Journal of Controlled Release 347 (2022) 308–313

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Perspective

Roadmap to next-generation cancer vaccines

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ARTICLE INFO

Keywords:
Cancer vaccine
Immunotherapy
Neoantigen
Targeted delivery
Nanovaccines

ABSTRACT

Cancer vaccines have emerged as powerful and clinically viable therapeutic modalities to reduce tumor burden, eradicate residual cancer cells and prevent relapse. The past years have witnessed rapid advances in various scientific and engineering approaches to next-generation cancer vaccines. This perspective highlights the cutting-edge technologies to elicit robust, durable and cancer-specific immune responses as well as interesting research directions in augmenting the therapeutic efficacies and reducing the systemic side effects of cancer vaccines. The featured technologies include (i) bottom-up high-throughput screening strategies to identify neoantigens as well as optimal delivery systems for tumor antigens and/or adjuvant; (ii) top-down knowledge-based strategies to de novo design effective delivery platforms and to engineer tissue-targeting specificity; and (iii) synergizing cancer vaccines with the clinical immunotherapeutic practices such as CAR-T and anti-PD-1 therapies.

1. Introduction

Therapeutic cancer vaccines have demonstrated encouraging potentials in treating both hematological malignancies and solid tumors. Cancer vaccines can be of clinical utilities in reducing tumor burden with rekindled hope for possible cures, eradicating residual disease, or preventing relapse. The current understanding of the anti-tumor immunity indicates that immunogenic tumor antigens (TAs), immunostimulatory adjuvants and effective delivery platforms are crucial to successful cancer vaccines. Delivery vehicles can maximize the amount of TAs and adjuvants sent to antigen presenting cells (APCs) such as dendritic cells (DCs) in the draining lymph nodes (LNs). Activated APCs then process and present TAs via Major Histocompatibility Complex (MHC) molecules to T cells for mounting anti-tumor immune responses [1].

The search for highly immunogenic and specific TAs have yielded promising results, but further improvements are still in urgent need. TAs can be broadly classified as tumor-associated antigens (TAAs) or tumor-specific antigens (neoantigens). TAAs are self-antigens abnormally expressed or overexpressed in tumors. Although TAAs can provide attractive therapeutic targets, the efficacy of TAA-based cancer vaccines might be compromised due to the central T cell tolerance as TAAs are self-antigens by nature. In addition, undesirable autoimmunity might be triggered due to the expression of TAAs in normal tissues [1]. Thus, TAA-based cancer vaccines alone yielded mostly mediocre outcome in the past clinical trials. However, when used in combination with immune checkpoint inhibitors (ICIs) such as anti-PD1 antibody, TAA-based vaccines could elicit strong and durable immune responses as ICIs amplified the anti-tumor immunity [2]. In addition, cancer vaccines can increase the response rates to ICI treatments due to the broadened endogenous repertoire of tumor-specific T cells generated.

Immunosuppressive molecules are emerging as new sources for TAAs. Tumor cells usually upregulate immune-modulatory molecules such as indoleamine 2,3-dioxygenase (IDO) and PD-L1 to hijack the immune checkpoint pathways of T cells to diminish anti-tumor immunity. Vaccines targeting these immunosuppressive molecules not only can eradicate tumor cells, but also mitigate the immunosuppression caused by the target cells. In a recent phase 1/2 clinical trial, vaccine against IDO/PD-L1 together with anti-PD1 was proven to be effective and yielded a high objective response rate of 80% [3].

On the other hand, neoantigens, generated by mutations in cancer cells, are usually tumor-specific and not subjected to central immune tolerance. Compared to shared non-mutated TAAs, neoantigens can induce stronger anti-tumor immunity and offer personalized treatments. In a recent clinical trial, neoantigens identified from individual patients' tumor, in combination with anti-PD1, achieved favorable therapeutic outcome in advanced solid tumor settings [4]. One disadvantage of neoantigen-based cancer vaccines is that neoantigens are difficult to be

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https://doi.org/10.1016/j.jconrel.2022.05.005
Received 14 March 2022; Accepted 2 May 2022
Available online 13 May 2022

obtained from patients with low tumor mutational burden (TMB). Thus, in patients with low TMB, TAA-based vaccines might be at an advantage [2]. Recently, the water insoluble components of tumor cell lysates were proven to be another effective source of antigens, raising hopes for identifying new immunogenic TAs from the expanded pools [5].

