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Newcastle disease virus-like particles induce dendritic cell maturation and enhance viral-specific immune response

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Abstract Circulating of genotype VII Newcastle disease virus (NDV) is a great threat to the poultry industry worldwide. Virus-like particles (VLPs) are increasingly being considered as potential viral vaccines due to their safety and efficacy. In this study, we analyzed in vitro the stimulatory effects of VLPs containing the matrix and hemagglutinin-neuraminidase of genotype VII NDV on dendritic cells (DCs) and evaluated their immunogenicity in mice. The results showed that immature bone marrow-derived dendritic cells (BMDCs) responded to stimulation with VLPs by up-regulating expressions of MHC II, CD40, CD80, and CD86 molecules and by increasing the cytokine secretions of TNF-α, IFN-γ, IL-6, and IL-12p70. Besides, VLPs enhanced the immunostimulatory capacity of DCs to stimulate autologous T cell proliferation. Furthermore, VLPs can induce efficient humoral and cellular immune responses, and recruit mature DCs to the spleen in C57BL/6 mice, as shown by an obvious increase in double-positive proliferation of splenic CD11c+CD86+ cells. These data indicate that NDV VLPs can be a valuable candidate for NDV vaccine development.

Keywords Newcastle disease virus · Virus-like particle · Dendritic cell · Immune response

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Introduction

Newcastle disease (ND) is one of the most seriously infectious diseases for poultry, especially in chickens, geese, and ducks [1]. Its etiological agent, virulent strains of Newcastle disease virus (NDV), is classified as members of the genus *Avulavirus* within the family *Paramyxoviridae*. The enveloped virus has a negative-sense, single strand, non-segmented RNA genome that contains six genes in the order of 3′-NP-P-M-F-HN-L-5′ encoding the major structural proteins, including nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and large protein

(L) [2]. The HN protein, a multifunctional type II membrane glycoprotein with HA and NA activities, functions to recognize cell receptors and to catalyze cleavage of these receptors [3, 4], which is favorable in vaccine construction for its good immunogenicity.

In China, the majority of epidemic and velogenic strains belong to genotype II and VII from Class II, affecting several domestic and wild avian species [5]. Although waterfowl such as ducks and geese are less susceptible to NDV than chickens, they play an intermediate role in the process of viral cross-species transmission [6]. Many genotype VII strains isolated from geese are also infectious to duck, chicken, and pigeon. Currently, the commercial vaccines are designed with genotype II strains (LaSota, B1, or clone 30) and genotype I strains (Ulster or VG/GA), rather than the genotype VII. These vaccines are effective protection from clinical symptom against genotype VII and they are not equivalent to genotyped matched vaccine strains in terms of virus loading and shedding [4, 7]. On the other hand, as the most popular vaccine in China, LaSota is alive and bears the risk of recombination with other wild strains. Thus, a non-productive novel vaccine is necessary for NDV control in the future.

Virus-like particles (VLPs), the assembly of viral structural proteins without the incorporation of genome, are increasingly being considered as potential viral vaccines [8]. Due to the non-genome nature, VLPs are incapable of infection or self-replication, yet remain the efficient antigenicity required for immune response [9]. At present, several licensed VLP vaccines are already being used clinically against pathogens such as papillomavirus, hepatitis B virus, and porcine circovirus type 2 and a number of other VLP vaccines have been evaluated in preclinical and clinical trials [10–13]. NDV VLPs could be quantitatively prepared from cells transiently transfected with cDNAs encoding the HN, F, NP, and M proteins and retained their attachment and fusion activities [14, 15]. Some studies have demonstrated that the sequence encoding foreign protein ectodomains, fused to the CT and TM domains of the NDV F or HN proteins, can be incorporated into NDV VLPs [16, 17]. Thus, NDV VLPs also can be used as a platform for the assembly of foreign sequences for other pathogens.

Dendritic cells (DCs) function as monitors supervising foreign pathogens or allergens through multiple pattern recognition receptors (PRRs) and are acknowledged as the professional antigen-presenting cells (APCs) [18]. Different status and subsets of DCs have their specific roles translating innate immune response to adaptive immune response. Immature DCs (imDCs) reside in the sites of potential antigen entry, with very limited ability of aiding T cell maturation. After challenged with antigens, imDCs in peripheral parts will be activated into mature DCs (mDCs)

expressing high levels of surface costimulatory molecules like MHC II, CD40, CD80, and CD86 [19]. In the meanwhile, the mDCs will migrate to lymphoid organs and acquire the ability to stimulate proliferation and differentiation of T cell, thus initiating the adaptive immune process [20].

The NDV VLPs were proved protective against lethal challenge in both mice and chicken [14, 21]. However, the effect of NDV VLP on DC maturation and subsequent innate immune response is poorly understood. In this study, NDV VLP containing the M and HN of genotype VII NDV were constructed and produced through the insect–baculovirus expression system. Their stimulatory effects on DCs were determined in vitro and the immunogenicity was evaluated in mice. These results contribute to a better understanding of the mechanisms involved in the immune response to NDV VLP and provide knowledge for the rational design and development of candidate vaccines.

