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Flagellin-expressing virus-like particles exhibit adjuvant effects on promoting IgG isotype-switched long-lasting antibody induction and protection of influenza vaccines in CD4-deficient mice

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Abstract

Incorporation of membrane-anchored flagellin molecules into the surfaces of influenza virus-like particles (VLP) was previously reported to promote T helper (Th) 1 IgG antibody production and protective efficacy of co-presented vaccine antigens. Herein, we investigated the potential adjuvant effects and mechanisms of flagellin-expressing VLP (FliC-VLP) as an independent component on influenza vaccination in wild-type and mutant mouse models. FliC-VLP adjuvanted influenza vaccination was highly effective in promoting the induction of Th1-biased IgG isotype switched antibodies, enhanced protection, and long-lasting IgG antibody responses in both wild-type and CD4-knockout mice. In contrast, the adjuvant effects of soluble flagellin were Th2-biased and required CD4 T helper cells. The adjuvant effects of FliC-VLP were less dependent on CD4 T cells and flagellin-mediated innate immune signaling pathways. The results suggest that FliC-VLP might play an effective adjuvant role in an immune competent condition as well as in a defect of CD4 T cells.

Keywords

Adjuvant; Flagellin; VLP; Influenza; CD4 T cells

1. Introduction

Influenza virus causes mild to severe systemic symptoms including a high fever, headache and inflammation in upper and lower respiratory systems. Young children, older adults, pregnant women and immune-compromised patients have high risk of influenza virus

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Conflict of interest

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infection. Vaccination is the most effective way to prevent influenza virus infection. World Health Organization predicts seasonal influenza vaccine strains and recommends vaccination annually. Inactivated split influenza virus vaccine is the most common platform but its immunogenicity and efficacy need to be improved particularly in naïve individuals and the elderly [1, 2].

To enhance the immunogenicity and efficacy of vaccines, adjuvants are an option to be included in the vaccine formulations for young children and the elderly. Most adjuvants stimulate the innate immune cells and antigen presenting cells (APCs) which result in subsequently activating CD4 T helper cells. B cells require CD4 T helper cells in producing IgG class-switched antibodies. Therefore, the pattern and the outcomes of adjuvant effects are dependent on the types of CD4 T cells that are being induced and activated by APCs [3–9]. Aluminum hydroxide (alum), the most common adjuvant used in human vaccines, promotes T helper (Th) 2 IgG antibody responses. As expected, the adjuvant effects of alum required the presence of intact CD4 T cells in a mouse model [10]. MF59, an oil-in-water emulsion form of adjuvant, is licensed for use in influenza vaccines. AS04, a combination of alum and toll-like receptor (TLR) 4 agonist monophosphoryl lipid A (MPL), is included in human hepatitis B virus and papilloma virus vaccines [11, 12]. MF59 and AS04 adjuvant effects were recently shown to be independent of or less dependent on the presence of CD4 T helper cells in contrast to alum in a mouse model [13, 14].

Flagellin, a bacterial component, is recognized by TLR5 [15] and also stimulates intracellular signals via an inflammasome-activating pathway involving NLRC4 (nucleotide-binding and oligomerization domain [NOD]-like receptor [NLR] member) [16, 17]. Flagellin has been utilized as adjuvant in a fusion conjugate with vaccine protein antigens or by delivery of co-mixed flagellin and vaccine antigens [18, 19] (reviewed in [20, 21]). The safety and efficacy of flagellin as an immune potentiating molecule in plague vaccine conjugates or influenza antigen conjugates were also tested in clinical trials [22] (reviewed in [20]).

Virus-like particles (VLP) have been developed as a new form of vaccines. VLP express antigenic virus proteins on lipid bilayer membranes but do not contain virus genetic material. Therefore, VLP can elicit antigen-specific immune responses without a risk of infection by the attenuated virus and show better immunogenicity than soluble subunit vaccines [23, 24]. Incorporation of membrane-anchored recombinant flagellin molecules into the surfaces of influenza hemagglutinin VLP was shown to enhance Th1-biased IgG antibodies and the breadth of immune responses [25, 26]. However, the potential adjuvant effects and mechanisms of flagellin expressing VLP (FliC-VLP) standalone have not been elucidated.

