Nanoparticles of Conformation-stabilized Canine Distemper Virus Hemagglutinin are Highly Immunogenic and Induce Robust Immunity

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Research

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Abstract

Background

Canine distemper virus (CDV) infection of ferrets, dogs, and giant pandas causes an acute systemic disease involving multiple organ systems, including the respiratory tract, lymphoid system, and central nervous system. In this study, we tested a new type candidate CDV vaccine–CDV nanoparticles–based on hemagglutinin protein.

Methods

The nanoparticles were generated from conformation-stabilized CDV hemagglutinin tetramers. Immune responses against CDV were evaluated in mice. Immunization was initiated 6 weeks after birth and boosted twice with 4-week intervals. The blood and mucosal samples were collected 2 weeks after each immunization.

Results

Vaccination with CDV nanoparticles elicited high levels of IgG antibody titers in mice (approximately seven- to eight fold higher than that obtained with soluble CDV H protein), as well as mucosal immune responses, and developed increased CDV-specific neutralizing antibody. The mice that received nanoparticles showed significantly higher IFN-γ- and IL-4-secreting cell population in the spleen and lymph node compared with mice immunized with soluble H protein. The co-stimulatory molecular expression of CD80 and CD86 on the surface of DCs were also upregulated.

Conclusion

The results demonstrate that self-assembly into nanoparticles can increase the immunogenicity of vaccine antigens, and nanoparticles assembled from conformation-stabilized CDV H protein has the potential to serve as a new type CDV vaccine.

Introduction

Canine distemper virus (CDV) is an enveloped, single-stranded RNA virus of the *Morbillivirus* genus and family *Paramyxoviridae*, closely related to measles virus (MV)[1]. The virus enters hosts through the respiratory tract and targets immune cells, and after amplification in lymphoid organs, it disseminates via the blood stream to multiple organs, leading to gastrointestinal, dermatological, and respiratory signs[2, 3]. Canine distemper (CD), which is caused by CDV, is a highly contagious and fatal disease in a wide range of mammals[4, 5]. CDV infections have been observed in the order Carnivora, as well as in nonhuman primates, rhesus monkeys, and even in giant pandas[6–8]. Four pandas infected with CDV died in Chongqing Zoo and Nanjing Zoo[9]. Six giant pandas with confirmed CDV infection in the Shanxi Rare Wild Animal Rescue and Research Center in China were isolated and named as giant panda/SX/2014 [8].

Previous studies with CDV have utilized purified hemagglutinin (H) and fusion (F) proteins to immunize small numbers of dogs against CDV challenge[10]. In addition, many kinds of CDV vaccine approaches, such as DNA vaccine, nonreplication-competent and replication-competent vector vaccines[11], and rationally attenuated vaccines[12], have been evaluated. The CDV live vaccine can elicit high protective titers of neutralizing antibody against CDV, but attenuated strains of CDV cannot be safely used in some exotic species and may cause symptomatic and sometimes fatal infections in mink and ferrets[13, 14]. Animals receiving the attenuated CDV vaccine became leukocytopenic and developed the erythematous rash typical of distemper[15], highlighting the limitations of arbitrary attenuation. The DNA vaccine that expresses the CDV H protein is sufficient to protect against *Morbillivirus* infections[16], and other vector vaccines also show superior immunogenicity compared with live-attenuated vaccine[17]. Wang et al. found through the limited application of canine distemper-attenuated live vaccines used in giant pandas that CDV vaccine is inadequate to stimulate enhanced immune responses in giant pandas[18], and inactivated or killed vaccine tend to stimulate a weak immune reaction and requires the administration of multiple dosages. More effective and specific immunological preparations than what is currently used should be developed to save endangered giant pandas.

Recently, nanoparticlebased vaccines have elicited high interest as they offer multiple advantages over inactivated viruses or subunit soluble antigens. These nanoparticle antigens are entirely from antigenic proteins of interest, exhibit high immunogenicity[19] and adjuvant effects, and stimulate antigen-presenting cells (APCs) upon binding or internalization[20], then, they elicit innate and adaptive immunity. In our study, we generated nanoparticles from CDV H stabilized with a tetramerization motif and investigated their roles as a potential giant panda CDV vaccine.

Materials And Methods

Ethics Statement

This study was approved by the Animal Care and Use Committee of the Jiaxing University with protocol number JUMC2019007. All animal experiments were performed in accordance with the guidelines of the Jiaxing University Animal Care and Use Committee. Immunization and sampling were performed under anesthesia.

Cell lines and virus gene

Sf9 insect cells were maintained in SF900II (Life Technologies, San Diego, CA, USA) at 27 °C in cell culture, and CDV H gene was obtained from giant panda/ SX/2014. A recombinant Onderstepoort strain expressing green fluorescent protein used for virus neutralizing (VN) was constructed and generated using a reverse genetics system based on RNA polymerase II for CDV by our laborotary.

