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Construction of expression vectors of capsid proteins from goose parvovirus and investigation of the immunogenicity

M. TU[1#], P. LIU[1#], F. LIU[3#], M. WANG[1,2,3], R. JIA[1,2,3], D. ZHU[2,3], M. LIU[1], K. SUN[1,2,3], Q. YANG[1,2,3], Y. WU[1,2,3], X. CHEN[2,3], A. CHENG[1,2,3*], S. CHEN[1,2,3#*]

[1]Institute of Preventive Veterinary Medicine, Sichuan Agricultural University, Wenjiang District, Chengdu City, Sichuan Province, 611130, P. R. China; [2]Avian Disease Research Center, College of Veterinary Medicine of Sichuan Agricultural University, Wenjiang District, Chengdu City, Sichuan Province, 611130, P. R. China; [3]Key Laboratory of Animal Disease and Human Health of Sichuan Province, Sichuan Agricultural University, Wenjiang District, Chengdu City, Sichuan Province, 611130, P. R. China

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Summary. – Goose parvovirus (GPV) is a highly contagious and lethal disease in goslings and Muscovy ducklings, and is of concern to the waterfowl industry. With the aim of comparing the cellular immunogenicity of three capsid proteins of GPV, plasmids of pcDNA3.1(+)-VP1, pcDNA3.1(+)-VP2, and pcDNA3.1(+)-VP3 were constructed, and the recombinant protein VPs were expressed using an eukaryotic expression system. We detected the levels of immune-related genes (CD4, CD8α, IL-1β, IL-6, IFNα, IFNγ, and IFNλ) in both goose embryo fibroblasts (GEF) and goose peripheral blood mononuclear cells (PBMCs) cellular models. The immune response conferred by a VP2 DNA vaccine *in vivo* was observed in a time course. Our data suggested that the cellular immune response to VP2 and VP3 was stronger than that to VP1, while VP2 and VP3 shared similar cellular immune reactivity. In addition, vaccination with VP2 plasmid can induce high level of IgY antibody that continued to increase through 28 days post vaccination. Therefore, our findings shed light on the host cellular immune response against GPV capsid proteins.

Keywords: GPV; capsid proteins; cellular immune response; humoral immunity

Introduction

Derzsy's disease is an acute infectious disease caused by GPV that can cause low spirits, loss of feathers, stunted growth, diarrhea and neurological symptoms in geese (Palya *et al.*, 2009). Infected geese have typical conditions such as hydropericardium, ascites, enteritis, and degenerative and inflammatory changes of the myocardium and liver (Jansson *et al.*, 2007). GPV is a small icosahedral virus with a diameter of 20–24 nm, that belongs to the *Dependovirus* genus of the *Parvoviridae* family (Cotmore *et al.*, 2014). It has an approximately 5-kb single genome that contains two open reading frames (ORF). The left ORF encodes a non-structural protein that is responsible for viral replication, and the right ORF encodes three capsid proteins, VP1, VP2, and VP3 (Gall-Reculé and Jestin, 1994). VP1 contains the entire sequence of VP2 and VP3, all three VPs share the same terminal codon. The VP1 N-terminal region harbors a nuclear localization signal and a PLA_2 domain, which is critical for viral infectivity (Lombardo *et al.,* 2002; Vihinenranta *et al.*, 2002; Ros *et al.*, 2006). VP2 has an "anti-receptor" that can interact with a cellular receptor on the cell surface (Chipman *et al.*, 1996; Summerford *et al.*, 1998). VP3 appears to be the most abundant of the three capsid proteins and may act as a scaffold protein (Ju *et al.*, 2011).

China is the world's largest waterfowl breeder. The regulations for producing poultry meat and eggs are increasing,

*Corresponding authors. E-mails: shunchen@sicau.edu.cn (Shun Chen); chenganchun@vip.163.com (Anchun Cheng); phone: +86-028-86291482. #These authors contributed equally as co-first authors of this work.

Abbreviations: GEF = goose embryo fibroblasts; GPV = goose parvovirus; HRP = horseradish peroxidase; PBMCs = peripheral blood mononuclear cells; PBS = phosphate buffered saline; RT-qPCR = real-time quantitative PCR; VLPs = virus-like particles

and the living standards are improving. GPV is of concern to the poultry industry because of its rapid transmission and high mortality rates in infected animals. At present, there are no effective drugs for GPV treatment, and preventative measures involve inoculation of traditional vaccines or hyperimmune serum in breeding geese. GPV poses a significant threat to the goose industry, and insight into its pathogenic mechanisms and molecular biology characteristics is needed. The humoral immunity responses of VPs have been widely discussed (Jaiwei *et al.*, 2010, 2013; Chen *et al.*, 2012; Xu *et al.*, 2014). However, there is still a lack of information on the cellular immunogenicity of VPs.