Post-translational modifications such as glycosylation, phosphorylation and citrullination of identified TAs can generate new epitopes to expand the existing library and can increase the immunogenicity of existing TAs [6]. For example, phosphorylated peptides from insulin receptor substrate 2 (pIRS2) and breast cancer antiestrogen resistance 3 (BCAR3) have been clinically verified for their safety and immunogenicity [7].

However, even with optimal TAs and adjuvants, the efficacy of cancer vaccines might still be hampered by their poor pharmacokinetics. Administered TAs and adjuvants may not even have the chance to encounter APCs to trigger immune responses. Biomaterial-based approaches have been exploited to increase the effective dose of TAs and adjuvants by improving their pharmacokinetics and by targeting them to organs where anti-tumor immunity is generated. Delivery platforms can also enhance the spatial-temporal co-localization of administered TAs and adjuvants for effectively mounting robust immune responses. For instance, nanoparticle-formulated RNA could significantly enhance the stability, bioavailability and clinical efficacy of RNA in both cancer [2] and COVID-19 vaccines [8].

Here, we highlight the recent progress and interesting research directions in augmenting the therapeutic efficacies of cancer vaccines

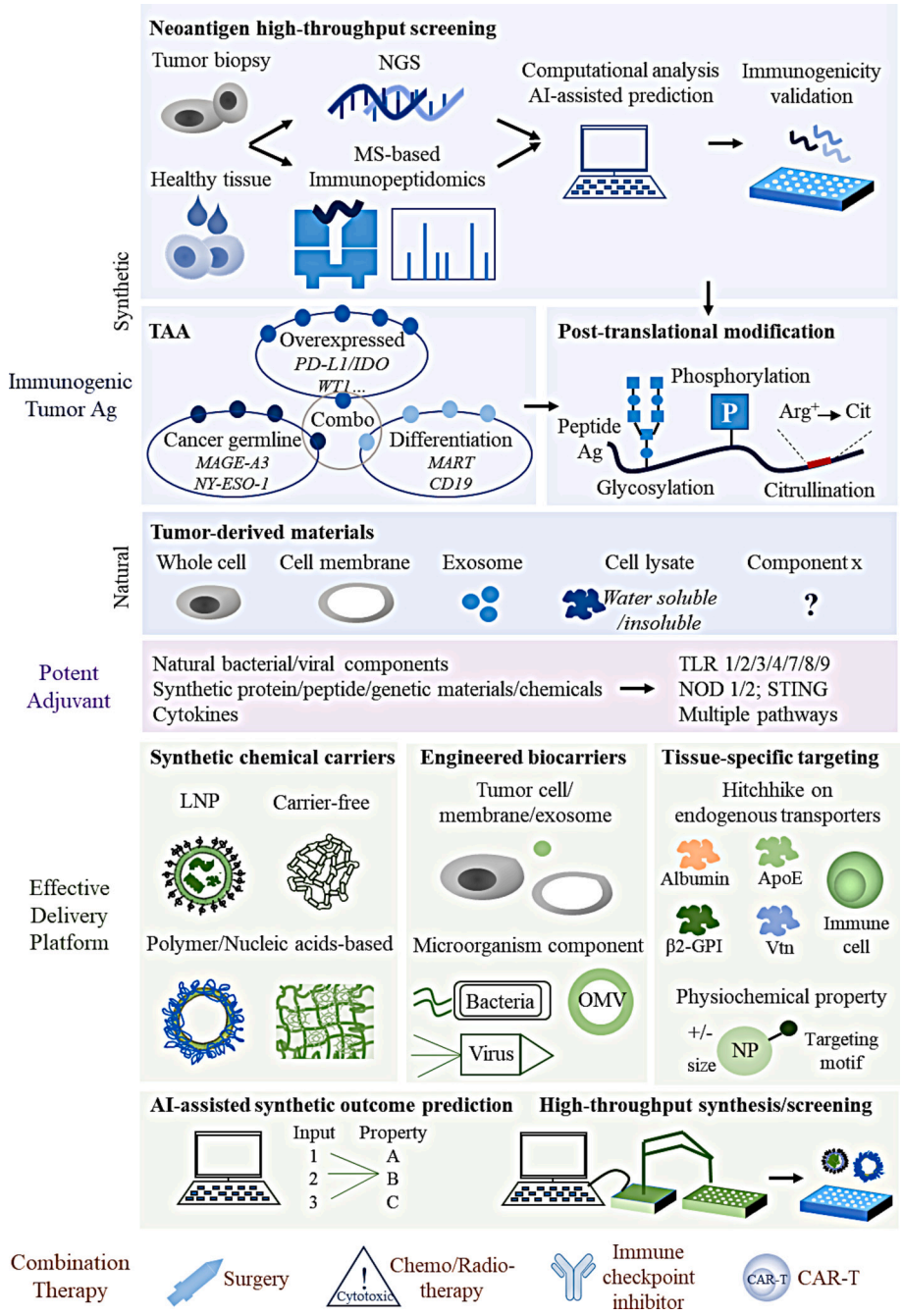

Fig. 1. Current strategies and future directions for developing next-generation cancer vaccines. Ag: antigen; NGS: next-generation sequencing; MS: mass spectrometry; AI: artificial intelligence; TAA: tumor associated antigen; Cit: citrulin; Arg: arginine; TLR: toll like receptor; NOD: nucleotide-binding oligomerization domain; LNP: lipid nanoparticles; NP: nanoparticles; OMV: bacteria outer membrane vesicles; ApoE: apolipoprotein E; β2-GPI: β2-glycoprotein I; Vtn: vitronectin.

(Fig. 1). Bottom-up high-throughput screening approaches to identify neoantigens and optimal delivery vehicles for the chosen TAs and adjuvant are discussed. On the other hand, top-down strategies to de novo design effective delivery platforms and to engineer tissue-targeting specificity are also examined. In addition, the potential of synergizing cancer vaccines with other treatment options such as CAR-T therapies is also analyzed.

2. High-throughput identification of neoantigens

