An all-in-one adjuvanted therapeutic cancer vaccine targeting dendritic cell cytosol induces long-lived tumor suppression through NLRC4 inflammasome activation

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Article

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Abstract

Therapeutic cancer vaccines (TCVs) should induce robust tumor-specific T cell responses. To achieve this, TCVs incorporate T cell epitopes and strong adjuvants. Here, we report an all-in-one adjuvanted cancer vaccine platform, which targets intracellular compartment of antigen presenting cells and subsequently induces effective cytotoxic T cell responses. We screened a novel peptide (DCpep6) that specifically binds and transmits into CD11c⁺ cells through *in vivo* phage biopanning. We then engineered a protein-based TCV (DEF) consisting of DCpep6 (D), an optimized HPV E7 tumor antigen (E), and a built-in flagellin adjuvant (F) as a single molecule. DEF was stably expressed and each component was functional. *In vivo* administered DEF rapidly biodistributed in draining LNs and internalized into CD11c⁺ cells. DEF immunization elicited strong anti-tumor T cell responses and provided long-term survival of TC-1 tumor implanted mice. The DEF-mediated anti-tumor effect was abolished in NLRC4^{-/-} mice. Taken together, we propose a protein-based all-in-one TCV platform that intracellularly co-delivers tumor antigen and inflammasome activator to DCs to induce long-lasting anti-tumor T cell responses.

Introduction

Therapeutic cancer vaccines (TCVs) that elicit de novo immune responses or amplify existing tumor antigen (TA)-specific T cell responses serve a crucial pillar of cancer immunotherapy (1–7). TCVs should induce regression of existing tumors and long-lasting anti-tumor immune responses (1). Though many types of TCVs were tested in diverse preclinical and clinical settings, they still need prodigious improvements for clinical application (2–7).

Dendritic cell (DC) is a professional antigen presenting cell (APC) priming and activating anti-tumor T cell responses (8, 9), and coordinate innate and adaptive immune responses through recognition of PAMP/DAMP (10). PRR-mediated DC activation induces high levels of co-stimulatory molecules and cytokines, which in turn enhance antigen-specific immune responses. The DC activation also results in enhanced antigen processing and presentation mostly through MHC class II restriction (11). DCs serve a major target for PRR-activating vaccine adjuvants. Many strategies have been developed to modulate them in cancer immunotherapy (12). To achieve efficacious anti-tumor responses, TCVs should induce TA-specific CD8⁺ cytotoxic T cells (CTLs). CD8⁺ CTLs are more robustly induced through cross presentation of TA epitopes by DCs. For the cross presentation, the antigen should be degraded by cytosolic proteasome and presented in association with MHC class I molecules. PRR-dependent activation of DCs would not be sufficient to activate the cross presentation, and rather have propensity to potentiate phagocytosis and processing of antigens towards MHC class II restriction. Introduction of protein TAs directly into cytosol, combined with DC activation through PRR stimulation, should significantly enhance the cross presentation. To this end, mRNA-based TCVs are tried against several tumors with limited efficacy (13, 14). Intracellularly translated TAs would be apt to be presented through MHC class I restriction and mRNA by itself would stimulate PRR-mediated immune cell activation. Selective targeting of mRNA carriers to antigen presenting cells will improve anti-tumor CTL responses

(13, 15). By the same token, specific targeting and intracellular transmission of protein antigens to DCs should significantly intensify cytotoxic immune responses.

Monoclonal antibodies specifically binding to DC markers significantly improved immune responses to co-formulated antigens (2, 16). Monoclonal antibody would be bulky and relatively expensive to be applied to versatile vaccines. DC-targeting peptides were also screened and applied to many types of vaccines (17, 18). But it is not sure whether those DC-targeting ligands would preferentially potentiate CTL responses against TAs. As for peptides, various panning techniques have been described for the delivery of cargo molecules to DC (19, 20). However, most of these techniques employed the *in vitro* panning of bacteriophage library. In general, despite the elaborate negative pre-selection of library-ligands against other immune cells such as macrophages, T cells, and B cells, there always exists the possibility of selecting non-functional peptides *in vivo*.

Given that flagellin is a unique PRR agonist capable of activating both extracellular TLR5 and intracellular NLRC4 inflammasome pathways (2, 21–24), engineered cell permeable flagellin would possibly activate both pathways. Furthermore, flagellin could be covalently fused with antigens to make built-in adjuvanted vaccines (25, 26). We previously reported that *Vibrio vulnificus* FlaB serves excellent adjuvant in combination with a variety of vaccine antigens of pathogens and cancer (27–30). We also showed that FlaB secreted in the tumor tissue by an engineered *Salmonella* was able to convert the tumor microenvironment towards immunostimulatory (31).

To develop a novel protein-based TCV platform, we first identified a novel DC-permeating peptide by *in vivo* biopanning of a bacteriophage library. Interestingly, the peptide named DCpep6 exhibited dual activity of specific DC targeting and permeation into the intracellular compartment. We then generated *in vivo* DC-targeting all-in-one built-in adjuvanted engineered TCV composed of DCpep6, HPV16 E7ΔNLS tumor antigen and FlaB (DCpep6-4xE7ΔNLS-FlaB; DEF). DEF induced a long-lasting tumor suppressive cellular immune response. And the NLRC4 inflammasome signaling pathway played a dominant role for the vaccine efficacy.

Results

Identification of CD11c⁺ cell-targeting DCpep6 peptide that specifically bind to intracellular compartment by *in vivo* biopanning

Molecules having *in vivo* DC-targeting function may serve an efficacious tool for eliciting more potent immune responses in the development of better vaccines and immunotherapies. In the present study, we carried out *in vivo* biopanning of bacteriophage peptide display library in search for a novel peptide that effectively targets and permeates into DCs *in vivo*. To identify DC-targeting peptides that specifically bind and permeate to intracellular compartment of DCs, 2.5×10^{10} phages were administered intranasally to BALB/c mice, and CD11c⁺ cells were separated from cervical LNs (cLNs) by magnetic cell sorting 6 hours after the phage administration. Surface-bound phage particles were removed by pH shift to harvest

intracellular phage, and then the cells were disrupted by 5 cycles of freezing and thawing. The eluted phages were expanded *in vitro* and another round of *in vivo* biopanning was performed (Figure 1A). After the biopanning of the bacteriophage library, phage recovery rate was calculated (Figure 1B), and the DNA sequence of randomly selected 50 phages was determined by dideoxy sequencing. As shown in Supplementary Figure 1, after two rounds of *in vivo* biopanning, the overall diversity of the peptide sequence was reduced, and over-presented peptide sequences were obtained. Among the sequenced 50 clones, we selected multiply sequenced 6 peptides (DCpep1~6) to test DC-targeting ability (Figure 1C). The DC-targeting efficiency of the 6 peptides was determined by measuring the cellular uptake of the biotinylated peptides by BMDCs using flow cytometry. Among the six peptides, DCpep 3, 4 and 6 efficiently targeted CD11c⁺-BMDCs, but the targeting ability of other three peptides didn't significantly differ from negative control (Supplementary Figure 2A). Among three positive peptides, DCpep6 exhibited highest specific signals in DCs from various sources (Figure 1D). DCpep6 was chosen for further studies. When BALB/c mice were intranasally administered with DCpep6 or scrambled peptides, only DCpep6 clearly localized in the cytoplasm of CD11c⁺ cells from cLNs (Figure 1E). Collectively, we identified a CD11c⁺ cell-targeting DCpep6 peptide that specifically permeates into the intracellular compartment through *in vivo* biopanning.

