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Targeting Tumor-Associated Antigens in Hepatocellular Carcinoma for Immunotherapy: Past Pitfalls and Future Strategies

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Hepatocellular carcinoma (HCC) is a deadly cancer with a 5-year survival of 18% for patients at all stages.[1] Systemic treatments for advanced-stage HCC, including targeted agents and immune checkpoint inhibitors, demonstrate suboptimal clinical efficacy.[2] The objective response rates (ORRs) of monotherapy by anti-CTLA4 (cytotoxic T lymphocyte antigen 4) monoclonal antibody (mAb) tremelimumab and anti–programmed cell death-1 (PD-1) mAb nivolumab or pembrolizumab in patients with advanced HCC range from 15%-20%,[3-5] and the ORR is only modestly increased (26.3%) when tremelimumab is combined with radiofrequency/chemoablation treatment.[6] A recent phase 3 trial (CheckMate-459) that tested nivolumab as first-line treatment in newly diagnosed unresectable HCC failed to meet its primary endpoint of improved overall survival (OS) compared with sorafenib.[7] However, combination of anti-PD-L1 atezolizumab plus anti-vascular endothelial growth factor-A bevacizumab in patients with unresectable HCC demonstrated some encouraging clinical efficacy over sorafenib.[8]

Tumor-associated and tumor-specific antigens serve as the primary targets for cancer immunotherapies. Tumor-associated antigens (TAAs) are overexpressed or re-expressed normal self-proteins. Targeting TAAs for cancer treatment has been investigated extensively in the therapeutic cancer vaccine field, with limited success to date.[9] Tumor-specific antigens, also termed neoantigens, are newly expressed antigens in tumors that can be generated, expressed, and presented from viral proteins, normal cellular proteins, or mutated host genes.[10] As T lymphocytes that are reactive to neoantigens are not negatively selected during thymic maturation and can be primed

Abbreviations: AFP, alpha-fetoprotein; APC, antigen-presenting cell; BCR, B cell receptor; CD, clusters of differentiation; CLIP, Class II-associated invariant chain peptide; CR, complete response; CTLA4, cytotoxic T lymphocyte antigen 4; DC, dendritic cell; DC-LAMP, DC lysosomal-associated membrane protein; DD, destabilizing domain; GPC3, glypican-3; HCC, hepatocellular carcinoma; HLA, human leukocyte antigen; hTERT, human telomerase reverse transcriptase; IFA, incomplete Freund's adjuvant; Ii, invariant chain; LAMP-1, lysosomal-associated membrane protein 1; mAb, mitochondrial antibody; MAGE-1, melanoma-associated antigen 1; MDSC, myeloid-derived suppressor cell; MHC, major histocompatibility complex; MIIC, MHC class II- containing compartment; MITD, MHC class I trafficking signal; MRP-3, multidrug resistance-associated protein 3; ORR, objective response rate; OS, overall survival; PD-1, programmed cell death-1; PR, partial response; SS, secretion signal; TAA, tumor-associated antigen; TAP, transporter associated with antigen processing; TMB, tumor mutation burden; TUMAP, tumor-associated peptide.

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into potent tumor-killing effector T cells, neoantigens are ideal targets for immunotherapy.[11]

Type of Antigens to Use in HCC for Immunotherapy

Targeting mutated neoantigens (hereafter referred to as neoantigens) for cancer immunotherapy is progressing rapidly due to advances in high-throughput omics technologies.[11] Clinical trials using personalized neoantigen cancer vaccines in melanoma have shown promise, setting the field into high gear.[12,13] Personalized cancer vaccines have also been tested in patients with newly diagnosed glioblastoma with mixed results.[14,15] With a surge of interest in neoantigen-based therapy, many clinical cancer trials, including three for HCC neoantigens, are registered at ClinicalTrials.gov. However, recent studies have shown that mutated neoepitopes are rarely detected in human leukocyte antigen (HLA) ligandomes from HCC and other cancers with intermediate and low tumor mutation burdens (TMBs). Löffler et al. probed the HLA ligandomes of HCC but failed to detect any mutated peptides out of 1,039 unique nonsynonymous mutations.[16] A similar study identified five neoantigens in HCC immunopeptidomes/ligandomes out of 5,498 predicted neoantigens.[17] Peptides from cancer-testis antigens (CTAs) and normal proteins were readily detected in the HLA ligandomes from HCC. Similar results were reported in other solid cancers with intermediate and low TMBs, including glioblastoma,[14] colorectal cancer,[18] and cholangiocarcinoma.[19] In contrast, neoepitopes were found in HLA ligandomes from the high TMB cancer melanoma.[16,20] The difficulty in detecting neoepitopes in HLA ligandomes from HCC, glioblastoma, colorectal cancer, and cholangiocarcinoma indicates that neoantigens are rarely presented on these tumor cells. Immunotherapy targeting neoantigens in these tumors may be a futile strategy due to low quantity and poor quality of this class of tumor antigens. In a recent review, we discussed the limits of using neoantigens as targets in immunotherapy for HCC and other solid tumors and suggest alternative strategies to overcome these limits.[21] Therefore, the identification of TAAs in HLA ligandomes from HCC and other cancers with low and intermediate TMBs suggests that targeting TAAs for immunotherapy is necessary for these cancers.