In this study, we investigated the adjuvant effects of FliC-VLP as an independent component in wild-type and mutant mouse models. FliC-VLP adjuvanted split influenza vaccination was discovered to be superior to soluble flagellin adjuvant in promoting the induction of Th1-biased IgG antibodies in wild-type mice, as well as IgG isotype switched antibodies and improved protection in CD4 knock-out (CD4KO) mice. IgG antibody responses induced by FliC-VLP adjuvanted influenza vaccination were long-lasting even in CD4KO mice. The

adjuvant effects of FLiC-VLP on influenza vaccine efficacy were also investigated in TLR5 and NLRC4 mutant mice, and potential adjuvant mechanisms have been discussed.

2. Materials and methods

2.1. Animals and reagents

Female C57BL/6 wild-type and mutant CD4 knock-out (CD4KO, B6.129S6-Cd4^{tm1Kw}/J) mice were purchased from the Jackson laboratory. Female TLR5 KO, NLRC4 KO (N4KO) and TLR5/NLRC4 double-KO (T5N4 DKO) mice were used in this study and previously described in detail [27]. All mice used in this study were maintained in Georgia State University (GSU) animal facility. All animal studies were conducted with the approval of the Institutional Animal Use and Care Committee at Georgia State University (Protocol A18001) implementing the humane care of experimental animals. Inactivated pandemic H1N1 A/California/07/2009 influenza split vaccine (Green Flu-S) commercially manufactured by Green Cross (South Korea) was used in this study. Flagellin-incorporated VLP (FLiC-VLP) was expressed in insect cells using the recombinant baculovirus expression system and prepared as previously described [25]. Soluble flagellin (SF) used in this study was purified from *Salmonella enterica* serovar Typhimurium through sequential cation- and anion-exchange chromatography including use of polymyxin B resins specifically designed to deplete LPS as described previously [15, 28].

2.2. Immunization and infection

C57BL/6 wild-type and mutant mice were intramuscularly immunized, two times at a 4-week interval, with split vaccine only (Vac only, 1 µg of hemagglutinin [HA]/mouse), split vaccine plus adjuvant of FLiC-VLP (10 µg/mouse) or SF (0.5 µg/mouse). At 7 or 16 weeks after boost immunization, the naïve and immunized mice were infected with a lethal dose (17×LD₅₀) of A/California/07/2009 H1N1 virus (A/Cal H1N1). A set of immunized mice was monitored body weight changes and survival rates for 14 days, and another set was euthanized at day 5 post infection for further analysis.

2.3. Antigen-specific antibody and cytokine ELISA

To determine antigen-specific antibody levels, serially diluted immune sera or lung extracts were added to the ELISA plates coated with inactivated virus (200 ng/well) and horseradish peroxidase-labeled anti-mouse IgG, IgG1 and IgG2c were used to detect antibodies. Tetramethylbenzidine (TMB) was used as a substrate and optical density was measured at 450 nm by an ELISA reader (BioRad). The total amount of antibody was quantified using the standard curve for each IgG isotype antibody. For analysis of antibody producing cell responses in the immunized mice, bone marrow cells were collected at day 5 post infection and cultured in inactivated A/Cal H1N1 virus-coated cell culture plates for 1 day. The binding and secreted antibodies were detected by ELISA. Interleukin (IL)-6 and tumor necrosis factor (TNF)-alpha ready-set-go kits (eBiosciences) were used by following manufacture's manual to measure the cytokines in lung extracts and cell culture supernatants.

2.4. Hemagglutination inhibition (HAI) assay

Immune sera were mixed with a receptor destroying enzyme (Sigma Aldrich) and then incubated at 37 °C for 18 hours. The samples were heat inactivated at 56 °C for 30 minutes for complement inactivation. Serially 2-fold diluted sera were incubated with 8 HA units of A/Cal H1N1 virus in V-bottom microplates. After 30 minutes, 0.5% chicken red blood cells were treated to the wells and hemagglutination was determined after 40 minutes to determine HAI titers as previously described [14].

2.5. Lung virus titration

Lung samples were harvested at day 5 post infection and lung extracts were prepared by using a mechanical tissue grinder with 1.5 ml of PBS per each lung. Embryonated chicken eggs were incubated for 9 to 10 days before inoculation. Serially diluted lung extracts were injected into the allantoic sac of the chicken eggs and incubated at 37°C for 3 days. The allantoic fluids of eggs were collected and hemagglutination assay was performed to determine viral titers. Virus titers as 50% egg infection dose (EID₅₀)/ml were evaluated according to the Reed and Muench method [29].