Although neoantigen-based cancer vaccine generated promising results in a recent clinical trial [4], the preparation time including tumor sample sequencing, neoantigen identification and vaccine manufacturing was still up to 12 weeks. Consequently, a significant portion of patients dropped out of the trial due to disease progression. Therefore, generalizable high-throughput screening (HTS) methods that can identify neoantigens in faster, more systemic and more robust manners are urgently needed to propel further development of personalized cancer vaccine.

With the technological advancement in sequencing techniques and artificial intelligence (AI), neoantigens can be discovered via HTS within clinically-relevant time frames. In a typical search for neoantigens, whole exome and RNA sequencing are conducted for patients' tumor biopsy and cells from normal tissue/blood. Sequences are then compared and ranked by bioinformatics algorithms [9].

Mass-spectrometry (MS)-based immunopeptidomics is a fast evolving tool used for improved HTS of neoantigens. Human leukocyte antigen (HLA)-bound peptides are obtained from tumors, subjected to MS analysis, and then computationally aligned with reference database. Instead of comparing genetic sequences of tumor samples with that of healthy tissues, MS-based immunopeptidomics narrow down the search scope to translated peptides which can bind to HLA (the MHC molecules in humans) and be presented to T cells. Furthermore, the technology allows the identification of neoantigens derived from sequences outside the protein-coding regions or TAs generated by noncanonical antigen-processing mechanisms, significantly expanding the current library of neoantigens which are mostly identified from the protein coding regions [10].

In addition to experimentally identify HLA-bound peptides, computational algorithms were also developed to predict peptide neoantigens that can fit into HLAs. A recent approach utilized MS-based immunopeptidomics to generate a large training dataset consisting of 185,000 high quality HLA-bound peptides and improved the prediction accuracy to over 75% in human tumor biopsies [11]. The neoantigen prediction accuracy could also be enhanced via a consortium approach. In this "algorithm of algorithm" method, neoantigens predicted by different algorithms were combined and a new algorithm could be generated by incorporating the existing varying algorithms [12].