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Clinical Trials of TAA-Based Cancer Vaccines in HCC: Disappointing Outcome

Several TAAs overexpressed in HCC were used as immunogens in cancer vaccine trials, and the results were largely disappointing[22,23] (Table 1). The rationale supporting these clinical trials was strong; the tested TAAs are not only overexpressed in HCC, but also appear to be immunogenic in patients. The clinically tested TAAs, including alpha-fetoprotein (AFP), glypican-3 (GPC3), human telomerase reverse transcriptase (hTERT), multidrug resistance-associated protein 3 (MRP-3), the cancer-testis antigens melanoma-associated antigen 1 (MAGE-1), and New York esophageal squamous cell carcinoma-1 (NY-ESO-1) are overexpressed in HCC tumor cells.[24-29] Furthermore, anti-TAA-specific T lymphocytes existed in unimmunized patients and were induced following immunization.[28-34] In spite of this evidence, clinical results did not match expectations.

AFP-based cancer vaccines in HCC immunotherapy were first tested in phase 1 and 2 trials by Butterfield et al.[35,36] The phase 1 trial used four HLA-A*02:01-restricted AFP peptides emulsified in incomplete Freund's adjuvant (IFA) to immunize 6 patients with HCC, and the phase 2 trial used the same AFP peptides delivered by autologous dendritic cells (DCs) to immunize 10 patients.

TABLE 1. Summary of Potential Pitfalls and Future Strategies for HCC Clinical Trials Targeting TAAs

Clinical Trials	TAA Used	Specific T-Cell Induction	Clinical Efficacy	Potential Pitfalls	Improvement Strategies	References
Phase 1	AFP/class I peptides in IFA	CD8 T in 6 of 6 patients	No OR	No CD4/B-cell stimulation	Enhance level I/II/III immunotherapy competence	Butterfield et al.[35]
Phase 1/2	AFP/class I peptides in DCs	CD8 T in 6 of 10 patients	No OR	No CD4/B-cell stimulation	Add prongs 1 and 2 plus ICI, cytokine	Butterfield et al.[36]
Phase 1	AFP/class I peptides in IFA	CD8 T in 5 of 15 patients	1 CR	No CD4/B-cell stimulation	Add prongs 1 and 2 plus ICI, cytokine	Nakagawa et al.[37]
Phase 1	GPC3/class I peptides in IFA	CD8 T in 39 of 44 patients	1 PR	No CD4/B-cell stimulation	Add prongs 1 and 2 plus ICI, cytokine	Sawada et al.[38] and Tsuchiya et al.[40]
Phase 2	GPC3/class I peptides in IFA	CD8 T in 35 of 41 patients	Lower RR at 1 year only	No CD4/B-cell stimulation	Three-pronged vaccine	Sawada et al.[39]
Phase 2	hTERT/class II peptides in IFA	CD4/CD8 T in 0 of 40 patients	No OR	No CD8/B-cell stimulation	Add prongs 1 and 3 plus ICI, cytokine	Greten et al.[41]
Phase 1	hTERT/class I peptides in IFA	CD8 T in 10 of 14 patients	Lower RR in T cell+ patients	No CD4/B-cell stimulation	Three-pronged vaccine	Mizukoshi et al.[43]
Phase 1	MRP3/class I peptides in IFA	CD8 T in 8 of 11 patients	1 PR	No CD4/B-cell stimulation	Add prongs 1 and 2 plus ICI, cytokine	Mizukoshi et al.[42]
Phase 1/2	AFP/GPC-3/MAGE-1 proteins in DCs	CD4/CD8 T in 5 of 5 patients	No OR	No B-cell stimulation	Add prong 1 plus ICI, cytokine	Tada et al.[44]
Phase 1/2a	AFP/GPC-3/MAGE-1 proteins in DCs	CD4/CD8 T in 11 of 12 patients	Longer TTP	No B-cell stimulation	Add prong 1	Lee et al.[45]
Phase 2	AFP/GPC-3/MAGE-1 proteins in DCs	CD4/CD8 T in 35 of 57 patients	No difference in RFS to controls	No B-cell stimulation	Add prong 1	Lee et al.[46]
Phase 2	HCV/multiple TAAs class peptides in IFA	CD8 T in 23 of 36 patients; anti-TAA IgG in 19 of 36 patients	Longer MST in some patients	No CD4 stimulation	Add prong 2 plus ICI, cytokine	Yutani et al.[47]
Phase 1	Multiple TAA long peptides in DCs	CD4/CD8 T in 11 of 13 patients	RFS correlate with T-cell response	No B-cell stimulation	Add prong 1	Han et al.[48]