In this study, we investigated the differences in cellular immune responses of the three GPV capsid proteins. Recombinant VPs were expressed in an eukaryotic expression system and used as antigens in the cellular models to determine which ones have the best reactivity. VP2 was selected as an immunogen and further characterized by examining its effects on expression of various cytokine genes in goose peripheral blood cells as well as in GEF and by determining the titer of antigen-specific antibodies in serum. Our data showed that the cellular immunity against VP2 and VP3 was stronger than that against VP1 and that the pcDNA3.1(+)-VP2 was able to induce GPV-specific humoral response in immunized goslings. This work provides essential data for the development of GPV vaccines.

Materials and Methods

Plasmid construction. To generate the plasmids pcDNA3.1(+)-VP1, pcDNA3.1(+)-VP2, and pcDNA3.1(+)-VP3, DNA fragments were amplified from the GPV genome (GenBank Acc. No. U25749) by PCR using specific primers (Table 1). The length of PCR products of VP1, VP2 and VP3 are 2216, 1785 and 1625 bp, respectively. The products were digested with the restriction enzymes *Kpn*I and *Not*I and cloned into the corresponding sites of pcDNA3.1 (+) (Invitrogen, Germany). Additionally, the His-tag sequences were inserted into the reverse primer. The plasmids were transformed into *Escherichia coli* DH5α and purified using the EndoFree plasmid purification Kit (OMEGA, USA).

Cell culture, transfection and the expression of VPs. GEF were grown in modified Eagle's medium supplemented with 10% FBS. GEF were transfected with 2.5 μg/well pcDNA3.1(+)-VP1, pcDNA3.1(+)-VP2, or pcDNA3.1(+)-VP3 using Lipofectmine™3000 (Invitrogen, USA) according to the manufacturer's instructions and incubated for 48 h. Cells were lysed with a lysis buffer containing 25 mmol/l Tris-HCl, pH 7.6, 150 mnol/l NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS for 20–30 min on ice, and whole cell lysate was obtained by subsequent centrifugation. Lysates were boiled for 10 min, separated on 12% polyacrylamide slab gels and transferred to polyvinylidene fluoride membrane (biorad, USA) for Western blot analysis. The blots were incubated with an anti-His monoclonal antibody (1:6,000, Protein, USA) or anti-GPV antiserum (1:1,000), followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (1:10,000, EARTH, USA). The immune complex was detected by Pierce™ ECL western blotting substrate (Thermo, MA, USA).

Goose PBMC infection model. Blood from healthy adult geese was collected in an anticoagulant-containing vial. PBMCs were isolated using the Goose Lymphocyte Separation Medium kit (TBD sciences, Tianjing, China) and grown in RPMI-1640 with 10% serum at a concentration of 1×10^7 cells/ml. Briefly, blood was overlaid on an equal volume of RPMI-1640, and then the mixture was slowly added into lymphocyte separation medium (2:1) with centrifugation at 800 × g for 30 min. The interface containing the PBMCs was collected and washed twice in wash buffer and then resuspended in RPMI-1640 medium. Forty-eight hours after the GEF were transfected with pcDNA3.1(+)-VP1, pcDNA3.1(+)-VP2, pcDNA3.1(+)-VP3, or negative control pcDNA3.1(+) vector, the cells were harvested and lysed by three freeze/thaw cycles, and then the supernatants were collected. PBMCs incubated with the supernatant from the transfected cells for 6 h were examined for differences in expression of immune-related genes (CD4, CD8α, IL-6, IL-1β, and IFNs) by real-time quantitative PCR (RT-qPCR).

GEF infection model. GEFs were transfected with pcDNA3.1(+)-VP1, pcDNA3.1(+)-VP2, pcDNA3.1(+)-VP3, and pcDNA3.1(+) for 48 h. The transfected cells were washed with cold phosphate buffered saline (PBS) three times and then resuspended in RNAiso Plus regent (Takara Bio, Otsu, Japan). RT-qPCR was used to detect the transcripts of goose immune-related genes (CD4, CD8α, IL-6, IL-1β, and IFNs).