Development and characterization of a protein-based all-in-one engineered TCV containing DCpep6, HPV E7ΔNLS and built-in flagellin adjuvant (DCpep6-4xE7ΔNLS-FlaB; DEF)

Previously, we had shown that FlaB significantly potentiated cell mediated immunity against MHC class I restricted E7 peptide epitope using TC-1 mouse tumor models (28, 30). Since peptide tumor vaccines should be matched with hosts' MHC class I types, universal peptide vaccine is very hard for clinical application. If tumor antigen could be directly permeated into the cytosol of DCs, CTL activating epitopes will be processed through proteasome pathway and subsequently presented on the surface in the context of MHC class I molecule. We also have demonstrated that the immunization with FlaB-antigen hybrid protein induced much more potent protective immunity than FlaB+antigen mixture formulation (25, 26). If it is possible to make the FlaB built-in adjuvanted fusion vaccine more efficiently cross-presented by DCs, we would expect significantly enhanced tumor suppression mediated by CTL responses. Fortunately, we identified the DCpep6 peptide having the ability of specific targeting and permeation to CD11c⁺ DCs. We hypothesized that a TCV platform composed of DCpep6, tumor antigen polypeptide and built-in flagellin adjuvant should induce stronger anti-tumor CD8⁺ CTL responses. To this end, HPV E7 polypeptide deleted of nuclear localizing sequence was selected as the model TA. HPV infection is associated with anogenital and oropharyngeal cancers (32), and E7 protein is an immunogenic TA (33, 34). It has been reported that N-terminal conserved regions (CR-1 and CR-2) are involved in the nuclear localization of the E7 protein (34, 35). Therefore, to avoid the risk of tumorigenesis by intracellularly introduced full length E7 (E7_{FL}), we deleted the N-terminal nuclear localization sequence to generate E7ΔNLS (Figure 2A, Figure 2B and Supplementary Figure 3). We then evaluated TA efficacy of the E7ΔNLS by measuring E7ΔNLS-mediated anti-tumor activity in a TC-1 mouse model. As shown in Figure 2C, E7ΔNLS and E7_{FL} induced similar level of tumor suppression. The FlaB-adjuvanted group (E_{FL}+F or E+F) showed significantly enhanced tumor

suppression compared with E7 Δ NLS (E) or E7_{FL} (E_{FL}) immunization. FlaB alone did not manifest any suppressive effect. These results attest that E7 Δ NLS will serve an optimal tumor antigen in combination with FlaB.

Next, we generated an all-in-one TCV composed of DCpep6, 4xE7 Δ NLS, and FlaB (Figure 2A, Figure 2B and Supplementary Figure 3). In the expression construct, we had the E7 Δ NLS repeated 4 times to make the antigen polyvalent and have comparable size with FlaB, which was supposed to render the resulting polypeptide more stable and consequently enhance immunogenicity. The resulting all-in-one TCV has the DCpep6-4xE7 Δ NLS-FlaB (DEF) structure. We also generated 4xE7 Δ NLS-FlaB (EF) as a non-DC targeting vaccine control. The recombinant proteins were verified by SDS-PAGE and Western blot analysis using E7_{FL}- or FlaB-specific mouse antiserum (Figure 2B). Because the fusion proteins contain structurally and functionally different polypeptides in a single molecule, maintaining the active conformation should be crucial for proper *in vivo* function. To prove proper structure formation and functionality, we determined TLR5-stimulating activity of the fusion proteins. The DEF or EF showed dose-dependent TLR5-stimulating activity (Figure 2D). The EC₅₀ of DEF (0.32 nM) or EF (0.38 nM) was lower than that of FlaB (0.73 nM), suggesting fusion partner protein does not interfere TLR5-binding activity and even has the TLR5 binding motif exposed more.

To investigate whether DCpep6 of the DEF facilitate permeation into intracellular compartment of CD11c⁺ cell, cellular uptake of EF or DEF into BMDCs was assessed by flow cytometry and confocal microscopy. Mouse BMDCs were incubated with various concentrations of EF or DEF for 120 min and intracellular EF or DEF was stained with anti-FlaB antibody for flow cytometric analysis. As shown in Figure 2E, the concentration-dependent cellular uptake of DEF was noted. In contrast to DEF, intracellular EF was scarcely detected with tested concentrations. To observe cellular uptake of DEF using confocal microscope, BMDCs or CD11c⁺ RAW274.7 cells which express CD11c on their surface (Supplementary Figure 4) were incubated with 20 μ g/ml of EF or DEF for 120 min. DEF located in the cytoplasm of CD11c⁺ cells and accumulated as speckles (Figure 2F). We also tested whether DEF could successfully target and permeate into human DC populations. Human immature DCs were prepared as previously described (36). To prepare human DCs, CD14⁺ monocytes were isolated from human peripheral blood mononuclear cells using a magnetic activating cell sorting system and then the CD14⁺ cells were differentiated into human immature DC populations by culturing in the presence of rhGM-CSF and rhIL-4 for 6 days (Supplementary methods). Resulting human immature DCs were incubated with EF or DEF and then cellular uptake of DEF was determined by FACS and confocal microscopy. As shown in Supplementary Figure 5, cellular uptake of DEF was significantly higher than that of EF by both FACS and microscopical analyses, indicating the DEF can serve a promising TCV platform for human use also. The *in vitro* generated human immature DCs, compare with mouse BMDCs, required lower concentration of DEF and EF for the internalization assay, suggesting the same TCV format will work more efficiently in human trials. Internalization of DEF was significantly decreased by cytochalasin B, suggesting energy consuming actin polymerization is a requisite. However, energy poison sodium azide or lowering temperature to 4°C could not fully abolish permeation, which strongly indicates that energy non-

consuming physical trespassing could have been mediated by DCpep6 (Figure 2G). These results indicate that DEF could be efficiently taken up by DCs and could permeate to cytosolic compartment efficiently, which will increase the chance for proteasomal degradation and subsequent MHC class I restricted presentation of protective epitopes to CD8⁺ T cells.

Biodistribution and *in vivo* DC targeting of DCpep6-4x Δ E7 Δ NLS-FlaB (DEF)

To visualize *in vivo*-administered EF or DEF, we generated fluorescein-conjugated proteins as previously described (28), and assessed the distribution in draining LNs and DC-targeting of DEF *in vivo*. Mice were subcutaneously injected with 50 μ g of EF-FNR675 or DEF-FNR675 in the groin, and draining inguinal LNs (iLNs) were isolated at 1, 6, 12, and 24 h post injection. As shown in Figure 3A, fluorescent signal was detected in the DEF-administered mice from 1-hour after administration. The fluorescent signal reached peak at 6 hours and dissipated at 24. To confirm *in vivo* DC targeting, fluorescein-conjugated proteins were administered and CD11c⁺ cell in the iLNs was analyzed by flow cytometry. Six hours after subcutaneous injection, fluorescence signal in CD11c⁺ cells was significantly higher in DEF- FNR675 group (Figure 3B). These results clearly testify that subcutaneously administered DEF efficiently reaches draining LNs (dNLs) and specifically targets CD11c⁺ cells there.