2.6. *In vitro* bone marrow derived dendritic cells (BMDCs)

BMDCs were generated from bone marrow cells of C57BL/6 wild-type, TLR5KO, N4KO, T5N4 DKO, and MyD88 KO mice with mouse granulocytes-monocyte colony stimulating factor (mGM-CSF) as described before [30, 31]. After 6 to 10 days culture, the generated immature DCs were collected and seeded in 96 well-plate with 5×10^4 cells/well. LPS (0.2 µg/ml), FliC-VLP (10 µg /ml), or SF (10 µg /ml) were treated and cultured for 2 days. Cell culture supernatants were collected, and cytokine ELISA was performed.

2.7. Statistical analysis

The data presented as the mean $\pm$ the standard deviation (SD). The statistical analysis was performed by one-way analysis of variance (ANOVA) with Tukey multiple-comparison test in GraphPad Prism. A p value less than 0.05 was regarded as statistically significant.

3. Results

3.1. FliC-VLP is superior to soluble flagellin adjuvant in promoting the IgG isotype switched antibodies to split influenza vaccination in CD4KO mice

Flagellin protein is known to play a role of adjuvant in enhancing the immunogenicity of co-administered antigen [21, 32, 33]. FliC-VLP was produced in insect cells by using recombinant baculoviruses expressing FliC and influenza virus M1 matrix proteins driving the VLP formation [25]. FliC-VLP contains recombinant flagellin in a membrane-anchored form and showed glycosylation between flagellin peptide backbone and TLR5 stimulating bioactivity comparable to that of soluble flagellin as determined by RAW264.7 cell-based *in vitro* assay [25]. ELISA results indicate that FliC-VLP contained 1.25% flagellin contents out of total VLP proteins (Supplementary Fig. S1).

Here, we investigated the adjuvant effects of FliC-VLP and determined whether FliC-VLP adjuvanted split influenza vaccination would overcome a defect of CD4 T helper cells in

inducing IgG isotype-switched antibodies. C57BL/6 and CD4 knock-out (CD4KO) mice were intramuscularly immunized with inactivated split influenza vaccine (Vac, 1 µg of HA/mouse) +/- FliC-VLP (10 µg/mouse) or Vac + soluble flagellin (SF, 0.5 µg/mouse) in a prime-boost strategy with a 4-week interval. After prime immunization of C57BL/6 mice, both the FliC-VLP and SF groups showed higher virus-specific serum IgG levels than Vac only (Fig. 1A). The FliC-VLP adjuvanted group induced more IgG2c production, representing Th1-biased immune responses, compared to Vac only or Vac+SF vaccination whereas the Vac only or Vac+SF group induced IgG1 (Th2)-biased antibody production. After boost immunization, the differences in IgG1 versus IgG2c isotype antibody levels were further expanded although the levels of serum IgG antibodies were increased in all groups. The titers of HAI were observed at substantially higher levels in the FliC-VLP- and SF-adjuvanted groups compared to the Vac only group which did not show HAI antibody responses despite the induction of antigen-specific binding IgG antibodies in C57BL/6 mice (Fig. 1A). These results provide evidence of significant adjuvant effects of both FliC-VLP and SF on enhancing IgG responses to vaccine in a different Th-biased pattern.

CD4 T cell help is required for B cell activation to generate isotype-switched IgG antibodies against T-dependent antigens. We determined the roles of CD4 T cells in inducing IgG antibodies by FliC-VLP- and SF-adjuvanted vaccination in a CD4KO mouse model. Vac only was not able to induce IgG antibodies in CD4KO mice, which indicates that inactivated split vaccine was T-dependent antigen (Fig. 1B). The Vac+SF group also showed no detectable IgG antibodies after prime immunization, and lower levels of IgG after boost in CD4KO mice. Interestingly, Vac+FliC-VLP immunization of CD4KO mice induced IgG isotype antibodies after prime immunization and further increased IgG isotype binding antibodies (IgG1, IgG2c) after boost immunization. In addition, significantly higher HAI functional antibodies were detected in Vac+FliC-VLP immunized CD4KO mice (Fig. 1B). Nonetheless, the levels of IgG and IgG2c isotype antibodies in CD4KO mice were lower than those in C57BL/6 mice after Vac+FliC-VLP immunization, indicating a partial role of CD4 T cells in promoting the IgG2c Th1-biased antibodies in FliC-VLP adjuvanted vaccination (Fig. 1B and Supplementary Table S1). These results suggest that FliC-VLP is superior to soluble flagellin adjuvant in promoting the IgG isotype switched antibodies to T-dependent influenza split vaccination in CD4KO mice.

