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

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GENERAL DISCUSSION

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Therapeutic vaccination against cancer is supported by a strong immunologic rational and by encouraging preclinical data, however the clinical translation of this tumor-specific therapy has been challenging. To date, only two therapeutic cancer vaccines have been approved in the clinic [1]: Sipuleucel-T, a dendritic cell-based vaccine against the prostatic acid phosphatase antigen for the treatment of prostate cancer, and T-VEC, a modified herpes virus that expresses GM-CSF and acts as *in situ* vaccine for melanoma. Apart from Sipuleucel-T and T-VEC, most cancer vaccines tested in the clinic in the last decades have been discontinued before or during phase 3 trials, failing to produce a significant benefit for patients. Concomitantly, immunotherapies using immune checkpoint blocking antibodies, such as anti PD-1/PD-L1 or anti-CTLA4 antibodies, have demonstrated promising outcomes in clinical trials, underlining the role of the immune system, including T cell immunity, in tumor regression [2]. The apparent discordance between the failed attempts of cancer vaccines and the success of immunotherapy can be harmonized by the knowledge gained during these studies: T cells can in fact recognize tumor cells and mediate tumor eradication, but they do so within a much more hostile environment than previously understood. Contemporarily, the vaccination field has also undergone a process of optimization in relation to vaccine formulation and delivery methods. The correlation between successful priming and the equipment of T cells with strong effector and memory functions was established, as well as their relationship with anti-tumor efficacy.

Compared to the initial attempts, there are now many lessons that have been learnt about the induction of effective anti-tumor T cell immunity and that can be applied to the current development of cancer vaccines. Optimal cancer vaccines should be targeted to DCs, should comprise a potent adjuvant that can skew towards Th1 response and should generate a response against a broad range of antigens, possibly inducing both cytotoxic CD8 T cells (CTLs) and CD4 helper T cells [3].

These features are key throughout different stages of the T cell responses:

- during **priming**, where antigen presentation by professional DCs together with potent co-stimulation by adjuvant and CD4 helper T cells will induce differentiation of robust effector and memory functions, maximizing T cell fitness;

- during the **effector phase**, where T cell responses will have to deal with tumor resistance mechanisms and the collaboration between CTLs and helper T cells is fundamental for an optimal anti-tumor response. In addition, the inclusion of multiple epitopes maximizes the chances to target relevant antigens as well to diversify the response in case of antigen loss by the tumor cells.

In this thesis, several of these concepts were explored and implemented for the design and formulation of novel well-defined cancer vaccines.

DC targeting and formulation

The physiological role of DCs is to integrate innate signals for the generation of an adaptive response. It is crucial for a vaccine to reach and activate DCs, and the formulation of vaccines plays a key role in DC targeting. In **Chapter 2** and **3**, conjugation of the TLR4 ligand CRX-527 to an antigenic peptide demonstrates

an enhancement of vaccine-induced T cell responses. The reasons may be multiple. On one hand, the uptake of the peptide and the presentation of both CD8 and CD4 epitopes appear to be increased upon conjugation to the TLR4 ligand. This could be due to interaction of CRX-527 with the co-receptor CD14. Interaction of the natural ligand LPS with this co-receptor facilitates binding with the TLR4 signaling complex at the cell surface [4]. Besides its chaperoning role, CD14 has been directly implicated in internalization of the TLR4-ligand complex by triggering a signaling cascade that activates endocytic pathways [5]. CRX-527 agonism was shown to be independent from the co-receptor CD14 [6], however the presence of CD14 at the cell surface was reported to potentiate downstream signaling, possibly indicating interaction of CRX-527 with the co-receptor, which could also stimulate internalization. In general, resemblance of the agonist to a PAMP, may implicate affinity for one or more scavenger receptor that are expressed by DCs [7, 8]. In fact, the facilitated uptake of antigen conjugated to PAMPS has been reported for other receptors: TLR2 in [9, 10] and **chapter 5**, TLR7 in **chapter 4,** TLR9 [8, 9], and various C-type lectin receptors [11, 12]. The scavenger receptor CD36 has affinity for diacylglycerides such as zymosan, which is also a TLR2/6 agonist [13].