All-in-one therapeutic cancer vaccine (DEF) induces long-lasting tumor suppression

We investigated the tumor suppressive efficacy of DEF vaccination using the previously described TC-1 implantation model (28, 30, 37). To compare anti-tumor efficacy of DEF with well characterized CTL-inducing peptide vaccine (28, 30), a group of mice was immunized with HPV16 E7 CTL peptide (amino acids 49–57: RAHYNIVTF) plus FlaB (E7 Pep+F). As shown in Figure 4, the E+F-immunized mice showed significantly longer survival than E-group (* $P < 0.05$ for E vs E+F). The E+F immunized mice showed comparable level of survival with E7 Pep+F ($P > 0.05$). Notably, the DEF-immunized mice showed significantly longer survival than E+F-group (* $P < 0.05$ for E+F vs DEF) with retarded tumor growth. The median survival of DEF group (102 days) was extended as much as 20 days compared with E+F (82 days) or E7 Pep+F (82 days). Collectively, DEF all-in-one vaccine exerted superior *in vivo* tumor suppression than antigen plus flagellin mixture formulation, and flagellin serves a potent built-in adjuvant enhancing therapeutic efficacy.

DEF-induced stronger anti-tumor immune responses were mediated by enhanced CTL activation

To investigate characteristics of DEF-induced immune responses, we treated mouse BMDCs with 500 ng/ml of DEF or EF for 24 hours then measured expression of costimulatory molecules on CD11c⁺ cells. DEF induced significantly enhanced expression of CD80 and CD86 molecules on CD11c⁺ BMDCs while EF did not (Figure 5A). Only DEF efficiently activated APCs while the EF showed negative control level CD80 and CD86, which suggested that NLR4 inflammasome pathway should have played major role for

the activation at given concentration. To assess cytoplasmic localization of DEF after DEF uptake, CD11c⁺ RAW264.7 cells (Supplementary Figure 3) were incubated with 10 µg/ml of DEF and stained with lysotracker and anti-E7 antibody. DEF uptake was gradually increased over time and most of cytosolic DEF co-localized with lysotracker and some DEF appeared outside the lysosomal compartment (Figure 5B). After 2 h of DEF incubation, the co-localization intensity decreased, indicating that DEF was dissociated from the lysotracker, which may have resulted in further permeation from lysosome to cytosol. These results strongly attest that cytosolic DEF should have been effectively processed by proteasome for cross-presentation.

Next, to determine DEF-mediated Ag-specific T cell immune responses, tumor bearing C57BL/6 mice were immunized with E, EF or DEF (Figure 5C) and measured representative CTL epitope-specific tetramer-positive CB8⁺ cells in the peripheral blood. As shown in Figure 5D, DEF treated mice showed significantly higher level of tetramer-positive cells compared with PBS, E, or EF treatment. We also determined CTL epitope (amino acids 49–57: RAHYNIVTF)-specific IFN-γ production in spleen and TDLNs in tumor-bearing mice. DEF immunization significantly enhanced systemic and local IFN-γ production by CTL peptide (Figure 5E). This tumor-specific T cell immune responses were also detected in tumor non-bearing C57BL/6 mice (Supplementary Figure 6), indicating the TCV can actively induce TA-specific immune responses *in vivo*. These results clearly indicate that DEF can induce strong anti-tumor CTL responses *in vivo* through cross presentation.

DEF-mediated long-term anti-tumor immune response is abolished in NLRC4^{-/-} mice

Flagellin can activate both extracellular TLR5 and intracellular NLRC4 pathways. To investigate which PRR signaling pathway played dominant role in the DEF-mediated long-term survival, we compared long-term survival of DEF- or EF-immunized tumor-bearing mice in WT, TLR5^{-/-} or NLRC4^{-/-} mice. Since EF did not efficiently permeate to the cytosol of CD11c⁺ cells (Figure 2F, 2G, & 2H), we employed the EF as a control vaccine, which may not activate intracellular NLRC4-inflammasome pathway. The tumor growths in TLR5^{-/-} or NLRC4^{-/-} mice were similar with wild-type mice, indicating TLR5 or NLRC4 knock out did not influence TC-1 tumor growth (Supplementary Figure 7A). EF or DEF immunizations significantly extended survival of tumor-bearing mice, and DEF induced significantly longer survival than EF (* $P = 0.0357$ for EF vs DEF in WT) in WT mice (Figure 6). No significant difference was detected between PBS and EF in the TLR5^{-/-} background, suggesting non-permeating EF dominantly utilize TLR5 for the adjuvant activity ($P = 0.0620$ for PBS vs EF in TLR5^{-/-}). DEF-immunization in TLR5^{-/-} mice induced significantly longer survival compared with PBS group (** $P = 0.0018$ for PBS vs DEF in TLR5^{-/-}). Notably, DEF-mediated long-term survival of the tumor-bearing mice was abolished in NLRC4^{-/-} mice ($P = 0.2538$ for PBS vs DEF in NLRC4^{-/-}). This result indicates that the adjuvant function in DEF would have been mainly executed through NLRC4 inflammasome-mediated activation, while TLR5 signaling played minor roles. As shown in Supplementary Figure 7B, in contrast to DEF-immunized WT mice showing 40% survival until 100 days, DEF immunization in TLR5^{-/-} mice resulted in 100% death at 85 days after the tumor implantation.

TLR5^{-/-} mice died faster than WT while statistical significance was not noted between two groups, which suggests TLR5 signaling may have played some role while the dominant player was NLRC4 signaling pathway.

Finally, we proved that the NLRC4 inflammasome pathway played crucial role in inducing the CD8⁺ CTL response using an *in vitro* co-culture experiment. DEF- or EF-pulsed BMDCs isolated from WT or NLRC4^{-/-} mice were co-cultured with CD8⁺ T cell isolated from TC-1 tumor-bearing mice and T cell proliferation was assessed by the CFSE dilution assay. As shown in Figure 6B, DEF-pulsed WT BMDCs significantly enhanced division index of CD8⁺ T cell from tumor-bearing mice compared to EF-pulsed WT BMDC (** $P < 0.01$ for EF-pulsed WT BMDCs vs DEF-pulsed WT BMDCs). Notably, DEF-mediated potentiation of CD8⁺ T cell proliferation was abolished in NLRC4^{-/-} BMDCs. Collectively, these results clearly corroborate that NLRC4-mediated inflammasome signaling pathway was responsible for the cross presentation of E7 antigens and subsequent CD8⁺ CTL responses in the DEF TCV immunized animals.

Discussion

Here, we report the development of a protein-based all-in-one engineered TCV platform consisting of DC-targeting peptide, optimized TA, and inflammasome activating built-in adjuvant to efficiently enhance cross presentation and subsequent robust CTL activation. First, we identified a novel CD11c⁺ cell-targeting DCpep6 peptide that specifically binds to intracellular compartment employing *in vivo* biopanning. An all-in-one engineered TCV consisting of DCpep6, a four-times repeated E7ΔNLS, and FlaB (DCpep6-4xE7ΔNLS-FlaB; DEF) was generated using the recombinant DNA technology. DEF recombinant protein maintained biological functionality and showed favorable pharmacokinetics distributing in draining LNs (dLNs) and internalizing into CD11c⁺ cells to be cross presented to CD8⁺ T cells. Flagellin molecules should have been stabilized to be expressed under very diverse physicochemical conditions throughout the long history of evolution. This may partly explain why flagellin fusion hybrid proteins exhibit good pharmaceutical properties (25, 26). We also confirmed that the all-in-one typed TCV (DEF) successfully internalize into human DCs with high efficiency. The DEF platform would be applied universally to the development of CTL-inducing vaccines/immunotherapeutics regardless of host HLA types since fused target antigens will be endogenously processed to be presented with MHC class I restriction.

