Vaccine 32 (2014) 2173–2180

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

As a genetic adjuvant, CTA improves the immunogenicity of DNA vaccines in an ADP-ribosyltransferase activity- and IL-6-dependent manner

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ARTICLE INFO

Article history:
Received 14 November 2013
Received in revised form 13 February 2014
Accepted 14 February 2014
Available online 11 March 2014

Keywords:
Adjuvant
DNA vaccine
Cholera toxin A
T cell immunity
IL-6

ABSTRACT

Cholera toxin (CT) and its subunits (A and B) have been intensively investigated as adjuvants for protein-based vaccines. Their underlying mechanisms vary with respect to the inoculation route used. By fusing the CTA gene to either the HIV-1-derived Tat-Rev-Vif-Integrase-Nef fusion gene or the OVA gene, our study showed that the fusion of CTA in these DNA vaccines had no cytotoxic effect *in vitro* and significantly improved both the quantity and quality of the elicited $CD8^+$ T cell responses. Further experiments identified that the fusion of CTA in these DNA vaccines augmented the secretion of IL-6 in a manner that was dependent on its ADP-ribosyltransferase activity, and protein kinase A (PKA) was found to be the major mediator of its downstream signaling. By site-directed mutagenesis of the ADP-ribosyltransferase catalytic center and *in vivo* RNAi, we demonstrated that the ADP-ribosyltransferase activity and the upregulation of IL-6 were required for the CTA gene-mediated adjuvant effect. These findings demonstrate that when fused to an immunogen gene, the CTA gene could serve as a potent genetic adjuvant, providing new insights into the mechanisms of CTA as an adjuvant.

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1. Introduction

Adjuvants are pivotal in boosting the protective immunity elicited by vaccines. However, only a few adjuvants are licensed for clinical use. Since the first report detailing the use of cholera toxin (CT) as an adjuvant [1], great efforts have been made to optimize its adjuvanticity, especially with respect to the development of non-toxic derivatives. Native cholera toxin (CT) consists of one A subunit (CTA) and five B subunits (CTB) [2]. Although it is non-toxic, CTB may not be a good adjuvant due to its negative immunomodulatory effect on macrophages [3] and its weak adjuvant effect [2]. In contrast, CTA was shown to be an indispensable component for the adjuvant activity of CT [4,5]. Nevertheless, concerns about the safety of CTA have hindered its use as an adjuvant, as it causes electrolyte imbalance *via* the ADP-ribosylation of G proteins [6]. Two major approaches were employed in previous studies to reduce the cytotoxicity of CTA, including the fusion of peptides to the CTA1 domain and the site-directed mutagenesis of its catalytic center [2]. CTA1-DD (the catalytic domain of CTA fused to a cell-binding peptide derived from the *Staphylococcus aureus* protein A [7]) is one of the most extensively evaluated modalities [8–23]. CTA and its non-toxic derivatives have often been studied as adjuvants for protein-based vaccines. Only recently was it shown that the use of CTA2 (the non-catalytic domain of CTA)/CTB as a genetic adjuvant could improve the protection rate of a *Toxoplasma gondii* vaccine in mice [24]. However, the effect of CTA as a genetic adjuvant on the antigen-specific $CD8^+$ T cell immunity induced by DNA vaccines remains unknown. By fusing the full-length *cta* gene with two immunogen-coding genes, we found that the fusion of CTA improves the quantity and quality of the elicited $CD8^+$ T cell responses and that these DNA vaccines induced antibody production without generating cytotoxicity (Fig. S1). A mechanistic study revealed that the genetic adjuvant effect of the fused CTA depends