Table 1. The list of primers for plasmid construction

Genes	The sequences (5'-3')	The site
VP1	F: ACTTAAGCTT***GGTACC***GCCACCATGTCTACTTTTTTAGATTC	*Kpn*|
	R: CTAGACTCGA***GCGGCCGC***TTA**GTGGTGGTGGTGGTGGTG**CAGATTTTGAGTTAGATATC	*Not*|
VP2	F: ACTTAAGCTT***GGTACC***GCCACCATGGCACCTGCAAA	*Kpn*|
	R: CTAGACTCGA***GCGGCCGC***TTA**GTGGTGGTGGTGGTGGTG**CAGATTTTGAGTTAGATATC	*Not*|
VP3	F: ACTTAAGCTT***GGTACC***GCCACCATGGCAGAGGGAGGAG	*Kpn*|
	R: CTAGACTCGA***GCGGCCGC***TTA**GTGGTGGTGGTGGTGGTG**CAGATTTTGAGTTAGATATC	*Not*|

Table 2. The list of primers for RT-qPCR

Target gene	Primer sequences (5'-3')	Products	Reference
GAPDH-F	CATTTTCCAGGAGCGTGACC	80 bp	
GAPDH-R	AGACACCAGTAGACTCCACA		
IL-1β-F	TCCGCCAGCCGCAAAGTG	136 bp	(Qi *et al.*, 2015)
IL-1β-R	CGCTCATCACGCAGGACA		
IL-6-F	AAGTTGAGTCGCTGTGCT	120 bp	(Qi *et al.*, 2015)
IL-6-R	GCTTTGTGAGGAGGGATT		
CD4-F	TTTCAACGCCACAGCAGA	127 bp	(Chen *et al.*, 2015)
CD4-R	GTGCCTCAACTGGATTTT		
CD8α-F	AGAGACGAGCAAGGAGAA	97 bp	(Chen *et al.*, 2015)
CD8α-R	GACCAGGGCAATGAGAAG		
IFNα-F	CAGCACCACATCCACCAC	98 bp	(Chen *et al.*, 2015)
IFNα-R	TACTTGTTGATGCCGAGGT		
IFNγ-F	TGAGCCAGATTGTTTCCC	146 bp	(Chen *et al.*, 2015)
IFNγ-R	CAGGTCCACGAGGTCTTT		
IFNλ-F	GAGCTCTCGGTGCCCGACC	165 bp	
IFNλ-R	CTCAGCGGCCACGCAGCCT		

Analysis of immune response genes by RT-qPCR. RNA extraction and cDNA synthesis: The cells were centrifuged for 5 min, and the resulting pellet was resuspended in 1 ml of RNAiso Plus regent (Takara Bio, Otsu, Japan); total RNA was isolated. Briefly, RNA from the resulting mixture was extracted with chloroform and precipitated with isopropanol. The RNA pellet was washed with 75% ethanol and resuspended in RNA-free water (TIAGEN, Beijing, China). cDNA was synthesized using a 5x All-In-One RT Master Mix Reagent Kit according to the manufacturer's instructions (Applied Biological Materials, Richmond, BC, Canada).

RT-qPCR: The mRNA expression levels of goose CD4, CD8α, IL-6, IL-1β, and IFNs were analyzed by RT-qPCR using the QuantiTect SYBR Green qPCR Kit (Qiagen, CA, USA) on a CFX96 real time system (Bio-Rad, CA, USA) using published gene-specific primers (Table 2). GAPDH was used as the reference gene. QPCR was performed in triplicate for each sample in a total volume of 10 µl consisting of 5 µl of QuantiTect SYBR Green Master Mix (Qiagen, CA, USA), 0.2 µl cDNA, and 0.2 µl (5 µmol/l) of each primer. Products were amplified during the following program: 1 cycle at 94°C for 3 min, followed by 39 cycles of 94°C for 10 s, and 1 cycle at 60°C for 30 s. The final step was to obtain a melt curve for the PCR products to determine the specificity of the amplicons. Expression levels were calculated relative to the GAPDH expression.

Immunization of goslings with plasmids. Goslings (9 per experimental group) were immunized with 200 µg of plasmid dissolved in 100 µl of sterile PBS by i.m. route twice, with two weeks between inoculations. As negative and untreated controls, the goslings were injected with empty pcDNA3.1 (+) vector or PBS alone, respectively. At 0, 7, 14, 21, and 28 days post immunization, blood samples were collected from the jugular vein, and one portion was mixed with RNAiso Plus reagent (Takara Bio, Otsu, Japan) for analysis of immune-related genes expression (CD4, CD8α, IL-6, IL-1β, and IFNs) by RT-qPCR. A second portion of the collected serum samples were stored at -20°C until they could be analyzed for antibodies against GPV by ELISA.