Purification and characterization of recombinant CDV hemagglutinin proteins

We constructed recombinant plasmid-GCN4 sequence-stabilized tetrameric H (tH), consisting of a signal peptide-encoding sequence from the honeybee melittin to facilitate protein expression in sf9 cells, full-length CDV H gene, a foreign tetramerization motif GCN4, and His tag gene at C-terminal (Figure 1, a). The full CDV H fusion gene was cloned into pFastbacI, and recombinant plasmid was transformed into DH10 Bac *Escherichia coli* (Life Technologies, San Diego, CA, USA) to obtain recombinant bacmid. The bacmid was transfected into sf9 to produce recombinant baculovirus (rBV) after bacmid was identified. rBV expression was generated using a Bac-to-Bac system (Invitrogen, Grand Island, NY, USA). Recombinant H protein was purified by infecting sf9 cells with rBVs at a MOI of 1 and incubated at 27 °C for 48 h. Supernatants were collected, and recombinant CDV H protein was purified using a His tag purification kit (Beyotime, Beijing, China). The sample of infected sf9 cells and recombinant CDV H protein purity was conformed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot analysis. Flagellin expression was determined using the Bac-to-Bac system, purified through his tag, and stored at -80 ℃ for further use.

Preparation of nanoparticles

Exactly 1 μg of recombinant CDV H pure protein was incubated at room temperature in the presence of Bis [sulfosuccinimidyl] (BS3) at final concentrations 6 mM for 30 min. Then, 1 M Tris-HCl (pH 8.0) was added to reach a final concentration of 50 mM and stop the crosslinking reaction. Then, the sample was separated via SDS-PAGE followed by Western blot analysis using an anti-his tag antibody (Beyotime, Beijing, China) to identify hemagglutinin tetramers (tetrameric hemagglutinin, tH).

Immunization and sampling

Female 6 week-old BALB/c mice were randomly divided into three groups. The mice were intranasally (i.n.) and intramuscularly (i.m.) immunized with 10 μg of soluble recombinant H protein (group 1, G1), 10 μg of nanoparticles tH (group 2, G2), or 10 μg of nanoparticles tH+1 μg flagellin (group 3, G3) at weeks 0, 4, and 8, respectively (Figure 1, b). Sera and nasal sample were collected 2 weeks after each immunization (We tested the samples collected at last time). Lymphocytes from the spleen and lymph node were collected after the last sample was collected and used for ELISPOT testing. Lymph nodes were collected after primary immunization for flow cytometry assays.

Neutralization assay and antibody ELISA

CDV H-specific antibody titers, IgG, IgG1, IgG2a, and IgA in immune samples were detected by ELISA using purified recombinant CDV H protein as coating antigens at 1 μg/ml. The diluted samples were

added to each well and incubated. After washing, the plates were incubated with HRP-conjugated goat anti-mouse IgG, IgG1, IgG2a, and IgA antibodies (Southern Biotechnology Associates, Birmingham, AL, USA). TMB was used to develop the color, and ELISA reader was used to read the OD value at 450 nm.

Neutralizing antibody (NA) was assessed by incubating the double dilution of serum with 100 $TCID_{50}$ CDV for 1 h and adding it into vero cells at 10^5 cells/well in 96-well plates. The NA titers were calculated using the method of Reed and Muench.

Cytokine ELISpot

Interferon gamma (INF-γ) and interleukin 4 (IL-4) secretions from immunized mouse splenocytes and lymph node cells were evaluated using ELISpot kits (eBioscience, San Diego, CA) in accordance with the manufacturer's instructions.

Flow cytometry assays for DCs

The lymph nodes were collected at 3, 6, and 9 days after primary immunization. Single-cell suspensions (1 $\times 10^6$ cells/mL) were prepared in PBS containing 2% FBS and stained with anti-mouse CD11c, CD80, and CD86 antibodies (BD Biosciences, Franklin, TN, USA) for 30 min at 4 °C. After staining, the labeled cells were washed twice with PBS containing 2% FBS and analyzed in a flow cytometer.

Statistical analysis

P-values of less than 0.05 (*$P < 0.05$) were considered to be statistically significant. **$P < 0.005$; ***$P < 0.001$; n.s., $P > 0.05$.

Results

Characterization of CDV H and nanoclusters

Figure 2 a, shows that the CDV H protein was expressed in sf9 cells. Lane 1 is the sf9 cell sample and lane 2 is the infected cells. The CDV recombinant H protein is predicted to have a molecular mass of about 70 kDa. Figure 2 b shows the Western blot results for purified recombinant H protein. The tetrameric structure of tH was confirmed by using BS3 for fixing, followed by cross-linking reaction. Western blot analysis showed a major band with a molecular mass of 280 kDa, representing the CDV H tetramer and a band with a molecular mass of 140 kDa, representing the dimers. A band with a molecular mass of 70 kDa represented the CDV H monomer (Figure 2 c). Thus, GCN4-stabilized recombinant H had a tetrameric form.