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http://dx.doi.org/10.1016/j.vaccine.2014.02.056
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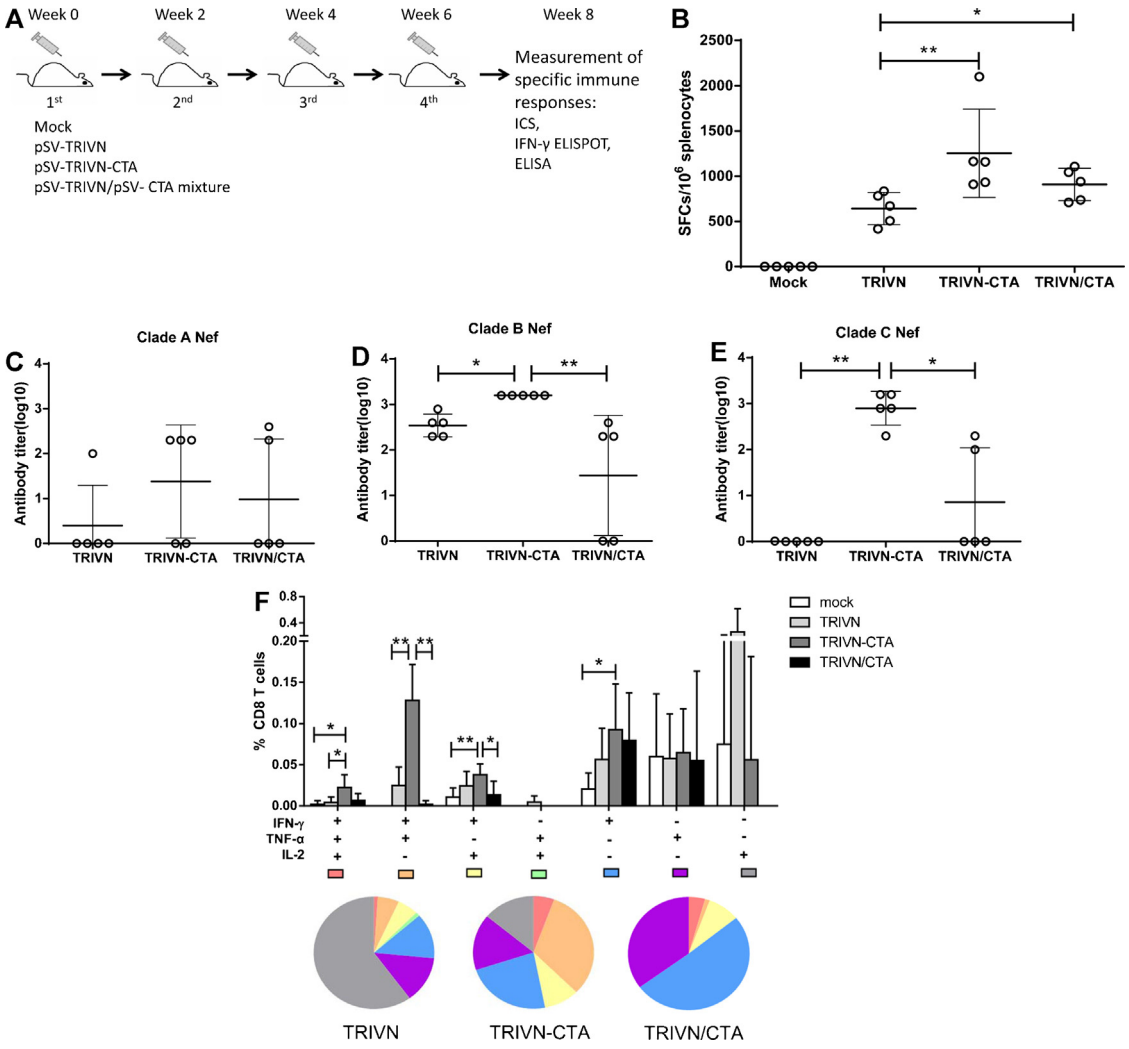

Fig. 1. The CTA fusion showed a potent genetic adjuvant effect. (A) Schematic illustration of the mouse immunization schedule. BALB/c mice (5 per group) were intramuscularly inoculated with mock, pSV-TRIVN, pSV-TRIVN-CTA or pSV-TRIVN mixed with pSV-CTA four times with an interval of 2 weeks. The total T cell responses were quantified by IFN-γ ELISPOT two weeks after the final vaccination. Data are shown as the mean of each group, and error bars represent the SD. (B) Both pSV-TRIVN-CTA and pSV-TRIVN mixed with pSV-CTA elicited significantly higher T cell responses than pSV-TRIVN. (C–E) Nef-specific antibody responses were measured by ELISA. pSV-TRIVN-CTA improved the binding antibody titers against HIV-1 clade B and clade C Nef. (F) CD8+ T cell polyfunctionality was analyzed by polychromatic flow cytometry. The frequencies of triple-positive (IFN-γ^+TNF-α^+IL-2^+), double-positive (IFN-γ^+TNF-α^+IL-2^-, IFN-γ^+TNF-α^-IL-2^+, IFN-γ^-TNF-α^+IL-2^+) and single-positive (IFN-γ^+TNF-α^-IL-2^-) CD8$^+$ T cells were all higher in the mice immunized with pSV-TRIVN-CTA. $^*P < 0.05$, $^{**}P < 0.01$.

on its ADP-ribosyltransferase activity. Thus, our results demonstrate that CTA can be used as a potent DNA vaccine adjuvant.

2. Materials and methods

2.1. Ethics statement

All mouse experiments were reviewed by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Public Health Clinical Center and were performed in strict accordance with the approved protocol (Permit Number: 2013-E013).

2.2. DNA vaccines and recombinant Tiantan vaccinia virus

Codon-optimized DNA vaccines encoding either a HIV-1 (AE recombinant subtype) Tat/Rev/Integrase (144 C-terminal amino acid residues)/Vif/Nef fusion protein (called TRIVN) [25] or OVA were constructed in our previous work. The *cta* gene used in this study was derived from *Vibrio cholerae* O1 biovar El Tor strain N16961 and was codon optimized for expression in humans. The TRIVN-CTA and OVA-CTA fusion genes were constructed by overlapping PCR. A CTA mutant with inactivated ADP-ribosyltransferase was constructed by introducing a K residue in place of the E residue in the catalytic center (E130K) *via* site-directed mutagenesis [26]. The DNA vaccines that were used for immunization were prepared with an Endofree Plasmid Giga Kit (Qiagen, #12391). The recombinant Tiantan vaccinia virus that encodes the Tat/Rev/Integrase (144 C-terminal amino acid residues)/Vif/Nef fusion protein was constructed using a previously described method [27].

2.3. In vitro cytokine transcription and secretion assays

NIH3T3 and RAW264.7 cells were transfected with either the mock control or a DNA vaccine encoding TRIVN, TRIVN-CTA, or CTA. Forty-eight hours later, the cells were harvested for total RNA extraction and the cell supernatant was collected for CBA analysis. Total RNA was extracted with an RNeasy Mini Kit (Qiagen, #74104). The intracellular mRNA levels of the cytokines IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12a, IL-12b, IL-17A, TNF-α and IFN-γ were quantified using a GoTaq^{TM} Real-Time qPCR Kit (Promega, #A6002). Reverse transcription was performed with

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