3.2. FliC-VLP is more effective than soluble flagellin in conferring protection in CD4KO mice after adjuvanted split influenza vaccination

To determine the protective efficacy, naïve and the immunized C57BL/6 and CD4KO mice were challenged with a lethal dose (17×LD₅₀) of A/Cal H1N1 virus intranasally at 16 weeks after boost immunization. The Vac+FliC-VLP and Vac+SF C57BL/6 groups showed minimum weight loss (1~5%) and 100% of survival rates. In contrast, the Vac only C57BL/6 group showed 16% of weight loss until 8 days post infection, and 46% of survival rates (Fig. 2A). Lung virus titers were measured at day 5 post infection by using embryonated chicken eggs. The Vac and Vac+SF C57BL/6 groups showed approximately 100 times lower virus titers compared to those in the naïve infected group. The Vac+FliC-VLP C57BL/6 group reduced lung viral loads by 1×10⁴ times less than naïve mice after infection.

Compared to C57BL/6 wild-type mice, the Vac only CD4KO group displayed severe weight loss (> 25%) and no mice survived (Fig. 2B). The Vac+SF CD4KO group showed 10~12% of weight loss and 75% of survival rates, indicating low adjuvant effects of SF in CD4KO mice. In contrast, the Vac+FliC-VLP CD4KO group showed the best protective efficacy (2.6% body weight loss and 100% of survival rate) with significantly lower virus titers by 1×10^4 times (Fig. 2B). These data suggest that FliC-VLP adjuvanted vaccination significantly enhanced protection against lethal challenge although FliC-VLP adjuvant effects were partially dependent on CD4 T cells in eliciting antigen-specific antibody production in CD4KO mice.

In both C57BL/6 wild-type and CD4KO mice, interleukin (IL)-6 was highly produced in the lungs from the naïve and Vac only groups, but not in FliC-VLP and SF-adjuvanted groups at day 5 post infection (Fig. 3). The levels of virus specific IgG antibodies in lung extracts appeared to be correlated with protective efficacy in vaccinated mice (FliC-VLP and Vac+SF C57BL/6, FliC-VLP CD4KO, Fig. 3). Taken together, FliC-VLP was significantly more effective than soluble flagellin in conferring protection in CD4KO mice after adjuvanted split influenza vaccination, whereas both adjuvants were effective in promoting protective efficacy of split influenza vaccination in C57BL/6 wild-type mice.

3.3. FliC-VLP adjuvanted split influenza vaccination induces long-term immune responses and protection even in CD4KO mice

To evaluate the antibody-producing long-lived plasma cell responses, we collected bone marrow cells from immunized C57BL/6 and CD4KO mice at day 5 post infection. The cells were cultured *in vitro* with inactivated virus and virus-specific IgG antibody levels were measured by ELISA (Fig. 4A). The Vac+FliC-VLP and Vac+SF C57BL/6 groups showed high antibody production in bone marrow cell cultures *in vitro* for 1 day. In contrast, the Vac+FliC-VLP CD4KO group showed *in vitro* IgG production in bone marrow cell culture but not the Vac+SF group.

It is critical to maintain long-lasting IgG antibody responses after vaccination. Therefore, we measured IgG antibody levels longitudinally in wild-type and CD4KO mice after Vac only or Vac+FliC-VLP vaccination (Fig. 4B). The Vac only group induced low levels of IgG (mostly IgG1 isotype) in wild-type mice, which reached a peak level at 3 months after vaccination and then waned to a low level. However, Vac+FliC-VLP vaccination led to maintaining high levels of IgG and IgG isotype (IgG1, IgG2c) antibodies in wild-type and CD4KO mice for 9 months. The IgG antibody levels were higher in wild-type than those in CD4KO mice. The mice were finally infected with the lethal dose of A/Cal H1N1 virus to determine the protective efficacy of FliC-VLP adjuvanted vaccination. Both WT and CD4KO mice immunized with Vac+FliC-VLP showed 0~2% of body weight loss, a trend of lowering PenH values and higher plasma cell responses (Supplementary Fig. S2). These results suggest that FliC-VLP adjuvanted split influenza vaccination led to maintaining high levels of IgG antibodies for long-term protection even in a condition of CD4 T cell deficiency.