Materials and methods

Virus and cloning of M and HN genes into recombinant baculovirus

NDV strain NA-1 (GenBank accession: DQ659677) was isolated from diseased goose by our laboratory and identified as velogenic genotype VII virus, which was highly pathogenic for chickens and geese (MDT = 59.6, ICPI = 1.65 for chickens and 1.60 for geese) [22]. The M and HN sequences were obtained from NA-1. Both genes were synthesized biochemically and cloned into the baculovirus pFastBac Dual transfer vectors (Life technologies corporation, Gaithersburg, MD, USA). The recombinant baculovirus (rBV) containing M and HN genes were generated by the Bac-to-Bac insect–baculovirus expression system (Life technologies corporation, Gaithersburg, MD, USA). The titers of rBV preparations were determined by plaque assay in Sf9 cells(ATCC, Manassas, VA) and expressed as plaque-forming units (PFU/ml). The rBV stocks were prepared by infecting cells at 0.2×10^6 cells/ml at a multiplicity of infection (MOI) = 0.5 and harvested at 72 h post-infection under 28 °C.

Preparation and characterization of NDV VLPs

For production of VLPs, Sf9 cells were adjusted to 2×10^6 cells/ml and infected at a MOI of 5 for 120 h with rBV co-expressing M and HN genes under 28 °C. VLPs were harvested and analyzed by sucrose gradient ultracentrifugation followed by Western blot as described by McGinnes et al. [23]. Briefly, pellet cell debris was removed by

centrifugation at $5000 \times g$ for 30 min. VLPs in the supernatant were filter sterilized using a 0.22 μm filter and then pelleted by centrifugation at $140,000 \times g$ for 6 h at 4 ℃ through 20% (w/v) sucrose to a 20 to 40% interface discontinuous sucrose gradient at $100,000 \times g$ for 6 h at 4 °C using SW41 rotor (Beckman Coulter, Miami, FL, USA). About 1 ml fractions were collected and pelleted by a second ultracentrifugation and then re-suspended in TNE buffer (0.150 M NaCl, 0.025 M Tris·Cl, 0.005 M EDTA, pH 7.4). Total protein concentrations of VLPs were evaluated by Pierce BCA Protein Assay Kit (Pierce Thermo Scientific, Rockford, IL, USA) and examined by SDS-PAGE using 10% polyacrylamide gels. For Western blot analysis, proteins in the polyacrylamide gels were transferred to polyvinylidene difluoride (PVDF) membranes and then detected with 1:250 dilution of chicken anti-NDV polyclonal serum (Weike biotechnology Co. Ltd, Harbin, China) as primary antibody followed by 1:5000 dilution of HRP-conjugated rabbit anti-chicken IgY(H+L) (EarthOx, San Francisco, CA, USA) as the secondary antibody.

The hemagglutination titer was determined by serially diluting VLPs at twofold increments in 25 μl in a 96-well plate. To each VLP dilution, 25 μl 1% chicken red blood cell (RBC) (collected from three adult male chickens) working solution was added. The plates were incubated at room temperature for 30 min before examination [24].

For negative staining electron microscopy, NDV VLPs were adsorbed onto freshly discharged 400 mesh carbon parlodion-coated copper grids. The grids were negatively stained with 1% phosphotungstic acid and then dried by aspiration. VLPs were visualized on a Hitachi H-7650 transmission electron microscope (Hitachi Ltd, Tokyo, Japan) operating at 80 kV.

Detection of DC activation

The bone marrow-derived DCs (BMDCs) were isolated from femur and tibia bones of C57BL/6 mice, and cultured in RPMI-1640 medium supplemented with 10% FBS (Gibco, Carlsbad, California, USA), 10 ng/ml rmGM-CSF, 10 ng/ml rmIL-4 (R&D Systems, Minneapolis, MN, USA) and 1% penicillin–streptomycin as described by Lutz et al. [25]. Furthermore, BMDCs were cultured with half of the medium being changed every 2 to 3 day, and collected on day 6 as imDCs for use.

The imDCs were harvested and incubated with VLP (256 HAU/ml, equivalent to 10 μg) or whole inactivated NDV (WIV, 256 HAU/ml, equivalent to 10 μg) in 6-well plates (5×10^6 cells/well) for 48 h under 5% CO_2 at 37 ℃. Cells were stained with anti-CD11c-FITC monoclonal antibody (mAb) and PE-conjugated mAbs against phenotypes of MHC II, CD40, CD80, and CD86 (eBioscience, San Diego, CA, USA). The samples were measured by flow cytometry in a BD FACSCalibur cytometer (BD Biosciences, San Jose, CA, USA) and data analysis was performed with FlowJo software (Version 7.6.1, Treestar, Ashland, OR, USA). Supernatants were collected and assayed for the secretions of TNF-α, IFN-γ, IL-6, and IL-12p70 by ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Autologous mixed leukocyte reaction

The imDCs were treated with VLPs or WIV for 48 h. The autologous naïve T cells were isolated from spleen of C57BL/6 mice and, respectively, cultured with these treated DCs at a ratio of 20:1 (naïve T cell : DC). The negative control was imDCs treated with PBS and then mixed with the autologous naïve T cells. After 48 h, cells in 96-well plates were incubated in 10 μl of CCK-8 solution (Dojindo, Kumamoto, Japan) for 4 h at 37 °C. The absorbance of each well at 450 nm was measured. The stimulation index (SI) was calculated using the following formula:

$$SI = (ODsample\ well - ODblank\ well)/(ODnegative\ well - ODblank\ well).$$

Animals and vaccinations

The 6–8-week-old female C57BL/6 mice were purchased from the Changchun Institute of Biological Products Co., Ltd, China. Mice were acclimated and randomly distributed into experimental groups, and water and food were provided ad libitum. Mice were randomly divided into three groups ($n = 6$ for each group). Mice immunized with immunogen were injected twice (day 0 and 14) by intraperitoneal (i.p.) with PBS, VLP (256 HAU/mouse, equivalent to 10 μg), or WIV (256 HAU/mouse, equivalent to 10 μg). WIV were generated from live virus through UV exposure for 30 min (254 nm, 2 mW/cm^2, distance 25 cm). WIV were subsequently inoculated to 9-day-old chicken embryos to confirm inactivation.