The concentration of serum IgY antibodies against GPV was determined by ELISA. Briefly, individual wells were coated with 30 ng/well of the purified GPV and blocked with 1% gelatin. The coated GPV antigens were incubated with serum samples diluted at 1:80 at 37°C for 1.5 h. After washing, the wells were incubated with HRP-conjugated goat anti-Bird IgY (abcam, USA) diluted 1:3,500. Bound antibodies were detected following the addition of 100 µl/well of 3, 3', 5, 5'-tetramethylbenzidine. The absorbances at 450 nm and 630 nm were read using a microplate reader.

Statistical analysis. Data are shown as the mean and standard deviation calculated using Prism 5 (GraphPad, USA), with Student's *t*-test analysis of variance.

Results

Expression of VP1, VP2, and VP3 in GEF

Forty-eight hours after transfection with pcDNA3.1(+)-VP plasmids, VP1, VP2, and VP3 recombinant proteins in the cell lysates of transfected GEF were analyzed by Western blotting. All three VPs were recognized by specific anti-His antibody and anti-GPV antiserum at approximately 80 kDa, 70 kDa and 60 kDa, respectively (Fig. 1).

VPs-induced cellular immune response in different cellular models

The immunogenicity of capsid proteins was examined in different cellular models. In the GEF model, cells were

transfected with pcDNA3.1(+)-VP1, pcDNA3.1(+)-VP2, pcDNA3.1(+)-VP3, and pcDNA3.1(+) for 48 h, and RNA was extracted using a RNAiso Plus reagent (Takara Bio, Otsu, Japan). The transcripts of the genes encoding CD4, CD8α, IL-6, IL-1β, and IFNs were measured. The results indicated that VP2 and VP3 significantly up-regulated the transcription of genes encoding IL-6 and IL-1β compared with the vector control ($P < 0.001$), while IL-6 was significantly increased by VP2 when compared with VP3 and the vector control ($P < 0.01$) (Fig. 2). The transcripts of the genes encoding CD4, CD8α, IFNα, IFNγ, IFNλ were nearly undetectable (data not shown) in both transfected and control cells.

In the PBMC model, none of the genes were altered by the treatment with VP1-containing extracts compared to the treatment with extracts from the control group. The transcripts of the genes encoding CD8a and IL-1β were significantly increased by the treatment with VP3-containing lysates compared with VP1 extracts. The transcript levels for IL-6, IL-1β, IFNγ, and IFNλ were significantly upregulated by VP2 extracts when compared to control and VP1 extracts (Fig. 3).

Expression levels of cytokines in the peripheral blood cells from vaccinated goslings

Based on the *in vitro* data, pcDNA3.1(+)-VP2 was used as a DNA vaccine to immunize goslings. Blood samples were collected at day 7, 14, 21, and 28 post-vaccination for cytokine expression analysis. Real time qPCR was performed to measure the mRNA expression levels of goose CD4, CD8α, IL-1β, IL-6, IFNα, IFNγ, and IFNλ. The results indicated that the transcriptional levels of the genes encoding CD4 and CD8 showed a steady increase through day 28 post immunization. At day 7 and day 14, the mRNA expression level of CD8 is up-regulated significantly compared to control

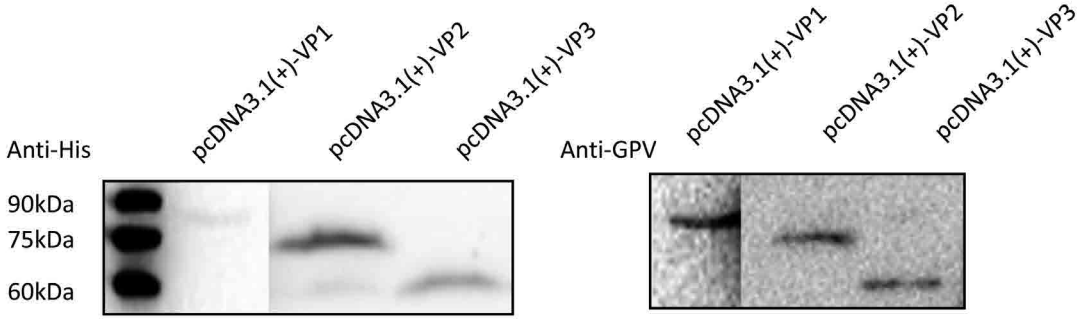

Fig. 1

Western blot analyses of VP1, VP2, and VP3 expression

Protein extracts from GEF transfected with the indicated plasmids were separated by gel electrophoresis and then transferred to PVDF membrane. The blot was incubated with anti-His antibody or anti-GPV antiserum, followed by a HRP-conjugated secondary antibody.

