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Recombinant lipidated dengue-3 envelope protein domain III stimulates broad immune responses in mice

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ABSTRACT

The linkage of an immunogen with a toll-like receptor ligand has great potential to induce highly potent immune responses with the initial features of antigen-presenting cell activation. In the current study, we expressed recombinant dengue-3 envelope protein domain III (D3ED III) in lipidated form using an *Escherichia coli*-based system. The recombinant lipidated dengue-3 envelope protein domain III (LD3ED III) augments the expression levels of IL-12 family cytokines. LD3ED III-immunized mice enhance wide ranges of T cell responses as indicated by IFN-γ, IL-17, IL-21 production. Additionally, LD3ED III-immunized mice increase the frequencies of anti-D3ED III antibody producing cells. The boosted antibody titers cover various IgG isotypes, including IgG1, IgG2a, IgG2b, and IgG3. Importantly, LD3ED III-immunized mice induce neutralizing antibody capacity associated with a reduction of viremia levels after challenges. In contrast, mice that are immunized with D3ED III formulated with aluminum phosphate (D3ED III/Alum) only enhance Th2 responses and boost IgG1 antibody titers. Neither neutralizing antibody responses nor the inhibition of viremia levels after challenge is observed in mice that are immunized with D3ED III/Alum. These results suggest that LD3ED III can induce broad profiles of cellular and humoral immune responses.

1. Introduction

Dengue fever or severe dengue hemorrhagic fever and dengue shock syndrome are caused by the infection of dengue virus, which includes four antigenically different serotypes. Although the true disease burden is not well known, a recent estimation reported that dengue infection reaches 390 million clinical infections annually [1] and results in a serious public health threat in more than 120 countries throughout tropical and subtropical areas [2,3]. It is believed that vaccination is an effective and successful tool to combat pathogens [4]. A licensed dengue vaccine is not currently available due to the complex interaction of four serotypes of dengue virus with the human immune system. Pharmaceutical companies and research laboratories have employed various technologies for dengue vaccine development. Several dengue vaccine candidates are in clinical development [5]. The leading vaccine candidate is Sanofi Pasteur's live chimeric virus vaccine. After three injections of tetravalent live chimeric virus vaccine, the vaccine efficacies were 56.5% [6] and 60.8% [7] in Asia and Latin America phase 3 studies, respectively. Thus, continued efforts are required to develop dengue vaccines.

Subunit vaccines provide an alternative approach for dengue vaccine development. Recombinant protein-based subunit vaccines do not contain live components of pathogens and are considered to be safe. In total, recombinant proteins are poor vaccine immunogens and require appropriate adjuvants to provoke the desired immune responses. When designing a subunit vaccine, the choice of immunogen is determined by the pathogen,

Abbreviations: D3ED III, ecombinant dengue-3 envelope protein domain III; ED III/Alum, D3ED III formulated with aluminum phosphate; FFU, focus-forming unit; FRNT, focus reduction neutralization tests; IMAC, immobilized metal affinity chromatography; LD3ED III, recombinant lipidated dengue-3 envelope protein domain III.

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whereas the selection of adjuvant depends on the desired immune response. Dengue envelope protein domain III is a critical region on the viron surface for viral attachment to cellular receptors [8,9]. Several neutralizing epitopes have been identified in dengue envelope protein domain III [10–12]. These results suggest that dengue envelope protein domain III is a suitable target for dengue vaccine development [13]. Several dengue envelope protein domain III subunit vaccine candidates have been evaluated in mice [14–21] and nonhuman primates [22–26]. A subunit vaccine candidate formulated with proper adjuvant is required to induce robust immunity. Unfortunately, formulations of dengue subunit vaccines using adjuvants containing aluminum, the most widely used adjuvants in human vaccines, are unable to induce complete protection against dengue virus infection [17,22,25]. Exploring exogenous adjuvant-independent approaches to enhance immunogenicity may provide a solution for dengue subunit vaccine design.

An efficient way to trigger immune responses is through the presentation of the antigen by antigen-presenting cells and simultaneously providing activation signals [27,28]. To mimic natural infection, recombinant protein is linked to pattern recognition receptor ligands to create immunogen-pattern recognition receptor ligand-conjugated subunit vaccines, ensuring that the same antigen-presenting cell receives immunogens and stimulation signals at the same time. In our previous studies, we developed a novel platform technology to express recombinant lipidated immunogens with intrinsic adjuvant properties [29]. The lipid moiety of recombinant lipidated immunogens provides a danger signal to activate antigen-presenting cells via toll-like receptor 2 [30] and induces an appropriate adaptive immunity in the absence of exogenous adjuvant formulation [19]. The lipidation strategy has been applied to dengue-1, -2, and -4 envelope protein domain III [17,18,20,31], but recombinant lipidated dengue-3 envelope protein domain III (LD3ED III) has not yet been characterized. In the present study, we further dissected the profile of T cell and antibody responses in mice that were immunized with LD3ED III. We confirmed and further extended the main findings of our previous works [17,18,20,31], namely, that recombinant lipidated immunogens can induce strong and persistent immune responses even without formulation with exogenous adjuvants.