Abbreviations: ICI, immune checkpoint inhibitor; HCV, hepatitis C virus; IgG, immunoglobulin G; MST, median survival time; OR, objective response; RFS, recurrence-free survival; RR, relative risk; TTP, time to progression.

Although anti-AFP-specific T cells were successfully induced in most patients, no objective response was achieved.[35,36] A recent phase 1 trial immunized 15 patients with HCC with two HLA-A*24:02-restricted AFP peptides emulsified in IFA, inducing anti-AFP peptide-specific T-lymphocyte responses in 5 patients, with one complete response (CR), at 6.7%[37] (Table 1).

GPC3-derived HLA-A*02:01-restricted and HLA-A*24:02-restricted peptides were also investigated in phase 1 and 2 trials as therapeutic vaccines in patients with HCC.[38,39] In the phase 1 study, 39 patients with advanced-stage HCC received either HLA-A*02:01-restricted or HLA-A*24:02-restricted GPC3 peptide in IFA. GPC3 peptide-specific T-cell responses were induced in 30 patients and a partial response (PR) in 1 patient.[38] Eleven additional treated patients with advanced-stage HCC demonstrated no CR/PR, despite induction of anti-GPC3 T cells in 9 patients.[40] In the phase 2 study, GPC3 peptide vaccines were used as an adjuvant (maintenance) therapy in 35 patients with HCC and showed clinical efficacy in delaying HCC recurrence at the 1-year, but not the 2-year, mark.[39]

hTERT-derived or MRP-3-derived peptides as therapeutic vaccines for patients with advanced HCC were tested in other clinical trials.[41,42] None of the 40 patients treated with hTERT peptide had a disease response (PR or CR), whereas 1 of 12 patients treated with MRP-3 peptide had a PR. Interestingly, when hTERT-derived HLA-A*2402-restricted peptide was used as an adjuvant therapy in 14 patients with HCC, the induction of hTERT-specific T cells was correlated with the absence of HCC recurrence,[43] suggesting a potential role for anti-hTERT cellular immunity in preventing recurrence.

Additional TAA-based clinical trials were conducted in patients with HCC. In three phase 1 and 2 clinical trials, patients with HCC received autologous DCs loaded with fusion proteins of three TAAs (AFP, GPC3, and MAGE-1) in different disease settings.[44-46] All 5 patients with advanced-stage HCC receiving the DC vaccines showed T-cell responses to the TAAs, but no disease response (CR or PR).[44] When used as an adjuvant in a multicenter randomized phase 2 trial, the difference in recurrence-free survival between the immunotherapy and control groups was not significant, despite induction of tumor-specific T-cell responses in 60%-85% of patients.[46] Other trials using multiple TAAs showed similar induction of antigen-specific T-cell responses and low clinical efficacy.[47,48] The outcomes from these clinical trials have made two points clear. First, TAAs have the capability to induce antigen-specific T cells in patients with HCC. The T-cell responses are likely weak and transient, as assessment of the vaccine-induced T-cell responses in most of these studies entailed *in vitro* stimulation for T-cell expansion. Second, TAA-based cancer vaccines as monotherapy have limited clinical efficacy in treatment of advanced-stage HCC and in prevention of disease recurrence as adjuvant therapy (Table 1).

