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Original Article

Engineering of a self-adjuvanted iTEP-delivered CTL vaccine

Shuyun DONG[1, #], Tiefeng XU[1, 2, #], Peng WANG[1], Peng ZHAO[1], Mingnan CHEN[1, *]

[1]*Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, Utah 84112, USA;* [2]*The First Affiliated Hospital of Hainan Medical University, Cancer Institute of Hainan Medical University, Haikou 570102, China*

Cytotoxic T lymphocyte (CTL) epitope peptide-based vaccines are widely used in cancer and infectious disease therapy. We previously generated an immune-tolerant elastin-like polypeptides (iTEPs)-based carrier to deliver a peptide CTL vaccine and enhance the efficiency of the vaccine. To further optimize the vaccine carrier, we intended to potentiate its function by designing an iTEP-based carrier that was able to deliver adjuvant and a vaccine epitope as one molecule. Thus, we fused a 9-mer H_{100}, a peptide derived from the high-mobility group box 1 protein (HMGB1) that could induce activation of dendritic cells (DCs), with an iTEP polymer to generate a new iTEP polymer named H_{100}-iTEP. The H_{100}-iTEP still kept the feature of reversible phase transition of iTEPs and should be able to be used as a polymer carrier to deliver peptide vaccines. The expression levels of CD80/CD86 on DCs were assessed using flow cytometry. The iTEP fusion-stimulated IL-6 secretion by DCs was measured with ELISA. Activation of antigen-specific $CD8^+$ T cells induced by iTEP fusions was examined through a B3Z hybridoma cell activation assay. *In vivo* CTL activation promoted by iTEP fusions was detected by an IFN-γ-based ELISPOT assay. The iTEP fused with H_{100} could induce maturation of DCs *in vitro* as evidenced by increased CD80 and CD86 expression. The iTEP fusion also promoted activation of DCs by increasing secretion of a proinflammatory cytokine IL-6. The N-terminus or C-terminus fusion of H_{100} to iTEP had a similar effect and a reduced form of cysteine in iTEP fusions was required for DC stimulation. iTEP fusions potentiated a co-administrated CTL vaccine by increasing an antigen-specific CTL response *in vitro* and *in vivo*. When the H_{100}-iTEP was fused to a CTL epitope to generate a one-molecule vaccine, this self-adjuvanted vaccine elicited a stronger antigen-specific CTL response than a vaccine adjuvanted by Incomplete Freund's Adjuvant. Thus, we have successfully generated a functional, one-molecule iTEP-based self-adjuvanted vaccine.

Keywords: iTEP; CTL vaccine; peptide adjuvant; dendritic cell activation/maturation; self-adjuvanted vaccine; CTL response

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Introduction

Cytotoxic T lymphocyte (CTL) epitope peptide-based vaccines are widely applied in cancer and infectious disease therapy[1–5]. Today they are attracting new interest as a combination partner with other immunotherapy like blockade of CTLA-4 and PD-1 T cell checkpoints[3, 6–9]. To maximize the CTL vaccine efficiency, an ideal vaccine adjuvant is absolutely indispensable. Adjuvants potentiate CTL vaccine response through different mechanisms. Examples include the following: Incomplete Freund's Adjuvant (IFA) and aluminum induce a Th response to further enhance a CTL response[10–13]. MPLA, CpG, Pam_3CSK_4, poly-ICLC, and imiquimod target toll-like receptors (TLRs) activate antigen-presenting cells[14–25]. Cytokines like IL-2, GM-CSF, and IFNs are also used as immunopotentiators[26–31]. Therefore, the selection of an appropriate adjuvant for a CTL vaccine is a key for the successful application of the vaccine.

We previously developed iTEPs to deliver and potentiate CTL vaccine efficacy[32, 33]. We intentionally designed the iTEPs to be immune-tolerant so that we can control the type of immune response by selecting appropriate adjuvants for incorporation. An ideal adjuvant for an iTEP-based vaccine could be a peptide which can be easily fused to the iTEP molecule, and thus a one-molecule self-adjuvanted vaccine is achieved. The advantage of a one-molecule self-adjuvanted vaccine is that this kind of vaccine is taken up and processed by the same antigen-presenting cell like dendritic cell (DC). This promises the exact antigen-presenting cell is activated by the adjuvant on the same molecule and thus presents the vaccine epitope on the cell surface, and non-specific DC stimulation and/or the possible induction of antigenic tolerance is avoided[34].

Among several peptide candidates, peptides derived from high-mobility group box 1 (HMGB1) captured our attention. H_{91}, a peptide corresponding to amino acids 91–108 in the

# These authors contributed equally to this article.
*To whom correspondence should be addressed.
E-mail Mingnan.chen@utah.edu
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HMGB1 protein, was found to mature and activate DCs and thus induced a potent antigen-specific CTL response *in vitro* and *in vivo*[35]. The function of H_{91} was confirmed in several delivery systems: peptides modified with N-terminal biotin[35], peptides engrafted by liposomes[36], and peptides encapsulated or conjugated to the surface of poly(*D*,*L*-lactic-co-glycolic) acid (PLGA)[37, 38]. A recent study showed that H_{91} executed its immunostimulatory function through a TLR4 pathway[39]. The helical C-terminal portion of H_{91}, a peptide corresponding to amino acids 100-108 of HMGB1 and thus was named as H_{100}, was responsible for its immunostimulatory function. H_{100} with N-terminal biotin modification induced a more potent antigen-specific CTL response than H_{91} and delayed tumor development in a prophylactic vaccine setting[40].

In this study, we fused H_{100} with an iTEP to generate a vaccine carrier augmenting a desired immune response. This H_{100}-iTEP carrier stimulated DCs to upregulate co-stimulatory molecules such as CD80 and CD86 and produce cytokines such as IL-6. The fusion of H_{100} at the N-terminus or C-terminus of the iTEP acquired similar activity. We further proved that the redox status of the H_{100}-iTEP fusion is a key for the adjuvant activity of the fusion. Reduced cysteine residue with a free thiol group in the fusion was required for its adjuvant potency. The iTEP fusions potentiated the effect of a co-administered CTL vaccine by increasing the antigen-specific CTL response both *in vitro* and *in vivo*. More importantly, when the H_{100}-iTEP carrier was fused to a CTL epitope to generate a one-molecule vaccine, this vaccine was self-adjuvanted and by itself led to a stronger antigen-specific CTL response than a vaccine co-administered with IFA, a commonly used vaccine adjuvant.