2. Materials and methods

2.1. Virus

Dengue-3/H-087 (laboratory-adapted virus) was used for this study and kindly provided by Dr. Yi-Ling Lin of the Institute of Biomedical Sciences, Academia Sinica, Taiwan. Propagation of virus was performed in C6/36 cell, and viral titers were determined by focus-forming assays with BHK-21 cells [21].

2.2. Experimental mice and immunization

Female BALB/c mice were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). The mice were maintained at the Laboratory Animal Center of the National Health Research Institutes (NHRI). All of the animal experiments were approved and performed in compliance with the guidelines of the Animal Committee of the NHRI. Groups of mice (6–8 weeks of age) were immunized with D3ED III, D3ED III/Alum, or LD3ED III via subcutaneous injection. Each mouse received a 10 μg/0.2-mL dose. Mice that were immunized with PBS alone (without antigens) served as controls. All of the animals were given 2 injections at a two-week interval with the same regimen. Blood samples were collected by tail bleeding for 0.1–0.2 mL from each mouse at different time points as indicated. The serum samples were prepared and stored at −20 °C until use.

2.3. Preparation of recombinant proteins and the other materials and methods

All the details were in Supplementary data.

3. Results

3.1. Preparation and characterization of dengue-3 envelope protein domain III recombinant immunogens

The dengue-3 envelope protein domain III gene was cloned into the expression vector pET-22b(+) (Novagen, Madison, WI), using Nde I and Xho I sites to produce the pD3DEIII plasmid for the production of recombinant dengue-3 envelope protein domain III (D3E3 III). To produce LD3ED III, the D3ED III gene was cloned into the pET-22b-based plasmid containing a lipid signal peptide using Bam HI and Xho I sites to produce the pLD3EDIII plasmid. As a result, both D3ED III and LD3ED III were contained an additional HHHHHH sequence (HisTag) at their C-terminus and were expressed under the control of the T7 promoter (Fig. 1A).

The D3ED III was purified using an immobilized metal affinity chromatography (IMAC) column (Fig. 1B, lanes 1–4). D3ED III was detected with anti-HisTag antibodies (Fig. 1B, lanes 5–8). The lipidated counterpart of D3ED III, LD3ED III was also purified using an IMAC column (Fig. 1B, lanes 9–12) and was detected with anti-HisTag antibodies (Fig. 1B, lanes 13–16). After removing the lipopolysaccharide (LPS), the residues of LPS in D3ED III and LD3ED III were less than 0.06 EU/mg. To have stable materials for further characterization and validation, we produced and lyophilized a sufficient amount of materials in one batch. Endotoxin-free D3ED III and LD3ED III were comparatively analyzed for their immunogenicity and efficacy in animal models.

Next, the exact mass of the N-terminal fragments of LD3ED III were measured. As shown in Fig. 1C, there were three major peaks with m/z values of 1452, 1466, and 1480 in the spectrum. These peaks have been identified in other lipidated proteins and can be considered as a lipidation signature [18,20,29,31,32]. We confirmed that the peaks of LD3ED III were associated with the lipidated cysteine residue and verified that LD3ED III contains a *N*-acetyl-*S*-diacyl-glyceryl-cysteine at its N-terminus.

3.2. Recombinant lipidated dengue-3 envelope protein domain III upregulates IL-12 family cytokines in bone marrow-derived dendritic cells

Cytokines are important mediators of immune responses. Dendritic cells produce IL-12, IL-23, and IL-27 to modulate T cell differentiation. Members of the IL-12 family are heterodimeric cytokines that consist of an α chain and a β chain. The p40 chain can heterodimerize with p35 or p19 to form IL-12 or IL-23, respectively, whereas Epstein-Barr virus-induced gene 3 (EBI3) can pair with p28 or p35 to form IL-27 or IL-35, respectively [33]. Therefore, IL-12 family cytokines were analyzed after stimulation. The expression levels of p40, p35, p19, p28, and EBI3 mRNA in dendritic cells 4 h after LD3ED III stimulation were higher than PBS stimulated dendritic cells for 7180-, 79-, 1895-, 1563-, and 17-fold, respectively (Fig. 2A). To verify the cytokine expression, heterodimeric form of IL-12, IL-23, and IL-27 levels in the supernatants were evaluated by ELISA. As shown in Fig. 2B, the highest levels of IL-12, IL-23, and IL-27 were induced after LD3ED III stimulation. In contrast, there were no significant IL-12, IL-23, and IL-27 production when dendritic cells were stimulated with 6 EU/mL LPS (equivalent to 10,000 folds of LD3ED III LPS residues) (Supplementary Fig. 1). These results indicate that LD3ED III activates dendritic cells and upregulates the production of IL-12 family cytokines in dendritic

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