Engineering of a self-adjuvanted iTEP-delivered CTL vaccine

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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 H100, 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 H100-iTEP. The H100-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 H100 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 H100 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 H100-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

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, 5-9]. 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, Pam3CSK4, 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. H100, a peptide corresponding to amino acids 91-108 in the
HMGB1 protein, was found to mature and activate DCs and thus induced a potent antigen-specific CTL response in vitro and in vivo. The function of H$_{100}$ was confirmed in several delivery systems: peptides modified with N-terminal biotin, peptides engrafted by liposomes, and peptides encapsulated or conjugated to the surface of poly(D,L-lactic-co-glycolic) acid (PLGA). A recent study showed that H$_{100}$ executed its immunostimulatory function through a TLR4 pathway. The helical C-terminal portion of H$_{100}$, a peptide corresponding to amino acids 100-108 of HMGB1 and thus 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$_{100}$ and delayed tumor development in a prophylactic vaccine setting.

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. Specifically, genes encoding peptides of GVLPGVG, GAGVPG, SAFFLFCSE, and SIINFEKL were generated by annealing the sense and antisense oligonucleotides together and then inserting them into the vector at the BseR 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 BamHI 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. 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. 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. 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 (Tc) 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$^{[40]}$. 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 by 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 solution 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 ITP-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: SAFFLCSE, corresponding to amino acids 100-108 in HMGBl protein) with an iTEP polymer (sequences: N-(GAGVPG)$_{35}$-(GVLPGVG)$_{15}$-(GAGVPG)$_{35}$-(GVLPGVG)$_{15}$ 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 ($T_1$) of the iTEP was 57.25°C, and its cooling $T_1$ was 50.49°C at 15 µmol/L. In contrast, the heating $T_1$ of H$_{100}$-iTEP was 46.48°C, and its cooling $T_1$ 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$^{[35]}$. 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$^{[36]}$. However, treatment of H$_{100}$-iTEP fusion led to a significant increase of CD80 expression on 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
to non-treatment, while the iTEPs or H100 peptides alone did not change the CD86 expression (Figure 1E, Supplementary Figure S1–S4), suggesting that H100-iTEP induced the maturation of DC cells. Also, DC2.4 cells treated by H100-iTEPs were activated to release more than 200 pg/mL of IL-6 while the untreated cells and cells treated by the iTEPs or H100 peptides virtually released no IL-6 (Figure 1F), suggesting that the H100-iTEP fusion stimulates DC cells to secrete proinflammatory cytokines. These results demonstrated that H100-iTEP induces phenotypic maturation of DCs and activates the DCs to secrete proinflammatory cytokines. The H100-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 H100 to iTEP had similar effect**

As shown above, we fused H100 on the N-terminus of the iTEP and successfully generated an iTEP-based self-adjuvanted vaccine carrier. We were curious whether H100 still keeps its function at other positions 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. 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 H100 in the iTEP fusion keeps or even promotes its adjuvant activity, we generated iTEP-H100, a fusion of H100 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-H100, kept the feature of reversible phase transition induced by temperature change. Its heating Tt and cooling Tt were 52.72 °C and 42.99 °C, respectively (Figure 2B). The iTEP-H100 increased the CD80 expression of DCs by 2 fold and CD86 by 1.5 fold (Figure 2C, 2D and Supplementary Figure S5). Also, iTEP-H100 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 H100-iTEP is a key of their function**

To further study the mechanism of DC activation by the H100-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. On the basis of finding that irreversible oxidation to sulphonates of C106 inhibits TNF production from macrophages, we reasoned that this also might be true in our case. To test this, we changed the redox status of the H100-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, oxidation and reduction had no effect
on the iTEPs without the H100 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 H100-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 H2O2) migrated to a position of around 100 kDa (lane 4). This SDS separation gel analysis suggested that oxidized H100-iTEPs form dimers because of the oxidation of the thiol group provided by C106. In the functional test, the H100-iTEP-triggered DC activation dropped around 20 fold after the H100-iTEPs were oxidized. When the H100-iTEPs were oxidized by exposure to H2O2, 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 H100 peptide, at either the N-terminus or the C-terminus, activated and matured DC cells suggested that the H100-iTEP fusion has the potential to be used as an adjuvant to enhance the response of CTL vac-

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**Figure 2.** N-terminus and C-terminus fusion of H100 to iTEP have similar effects. (A) Schematic showing polymer composition of iTEP-H100. (B) Turbidity profiles (OD350) of iTEP-H100 as 15 μmol/L of iTEP-H100 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 H100. DC2.4 cells were incubated with 15 μmol/L of iTEP-H100 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 H100. Supernatants from DC2.4 cells incubated with 15 μmol/L of iTEP-H100 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.

