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# A novel method for synthetic vaccine construction based on protein assembly

SUBJECT AREAS:

PROTEIN VACCINES

IMMUNOLOGICAL TECHNIQUES

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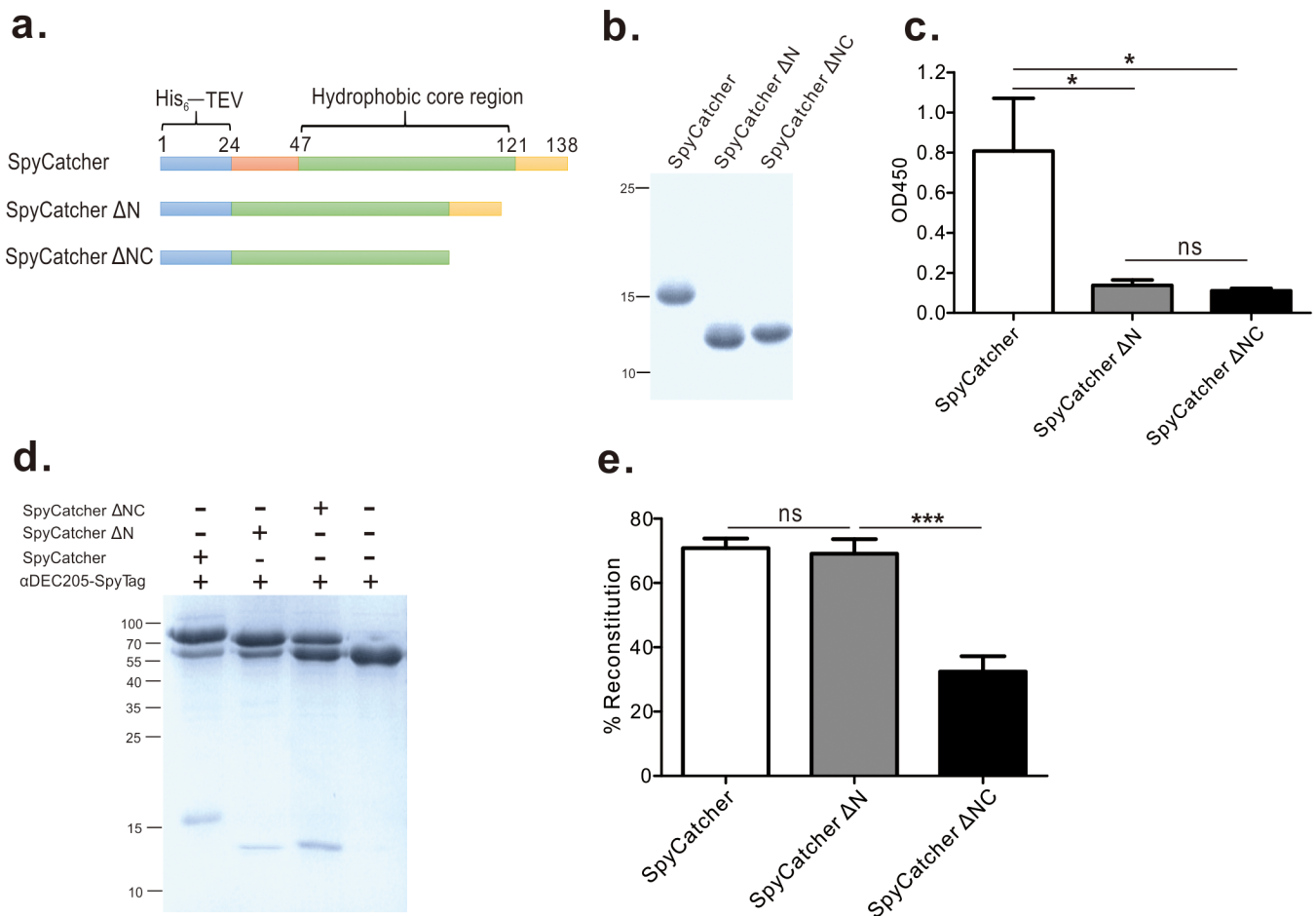
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In the history of vaccine development, the synthetic vaccine is a milestone that is in stark contrast with traditional vaccines based on live-attenuated or inactivated microorganisms. Synthetic vaccines not only are safer than attenuated or inactivated microorganisms but also provide the opportunity for vaccine design for specific purposes. The first generation of synthetic vaccines has been largely based on DNA recombination technology and genetic manipulation. This *de novo* generation is occasionally time consuming and costly, especially in the era of genomics and when facing pandemic outbreaks of infectious diseases. To accelerate and simplify the R&D process for vaccines, we developed an improved method of synthetic vaccine construction based on protein assembly. We optimized and employed the recently developed SpyTag/SpyCatcher technique to establish a protein assembly system for vaccine generation from pre-prepared subunit proteins. As proof of principle, we chose a dendritic cell (DC)-targeting molecule and specific model antigens to generate desired vaccines. The results demonstrated that a new vaccine generated in this way does not hamper the individual function of different vaccine components and is efficient in inducing both T and B cell responses. This protein assembly strategy may be especially useful for high-throughput antigen screening or rapid vaccine generation.

Since the creation of the first vaccine, for cowpox, by Edward Jenner in the late eighteenth century<sup>1</sup>, immunological research on vaccines has focused on deconstruction analysis, or evaluation of the importance and mechanisms of each component of a vaccine that may determine its effect. This research strategy has led to the discovery of a large, increasing number of functional elements of different categories including antigens, immune modulators and adjuvants, and delivery systems, among others<sup>2</sup>. A successful vaccine is usually composed of multiple elements, such as those listed above. Given the multitude of choices, the construction of different elements into an integrated, functional whole has become a new challenge in the field. Although gene-based synthetic and recombinant DNA technologies provide great flexibility for construction, certain limitations still exist: (1) large fusion proteins containing multiple functional elements are occasionally technically difficult to express or purify, and (2) *de novo* generation is usually a tedious and long process that is especially inadequate in the face of emergent pandemics of infectious diseases, when screening and identification of antigens are crucial for vaccine development<sup>3,4</sup>. Facing such difficulties and demands, instead of making complex fusion-protein candidate vaccines *de novo* every time, it would be easier, faster, more flexible and more efficient to prepare the smaller building blocks first and then to assemble them into a whole, as needed.

