, which contains multiple CD8 and CD4 T cell epitopes.

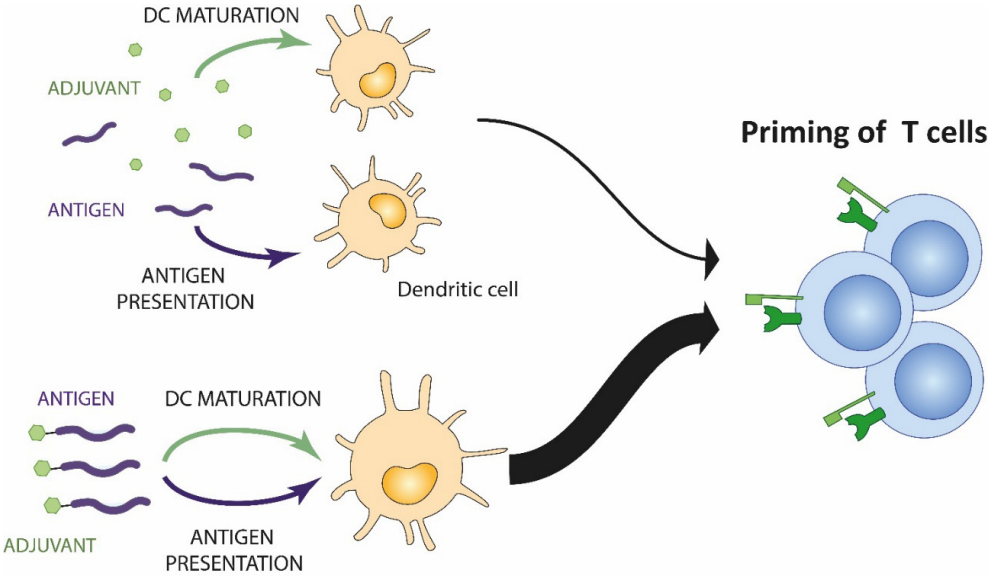

Figure 2: Covalent attachment of adjuvants to antigen results in improved immunological activation of T cells

Formulated peptide vaccines

Another approach to optimize peptide vaccines is their encapsulation in nano- or micro-sized particles. The benefits of these methods consist of increased half-life of the vaccine and reduced dispersal of the components. Different encapsulation strategies have been reported, among which liposomes, polymeric nanoparticles and nanogels. One interesting feature of these carriers is that their properties (such as size, composition, surface charge or tags) can be manipulated to maximize uptake and DCs targeting. For example, it has been shown that nano-sized particles are better internalized by DCs via pinocytosis or endocytosis compared to micro-sized particles [51, 52], which are preferentially taken up via phagocytosis by macrophages. Furthermore, positively charged particles seem to facilitate DC uptake as well as maturation [53, 54]. For example, the loading of peptide vaccines in nanoparticles or liposomes enhanced the induction of T cell responses in different pre-clinical studies. In many cases, this was also associated to enhanced control tumor rejection [55-58]. Another property that can be modulated is content release. For example, vaccine components can be conjugated to the particles via reduction sensitive linkers, which will be hydrolyzed only in highly reducing environments such as cellular lysosomes. This idea has been applied to dextran nanogels for the delivery of a full protein vaccine [59]. Covalent loading

of protein into positively charged nanogels led to the highest DC uptake in vitro, and to induction of strong specific T cell responses that could mediate rejection of tumors [60].

DNA vaccines

DNA vaccination consists in the delivery of the epitope encoded in its genetic form as linear DNA sequences, and it relies on DNA transcription followed by antigen production by host cells upon injection. The advantage of this methodology is the extreme versatility in accommodating any type of sequence, the stability of the molecule and an inherent adjuvanticity thanks to activation of PAMPs or DAMPS receptors [61, 62].

The ability of DNA vaccines to induce adaptive immunity was proven nearly 30 years ago [63-65]. Since then, many studies have investigated the possibility of gene immunization. Numerous preclinical and clinical studies have explored multiple methods for optimizing vector design, delivery and routes of administration [66]. Immune responses by DNA vaccines can be induced by intramuscular, intradermal or intravenous administration [64, 67, 68] and original administration devices such as gene gun [69], electroporation [70] and tattooing [71] have been developed to increase transfection efficiency and induction of both T cell and antibody-mediated immune responses.