In addition, several lines of evidence underline how, the presence of antigen and signaling of TLR ligands in the same endosomes promote processing and presentation of the endosomal cargo rather than degradation [14, 15]. Furthermore, especially upon injection *in vivo*, it is likely that antigen-adjuvant conjugates enable antigen presentation and co-stimulation to be delivered to T cells by the same DCs, resulting in improved T cell priming as demonstrated in **chapter 2** by the increased differentiation of T cells into effector memory T cells, by the enhanced anti-tumor efficacy reported in **chapter 3**, and by the higher numbers of specific T cells induced reported in **chapter 5**.

Encapsulation of antigen into biodegradable nanoparticles is a promising strategy employed to improve vaccine targeting and efficacy. In fact, **chapter 6** shows that loading of peptide antigens into cationic dextran nanogel improves uptake and antigen presentation of the epitopes by DCs, *in vitro*. *In vivo*, this translates into enhanced induction of antigen-specific CD8 and CD4 T cells. The quality of these responses is also improved as indicated by the increased poly-functional cytokine profile observed. Cationic nanoparticles have gained growing interest in recent years, as evidence accumulates on their natural affinity towards DCs and their maturing properties [16-18], as well as their ability to allow antigen depot formation upon subcutaneous or intradermal injection and prolonged antigen presentation [18-20], which results in an overall improvement of the T cell responses. These properties are connected to the cationic nature of the molecules. Vaccinations with peptide encapsulated into cationic liposomes, for example, results in a similar improvement of T cell induction and translates into superior preclinical anti-tumor efficacy [19]. The reduction-sensitivity of the nanogels described in this thesis, which allows peptide release only in reducing

conditions, seems to be intimately linked to the observed improvement. Antigen covalently bound to self-assembling nanoparticles have been developed and successfully employed in recent studies. For examples, peptide antigens and adjuvant coupled with lipoprotein-mimicking molecules that assemble in nanosized discs could improve antigen delivery to lymphoid organs and sustain presentation by dendritic cells [21]. Similarly to what was observed in **chapter 6**, this resulted in up to 45-fold increase of antigen specific T cells. Another self-assembling nanoparticle system containing covalently bound peptide and TLR7 agonist was applied to putative neoantigens and was shown to expand the breadth of CD4 and CD8 responses in mice and primates [22].

While *in vitro* studies allow characterization of the vaccine in a controlled setting, it is evident that the success of cancer vaccination is also influenced by the route of administration. While most vaccinations are typically injected intramuscularly, the vaccinations reported in this thesis are performed intradermally. Compared to the intramuscular route, which is relatively inefficient and require high doses to achieve immunogenicity, intradermal vaccination represents a safe and accessible route, that could improve vaccine immunogenicity for T cells and lower the required dose per injection. This is based on the notion that the dermis is patrolled by many DC subsets, which are involved in antigen uptake and transport to the draining lymph nodes. In particular, CD103+ migratory DCs have sparked the interest of tumor immunologist for their increasingly evident role in anti-tumor T cell immunity [23]. CD103+ DCs are found intratumorally and mediate attraction and stimulation of CTLs [24-26], playing a role also in immune checkpoint therapies. Therefore, the involvement of these DC subset for the stimulation of an anti-tumoral immune response is auspicated. In **chapter 3**, the mobilization of this subset was described upon vaccination with CRX-527-peptide conjugates.

Adjuvants

Proper polarization of the immune response is determined by the instructions given by DCs, which in turn integrate environmental cues to distinguish between different types of threats. The same concept can be exploited during vaccination through the choice of adjuvants.