Materials and methods

Animals

6–8 week old C57BL/6 female mice were purchased from Jackson Laboratories. This study followed an approved protocol by the Institutional Animal Care and Use Committee (IACUC) at the University of Utah.

Cell lines

The DC2.4 cell line (H-2K^b) was a gift from Dr Kenneth ROCK (University of Massachusetts, USA). The DC2.4 cells were cultured in RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum, 2 mmol/L glutamine, 1% non-essential amino acids, 1% Hepes, 50 μmol/L β-Mercaptoethanol, 100 units/mL penicillin, and 100 μg/mL streptomycin (ThermoFisher Scientific, USA). The B3Z T-cell hybridoma specific for H-2K^b, OVA257-264 (SIINFEKL, also known as pOVA), was kindly provided by Dr Nilabh SHASTRI (University of California, USA). The B3Z cells were cultured in RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum, 2 mmol/L glutamine, 1 mmol/L pyruvate, 50 μmol/L β-Mercaptoethanol, 100 units/mL penicillin, and 100 μg/mL streptomycin (ThermoFisher Scientific, USA).

Construction of the expression plasmids of the iTEP and iTEP fusions

The genes encoding the iTEP, H_{100}-iTEP, iTEP-H_{100}, iTEP-pOVA, H_{100}-iTEP-pOVA were synthesized on a modified pET25b(+) vector using a previously described PRe-RDL method[33, 41]. Specifically, genes encoding peptides of GVLP-GVG, GAGVPG, SAFFLFCSE, and SIINFEKL were generated by annealing the sense and antisense oligonucleotides together and then inserting them into the vector at the *Bse*R I site. Then, the iTEP gene was polymerized by the PRe-RDL method until a desired length of the iTEP gene was achieved. The iTEP sequences contain 35 repeats of GAGVPG, 16 repeats of GVLPGVG, and then another 35 repeats of GAGVPG, 16 repeats of GVLPGVG. Genes encoding H_{100} (SAFFLFCSE) and pOVA (SIINFEKL) were inserted to the iTEP at the desired position in a similar manner. The sequences of the oligonucleotides used for constructing these genes are listed in Supplementary Table S1. After the resulting expression vectors were transformed into DH5α for their amplification, the lengths of the coding genes were confirmed by an *Xba* I and *Bam*H I double digestion and followed agarose gel analysis. The coding genes were also verified by DNA sequencing (Genewiz, USA).

Production and purification of the iTEP and iTEP fusions

The iTEP and iTEP-pOVA were produced from BL21 competent cells and purified as previously described[33]. H_{100}-iTEP, iTEP-H_{100} and H_{100}-iTEP-pOVA were generated from BL21 competent cells and purified using buffer in a reductive condition: PBS with 10 mmol/L TCEP-HCl, pH 7.0. The purity of the proteins was assessed by SDS-PAGE. The endotoxin was removed by 1% Triton X-114 3 times as previously described[42, 43]. Then Triton X-114 was removed using Amicon Ultra-15 (10k) centrifugal filters (Millipore, USA). The residual endotoxin in the samples was determined by Limulus Amebocyte Lysate (LAL) PYROGENT Single Test Vials (Lonza, Allendale, NJ, USA). All samples used for *in vitro* and *in vivo* immune assays had their endotoxin level below 0.25 EU per mg protein. Final purified proteins were dissolved in PBS without TCEP before assays.

Characterization of thermally-induced, reversible, inverse phase transition of the iTEP and iTEP fusions

The phase transitions of the iTEP or iTEP fusions were characterized by turbidity changes of sample solutions as a function of temperature[33]. Briefly, the OD350 of a sample as indicated was dynamically recorded using a UV-visible spectrophotometer equipped with a multi-cell thermoelectric temperature controller (Cary 300, Varian Instruments, Walnut Creek, CA, USA), during which the sample was heated from 25 °C to 75 °C and then cooled to 25 °C at a rate of 1 °C/min. The maximum first derivative of the turbidity curve of a sample was identified. The transition temperature (T_t) of the sample is the temperature that corresponds to the maximum derivative.

DC maturation analysis

1.5×10^5 DC2.4 cells/well were set in 48-well plates and cul-

tured with lipopolysaccharide (LPS, Sigma, St Louis, MO, USA), H_{100} peptide (Biomatik, LLC, USA) or iTEP fusions as indicated, for 16 h. The cells were then collected and washed before staining with APC anti-mouse CD80 Antibody and Alexa Fluor® 488 anti-mouse CD86 Antibody (Biolegend, San Diego, CA, USA). DAPI was added to the labeled cells for gating live cells. The cells were then analyzed using Cytek DxP Analyzer (Cytek Biosciences Inc, Fremont, CA, USA).

IL-6 release assay

1×10^5 DC2.4 cells/well were set in 96-well plates and cultured with different treatments for 24 h. Cell culture supernatants were collected and analyzed for the amount of IL-6 by ELISA (Biolegend, San Diego, CA, USA).

Activation of B3Z hybridoma ($CD8^+$ T) cells

The B3Z cell is a $CD8^+$ T-cell hybridoma engineered to secrete β-galactosidase when its T-cell receptors are engaged with a SIINFEKL:H-$2K^b$ complex[44]. This assay was done by a protocol described previously[33]. Briefly, 1×10^5 DC2.4 cells/well were set in 96-well plates. iTEP fusions at indicated concentrations were loaded into the DC culture for 16 h and then washed away. 1×10^5 B3Z cells/well were added to the DC culture and co-cultured with DC2.4 cells for 24 h. The cells were washed and lysed with 100 μL of lysis buffer (PBS with 100 mmol/L 2-mercaptoethanol, 9 mmol/L $MgCl_2$, and 0.125% NP-40) together with 0.15 mmol/L chlorophenol red β-galactoside substrate (Sigma, St Louis, MO, USA). After a 4 h incubation at 37 °C, the reaction was stopped with 50 μL of 15 mmol/L EDTA and 300 mmol/L glycine. OD_{570} of the solutions was measured, and OD_{630} was used as a reference using an Infinite M1000 PRO plate reader (Tecan Trading AG, Switzerland).