To achieve this goal, in the present study, we have developed a new method for synthetic vaccine construction based on a novel protein-protein conjugation technique. The SpyTag/SpyCatcher conjugation technique was recently developed based on the split protein CnaB2 from *Streptococcus pyogenes*<sup>5-7</sup>. This protein contains two fragments: one named SpyTag (13aa), and the other named SpyCatcher (138aa). Once combined under nearly any common conditions, SpyTag and SpyCatcher can rapidly and efficiently covalently conjugate to each other through an isopeptide bond<sup>5</sup>. We hypothesized that this conjugation technique could allow us to achieve our goal and assemble vaccines based on different pre-prepared functional components.

Dendritic cell (DC) targeting has emerged as an important strategy for vaccine development due to the increasing recognition of this small population of cells in both cellular and humoral immune responses<sup>8-13</sup>. DEC205 is a C-type lectin endocytic receptor that is highly expressed on CD8 $\alpha^+$  DCs in mice<sup>14</sup> and on CD141<sup>+</sup> DCs in humans<sup>15</sup>. An antibody against DEC205 has been developed as a useful targeted delivery molecule. When conjugated to this antibody, an antigen can be efficiently delivered to DCs, an approach that has been found to be superior in mediating both cytotoxic T cell responses<sup>16-18</sup> and antibody responses<sup>19,20</sup>.



**Figure 1 | SpyCatcher truncation design, production and evaluation.** (a) According to the immunogenicity and structural analyses, SpyCatcher was truncated into two forms, with a single deletion at the N-terminus (24–47aa) or a double deletion (at the N- and C-termini; 121–138aa). (b) The full-length and truncated SpyCatchers were expressed in *E. coli*, and the purified SpyCatchers were analyzed by SDS-PAGE. (c) C57BL/6 mice were immunized subcutaneously with 1.5 nmol of full-length SpyCatcher or truncated SpyCatcher, along with 30 μg CpG1826 and 30 μg Poly I:C as an adjuvant. The serum antibody response was measured 14 days later by ELISA. Mean ± SD (n = 5). \*, P < 0.05. (d, e) To assay binding, αDEC205-SpyTag was mixed with truncated SpyCatcher at a 1 : 1 molar ratio at 4 °C for 10 min. The binding efficiency was visualized by SDS-PAGE (d) and quantified by ImagePro Plus (e) Mean ± SD (n = 4). \*\*\*, P < 0.001.

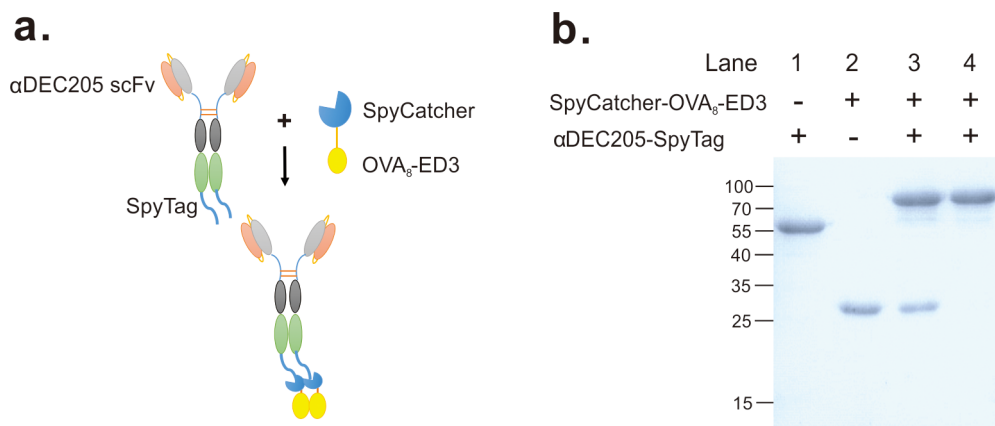
Recently, CDX1401, a vaccine composed of an anti-human DEC205 mAb fused with the tumor antigen NY-ESO-1, demonstrated promising biological activity in a phase I clinical trial<sup>21</sup>.

In the current work, we have tested the novel method of protein-assembly based vaccine construction. Employing the optimized SpyTag/SpyCatcher system, we have assembled vaccines composed of a single-chain antibody against DEC205 and model antigens (including the model T-cell epitope chicken ovalbumin<sub>257-264</sub> (OVA<sub>8</sub>) and tick-borne encephalitis virus envelope protein domain 3 (TBEV ED3))<sup>22,23</sup>. This new synthetic vaccine was shown to be fully functional and to generate efficient cytotoxic T-cell and antibody responses. Thus, this protein-based synthetic vaccine strategy may be a significant improvement over the conventional gene-based synthetic vaccine strategy and may serve as a useful platform for faster and easier vaccine development.

## Results

**Optimization of SpyCatcher.** The current SpyTag/SpyCatcher system consists of a 13 aa SpyTag and a 138 aa SpyCatcher. To further simplify this system for engineering purposes and to minimize its immunogenicity, we tried to truncate the SpyCatcher protein while maintaining its conjugation activity. A structural analysis found that aa 53–118 are probably essential for the

conjugation activity. An immunogenicity analysis revealed four major immune epitopes at the N-terminus (aa 32–50, aa 57–66) and C-terminus (aa 104–112, aa 121–138) (Figure S1). Considering these together, we performed several truncations, as shown in Figure 1a. The truncation with deletion at the N-terminus (24–47 aa) was named SpyCatcherΔN, and the truncation with deletions at both the N-terminus and the C-terminus (24–47 and 121–138 aa) was named SpyCatcherΔNC. The truncated SpyCatcher proteins were expressed in *Escherichia coli* and purified by Ni-NTA chromatography (Figure 1b). The full-length and truncated SpyCatcher proteins were then used to immunize C57BL/6 mice. Fourteen days later, antibody levels in the sera were determined by ELISA. As shown in Figure 1c, the antibody levels induced by SpyCatcherΔN and SpyCatcherΔNC were significantly lower than those induced by full-length SpyCatcher. No significant difference was found between SpyCatcherΔN and SpyCatcherΔNC. Next, we further tested the efficiency of the binding of the truncated SpyCatcher proteins to the SpyTag fusion protein, αDEC205-SpyTag, which was made by genetic fusion of SpyTag with a single-chain antibody against murine DEC205 (αDEC205) at its C-terminus. As shown in Figures 1d and 1e, there was no significant difference in binding efficiency between SpyCatcherΔN and full-length SpyCatcher, consistent with the results of a recent study<sup>24</sup>. However, the binding efficiency of SpyCatcherΔNC was obviously lower than that of full-length



