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FOCUSSED RESEARCH REVIEW

Personalized cancer vaccines: adjuvants are important, too

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Abstract
Therapeutic cancer vaccines have shown limited clinical efficacy so far. Nevertheless, in the meantime, our understanding of immune cell function and the interactions of immune cells with growing tumors has advanced considerably. We are now in a position to invest this knowledge into the design of more powerful vaccines and therapy combinations aimed at increasing immunogenicity and decreasing tumor-induced immunosuppression. This review focuses essentially on peptide-based human vaccines. We will discuss two aspects that are critical for increasing their intrinsic immunogenicity: the selection of the antigen(s) to be targeted, and the as yet unmet need for strong adjuvants.

Keywords Cancer · Vaccine · Clinical trial · Peptide · Adjuvant · CITIM2017

Abbreviations
PAMP Pathogen-associated molecular pattern
TSA Tumor-specific antigen

Introduction: two decades of vaccines against cancer

The success and enormous potential of immune checkpoint modulation demonstrates how powerful our immune system is (T cells in particular) in controlling and eradicating tumors, even at an advanced stage of disease. Therapeutic vaccination aims at inducing or boosting T cells that can specifically recognize and eliminate tumor cells, and at establishing long-term immunological memory. The molecular basis of this recognition is the interaction between T cell receptors on the T cell and MHC-peptide ligands derived from tumor-associated or tumor-specific antigens (TAAs and TSAs, respectively) that are expressed by the malignant cell. A number of immunization approaches have been applied in patients; they can be classified according to the nature of the product administered (i.e., cellular vs. non-cellular) or of the antigen(s) targeted (defined vs. non-defined). Antigen(s) can be administered per se or preloaded in vitro onto DCs; several formats (peptides, recombinant viruses, DNA and more recently RNA), routes (mostly subcutaneous or intradermal applications) and schedules have been tested (reviewed in [1]). Synthetic peptides have the advantage of being well defined and relatively inexpensive to synthetize. It is still debated whether short peptides representing exact $CD4^+$ or $CD8^+$ T cell epitopes should be used, or rather long peptides which need to be processed intracellularly before presentation on MHC molecules. In our view, one decisive advantage of short peptides is their versatility: they are easy to produce in GMP quality and can be mixed for each individual patient either from a pre-existing warehouse or after de novo synthesis [2]. Since tumor cells may escape vaccine-induced T cells by losing either a specific antigen or a particular MHC allele, a vaccine tailored to each patient and composed of peptides derived from several tumor antigens that bind to different HLA allelic products appears to be the most suitable strategy. Another crucial advantage of short peptides is that they allow a straightforward in vitro T cell monitoring for both $CD4^+$ and $CD8^+$ T cell responses, a key element in vaccine development [3].

After more than 20 years of experimental cancer vaccines, three main conclusions can be drawn. First, immunization

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with defined antigens in the form of synthetic peptides has demonstrated excellent feasibility and safety. Second, tumor regressions have been repeatedly observed in various malignancies and when targeting various antigens. Third, overall clinical response rate is low, and was estimated to be less than 5% [4, 5]. Hence, despite sustained efforts, the outcome of therapeutic vaccination attempts has been disappointing so far. There are, however, sound reasons for still considering vaccination as one meaningful weapon against cancer: (1) currently, only a fraction of treated patients benefits from checkpoint inhibitor modulation and response to treatment is limited to certain tumor entities; (2) vaccination with peptides selects for desired anti-tumor T cell specificities and can establish long-lasting immunological memory; (3) there is a strong rationale for expecting synergistic effects when combining vaccination with other therapies, especially with checkpoint receptor inhibition; (4) vaccination in combination with efficient adjuvantation should give stronger immune responses as observed so far; and finally (5) vaccination is safe, highly practicable, and relatively inexpensive as compared to other approaches. It could therefore be made available for early cancer stages and even as a preventive option for high-risk individuals [6].

