Original Article

Synthetic toll-like receptor 7 agonist as a conjugated adjuvant enhances the Th1 type immunogenicity of influenza virus vaccine

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Abstract: Vaccination, one of the most effective and cost-efficient strategy in preventing and controlling influenza virus infection, also has poorly immunogenicity and side effects. Thus, adjuvants are employed to augment the immune response and overcome the drawbacks of vaccine. Here, we present a novel Toll-like receptor agonist (SZU-101, T7) to evaluate the adjuvanticity for inactivated H1N1 influenza vaccines. Chemical conjugation of T7 agonist with Flu (Flu-T7) significantly increased the release of IFN-γ, IL-12, IL-6 and TNF-α in mouse lymphocytes in vitro. Immunization with Flu-T7 elicited a more potent Th1-polarized immune response, represented by higher IgG2a titers compared to Flu alone. More importantly, immunization with Flu-T7 had no adverse effects. These observations have significant implications for the development of new and promising alternative adjuvant against inactivated H1N1 influenza vaccines.

Keywords: TLR7 agonist, influenza vaccine, conjugated adjuvant, Th1 immunity

Introduction

Influenza (commonly called flu) is an acute respiratory infectious disease, with highly contagious, significant morbidity and mortality [1]. There are three main types of influenza virus: Types A, B and C based on different nucleoprotein (NP) and matrix (M1) protein [2]. Influenza A (H1N1) virus is the most common subtype of influenza A virus that caused devastating outbreaks in Spanish from 1918 to 1919 and at least 20 million people dead [3]. The most effective means of preventing the outbreak of influenza pandemic is vaccination [4]. However, the efficacy of vaccines is affected by a multitude of factors and different every year [5]. Influenza vaccines are based on the conserved regions of haemagglutinin (HA), the major surface glycoprotein antigen [6]. Antigenic drift in HA exhibits high plasticity, and it is desirable to develop novel antigen and use adjuvants to enhance vaccine immunogenicity against influenza virus infections [7].

Vaccine adjuvants are an attractive option to enhance the vaccine efficacy, spare antigen dose and sufficiently induce immune responses [8]. CD4+ effector T cells and cytotoxic CD8+ T cell responses kill intracellular pathogens and play a key role in anti-influenza immunity responses [6]. Therefore, Toll-like receptors (TLRs) targeting innate immune receptors are one of the ideal adjuvant candidates. Small synthetic molecules TLR agonist has been reported to induce cytokine production and immune activation [9].

Here, we introduced a novel TLR7 agonist (SZU-101, T7) as an influenza adjuvant to enhance the immunologic effect of inactivated H1N1 vaccine. Moreover, SZU-101 demonstrates no in vivo toxicity. So, SZU-101 might be used as an effective adjuvant for H1N1 influenza vaccine.

Materials and methods

Animals

Female 4- to 6-week-old BALB/c mice were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, Guangdong, China).
TLR7 agonist as adjuvant and influenza virus vaccine

All animal experiments were approved by the Laboratory Animal Ethics Committee of Shenzhen University.

Reagents

Compound SZU-101 (T7, Figure 1A) was synthesized in our laboratory as previously described [9]. Inactivated H1N1 influenza vaccines were kindly provided by SINOVAC BIOTECH CO. (Beijing, China). Flu-T7 was prepared in our lab as described before [10].

In vivo immunization studies

32 female MALB/C mice (4-6 weeks) were randomly divided into four groups (n=8) and intraperitoneally (i.p.) immunized with 100 μl T7 (1 mg/ml), Flu (3 μg), Flu-T7 (3 μg) three times at 2-week intervals. Vehicle (PBS in PH 7.4) was used as control. Blood were collected from the tail on days 7, 21, 35 and centrifuged at 3000 g for 30 min to obtain sera. Mice were sacrificed on day 35 and the spleens were harvested for weight and cytokine responses.