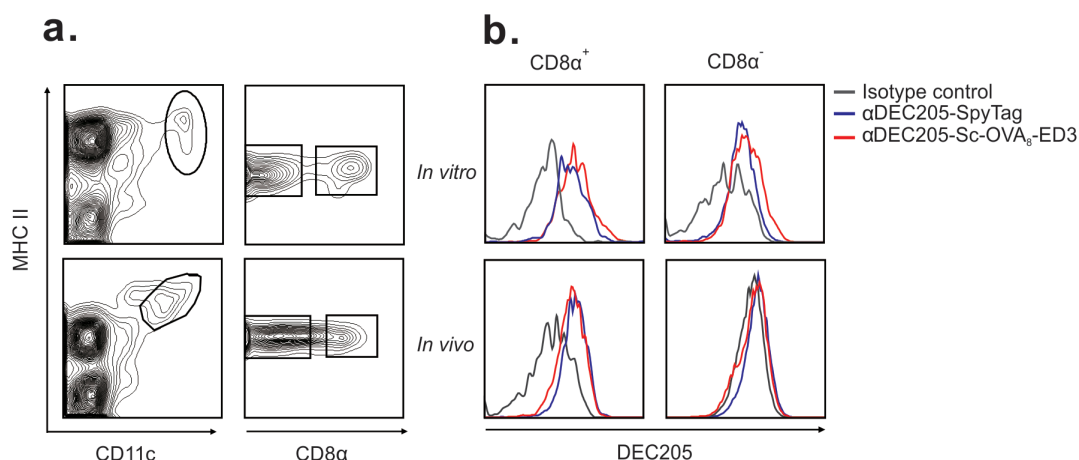
**Figure 2 | Design and generation of SpyTag/SpyCatcher-based DC-targeted synthetic vaccine.** (a) Schematic diagram of the synthetic vaccine design and assembly. The cDNA encoding the single chain of  $\alpha$ DEC205 was genetically fused to SpyTag at the C-terminus, and the antigen containing the OT1 epitope and TBEV ED3 was fused to the C-terminus of SpyCatcher $\Delta$ N. Once mixed, an amide bond efficiently forms between SpyTag and SpyCatcher. (b) Synthetic vaccine production and purification. Purified  $\alpha$ DEC205-SpyTag was mixed with SpyCatcher $\Delta$ N-OVA<sub>8</sub>-TBEV ED3 at a molar ratio of 1:1.5 at 4°C for 2 h. The assembled  $\alpha$ DEC205-Sc-OVA<sub>8</sub>-ED3 adduct was purified by Protein A chromatography. Both the efficiency of the reaction and the purified adduct can be analyzed by SDS-PAGE.

SpyCatcher. Therefore, SpyCatcher $\Delta$ N was chosen for further studies.

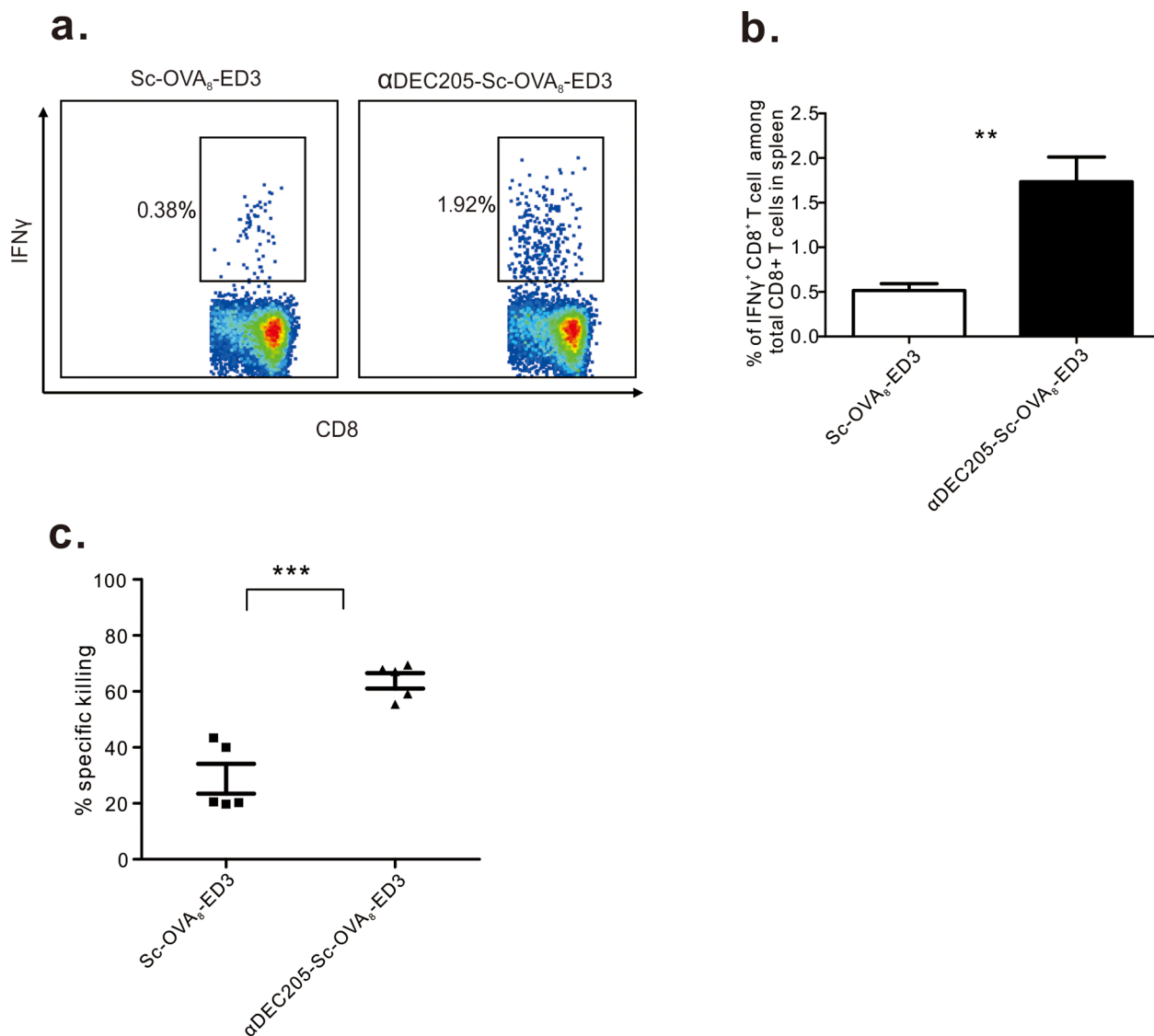
**Assembly of synthetic DEC205-targeted vaccine using optimized SpyTag/SpyCatcher system.** A full vaccine usually consists of at least an immunoregulatory functional unit in addition to the antigen. The modified single-chain antibody  $\alpha$ DEC205-SpyTag was used as a functional unit in the present study. The OVA<sub>8</sub>-TBEV ED3 DNA sequence, which encodes model antigens including both a CD8 T-cell epitope (ovalbumin<sub>257-264</sub>) and a B-cell epitope (TBEV ED3), was genetically fused to the C-terminus of SpyCatcher $\Delta$ N (Sc-OVA<sub>8</sub>-ED3). Conjugation of  $\alpha$ DEC205-SpyTag and Sc-OVA<sub>8</sub>-ED3 would result in a fully functional vaccine (Figure 2a).  $\alpha$ DEC205-SpyTag was expressed in FreeStyle™ 293-F cells and purified by Protein A chromatography (Figure 2b, Lane 1). The Sc-OVA<sub>8</sub>-ED3 fusion protein was expressed in *E. coli* and purified by Ni-NTA chromatography (Figure 2b, Lane 2). The covalent binding reaction was tested under different conditions and with different molar ratios. A 1:1.5 molar ratio of  $\alpha$ DEC205-SpyTag:Sc-OVA<sub>8</sub>-