Tetrameric CDV H nanoparticles induced strong humoral responses

The efficiency of tH as an immunogen was examined by immunizing mice with soluble CDV H protein and nanoparticle tH with or without flagellin. Then, immune responses, including systemic and mucosal immune responses, were tested. Serum and mucosal samples were evaluated for CDV H-specific IgG and IgA titers by using ELISA. In Figures 3 a and c, nanoparticle tH with or without flagellin group, elicited significantly higher IgG and IgA titers compared with the soluble CDV H group. Mice immunized with nanoparticles achieved between seven and eightfold higher IgG titers than mice from G1. The mice from G3 (nanoparticles+flagellin) also showed enhanced immune responses compared with G2 but without significant difference.

We also compared the IgG isotype. As shown in Figure 3 b, in G3, the high levels of antibody, mainly of the IgG2a isotype (IgG1/ IgG2a around 0.5; $P<0.05$), were induced. In G1, predominantly IgG1 dominant humoral antibody responses were induced (IgG1/IgG2a around 2; $P<0.05$). In G2, tH induced both Th1 and Th2 immune responses (IgG1/ IgG2a around 0.93; $P>0.05$).

When sera were examined for virus neutralization activity, only the mice immunized with tH, in G2 and G3, had increased neutralizing antibody titers (Figure 3 d), and only background levels of neutralizing antibody were detected in G1.

Tetrameric CDV H nanoparticles induce robust mucosal antibody responses

We tested CDV H-specific IgA antibody in nasal wash by ELISA to evaluate whether immunization with nanoparticles can elicit mucosal antibody responses. As shown in Figure 3 c, in G2 and G3, significantly higher levels of IgA were observed, and nanoparticles dramatically improved IgA immuno responses compared with G1. Mice immunized with nanoparticles achieved more than 10-fold higher IgA titers than those from G1, and IgA antibody responses in G2 were significantly increased compared with those in G1.

Tetrameric CDV H nanoparticles activate CDV H-specific T cell responses

T-cell responses are important and known to contribute to broad cross protection. IFN-γ- and IL-4-secreting cells in both the spleens and lymph node of immunized mice were evaluated using cytokine ELISpot. As shown in Figure 4, in G2 and G3, the IFN-γ-secreting cell populations in the spleens and lymph node were significantly higher after stimulation with tH compared with mice immunized with soluble CDV recombinant H protein. The IL-4 secreting cells were also detected in the spleens and lymph node in G2 and G3, and the difference was significant. Only background levels of cytokine-secreting cells were

detected in G1, and these results show that nanoparticles induce enhanced CDV H-specific T-cell responses.

CDV H nanoparticle activation of DCs in lymph nodes

Lymph node cells were analyzed by flow cytometry to investigate whether nanoparticles can stimulate DC activation. Figure 5 shows that more DCs (CD11c+CD86+ and CD11c+CD80+ cells) were detected in mice in G2 and G3 than in G1. In G2 and G3, CD86 and CD80 expression was higher compared with G1, and the difference was statistically significant. These results indicate that nanoparticles elicit a higher percentage of co-stimulatory expression DCs compared with soluble CDV H protein-immunized group.

Discussion

Many studies have shown that protein in particle form can enhance immunogenicity because it mimics the natural conformation of this protein in viral particles, and these particles induce high levels of humoral and cellular responses, conferring complete protection against virus lethal challenge[21, 22]. Given the limitations of the current CDV vaccine, we generated a terminal tetramerization module based on leucine-zipper domain, which spontaneously assembles into a parallel four-helix bundle-tetrameric CDV H nanoparticles and tested its immunogenicity.

In the present study, we examined the effects of CDV H nanoparticles. Mice were immunized with soluble or tetrameric nanoparticles, and overall, high IgG titers were observed in the three groups. The antibody titer in G2 and G3 was compared with that in G1, and the difference was statistically significant. The IgG isotype profiles may reflect which kinds of Th cells are activated in the early stage of an immunization or infection, and suggest which mechanism of antibody-mediated effector functions may be employed[23]. In our study, G1 showed a high IgG1/IgG2a ratio, indicating a Th2 response, while inoculation of nanoparticles in G2 induced a Th1/Th2 balanced response. Th1-based responses with an IgG2a-dominant IgG isotype was observed in G3.