3.4. The adjuvant effects of FliC-VLP on split influenza vaccination do not require but are partially dependent on TLR5 and NLRC4 signaling pathways

We determined whether TLR5 would be required for the adjuvant effects of FliC-VLP on split influenza vaccination using a TLR5KO mouse model. TLR5KO mice were immunized with Vac (1 µg of HA) only, Vac+FliC-VLP (10 µg) or Vac+SF (0.5 µg) in an independent experiment under the same immunization condition as we described for C57BL/6 and CD4KO mice (Fig. 5A). Virus-specific IgG and isotype-switched (IgG1, IgG2c) antibodies were induced at higher levels in the Vac+FliC-VLP group than those in the Vac only group after prime immunization of TLR5KO mice (Supplementary Table S1), suggesting that the adjuvant effects of FliC-VLP were retained in TLR5KO mice. SF-adjuvanted immunization in TLR5KO mice induced lower antibody production than FliC-VLP, but higher than Vac only (Fig. 5A). Boost immunization further increased IgG antibody responses in TLR5KO mice. TLR5KO mice with Vac only vaccination displayed severe weight loss (20–22%) and 40% survival rates after lethal challenge (Fig. 5B and C), which are comparable to wild-type mice with Vac only vaccination. Vac+SF immunized TLR5KO mice showed 20% weight loss at day 7 post infection, but faster weight recovery than Vac only. The survival rate of SF-adjuvanted TLR5KO mice was 70%, which was lower than that in WT mice (Fig. 2). These results suggest that the adjuvant effects of SF are partially dependent on the TLR5 signaling pathway.

TLR5KO mice with Vac+FliC-VLP vaccination did not show severe weight loss (3.7% loss at day 7) and were 100% protected against lethal challenge (Fig. 5B and C), which is a similar level of protection observed in wild-type mice. Flagellin is also known to activate immune responses via an NLRC4 inflammasome signaling pathway [34]. We determined the FliC-VLP adjuvant effects in NLRC4 KO (N4KO) and NLRC4/TLR5 double KO (T5N4DKO) mice. Vaccination of N4KO and T5N4DKO mice with Vac+FliC-VLP induced IgG1 and IgG2c antibodies at a similar or slightly lower level than those observed in TLR5KO (Supplementary Table S1). The levels of IgG and isotype antibodies in TLR5KO, N4KO, and T5N4DKO mice CD4KO mice were lower than those in C57BL/6 mice after Vac+FliC-VLP immunization (Supplementary Table S1). Vac+ FliC-VLP immunized N4KO and T5N4DKO mice showed a moderate loss of 7~13% and survived from lethal challenge whereas unvaccinated and Vac only mice lost weight over 20% and did not survive (Fig. 5B and C, Supplementary Table S1). The deficiency of both NLRC4 and TLR5 signaling pathways might moderately reduce the protective efficacy of Vac+FliC-VLP vaccination compared to those in wild-type C57BL/6 mice. Overall, these results suggest that the adjuvant effects of FliC-VLP on split influenza vaccination were partially dependent on TLR5 and NLRC4 signaling pathways.

4. Discussion

Flagellin was reported to activate human DCs, resulting in up-regulation of maturation markers and chemokine production via a TLR5 signaling [35], suggesting its potential vaccine adjuvant mechanism. Also, previous studies demonstrated that influenza VLPs co-presenting flagellin and hemagglutinin antigens were more effective in inducing virus-specific Th1-biased humoral immune responses than VLP vaccines without flagellin [25, 26,

36, 37]. Herein, we investigated whether flagellin only expressing VLP (FliC-VLP) would be an independent and effective vaccine adjuvant to promote the immune responses to co-administered vaccine antigens in wild-type and mutant mice in comparison with soluble flagellin. FliC-VLP was found to contain approximately 1.25% flagellin (0.125 µg Flagellin in 10 µg FliC-VLP), indicating that the dose of 0.5 µg soluble flagellin was 4 folds higher than that in FliC-VLP (Supplementary Fig. S1). Even though the displayed flagellin was 4 folds less on FliC-VLP, both FliC-VLP and soluble flagellin adjuvanted vaccinations showed similar HAI titers in wild-type mice, which were correlating with protection (Fig. 1A). Due to the similar antibody responses in wild-type mice, we have used 10 µg of FliC-VLP and 0.5 µg of soluble flagellin in further experiments. Both IgG1 and IgG2c isotype antibodies were effectively produced by inclusion of FliC-VLP in the influenza split vaccination in wild-type and CD4KO mice whereas Vac+soluble flagellin vaccination induced IgG1 isotype antibodies in wild type mice but not in CD4KO mice (Fig. 1). In contrast to soluble flagellin, this study demonstrated that flagellin on a VLP platform endowed unique adjuvant properties of promoting the induction of Th1-biased IgG isotype antibodies (IgG2c) as well as enhancing the efficacy of vaccination, which is partially dependent on conventional CD4 T cell help. Despite of lower antibody production in FliC-VLP adjuvanted CD4KO mice compared to those in WT mice, both WT and CD4KO mice adjuvanted with FliC-VLP showed better protection against lethal influenza virus infection (Fig. 1 and 2), indicating that FliC-VLP could induce protective immune responses in CD4KO mice. In addition, the levels of IgG antibodies induced in CD4KO mice with Vac+FliC-VLP vaccination were maintained for 9 months, comparable to those in wild-type mice (Fig. 4).