In **chapter 3**, the TLR4 agonist CRX-527 was successfully employed for the first time to generate anti-tumor immunity. TLR4 represents an interesting target for stimulation during vaccination as it has a wide expression pattern and a broad immunological effect. The signaling complex TLR4 is well studied and it triggers two independent signaling cascades. Upon ligand binding, TLR4 requires heterodimerization with the co-receptor MD-2, which mediates activation of the MyD88-TRAF6 pathway at the cell surfaces and consequent NF-kB-mediated transcription of pro-inflammatory cytokines [4]. Internalization of the TLR4 complex initiates signaling via the TRIF pathway, which activates IRF3-mediated transcription of type I interferons [27]. Exploitation of these potent pathways for

vaccination purposes has always been cautious, because the native ligand LPS is associated with toxicity and sepsis [28]. However, the molecularly defined agonist employed in this thesis allows controlled used of this adjuvant as well as strong immunological activity (as low as 1 nmol per mouse) showing no side-effects. Moreover, it could generate a Th1 skewed response which efficiently mediated protection from tumor challenge upon prophylactic vaccination. Therapeutically, the vaccine could significantly control tumor growth, however vaccination may be combined with another therapy to reach full effectiveness. Recently, a similar synthetic TLR4 agonist has also been described. Glucopyranosil lipid A (G100) also shows Th1 skewing and effective adjuvanticity in cancer vaccines [29], where combination with PD-1/PD-L1 blockade demonstrates synergism in pre-clinical setting. In addition, this adjuvant showed excellent activity as intra-tumoral monotherapy [30], and this therapy is currently under clinical investigation for the treatment of Merkel cell skin carcinoma [31]. TLR4 expression in DCs has also been implicated in recognition of DAMPS in anti-tumor immunity [32]. Altogether, these reports support the rational for triggering TLR4 during cancer vaccination.

Stimulation of other TLRs can also be exploited during cancer vaccinations. For example, the triggering of TLR7 is attractive in the context of anti-tumoral immunity because it drives production of type I interferons and the induction of an anti-viral response, which, among all types, most resembles the response required for the elimination of tumor cells. In **chapter 4**, it was explored whether the dual conjugation of a TLR7 agonist and the ligand for an intracellular trafficking receptor to antigenic peptides could improve vaccine efficacy. The M6P receptor mediates physiological trafficking of lysosomal enzymes [33] between Golgi and pre-lysosomes. Even though it was reported an enhancement of DC maturation, the antigen presentation was hampered by the addition of Mannose-6 phosphate (M6P). While it may not represent a strategy to improve vaccine efficacy, these observations underline the complexity of routing that exogenous antigen undergoes, from initial uptake to processing and MHC presentation rather than degradation. In this case, conjugation of the TLR7 ligand potentiated antigen presentation which was abolished by the addition of the M6P.

In **chapter 5**, the same TLR7 agonist was tested in combination with the TLR2 ligand Pam_3CysSK_4. Dual conjugation preserved the immunological activity of the ligands and the antigen, and represents a promising strategy to further investigate, especially regarding the type of immunity raised by dual TLR stimulation. This approach has potentially endless possibilities of combination, for example with other TLR ligands or other PAMP receptors ligands such as NOD-like receptors, CLRs, STING agonists or ligands for uptake and trafficking receptors. The system that vaccination tries to artificially replicate, is governed by the triggering of different receptors by the various PAMPS present on pathogens, therefore a vaccine may be potentiated by the integration of different signaling pathways. For example, dual conjugation of Pam3CysSK4 and NOD2 agonist to antigenic peptides, synergizes in the induction of DC maturation as well as Th1 cytokine production by T cells [34].