When mice were vaccinated with nanoparticles, they developed CDV-neutralizing antibody titer comparable with the soluble protein immunized group. Neutralizing antibodies are critical factors in eliminating free viral particles, as well as for the clinical prognosis of infected animals. Neutralizing antibodies are responsible for preventing intra and extracellular viral dissemination. In G1, only background neutralizing antibody response was tested. tH nanoparticles elicited high neutralizing antibody titer in G2 and G3, but the difference was not statistically significant compared with that in G1. Brindley et al. engineered headless MeV-H stem constructs capable of efficiently inducing MeV F refolding; combined with previous work, the findings indicate that introduction of terminal tetramerization tag to the MeV H stalk can induce covalent H tetramerization[24, 25]. This result further confirms that the four-helix bundle structure represents the conserved physiological configuration of paramyxovirus attachment protein stalk[26]. Maintaining or mimicking the native conformation of CDV H may explain

the mechanism through which nanoparticle tH induced high levels of spatial conformation-dependent neutralizing antibody titer in our study.

CDV is transmitted by aerosols and infects the upper respiratory tract, replicated in the macrophages and lymphocyte, and viral particles spread to bronchial lymph nodes and tonsils; the viral replication in lymphoid tissues lead to lasting and severe immunosuppression[27, 28]. Thus, mucosal immune responses are important for CDV immune protection; IgA in upper respiratory tract secretions plays a major role in antiviral immunity[29] and contributes to protective immunity. We found that the IgA levels in G2 and G3 were enhanced. The IgA antibody titer in G2 and G3 was significantly different compared with that in G1. Flagellin is an effective adjuvant used in many studies and induces strong systemic and mucosal immune responses. Our study also found that mixed flagellin elicited high titers of IgG and IgA.

DCs play an important role in stimulating the proliferation and differentiation of naive and memory T cells; DCs are the targets of flagellin in initiating the TLR5-associated innate signaling pathway[30]. In G3, nanoparticles with flagellin may efficiently utilize the innate-signaling function of flagellin, and the activation of these APCs promotes antigen presentation and cytokine production, which drives antigen-specific adaptive responses. DC activation in the lymph node in G2 and G3 was remarkable. The co-stimulatory CD80 and CD86 expression was upregulated; such molecules deliver a signal to T cells and enhance T cell responses. In addition to flagellin activation, nanoparticles that are highly symmetrical, stable, structurally organized, mimic the repetitive surface architecture of a natural microbe, and with diameters of 100–200 nm are highly suitable for optimal interactions with various cells of the immune system[31]. These in vivo data indicate that nanoparticles itself can induce high levels of cytokine secretion and enhance the co-stimulatory molecular expression on the surface of DCs, thereby stimulating DC maturation. In brief, nanoparticles can enhance antigen adsorption and uptake by APCs, facilitate antigen processing, induce maturation of DCs, promote antigen cross-presentation, and induce the production of innate cytokines that regulate humoral and cellular immune responses.

The host defense depends on the innate immune system, which is also responsible for producing signals that activate the adaptive immunity; the interferons are critical elements in the innate immune defense against viruses. Cytokine analysis shows that G2 and G3 produce more IFN-γ and IL-4-secreting T cells, especially in G3, demonstrating that nanoparticles enhance T cell responses in mice. Cellular immune response is critical in virus clearance, and a vigorous and continued cellular immunity, including cytotoxic T and killer cells can determine CDV elimination in infected animals[32].

Conclusions

In this work, the significantly higher IgG and IgA antibody titers were detected in nanoparticle-immunized mice. Moreover, the nanoparticle-immunized group also showed stronger T cell response and neutralizing antibody compared with the other group. Taken together, the results showed that the vaccine is capable of inducing increased immunogenicity and strong immune response and is a promising novel vaccine candidate for giant panda CDV. In addition, MV and CDV belong to the *Morbillivirus* genus, have very

similar pathogenesis, and enter hosts through respiratory tract and target immune cells residing within the airways, our results also provide the vaccine antigen design for MV.

Abbreviations

CDV, Canine distemper virus; MV, measles virus; APC, antigen-presenting cells; VN, virus neutralizing; NA, neutralizing antibody; tH, tetrameric hemagglutinin; CD, Canine distemper; INF-γ, Interferon gamma; IL-4, interleukin 4;

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Jiaxing University. The committee's reference number was No.JUMC2019007.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare they have no competing interests.

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Authors' contributions

H.F., N.F., X.Z.X., conceived the study, participated in its design and coordination, drafted and edited the manuscript. J.J.D., performed the animal experiments, preparation of the samples and data analysis. L.L.S., assisted with the design of study, data analysis. Y.C., participated in animal experiments and sample preparation. B.S., and X.L,S., assisted with the analysis of the ELISPOT data. All authors have read and approved the final manuscript.