Soluble flagellin in influenza split vaccination promoted the induction of IgG1 isotype antibodies and serum HAI activity as well as protective efficacy of preventing weight loss in wild-type mice (Fig. 1 and 2). The Th2-biased responses might be associated with maturation of DCs, driven by MyD88-TLR5 dependent pathways and by induction of CD4 T cells producing IL-4 and IL-13 but not the Th1-promoting IL-12 p70 cytokines [38]. In consistent, the adjuvant effects by soluble flagellin in influenza vaccination of mice were more dependent on CD4 T cells as shown in comparison with vaccination in CD4KO and wild-type mice (Fig. 1). This observation of CD4 dependency of soluble flagellin adjuvant is consistent with that of aluminum hydroxide (alum) adjuvant effects that required the CD4 T cells [10]. In partial, this might be due to the lack of effective stimulation of murine DCs to secrete inflammatory cytokines (IL-6, TNF-α) by soluble flagellin (10 µg) compared to FliC-VLP (10 µg) as shown in supplementary data (Supplementary Fig. S3) while T84 cells (a cell line derived from human colonic adenocarcinoma) were highly stimulated by the same flagellin preparation in as low as 10 ng/ml concentrations [15, 28].

It is notable that FliC-VLP was highly effective in inducing both IgG1 and IgG2c isotype antibodies in wild-type C57BL/6, and a similar pattern of isotype IgG antibodies was also observed in CD4KO mice after Vac+FliC-VLP vaccination. CD4-independent adjuvant effects were also demonstrated with oil-in-water emulsion MF59 and TLR4 agonist monophosphoryl lipid A (MPL) adjuvants [10, 13]. A difference is that FliC-VLP displayed adjuvant effects on promoting a balanced IgG1 and IgG2c whereas MF59 and MPL adjuvant effects were biased on inducing higher levels of IgG1 versus IgG2c isotype antibodies [10, 13]. In contrast to MPL, we observed that adjuvant effects of oligodeoxynucleotides with

CpG motifs, a TLR9 agonist, on producing co-administered antigen specific IgG antibodies were dependent on the presence of CD4 T cells (Ko et al., unpublished data). MF59 does not contain any TLR stimulating agonists, resulting in no stimulation of DCs *in vitro* [10]. Previous studies suggest that there is no direct correlation between *in vitro* stimulation of DCs via TLR signaling and *in vivo* adjuvant effects of CD4 dependency and independency. Despite of no *in vitro* stimulation of DCs by MF59 adjuvant, MyD88 mediated signaling was required for the adjuvant effects of MF59 *in vivo* [39]. FliC-VLP induced IL-6 and TNF- α cytokine production by bone marrow derived DCs from WT, TLR5KO, N4KO, T5N4DKO, and even MyD88KO mice (Supplementary Fig. S3). Even though stimulation of MyD88KO DCs produced cytokines at lower levels compared to WT DCs, MyD88KO DCs still showed some cytokine production after FliC-VLP treatment. The immune signaling mechanisms of VLP itself has not been fully elucidated yet, VLP could stimulate MAPK-Erk1/2 pathways for DC activation{Martinez, 2007 #125} as well as MyD88 pathway{Pitoiset, 2017 #126}. Therefore, the immune-stimulatory effects of FliC-VLP on DCs might be derived from both MyD88-NF-KB and MAPK-Erk1/2 pathways. The immunogenicity and efficacy of influenza VLP vaccines were shown to be compromised in MyD88KO mice{Kang, 2011 #124}, suggesting that adjuvant effects of FliC-VLP are partially dependent on MyD88 signaling pathways.