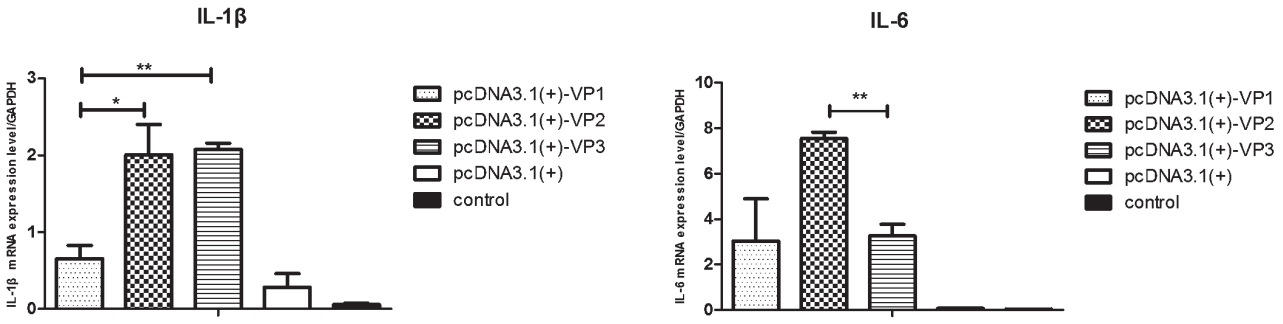

Fig. 2

mRNA expression levels of proinflammatory cytokines in goose embryo fibroblast after transfection with pcDNA3.1(+)-VPs and pcDNA3.1(+)

Gene expression relative to the housekeeping gene GAPDH was assessed using qPCR. The data are expressed as the mean ± SEM (n = 4), and the difference between groups was analyzed with a t-test. Groups denoted by (*) indicate a significant difference at $P < 0.05$, and groups denoted by (**) indicate a significant difference at $P < 0.01$.