Personalized target antigens for optimized vaccines

Until recently, many of the tumor-associated proteins targeted by vaccination belonged to the category of overexpressed or differentiation antigens [7]. Tumor-associated antigens (TAAs) are expressed at a certain level in normal cells, and the T cell repertoire available for their specific recognition is expected to be deleted of high-avidity effector cells. Indeed, peripheral T cells specific for TAAs are generally of moderate to low affinity [8]. It is assumed that vaccination against this category of antigens leads to weak T cell responses with poor anti-tumor activity. Another reason which might explain the limited clinical efficacy of earlier vaccines is the fact that selection of vaccine antigens was essentially based on their reported expression pattern in the tumor entities considered; expression of these antigens was in most cases not verified on each individual patient's tumor (either the tumor tissue or the specific tools for analysis were unavailable).

Great efforts are being invested worldwide in the development of approaches that are tailored to each patient and that target tumor-specific antigens (i.e., not expressed in normal adult tissues). Known TSAs are cancer-germline proteins, virus-derived products and neoantigens. Cancer-germline antigens are found across a number of tumor types and in germ cells [7]; since they are also expressed ectopically in the thymus, high-affinity T cells might just be deleted from the mature repertoire [9]. Human papilloma virus-derived antigens are being successfully targeted in patients with premalignant lesions [10]; however, only very few tumor-associated pathogens are identified, with the notable exceptions of EBV (associated with Burkitt lymphoma or nasopharyngeal carcinoma) and HPV (associated with cervical and head and neck carcinomas).

Neoantigens are currently gaining most of the attention; since they are tumor-specific per se, it is believed that they are highly immunogenic. Neoantigens can originate from point or frameshift mutations, fusion proteins, and spliced peptides. In most cases, they appear randomly and are tumor and patient specific. Notable exceptions are, for example, K-Ras mutations, which are present in approximately 60% of pancreatic carcinomas and 20% of all cancers [11]. It has long been known that tumor neoantigens can be recognized by T cells and they have also been included in early peptide-based vaccines [7, 12–14], but their clinical impact was only highlighted recently. Indeed, data indicates that T cells from patients who respond to checkpoint inhibitory Abs recognize tumor-specific neoantigens [15–18]. Along with technical progress in the sequencing of whole tumor genomes, and based on in silico predictions for MHC binding, the first personalized vaccines composed of patient's specific, predicted neoantigens (as peptides or RNA) have shown encouraging results in melanoma, especially when the vaccine was combined with checkpoint blockade [19–21].

Mutated antigens hold great promise, but they are unfortunately rare [22]. The frequency of intrinsic mutations varies greatly among tumor entities: melanoma or lung carcinoma harbor on average approximately 200 different mutations, in contrast to leukemia or pediatric tumors that display less than 10 [23]. In addition, and even with a consequent number of non-synonymous mutations at the exome level, it is very likely that, at best, only a few mutated sequences will finally be presented onto tumor MHC molecules for T cell recognition [24, 25]. Hence, targeting mutations with T cells will simply not be possible for many tumor types. Recent reports revealed that spliced peptides or frameshift products could constitute a substantial source of tumor MHC ligands [26, 27]. Feasibility and clinical benefit of targeting such sequences in anti-cancer vaccination still needs to be established.

We analyzed the HLA ligandome from a number of malignancies, in particular renal cell carcinoma, ovarian carcinoma and leukemia [28–30]. Our peptidome database now contains more than 2 million "wildtype" peptide identifications obtained from over 380 tumors and a number of healthy tissues that we studied during recent years. We can also detect neoantigens by mass spectrometry [31], but their frequency is far lower than the frequency of exome mutations. Instead, we found that many HLA-ligands with germline sequence appear as tumor-specific as neoantigens