Animal immunization and splenocyte isolation

C57BL/6 mice were randomly separated into 3 groups and immunized subcutaneously twice. The first immunization was on the left flanks of mice. Group 1 was immunized with 2 nmol of iTEP-pOVA together with an equal volume of IFA (Sigma, St Louis, MO, USA). Group 2 was immunized with 2 nmol of iTEP-pOVA together with 2 nmol of H_{100}-iTEP. Group 3 was immunized with 2 nmol of H_{100}-iTEP-pOVA. The immunization was repeated on the mice's right flanks one week later. At 10 d after the second immunization, the mice were sacrificed, and the spleens were harvested. Single splenocytes were isolated and counted using a Countess™ Automated Cell Counter (ThermoFisher Scientific, USA).

IFN-γ-based Enzyme-linked immunospot (ELISPOT) assay

The assay was done using a protocol described in a previous study[33]. Briefly, Splenocytes were reactivated by pOVA (SIINFEKL peptide, 2.5 mg/mL) for 48 h before being loaded into wells of 96-well filtration plates (Millipore, Billerica, MA, USA) coated with 5 mg/mL of capture anti-mouse IFN-γ mAb (Clone: R4-6A2, Biolegend, San Diego, CA, USA). Triplicates were set up for each condition. Cells were discarded after 24 h of culture, and the wells were incubated overnight with 2 mg/mL of biotinylated detection anti-mouse IFN-γ mAb (Clone: XMG1.2-Biotin, Biolegend, San Diego, CA, USA). After washing, the bound mAb was detected using horseradish peroxidase (HRP Avidin, Biolegend, San Diego, CA, USA) together with a 3-amino-9-ethyl-carbazole (AEC) substrate (Sigma, St Louis, MO, USA). The spots were scanned and automatically counted using ImageJ software.

Statistical analysis

Data were analyzed for statistical significance using unpaired Student's *t*-test. GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis and figure construction. A P value <0.05 was statistically significant.

Results

Generation of an iTEP-based self-adjuvanted vaccine carrier

Previously, we generated an iTEP-based carrier to deliver a peptide CTL vaccine and enhanced the efficiency of the delivered vaccine[32, 33]. To further optimize the vaccine carrier, we intended to potentiate its function by designing an iTEP-based carrier that was able to deliver adjuvant and a vaccine epitope as one molecule. To this end, we fused a 9-mer H_{100} peptide (sequences: SAFFLFCSE, corresponding to amino acids 100–108 in HMGB1 protein) with an iTEP polymer (sequences: N-$(GAGVPG)_{35}$-$(GVLPGVG)_{16}$-$(GAGVPG)_{35}$-$(GVLPGVG)_{16}$-C) to generate a new iTEP polymer named H_{100}-iTEP (Figure 1A). H_{100}-iTEP was characterized by its thermally-induced, reversible phase transition feature. Similar to the iTEPs without the H_{100} fusion, the H_{100}-iTEPs were in solution when the temperature was low and started to form coacervates with the increase of temperature. When the temperature dropped, they dissolved again (Figure 1B and 1C). The heating transition temperature (T_t) of the iTEP was 57.25 °C, and its cooling T_t was 50.49 °C at 15 μmol/L. In contrast, the heating T_t of H_{100}-iTEP was 46.48 °C, and its cooling T_t was 35.77 °C, suggesting that the fusion with H_{100} increases hydrophobicity of the iTEP. However, the H_{100}-iTEP still kept the feature of reversible phase transition of iTEPs and should still be able to be used as a polymer carrier to deliver peptide vaccines.

To test if the H_{100}-iTEP is functional, we examined how the maturation and activation of DC2.4 cells were affected by H_{100}-iTEPs. DC2.4 cells presenting H-$2k^b$ are an immature murine dendritic cell line developed by the Dr Rock lab[45]. First, after the DC2.4 cells were cultured with the iTEPs without the H_{100} fusion for 16 h, their surface CD80 expression level had no apparent change (Figure 1D and Supplementary Figure S1). Free H_{100} peptides also did not affect the CD80 expression on DC cells (Figure 1D and Supplementary Figure S2), which was also observed previously[46]. However, treatment of H_{100}-iTEP fusion led to a significant increase of CD80 expression in DC cells compared to the untreated control. The magnitudes of the increase were at least 2.5-fold and equivalent to the effect of LPS, which served as a positive control (Figure 1D, Supplementary Figure S3 and S4). H_{100}-iTEPs also increased CD86 expression in DC cells by 1.8-fold compared