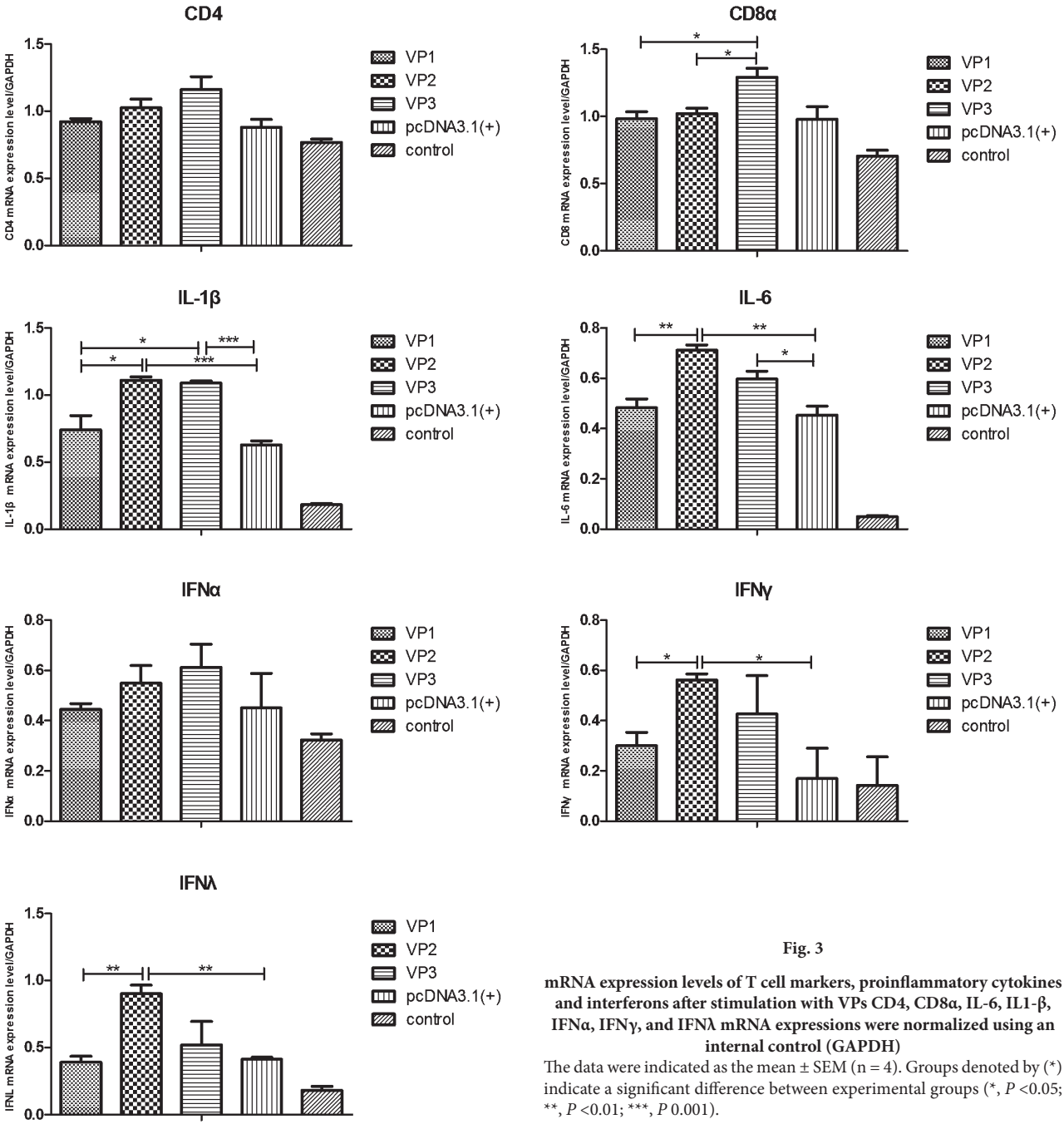

Fig. 3

mRNA expression levels of T cell markers, proinflammatory cytokines and interferons after stimulation with VPs CD4, CD8α, IL-6, IL1-β, IFNα, IFNγ, and IFNλ mRNA expressions were normalized using an internal control (GAPDH)

The data were indicated as the mean ± SEM (n = 4). Groups denoted by (*) indicate a significant difference between experimental groups (*, P <0.05; **, P <0.01; ***, P 0.001).

(Fig. 4). However, the mRNA expression levels of IL-1β and IFNγ experienced a decline after 14 days. Additionally, the expression level of IL-18 significantly increased on day 21. The data for IFNα and IFNλ transcripts showed a similar trend in that they both peaked on day 14 and then declined to very low levels on day 21 through day 28 (Fig. 4).

Detection of GPV-specific antibody in vaccinated goslings

To determine the efficacy of vaccination with the VP2 DNA vaccine, ELISA was performed to measure specific antibodies. Our results demonstrated that at day 14 post-immunization, the GPV-specific antibody was highly abundant. In addition, the GPV-specific antibody in pcDNA3.1(+)-VP2 immunized goslings was significantly increased at day 21 and