Potential Pitfalls of TAA-Based HCC Immunotherapy Trials

To achieve efficient antigen-specific lymphocyte-mediated eradication of established tumors, three levels of immunotherapeutic competence appear to be essential (Fig. 1). First, all three cellular components of adaptive immunity, clusters of differentiation (CD) 4^+ T, $CD8^+$ T and B lymphocytes, are activated to become effectors (CD4 help, CD8 CTL, and anti-tumor antibody producers and other B lymphocyte functions), and the effectors must have full access to tumor cells. Activated DCs and anti-TAA antibody further enhance natural killer (NK) function in HCC immunotherapy.[49,50] Second, the immunosuppressive factors in the tumor microenvironment, represented by programmed death ligand 1 (PD-L1) and others, are neutralized. Third, the tumor cells express relevant targets recognized by the effectors and are sensitive to immune cell–mediated killing. A study on mouse tumor models highlights the importance of meeting all three conditions for tumor eradication.[51] To eradicate large established tumors, four components of the host immunity consisting of a tumor antigen targeting antibody, an immune checkpoint blocking antibody, a powerful T-cell vaccine, and a T-cell stimulating cytokine, were required.[51] However, none of these discussed HCC vaccine trials meet the three levels of immunotherapeutic competence (Table 1).

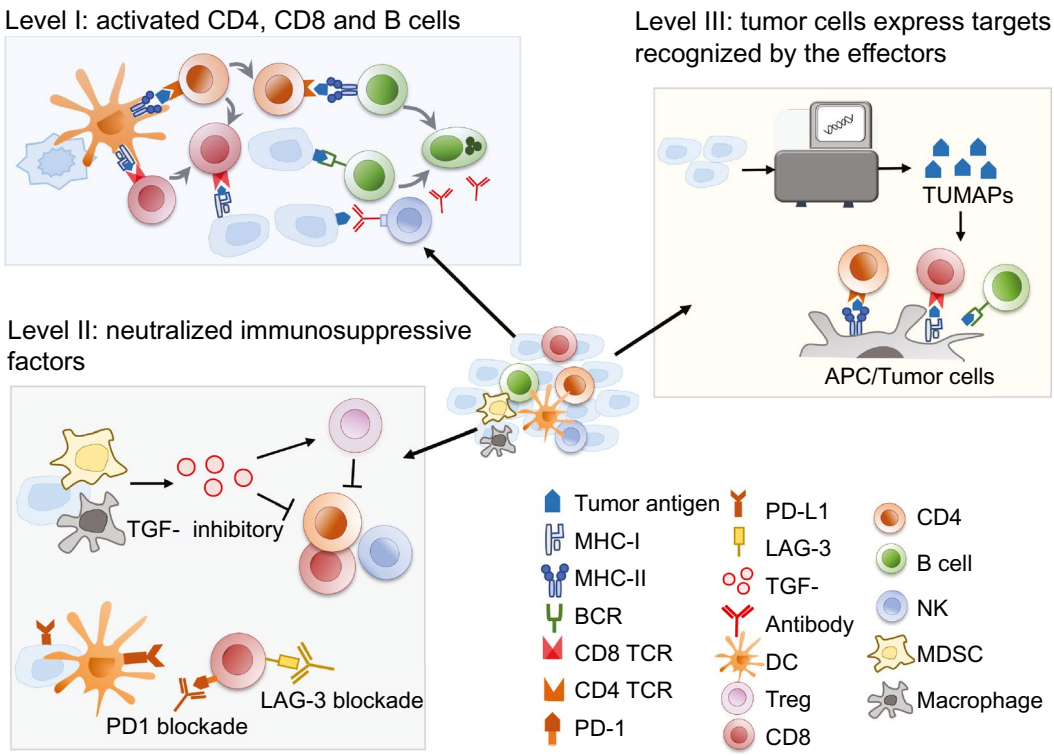

FIG. 1. Three levels of immunotherapeutic competence are essential for HCC immunotherapy. On level one, $CD4^+$, $CD8^+$, and B lymphocytes are activated to become effectors, and the effectors have full access to tumor cells. On level two, the immunosuppressive factors in the tumor microenvironment are neutralized, such as PD-1, lymphocyte activation gene-3, and transforming growth factor β. On level three, the tumor cells express relevant targets recognized by the effectors. Abbreviations: BCR, B cell receptor; LAG-3, lymphocyte activation gene-3; MDSC, myeloid-derived suppressor cell; TCR, T cell receptor; TGF-β, transforming growth factor β; Treg, regulatory T cell.