ED3 at 4°C for 2 h was found to give rise to an optimal binding efficiency for the proteins. SDS-PAGE analysis showed that more than 90% of the input  $\alpha$ DEC205-SpyTag was conjugated (Figure 2b, Lane 3). The synthetic DEC205-targeted vaccine ( $\alpha$ DEC205-Sc-OVA<sub>8</sub>-ED3) was then purified by Protein A chromatography, with a purity above 90% (Figure 2b, Lane 4).

**Synthetic  $\alpha$ DEC205-Sc-OVA<sub>8</sub>-ED3 vaccine can bind to DEC205<sup>+</sup> DCs both *in vitro* and *in vivo*.** To test the DC-targeting ability of the synthetic  $\alpha$ DEC205-Sc-OVA<sub>8</sub>-ED3 fusion protein, splenocytes isolated from naïve WT C57BL/6 mice were incubated with the fusion protein or an isotype-control protein. After staining with a fluorescent secondary antibody, the cells were analyzed by flow cytometry. As shown in Figure 3a, conventional DCs were gated as MHC class II<sup>high</sup> and CD11c<sup>high</sup> cells, and the CD8 $\alpha$ <sup>+</sup> DC and CD8 $\alpha$ <sup>-</sup> DC subsets were then each further gated and analyzed. As shown in Figure 3b (top panel), the  $\alpha$ DEC205 fusion protein preferentially bound to CD8 $\alpha$ <sup>+</sup> DCs, rather than to CD8 $\alpha$ <sup>-</sup> DCs or other immune cells (data not shown), which is consistent with the



**Figure 3 | Synthetic vaccine targets DEC205<sup>+</sup> DCs both *in vitro* and *in vivo*.** For the *in vitro* assay,  $2 \times 10^6$  splenocytes from C57BL/6 mice were incubated with 10 pmol of isotype-control antibody or  $\alpha$ DEC205-SpyTag protein, respectively.  $\alpha$ DEC205 targeting was then visualized using a fluorescent secondary antibody. For the *in vivo* assay, C57BL/6 mice were injected with 200 pmol of isotype-control antibody or  $\alpha$ DEC205-SpyTag protein, along with 30  $\mu$ g CpG1826 and 30  $\mu$ g Poly I:C as an adjuvant. Twenty hours later, cells were isolated from DLNs and stained. (a) In both assays, anti-CD8 $\alpha$ , anti-MHC II and anti-CD11c antibodies were used to gate the CD8 $\alpha$ <sup>-</sup> and CD8 $\alpha$ <sup>+</sup> DC populations. (b) A fluorescent secondary antibody was then used to visualize the binding ability of the synthetic vaccine.



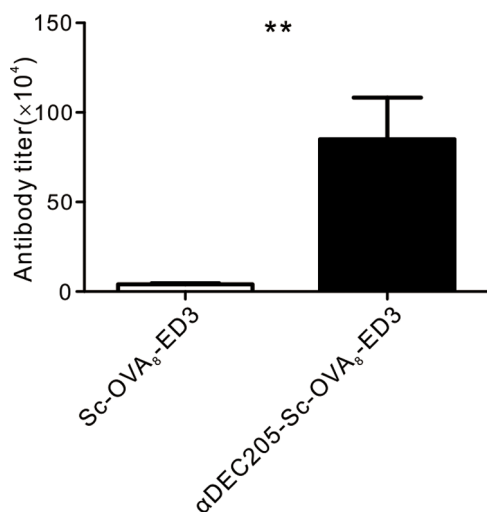
**Figure 4 | Synthetic  $\alpha$ DEC205-Sc-OVA<sub>8</sub>-ED3 vaccine generates increased cytotoxic T cell response.** C57BL/6 mice were subcutaneously immunized twice with 30 pmol of  $\alpha$ DEC205-Sc-OVA<sub>8</sub>-ED3 or Sc-OVA<sub>8</sub>-ED3, along with 30  $\mu$ g CpG1826 and 30  $\mu$ g Poly I:C, at a 7-day interval. Five days later, cells were isolated from the spleens and restimulated with OT1 (OVA<sub>257-264</sub>) peptide (5  $\mu$ g/ml) for 6 h in the presence of brefeldin A. (a) The frequencies of IFN $\gamma$ <sup>+</sup> cells among CD8 T cells were analyzed. The statistical results are shown in panel (b). Mean  $\pm$  SD (n = 4). \*\*, P < 0.01. (c) To assay *in vivo* cytotoxicity, C57BL/6 mice were immunized as described above. Five days after the secondary vaccination, the immunized mice were injected intravenously with peptide-pulsed, CFSE-labeled target cells. Twenty hours later, the spleens were harvested, and single-cell suspensions were analyzed by flow cytometry. The percentage of specific killing is shown. Mean  $\pm$  SD (n = 5). \*\*\*, P < 0.001.

specific expression of DEC205 on the former subset of DCs. To further test its targeting ability *in vivo*, naïve WT mice were immunized with the  $\alpha$ DEC205 fusion protein, together with CpG/Poly I:C. Next, 24 h after immunization, draining lymph nodes (DLNs) were isolated and digested to form single-cell suspensions.  $\alpha$ DEC205 fusion protein-bound cells were visualized with fluorescent secondary antibody staining followed by flow cytometry. Preferential targeting to CD8 $\alpha$ <sup>+</sup> DCs was confirmed (Figure 3b, bottom panel). Thus, the SpyTag/SpyCatcher system allows assembly of SpyCatcher fusion proteins with  $\alpha$ DEC205-SpyTag, without influencing its DC-targeting ability.