Antigen selection

The vaccination efficacy of a determined tumor antigen is defined by a number of immunological and tumor-related factors. The immunogenicity of an antigen may vary based on a) the processing pattern regulated by the proteasome or other proteolytic enzymes, b) the binding affinity of the epitope for the MHC molecules, which can influence the strength of the immune response, c) the nature of the affinity for MHC class II or I, which determines CD4 versus CD8 T cell induction, and d) the T cell receptor repertoire i.e. the presence and frequency of specific precursor T cells. On the tumor side, the immunological efficacy of an antigen will vary depending on the antigen and MHC expression levels within single tumor cells but also on clonal distribution in the primary tumor and metastasis. All these factors are difficult to predict and the potency of most antigens can thus far only be determined empirically. Therefore, selection of the right antigen for cancer vaccines is an open challenge.

An optimal cancer vaccine should be able to evoke both CTL and T helper responses. This is important during priming of vaccine-induced T cells, as T helper cells actively support CTL differentiation [35] as well as on tumor site, where CTLs mediate direct recognition and killing of tumor cells while specific helper T cells modulate the tumor microenvironment and support CTLs activity.

In **chapter 3** it was clearly depicted with two antigen models how the two responses complement each other for full anti-tumor efficacy. The inclusion of a helper epitope plays a crucial role during CTL priming. In fact, help presence impacts the development of CTL effector functions, the breadth of the response and the formation of memory precursors [35, 36]. This is believed to happen in a two-step priming process. This model postulates that CD8 and CD4 are independently primed by migratory conventional (c) DC1 and cDC2, respectively [37]. After this initial activation, primed CD8 and CD4 T cells produce chemokines that attracts plasmacytoid (p) DCs accumulation, via CCL3, and cDC1, via XCL1 [38]. In this second step, pDCs promote further stimulation via type I interferons, while CD4 T cells interact with cDC1 via CD40L, which amplifies upregulation of CD80/CD86 and CD70 co-stimulatory molecules and IL-12 and IL-15 production by DCs [39]. This in turn potentiates CD8 T cell expansion and differentiation of memory and effector functions upon antigen-specific interaction with cDC1 [35, 40]. Lack of help-induced signals during priming impairs CTL differentiation and coveys them to predysfunction, which can still be rescued, and eventually terminal exhaustion [41]. This argues that inclusion of help, even tumor non-specific, is advisable in the design of a cancer vaccine to potentiate T cell priming. During anti-tumor activity, tumor-specific help can exert additional functions through recognition of MHC class II positive tumors or by locally presented antigen by dendritic cells in tumor or draining LN, or by activation of tumor-specific macrophages [42], production of cytokines that recruit and support CTL proliferation and effector functions [43, 44], or even direct cytotoxicity activity [45].

The latest technological advances in high throughput techniques have played an important role in substantiating molecular and biological details in cancer

vaccination. Sequencing techniques allow mutational profiling of individual tumors, uncovering all putative mutation-derived antigens [46]. RNA sequencing and proteomic analysis enable the verification of antigen expression while immune-peptidome techniques can detect epitope presentation [47, 48]. These innovations have further broadened antigen repertoire for cancer vaccines and directed research towards personalized vaccines.

In **chapter 7**, a possible approach to meet this new challenge was explored. Nucleotide-based vaccines have gained wide interest since the emergence of personalized vaccines because they can more easily accommodate diverse sequences and include multiple antigens. This is advantageous not only in the light of personalized therapy but also for broadening the immune response induced. A DNA plasmid containing multiple epitopes in a similar design to the one reported in this thesis has also been developed in pre-clinical setting with epitopes targeting three different tumor models and was shown to also generate T cell immunity successfully and to mediate tumor control [49]. RNA-based and adenoviral vectors with mini-genes are also under investigations for these purposes [50, 51]. Nucleotide-based vaccines are easy to manufacture and their physicochemical properties are not altered by the sequence encoded. In contrast, the production and the solubilization of peptide-based vaccines under GMP conditions is challenging due to the unique properties of every amino acid sequence, creating extra barriers for optimal antigen selection. An exemplification of the swiftness of genetic vaccines was the quick development of RNA- and adenoviral-based vaccines containing the Spike protein of the SARS2-coronavirus during the COVID19 pandemic [52-55]. These vaccines were able to awake both B-cell mediated antibody responses as well as CD4 and CD8 T cell responses with a Th1 profile [54, 56].