Genetic mutation in the encoding CD4 gene expression in CD4KO mice might have resulted in developing compensatory mechanisms such as MHC class II-restricted CD8 T cells and double negative T cells [40] and MHCII^{high} macrophages and CD11b⁺ DCs [10], possibly providing a helper T cell-like function. The wild-type C57BL/6 mice that were acutely depleted of CD4 T cells and then vaccinated with Vac+FliC-VLP were protected against lethal challenge despite of substantial weight loss (Supplementary Fig. S4). The ineffective protection in acutely CD4-depleted mice might be due to the failure of inducing virus specific IgG antibody responses after vaccination with Vac+FliC-VLP, suggesting no significant adjuvant effects in acutely CD4-depleted mice although survival protection was observed (Supplementary Fig. S4). The compensatory immune components such as double negative T cells developed in CD4KO mice might be required for the conventional CD4 independent adjuvant effects of FliC-VLP on inducing vaccine-specific IgG responses. Survival protection was observed in FliC-VLP immunized CD4-depleted mice after lethal challenge even though there were substantial weight loss and no antigen-specific antibodies detected (Supplementary Fig. S4). It might be possible that CD4-independent cellular immune responses would have contributed to survival protection in CD4-depleted WT mice after FliC-VLP adjuvanted vaccination. In contrast, MF59 and MPL adjuvants were shown to stimulate the induction of IgG antibodies at low levels and comparable protection in acute CD4-depleted mice although a lower level of isotype-switched IgG antibodies was induced in the case of MPL [10, 13]. It is likely that the requirement of intact CD4 immune components may be different among the adjuvants (MF59, MPL, FliC-VLP) even though these adjuvants apparently displayed CD4 T helper cell independent effects on promoting IgG antibody production and conferring protection in CD4KO mice. Therefore, the results in CD4KO and CD4-depleted WT mice suggest that FliC-VLP full adjuvant efficacy on inducing vaccine-specific IgG antibodies requires intact CD4 T cell help despite significant

adjuvant effects of FliC-VLP on inducing protective immune responses to vaccination in CD4KO mice.

Extracellular flagellin recognizes TLR5 whereas flagellins present intracellularly primes the activation of cytosolic multiprotein inflammasome complexes via NLRC4 [16, 17, 41]. Vaccination of TLR5KO mice with Vac+FliC-VLP induced significant levels of IgG and isotype-switched antibodies, which were lower than those in the corresponding group in wild-type mice (Supplementary Table S1), suggesting a partial role of TLR5 in inducing IgG antibodies mediated by FliC-VLP adjuvant. Similar levels of IgG and IgG2c isotype antibodies were induced after vaccination of NLRC4KO mice and TLR5KO mice with Vac+FliC-VLP, which were significantly higher than those in the Vac alone NLRC4KO and TLR5KO groups. Thus, the extracellular TLR5 or intracellular NLRC4 signaling pathways were not required for the adjuvant effects of FliC-VLP. Double knockout (T5N4DKO) mice induced only moderately lower levels of IgG and IgG isotypes compared to NLRC4KO or TLR5KO mice after vaccination with Vac+FliC-VLP. It is possible that the adjuvant effects of FliC-VLP could be from VLP particulate nature. To evaluate the adjuvant role of VLP itself, we tested M1-VLP, an empty VLP without surface FliC, in WT, CD4KO and TLR5KO mice. Vac+M1-VLP immunization induced significantly higher levels of antigen-specific IgG antibodies than Vac only and Vac+SF immunized groups, but moderately lower levels of IgG production than Vac+FliC-VLP immunization in WT mice (Supplementary Fig. S5). Moreover, M1-VLP showed more CD4 dependency than FliC-VLP in CD4KO mice, which shows that FliC-VLP was less dependent on conventional CD4 T cells than M1-VLP in inducing immune responses. Nonetheless, the adjuvant effects of FliC-VLP in TLR5KO mice were largely from the nature of VLP since IgG antibody levels were similar between the FliC-VLP and M1-VLP groups of TLR5KO mice (Supplementary Fig. S5). These data indicate that VLP itself play a role as an independent adjuvant partially in CD4KO mice and with no significant defects in TLR5KO mice. Taken together, TLR5 and NLRC4 pathways were found to be partially involved in, but not required for exhibiting adjuvant effects of FliC-VLP. This observation is in line with alum adjuvant. Alum adjuvant was shown to stimulate caspase-1 activation in macrophage cells and IL-1 β secretion *in vitro* via the NLR family, pyrin domain containing 3 (NLRP3) protein [42]. Nonetheless, induction of IgG antibodies against co-immunized antigens with alum adjuvant was not impaired in mice deficient in Nlrp3 [42]. This is additional evidence that activating pathways predicted from *in vitro* cell culture studies might be only a part in adjuvant effects *in vivo* and represent a pathway out of many possible yet unknown *in vivo* mechanisms.