**Synthetic  $\alpha$ DEC205-Sc-OVA<sub>8</sub>-ED3 vaccine generates enhanced cytotoxic T-cell response.** We next tested whether the synthetic vaccine could generate an efficient cytotoxic T-cell response. This was measured by IFN $\gamma$  intracellular staining and *in vivo* specific killing assays. Naïve WT C57BL/6 mice were immunized twice

with the  $\alpha$ DEC205-Sc-OVA<sub>8</sub>-ED3 fusion protein or the Sc-OVA<sub>8</sub>-ED3 protein at a 7-day interval. CpG1826/Poly I:C was used as adjuvant. For the IFN $\gamma$  intracellular staining assay, 5 days after the last injection, splenocytes were isolated from the immunized mice and stimulated with 5  $\mu$ g/ml OT1 peptide in a U-bottom 96-well plate for 6 h. The IFN $\gamma$ -producing CD8 T cells were then stained and analyzed by flow cytometry. The splenocytes from the  $\alpha$ DEC205-Sc-OVA<sub>8</sub>-ED3-vaccinated mice showed a significantly higher percentage of IFN $\gamma$ -producing CD8 T cells compared with the splenocytes from Sc-OVA<sub>8</sub>-ED3-immunized mice (Figures 4a and 4b). For the *in vivo* specific killing assay, 4 days after the secondary injection, target cells (an equal mixture of OVA<sub>8</sub> peptide-loaded, CFSE-high, labeled naïve splenocytes and non-loaded, CFSE-low, labeled naïve splenocytes) were transferred to the immunized mice. Next, 20 h later, the proportion of CFSE-labeled cells in the spleen was analyzed. A significantly higher specific killing rate was found in mice immunized with the  $\alpha$ DEC205-Sc-OVA<sub>8</sub>-ED3 vaccine





**Figure 5 | Synthetic  $\alpha$ DEC205-Sc-OVA<sub>8</sub>-ED3 vaccine generates enhanced antibody response.** C57BL/6 mice were subcutaneously immunized twice with 300 pmol of  $\alpha$ DEC205-Sc-OVA<sub>8</sub>-ED3 or Sc-OVA<sub>8</sub>-ED3, along with 30  $\mu$ g CpG and 30  $\mu$ g Poly I:C as an adjuvant, at a 14-day interval. Seven days after the last vaccination, the mice were bled, and the serum antibodies against TBEV ED3 were measured by ELISA. The statistical results are shown. Mean  $\pm$  SD (n = 8). \*\*, P < 0.01.

than in control-vaccinated mice (Figure 4c). These data suggest that the synthetic DC-targeted vaccine is superior to the non-DC targeted vaccine in inducing CTL responses.

**Synthetic  $\alpha$ DEC205-Sc-OVA<sub>8</sub>-ED3 vaccine induces better antigen-specific antibody response.** It has been shown that targeting antigens to DEC205 can induce strong antibody responses in the presence of adjuvants. We next tested whether synthetic  $\alpha$ DEC205-Sc-OVA<sub>8</sub>-ED3 can elicit a better antibody response against the antigen TBEV ED3. Naïve WT C57BL/6 mice were immunized twice with 300 pmol  $\alpha$ DEC205-Sc-OVA<sub>8</sub>-ED3 fusion protein or Sc-OVA<sub>8</sub>-ED3 protein, together with CpG1826/Poly I:C as an adjuvant, at a 14-day interval. Seven days after the last vaccination, the mice were bled, and anti-TBEV ED3 antibody was measured by ELISA. Compared with the Sc-OVA<sub>8</sub>-ED3 control vaccine,  $\alpha$ DEC205-Sc-OVA<sub>8</sub>-ED3 elicited a significantly increased antibody response against TBEV ED3 (Figure 5). Thus, the synthetic DC-targeting vaccine is efficient in inducing both T- and B-cell responses.

## Discussion

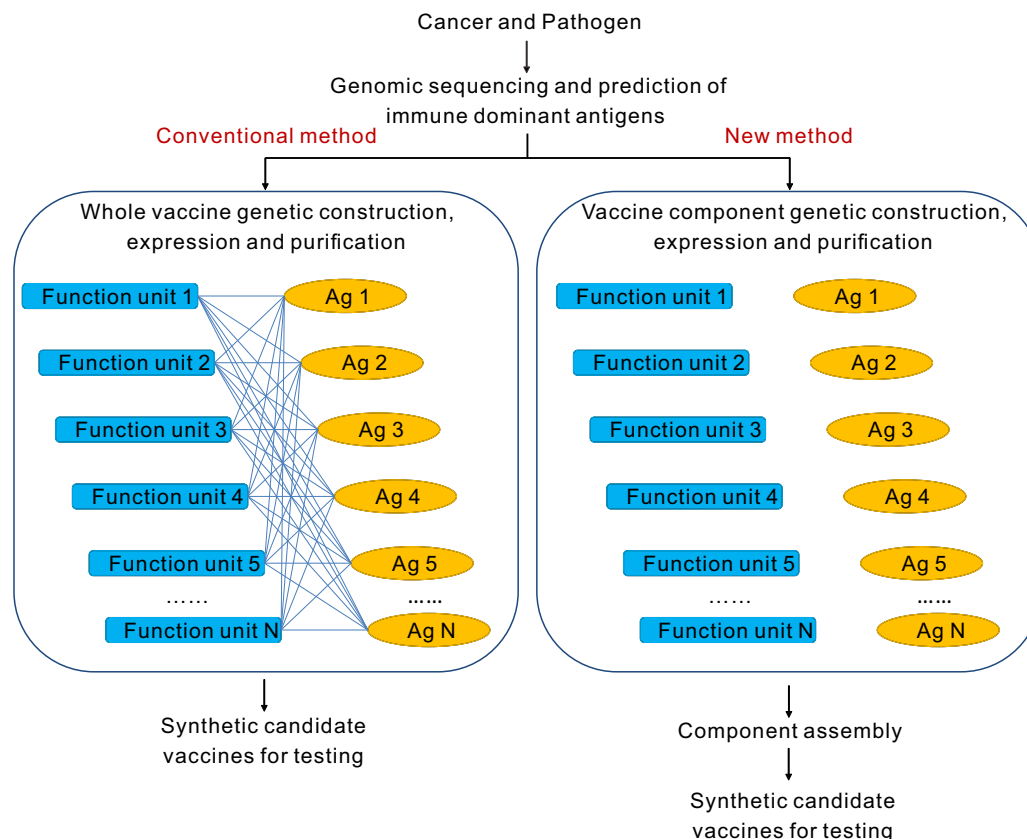
A successful vaccine is an integral, functional whole composed of different elements. The discovery of effective antigens and immunomodulatory molecules is a major challenge in the field of vaccine development<sup>3,4</sup>. Modern immunology and genomics provide a large amount of candidates for each. Simple, rapid and efficient experimental screening of the optimal composition of a vaccine is generally in demand, especially when facing pandemic outbreaks of infections, such as SARS-CoV in 2003<sup>25</sup>, H1N1 influenza in 2009<sup>26</sup> and MERS-CoV in 2012<sup>27</sup>. The traditional *de novo* generation of gene-based synthetic vaccines is both time consuming and costly. In our present study, based on the SpyTag/SpyCatcher technique, we have developed a novel method of vaccine construction by protein assembly based on pre-prepared vaccine components. We have employed this technique for the construction of a DC-targeting vaccine and have found it highly efficient to induce both T- and B-cell responses. Although this strategy still relies on genetic manipulation and protein production, it does not require synthesizing a large, complex fusion protein *de novo* every time; the strategy only produces smaller