On level one of immunotherapeutic competence, the major histocompatibility complex (MHC) class I ligands from AFP, GPC3, MRP3, and hTERT applied in the HCC trials were used to prime only $CD8^+$ T cells. However, differentiation of effector and memory $CD8^+$ T cells requires $CD4^+$ T-cell help.[52] In the absence of $CD4^+$ T-cell priming, anti-AFP epitope-sensing $CD8^+$ T cells were transiently detected in immunized patients with HCC.[35] In contrast, clinical trials using long peptides encoding both $CD4^+$ and $CD8^+$ T-cell epitopes in therapeutic human papillomavirus (HPV) vaccines showed promising clinical efficacy.[53] Thus, future TAA-based therapies should include antigenic epitopes activating both $CD4^+$ and $CD8^+$ T cells. Furthermore, recent studies demonstrate the essential roles of B lymphocytes and antitumor antibodies in eradicating tumors and mediating immune checkpoint blockade-based therapy,[51,54-57] suggesting that anti-TAA-specific B cells and antibodies need to be induced for effective cancer vaccination (Fig. 1).

On level two of immunotherapeutic competence, the TAA-based cancer vaccine trials in patients with HCC were mostly monotherapy-based approaches without concomitant mitigation of immunosuppressive factors. The HCC microenvironment is highly immunosuppressive, consisting of many regulatory layers, such as regulatory T cells and myeloid-derived suppressor cells, inhibitory soluble factors such as transforming growth factor β, and cell surface immune checkpoint receptors such as PD-1 and lymphocyte activation gene-3.[58,59] Although antigen-specific T cells were induced in most patients with HCC after vaccination, the lack of clinical efficacy suggests that an inhibitory HCC tumor microenvironment prevents T-cell killing. Thus, it is critical for future TAA-based therapies to be combined with other strategies such as immune checkpoint blockade, oncolytic viruses,

targeted therapy, and radiotherapy.[60] Importantly, the adjuvants used in the previous trials were not optimized for T-cell vaccines. It is imperative to combine vaccine adjuvants optimized for T-cell responses in future clinical trials for HCC immunotherapy.[61]

On level three of immunotherapeutic competence, it remains unclear whether HCC tumor cells present the same antigenic epitopes as those used in vaccines or are intrinsically resistant to immune cell–mediated cytotoxic killing. Recent studies analyzing HLA class I ligandomes from HCC samples reported detection of CTAs including ARMC3, ATAD2, MAEL, PRAME, TFDP3, and several SSX family members.[16,17] Thus, an important unanswered question is whether the TAAs tested in the various clinical trials (AFP, GPC3, hTERT, MRP-3, and MAGE-1) are the optimal TAAs for HCC cancer vaccines. For effective killing of tumor cells, it is critical to match the antigenic epitopes in cancer vaccines with those presented by tumor-cell MHC complexes. For this purpose, an integrative multiomics high-throughput strategy termed HEPAVAC is used to identify MHC-I-restricted and MHC-II-restricted tumor-associated peptides (TUMAPs) from primary tumor tissues of patients with HCC.[23,62] The identified TUMAPs are used to generate personalized cancer vaccines for combinatorial immunotherapies[63] (Fig. 1).

Three-Pronged Cancer Vaccine Platform to Enhance TAA-Based Cancer Vaccine in HCC Immunotherapy: A Perspective

To achieve effective immunotherapies in HCC, numerous excellent strategies have been proposed.[9,60,63] Although combinatorial approaches including immune checkpoint inhibition, cancer vaccines, immune activating cytokines, and adaptive cellular therapy appear to be essential to meet these discussed three levels of immunotherapeutic competence (Fig. 1), cancer vaccines themselves also need to be improved for future clinical applications. We propose a three-pronged cancer vaccine platform that induces simultaneous activation of the three important cellular components of adaptive immunity: B lymphocytes, $CD4^+$ T, and $CD8^+$ T lymphocytes (Fig. 2).

PRONG 1: B-CELL ACTIVATION AND ANTIBODY INDUCTION TO TAAs

Prong 1 of the new vaccine platform is to include soluble tumor antigens in the vaccine. To induce B-cell activation and antitumor antibody production, intracellular and cell surface tumor antigens are modified into secreted forms. Therapeutic antitumor antibodies have long been used in cancer treatment,[64] most recently combining them with other approaches to increase clinical efficacy.[65] One study highlighted the importance of antitumor antibodies in conjunction with antitumor-specific $CD4^+$ and $CD8^+$ T lymphocytes. In mice with large established tumors, tumor antigen-targeting antibodies are essential to achieve maximal antitumor efficacy when combined with a T-cell vaccine, anti-PD-1, and recombinant IL-2.[51] Several recent studies showed that both the B-cell population and antitumor antibodies are essential for immune checkpoint blockade (anti-PD1/CTLA-4)–based therapy.[54-57] The tumor antigen-activated B cells and antibodies may play important roles in mediating immune checkpoint blockade–based therapy by enhancing antigen presentation, cross-presentation, activation of T helper cells, and antibody-dependent cytotoxicity.