Direct targeting of vaccine antigens to DCs *in vivo* should provide attractive way to generate appropriate responses in targeted immune compartments. In general, cell targeting peptides are screened using phage or phagemid-displayed peptide libraries (38, 39) through *in vitro* biopanning (19, 20). However, substantial fraction of the candidate peptides identified by *in vitro* panning would be dropped after animal studies due to ineffectiveness *in vivo*. In this study, we established an *in vivo* bacteriophage panning method that resulted in enrichment of DC permeating DCpep6 peptide by just two rounds of panning. We used a pCL3-based phagemid library that has 14-mer peptide display with the pIII gene of M13 phage. The precision of the selection and the amplification process was demonstrated by the fact

that none of the peptide sequences obtained in round 1 repeated (Supplementary Figure 1B). After both rounds of biopanning, unamplified phage pool was used for sequence analysis to avoid amplification advantage that some phage may have. The stringency of *in vivo* biopanning was reflected in the small number of phage pool that was obtained after the first round of panning. To select intracellular phages only, we harvested bacteriophage located only inside of CD11c⁺ cells while surface-bound phage particles were rigorously removed by pH shifting (Figure 1A). The intracellular phages should be actually targeting DCs or merely taken into the cell through phagocytosis. The probability of latter would be diminished in the second round of panning where the actual targeting phage would accumulate better into the intracellular compartments than the phage that accumulates by phagocytosis only. Among the selected 6 peptides, DCpep6 had highest targeting ability and better cytoplasmic accumulation in DCs.

Adjuvant can potentiate and modulate immune reactions triggering specific CTL responses against Ags (2, 40). Broad range of compounds has adjuvant activity and adjuvants can act in several nonmutually exclusive ways; majority of the adjuvant effects seems to employ APCs. It has been reported that various commonly used adjuvants, such as alum, saponin, emulsions and TLR agonists happened to activate inflammasomes pathways (41–46). Intracellular flagellin molecules are sensed by cytosolic NAIP proteins, which physically associate with NLRC4 to form a inflammasome complex (47). The matured NAIP/NLRC4 inflammasome activates caspase-1 and IL-1 β and IL18 secretion (47, 48). NAIP5 or 6 protein can detect only intracellular flagellin that derived from intracellular bacteria or delivered through specialized secretion system by virulent bacteria (49, 50). In this study, we clearly showed that DCpep6 deliver 4xE7 Δ NLS-FlaB cargo into CD11c⁺ cell cytoplasm and subsequently activate NLRC4-inflammasome. The DEF-mediated long-term anti-tumor immune response is abolished in NLRC4^{-/-} mice but not in TLR5^{-/-} mice, which indicate NLRC4-inflammasome pathway plays a pivotal role for the induction of specific CTL responses. DEF should be the first, to our knowledge, all-in-one NLRC4-stimulating adjuvanted TCV.

Triggering of TA-specific CTL responses serves an important requisite for successful combinatorial cancer immunotherapy against established tumors (51). The triggered TA-specific CTL responses help epitope expansion when combined with appropriate immunotherapeutic modalities. As far as TA-specific CTL responses are required for tumor suppression, TCV should serve an essential component of cancer immunotherapy (51). The major type of TCVs include peptide-, mRNA-, DNA-, viral vector containing mRNA-, or yeast-based formulations (52). However, many clinical trials of TCVs were largely unsuccessful to bring clinical benefits (52–54). Peptide-based vaccines have intrinsic limitations for use in clinical setting because of their poor *in vivo* pharmacokinetics and dependence on host HLA types. To develop effective peptide-based cancer vaccine, MHC epitope prediction and further wet-bench validation based on *in vitro* binding assay should be accompanied. In this study, we showed that functionally optimized protein-based TCV, encoding all the possible T cell epitopes that would be presented by a wide variety of MHC class I types, could induce strong anti-tumor activity and long-term survival of tumor-bearing mice (Figure 4). The HPV E7 protein, a transcriptional regulator modulating the retinoblastoma protein (pRb)/E2F system, is constitutively expressed in most HPV-induced neoplasias (33, 34). Endogenous E7

is predominantly observed in the nucleus (34, 55), and the N-terminus of E7 are similar to those in other viral oncoproteins, reflecting a possible role in transformation and transactivation. To avoid of safety issue regarding unexpected cellular responses induced by wild-type E7 oncogene, we generated HPV16 E7 E7 Δ NLS variant with deletion of the N-terminal nuclear localization sequence (i.e., E7 Δ NLS). E7 Δ NLS showed no difference compared with WT E7 for the induction of tumor suppressing immune responses in C57BL/6 mice. In an effort to develop optimal tumor antigen for the DCpep6- and FlaB-based all-in-one cancer vaccine, we tried to develop 2x-, 3x-, 4x-, and 6x-E7 Δ NLS antigens. Of the several combinations of recombinant proteins, DCpep6-4xE7 Δ NLS-FlaB showed the most stable and efficient protein expression in an *Escherichia coli* system.

Collectively, we offer a vaccine platform; an *in vivo*-DC-targeting all-in-one cancer vaccine (DEF). DEF administered *in vivo* preferentially targeted the intracellular compartment of CD11c⁺ cells and activated anti-tumor immune response via NLRC4-inflammasome signaling-mediated cross presentation.

Material And Methods

Mice, phagemid library, cell lines and media

BALB/c female mice (6-8 weeks) were purchased from Charles River Laboratories and housed under SPF conditions at CVRDC, Chonnam National University Hwasun Hospital, South Korea. Seven- to eight-week-old female SPF C57BL/6 mice were purchased from the ORIENT (Seongnam-si, Gyeonggi-do, Korea). TLR5^{-/-} (28, 30) and NLRC4^{-/-} mice, all on the C57BL6 background were bred in our animal facility. The NLRC4^{-/-} mice were obtained from Dr. Tatsuya Saitoh of Osaka University. The CPL3 peptide phagemid library (Cosmix Molecular Biologicals GmbH, Braunschweig, Germany) (56) was used for two rounds of biopanning. Phages were rescued with M13K07 helper phage and amplified in *Escherichia coli* TG1(λ). TC-1 cell line was maintained cell line was maintained in RPMI 1640 medium (Life Tech-nologies, Grand Island, NY, USA) containing 10% fetal bovine serum (Hyclone, Logan, UT) and 1% penicillin/streptomycin (Life Tech-nologies, Grand Island, NY, USA). Raw264.7 cell line was purchased from the ATCC (Rockville, MD, USA) and maintained in RPMI 1640 medium (Life Tech-nologies, Grand Island, NY, USA) containing 10% fetal bovine serum (Hyclone, Logan, UT) and 1% penicillin/streptomycin (Life Tech-nologies, Grand Island, NY, USA).

Ethics statement

All animal experimental procedures were performed with approval from the Chonnam National University Institutional Animal Care and Use Committee under protocol CNU IACUC-H-2020-31. Animal research facility maintenance and experimental procedures were carried out in strict accordance with the guidelines of the Animal Welfare Act legislated by Korean Ministry of Agriculture, Food and Rural Affairs.