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Figures

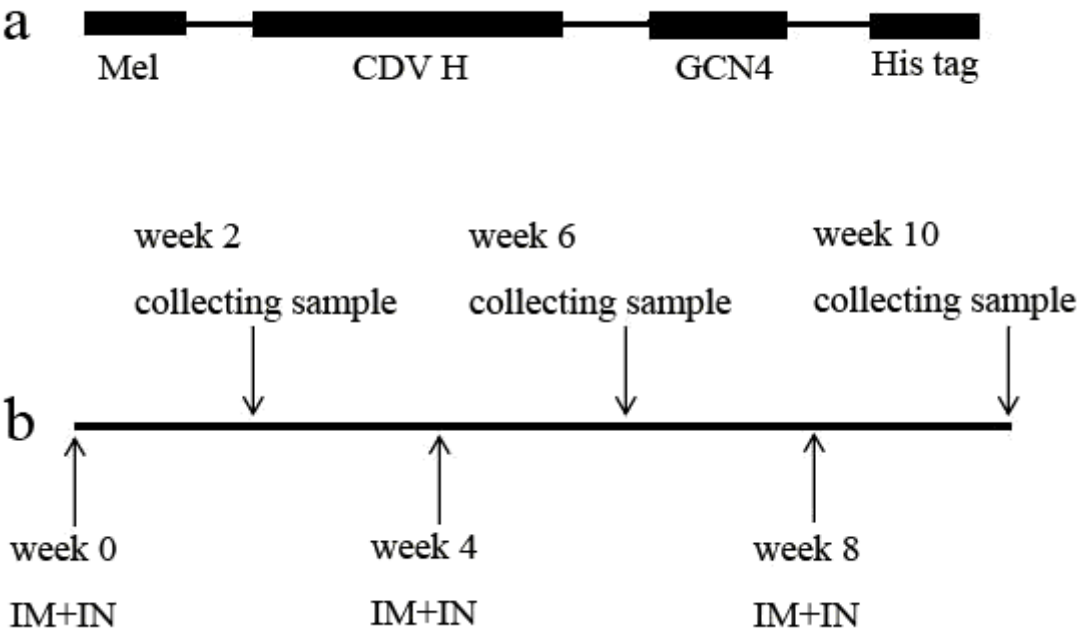

Figure 1

Schematic of constructs and immunization schedule a, schematic of constructs expressing full length CDV H containing mellitin SP, CDV H, GCN4, and his tag gene. b, the mice were immunized three times at 4-week intervals via i.m. and i.n., and samples were collected 2 weeks after each immunization.