For intracellular and cell surface tumor antigens, secretion signal (SS) sequences are fused with partial or full-length tumor antigens to generate soluble antigens for B-cell and antibody induction (Fig. 2A). A soluble GPC3 antigen can be generated by fusing SS sequences from other soluble proteins, such as cytokines or immunoglobin, to transmembrane domain-lacking GPC3. Biological activity of naturally secreted tumor antigens may prevent their direct use as vaccine components. For example, tumor-derived AFP, a naturally secreted plasma protein, diminishes host immunity by impairing DC function and promoting DC and NK cell death.[66-69] In such cases, a truncated version may be used. The soluble tumor antigens generated from intracellular and cell surface tumor antigens not only activate B cells and

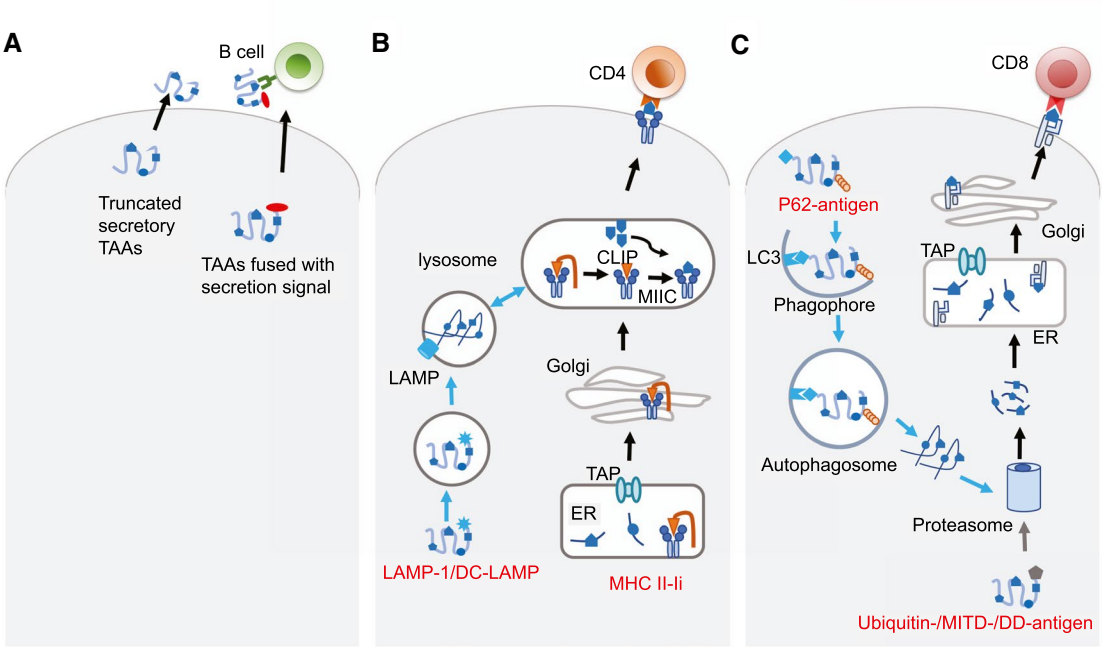

FIG. 2. Three-pronged new cancer vaccine platform to enhance adaptive immunity. (A) Prong 1: Tumor antigens are modified to be soluble to induce B-cell activation and antibody induction. (B) Prong 2: Molecules associated with the MHC-II pathway, such as LAMP-1, DC-LAMP and Ii signals, are linked to antigens in professional APCs to enhance antitumor $CD4^+$ T-cell priming. (C) Prong 3: Molecules associated with the MHC-I pathway, such as ubiquitin, p62, MITD, γ-tubulin and DD signal, are linked to antigens in professional APCs to enhance antitumor $CD8^+$ T-cell priming. Abbreviation: CLIP, Class II-associated invariant chain peptide; ER, endoplasmic reticulum; MIIC, MHC class II- containing compartment; TAP, Transporter associated with antigen processing.