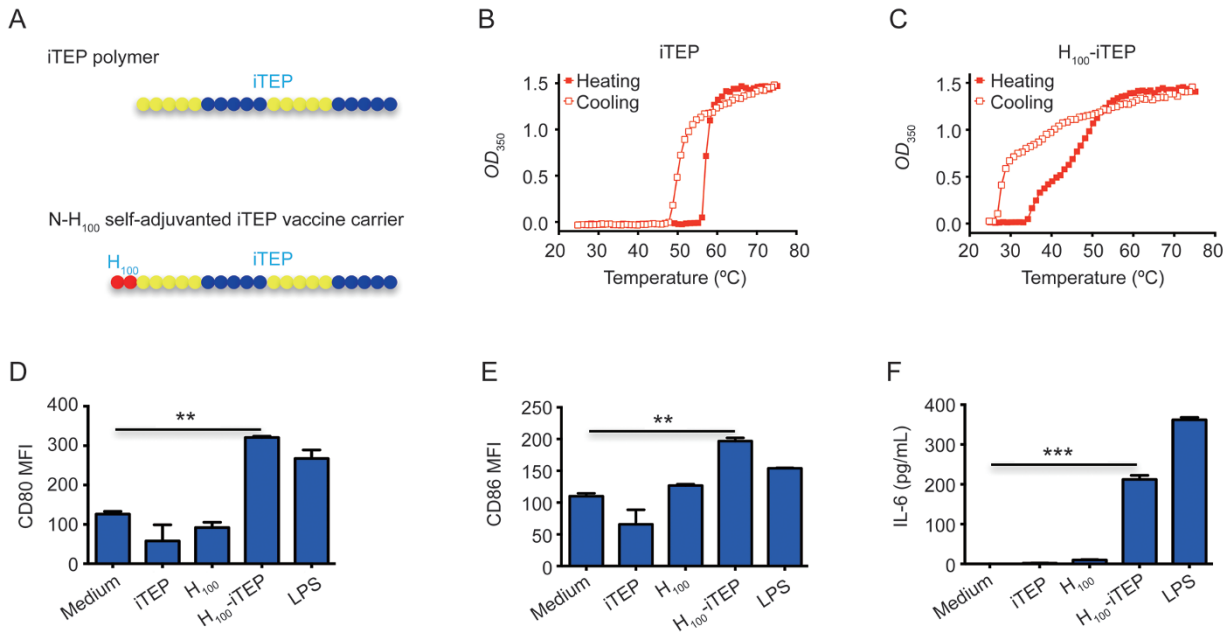

Figure 1. H_{100}-iTEP causes phenotypic maturation and proinflammatory cytokine release from dendritic cells. (A) Schematic showing polymer composition of the iTEP and H_{100}-iTEP. (B-C) Turbidity profiles (OD_{350}) of the iTEP (B) and H_{100}-iTEP (C) as 15 μmol/L of the samples were heated and then cooled between 25 °C and 75 °C in PBS. (D-E) Analysis of DC maturation in response to different treatments. DC2.4 cells were incubated alone or in the presence of 15 μmol/L of the iTEP, H_{100}, H_{100}-iTEP, or LPS (100 ng/mL) for 16 h. Surface molecules of the cells were labeled by APC-CD80 (D) and Alex488-CD86 (E) and analyzed using flow cytometry. (F) Cytokine release from DC induced by different treatments. Supernatants from DC2.4 cells treated with 15 μmol/L of the iTEP, H_{100}, H_{100}-iTEP, or LPS (100 ng/mL) for 24 h were collected and analyzed for IL-6 by ELISA. Data in all panels are representative of 3 independent repeats. Bars, mean±SD, n=3. Analysis of variance (Student's t-test). $^{**}P$<0.01, $^{***}P$<0.001.

to non-treatment, while the iTEPs or H_{100} peptides alone did not change the CD86 expression (Figure 1E, Supplementary Figure S1–S4), suggesting that H_{100}-iTEP induced the maturation of DC cells. Also, DC2.4 cells treated by H_{100}-iTEPs were activated to release more than 200 pg/mL of IL-6 while the untreated cells and cells treated by the iTEPs or H_{100} peptides virtually released no IL-6 (Figure 1F), suggesting that the H_{100}-iTEP fusion stimulates DC cells to secrete proinflammatory cytokines. These results demonstrated that H_{100}-iTEP induces phenotypic maturation of DCs and activates the DCs to secrete proinflammatory cytokines. The H_{100}-iTEPs are potentially self-adjuvanted and could be used to deliver CTL peptide vaccines which especially require sensitization of dendritic cells.

N-terminus and C-terminus fusions of H_{100} to iTEP had similar effect

As shown above, we fused H_{100} on the N-terminus of the iTEP and successfully generated an iTEP-based self-adjuvanted vaccine carrier. We were curious whether H_{100} still keeps its function at other positons of the fusion. Sometimes, the fusion of a small peptide might have an effect on the tertiary structure and biological activity of fusion proteins, depending on the location and the amino acid composition of the peptide[47]. In our experience, the fusion of some other peptides at different positions of iTEPs made the new fusion lose the physical thermal-reversible transition specificity and/or biological functions. To test if changing position of H_{100} in the iTEP fusion keeps or even promotes its adjuvant activity, we generated iTEP-H_{100}, a fusion of H_{100} to the C-terminus of the iTEP (Figure 2A), and then determined its characteristic and function. Like the N-terminus fusion, the C-terminus fusion, iTEP-H_{100}, kept the feature of reversible phase transition induced by temperature change. Its heating T_t and cooling T_t were 52.72 °C and 42.99 °C, respectively (Figure 2B). The iTEP-H_{100} increased the CD80 expression of DCs by 2 fold and CD86 by 1.5 fold (Figure 2C, 2D and Supplementary Figure S5). Also, iTEP-H_{100} stimulated the DC cells to secrete around 150 pg/mL of IL-6 (Figure 2E). The iTEP C-terminus fusion promoted IL-6 secretion and maturation of DCs like the N-terminus fusion. Their effects were comparable and both can be used as self-adjuvant carriers.

Redox status of H_{100}-iTEP is a key of their function

To further study the mechanism of DC activation by the H_{100}-iTEP fusion, we analyzed its sequences and noticed that there is a cysteine corresponding to the 106th amino acid of the full-length HMGB1. This C106 is essential for the interaction of the full-length HMGB1 with TLR4/MD-2[48, 49]. On the basis of finding that irreversible oxidation to sulphonates of C106 inhibits TNF production from macrophages[49], we reasoned that this also might be true in our case. To test this, we changed the redox status of the H_{100}-iTEP fusion and assessed its ability to induce IL-6 secretion from DC2.4 cells. First, the redox status of the fusion was changeable and observable. As shown in Figure 3A, oxidization and reduction had no effect