**Figure 3.** Reductive status is necessary for function of H100-iTEP. H100/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% H2O2 or 1 mmol/L of DTT for 2 h before loading to a SDS-PAGE. (B) Analysis of IL-6 secreted by DC cells. H100-iTEP was treated with or without 3% H2O2 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 H100-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.
cines. To investigate this, we used iTEP-pOVA as a CTL vaccine and the H100-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-2Kb/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. H100-iTEPs significantly increased the response of iTEP-pOVA by an additional 2 fold, while iTEPs without the H100 fusion did not (Figure 3C). We then compared the adjuvant activity of H100-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 H100-iTEP-pOVA. 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 H100-iTEP-pOVA-induced significantly 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 H100-iTEP-pOVA 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 H100 with the iTEP on its N-terminus and pOVA on its C-terminus to generate H100-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 \(T_c\) were 49.42 °C and 43.99 °C respectively (Figure 4B). Like the fusion without pOVA, H100-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). H100-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 H100-iTEP-pOVA was at least 2-fold higher than iTEP-pOVA, the vaccine without adjuvant (Figure 5A). In the in vivo ELISPOT assay, we immunized mice with the same amount of H100-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 H100-iTEP-pOVA immunized mice produced significantly higher responses than the control group. The results are consistent with our previous study [44].
More importantly, when a model...and elastin respectively. The adjuvant augmented are derived from sequences of natural endogenous proteins, produced by a recombinant approach. 3) Both H-100t-ipeps are versatile application for drug delivery into the innate design immune-tolerant polypeptide carrier. This self-adjuvanted vaccine administration ensures that the antigen-specific CTL response by itself. (A) Activation of B3Z cells after presentation of pOVA as derived from HMGB1, an essential, ubiquitous DNA-binding nucleoprotein, which was also found to translocate to the cytoplasm, and even the extracellular space as a pro-inflammatory cytokine (IL-6) secretion. 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[52], later studies reported that free peptides like H$_{89}$ 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[53]. 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$_{89}$ 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 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.

Discussion

Previously, we delivered CTL peptide vaccine using iTEP, an immune-tolerant polypeptide carrier[32–34]. 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[35, 36]. 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[37, 40]. 2) It is easily fused at a desired position of the iTEP's 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 CD86) 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 vivo and in vitro. 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 nucleoprotein, 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[52], later studies reported that free peptides like H$_{89}$ 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[53]. 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$_{89}$ 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 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.

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.
We noticed that fusion of H\textsubscript{100} to iTEP changed the $T_c$ of iTEP. The heating $T_c$ of iTEP was 57.25°C at 15 µmol/L, while the heating $T_c$ for H\textsubscript{100}-iTEP, the N-terminus fusion, and iTEP-H\textsubscript{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\textsubscript{100} is SAEFFLFCSE. The grand average of hydropathicity value of H\textsubscript{100} is 1.267, thus H\textsubscript{100} is hydrophobic\cite{55}. It has been reported that hydrophobic patches on ELP fusions, similar to H\textsubscript{100} on our iTEP fusion, depress $T_c$ of the ELPs. This effect was termed “fusion $T_c$ effect”. The effect is due to interactions between hydrophobic surface endowed by the patches and ELPs close to the patches\cite{38}. It is interesting that the N-terminus fusion depressed $T_c$ more than the C-terminus fusion. The iTEP used in this study has a sequence as of N-(GAGVPG)\textsubscript{10}(GVLPGVG)\textsubscript{10}(GAGVPG)\textsubscript{10}(GVLPGVG)\textsubscript{10-SH}. The N-terminus is hydrophilic while the C-terminus is hydrophobic. It appears that the hydrophobic patch, H\textsubscript{100}, exerts a great “fusion $T_c$ 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\cite{37-39}. NP delivery of H\textsubscript{100} by several systems were tested. Liposomal engrafted H\textsubscript{100}, PLGA encapsulated H\textsubscript{100}, or H\textsubscript{100} conjugated with PLGA showed adjuvant activity of H\textsubscript{100} 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\textsubscript{100} fusion was versatile and had great potential for NP delivery of H\textsubscript{100}. The iTEP/H\textsubscript{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\textsubscript{100} had similar activity suggests that H\textsubscript{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\cite{53}. 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\textsubscript{100}, the longer form of H\textsubscript{100} promoted DC binding and uptake to activate DCs. Deletion and overlapping analysis of H\textsubscript{100} showed that H91-98, H94-101, or H97-104 did not bind or induce DCs to release IL-6. Only H91-108 (H\textsubscript{100}) or H100-108 (H\textsubscript{100}) had the DC stimulatory function, and the effect of H\textsubscript{100} was even stronger than that of H\textsubscript{100} \cite{40}. It is noteworthy that a cysteine (C106 of HMGB1) is present in both H\textsubscript{100} and H\textsubscript{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/MC-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\cite{48, 49, 60}. Like the full-length HMGB1, the H\textsubscript{100}-iTEP fusion containing C106 activated DCs for IL-6 production only when C106 was at reduced status, while oxidation made the fusion completely lose function (Figure 3B). Together with the report of H\textsubscript{100} induced DC activation through the TLR4-dependent pathway\cite{39}, our data suggested that the iTEP/H\textsubscript{100} fusions mimic HMGB1 to stimulate DC maturation and activation via the TLR4 system. However, the iTEP/H\textsubscript{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\textsubscript{100} fusions are optimal adjuvants without irrelevant immune stimulation. It was proposed that a dimer of H\textsubscript{100}, the larger form of H\textsubscript{100} enhanced the ability to bind and activate DCs possibly through C106\cite{49}. However, based on our observation, the C106 in iTEP-H\textsubscript{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\textsubscript{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.

Acknowledgements

The authors thank Dr Kenneth ROCK and Nilabh SHASTRI for providing cell lines and Mr James MARVIN for flow cytometry work. The flow cytometry work was supported by the University of Utah Flow Cytometry Facility in addition to the National Cancer Institute through Award Number 5P30CA042014-24. The research work was supported by Ministry of Human Resources and Social Security of China Fund: 2013-277 to Tie-feng XU, the National Natural Science Foundation of China: 81160297 to Tie-feng XU, the University of Utah Start-up Fund to Ming-nan CHEN, Huntsman Cancer Institute Pilot Grant 170301 to Ming-nan CHEN, and NIH R00CA153929 to Ming-nan CHEN.

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.
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