Serological assays

The levels of antigen-specific serum antibodies (total IgG, IgG1, IgG2a, IgG2b, and IgG3) were measured by indirect ELISA. Briefly, 5 μg of VLPs or inactivated NDV was used as antigens on 96-well microplates (Costar, Corning, NY, USA) in coating buffer (0.05 M sodium carbonate, pH 9.5) at 4 °C overnight. Followed by washing with PBS containing 0.5% Tween 20 (PBST), the plates were blocked with PBS containing 1% BSA at 37 °C for 1 h and incubated with 1:1000 dilution of each serum sample at

37 °C for 1 h. After washed with PBST for five times, all the samples were incubated with 1:4000 dilution of HRP-conjugated goat anti-mouse IgG (H+L) (TransGen Biotech Co. Ltd, Beijing, China); IgG1, IgG2a, IgG2b, and IgG3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as secondary antibodies and incubated for 1 h followed by washing with PBST for five times. 100 μl of TMB substrate solution (Tiangen Biotech Co. Ltd., Beijing, China) was then added and incubated at 37 °C for 15 min in the dark. The enzyme reaction was stopped after 15 min by addition of 50 μl stopping solution (2 M H_2SO_4), and the absorbance was measured at 450 nm.

The HI assay was performed using 1% chicken RBC with 4 hemagglutination units (HAU) of homologous viruses using the standard methods as described [24].

Analysis of splenic cells by flow cytometry

Six weeks after the second vaccination, splenic cell suspension was isolated and cultured as previously described [16]. Briefly, the spleen was prepared by grinding in a 200-mesh nylon sieve with 4 ml lymphocyte separation liquid (Dakewe Biotech Co., Shenzhen, China). Cell suspension was added to a 15 ml centrifuge tube and 1 ml cold RPMI-1640 medium was gradually added. Cell pellets were mixed with 5 ml RBC lysis buffer (0.15 M Tris-NH_4Cl solution) and then washed twice with cold PBS, resulting in cell suspension at 2×10^6 cells/ml in RPMI-1640 with 10% FBS.

The cell suspension was labeled with anti-mouse PerCP-Cy5.5/CD3+, FITC/CD4+, PE/CD8+, FITC/CD11c+, PE/MHC II+, PE/CD40+, PE/CD80+, PE/CD86+, PE-Cy5.5/IFN-γ, PE-Cy7/IL-4 (eBioscience, San Diego, CA, USA) for analysis of T lymphocyte cells and DCs by flow cytometry. For intracellular stain, splenic cells were treated with WIV and ionomycin (Sigma-Aldrich) in presence of monensin for 24 h and then performed with anti-CD4 and anti-CD8 antibodies. The cells were permeabilized with Intracellular Fixation and Permeabilization Buffer Set (eBioscience, San Diego, CA, USA) and then stained with anti-IFN-γ and anti-IL-4 antibodies.

Statistical analysis

The experimental data between two groups were analyzed using one-way analysis of variance (ANOVA) in SPSS (version 13.0) and accomplished using GraphPad Prism (Version 6) software. P value <0.05 was considered significantly different (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). P value greater than 0.05 stands for no significant difference (n.s). All results are representative of at least three repeated experiments. Error bars mean standard deviation (SD).

Ethics statement

Animals were treated humanely and with regard for alleviation of suffering. All animal experiments were carried out according to the experimental practices and standards approved by the Animal Welfare and Research Ethics Committee at Jilin University (Approval ID: 2015047815-1).

Results

Characterization of NDV VLPs

NDV VLPs were constructed in a rBV expression system for M and HN genes of the genotype VII NA-1strain (Fig. 1a). The protein concentration was 3.5 mg/ml of the purified NDV VLPs. The presence of M and HN proteins in NDV VLPs was detected by Western blot with chicken anti-NDV serum (Fig. 1b). The M protein is 364 amino acids in length with the expected molecular mass of approximately 40 kilodaltons (kDa). The expressed HN glycoprotein of the VLPs represented 63–68 kDa. The morphology and size of VLPs were further examined by TEM. As shown in Fig. 1c, we observed enveloped virus particles with surface peplomers in the diameter of approximately 100 nm, which was slightly smaller than the wild-type virus (100–320 nm). The HA titers of purified NDV VLPs were as high as 7–9 log2 (data not shown), confirming that the incorporated HN protein retained its hemagglutination activity. These results showed that the M and HN proteins can be assembled into VLPs with functional activity.

NDV VLP induces DC maturation in vitro

To determine whether NDV VLPs can activate imDCs, murine BMDCs were cultured on day 6 as imDCs (Fig. 2a) and treated with VLP WIV for 48 h. As shown in Fig. 2b, imDCs expressed high levels of CD11c (approximately 88.0%). We measured and compared the expression of surface molecules by flow cytometry, demonstrating that treatment with VLP or WIV led to up-regulated expression of MHC II, CD40, CD80, and CD86 molecules on DCs. There was no significant difference on MHC II, CD40, and CD80 molecules between treatments with VLP ($p > 0.05$) (Fig. 3a–d). In addition, CD40 and CD86 expressions of DCs with VLP were significantly increased, compared to WIV ($p < 0.01$) (Fig. 3b, d), but not for MHC II (Fig. 3a) and CD80 (Fig. 3c). We further detected the levels of TNF-α, IFN-γ, IL-6, and IL-12p70 in the culture supernatants by ELISA. The results after stimulation for 48 h in vitro indicated that VLP increased the production of these