Nevertheless, peptide-based systems have also been successfully applied to personalized vaccines in pre-clinical and early clinical settings [21, 47, 57] and advancement in peptide synthesis methods are also under development [58]. Promising results were obtained in a recent Phase I clinical study named HESPECTA, using the optimized TLR2 ligand Amplivant conjugated to two HPV16 E6 long synthetic peptides. This study showed safety and robust immunogenicity in HPV16+ cancer patients which were intradermally vaccinated in four doses groups (unpublished, manuscript in preparation).

All platforms represent valid alternatives to investigate for the potency of cancer vaccines. In addition, the existence of these different platforms allows for testing heterologous prime/boost protocols to further potentiate vaccine efficacy [59, 60].

Combination therapies

Several lines of evidence suggest that the limited success of cancer vaccines may be due to its use as monotherapy. In **chapter 7**, the combination of vaccination and anti-PD1 blockade resulted in most optimal therapeutic effect, while separately both therapies had limited effect. A growing number of reports successful-

ly combines immunotherapeutic treatments to improve T cell-mediated tumor control. In particular, combinations with immune checkpoint inhibitors (ICI), such as anti-PD1/PD-L1, seem to be complementary to vaccination: on one side vaccination increases the pool of tumor-specific T cells on which ICI can act, and on the other side ICI helps preventing the inhibition of effector functions of vaccine-induced T cells in the tumor microenvironment [29, 51, 61]. Also in the clinic, the synergism between ICI and vaccination is becoming evident as reports show how PD-1 treatment could rescue vaccinated cancer patients with progressing tumors in melanoma [50, 62, 63] and HPV-16 malignancies [64], and vice versa, how vaccination before anti-PD1 treatment could double the response rate and survival to treatment in patients bearing HPV16-related malignancies [65]. Moreover, key activating or inhibitory receptors involved in cancer immunity are being identified. For example, the expression of the CD8 T cell inhibitor NKG2A was found to correlate with unresponsiveness to anti-PD1 treatment in patients with HPV16 malignancies [65, 66], while the activator ICOS was found to be positively expressed by tumor-specific T cells in mice responding to PD-L1 treatment. Based on this analysis, the targeting ICOS with activating antibodies was able to double the survival rate in tumor models by synergizing with PD-L1 therapy [67]. These observations set the basis for the rational combination of ICIs and vaccination.

Next to immunotherapies, vaccination can synergize with other therapies. Chemotherapy for example, can cause immunogenic cell death of tumor cells causing the induction of tumor-specific T cells [68]. Vaccination can boost these responses and increase therapeutic efficacy of the treatments as shown for the combination of cisplatin and peptide vaccination in mouse models and patients for HPV16+ tumors [69, 70]. Ablative therapies such as radiotherapy and photodynamic therapy were also reported to synergize with vaccines [71, 72]. Combination of cancer vaccine with these therapies have the added value of partially debulking the tumor mass, creating a damaged environment that is easy to infiltrate and control by T cells, which could eliminate residual cancer cells and establish immunological memory to prevent recurrences and metastases. Abscopal effects on distant secondary tumors have been described in pre-clinical models for combination of photodynamic therapy or radiotherapy with vaccination [73, 74].

Concluding remarks

After more than twenty years of break-in, we just started to disclose the real potential of cancer vaccination. New challenges and possibilities are awaiting to be tackled. In this thesis, different strategies were explored to refine the formulation of cancer vaccines, to maximize vaccine performance and to address current demands. This constitutes only one building block of a much wider task, which is the rational integration of cancer therapies for the successful treatment of cancer.

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