***In vivo* biopanning**

For *in vivo* biopanning, 10 µl of phagemid library (10^{12} pfu/mL) was given by intranasal instillation into each nostril of BALB/c mice. Library phage solution was instilled into three mice. After 6 hours of *in vivo* panning mice were euthanized and cervical lymph nodes (cLNs) were harvested and kept on ice until processed. cLNs were washed at least three times in 0.2% PBST (phosphate buffered saline containing 0.2% Tween 20) followed by one washing in 100 µl of 0.2 M glycine-HCl buffer, pH 2.2. Finally, cLNs were placed in same buffer and incubated at RT/10 min. Glycine buffer was replaced by cold 1 x PBS. Capsules of cLNs were broken gently and cell suspension was passed through 40 µm cell strainer (Falcon, 352340). Resulting single cell suspension was centrifuged and treated with glycine buffer for 5 min after which cells were washed in PBS and suspended in MACS buffer. Cell suspension was admixed with CD11c⁺ MicroBeads MACS (Miltenyi Biotec, 130-052-001) and DCs were purified by application of a magnetic field, as per manufacture's recommendations. Purified DCs were subjected to glycine buffer for another 5 min after which cells were washed in PBST (1 x PBS plus 0.05% TWEEN20) at least three times. Finally, to elute the intracellular phage, cells were suspended in 100 µl glycine buffer and disrupted by five freeze thaw cycles with in-between high-speed centrifugations (13000 rpm/10 min). Disruption of cells was confirmed by trypan blue staining. At the end of cycle, glycine buffer was neutralized by addition of equal volume of 1x PBS (pH 7.5). The resulting preparation was centrifuged and supernatant containing the eluted phage was harvested and stored at -20°C until use. Phage titer in the preparation was estimated by standard protocols. Individual bacterial clones containing phagemid were grown to an optical density (OD₆₀₀) equivalent to 0.5 in ampicillin (200 µg/mL) containing Luria Broth. Equal volumes of all 45 bacterial clones were mixed together and phages were eluted using M13K07 helper phage for 13 hours. Phage from cell free supernatant was recovered by PEG/NaCl precipitation (4°C/overnight). In the similar manner to round 1 (R1), round 2 (R2) panning was performed using amplified phage harvested after R1.

Preparation of CD11c⁺ mouse BMDC cells

Bone marrow–derived dendritic cells (BMDCs) were prepared as previously described (37, 57). Briefly, bone marrow (BM) cells were isolated by flushing femurs and tibias of C57BL/6 mice and red blood cells (RBC) were depleted by ACK lysis (Gibco, A10492-01). The cells were centrifuged again and strained through a 70 µm filter before being re-suspended in RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin. The cells were resuspended in complete RPMI 1640 culture medium containing GM-CSF (10 ng/mL; R&D systems, 415-ML-010) and IL4 (10 ng/mL; R&D systems, 415-ML-010), inoculated into a cell culture dish, and cultured at 37°C, 5% CO₂. The culture medium was refreshed every two days. On day 7, loosely bound DCs were collected by gentle pipetting. Splenic or cLNs-derived DCs were prepared by CD11c⁺ MicroBeads MACS (Miltenyi Biotec, 130-052-001) following the manufacturer's instruction.

DC-targeting of peptides

DC-targeting of the selected peptides was determined by flow cytometry. To visualize intracellular peptides in CD11c⁺ cells, BMDCs were treated with biotinylated peptides and then stained with fluorescence-labelled streptavidin. For side chain biotinylation, lysine (K) residue was required in the peptides. Since not all peptides contained lysine, peptides (DCpep1, 2, 3, 4, and 5) were modified and arginine (R) in those peptides was replaced with lysine. Peptides were synthesized using 9-fluorenylmethoxycarbonyl chemistry, purified using high pressure liquid chromatography to >95% purity, and biotinylated in the side chain by AnyGen Inc. (Gwangju, South Korea). Peptides were dissolved in appropriate solvents as per manufacturer's instructions to the final concentration of 0.5 mg/ml. To test CD11c⁺ cell targeting by the synthesized peptides, cLNs cells, splenic DCs and BMDCs were treated with peptides for 2 hours in RPMI 1640 medium. Splenic CD11c⁺ and BMDCs cells were prepared as described above. After incubation with peptides, cells were harvested, washed and stained with anti-mouse CD11c⁺ APC-conjugated Ab (eBioscience, Clone: N418, 170114-82) and streptavidin-AF488. Cells were FACS analyzed by gating upon CD11c⁺ cell population. To determine targeting of DCpep6 peptide to cLN after intranasal administration, peptide at appropriate concentrations in a total volume of 10 μ l/nostril was given intranasally. A scrambled peptide previously shown to be non-specific to DCs was used as a negative control. At 6.5 hours post-inoculation, mice were sacrificed and their cLNs were harvested. Single cell suspensions were prepared by gentle tearing of the LN capsule. After gentle washing in 1 x PBS, cells were stained with anti-mouse CD11c⁺ APC-conjugated Ab (eBioscience, Clone: N418, 170114-82) and streptavidin-AF488, and FACS analyzed.

Construction and purification of 4xE7 Δ NLS-FlaB (EF) and DEF (DCpep6-4xE7 Δ NLS-FlaB; DEF)

To produce recombinant E7 Δ NLS protein, an HPV16 E7 variant with deletion of the N-terminal nuclear localization sequence (amino acids 1-37), we constructed a pET30a⁺ plasmid (Novagen, 69909 - Merck Millipore) containing a DNA fragment corresponding to the truncated E7 sequence (amino acids 38-98). The insert DNA fragment was amplified by PCR using codon-optimized DNA template and the following primer set: forward 5' GG GAA TTC CAT ATG ATC GAT GGG CCG GCC GGT 3' and reverse 5' CCG CTC GAG TGG TTT CTG GGA GCA TAT 3' (Supplementary Table 1). Recombinant FlaB was produced as previously (27). For the production of recombinant fusion protein of 4xE7 Δ NLS-FlaB (EF) (Figure 2A), we firstly cloned the synthesized 4xE7 Δ NLS DNA fragment (*Nde*I-E7 Δ NLS-*Eco*R-E7 Δ NLS-*Sal*I-E7 Δ NLS-*Sal*I-E7 Δ NLS-*Hind*III) into a pET30a⁺ plasmid with overhangs recognized by specific restriction enzymes (REs) of *Nde*I and *Hind*III. Next, a DNA fragment of FlaB was generated by PCR using a primer set: forward 5' CCC AAG CTT ATG GCA GTG AAT GTA AAT AC 3' and reverse 5' CCG CTC GAG GCC TAG TAG ACT TAG CGC TG 3' with *Hind*III and *Xho*I overhangs, respectively (Supplementary Table 1). The DNA fragment of FlaB was further digested by *Hind*III and *Xho*I REs and fused with