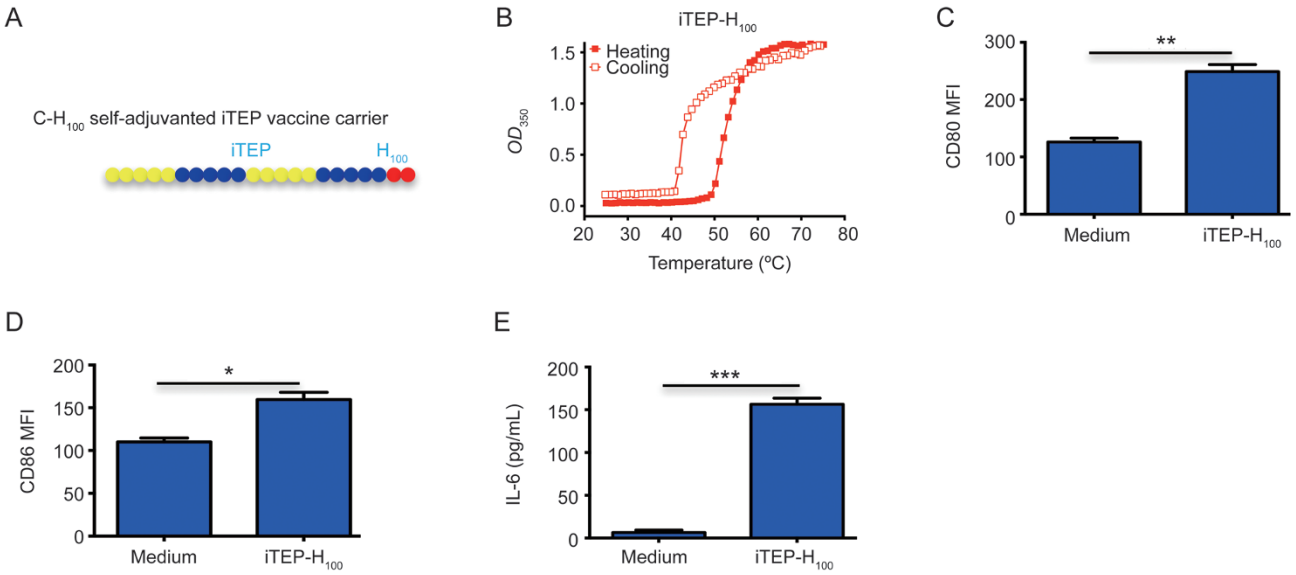

Figure 2. N-terminus and C-terminus fusion of H_{100} to iTEP have similar effects. (A) Schematic showing polymer composition of iTEP-H_{100}. (B) Turbidity profiles (OD_{350}) of iTEP-H_{100} as 15 μmol/L of iTEP-H_{100} was heated and then cooled between 25 °C and 75 °C in PBS. (C-D) DC maturation induced by C-terminus fusion of the iTEP and H_{100}. DC2.4 cells were incubated with 15 μmol/L of iTEP-H_{100} for 16 h. Surface molecules of the cells were labeled by APC-CD80 (C) and Alex488-CD86 (D) and analyzed using flow cytometry. (E) Cytokine release from DC induced by C-terminus fusion of iTEP with H_{100}. Supernatants from DC2.4 cells incubated with 15 μmol/L of iTEP-H_{100} for 24 h were collected and analyzed for IL-6 by ELISA. Data in all panels are representative of 3 independent repeats. Bars, mean±SD, n=3. Analysis of variance (Student's t-test). $^{*}P$<0.05. $^{**}P$<0.01. $^{***}P$<0.001.

on the iTEPs without the H_{100} fusion, and the iTEPs migrated to the position of 50 kDa on an SDS gel, which matched their theoretical MW (49.5 kDa) (Figure 3A, lane 2 and lane 3). On the contrary, most of the reduced form of the H_{100}-iTEPs (treated by DTT) migrated to their MW position of 50.6 kDa (lane 5), but the major fraction of the oxidized form (treated by H_2O_2) migrated to a position of around 100 kDa (lane 4). This SDS separation gel analysis suggested that oxidized H_{100}-iTEPs form dimers because of the oxidization of the thiol group provided by C106. In the functional test, the H_{100}-iTEP-trigged DC activation dropped around 20 fold after the H_{100}-iTEPs were oxidized. When the H_{100}-iTEPs were oxidized by exposure to H_2O_2, the oxidized form was no longer capable of stimulating IL-6 released from cultured DC2.4 cells, as shown in Figure 4B. Therefore, keeping the C106 in the iTEP fusions in its reduced status with the free thiol group is a key for their application of stimulating DC cells.

Self-adjuvanted iTEP carrier enhances antigen-specific CD8 T cell responses

The data that iTEPs fused with the H_{100} peptide, at either the N-terminus or the C-terminus, activated and matured DC cells suggested that the H_{100}-iTEP fusion has the potential to be used as an adjuvant to enhance the response of CTL vac-

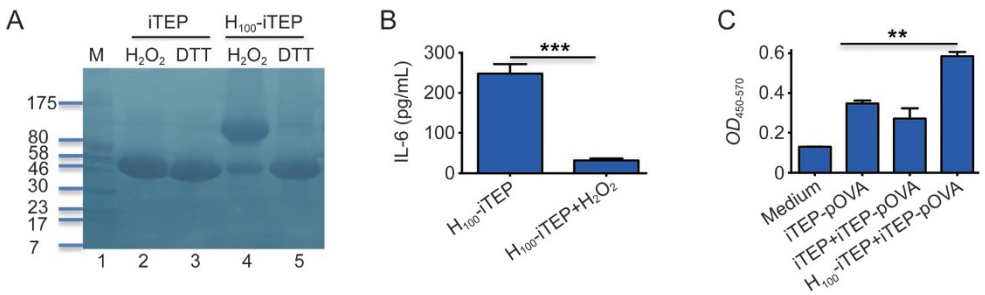

Figure 3. Reductive status is necessary for function of H_{100}-iTEP. H_{100}-iTEP enhances antigen-specific CD8 T cell activation *in vitro*. (A) An SDS-PAGE analysis showing redox status of iTEP fusions. The samples were treated with 3% H_2O_2 or 1 mmol/L of DTT for 2 h before loading to a SDS-PAGE. (B) Analysis of IL-6 secreted by DC cells. H_{100}-iTEP was treated with or without 3% H_2O_2 for 2 h and clean up by centrifugal filters before they were used to treat DC2.4 cells for 24 h. Supernatant from the DC cells were analyzed by ELISA. (C) Activation of B3Z cells after they were incubated with DCs that presented pOVA. The DC2.4 cells were pre-incubated with antigen (5 μmol/L iTEP-pOVA) alone or together with 5 μmol/L of iTEP or H_{100}-iTEP. Data shown in (B) and (C) are representative of 3 independent repeats. Bars, mean±SD, n=3. Analysis of variance (Student's t-test). $^{**}P$<0.01. $^{***}P$<0.001.