The development of high-throughput technologies in both experimental identification and computational prediction will constitute a positive feedback loop in neoantigen discovery. Experimental approaches can generate more high quality datasets to increase the prediction accuracy of algorithms while more precise and faster predictions will accelerate the experimental validation of neoantigens. The potency of cancer vaccine are believed to be enhanced with the discovery of more immunogenic neoantigens.

3. High-throughput and AI-assisted development of delivery systems

The accelerated discovery of TAs and adjuvants demands speedy development of customized platforms to deliver them. However, the search for the most suitable delivery vehicles might be a laborious and tedious task. High-throughput approaches can enable systemic exploration of design configurations to identify the optimal carrier structure within reasonable timeframes, thus boosting the efficacy of cancer vaccines.

Yamankurt et al. synthesized a carrier system named spherical nucleic acids (SNAs) based on three modular components—the nanoparticle core, oligonucleotide shell and peptide antigen. By varying 11 design parameters, the authors generated a library of ~1000 SNAs and evaluated their immunostimulatory activities by MS-based high-throughput approaches. The identified structure indeed significantly enhanced the immune stimulation over random structures [13]. Li et al. screened a library of biomimetic materials, which are composed of a biomimetic head (phosphate- or glycol-head), an ionizable amino core, and multiple hydrophobic tails. The phospholipid NPs, formed by identified biomaterials and mRNA coding for T cell costimulatory receptor OX-40, substantially enhanced T cell activation when used in combination with anti-OX40 antibody [14].

In addition to generate delivery vehicles in high-throughput ways, high-throughput evaluation methods were also developed to test the delivery platforms made. A high-content and high-throughput imaging bioassay was established to quantitatively gauge endocytosis and endosomal escape of mRNA containing nanoparticles. The assay was fast and could test ~100 nanoparticle formulations in just a few hours. More importantly, the results of the assay matched with the *in vivo* performance of the respective nanoparticles and it could be used as a predictive assay to decrease the amount of animal testing required [15].

Machine learning algorithms have also been adopted to predict the nanoparticle synthetic outcome. With the assistance from AI, the design parameters and range of parameters required for experimental verification could be significantly narrowed down. Therefore, the identification of the optimal delivery system could be achieved much faster. In the same time, with the same synthetic capabilities, more design space could be covered to increase the chance of locating the best delivery system [16].

4. Knowledge-based de novo design of delivery systems

Complimentary to the HTS approaches which focus less on prior knowledge, delivery systems could be rationally synthesized by leveraging on the known properties of TAs, adjuvants and the microenvironments involved to realize pre-defined functions.

To enhance antigen presentation by DCs, peptide TAs as well as agonists for Toll Like Receptor (TLR) 3 and TLR9 were co-loaded into the inner space of pH-responsive nanostructures made by DNA templates. The platform allowed efficient uptake of nanostructures by DCs and release of cargos specifically after internalization. In this system, a substantially low amount of TAs and adjuvant managed to elicit strong anti-tumor immune response [17].

To achieve sustained release of TAs and adjuvant for prolonged activation of tumor-specific immunity, alginate and PEG polymers were crosslinked to form injectable macroporous gel. In mice, the gel served as depots for prolonged release of loaded cytokine granulocyte–macrophage colony-stimulating factor (GM-CSF), the TLR9 agonist CpG and leukemia antigens to attract and then activate DCs for acute myeloid leukemia (AML) treatment [18].

To increase the loading amount and loading efficiency of TAs and adjuvants, a few approaches were explored to form the delivery platforms by using TAs and adjuvants themselves as the building blocks. In these carrier-free methods, TAs and adjuvants were engineered so that the initial uncomplexable mixtures of the two can form nanoparticles themselves. Lynn et al. flanked TA peptides with charge-modifying peptide blocks and hydrophobic adjuvant blocks at the N and C terminus, respectively. The peptide-adjuvant fusion self-assembled under physiological condition to form ~20 nm nanoparticles. The system worked well for different TA peptide sequences and several TLR7/8 agonists or STING agonists, exhibiting broad application potentials [19]. Wei et al. modified both TA peptides and adjuvant Pam3CSK4, a TLR1/2 agonist, with multiple lysines at one end. Reversible linkers with NHS functional group at both ends crosslinked TAs and adjuvants together

via polycondensation to form ~8 nm nanoparticles [20].