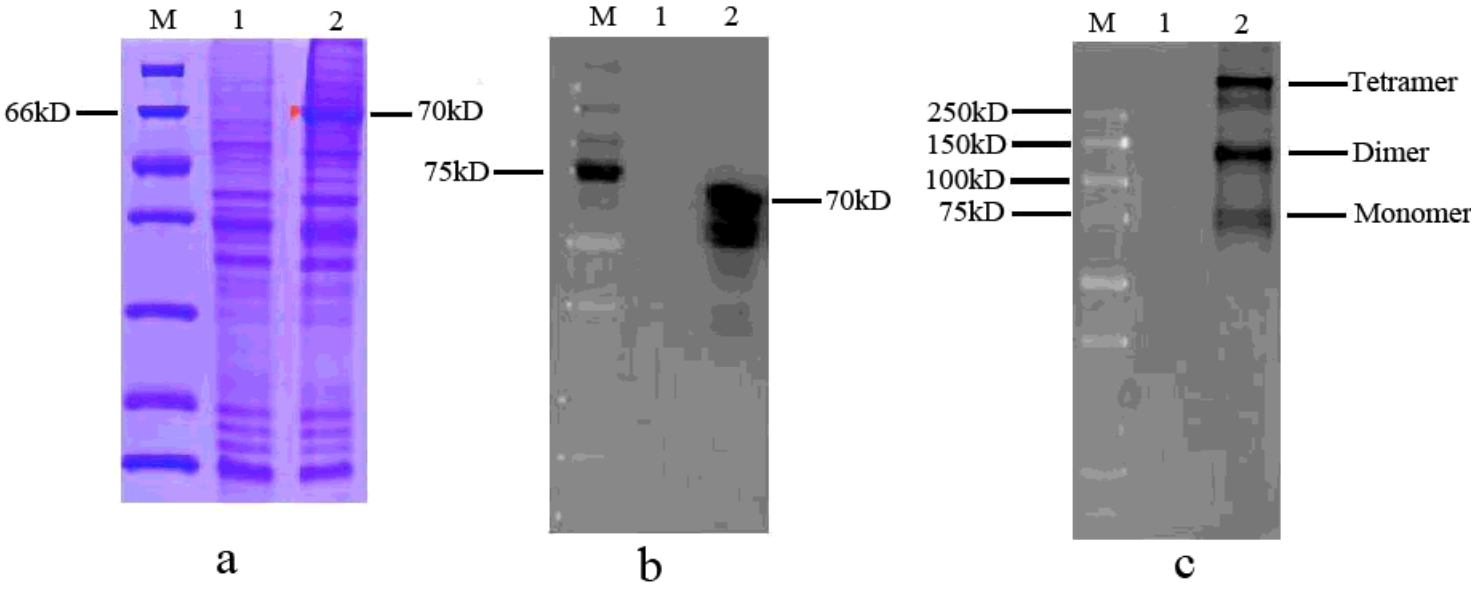

Figure 2

Generation and characterization of CDV H protein and nanoparticle tH a and b, Coomassie blue staining and Western blot analysis of CDV H protein expression, M, molecular weight (kD), lane 1, sf9 cells, lane 2, sf9 cells infected with recombinant baculovirus; c, CDV H protein was purified, and cross-linked CDV H samples were applied to Western blot analysis, M, molecular weight (kD), lane 1, sf9 cells, lane 2, cross-linked CDV H protein.