induce antibody production, but also enhance antigen cross-presentation.[70]

PRONG 2: TARGETING TAAS TO MHC CLASS II PATHWAY FOR $CD4^+$ T CELL PRIMING

Prong 2 of the new vaccine platform is to specifically target tumor antigens into the MHC class II processing and presentation pathway in professional antigen-presenting cells (APCs) to enhance antitumor $CD4^+$ T-cell priming (Fig. 2B). Antigenic peptides presented by MHC class II complexes are derived from acid protease–degraded proteins in endosomal/lysosomal compartments.[71] Antigen presentation through the MHC class II pathway can be enhanced by linking the antigen to the sorting signal of lysosomal-associated membrane protein 1 (LAMP-1), DC-LAMP, and the invariant chain (Ii).[72-75] Targeting LAMP-1-linked antigens from microbial pathogens and tumor cells to the MHC class II pathway has been studied extensively in mice and primates.[72,73,75-80] Several patterns from these preclinical studies emerged. First, LAMP-1-mediated antigen targeting the MHC class II pathway increases antigen-specific responses of not only $CD4^+$ T cells, but also $CD8^+$ T cells, and increases antibody production. Second, direct comparison of the three types of molecular modifications revealed that LAMP-1 and DC-LAMP are better than Ii in their capacity to target antigens to the MHC class II pathway and induce antigen-specific immune responses. Third, inserting an antigen between the luminal domain and sorting signal (transmembrane and cytoplasmic domains) of LAMP-1 elicited stronger cellular and humoral immune responses than fusing antigen with only its sorting signal.[78]

Both LAMP-1-mediated and DC-LAMP-mediated antigen targeting have been tested in clinical trials to treat cancer patients with encouraging clinical outcomes. In a recent personalized cancer vaccine trial, autologous DC vaccines transfected with mRNAs encoding TAAs with LAMP-1 modification were delivered to solid cancer patients.[81] Most

of the immunized TAAs induced antigen-specific $CD4^+$ and/or $CD8^+$ T-cell responses and were associated with favorable overall survival.[81] Additionally, patients with newly diagnosed glioblastoma treated with autologous DCs pulsed with cytomegalovirus phosphoprotein 65 (pp65) RNA linked to the sorting signal of LAMP-1 as adjuvant (maintenance) therapy showed prolonged progression-free and overall survival.[82,83] Several clinical trials using DC-LAMP to target TAAs into the MHC class II pathway were conducted in patients with melanoma.[84-86] Anti-TAA-specific $CD4^+$ and $CD8^+$ T cells were induced in most patients. Notably, a phase 2 study showed that patients with pretreated advanced melanoma had a 38% ORR, including 8 CRs and 7 PRs (n = 39) after receiving combined treatment of DC-LAMP-TAA-loaded autologous DCs plus the anti-CTLA4 mAb ipilimumab.[86] These studies demonstrate that LAMP-1-based and DC-LAMP-based antigen targeting to the MHC class II presentation pathway is promising. We propose to incorporate LAMP-1-mediated or DC-LAMP-mediated TAA targeting to the MHC class II pathway to enhance $CD4^+$ T-cell priming into the new vaccine platform (Fig. 2B).