ELISA

BALB/C mouse splenocytes were harvested using Mouse Lymphocyte Separation Medium (Dakewe, Beijing, China) according to the manufacturer's instructions. Then, lymphocytes (10⁶ cells per well) were cultured in 24-well plates in 100 μl of RPMI 1640 (Gibco, CA, USA) with 10% fetal bovine serum (Gibco, CA, USA). The cells were incubated with 1 μg/ml, 5 μg/ml, 10 μg/ml Flu or Flu-T7 for 24 h. The supernatants were collected and cytokine levels of IFN-γ, IL-12, TNF-α, IL-6 concentration in the culture supernatant of mouse splenocytes stimulated with SZU-101 was performed by ELISA assay. Data are mean ± SEM of triplicates and are representatives of three independent experiments. *P<0.05.
IL-12 and IL-6 were measured by Mouse ELISA Ready-SET-Go reagent sets (eBioscience, San Diego, CA) according to the manufacturer’s instructions.

**Measurement of antigen-specific antibodies**

Antigen-specific IgG and IgG subclasses IgG1, IgG2a in the serum were quantitatively determined by ELISA as described previously [11]. Briefly, each 96-well ELISA plate (Costar, Corning, New York, USA) was coated with 1 μg/ml Flu overnight at 4°C overnight. Plates were washed with PBST (0.01 M PBS containing 0.05% Tween 20, pH 7.4) for three times. Serum samples were detected at 1:500 dilution for 2 hours. Alkaline phosphate-conjugated detection antibody for IgG total, IgG1, IgG2a (Sigma-Aldrich, St. Louis, MO, USA) were incubated for 1 h at room temperature. Thereafter, p-NPP substrate (Millipore, Billerica, U.S.A.) and stop solution (50 μL of 3 M NaOH) were added to each well, and the optical density (OD, 405 nm) was measured by a spectrophotometer (BioTek, Winooski, U.S.A.).

**Statistical analysis**

All Data in this study were expressed as means ± standard errors of the mean (SEM). Graphpad Prism 5.0 software (San Diego, CA, USA) was used for statistical analysis. A two-tailed Student’s *t* test was used to compare two groups, and one-way ANOVA with Kruskal-Wallis test was used for the determination of multiple comparisons. Significant differences between groups were expressed as: *P*<0.05.
Results

Flu-T7 activated T cells and induced cytokine production

Previous studies have shown T7 induced cytokine production, enhanced dendritic cell maturation and antigen-specific cellular immune responses [9]. Hence, we evaluated the adjuvant activity of T7 for Flu. First, T7 was synthesized and used in the preparation of Flu vaccine (Figure 1A). T7 was also conjugated with Flu by chemical synthesis (Flu-T7). Then, we evaluated cellular immune responses by determining cytokines secretion in the splenocytes exposed to vaccines at indicated concentrations. As shown in Figure 1, Flu-T7 induced significantly more IL-12 (Figure 1B), IFN-γ (Figure 1C), TNF-α (Figure 1D) and IL-6 secretion (Figure 1E) compared with Flu alone (P<0.05). These findings indicated that Flu-T7 could induce both Th1- and Th2-associated immune response, suggesting T7 provide better protection against Flu virus infection.

Serum antibody responses in vaccinated mice

In order to assess the efficacy of T7 as Flu vaccine adjuvant, BALB/c mice were immunized with 3 μg of Flu or Flu-T7 on day 0, 14, 28 three times and bled on day 7, 21, 35, as schematized in Figure 2A. Antigen-specific total IgG (Figure 2B), IgG1 (Figure 2C), and IgG2a (Figure 2D) as early as 7 days after the first immunization. As shown in Figure 2B, from the second immunization, Flu-T7 induced rapid seroconversion to the Flu alone, and elicited the highest IgG titer. IgG is consisted of different subclasses based on structure and function, and IgG1 and IgG2a are main IgG subclasses induced by protein antigens [12]. We also detected the IgG subclass profiles, IgG1 and IgG2a titers in sera and ratio of IgG2a/IgG1 was determined for indicating the Th1/Th2 polarization [13]. Flu-T7 produced predominantly IgG2a in a Th1-type response. Taken together, T7 evoked stronger Th1-associated immunity than Flu alone.

Adverse effects

We also monitored both body weights and spleen weights and spleen/body weight ratio were calculated at the end of the experiment. There were no significant differences in toxicity between mice with Flu-T7, compared to Flu, T7 alone and the saline control (Figure 3).