4xE7ΔNLS at the C-terminal, generating pET30a⁺::4xE7ΔNLS::FlaB (Supplementary Figure3). To generate the DCpep6-4xE7ΔNLS-FlaB (DEF), an DNA fragment of 3xE7ΔNLS::FlaB was cut out from pET30a⁺::4xE7ΔNLS::FlaB by *EcoRI*-*XhoI* REs and cloned into a pET30a⁺ plasmid, making pET30a⁺::3xE7ΔNLS::FlaB. Then, the DNA fragment of DCpep6-E7ΔNLS (*NdeI*-DCpep6-E7ΔNLS-*EcoRI*) was amplified by PCR following primer set: forward 5' GG GAA TTC CAT ATG CGC TTC TTC TGT TTG GGT CCA 3' and reverse 5 CCG GAA TTC AAT TGG ATC GAT GTA CGC TA 3' with *NdeI* and *EcoRI* overhangs, respectively (Supplementary Table 1) and cloned into an pET30a⁺::3xE7ΔNLS::FlaB at the N-terminal to produce pET30a⁺::DCpep6::4xE7ΔNLS::FlaB (Figure 2A and Supplementary Figure3). FlaB was prepared as previously (27, 28). The DNA sequences of the expression vectors were confirmed by the dideoxy-chain termination sequencing method via the MacroGen Online Sequencing Order System (<http://dna.macrogen.com/kor/>). The resulting plasmid was transformed into competent *Escherichia coli* BL21 cells. Protein expression was induced by incubation with 0.2 mM isopropyl-β-D-thiogalactoside (IPTG) for 18 hours at 20°C, and cells were pelleted by centrifugation and stored at -80°C until use. The bacterial cell pellets were lysed with 50 ml of lysis buffer (pH 8; 50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 0.1% Triton X-100, 0.1% Tween and 20 μM phenylmethylsulfonyl fluoride). After centrifugation at 18,000 rpm for 30 minutes, the cell-free supernatant was loaded on a column containing Ni-NTA agarose beads (Qiagen, Hilden, Germany) following the manufacturer's instructions. The purity of the recombinant proteins was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent Western blot analysis with an anti-E7 or anti-FlaB antibodies raised in BALB/c mice. Lipopolysaccharide (LPS) contamination was removed by treatment with TritonX-114 (Sigma-Aldrich, St. Louis, MO) and traces of Triton X-114 were removed by treatment with Bio-Beads™ SM-2 (Bio-Rad Laboratories, Inc., Hercules, CA) according to the manufacturer's instructions by incubation with 0.3 g of Bio-beads™-2 for 1 ml of protein. The residual LPS content was determined by using a gel-clotting Endosafe LAL kit (Charles River, Charleston, SC). The LPS levels in protein preparations were kept below the Food and Drug Administration (FDA) guidelines (less than 0.15 EU/30 g per mouse).

Cellular uptake of EF or DEF

To test uptake of EF or DEF into CD11c⁺ cells, cells were treated with respective concentrations of EF or DEF for 2 hours in free RPMI 1640 medium. The mock cells (treated with PBS) were used as a negative control. After incubation with recombinant proteins, cells were washed twice with cold PBS containing 3% FBS then stained with anti-mouse CD11c⁺ antibody (eBioscience, Clone: N418, 25-0114-82) for 1h at 4°C and fixed with 4% paraformaldehyde (T&I, BPP-9004) for 10 minutes at RT. To detect intracellular localization of the recombinant proteins, the cells were permeabilized using permeabilization kit according to the manufacturer's instructions (Invitrogen, 00-8333-56). Then the cells were stained with anti-FlaB antibodies raised in rabbit and goat anti-rabbit IgG secondary Ab (Invitrogen, A21429). Cells were FACS analyzed by gating upon CD11c⁺ cell population.

Cellular uptake of the engineered cancer vaccine was also determined by confocal microscopy. Various cells were treated with respective concentrations of EF-FNR675 or DEF-FNR675 for 120 minutes in free RPMI 1640 medium. After incubation with the fluorescence-conjugates recombinant proteins, cells were harvested, washed, fixed with 100% methanol for 15 minutes at RT, and stained with anti-mouse CD11c⁺ APC-conjugated Ab (eBioscience, Clone: N418, 170114-82) and DAPI for 1h at RT. After two washes with cold PBS, the cells were mounted with counting solution (Fisher Chemical, SP15-100) and determined the colocalized Ags and CD11c⁺ by confocal microscopy (LSM800).

Intracellular distribution of DEF

To determine the intracellular distribution of DEF in CD11c⁺ cells, RAW264.7 cells were treated with 20mg of DEF for respective time courses in free RPMI 1640 medium. The mock cells were used as a negative control. After incubation with recombinant proteins, cells were washed twice with cold PBS containing 3% FBS then stained with LysoTracker Red DND-99 (Life technologies, L7527) and DAPI for 30 minutes at RT and fixed with 4% paraformaldehyde (T&I, BPP-9004) for 10 minutes at RT. To visualize the intracellular distribution of the recombinant proteins, the cells were then permeabilized using permeabilization kit according to the manufacturer's instructions (Invitrogen, 00-8333-56) and stained with anti-E7 antibodies raised in mouse and goat anti-mouse IgG Secondary Ab (Life technologies, A21424). Cells were further washed and mounted with counting solution (Fisher Chemical, SP15-100). The intracellular distribution of DEF was determined by confocal microscopy (LSM800).

Determination of TLR-5-stimulating activity

To test whether the EF or DEF proteins maintain TLR5-stimulating activities, we measured TLR5-depedent NF-κB stimulating activity of the recombinant proteins by using HEK-BlueTM hTLR5 cells (InvivoGen, hkb-htlr-5) and HEK-BlueTM Detection (InvivoGen, hb-det2) assay systems following manufacturer's instruction. EC₅₀ was calculated using triplicate OD 620nm values for each protein concentration over a wide range of protein concentrations (0.0375 nM to 19.29 nM at the AAT Bioquest website [<https://www.aatbio.com/tools/ec50-calculator>]).

Preparation of FNR675-conjugated EF or DEF

Purified EF (4XE7ΔNLS-FlaB) or DEF (DCpep6-4xE7ΔNLS-FlaB) was mixed with FNR675-NHS ester (BioActs, Korea) at 4 °C and maintained overnight in the dark with stirring. The labeled EF- or DEF-FNR675 was then separated from the unconjugated dye using a centrifugal filter (10 kDa cutoff) (Amicon Ultra®-4, UFC801024), followed by washing (5x) in PBS. Next, the amount of conjugated protein was

determined from the calibration curve of the FNR675-NHS ester using a UV-Vis spectrophotometer (UV-2700, Shimadzu, Japan).

***In vivo* tracing of EF or DEF**

To test *in vivo* distribution of the cancer vaccines, C57BL/5 mice were subcutaneously administered in the inguinal region with PBS, 50 µg EF-FNR675 or 50 µg DEF-FNR675. The fluorescence signals in the draining lymph nodes were determined in appropriate time intervals. The imaging signals were collected using a fluorescence labeled organism bioimaging instrument (FOBI; NeoScience) and the signal intensities were assessed quantitatively in the tumor regions by measuring the maximum photons per second per centimeter squared per steradian (p/s/cm²/sr). To determine *in vivo* DC targeting of EF-FNR675 or DEF-FNR675, draining lymph node cells were further prepared and analyzed by gating upon CD11c⁺ cell population using flow cytometry.

APC activation

Mouse BMDCs were prepared as described above. On day 7–8, loosely bound DCs were harvested by gentle pipetting and were activated with PBS, EF (0.5µg/mL), or DEF (0.5µg/mL) for 24 hours. The cells were stained on ice with fluorophore-labeled antibodies against CD11c, CD80, and CD86 then analyses by flow cytometry.