Fig. 1 Construction diagram of recombinant baculovirus (rBV) and identification of NDV VLPs. **a** The NDV M and HN genes were combined within baculovirus so that each gene was expressed from its own p10 or polyhedrin (polh) promoter. **b** Western blot analysis of NDV VLPs that were incubated with chicken anti-NDV polyclonal serum. The molecular weight (MW) of M and HN were approximately 40 and 63–68 kDa, respectively. **c** Negative staining electron microscopy of NDV VLPs. *Bar* represents 100 nm
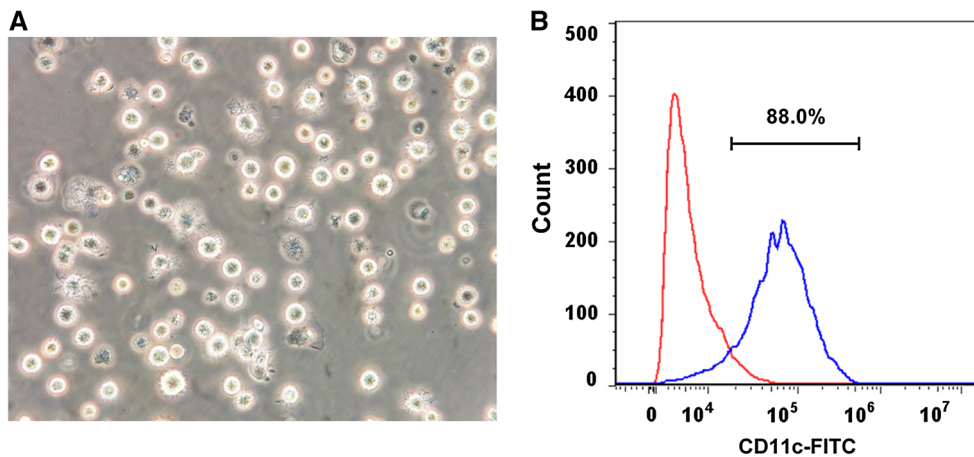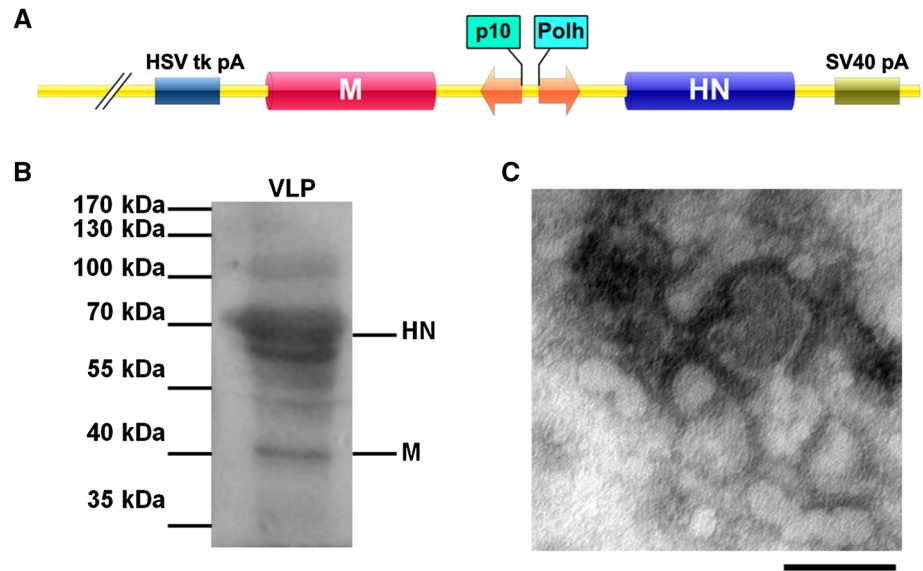

Fig. 2 Analysis of murine BMDCs by optical microscope and flow cytometry. **a** At day 6, cells were observed dendritic branches on cell surface and called imDCs. **b** These imDCs were harvested and stained with the anti-mouse CD11c-FITC mAb (N418) (*blue* histograms) or an appropriate isotype control antibody (*red* histograms)

cytokines, compared to the PBS and WIV controls ($p < 0.001$) (Fig. 3e–h). Thus, it is conceivable that NDV VLPs can activate DC maturation by improving the expression levels of surface molecules on DCs and inducing the cytokine production.

To determine whether VLP-activated mDCs have the capacity to stimulate autologous T cell proliferation, we tested the mixed leukocyte reaction by CCK-8 assay and SI value. As shown in Fig. 4, the DCs treated with VLP had a stronger capacity to activate naïve T cells than that treated with PBS ($p < 0.001$) or WIV ($p < 0.05$). These results demonstrated that the constructed VLPs had potential to modulate DC maturation and enhance its immunostimulatory capacity.