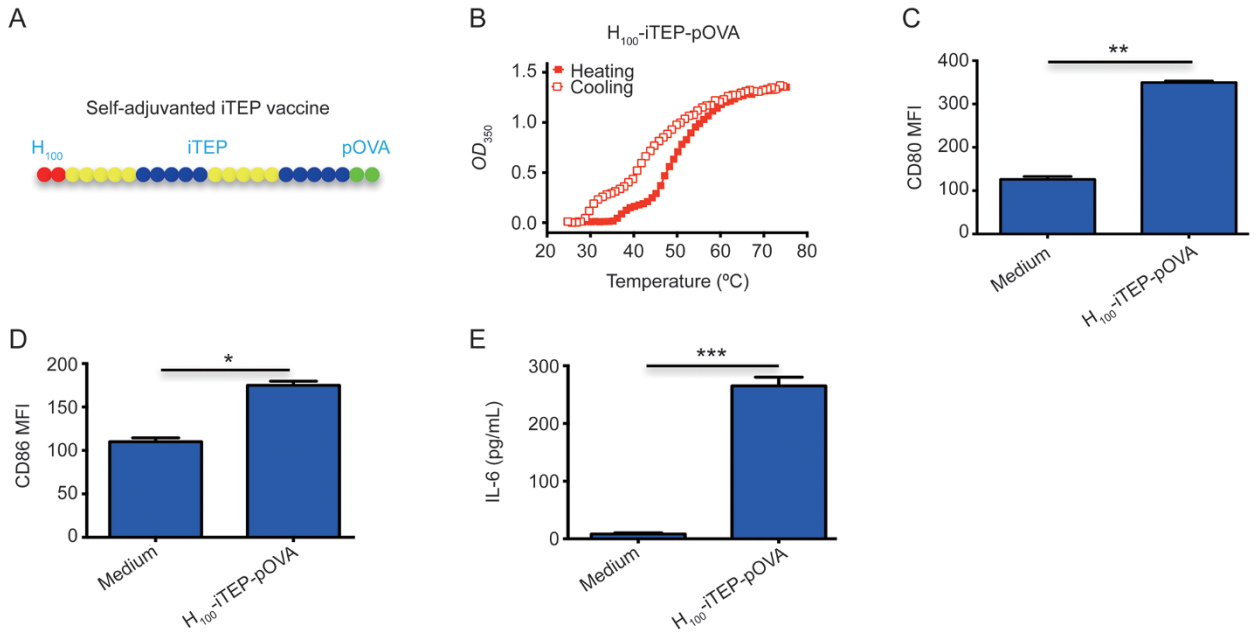

Figure 4. Generation of iTEP-based self-adjuvanted CTL vaccine. (A) Schematic showing polymer composition of iTEP-based self-adjuvanted CTL vaccine. (B) Turbidity profiles (OD_{350}) of H_{100}-iTEP-pOVA as 15 μmol/L of the sample was heated and then cooled between 25 °C and 75 °C in PBS. (C-D) DC maturation induced by iTEP-based self-adjuvanted CTL vaccine. DC2.4 cells were incubated alone or in the presence of 15 μmol/L of H_{100}-iTEP-pOVA for 16 h. Surface molecules of the cells were labeled by APC-CD80 (C) and Alex488-CD86 (D) and analyzed using flow cytometry. (E) IL-6 release from DC induced by iTEP-based self-adjuvanted CTL vaccine. Supernatants from DC2.4 cells treated with or without 15 μmol/L of H_{100}-iTEP-pOVA for 24 h were collected and analyzed for IL-6 by ELISA. Data in all panels are representative of 3 independent repeats. Bars, mean±SD, n=3. Analysis of variance (Student's t-test). $^{*}P$<0.05. $^{**}P$<0.01. $^{***}P$<0.001.

cines. To investigate this, we used iTEP-pOVA as a CTL vaccine and the H_{100}-iTEP as an adjuvant to treat DCs. We then determined the activation of B3Z cells incubated with the pretreated DCs. iTEP-pOVA is a fusion of iTEPs with the C-terminus SIINFEKL peptide, a CD8 epitope. B3Z cells are a genetically engineered $CD8^+$ T cell hybridoma line restricted to the H-$2K^b$/SIINFEKL complex[44]. As shown in Figure 3C, the iTEP-pOVA alone induced a SIINFEKL specific $CD8^+$ response to activate B3Z cells, which was 2.5-fold of the control. H_{100}-iTEPs significantly increased the response of iTEP-pOVA by an additional 2 fold, while iTEPs without the H_{100} fusion did not (Figure 3C). We then compared the adjuvant activity of H_{100}-iTEPs with IFA, a commonly used adjuvant for cell-mediated immune response, for a CTL response *in vivo*. Mice were immunized with iTEP-pOVA together with IFA or the H_{100}-iTEPs. Then splenocytes were collected and restimulated with pOVA before the number of antigen-specific IFN-γ-secreting T cells were estimated by an ELISPOT assay. Compared to mice vaccinated with iTEP-pOVA together with IFA, mice vaccinated with iTEP-pOVAs together with H_{100}-iTEPs induced a statistically significant increase in the number of $CD8^+$ cells that produce IFN in response to stimulation with pOVA. There was an average of 270 spots per million of splenocytes from each mouse adjuvanted with H_{100}-iTEPs versus 167 spots from IFA adjuvantation (Figure 5C). Therefore, the iTEP adjuvants successfully enhanced the antigen-specific CTL response both *in vitro* and *in vivo*.