In these de novo synthetic approaches, materials used in delivery systems might trigger undesirable immune response against the carriers after repeated dosing, leading to the generation of tumor-irrelevant immunity or premature removal of vaccine content. Heterologous prime-boost by using different carriers might minimize the immunogenicity of carrier materials and circumvent the issues [21]. The search for low-immunogenic and biocompatible materials should still be ongoing.

In addition to synthetic materials, natural materials such as microorganisms, cells or components derived from them have been engineered for TA and adjuvant delivery. Using modified cells or biocomponents of cells can endow these delivery vehicles with biological functions hard for synthetic materials to mimic. Cheng et al. engineered gram-negative bacteria to express TAs, as fusion proteins with bacteria protein ClyA, on bacteria outer membrane vesicles (OMV) surface. The ~30 nm OMVs efficiently drained to lymph node (LN) and acted as both TA-displaying platforms and potent adjuvants. The system was further expanded to express ClyA-catcher fusion proteins which can conjugate with and simultaneously display different TAs [22]. Liquid-nitrogen-treated AML cells lost pathogenicity, but maintained cell surface CXCR4 and CD44, thus exhibiting similar bone marrow homing capabilities compared to non-treated cancer cells. Not only these cryoshocked AML cells could be used as drug carriers for targeted delivery of doxorubicin, they could also serve as sources of TAs for vaccine with the support from adjuvant monophosphoryl lipid A (MPLA) [23]. Tumor cells could also be cryogenically silicified to preserve TAs, followed by surface modification with adjuvant CpG and MPLA to serve as a vaccine platform [24]. Li et al. specifically knocked out CD47 from tumor cells by using CRISPR-Cas9 technology and then killed tumor cells with chemotherapeutics or irradiation *ex vivo*. Without the "do-not-eat-me" signal from CD47, internalization of the dead tumor cells by splenic $CD11c^+$ DCs was significantly enhanced, leading to amplified DC activation and antigen presentation [25].

Despite the inherent advantages of these biological delivery vehicles made from natural materials, clinical translation of these platforms might require improvements in reproducibility and quality control due to the heterogeneous status of living organisms. With advances in the synthetic and biological delivery platforms, the separation of the two sectors are getting blurred. Fusion systems with clear defined modularity as well as biomimetic functions can reap benefits from both sides and will most likely to be the future trend.

With rekindled interests in therapeutic cancer vaccine, substantial number of novel delivery systems have emerged. However, it is difficult to fairly compare these delivery systems, regardless of being synthetic or biological, in parallel. Standardized evaluation methods are in urgent need to facilitate the selection process so that the already strained clinical resources will not be further diluted.

5. Hitchhiking on endogenous transporters for tissue-specific vaccination

Given the current understanding of the human physiological system and availability of synthetic tools, it is difficult to develop vaccine carriers that are simple enough for clinical translation and yet with high targeting specificity to the chosen tissues/organs. Fortunately, in human body there are millions of native transporters that deliver all sorts of materials such as nutrients, hormones, metabolic waste and even foreign intruders to various destinations. Vaccine carriers that can hitchhike on these endogenous transporter proteins or cells can be endowed with the targeting specificity and biodistribution property of these native delivers.

In order to induce robust and specific anti-tumor immunity, tumor antigens and adjuvants need to be delivered to where immune responses are initiated. Thus, most of the delivery platforms were designed to target to secondary lymphoid organs as DC activation and cross-presentation for T cell priming usually take place there.

Albumin has been known as an essential lipid transporter that frequently transverse to LNs. Thus, chemically linking TAs or adjuvant to diacyllipids could allow them to hitchhike on albumin via the lipid-albumin interactions and to be transported to LNs together with albumin [26]. Peptide TAs could also be directly linked to albumin by expressing the two as fusion proteins [27]. As albumin can also transverse across the airway epithelium and accumulate in the lung area, TAs or adjuvants chemically modified with albumin-binding tail could significantly accumulate in lungs and improve the induction of lung-resident memory T cells for lung-targeted vaccination [28].