Immunization with NDV VLPs elicit higher IgG and HI titers

To test the in vivo immunogenicity and efficacy of NDV VLPs, serum antibody responses in immunized mice were analyzed using HI assay and ELISA. Mice immunized with VLP demonstrated significantly increased HI titers (Fig. 5a) and specific IgG levels (Fig. 5b), compared to mice immunized with WIV. In addition, we found that the trend of specific IgG levels was similar to those of HI titers during 8 weeks, suggesting that there was a positive correlation between IgG levels of ELISA and HI titers. Mice immunized with VLP maintained a high IgG level for at least 6 weeks and displayed enhanced antibody production
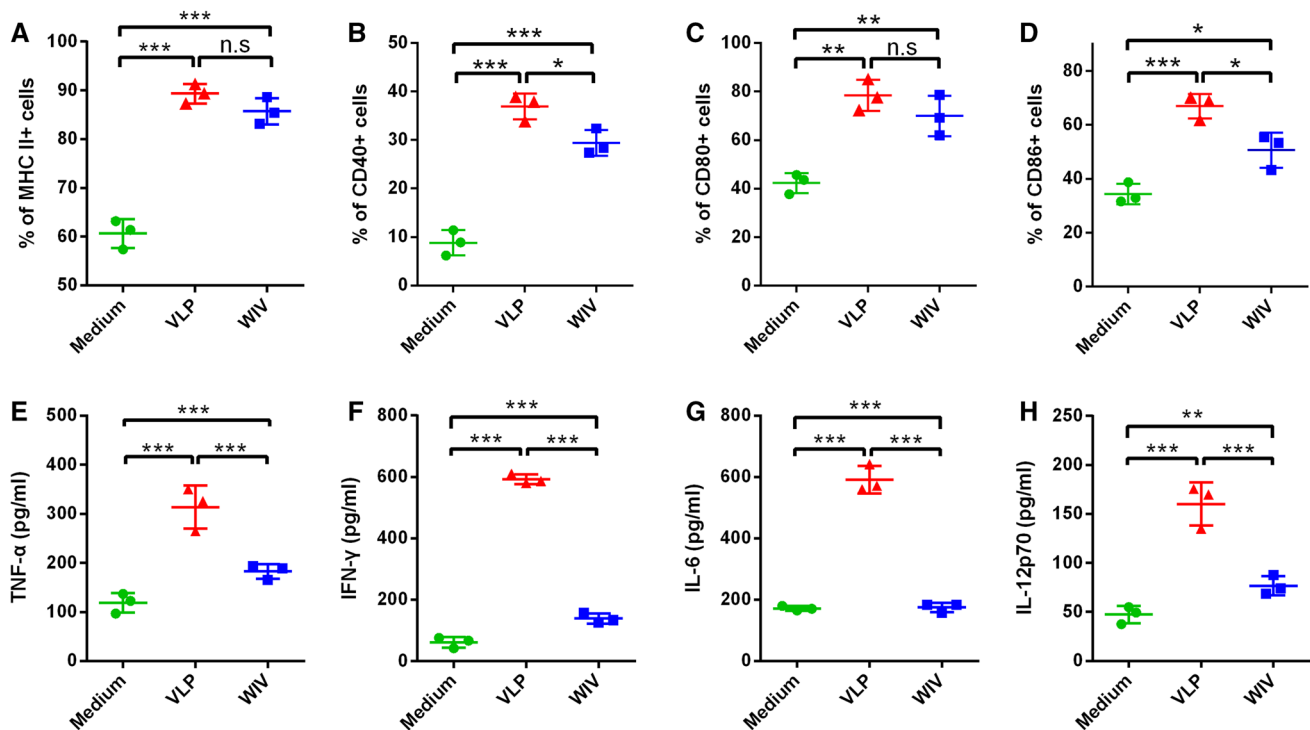
Fig. 3 NDV VLPs induced DC maturation. DCs were treated with VLP (10 µg/ml) or WIV (10 µg/ml) for 48 h. The expression levels of MHC II (a), CD40 (b), CD80 (c), and CD86 (d) molecules on DCs were analyzed by flow cytometry. The secretions of TNF-α (e), IFN-γ (f), IL-6 (g), and IL-12p70 (h) in culture supernatants were measured by ELISA. The experiments were repeated for three independent experiments. The data are presented as the mean ± SD ($n = 3$). P value <0.05 was considered significantly different (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). P value greater than 0.05 stands for no significant difference (n.s)
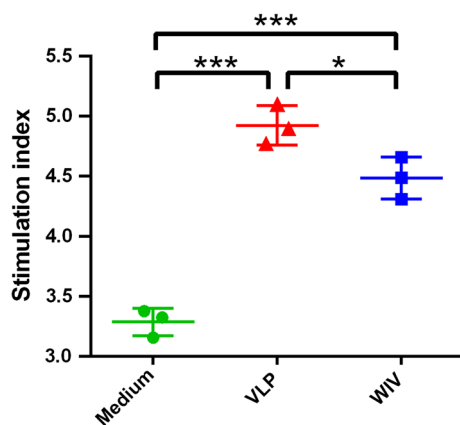
Fig. 4 Mixed leukocyte reaction (MLR) of the mature DC to allogeneic T cells. DCs were treated with VLP or WIV that has the capacity to stimulate autologous T cell proliferation. (DC:T = 1:20). The data are presented as the mean ± SD ($n = 3$). P value <0.05 was considered significantly different (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). P value greater than 0.05 stands for no significant difference (n.s)

against NA-1 (homologous) virus and LaSota (heterologous) virus, compared to the WIV control (Fig. 5c, d), suggesting that VLPs led to enhanced anti-homologous and anti-heterologous virus antibody responses.