protein components as building blocks, which is easier and faster. This block-building strategy may allow people to construct various formulations of vaccines conveniently and efficiently when needed. The approach may simplify the whole process of vaccine generation and accelerate antigen screening and verification (Figure 6). In addition, certain vaccine components (building blocks) may be easily reused for the construction of other vaccines.

Different methods have been developed to construct synthetic vaccines. The traditional way is genetic manipulation, which provides great flexibility. However, this *de novo* generation usually takes a long time, and it is occasionally difficult to express large molecules. Chemical conjugation offers an alternative, fast way to overcome the shortcomings mentioned above. However, the chemical conjugation process is usually not easily controlled, resulting in poor homogeneity in terms of the degree of conjugation and the number of conjugation sites. An isopeptide bond is an amide bond formed between the side-chain amine of lysine and the side-chain carboxyl group of either glutamate or aspartate. This bond has been shown to be very useful for protein-protein coupling and protein modification<sup>28</sup>. SpyTag/SpyCatcher was recently developed and found to be highly efficient for site-specific protein conjugation. Impelled by the limitations of current methods for vaccine construction and inspired by the concept of synthetic vaccinology, we hypothesized that this new technique might be useful for easily and precisely assembling different vaccine components at the protein level. This approach may possess several advantages: (1) In contrast to genetic manipulation, this system does not generate a whole vaccine *de novo*; instead, it produces different protein components and assembles them as needed. This method may thus significantly reduce the time spent and the cost, especially when many designs need to be tested. (2) In contrast to chemical conjugation-based approaches, this system allows quantitative and site-specific installation of antigens at a designated position in the antibody that does not influence the antibody-targeting ability. (3) SpyTag and SpyCatcher can efficiently react with each other to form a stable covalent bond under diverse conditions. (4) SpyTag is reactive no matter it is at the N-terminus, the C-terminus, or the internal site of a protein and therefore offers greater flexibility than other split protein-derived systems or intein/sortase tag systems do<sup>29,30</sup>. In addition, recent work by the Howarth group has optimized this system to potentially provide further advantages<sup>31</sup>: (1) SpyCatcher can be shortened to KTag, allowing SpyTag and KTag to form a peptide-peptide ligation. Thus, this new system could further reduce the influence of SpyCatcher's own immunogenicity, if any. (2) The SpyTag-and-KTag system allows cycles of conjugation, which enables assembly of a vaccine with multiple components, such as protein-based adjuvants and multivalent antigens. (3) The short KTag makes high-throughput screening of epitopes feasible because the short KTag-epitope fusion peptides can be easily prepared by total synthesis.

The results of the present study showed that the optimized SpyCatcher $\Delta$ N exhibits much lower immunogenicity than wild-type SpyCatcher does, without influencing the ability to bind to  $\alpha$ DEC205-SpyTag. The antigen-containing fusion protein SpyCatcher $\Delta$ N-OVA<sub>8</sub>-ED3 can also efficiently bind to  $\alpha$ DEC205-SpyTag, without affecting the targeting ability of DEC205 either *in vitro* or *in vivo*. Subsequent results from the animal study showed that an  $\alpha$ DEC205 adduct vaccine can elicit enhanced T-cell and B-cell responses. Furthermore, this new method is highly translatable; in fact, our unpublished data demonstrated its usefulness and efficacy in the construction of other vaccines.

In summary, we have established a convenient and efficient platform for conjugating antigens of interest to specific antibodies using the optimized SpyTag/SpyCatcher system. This platform could be very useful in antigen screening and vaccine development against infectious diseases and cancers. Furthermore, the use of this method is probably not limited to vaccine development; it may have broader



**Figure 6 | Perspective on protein assembly-based synthetic vaccine R&D.** During the R&D of vaccines against cancer and infectious diseases, especially in the era of big data, many functional units (such as immune molecules) and antigens are available for screening and optimization. The conventional way (left) of making synthetic vaccines usually completely depends on *de novo* construction, and each combination has to be made individually, which is time consuming and costly. By taking advantage of a protein assembly-based method (right), each vaccine component can be prepared and then simply assembled into a full vaccine, as needed. This procedure could be easier, faster, more flexible and more efficient. Ag, antigen.

applications. Antibodies and recombinant proteins have been extensively studied in animal and clinical studies of various diseases for preventative and therapeutic purposes. The strategy proposed in our current study may provide an easy and efficient protein-engineering method for generating bi-specific antibodies and producing multi-functional fusion proteins or antibodies.

## Methods

**Mice.** Female C57BL/6 mice (6–8 weeks old) were purchased from Vital River Laboratory Animal Technology Co. (Beijing, China). All mice were housed under specific pathogen-free conditions in the animal care facilities at the Institute of Biophysics, Chinese Academy of Sciences. All animal experiments were performed in accordance with the guidelines of the Institute of Biophysics, Chinese Academy of Sciences, using protocols approved by the Institutional Laboratory Animal Care and Use Committee.