Tumor implantation and determination of anti-tumor activities of the therapeutic cancer vaccines

Seven- to eight-week-old female SPF C57BL/6 mice were purchased from the ORIENT (Seongnam-si, Gyeonggi-do, Korea). TC-1 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin (Gibco, NY, USA) at 37°C and 5% CO₂. Tumors were established in mice by subcutaneous injection of 5×10^4 TC-1 cells in 100 µl of PBS into the right midflank of each mouse as previously described (30). When the tumor diameter reached approximately 3-5 mm, the tumor-bearing mice were randomly assigned to different treatment groups and the tumor-bearing mice were then peritumorally vaccinated with recombinant proteins under anesthesia (intraperitoneal, i.p.) injection of diluted Zoletil®; tiletamine, zolazepam and Rompun®) at five-day intervals as previously. Tumor growth was monitored every 3 days. The tumor volume was calculated with the formula $V = (\text{tumor length}) \times (\text{tumor width}) \times (\text{tumor height}) / 2$ as previously described (30, 31, 58).

IFN- γ ELISpot assay

Single-cell suspensions from spleen (SPL) or tumor draining lymph nodes (TDLNs) were prepared. 1×10^6 of SPL or 2.5×10^5 of TDLNs cells were seeded into 96-Well Filtration ELISpot plates (Merk, HAMAS4510) and stimulated with 1 μ g/ml E7 CTL short peptide (amino acids 49–57: RAHYNIVTF). Cells stimulated with 10 ng/ml concanavalin A was used as a positive control. After two-day culture of SPL cells or five-days culture of TDLNs cells, IFN- γ -producing cells were detected by using Mouse IFN- γ ELISpot Set (BD Bioscience, 551083) in accordance with the manufacturer's instructions. The spots were developed by using AEC substrate Set (BD, 551951). IFN- γ -producing cells were analyzed using a CTL-ImmunoSpot Analyzer and ImmunoSpot Professional Software version 5.0 (Cellular Technology, Shaker Heights, OH, USA).

Tetramer staining

To analyze E7-specific CD8⁺ T cells, peripheral blood was collected from mice and stained with PE-conjugated HPV16 H-2Db-RAHYNIVTF tetramer reagent (TB-5008-1, MBL) following the manufacturer's instruction. Tetramer staining was combined with surface staining using APC-conjugated anti-mouse CD8 α (BioLegend, Cat # 100712, Clone: 53-6.7) and analyzed by flow cytometry.

CD8⁺ T cell proliferation assay

To determine CD8⁺ T cell proliferation induced by EF- or DEF-pulsed BMDC, CD8⁺ T cells prepared from the TC-1 tumor-bearing mice were treated with CFSE and then co-cultured with the BMDCs. BMDCs were prepared as described above. Briefly, on day 7–8, loosely bound DCs were harvested by gentle pipetting and were activated with 1 μ g/mL of EF or DEF for 24 hours. The CD8⁺ splenocytes from TC-1 tumor-bearing mice were prepared using MagniSortTM Mouse CD8 T Cells Enrichment kit (Invitrogen, 8804-6822) following manufacture's instruction and the CD8⁺ cells were further labeled with 5 μ M CFSE. EF- or DEF-pulsed BMDCs were co-cultured with CFSE-stained CD8⁺ cells at a ratio of 1:5 (BMDC:CD8⁺ T cell) for 3 days in RPMI 1640 medium (Life Technologies, Grand Island, NY) containing 10% fetal bovine serum (Hyclone, Logan, UT) and 1% penicillin/streptomycin (Life Technologies, Grand Island, NY) with supplementation of 50 mM 2-mercaptoethanol (Sigma, 516732). CD8⁺ T cell proliferation was assessed by CFSE dilution using flow cytometry.

Flow cytometry

Single-cell suspensions were prepared for flow cytometric analyses. To stain cell surface markers, cells were treated with FcBlock (2.4G2, BD Bioscience, 55–3142) and stained with the appropriate combinations of the following antibodies: PB-anti-mouse CD3 (145-2C11, BioLegend, 100333); FITC-anti-mouse CD8 (53-6.7, BioLegend, 100705); PE-anti-mouse CD4 (GK1.5, BioLegend, 100407); PE-anti-mouse CD80 (BD Bioscience, 553769); PE-anti-mouse CD86 (BD Bioscience, 553692). Flow cytometry data were acquired using a BD FACS Canto flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR).

Statistical analyses

The results are expressed as the mean $\pm$ standard error of the mean (SEM) values unless otherwise stated. The Student's *t*-test was used to compare two groups. The statistical significance of differences in survival and tumor volume was determined by Log-rank (Mantel-Cox) test and two-way ANOVA, respectively. Statistical analyses were performed using the Prism 8.00 software for Windows (GraphPad software, San Diego, CA). *P* values < 0.05 were considered statistically significant.

Abbreviations

Ag; antigen

Ab; antibody

APC; antigen presenting cell

CFSE; 6-Carboxyfluorescein succinimidyl ester

DAMP; damage associated molecular pattern

DC; dendritic cell

DEF; DCpep6-4xE7ΔNLS-FlaB

EC50; Half maximal effective concentration

ELISA; enzyme-linked immunosorbent assay

ELISpot; enzyme-linked immune absorbent spot

FlaB; *Vibrio vulnificus* Flagellin B

HPV; human papillomavirus

LN; lymph node

MHC; *major histocompatibility complex*

NLR; NOD-like receptor

NLRC4; NLR containing a caspase activating and recruitment domain (CARD) protein 4

NOD; nucleotide-binding oligomerization domain

PAMP; pathogen associated molecular pattern

PRR; pattern recognition receptor

TA; tumor antigen

TAA; tumor associated antigen

TCV; therapeutic cancer vaccine

TDLN; tumor draining lymph node

TLR; Toll-like receptor

TSA; tumor specific antigen

Declarations

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Competing interests

The authors declare no competing interests.

Author contributions sections

Substantial contributions to conception and design of this study: VV, SP, SEL, JHR

Acquisition of data: VV, SP, SHH, WT-

Analysis and interpretation of data: VV, SP, SHH, SEL, JHR

Drafting the article: VV, SP, SEL, JHR

Revising it critically for important intellectual content: SEL, JHR

Final approval of the version to be published: SEL, JHR

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Figures

Figure 1

Figure 1

Identification and characterization of DC-targeting peptide that specifically binds to intracellular compartment of CD11c⁺ cells. (A) Schematic representation of the in vivo phage biopanning processes in mice. (B) Enrichment number and frequency of phagemid particles after two rounds of panning are depicted. Biopanning enrichment was expressed in either number of plaques per 1×10^6 cells or phage output/input ratio ($\times 10^{-8}$). (C) Name, sequence and frequency of 6 peptides selected from the phage pool obtained after second round of selection. (D) Binding of biotinylated synthetic peptides on CD11c⁺ cells. BMDCs, or CD11c⁺ cells from cLNs and spleen cells were treated with DCpep3, DCpep4, and DCpep6 for 2 hours and cellular uptake of peptides in CD11c⁺ cells was determined by FACS analysis gating upon CD11c⁺ cell population. (E) Determination of in vivo DC-targeting ability of DCpep6. BALB/c mice were intranasally administered with DCpep6 or scrambled peptide. At 6 hours after peptide administration, cLNs cells were harvested, washed and stained with CD11c⁺ or streptavidin-AF488. Statistical significance was calculated by Student's t-test. *, $P < 0.05$; and ns, non-significant.

