Vaccine 29 (2011) 2561–2567

Contents lists available at ScienceDirect

Vaccine

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

The virus-induced signaling adaptor molecule enhances DNA-raised immune protection against H5N1 influenza virus infection in mice

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ARTICLE INFO

Article history:
Received 18 July 2010
Received in revised form 4 January 2011
Accepted 21 January 2011
Available online 16 February 2011

Keywords:
DNA vaccines
VISA
TRIF
H5N1 influenza virus
Genetic adjuvant

ABSTRACT

As an adaptor molecule in the retinoic acid-inducible gene-I (RIG-I) signaling pathway, the virus-induced signaling adaptor (VISA) molecule activates NF-κB and IRF3 and thereby leads to the production of type I interferons (IFNs). To explore the potential of VISA as a genetic adjuvant for DNA vaccines, a eukaryotic expression plasmid, pVISA, was generated by cloning the VISA gene into the pVAX1vector. For comparison, the pTRIF plasmid was similarly constructed, encoding the known genetic adjuvant TRIF (TIR-domain-containing adapter-inducing interferon-β), an adapter in the Toll-like receptor (TLR) signaling pathway. Mice were immunized with the chimeric DNA vaccine pHA/$NP_{147–155}$, which encodes the HA (hemagglutinin) fused with NP (nucleoprotein) CTL epitope ($NP_{147–155}$) of H5N1 influenza virus, either alone or in combination with pVISA or pTRIF. Antigen-specific immune responses were examined in immunized mice. Our results demonstrate that co-immunization of the pHA/$NP_{147–155}$ plasmid with the VISA adjuvant augmented DNA-raised cellular immune responses and provided protection against H5N1 influenza virus challenge in mice. In addition, our data suggest that VISA acts as a stronger adjuvant for DNA immunization than TRIF. We conclude that co-inoculation with a vector expressing the adaptor molecule VISA enhanced the protective immunity against H5N1 infection induced by pHA/$NP_{147–155}$ and that VISA could be developed as a novel genetic adjuvant for DNA vaccines.

1. Introduction

The influenza A virus is able to cause severe morbidity and mortality to susceptible humans, making it one of the most deadly infectious pathogens. In 1997, a highly pathogenic avian H5N1 influenza virus was first reported to be transmitted from poultry to humans in Hong Kong, resulting in 18 infected people and six deaths [1]. During the period from 2003 to December 2010, 510 confirmed cases of human infection with H5N1 influenza viruses have been reported around the world, resulting in 303 deaths (http://www.who.int/csr/disease/avian_influenza). Although there have only been a few reports of direct human-to-human H5N1 transmission to date, H5N1 could potentially overcome this obstacle by reassortment with co-circulating human H1N1 or H3N2 influenza viruses [2], which may lead to the emergence of a pandemic influenza virus that could spread rapidly in the human population [3,4].

Vaccination is currently the most effective method to reduce influenza virus transmission, as well as the associated socio-economic burden [5,6]. The main forms of licensed human influenza virus vaccines are the traditional trivalent inactivated influenza vaccines, which are comprised of antigens from three circulating virus strains, currently including H1N1, H3N2 and influenza B. However, the difficulties associated with the egg-based production of traditional avian influenza vaccines, including the low yield of candidate vaccine viruses in chicken embryos, the necessity for biosafety level 3 containment facilities, and the poor immunogenicity of H5 hemagglutinin (HA) [7], have encouraged the development of new H5N1 vaccine strategies. As novel vaccine candidates, DNA vaccines have been proven to induce effective antibody response and long-term cell-mediated immunity in animal models [8–13]. DNA vaccines are also stable, inexpensive and relatively simple to prepare. However, the magnitude of the immune response elicited by DNA vaccines in larger animals, including humans, has been disappointing [14]. Considerable effort has been made to improve the efficacy of DNA vaccines, including optimizing the plasmid delivery system [15,16], improving antigen uptake and/or presentation [17,18], combining DNA vaccines with different prime–boost vaccination strategies [19], and co-delivering cytokines to up-regulate the immune responses to the antigens [20–25].

Viral infection results in induction of type I IFNs, including IFN-β and IFN-α family cytokines [26–28]. IFN-α/β play a key role in mediating both the induction of the innate immune response and the subsequent development of adaptive immunity to viruses [29–31]. At least two distinct mechanisms have

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doi:10.1016/j.vaccine.2011.01.060

been proposed for the production of IFN-α/β by virus-associated molecules [29,30]. One mechanism is mediated by TLRs, such as TLR3, which recognizes viral dsRNA released by infected cells [32]. Viral dsRNA binds to TLR3 and triggers TRIF-mediated signaling pathways, leading to IRF-3 and NF-κB activation [33–35]. Another mechanism involves retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), which function as cytoplasmic viral RNA sensors [36,37]. It has been shown that RIG-I-mediated signaling is independent of TLR3 and TRIF [36]. In the RIG-I pathway, the virus-induced signaling adaptor molecule (VISA, also known as MAVS, IPS-1, or Cardif) plays an essential role in the activation of antiviral signaling [38–41]. As an adaptor molecule in the RIG-I pathway, VISA is required for the activation of NF-κB and IRF-3 (interferon regulatory factor-3), and its activation therefore leads to the production of type I IFNs. VISA, which localizes to the mitochondria, triggers downstream signaling and is a critical link between mitochondria and innate antiviral immunity [41]. The overexpression of signaling molecules can be used to mimic infection by microbes, thereby eliciting the innate immune responses required for subsequent activation of adaptive immune responses against antigens. It has been found that incorporating the TLR adaptor molecule TRIF into a DNA vaccine triggers TLR signaling and thereby augments DNA-raised adaptive immune responses against the virus [42–44]. DNA vaccine immunogenicity is also enhanced by co-transfection of IRFs [45]. Adjuvanting a DNA vaccine with a TLR9 ligand together with Flt3 ligand results in enhanced cellular immunity against the corresponding virus [46]. In the present study, we used a mouse model to investigate the potential use of VISA as an adjuvant to augment DNA-raised immune responses against H5N1 and compared this with the TRIF genetic adjuvant.