by their virtual absence on normal tissues. Hence, dysregulated processes within tumor cells, possibly switched on by mutated proteins, might change the expression level of non-mutated proteins, thereby influencing the HLA-ligandome. Our current approach to vaccination builds on the great potential of all these wildtype, tumor-specific peptides that could be targeted in many, if not all, tumor types. Combination of the information delivered by ligandomics and by genomic expression allows selecting the best HLA-ligands for vaccination; in addition, in vitro immunogenicity testing using T cells from HLA-matched healthy donors can identify those sequences which are more likely to induce an immune response upon vaccination [32]. Such workflow leads to the design of an "off-the-shelf" peptide warehouse from which patient-individual multipeptide vaccines can be rapidly assembled [33]. This strategy has been successfully applied in a patient with cholangiocarcinoma who received a personalized, multipeptide vaccination based on HLA ligandome and gene expression analyses of the patient's own tumor. Despite the poor prognosis, this patient is stable more than 5 years after initiation of the vaccination [34]. As an additional level of vaccine personalization and possibly combined with the warehouse approach, a vaccine cocktail can be assembled from patient-individual peptides derived from mutated and/or wildtype, tumor-specific HLA ligands. Such an approach is being tested by two consortia supported by the European commission (http://www.gapvac.eu and http://www.hepavac.eu for glioma and hepatocellular carcinoma, respectively).

The need for strong adjuvants

Even if incorporating strongly immunogenic antigens, cancer vaccines need to be appropriately sensed by the immune system. It is well documented that peptides delivered alone in PBS are poorly immunogenic. Current studies therefore combine peptides with adjuvants: ideally, these should: (1) protect the antigen from immediate degradation and ensure its prolonged release; (2) support efficient uptake of the antigen by local antigen-presenting cells (APCs); and (3) induce full APC activation to initiate robust anti-vaccine Th1/CTL responses and long-term immunological memory. Aluminum salts (Alum), which are included in most licensed vaccines against pathogens, are known to activate various immune pathways (reviewed in [35, 36]). Alum preferentially supports Th2 responses and antibody formation, and is therefore not the adjuvant of choice for cancer vaccines.

A common practice is to prepare an oil-in-water emulsion with Montanide ISA™51 (incomplete Freund's adjuvant analogue, generally given s.c.) that will protect the peptides and ensure their slow release. Montanide is generally well tolerated, but sterile abscesses at the injection site have been reported [37, 38]. We have vaccinated more than 40 cancer patients and did not observe any severe systemic toxicity of Montanide so far ([34, 39, 40] and unpublished results). A point of debate about Montanide is based on data gained in mice: a single injection of a peptide together with a high dose of Montanide led to local sequestration and dysfunction of effector T cells [41]. In patients, repeated injections of peptides alone in Montanide induced the formation of organized lymphoid aggregates resembling tertiary lymphoid structures; however, infiltrating T cells were found to be dysfunctional [42, 43].

Another popular adjuvant is GM-CSF, which is employed to recruit and activate APCs at the injection site. GM-CSF has been applied to a very large number of patients either s.c. or i.d. In most studies, the adjuvant effect of GM-CSF is weak, both in terms of induced T cell responses and of clinical efficacy [44, 45]. In some studies, the addition of GM-CSF to peptides emulsified in Montanide did not appear to improve immune responses as compared to Montanide alone [46, 47]. It is important to note that low doses of GM-CSF support immune responses, whereas high doses (> 100 µg per application) might in fact promote the expansion of MDSCs and inhibit T cell function [48].

Hence, there is an urgent need for more efficient adjuvants, and high expectations have been placed on toll-like receptor (TLR) ligands (reviewed in [35, 49]). During the natural antimicrobial immune response, the binding of pathogen-associated molecular patterns (PAMPs) to TLRs initiates a strong APC activation, with upregulation of cell surface MHC, adhesion and costimulatory molecules together with the production of inflammatory cytokines and chemokines. All of these are essential for adequate antigen presentation, DC migration to the lymph node, and ultimately efficient T cell priming. These are all qualities that should be expected from an optimal cancer vaccine adjuvant. Synthetic compounds that bind to TLRs and can be co-administrated together with the vaccine peptides are being tested in clinical trials; in addition to their ability to induce strong T cell responses, such molecules should demonstrate good safety and be available at GMP grade. At present, agonists of TLR9, TLR7, TLR3 and TLR2 are or have been in clinical testing in combination with peptides. Most of these trials are early phase studies with limited numbers of patients. It is therefore difficult to address clinical efficacy, but two essential endpoints can be monitored: toxicity (by measuring adverse events) and immunogenicity (by assessing anti-vaccine T cells).