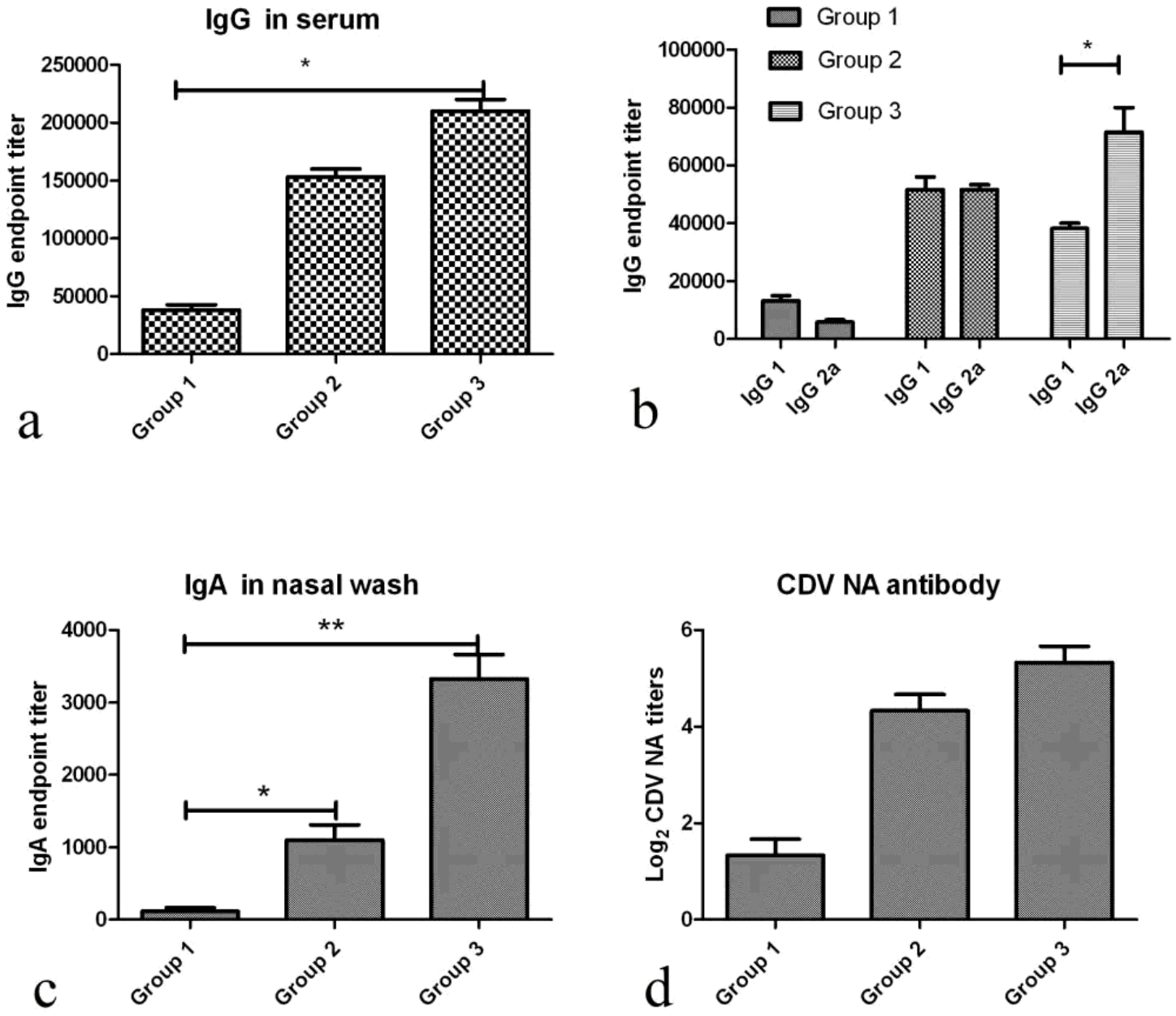

Figure 3

Systemic and mucosal antibody responses against CDV The mice were immunized with soluble CDV H and nanoparticles, and sera and nasal wash samples were collected 2 weeks after each immunization. The final samples of tested data are shown. ELISA plates were coated with soluble CDV H protein, and the samples were diluted 2×stepwise. Bound antibody was detected by binding HRP-conjugated goat anti-mouse IgG, IgG1, IgG2a, and IgA. a, serum IgG titers; B, IgG isotypes; c, IgA titers of nasal wash; d, CDV NA titers. Assays were performed as described in materials and methods. Results are expressed as means ± standard deviations. $P<0.05$ was considered statistically significant.**$P<0.01$, * $P<0.05$.

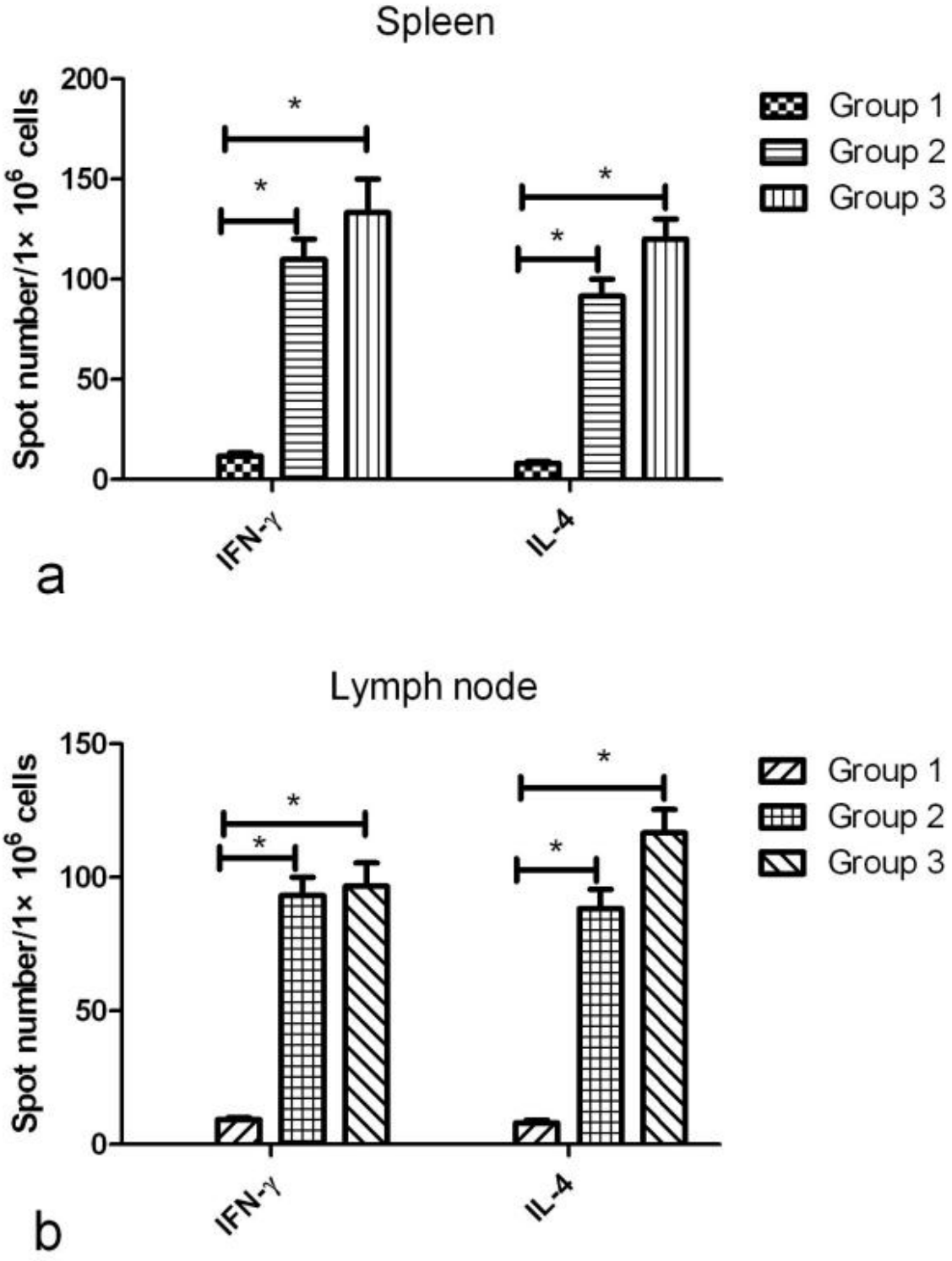

Figure 4

ELISpot analysis of CDV H-specific IFN-γ and IL-4 secretion. The spleen and lymph node were collected from each group 2 weeks after the final immunization. Lymphocytes from the spleen and lymph node were stimulated with soluble CDV H protein, tH, and flagellin. The IFN-γ and IL-4 cytokine-secreting cells were determined using ELISpot assay. The secretion of IFN-γ and IL-4 in the spleen (a) and lymph node (b). * $P<0.05$, ** $P<0.01$.