Figure 2

Generation and characterization of a protein-based all-in-one cancer vaccine (DCpep6-4xE7ΔNLS-FlaB; DEF). (A) The vector maps and amino acid sequences of recombinant proteins are shown; in vivo DC-targeting peptide (DCpep6), tumor antigen (E7ΔNLS), and built-in flagellin adjuvant (FlaB). E7ΔNLS tumor antigen was prepared by deleting N-terminal nuclear localization sequence (NLS) of HPV16 E7 protein. An all-in-one type TCV containing DCpep6, HPV E7ΔNLS and built-in FlaB adjuvant was constructed by recombinant DNA technology in pET30a⁺ plasmid. 4xE7ΔNLS-FlaB (EF) was also generated as a non-CD11c⁺ cell targeting TCV. (B) Characterization of the recombinant proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent Western blot analysis using mouse anti-FlaB or anti-E7 serum. Anti-FlaB or anti-E7 serum was induced by intraperitoneal immunization of FlaB or E7FL with complete Freund's adjuvant. (C) Selection of optimal tumor antigen (E7ΔNLS) for the all-in-one cancer vaccine. C57BL/6 mice were implanted with TC-1 cells in the right midflank. When the tumor size reached approximately 3-5 mm in diameter, the tumor-bearing mice were subcutaneously vaccinated with

200 μ l of PBS only (n=5), 4 μ g of FlaB (n=5), 10 μ g of E7 full length (E7FL; n=5), 10 μ g of E7FL plus 4 μ g of FlaB (n=5), 8 μ g of E7 Δ NLS (n=5), and 8 μ g of E7 Δ NLS plus 4 μ g of FlaB (n=5) in the peritumoral region three times at five-day intervals. Statistical significance of tumor suppression was calculated by two-way ANOVA test. (D) Determination of TLR5-dependent NF- κ B stimulating activity by FlaB, EFB or DEF. The relative NF- κ B activities were analyzed by using HEK-BlueTM hTLR5 cells and HEK-BlueTM detection assay systems. (E) Determination of dose-dependent cellular uptake of DEF in CD11c⁺ mouse BMDCs by FACS. BMDCs were incubated with various concentrations of EF or DEF for 2 hours, and then intracellular localization of the recombinant proteins was determined by FACS analysis using anti-FlaB serum gating upon CD11c⁺ cell population. (F) Determination of intracellular localization of DEF in BMDCs or Raw264.7 cells by confocal microscopical observation. BMDCs or Raw264.7 cells were incubated with 20 μ g/ml of DCpep6 for 2 hours, and intracellular localization of the recombinant proteins was determined by confocal microscopical observation. (G) Determination of energy-dependent cellular uptake of DEF in CD11c⁺ cells. BMDCs were incubated with 5 μ g/ml of DCpep6 at 37 °C, 4 °C, or 37 °C in the presence of 0.1% NaN₃ for 2 hour, and then intracellular localization of the recombinant proteins was determined by FACS analysis gating upon CD11c⁺ cell population. *, P < 0.05; **, P < 0.01; ***, P < 0.001; and ns, non-significant.

Figure 3

Biodistribution and in vivo-DC targeting of DEF. (A) Biodistribution of in vivo administered DEF. C57BL/6 mice were subcutaneously injected on the right side of the groin with PBS, 50 μ g of FNR675-conjugated EF (EF), or 50 μ g of FNR675-conjugated DEF. Six hours after the administration, the iLNs were prepared to determine the biodistribution of the EF-FNR675 or DEF-FNR675 by measuring fluorescence intensity. (B) Determination of in vivo DC-targeting of DEF. C57BL/6 mice were subcutaneously injected at the right side of the groin with PBS, 50 μ g of EF-FNR675, or 50 μ g of DEF-FNR675. Six hours after the administration, the iLNs-cells were prepared and then in vivo DC-targeting was determined by FACS analysis gating upon CD11c⁺ cell population.

Figure 4

Determination of the long-term anti-tumor immune response induced by DEF immunization. Groups of C57BL/6 mice were implanted with TC-1 cells in the right midflank. When the tumor size reached approximately 3-5 mm in diameter, the tumor-bearing mice were subcutaneously vaccinated with 200 μ l of PBS only (P; n=5), 8 μ g of E7 Δ NLS (E, n=5), 8 μ g of E7 Δ NLS plus 4 μ g of FlaB (E+F; n=5), and 20 μ g of Dcpep6-4xE7 Δ NLS-FlaB (DEF; n=5) in the peritumoral region three times at five-day intervals, and the

tumor volume and survival of the tumor-bearing mice was measured. Statistical significance of survival was determined by Log-rank (Mantel-Cox) test. *, $P < 0.05$; and ***, $P < 0.001$.

Figure 5

Characterization of DEF-mediated anti-tumor immune responses. (A) Flow cytometric analysis of CD80 and CD86 expression on CD11c⁺ cells. BMDCs were treated with 500 ng/ml of EF or DEF for 24 hours, and CD80 or CD86 expression on the BMDC surface was determined by FACS gating upon CD11c⁺ cell population. (B) Cellular localization of DEF. Raw264.7 cells were incubated with 10 µg/ml of DEF (green) for 30, 60, 90 or 120 min, followed by staining with DAPI (nuclei, blue) and LysoTracker (lysosome, red). (C) Experimental schedule for the analysis of tumor-specific immune responses in tumor bearing mice. (D) Determination of HPV16 H-2Db-RAHYNIVTF CTL epitope-specific tetramer-positive cells in the peripheral blood CD8⁺ cells. (E) ELISPOT analysis of HPV16 H-2Db-RAHYNIVTF CTL epitope-specific IFN-γ spot forming cells in 1x10⁶ spleen cells or 2.5x10⁵ TDLN cells.

Figure 6

NLR4-inflammasome activation plays a pivotal role in the DEF efficacy. (A) Determination of the long-term anti-tumor immune response induced by DEF immunization in WT, TLR5^{-/-}, or NLR4^{-/-} mice. Groups of WT, TLR5^{-/-} and NLR4^{-/-} mice were implanted with TC-1 cells in the right midflank. When the tumor size reached approximately 3-5 mm in diameter, the tumor-bearing mice were subcutaneously vaccinated with 200 μ l of PBS only (P; n=5), 20 μ g of 4xE7 Δ NLS-FlaB (EF; n=5), 20 μ g of Dcpep6-4xE7 Δ NLS-FlaB (DEF; n=5) in the peritumoral region three times at five-day intervals, and the tumor volume and survival of the tumor-bearing mice was measured. (B) CD8⁺ T cell proliferation assay. BMDC isolated from the wild type C57BL/6 or NLR4^{-/-} mice were pulsed with 1 μ g/ml of EF or DEF for 24 hours then the BMDCs were co-cultured with CFSE-stained CD8⁺ T cells prepared from TC-1 tumor-bearing mice for 72 hours. CD8⁺ T cell proliferation was assessed by CFSE dilution using flow cytometry. Statistical significance of survival was determined by Log-rank (Mantel-Cox) test. *, P < 0.05; **, P < 0.01; and ns, non-significant.

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