The first clinical trial of CpG ODNs (class B CpG 7909, a TLR9 ligand) combined with a MHC-class I peptide (modified MelanA-derived epitope) and Montanide s.c. showed impressive T cell responses detectable ex vivo after only four vaccinations [50]. The strong adjuvant effect of CpG was recently confirmed in combination with a long NY-ESO-1

peptide, also in melanoma patients [51]. Unfortunately, CpG at clinical grade is not easily available, which impairs its broad application, including in randomized trials.

TLR7/8 ligands such as Imiquimod or single-stranded RNA (under clinical development) are also an option. Imiquimod, as Aldara cream 5%, is an approved treatment for basal cell carcinoma and genital warts. In a human skin explant model, topical application of Aldara induced significant activation and migration of skin-resident DCs [52]. As an adjuvant, Imiquimod can be applied topically either before or shortly after the vaccine at the injection site [39, 53, 54]. In a randomized multicenter study for HPV-16$^+$ patients suffering from high-grade vulvar and vaginal intraepithelial neoplasia, Aldara application did not improve immunological and clinical responses to long peptides emulsified in Montanide [54]. In contrast, in a multipeptide-based study for prostate cancer patients with biochemical relapse, we found that TLR7-ligand application associates with improved clinical course. However, induced T cells were only detectable after peptide presensitization in vitro and were not significantly stronger in patients receiving the TLR-7 ligands ([39] and unpublished results). Altogether, Imiquimod appears to be a relatively weak adjuvant, but might well synergize with other TLR ligands.

More recently, stimulation of TLR3 has also been a topic of interest. Poly-ICLC (Hiltonol) is a polyinosinic–polycytidylic acid (poly-IC) stabilized by lysine and carboxymethylcellulose. Poly-ICLC can be applied intramuscularly or s.c. and is generally very well tolerated [55]. Worthy of mention is a three-cohort trial in which patients with ovarian cancer received repeated s.c. injections of overlapping long peptides either alone, emulsified in Montanide, or in Montanide plus Poly-ICLC. Montanide was required to induce T cells, and the addition of Poly-ICLC enhanced and stabilized these responses. Because of the limited number of patients enrolled in each cohort, no conclusion on clinical outcome could be drawn [56].

Another interesting TLR that could be targeted by vaccine adjuvants is TLR2. Upon ligand binding, TLR2 forms heterodimers with either TLR1 or TLR6; TLR2/TLR1 and TLR2/TLR6 interact with different lipopeptides, i.e., tryacetylated (e.g., Pam3CSK4) and diacetylated (e.g., Pam2CSK4), respectively. Whereas Pam2CSK4 has been shown to favor Th2 response in certain mouse models, Pam3CSK4 induces Th1 polarization of human T cells [57]. We demonstrated previously that a synthetic analogue of the bacterial lipopeptide Pam3Cys-Ser-Ser is a strong adjuvant for priming virus-specific T cells in mice. Intraperitoneal injection of the lipopeptide covalently coupled to a Flu-derived peptide induced CTLs with an affinity similar to that of T cells expanded by the virus itself [58]. We learned only later that this lipopeptide is a TLR1/TLR2 ligand. More recently, a similar conjugate was shown to support human DC maturation in vitro and to increase Th1 response of patients' T cells against HPV16-derived long peptides. In this model, the conjugate peptide-Pam3CSK4 appeared to be superior to the mere mix of both substances for stimulating recall CD4$^+$ and CD8$^+$ T cells. Clinical testing is ongoing.