To further identify which subtype of IgG was generated, the virus-specific IgG antibody subtypes were examined by ELISA. As shown in Fig. 6, VLP induced the highest IgG1 response among the four IgG subtypes. Immunization with VLP led to a 1.5-fold higher anti-NDV NA-1 virus IgG1 level than WIV (Fig. 6a). In contrast, VLP resulted in a 2.6-fold enhancement of anti-NDV LaSota virus IgG1 level compared to WIV. At the same time, VLPs elicited augment 1.3-fold anti-NDV homologous virus-specific IgG3 and 1.8-fold anti-NDV heterologous virus-specific IgG3. Besides, VLP immunization enhanced twofold anti-NDV homologous virus-specific IgG2b antibody response as similar as increased 1.9-fold anti-NDV heterologous virus-specific IgG2b response. Of note, the C57BL/6 mouse strain does not have the gene for IgG2a [26], and the data about IgG2a were not analyzed. Thus, NDV VLPs were immunogenic in mice and could induce an IgG1-dominant response, suggesting an enhancement of humoral immune responses against homologous and heterologous virus.

Positive proliferation of splenic cells after immunization in vitro

To further characterize cell-mediated immune responses induced by NDV VLP, we evaluated the levels of T cell
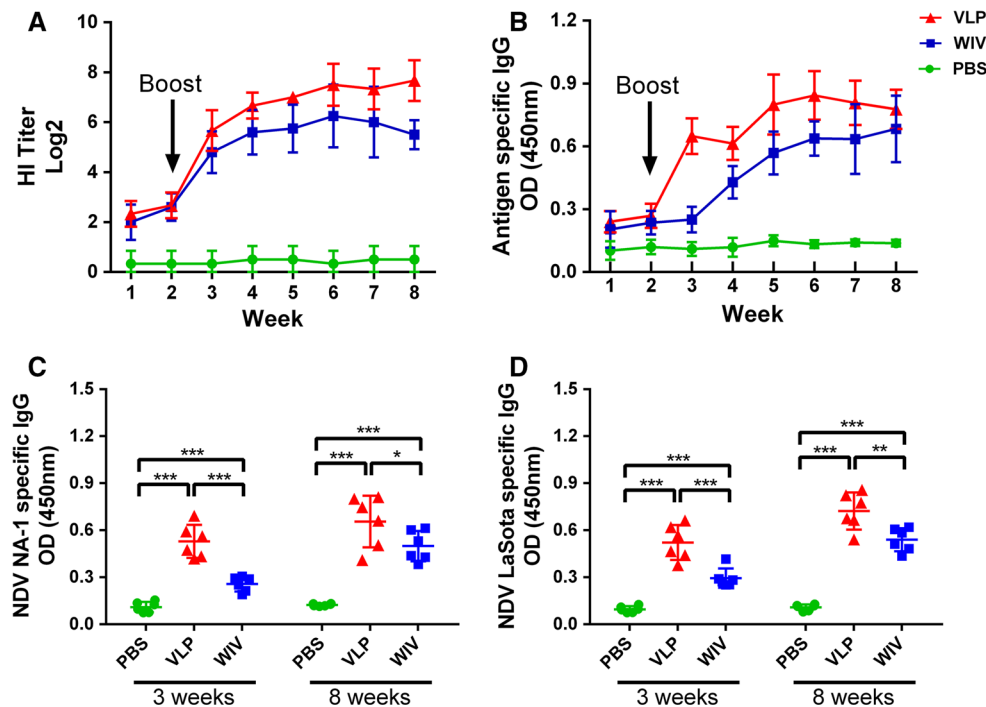
A
Homologous NDV
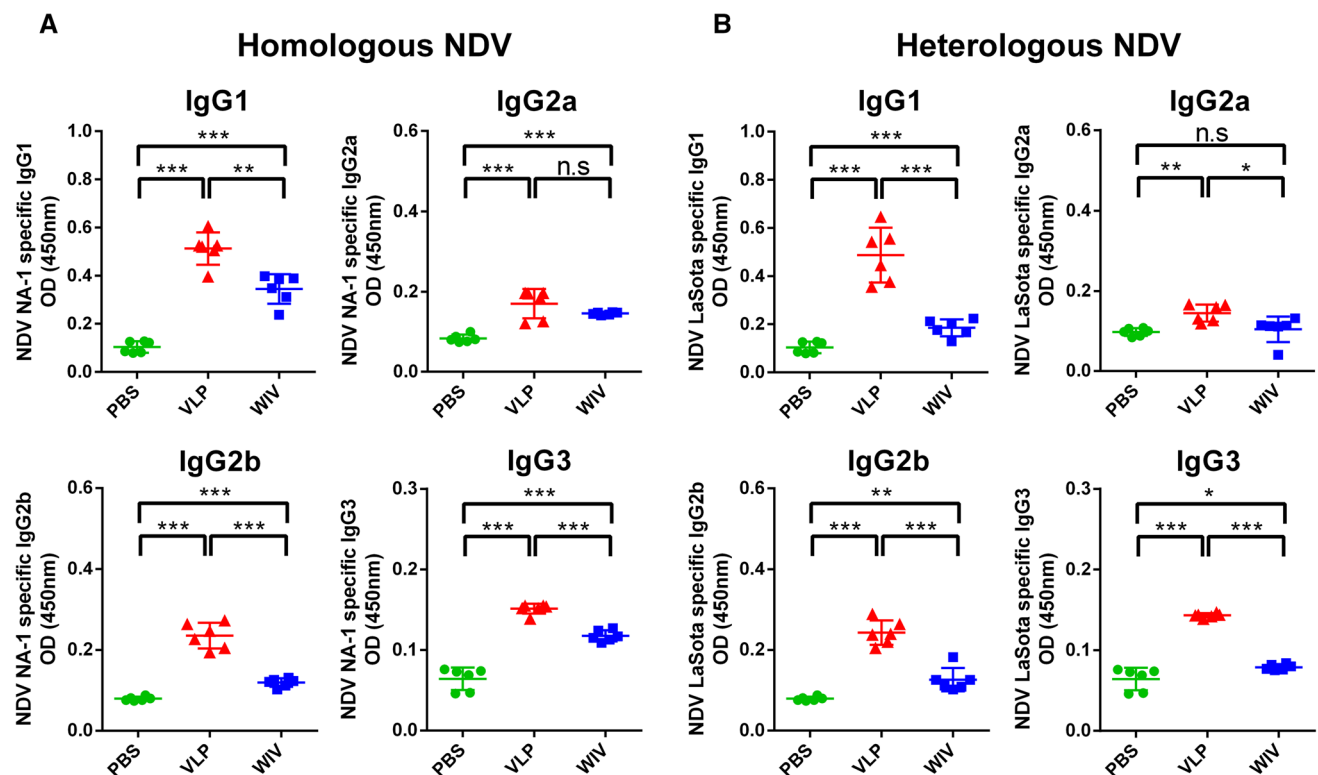