Discussion

Vaccines play important roles in protecting individuals against influenza virus infections. However, available influenza vaccines have low immunogenicity and the genetic variation in HA allows them to escape immune responses. Therefore, adjuvants have to be added to augment the immune response. Aluminum salts, oil-in-water emulsions (MF59, AS03 and AF03), virosomes and AS04 (monophosphoryl lipid A preparation (MPL) with aluminum salt) are currently employed in human vaccines. The new generation of vaccines often uses small synthetic ligands for Toll-like receptors (TLRs) and other innate immune receptors as vaccine adjuvants [14].

The Toll-like receptors (TLRs) are a family of evolutionally conserved pattern recognition receptors (PRRs) that recognize highly conserved components of diverse pathogens [15]. They are involved in induction of host innate and adaptive immune responses. More and more TLRs are used as adjuvants for viral vaccines and demonstrated efficacy. MPL (monophosphoryl lipid A), a TLR-4 ligand is approved by FDA as an adjuvant for anti-cervarix HPV vaccine [16]. The TLR9 ligand CpG oligodeoxynucleotide (ODN) has completed a phase 3 clinical trial as adjuvant for Hepislab vaccine against HBV [17]. MF59 is the first oil-in-water influenza vaccine adjuvant and boost overall immune responses. The addition of TLR9 agonist CpG or
TLR agonist E6020 to MF59 resulted in higher antibody titers as well as Th1 cellular immune response [18, 19]. Chemical conjugation of TLR7 agonist (UC-1V150) and mouse serum albumin (MSA) resulted in 10- to 100-fold potent cytokine production in vitro and significant delay in mortality in BALB/c mice inoculated with H1N1 influenza A virus [20]. Accordingly, a novel TLR7 agonist, SZU-101 might be a potent adjuvant for influenza vaccine.

CD4+ and CD8+ T cell immunity play important roles in the control and long term protective immunity for influenza virus infection [21]. Since influenza virus induces Th1 responses and IFN-γ and TNF-α have antiviral effect. It was reported that SZU-101 activation induced a high level of proinflammatory cytokines, such as TNF-α, IFN-γ and IL-12, as well as Th2 cytokines IL-6 [9]. Indeed, when T7 was conjugated with Flu (Flu-T7), Flu-T7 significantly amplified TNF-α, IFN-γ, IL-12 and IL-6 cytokines compared to Flu alone. These results here suggested that Flu-T7 showed better efficacy in augmenting cellular immune response, because the Th1 cytokine IFN-γ could improve cell-mediated immunity [22]. However, further studies should be carried out to confirm whether the improved cellular immunity could provide better protection against influenza virus infection.

IgG is the main antibody in blood and extracellular fluid to control infection of body tissues. Most vaccines mediated humoral protection against a variety of pathogens through induction of serum IgG [23]. In vivo studies showed that Flu-T7 induced more potent immune responses, including higher IgG titer than Flu alone, and no adverse effect on the safety profiles.

IgG subclass profile indicated that Flu-T7 elicited more Th1-polarized immune response, while Flu alone elicited Th2-polarized immune response. IgG is consisted of four substantial different subclasses and have differential affinities for activatory and inhibitory Fcγ receptors (FcyRs). The activating-to-inhibitory (A/I) ratios of IgG1, IgG2a, and IgG2b are 0.1, 69, and 7, respectively, so IgG2a, and IgG2b are the most potent for activating effector responses [12] and the ratio of IgG2a/IgG1 is considered as indicators of Th1/Th2 polarization [24].

Toxicity and safety concerns are crucial issues for vaccines adjuvants development. Although aluminum hydroxide is widely used for over 80 years, it also has some side effects, such as macrophagic myofasctis [25], IgE-associated allergic reaction [26] and cumulative alum toxicity [27]. Squalene-based oil-in-water emulsions MF59 [28] and AS03 [29] also caused local inflammatory reactions although they have been approved by Europe for broadly use. TLR7 agonist, as a novel viral vaccine adjuvant has been demonstrated to have excellent safety profile [30]. In our studies, spleen/body weight ratio is similar between Flu alone and Flu-T7 group. However, histological examination should also be evaluated to whether T7 caused vaccine-associated local inflammation in vivo at injection sites.

Considering its efficacy and safety profile, we conclude that SZU-101 is promising candidate adjuvant for inactivated H1N1 influenza vaccines, induced stronger IgG antibody and elicited strong IgG2a responses in mice compared to Flu alone.

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Disclosure of conflict of interest

None.

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