Generation of a self-adjuvanted iTEP-delivered CTL vaccine

The self-adjuvanted iTEP carrier described above can be used as a universal adjuvant for any co-administered CTL vaccine. However, our eventual goal was to generate an iTEP-based self-adjuvanted CTL vaccine. To this end, we directly fused H_{100} with the iTEP on its N-terminus and pOVA on its C-terminus to generate H_{100}-iTEP-pOVA (Figure 4A). Even though the iTEP was modified by both the N and C terminus, its feature of having a thermal reversible transition was still kept. Its heating T_t and cooling T_t were 49.42 °C and 43.99 °C respectively (Figure 4B). Like the fusion without pOVA, H_{100}-iTEP-pOVA induced phenotypic maturation of DC2.4 cells as evidenced by the 2.8 fold increase of CD80 and the 1.6 fold increase of CD86 (Figure 4C, 4D and Supplementary Figure S6). H_{100}-iTEP-pOVA also induced 265 μg/mL of IL-6 secretion from DC2.4 cells (Figure 4E). Therefore, the CTL vaccine was proved to trigger maturation and activation of the antigen-presenting cells by itself. Finally, we evaluated the adjuvant activity of the self-adjuvant vaccine to induce an antigen-specific immune response *in vitro* and *in vivo*. In the *in vitro* B3Z assay, the response of H_{100}-iTEP-pOVA was at least 2-fold higher than iTEP-pOVA, the vaccine without adjuvant (Figure 5A). In the *in vivo* ELISOT assay, we immunized mice with the same amount of H_{100}-iTEP-pOVA or iTEP-pOVA plus IFA and compared their induction on the pOVA-specific CTLs secreting IFN-γ in splenocytes from the mice. We found that the H_{100}-iTEP-pOVA immunized mice produced significantly

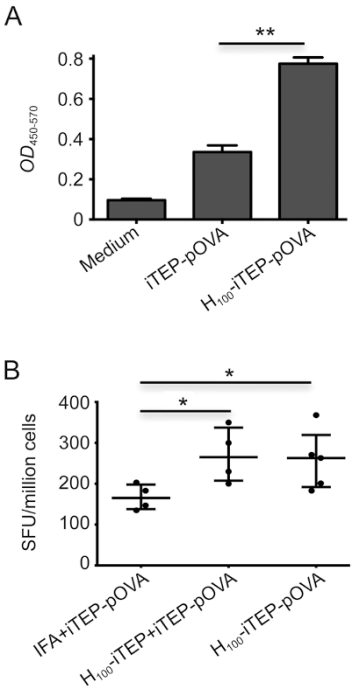

Figure 5. iTEP-based self-adjuvanted CTL vaccine promotes a strong antigen-specific CTL response by itself. (A) Activation of B3Z cells after they were incubated with DCs that pre-incubated with 5 μmol/L of normal or self-adjuvanted CTL vaccines. Data are representative of 3 independent repeats. Bars, mean±SD, n=3. (B) *In vivo* analysis of active, pOVA-restricted splenocytes cells from mice (n=4–5) immunized with iTEP–pOVA plus IFA, iTEP–pOVA plus H_{100}-iTEP, or H_{100}-iTEP-pOVA. The activation of the cells was characterized by using an IFN-γ-based ELISPOT assay. Data are presented as Spot Forming Units (SFU)/million cells. Each dot represents result of one mouse. The medians and interquartile ranges of the titers are shown. For both (A) and (B), analysis of variance (Student's t-test). $^{*}P$<0.05. $^{**}P$<0.01.

more pOVA specific CTLs in their spleen than the mice co-administrated with iTEP-pOVA and IFA, with an average of 258 spots versus 167 spots per million splenocytes from one mouse (Figure 5B). Actually, the H_{100}-iTEP-pOVA response was comparable to the effect of iTEP-pOVA plus H_{100}-iTEP.

Discussion

Previously, we delivered CTL peptide vaccine using iTEP, an immune-tolerant polypeptide carrier[32, 33]. We incorporated versatile application for drug delivery into the innate design of iTEPs. They have been used to deliver a small molecule drug as a good inert carrier[50, 51]. They have also been used to deliver CTL vaccine, and the vaccine potency was exaggerated by loading with immunogenic adjuvant, in this case the H_{100} peptide, to trigger a desired immune response. The H_{100} peptide is specifically suitable in our system because: 1) H_{100} is a proven TLR4 agonist and inflammatory mediator[39, 40]. 2) It is easily fused at a desired position of the iTEPs and readily produced by a recombinant approach. 3) Both H_{100} and iTEPs are derived from sequences of natural endogenous proteins, HMGB1 and elastin respectively. The adjuvant augmented immune response from the iTEP/H_{100} fusion, if achieved, will occur via innate immunity. In fact, in this study, we found that iTEPs fused with H_{100} at both their N-terminus and C-terminus stimulated DCs to be ready for antigen presentation by promoting DC maturation (upregulated CD80 and D86) and inducing a proinflammatory cytokine (IL-6) secretion. We further identified a reduced cysteine in the iTEP/H_{100} fusions required for their DC stimulation function. The iTEP/H_{100} fusions potentiated efficiency of an iTEP-delivered CTL vaccine by increasing an antigen-specific CTL response both *in vitro* and *in vivo*. More importantly, when a model CTL epitope was fused to H_{100}-iTEP, a self-adjuvanted vaccine was generated. This vaccine itself exhibited a stronger antigen-specific CTL response than a vaccine co-administered with IFA, a frequently used vaccine adjuvant. These findings clearly demonstrated that use of the one-molecule H_{100}-iTEP-pOVA vaccine is an effective strategy for inducing an antigen-specific CTL response.

Our work demonstrated that iTEP delivery markedly enhances the dendritic cell-stimulatory capacity of H_{100}. H_{100} was derived from HMGB1, an essential, ubiquitous DNA-binding nucleus protein, which was also found to translocate to the cytoplasm, and even the extracellular space as a pro-inflammatory cytokine[52–54]. However, even though a wholly synthetic 20-mer peptide corresponding to amino acids 89-109 of HMGB1 was reported to mediate TLR4-dependent activation of macrophage TNF release[48], later studies reported that free peptides like H_{91} have almost no activity. Interestingly, the peptide with N-terminal biotination was reported for the DC stimulatory effect. The possible reason is that binding of biotin to the proteins in the culture medium containing FBS could promote multimerization of the peptides and lead to receptor cross-linking[35]. Our data that iTEP/H_{100} fusion but not free H_{100} peptide elicited DC maturation and activation also supported the notion that multimeric binding is required for effective binding of the peptides to DC receptors. Compared to N-terminally biotinylated H_{100}, iTEP/H_{100} fusions have more distinctive advantages besides promoting receptor cross-linking for multimerization. First, iTEPs greatly decrease the dosage required for adjuvant activity. The typical concentration for observing obvious effect of biotinylated H_{100} or other HMGB1-derived peptides like H_{91} is 90 μmol/L *in vitro*, whereas that used for iTEP/H_{100} fusion was only 5-15 μmol/L. The drug amount for eliciting an antigen-specific CTL response in *in vivo* experiments was 167 nmol per mouse for biotinylated H_{100} but only 2 nmol for the iTEP/H_{100} fusion (Figure 5C). Second, the iTEP/H_{100} fusion was further fused to a CTL epitope to form a one-molecule self-adjuvanted vaccine. This self-adjuvanted vaccine administration ensures that the H_{100} adjuvant effect is exerted on the same antigen-presenting cell that takes up and presents the CTL antigen. On the contrary, co-administration of H_{100} with antigen might induce nonspecific DC stimulation followed by possible induction of antigenic tolerance. Third, the iTEP polymer protects H_{100} from degrading during its delivery in the body and elongates its effective duration *in vivo*.