B
Heterologous NDV

Fig. 6 Specific IgG antibody subtype responses in mice. Serum was collected from groups of vaccinated mice ($n = 6$) 6 weeks after boost. A 1:1000 diluted serum was used in an ELISA coated with homologous virus (**a**) and heterologous virus (**b**). HRP-conjugated goat anti-mouse IgG1, IgG2a, IgG2b, and IgG3 at a dilution of 1:4000 were used as secondary antibodies. The data represent the mean ± SD from six mice in each group and were analyzed by one-way ANOVA. P value <0.05 was considered significantly different (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). P value greater than 0.05 stands for no significant difference (n.s)

baculovirus (rBV) expression system to the field of Newcastle disease VLPs, the resultant VLPs showed IgG antibody responses comparable to the commercial vaccines, in vitro and in vivo in chickens [28]. Here, we developed a rational approach to construct NDV VLPs with a density of 1.18 to 1.08 g/cm^3 (20–40% sucrose gradient) similar to NDV viral particles which ranged from 1.21 to 1.19 g/cm^3, and the incorporated viral protein expressed on the VLPs had biological activity. Along with the VLPs containing M and HN protein (M-HN), we also successfully generated VLPs of different assembly strategies (M, M-F, M-F-HN).

In vitro results showed that all the NDV VLPs can improve expression of MHC II, CD40, CD80, and CD86 molecules on DC surface, and we found among the four combinations M-HN VLPs was most potent in inducing cytokine production in DCs (Supplementary Fig. S1). Therefore, the M-HN VLPs were chosen for further investigation. When compared with WIV, it showed that the DCs treated with VLPs exhibited a better ability to induce the secretions of TNF-α, IFN-γ, IL-6, and IL-12p70 than WIV (Fig. 3e–h). TNF-α is a rapid proinflammatory cytokine that can stimulate DC maturation [29], and IL-6 is essential in B cell proliferation [30]. Thus, the NDV VLPs

might play a role in the innate immune response through modulating proinflammatory cytokine production of DCs. It had been also reported that IFN-γ is a type II that possesses various biological activities including antivirus effect and antitumor effect, and can augment IL-12p70 production [31], which involves in the differentiation of Th0 cells into Th1 cells. Additionally, the result of MLR assay also indicated that the DCs treated with VLP significantly improved the proliferation capacity of autologous T cells. Therefore, the data suggested that DC maturation induced by NDV VLPs can improve the antiviral ability through secretion of proinflammatory cytokines and may subsequently play an indirect influence in the process linking innate and adaptive immunity.