Most adjuvants activate the innate immune system, which subsequently determines a pattern of CD4 T helper cells (Th1, Th2, Th17, follicular T cells), providing differential help for B cell activation [5, 11]. Influenza VLP vaccines produced in insect cells were tested and proven to be safe and efficacious in clinical trials [43–45]. Presenting influenza virus antigens on a VLP platform was shown to be effective in inducing IgG isotype-switched antibodies in CD4KO mice [46]. Similarly, FliC-VLP was found to be effective in promoting the Th1-biased IgG isotype antibodies and the vaccine efficacy in wild type and immune-defective mutant mice. This study provides the potential of developing FliC-VLP as a vaccine adjuvant to stimulate the immune system in healthy adults as well as in immune-compromised patients, the elderly and young children. Further studies are required to better

understand and extend the vaccine adjuvant effects of FLiC-VLP in combination with other vaccines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A. C57BL/6 wild-type **B. CD4KO** **Fig. 1. Antibody responses in C57BL/6 and CD4KO mice after immunization.**

The mice were immunized with split influenza virus vaccine (Vac, 1 μg of HA/mouse) with or without FliC-VLP (10 μg /mouse) or SF (0.5 μg /mouse). The intramuscular immunizations were given 2 times with a 4-week interval. The immune sera were collected 3 weeks post each immunization. Inactivated A/Cal H1N1 virus (200 ng/well) were used to determine antigen-specific IgG, IgG1 and IgG2c levels. P; 3-weeks post prime immunization. B; 3-weeks post boost immunization. HAI titers were measured with boost sera. For statistical analysis, One-way ANOVA and Tukey's post-multiple comparison test were performed. ns; not significant, *, $p < 0.033$, **, $p < 0.002$ and ***, $p < 0.001$ between the indicated groups.

A. C57BL/6 wild-type **B. CD4KO**

Fig. 2. protective efficacy of FliC-VLP adjuvanted vaccination in C57BL/6 and CD4KO mice.

At 16 weeks after boost immunization, the naïve and immunized mice were infected with a lethal dose of A/Cal H1N1 virus (17×LD₅₀) and monitored body weight changes and survival rates for 14 days. For lung virus titer, the infected mice were euthanized at day 5 post infection and lung samples were collected. For statistical analysis, One-way ANOVA and Tukey's post-multiple comparison test were performed. **, p<0.002, and ***, p<0.001 between the indicated groups.

A. C57BL/6 wild-type

B. CD4KO

Fig. 3. Inflammatory cytokines and antigen-specific antibody levels in the infected lung. The immunized and infected C57BL/6 wild-type and CD4KO mice were euthanized at day 5 post infection and lung samples were collected. Lung extracts were used to determine the IL-6 levels and antigen-specific IgG antibody levels by ELISA. For statistical analysis, One-way ANOVA and Tukey's post-multiple comparison test were performed. ***, $p < 0.001$ between the indicated groups.

Fig. 4. Memory and long-term antibody responses after FliC-VLP adjuvanted split influenza vaccination.

(A) Bone marrow cells were collected from the immunized mice (16 weeks post boost immunization + 5 days post lethal infection). The cells were cultured in inactivated A/Cal H1N1 virus coated plates for 1 day to determine IgG antibody production after *in vitro* antigen stimulation. For statistical analysis, One-way ANOVA and Tukey's post-multiple comparison test were performed. *, $p < 0.033$, and ***, $p < 0.001$ between the indicated groups. (B) The C57BL/6 wild-type (WT) and CD4KO (KO) mice were immunized with split influenza virus vaccine (Vac, 1 μ g of HA/mouse) with or without FliC-VLP (10 μ g/mouse). The intramuscular immunizations were given 2 times with a 4-week interval. The immune sera were collected at 1-, 3-, 6- and 9-months post boost immunization and antigen-specific antibody levels of 1000 times diluted sera were determined by ELISA.

Fig. 5. TLR5-independent adjuvant responses of FliC-VLP.

TLR5KO mice were immunized with split influenza virus vaccine (Vac, 1 μ g of HA/mouse) with or without FliC-VLP (10 μ g/mouse) or SF (0.5 μ g/mouse). The intramuscular immunizations were given 2 times with 4-weeks interval. **(A)** Antigen-specific IgG levels of the immunized mice sera. **(B and C)** After 7 weeks from the boost immunization, the mice were infected with a lethal dose of A/H1N1 virus intranasally and monitored body weight change and survival rates for 14 days.