One drawback of such lipopeptide molecules is their amphiphilic nature which renders them laborious to produce in GMP quality for human application. Moreover, vaccine peptides linked to lipopeptides are poorly adapted to personalized approaches in which each patient receives an individualized vaccine possibly containing patient-unique (e.g., tumor-specific) peptides. To circumvent these pitfalls, we (H. G. Rammensee and K. H. Wiesmüller) have designed a Pam3Cys derivative, whereby the Pam3Cys moiety is linked to a nine amino acid peptide derived from *Mycoplasma salivarium*. This compound is water soluble, simple to synthetize and to purify, and therefore can easily be produced in clinical grade quality. In vitro, we found that it induces DC maturation and activation of several immune cell subsets. Moreover, after a single s.c. injection of virus-derived peptides, Montanide, and the new TLR2 ligand in a healthy volunteer, we could detect a strong, ex vivo measurable, anti-vaccine T cell response in the PBMCs. Multifunctional vaccine-specific T cells were present both in the granuloma that formed at the injection site and in the blood, where they persisted more than 1 year after injection (manuscript in preparation). Our aim is now to combine this new adjuvant with tumor-specific peptides identified by our mass spectrometry pipeline in a patient-individualized vaccine setting. A large GMP-grade batch of this adjuvant is currently being produced.

Combinations of adjuvants and more

Thus, by providing peptides (the antigen), Montanide (for depot effect) and a TLR agonist (for APC activation), we hope to come up with one combination of choice for inducing strong anti-vaccine T cells and durable clinical responses in cancer patients. The next step could involve a combination of several TLR agonists, chimeric ligands designed to activate at least two TLRs simultaneously [59], or the co-application of TLR ligand(s) and agonist anti-CD40 Abs as already tested in preclinical models [60, 61]. In addition, systemic immunomodulators such as interleukin 2, cyclophosphamide or antibodies that target immune checkpoints can be combined with peptide vaccines. It is likely that the optimal combination(s) will differ for various tumor entities, possibly tumor stages or even individuals. Since it will be impossible to test every combination in every clinical setting, we need to rely not only on preclinical work for identifying the most promising settings, but also on the identification of tumor and immune predictive biomarkers. The

clinical translation of findings obtained from mouse tumor models has often been disappointing, probably because of multiple differences between humans and mice (e.g., expression of immune cell receptors including TLRs, skin biology, intrinsic tumor properties, etc.). In vitro systems with human cells are useful, but have obvious limitations since they are not able to recapitulate the complex events and cell interactions that ultimately lead to T cell activation in vivo. Human skin explants seem to be a good system to test the local effect of adjuvants and administration routes on APCs [52]. In our view, small-scale clinical trials designed to test immunogenicity of peptides, adjuvants and immunomodulator combinations should aid in selecting the most immunogenic combinations to be evaluated for anti-tumor activity in larger studies. Since more adverse events might be expected from synergistic combinations, a careful monitoring of induced toxicities is essential.

Conclusion

Our basic knowledge of T cell activation and of the interplay between tumors and the immune system has considerably improved in the last few years. Immunotherapy, i.e., checkpoint inhibitors, has now seized the oncology field. However, the majority of cancer patients do not profit from this success yet. For these, therapeutic vaccination, possibly followed by checkpoint inhibition, could be an option worthwhile for clinical testing. Several obstacles to successful vaccines have now been clearly identified and can be addressed concurrently following a clear roadmap that takes into account: (1) the crucial choice of the antigens; (2) the need for stronger adjuvants; (3) adequate measures to counterattack immune suppression; (4) rationale combinations for achieving synergistic effects; and (5) identification of the patients most likely to benefit from vaccination, e.g., those with tumors still expressing HLA. Next generation cancer vaccines are definitely on the march.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval, ethical standards and informed consent All studies mentioned were formally approved by the local ethics committee of the University of Tübingen, and informed consent obtained from all patients.

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