Especially in light of personalized cancer vaccines, it represents a platform that can guarantee the necessary freedom to include multiple potential antigens in a rapidly manufactured vaccine encoding a string of multiple minigenes. Therefore, linear DNA vaccination is a versatile approach which has currently gained a lot of attention for specific immunotherapy of cancer.

Scope of the thesis

Cancer vaccines have the potential of raising T cell responses that can specifically eradicate tumors. However, multiple challenges are hampering clinical translation of this therapy. Next to identifying the correct antigens, it is important to rationally design vaccines to increase vaccine potency, DC targeting and, finally, the quality of the T cell response generated. In this thesis, three different approaches to improve the current vaccination strategies were explored.

In **chapter 2**, the possibility of conjugating the synthetic TLR4 ligand CRX-527 to peptide antigens was investigated. This novel adjuvant represents an attractive option to increase vaccine potency as it represents a detoxified version of

LPS, one of the most potent immune-stimulatory molecules known. A synthetic route for this conjugate and different potential linkers and positions for peptide conjugation were established. It was demonstrated that the use of a glycol linker preserves the immune-stimulatory activities of the ligand without affecting epitope processing and presentation of a model peptide. Moreover, not only it has shown effective T cell priming *in vivo*, but also increased differentiation into effector memory T cells. This study presents and validates CRX-527-peptide conjugates for their potential use in cancer immunotherapy.

After validating the molecular and immunological quality of CRX-527 peptide conjugates, three distinct conjugates bearing different antigens were synthetized, and their ability to induce specific CD8 or CD4 T cell responses and eradicate tumors was tested. In **chapter 3**, it was demonstrated that the conjugated vaccine improved T cell activation compared to a mixture of peptide and CRX-527, resulting in improved protection upon prophylactic and therapeutic vaccination. This study marks the use of CRX-527 as a potential adjuvant for cancer vaccination as well as the efficacy of conjugation as a strategy to improve vaccines in two different tumor models.

Conjugated vaccines could benefit from the integration of more than one signaling pathway. To explore this hypothesis, peptide conjugates bearing two different ligands were designed. In **chapter 4**, the TLR7 agonist derived from 2-butoxy-8-hydroxyadenine was connected to mannose-6 phosphate, a ligand that normally mediates intracellular trafficking via its endosomal receptor. This combination showed increased potency in inducing DC maturation, however antigen presentation was dampened. This study illustrates the interplay between signaling and trafficking pathways in dendritic cells. In **chapter 5**, the possibility of combining the TLR1/2 ligand Pam_3CysSK_4 with the synthetic TLR7 ligand based on the 2-butoxy-8-hydroxyadenine derivative was tested. Two dual peptide-conjugates were synthetized and validated for their ability to activate DCs and induce CD8 and CD4 T cell responses

In **chapter 6**, the formulation of antigenic peptides into reduction-sensitive cationic nanogels was explored. This vaccine formulation was shown to enhance antigen uptake and presentation by DCs more efficiently than free synthetic peptide. In addition, cationic nanogel were able to mature DCs. Injection of peptide-loaded nanogels carrying either a CD8 or a CD4 epitope increased the breadth and the quality of the T cell response generated. This study shows the potential of nanogels as a promising vaccine delivery platform for peptide antigens.

The targeting of tumor-specific neoantigens for cancer vaccination requires a platform that can easily support the production of personalized vaccines. In **chapter 7**, a DNA vaccine containing a string of multiple neoantigens was developed and tested as a potential approach for personalized vaccines. The DNA vaccine was capable to mobilize neoantigen-specific T cell responses *in vivo* and, in combination with anti-PD-1 immunotherapy, mediate tumor control. This study provided proof of concept for a feasible design of personalized vaccines targeting multiple neoantigens in a single vaccine entity. In addition, it demonstrated the complementary efficacy of distinct immunotherapies to established tumors. In **chapter 8** the findings of this thesis are summarized and discussed.

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