**Cloning, expression, and purification of fusion proteins.** The full-length SpyCatcher expression plasmid pDEST14-SpyCatcher was kindly provided by Dr. Mark Howarth (University of Oxford, UK). DNA sequences of truncated forms of SpyCatcher were first amplified by PCR using the following primers: common forward primer  $\Delta$ Sc-F1 (GATTACGACATCCCAACGACCGAAAACCTGTAT-TTTCAGGGCGATAGTGCTAC) and reverse primers  $\Delta$ Sc-R1 (CGCGGA-TCCCTTAAT TAACTGTAAGGTAATAGCAGTTGCT, for SpyCatcher $\Delta$ N) and  $\Delta$ Sc-R2 (CGCGGATCCTTAAATATGAGCGTCACCTTTAGTTGCTTT, for SpyCatcher $\Delta$ NC). A 6 $\times$ -histidine tag coding sequence was further added by secondary PCR using  $\Delta$ Sc-F2 (GGAATTCATATGTCGACTACCATCACC ATCACCATCAGGATTACGACATCCCAA) and  $\Delta$ Sc-R1 or  $\Delta$ Sc-R2. The bold font indicates an NdeI or BamHI site. The underlined letters encode a tobacco etch virus (TEV) protease cleavage site or 6 $\times$ -histidine tag. The PCR products were cloned into pDEST14 using the NdeI and BamHI sites.

For protein expression, BL21(DE3) competent *E. coli* cells were transformed with respective plasmids, and single colonies were picked and cultured in 5 ml LB at 37 $^{\circ}$ C overnight. The cultures were then amplified to 400 ml and induced by 1 mM IPTG for 6 h. The bacterial cultures were harvested and lysed, and the targeting proteins

were purified using a Ni-NTA agarose column (ComWin Biotech, Beijing, China) according to the manufacturer's protocol.

To generate a SpyCatcher $\Delta$ N-OVA<sub>8</sub>-TBEV ED3 expression plasmid, cDNA encoding TBEV ED3 was PCR amplified with the plasmid pET-30a(+)-TBEV ED3, provided by Dr. Xiaoping Kang (Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences, China), and G<sub>4</sub>S linker and the OVA<sub>8</sub> epitope (chicken ovalbumin<sup>257-264</sup>, or SIINFEKL) were added at the N-terminus during PCR. The primers used in the PCR were as follows: OE F (CGGCTCGAGGGCGGTG-GTGGCAGCCAGCTTGAGAGTATAATCAACTTTGAAAAAC TGACTG-AATGGACATAC ACAATGTGCG) and OE R (CGCGAGCTCTTATCA TTTTGGAAACCATTG), in which the bold font indicates an XhoI or SacI site. The SpyCatcher $\Delta$ N fragment was amplified using the primers  $\Delta$ NSc-F (GCAATTCATATGTC GTACTACCATCAC) and  $\Delta$ NSc-R (CCGCTCGAGAATATGAGCGTCACCTTTA G), in which the bold font indicates an NdeI or XhoI site. The SpyCatcher $\Delta$ N and OVA<sub>8</sub>-TBEV ED3 fragments were then ligated and cloned into pDEST14 at the NdeI and SacI sites. Expression and purification of the SpyCatcher $\Delta$ N-OVA<sub>8</sub>-TBEV ED3 protein were performed as described above.

A plasmid encoding the DNA sequence of the variable regions of the heavy and light chains of anti-DEC205 (clone NLDC145) was kindly provided by Dr. Ralph Steinman (The Rockefeller University, USA). The single chain-encoding DNA was PCR amplified using the following primers: DEC205 F, GCG CGTACGGAGG-TGAAGCTGTTGGAATC and DEC205 R, GCGTTCGAACCGTTT CAATT-CCAGCTTGG. The DNA was then cloned into the pEE12.4 expression plasmid (Lonza, Basel, Switzerland) between the IgGκ leading sequence and the human IgG Fc sequence using BsiWI and BstBI (bold font). The SpyTag-encoding sequence (GCTCACATCGTATGGTGAGCGCCTACAAGCCACCAAG) and a GSGESG linker (GGATCCGGCGAGTCCGGC) were then genetically fused to the C-terminus of the human IgG Fc fragment by PCR to construct the final plasmid pEE12.4  $\alpha$ DEC205-SpyTag.

The  $\alpha$ DEC205-SpyTag fusion protein was transiently transfected into and expressed in FreeStyle<sup>TM</sup> 293-F cells (Invitrogen Life Technologies, Carlsbad, CA, USA). Briefly, FreeStyle<sup>TM</sup> 293-F cells (293F) were inoculated at 0.8  $\times$  10<sup>6</sup>/ml in CD OptiCHO<sup>TM</sup> media (Gibco Life Technologies) 2 days before transfection. The cells were washed once with the FreeStyle 293 media (Gibco Life Technologies) right before transfection and inoculated into 200 ml pre-heated FreeStyle 293 media.



Polyethylenimine (PEI) (Polysciences, Warrington, UK) and a plasmid DNA mixture (plasmid DNA (500 µg) and PEI (1.5 mg) in 10 ml FreeStyle 293 media) were added to the culture. Long™ R3IGF-1 (SABC biosciences, Carlsbad, CA, USA) was then added at a final concentration of 50 µg/l. The cells were cultured at a shaking speed of approximately 90 rpm. Four hours later, an equal volume (200 ml) of EX-CELL™ 293 media (SABC biosciences) was added, and the shaking speed was increased to 130 rpm. Next, 24 h after transfection, valproic acid (VPA) (Sigma- Aldrich, St. Louis, MO, USA) was added to the culture, with a final concentration of 3.8 mM, to inhibit the cells' growth. Seven days later, the culture media were collected for fusion protein purification by Protein A chromatography according to the manufacturer's protocol (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

**Generation and purification of αDEC205 fusion-protein adducts.** The purified truncated SpyCatcher and SpyCatcherΔN-OVA<sub>8</sub>-ED3 fusion proteins were conjugated to αDEC205-SpyTag *in vitro* to construct αDEC205 fusion-protein adducts. To assay reconstitution, αDEC205-SpyTag (10 µM) was reacted with truncated SpyCatcher (10 µM) in PBS at a molar ratio of 1 : 1 at 4°C for 10 min. SDS-PAGE and gray-intensity analysis with ImagePro Plus software (Media Cybernetics, Rockville, MD, USA) were used to evaluate the reconstitution efficiency. For vaccine generation, αDEC205-SpyTag (10 µM) was mixed with SpyCatcher-OVA<sub>8</sub>-ED3 (15 µM) in PBS at a molar ratio of 1 : 1.5 at 4°C for 2 h. The conjugated αDEC205-Sc-OVA<sub>8</sub>-ED3 adduct was then purified by Protein A chromatography according to the manufacturer's protocol (GE Healthcare Life Sciences). The product was desalted by buffer exchange (PBS) using Zeba Spin Desalting Columns (Thermo Scientific Pierce, Rockford, IL, USA) according to the manufacturer's protocol.