2. Materials and methods

2.1. Mice and viruses

Six-week-old female BALB/c mice were used for immunization and challenge experiments. All mice were maintained with free access to sterile food and water.

The avian H5N1 influenza virus isolate A/chicken/Hubei/489/2004(H5N1) (A/chicken/Hubei/489) and a reassortant influenza virus (A/Viet Nam/1194R) with HA and NA genes of human-isolated influenza virus (A/Viet Nam/1194/2004 (H5N1)) and the internal protein genes of A/Puerto Rico/8/1934 were used in this study. A/Viet Nam/1194R was kindly provided by Dr. N. Robert (Virology Department, NIBSC, UK). Both virus stocks were mouse-adapted by passage through mouse lungs [18,47]. Viruses were propagated in the allantoic cavity of 10-day-old embryonated chicken eggs at 37 °C for 24 h or 48 h. The fifty-percent infectious dose (EID_{50}) of A/chicken/Hubei/489 was determined by serial titration in eggs and calculated using the Reed-Muench method. Median lethal dose (LD_{50}) titers of A/Viet Nam/1194R were determined by serial titration in BALB/c mice. All experiments with infectious H5N1 virus were conducted under BSL-3 containment.

2.2. Synthetic peptide

The synthetic peptide TYQRTRALV, corresponding to amino acid residues 147–155 of the NP protein of the H5N1 influenza virus, was purchased from HD Biosciences (Shanghai, China) at a grade of greater than 95% purity. The peptide was dissolved in DMEM for use in subsequent assays.

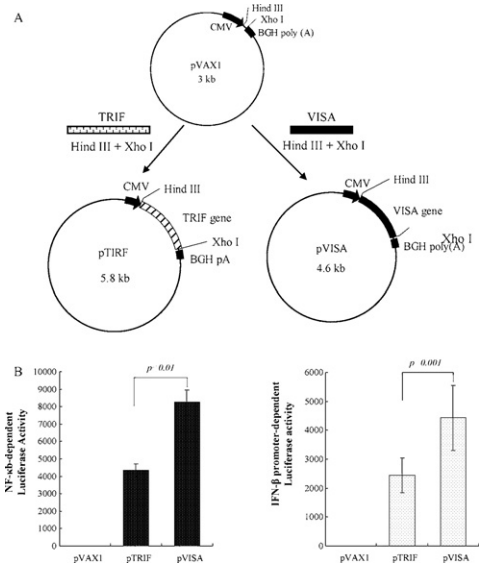

Fig. 1. VISA and TRIF activate IFN-β and NF-κB promoters. (A) A schematic diagram of plasmids expressing adjuvant molecules. (B) Vero cells ($\sim 2 \times 10^5$) were co-transfected with one of the reporter constructs (pNF-κB-luc or pIFN-β-luc) (200 ng) and 20 ng of a *Renilla* luciferase-expressing plasmid pRL-TK (for normalization), plus 600 ng of each indicated expression plasmid. Data represent the mean values with standard deviations for 3 independent experiments. The statistical significance of the differences between the relative luciferase activity in cells co-transfected with either pNF-κB-luc or pIFN-β-luc, together with either pVISA or pTRIF, is shown (VISA *versus* TRIF, $p < 0.01$ for NF-κB-luc activity; $p < 0.001$ for IFN-β-luc activity).

2.3. Construction of recombinant DNA plasmids

The chimeric HA DNA vaccine, pHA/$NP_{147–155}$, has been previously described [48]. The mammalian expression plasmids encoding human TRIF [49] (pRK-TRIF) and human VISA [38] (pHA-VISA), and the NF-κB-luciferase (pNF-κB-luc) and IFN-β-luciferase (pIFN-β-luc) [33] reporter constructs were kindly provided by Dr. H.B. Shu (Life Science College, Wuhan University, China). The VISA gene fragment was amplified from the pHA-VISA template by PCR using the primers 5′-CTGAAGCTTGACAATGCCGTTT-3′ (sense) and 5′-AGTCTCGAGAGGGCTTCACTAGTG-3′ (anti-sense). The PCR product was first digested using *HindIII* and *XhoI* and was then cloned into pVAX1 to generate pVISA. Similarly, the TRIF gene fragment was amplified from the pRK-TRIF plasmid by PCR using the primers 5′-TAGAAGCTTTACGTCGACCATGGCC-3′ (sense) and 5′-CAACTCGAGTCATTCTGCCTCCTGCG-3′ (anti-sense) and was then cloned into pVAX1 to create pTRIF (Fig. 1A). All constructs were sequenced to confirm their identities.

2.4. Reporter gene assay

Reporter gene assays were performed as previously described [42,50]. In brief, Vero cells were seeded in 24-well plates at a density of 2.0×10^5 cells per well 16–24 h prior to transfection. In each well, cells were co-transfected with 3 kinds of plasmids using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, USA), according to the manufacturer's protocol: (1) one of the reporter constructs (either pNF-κB-luc or pIFN-β-luc; 200 ng), (2) *Renilla* luciferase-expressing plasmid pRL-TK for normalization (20 ng), and (3) either pVISA, pTRIF or empty vector pVAX1 (600 ng). Cells were harvested 24 h post-transfection, lysed in passive lysis buffer and assayed for reporter activities using a reporter assay system (Promega, Madison, USA). All reporter gene assays were independently repeated at least 3 times.