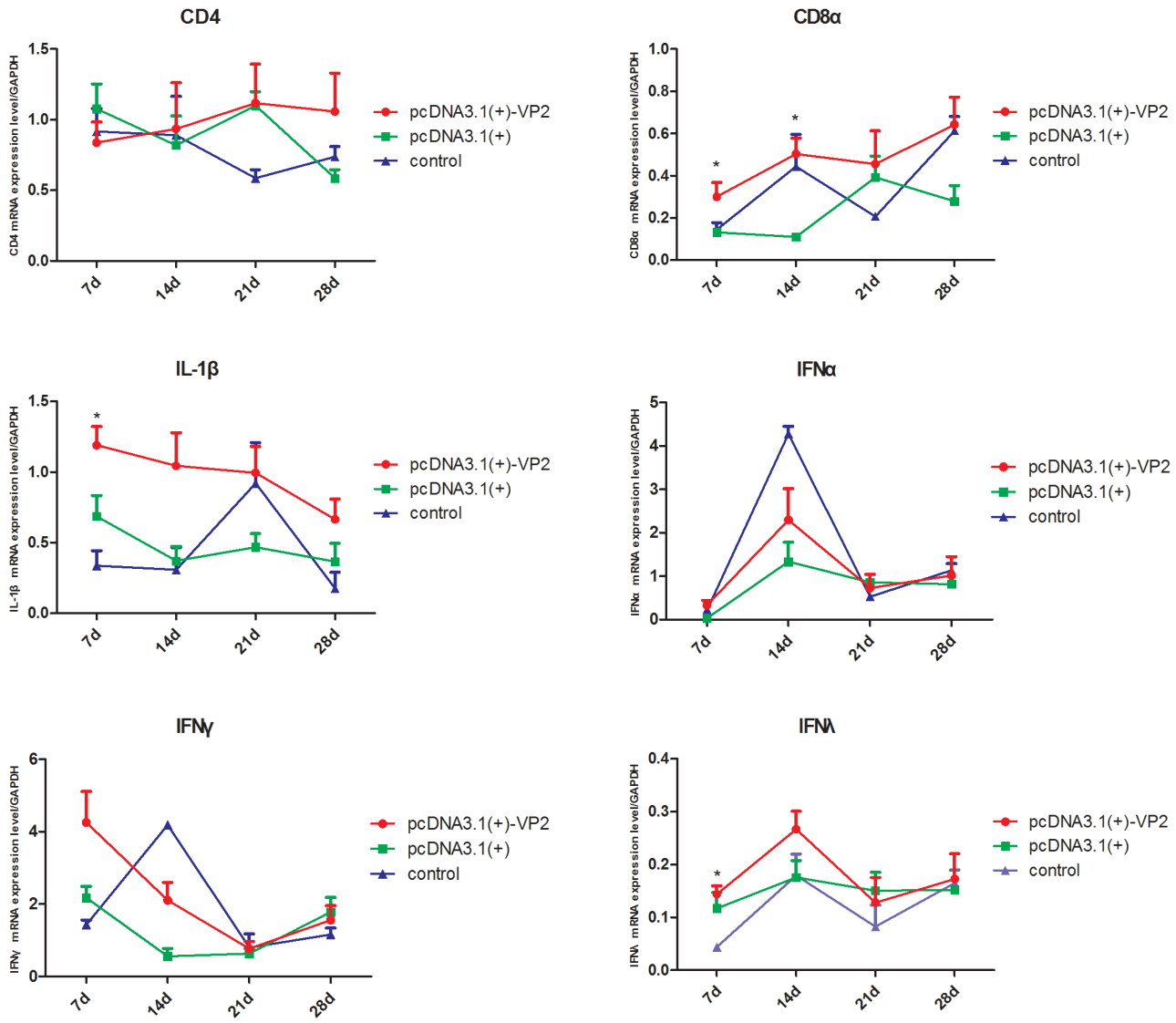

Fig. 4

pcDNA3.1(+)-VP2 plasmid induced different expression level of cytokines in goose peripheral blood cells

At day 7, 14, 21, and 28 post-immunization, transcripts were detected by RT-qPCR, and GAPDH was used as an internal control. The significance between mRNA cytokine levels in goose peripheral blood cells was determined by a *t*-test.

day 28 compared to control groups, and the IgY antibody levels were higher at day 28 than at any other time during the experimental phase (Fig. 5).

Discussion

Here, we examined the immunogenicity of recombinant VPs in different cellular models by assaying the expression level of various immune-related genes and the antibody titers in order to gain insight into the molecular mechanisms involved in the protective responses conferred by a GPV-specific DNA vaccine. The hallmark cytokine of the Th1 response, IFNγ, plays a major role in both innate and adaptive immunity. The results of this study indicate that IFNγ is significantly upregulated in PBMCs following treatment with VP2-containing extracts. Pro-inflammatory cytokines such as IL-1β play an important role in the host inflammatory and immune reactions. In assessing the transcriptional levels of genes encoding several cytokines, our results suggest that capsid proteins VP2 and VP3 significantly induced expression of IL-1β in GEF and goose PBMCs. A previous study

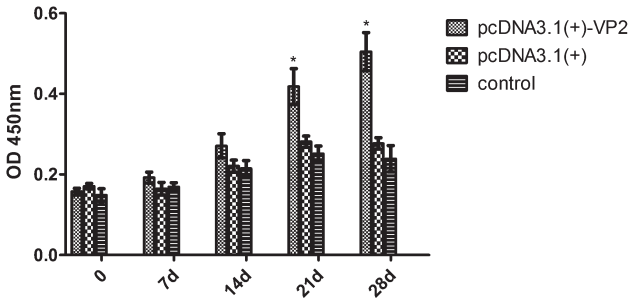

Fig. 5

Detection of serum-specific IgY antibody against GPV by indirect ELISA

Goslings were immunized with pcDNA3.1(+)-VP2 or pcDNA3.1(+) vector as a control. Blood samples were obtained from individual goslings at the indicated time points post immunization, and the levels of serum IgY antibodies against GPV were determined by indirect ELISA. Data are indicated as the mean ± SEM (n = 4).