We noticed that fusion of H_{100} to iTEP changed the T_t of iTEP. The heating T_t of iTEP was 57.25 °C at 15 μmol/L, while the heating T_ts for H_{100}-iTEP, the N-terminus fusion, and iTEP-H_{100}, the C-terminus fusion were 46.48 °C and 52.72 °C, respectively (Figure 1B, 1C and 2B). The amino acid sequence of H_{100} is SAFFLFCSE. The grand average of hydropathicity value of H_{100} is 1.267, thus H_{100} is hydrophobic[55]. It has been reported that hydrophobic patches on ELP fusions, similar to H_{100} on our iTEP fusion, depress T_t of the ELPs. This effect was termed "fusion ΔT_t effect". The effect is due to interactions between hydrophobic surface endowed by the patches and ELPs close to the patches[56]. It is interesting that the N-terminus fusion depressed T_t more than the C-terminus fusion. The iTEP used in this study has a sequence as of N-$(GAGVPG)_{35}$($GVLPGVG)_{16}$$(GAGVPG)_{35}$$(GVLPGVG)_{16}$-C. The N-terminus is hydrophilic while the C-terminus is hydrophobic. It appears that the hydrophobic patch, H_{100}, exerts a great "fusion ΔT_t effect" to the hydrophilic iTEP section close to the patch, but not to the hydrophobic one close to the patch. We suspect the effects of hydrophobic patches are dependent on their local environments although a separated, carefully-designed study is needed to conclude.

Nanoparticles (NPs) were widely utilized to deliver CTL vaccine and improve vaccine potency[57–59]. NP delivery of H_{91} by several systems were tested. Liposomal engrafted H_{91}, PLGA encapsulated H_{91}, or H_{91} conjugated with PLGA showed adjuvant activity of H_{91} in these forms. However, these NPs composed with multiple-dispersed material either had a low and inconsistent engagement efficiency or had a low release efficiency which limits their application. Compared to these strategies, the iTEP/H_{100} fusion was versatile and had great potential for NP delivery of H_{100}. The iTEP/H_{100} was composed of a clearly defined and mono-dispersed material: a polypeptide. The observation that both the N-terminus and C-terminus fusion of iTEPs with H_{100} had similar activity suggests that H_{100} can be fused to the desired position to facilitate NP formation and still keep its biological function. Furthermore, , iTEP-based NPs for delivering CTL vaccine and potentiating the vaccine efficiency have been previously achieved[32]. Further work to develop this vaccine in a self-assembled NP form to further boost its vaccine activity is in progress.

The mechanism by which HMGB1-derived peptides execute their immune-stimulatory function remains to be elucidated. H_{91}, the longer form of H_{100}, promoted DC binding and uptake to activate DCs. Deletion and overlapping analysis of H_{91} showed that H91-98, H94-101, or H97-104 did not bind or induce DCs to release IL-6. Only H91-108 (H_{91}) or H100-108 (H_{100}) had the DC stimulatory function, and the effect of H_{100} was even stronger than that of H_{91}[40]. It is noteworthy that a cysteine (C106 of HMGB1) is present in both H_{91} and H_{100} but not in the other non-functional HMGB1-derived peptides. The redox status of C106 is important for the HMGB1 proinflammatory activity. Full-length HMGB1 signals via multiple receptors including RAGE and TLR4. TLR4/MD-2 is a mandatory HMGB1 receptor complex for cytokine production for immune cells. The redox status of the three cysteines (C23, C45 and C106) in HMGB1 is important for their binding to MD-2. Only the isoform of HMGB1 with disulfide paired C23-C45 and C106 with a free thiol group can bind to MD-2 and hence activate the TLR4 system. The HMGB1 isoform with 3 fully reduced or completely oxidized cysteines cannot induce cytokine release[48, 49, 60]. Like the full-length HMGB1, the H_{100}-iTEP fusion containing C106 activated DCs for IL-6 production only when C106 was at reduced status, while oxidization made the fusion completely lose function (Figure 3B). Together with the report of H_{91} induced DC activation through the TLR4-dependent pathway[39], our data suggested that the iTEP/H_{100} fusions mimic HMBG1 to stimulate DC maturation and activation via the TLR4 system. However, the iTEP/H_{100} fusion does not have other danger signals as in HMGB1 due to its lack of all the other functional domains present in HMGB1, suggesting that iTEP/H_{100} fusions are optimal adjuvants without irrelevant immune stimulation. It was proposed that a dimer of H_{91}, the longer form of H_{100}, enhanced the ability to bind and activate DCs possibly through C106[40]. However, based on our observation, the C106 in iTEP-H_{100} fusions is unlikely to form disulfide bonds for dimerization because the thio group needs to be reduced for the DC activation effect.

In conclusion, the present work shows that iTEPs deliver the H_{100} peptide and a CTL epitope peptide vaccine as one molecule. This one-molecule vaccine promotes its antigen-specific immune response by itself and therefore is a self-adjuvanted vaccine. This strategy could be an effective approach for developing novel CTL vaccines for cancer immunotherapies or infectious disease therapies.

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Authors contribution

Shuyun DONG designed, performed all the experiments and prepared the manuscript; Tiefeng XU contributed to writing, protein purification and *in vivo* study; Peng WANG contributed to flow cytometry analysis and ELISPOT assay; Peng ZHAO contributed to ELISA analysis, discussing and editing of the manuscript; Mingnan CHEN initiated the project, joined in experiment design, and contributed to writing and editing of the manuscript.

Supplementary information

Supplementary files are available at the website of Acta Pharmacologica Sinica.

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