The animal experiment indicated that, apart from enhanced anti-homologous IgG response, mice immunized with VLP also exhibited a stronger anti-heterologous response, compared to the WIV-immunized mice. Antibody responses are considered to depend on the presence of T cell help [32]. For example, IgG1 antibody correlates with a Th2 response, whereas IgG2a subtypes are involved in a Th1 response. In the present study, immunization with VLP showed augmented viral-specific IgG1-dominant
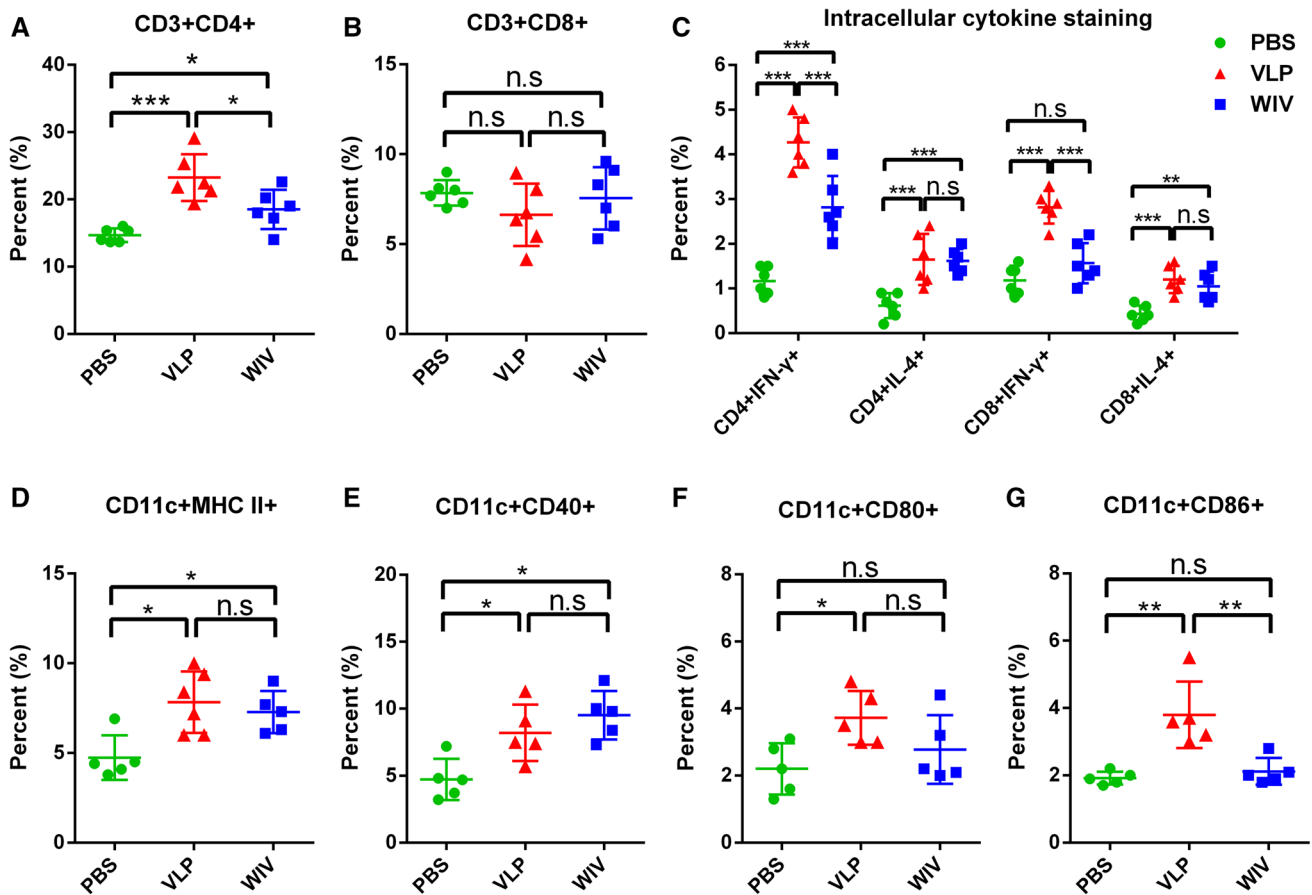
Fig. 7 Phenotypes of T cells and DCs after immunization by flow cytometry. Splenocytes from immunized mice in each group 6 weeks after boost were isolated and pretreated. Spleen lymphocytes were stained with mouse anti-CD3, anti-CD4, and anti-CD8 mAbs (n = 6). CD3 + CD4 + T cells (**a**), CD3 + CD8 + T cells (**b**), and intracellular cytokine staining (**c**) were shown. Splenocytes were stained with anti-CD11c, anti-MHC II, anti-CD40, anti-CD80, and anti-CD86 mAbs (n = 5). The percent of double-positive CD11c + MHC II + (**d**), CD11c + CD40 + (**e**), CD11c + CD80 + (**f**), and CD11c + CD86 + (**g**) cells was plotted. The data represent the mean ± SD of double-positive cells percentages and were analyzed by one-way ANOVA. P value < 0.05 was considered significantly different (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). P value greater than 0.05 stands for no significant difference (n.s).

response, suggestive of a Th2 phenotype. However, viral-specific IgG2a subtype was not dependable due to lack of IgG2a in C57BL/6 mice [26]. To further monitor the cellular response, the spleen T lymphocyte subsets were analyzed. Although, the CD3+CD8+ T cell population in the spleen following VLP treatment seems to be lower than the PBS and the inactivated NDV group, IFN-γ and IL-4-secreting CD8+ T cells of VLP group were significantly more than PBS group; IFN-γ-secreting CD4+ and CD8+ T cells of VLP were also higher than WIV group. The results showed that NDV VLPs induced a significantly higher percentage of CD3+CD4+ T cells and a stronger IFN-γ-secreting T cells compared with that of WIV, suggesting an effective T cell immune response.

To evaluate the impact of NDV VLPs on DCs in vivo, the surface molecular characteristics of DCs were analyzed. The results showed that at 8 weeks after immunization, VLP induced remarkably higher DCs recruitment (including DC11c+MHC II+ cells, CD11c+CD40+ cells, DC11c+CD80+ cells, and CD11c+CD86+ cells) in spleen compared to the PBS-treated mice. CD80 and CD86, members of the B7 family, worked as the second signal, playing a critical role in T cell activation and proliferation [33, 34]. Compared with WIV, VLP induced enhancement of the double-positive CD11c+CD86+ cells counting, suggesting that NDV VLPs may recruit more mDC.

In this study, we chose BMDCs from mice and compared VLP to an inactivated NDV mediated immune response in a mouse model. It has been shown that the immune responses of birds are the similar as those of other species, but there are differences in details such as immune organs and cell subsets. Wu, Z et al. firstly cultured functional chBM-DCs and these mature cells treated with LPS or CD40L showed high expression of MHC II and costimulatory molecules and proinflammatory cytokines,

which were the same as murine BMDCs [35]. Additionally, Th1 and Th2 cells derived from both chickens and mice can secrete IFN-γ and IL-4, respectively [36].

In conclusion, our results indicate that genotype VII NDV M and HN proteins can be assembled into VLPs, most importantly, which can activate DCs to initiate innate immune response by up-regulated expression of DC maturation molecules, secretion of proinflammatory cytokines in vitro, and DCs recruitment in spleen in vivo. It is also able to enhance viral-specific humoral and cellular immune responses. Therefore, NDV VLP has a potential ability of antivirus immune response and can be developed into a safe and effective vaccine candidate or platform carrying other proteins. For further study, the results will be verified in chicken and other heterologous antigens or proteins will be inserted and tested.

Compliance with ethical standards

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