indicated that VP1, VP2, and VP3 from GPV expressed from a baculovirus expression vector in insect cells were detected and that the formation of virus-like particles (VLPs) was observed. It was also found that the recombinant proteins elicited an immunoreactive response with specific antibodies and that the neutralizing activities of the antibodies against VP2 and VP3 were higher than that of antibodies against VP1 (Ju *et al.*, 2011). In this study, we found that both VP2 and VP3 can significantly upregulate the expression of genes encoding IL-6 and IL-1β in the GEF model, while the expression levels of IL-6, IL-1β, IFNγ, and IFNλ were significantly upregulated in PBMCs following the treatment with VP2-containing extracts. None of the tested genes showed any changes in response to VP1 in either the GEF or PBMCs. These results indicated that the cellular immune reactivity of VP2 and VP3 was stronger than that of VP1 and that VP2 is stronger than VP3. Since VP2 and VP3 are sub-fragments of VP1, VP1 theoretically contains all the antigenic epitopes on the VP2 and VP3 proteins. However, it is interesting that, based on our data, VP2 and VP3 appear to have higher immunogenicity than VP1. Among these three proteins, VP2 contains the immunodominat region the antibody can interact with. The VP2 proteins of many parvoviruses, including human parvovirus B19 (Lowin *et al.*, 2010), canine parvovirus (Jin *et al.*, 2012), porcine parvovirus (Zhou *et al.*, 2010), and goose parvovirus (Chen *et al.*, 2012) are able to form of VLPs in the baculovirus expression system. The VLPs always share similar immunogenicity with natural viral particles. Thus, VP2 was selected for the *in vivo* experiment.

The host innate immune response is the primary mechanism for resisting and clearing viruses during the early stage of infection (Barjesteh *et al.*, 2015). The acquired immunity in vaccinated animals likely alters the typical innate immune response profile (Pereiro *et al.*, 2014). Many papers have been published on GPV humoral immune responses, and the selection of VP proteins as antigen targets for a GPV vaccine has been previously established. However, little is known about cellular immunity against GPV. The pcDNA-GPV-VP1 DNA vaccine was shown to induce both cellular and humoral responses, and it can induce higher cellular and humoral immunity than live attenuated vaccine (Deng *et al.*, 2014). The codon-optimized VP2 (*opt*VP2) is capable of forming VLPs, which are highly immunogenic. Immunogenicity assays revealed that VLPs with *opt*VP2 could induce a high level of antibody production and provide effective protections against lethal challenge (Chen *et al.*, 2012). Moreover, Lee *et al.* (2010) selected some different adjuvants formulated with VP2 from GPV. Those results suggested that the percentage of $CD4^+/CD8^+$ cells in the PBMCs was significantly increased in ducks immunized with VP2 that was formulated with CpG ODNs. The major capsid protein VP3 was also tested in animals. Wang *et al.* (2015) constructed a recombinant VP3 vaccine (rmNA-VP3) by using the avirulent strain LaSota as a vaccine vector. Goslings inoculated with rmNA-VP3 showed no apparent signs of disease and had high levels of anti-GPV and anti-NDV neutralizing antibodies. Additionally, the localization of B-cell epitopes on GPV capsid proteins has been defined (Yu *et al.*, 2012). In other parvoviruses, for example human bocavirus species, the immunodominant epitopes of VP2 protein were also determined. Two of the peptides contain conserved epitopes among human bocavirus 1-4; these were recognized by a human polyclonal antibody and elicited high titer of antibodies in mice (Zhuo *et al.*, 2014).

Based on the immunobiological activity of VPs *in vitro*, humoral immune responses induced by GPV-VP2 were further explored *in vivo*. Here, we measured transcripts of some immune-related genes, such as T lymphocytes markers (CD4 and CD8α), IL-1β, IL-6, and IFNs after vaccination in goslings. T cell-mediated immunity is the central element of the adaptive immune system. Virus-specific $CD8\alpha^+$ T cells have been shown to be important for the elimination of viral shedding (Reusser *et al.,* 1991; McNeal *et al.*, 1995). In our study, the CD4 and CD8α mRNA expression levels increased over time after immunization. The expression of CD8α significantly increased on day 7 and day 14, suggesting that our VP2 vaccine induced a strong immune response at the early stage. Pro-inflammatory cytokines such as IL-1β play an important role in host immune and inflammatory reactions. In this study, we found that the mRNA levels of CD8a, IL-1β, IFNγ, and IFNλ in goose PBMCs were highly increased in the early phase of vaccination with pcDNA3.1(+)-VP2, which could compensate for the low humoral immunity (low titers of GPV-specific antibody) in the early post-vaccination phase. The antigen-specific humoral responses, particularly against VP2 and VP3, are

crucial for the prevention of viral assembly. The enhanced T cell population should promote stronger humoral responses. These data suggested that vaccination with pcDNA3.1(+)-VP2 had a strong cellular immune reactivity and induced strong humoral responses. These data will serve as a basis for better understanding of the molecular processes needed for successful vaccination development.

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