As prolonged antigen presentation in uninflamed distal lymphoid organs might promote tolerance, the long half-life of albumin might actually decrease the immunogenicity of TAs in the absence of stimulatory signals. To address this issue, transthyretin, a similar protein to albumin but with faster rate of clearance from circulation, was fused with peptide TAs. The optimized pharmacokinetics of transthyretin-TA fusion decreased systemic TA exposure while maintaining accumulation in target sites, thus significantly enhancing the vaccine immunogenicity [27].

To mediate specific targeting to liver, spleen and lung respectively, a selective organ targeting (SORT) lipid nanoparticle (LNP) system were designed to hitchhike on serum protein apolipoprotein E (ApoE), β2-glycoprotein I (β2-GPI), and vitronectin (Vtn) respectively. Interestingly, addition of various SORT molecules to conventional lipid components allowed the formed LNPs to have targeting specificity to different organs. Importantly, the hitchhiking ability depended on the biophysical property, instead of the exact chemical structure, of SORT molecules. Thus, a class of SORT molecules could achieve similar targeting ability, making the strategy generalizable and customizable to various delivery systems [29,30].

On top of hitchhiking on proteins, riding on cells could also utilize mobile cells to transport vaccine content to various destinations. Based on T cell homing property, genetically-engineered T cells that express TAs on the surface could travel to lymphoid tissues after intravenous (*i.v.*) injection and bring TAs to lymphoid tissues at the same time [31].

Tissue-specific vaccination can significantly enhance the effective doses of TAs and adjuvant delivered to the chosen destinations, thus augmenting the therapeutic efficacy of cancer vaccines. On the other hand, targeted cancer vaccine can decrease systemic exposure to TAs and adjuvants, decreasing immune-related adverse events as well as other systemic toxicities. Furthermore, tissue-specific vaccination might alleviate the complications from other diseases. For example, non-targeted cancer vaccines might exacerbate the conditions of patients suffering from autoimmune diseases such as type 1 diabetes (T1D) and rheumatoid arthritis (RA) due to systemic immune system activation.

6. Conclusion and future perspective

Despite the fact that the therapeutic outcome of cancer vaccines could be radically improved as monotherapies with technological advances, cancer vaccines will most likely be used in combination with other existing treatments, especially immunotherapies, in the foreseeable future. As immune responses induced by cancer vaccine could be dwindled by the immunosuppressive tumor microenvironment, ICIs which can counter immunosuppression have been rigorously tested and yielded promising results in combination with cancer vaccines in both pre-clinical and clinical studies. In addition, synergizing cancer vaccine with Chimeric Antigen Receptor T cell (CAR-T) therapy has demonstrated striking potential. Instead of generating endogenous tumor-specific T cells, these cancer vaccines aimed to stimulate activation and proliferation of exogenously prepared anti-tumor CAR-T cells. The cancer vaccines specifically delivered CAR-T cell targets to lymphoid compartments and radically enhanced DC presentation of the targets to CAR-T cells [32,33]. The synergistic approaches could overcome the limitation of poor *in vivo* functional persistence of CAR-T cells in solid tumor, boosting therapeutic efficacies.

In the past decade, the development of high-quality neoantigens and effective carriers have rekindled hope and interests in therapeutic cancer vaccines. High-throughput technologies in generating neoantigens and delivery platform, knowledge-based de novo design of delivery vehicles, and rationally engineered tissue-specific targeting ability might empower the next wave of cancer vaccine advancement. Hopefully, therapeutic vaccines will realize the dream of curing cancer in the near future.

Acknowledgements

This work was supported by the National Key R&D Program of China (2021YFB3800900), the National Natural Science Foundation of China (NSFC 51633005, 82150410455), the Natural Science Foundation of Jiangsu Province (BK20200861), and a Project Funded by the Priority Academic Program Development (PAPD) of Jiangsu Higher Education Institutions.

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