**Assessment of DC targeting *in vitro* and *in vivo*.** For the *in vitro* assay, the spleens of C57BL/6 mice were harvested and processed into single-cell suspensions in PBS containing 2% fetal bovine serum (FBS). Next,  $2 \times 10^6$  splenocytes were incubated with an anti-FcγR mAb (clone 2.4G2) for 10 min at 4°C to block nonspecific binding. Then, 10 pmol of αDEC205-Sc-OVA<sub>8</sub>-ED3 adduct, αDEC205-SpyTag or isotype-control antibody (human IgG Fc fusion protein prepared in-house) was added and incubated for 0.5 h at 4°C. The cells were stained with fluorescent secondary antibody (PE-conjugated anti-human IgG, polyclonal) or antibodies against cell surface markers, including anti-CD8α-PE-Cy7 (53-6.7), anti-CD11c-APC (N418) and anti-I-A/I-E-FITC (M5/114.15.2). Flow cytometry analysis was performed with a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA), and the data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA). All antibodies were purchased from eBioscience (San Diego, CA, USA) or Tonbo Biosciences (San Diego, CA, USA).

For the *in vivo* assay, naive WT C57BL/6 mice were injected subcutaneously in the tail base with 200 pmol of the αDEC205-Sc-OVA<sub>8</sub>-ED3 adduct, αDEC205-SpyTag or isotype-control antibody. After 20 h, inguinal DLNs were collected and digested to form a single-cell suspension. The cells were stained with an anti-FcγR mAb, followed by PE-conjugated anti-human IgG antibody staining before flow cytometry analysis.

**ELISA.** Microtiter plates (Corning Life Sciences, Tewksbury, MA, USA) were coated with 2 µg/ml (100 µl/well) full-length SpyCatcher or TBEV ED3 protein in carbonate buffer at 0.1 M (pH 9.5) overnight at 4°C. After washing away the unbound proteins, the plates were incubated with PBS containing 5% FBS at 37°C for 1.5 h. Serially diluted serum samples from immunized mice were added and incubated at 37°C for 1.5 h. HRP-labeled anti-mouse IgG was used as the detection antibody (Zhong Shan-Golden Bridge Biological Technology Co., Ltd, Beijing, China). The plates were visualized by adding 100 µl TMB (eBioscience) and were read at 450 nm using a SpectraMax Plus (Molecular Devices, Sunnyvale, CA, USA).

**Intracellular cytokine staining assay.** Naive WT C57BL/6 mice were subcutaneously immunized twice with 30 pmol of αDEC205-Sc-OVA<sub>8</sub>-ED3 or Sc-OVA<sub>8</sub>-ED3, along with 30 µg CpG1826 (Invitrogen Life Technologies, Beijing, China) and 30 µg Poly I:C (InvivoGen, San Diego, CA, USA) as an adjuvant, at a 7-day interval. Five days after the secondary vaccination, the spleens of the immunized mice were harvested and processed into single-cell suspensions. Splenocytes ( $1 \times 10^6$  cells/well in triplicate) were restimulated in U-bottom 96-well plates with 5 µg/ml OT1 peptide (SIINFEKL) (ChinaPeptides, Suzhou, China) for 6 h in the presence of brefeldin A (5 µg/ml) at 37°C with 5% CO<sub>2</sub>. After restimulation, the cells were first surface stained with APC-conjugated anti-mouse CD8α antibody (53-6.7) before fixation/permeabilization and intracellular staining for IFNγ (XMG1.2). All of the reagents and antibodies were purchased from eBioscience, and the manufacturer's protocol was followed for the surface and intracellular staining.

***In vivo* cytotoxicity assay.** Naive WT C57BL/6 mice were immunized subcutaneously with 30 pmol of αDEC205-Sc-OVA<sub>8</sub>-ED3 or Sc-OVA<sub>8</sub>-ED3, along with CpG1826 and Poly I:C as an adjuvant, as described above. Five days after the secondary vaccination, the immunized mice were injected intravenously with the peptide-pulsed target cells for *in vivo* killing. Briefly, congenic naive splenocytes ( $2 \times 10^7$  cells/ml) were pulsed with 10 µg/ml OT1 peptide for 1.5 h at 37°C. After one wash in PBS, the cells were labeled with 5 µM CFSE (CFSE<sup>high</sup>) (Invitrogen, Carlsbad, CA, USA) for 10 min, after which 20% FBS was immediately added to terminate the labeling reaction. The cells were then washed twice in PBS before use. Meanwhile, unpulsed splenocytes were labeled with 0.5 µM CFSE (CFSE<sup>low</sup>) following the same procedure. A 1 : 1 mixture of CFSE<sup>high</sup> and CFSE<sup>low</sup> cells ( $2.5 \times 10^6$  cells from each population) was co-transferred to the immunized mice. Twenty hours later, the

spleens were harvested, and single-cell suspensions were prepared and analyzed by flow cytometry. The percentage of specific killing was evaluated with the following formula: % specific killing =  $1 - \% \text{ survival}$ , in which % survival = (the number of OT1-pulsed target cells remaining)/(the number of unpulsed target cells remaining).

**Protein structure and immunogenicity analysis.** BepiPred Linear Epitope Prediction was used for immunogenicity analysis<sup>32</sup>, and the crystal structure data from the RCSB Protein Data Bank were used for structural analysis.

**Statistical analysis.** All data were analyzed using an unpaired two-tailed *t* test and GraphPad Prism statistical software (GraphPad Software Inc., San Diego, CA, USA). A value of  $P < 0.05$  was considered statistically significant (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; and \*\*\*,  $P < 0.001$ ).

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## Author contributions

Z.L. and M.Z. designed the experiments and analyzed the data; Z.L., H.Z. and W.W. conducted the experiments; W.T. and Y.X.F. contributed to reagents/materials; M.Z. supervised the experiments; Z.L. and M.Z. wrote the manuscript.

## Additional information

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