PRONG 3: TARGETING TAAs TO MHC CLASS I PATHWAY FOR $CD8^+$ T-CELL PRIMING

Prong 3 of the new vaccine platform is to specifically target tumor antigens into the MHC class I processing and presentation pathway in professional APCs, to enhance antitumor $CD8^+$ T-cell priming (Fig. 2C). Antigenic peptides presented by MHC class I complexes are derived from proteasome-degraded proteins in the cytosol,[71] motivating the study of strategies to target tumor antigens for increased proteasome degradation. An early study demonstrated enhanced MHC class I antigen presentation with a chimeric protein of γ-tubulin fused with a model HPV-16 E7 antigen.[87] γ-Tubulin is localized in the centrosome, which contains a high density of proteasomes.[88] Vaccination with chimeric γ-tubulin/E7 DNA plasmid enhanced E7-specific $CD8^+$ T-cell response and tumor rejection, independent of $CD4^+$ T cells.[87] A second strategy was to provide a proteasome degradation signal to TAAs by fusing them with ubiquitin.[89] Vaccines consisting of DCs transfected with mRNA encoding ubiquitin-MART-1 induced more efficient priming and expansion of TAA-specific $CD8^+$ T cells than those transfected with unmodified MART-1. Autophagy, a fundamental intracellular degradation system, is involved in antigen processing and presentation for both MHC class I and II pathways.[90] A third strategy was to fuse human immunodeficiency virus Gagp24 antigen to the C-terminus of the autophagy receptor sequestosome 1(SQSTM1)/p62,[91] as SQSTM1/p62 delivers ubiquitinated cargo for autophagic degradation.[92] Vaccination with DNA encoding SQSTM1/p62-Gagp24 significantly enhanced Gagp24-specific $CD4^+$ and $CD8^+$ T-cell responses.[91] A fourth reported strategy was to attach an MHC class I trafficking signal (MITD) (MHC I amino acid 308-362) to the C-terminus of antigens.[93] Fusion of MITD to viral and tumor antigens strongly enhanced antigen-specific $CD4^+$ and $CD8^+$ T-cell responses. In patients with melanoma, this strategy was used to enhance both TAA-based and neoantigen-based vaccines, and antigen-specific $CD4^+$ and $CD8^+$ T cells were induced to TAAs and neoantigens.[13,94] Finally, we devised a new strategy to enhance MHC class I antigen presentation by targeting antigen to proteasomal degradation. Banaszynski et al. reported that human FKBP12 mutants are unstable and subject the proteins fused with the mutants to rapid proteasomal degradation, therefore functioning as a destabilizing domain (DD).[95] We tested antigen presentation by MHC class I complexes with TAAs fused with DD, which resulted in rapid proteasomal degradation and enhanced MHC class I antigen presentation. We found that DCs transfected with DD-chicken ovalbumin had dramatically enhanced surface expression of K^b-SIINFEKL peptide complexes. Furthermore, we found that autologous DC vaccines expressing TAAs, including AFP, GPC-3, CDKN2A and AKR1B10, modified with DD induced potent antigen-specific $CD8^+$ T-cell responses in patients with HCC (unpublished observations).

This new three-pronged vaccine platform may be adapted for clinical application in a TAA-specific manner. Certain TAAs may only need one or two components of the three modifications to generate a potent vaccine. To make good use of this platform, several major issues need to be further investigated. First, what are the optimal combinations among the different molecular modifications? Preclinical and clinical testing is required to determine the vaccine efficacies of different combinations of SS-mediated, LAMP-1-/

DC-LAMP-mediated, or ubiquitin-/p62-/MITD-/γ-tubulin/DD-mediated molecular modifications. Second, what is an optimal format to deliver the three-pronged vaccine platform? To maximize presentation of antigenic epitopes derived from enhancing degradation and trafficking, full-length or near-full-length TAAs may be preferred over short stretches of peptides. Furthermore, DNA or mRNA encoding the molecularly modified TAAs may be the choice of vaccine format given their advantages for generation and manufacture. Third, which vaccine adjuvants will produce potent vaccines that induce responses in all three cellular compartments? Given the intense interest in developing TLR ligands and other innate immune activators into clinically approved adjuvants for cancer immunotherapy,[96] it is expected that new adjuvants will be available for optimal combination with our proposed new vaccine platform.

Conclusions

Recent studies on neoantigen identifications demonstrate that neoantigens are rarely presented on HCC tumor cells and suggest that it is necessary to target TAAs for immunotherapy of HCC and other cancers with intermediate and low TMBs.[21] However, numerous previous clinical trials in HCC and other cancers targeting TAAs largely failed to obtain clinical efficacy. We analyzed the potential pitfalls of TAA-based HCC immunotherapy trials and proposed a perspective on a new three-pronged vaccine platform to enhance TAA-based immunotherapy. Given that other tumors with intermediate and low TMBs face similar challenges, our proposed strategy is likely widely applicable in other types of tumors. We envision that a powerful cancer vaccine platform targeting TAAs can be used for almost all types of tumors, including solid and blood cancers at all stages. For cancer patients at early stage, cancer vaccines may be used alone to induce specific antitumor immunity to prevent recurrence. For patients at intermediate stage, cancer vaccines may be used in combination with other immunotherapeutic approaches to produce PR and preferably CR effects. For patients at late stage, cancer vaccines, combined with other therapeutic means, serve to prolong their life.

Author Contributions: Y-W.H., J.O.L., and L.L. were responsible for the conception. Y-W.H. was drafting and revising the paper. H.Z. generated the figures, M.Z., Q-T.W., S-N.S., X-K.G., H.Y., Y.W., S-Y.L., and Y.L. contributed to conception, discussion and review of the paper.

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