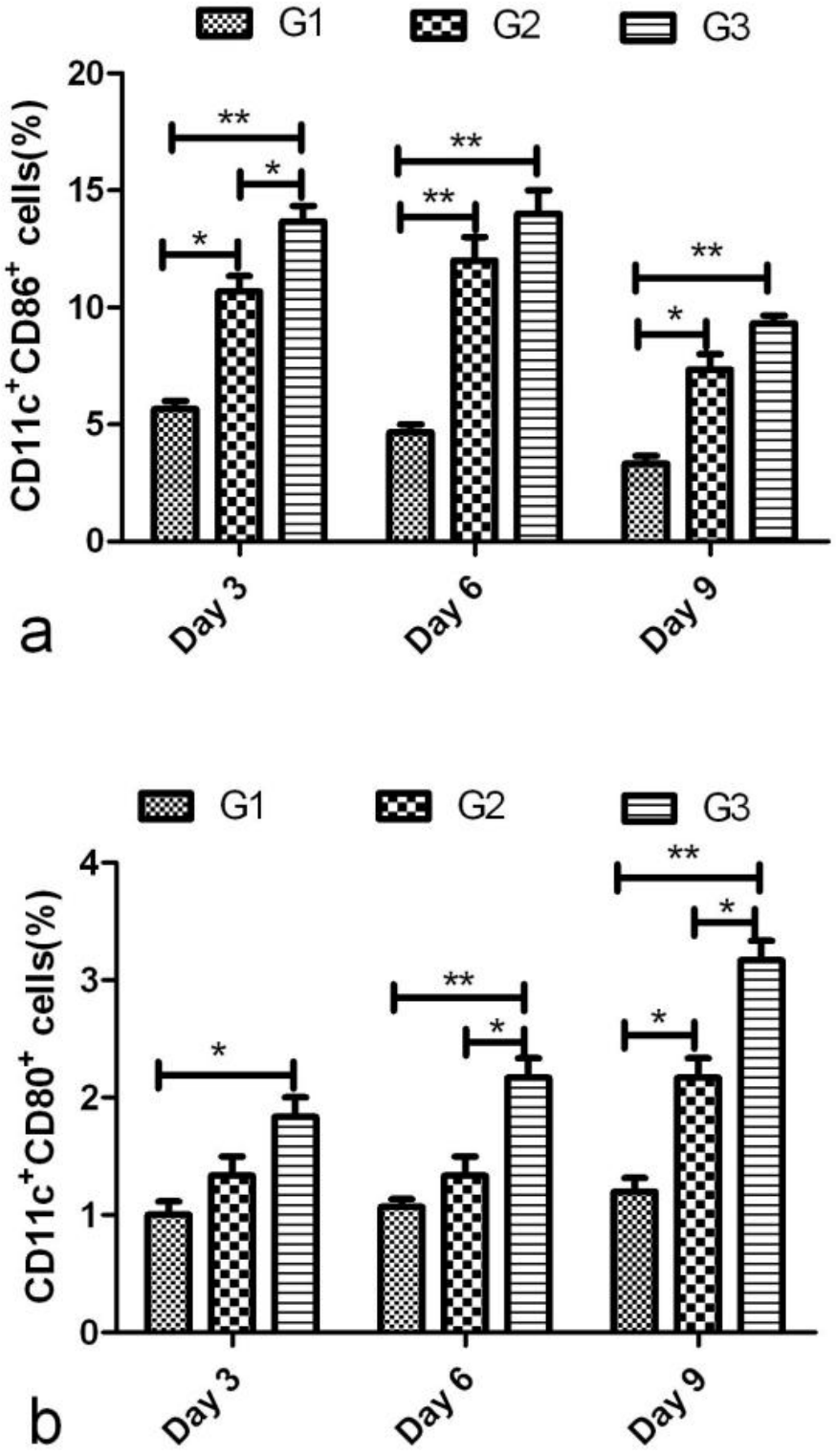

Figure 5

Flow cytometry assay of DCs in lymph nodes. The lymph nodes were collected from each group 3, 6, and 9 days after the first immunization. The cells were stained with mouse anti-CD11c, -CD86, and -CD80 monoclonal antibodies. Double positive cells that CD11c+CD86+ (a); CD11c+CD80+ (b) were plotted, and the data represent the means of double positive cells percentage, * $P < 0.05$, ** $P < 0.01$.