

Influence of porcine reproductive and respiratory syndrome virus GP5 glycoprotein N-linked glycans on immune responses in mice

Wenming Jiang · Ping Jiang · Xinglong Wang ·
Yufeng Li · Xianwei Wang · Yijun Du

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Abstract Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most economically significant viral diseases in the swine industry. Infection with PRRSV following vaccination is common, since protection is incomplete. Persistent infection may be one of the biggest obstacles to control of the disease. “Glycan shielding” was postulated to be a primary mechanism to explain evasion from neutralizing immune response, ensuring in vivo persistence of virus, such as HIV, SIV, and HBV. The objective of this study was to construct recombinant adenoviruses expressing single or multiple N-linked glycosylation site (NGS) mutant GP5 of PRRSV, and evaluate the expression in cell culture, and potential to induce immune responses in BALB/c mice. Six recombinant adenoviruses were constructed each expressing wild-type GP5 and 1-4 NGS mutants: N44S, N44/51S, N30/44/51S, N30/33/44/51S and N30/33S. Inoculation of BALB/c mice with all five recombinants expressing NGS mutant GP5 resulted in a significant neutralizing antibody responses which were significantly higher than that of recombinant adenovirus expressing wild-type GP5. But there were no significant difference in lymphocyte proliferation responses induced by wild type and NGS mutant GP5. It indicated that glycosylations of GP5 at residues N30, N33, N44 and N51 are critical for induction of neutralizing antibodies. These NGS mutant PRRSV GP5 will be useful to characterize the effects of glycosylation on immunogenicity in the natural host, and may lead to a new approach for the generation of PRRSV vaccines.

Keywords PRRSV · GP5 · Recombinant adenovirus · Glycosylation · Neutralizing antibodies

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a reproductive and respiratory disease of pigs that occur in the world and currently accepted as the most important infectious disease of swine. Porcine reproductive and respiratory syndrome virus (PRRSV), the causative agent of PRRS, is an enveloped, positive-stranded RNA virus. It belongs to the *Arteriviridae* family within the order *Nidovirales* [1], along with equine arteritis virus (EAV), simian hemorrhagic fever virus (SHFV), and lactate dehydrogenase-elevating virus (LDV) [2]. The PRRSV genome is approximately 15.0 kb in length and contains nine open reading frames (ORFs) [2–4]. The viral structural proteins, encoded in ORFs 2–7, are expressed from seven subgenomic capped and polyadenylated mRNAs that are synthesized as a 3′-coterminal nested set of mRNAs with a common leader sequence at the 5′ end [5]. They are the nucleocapsid (N, 15 kDa), matrix (M, 19 kDa), and envelope protein (GP5, 25 kDa), which have been consistently identified in virion and/or virus-infected cells [6, 7]. Other four proteins designated as GP2a, GP3, and GP4 envelope glycoproteins were encoded by ORF2a, ORF3, ORF4, respectively [8, 9]; and a nonglycosylated envelope protein E, that is expressed from a second ORF (ORF2b) entirely contained within ORF2 [3].

The major viral envelope protein is glycoprotein 5 (GP5), which is encoded by ORF5 of the viral genome and is the most important glycoprotein of PRRSV involved in the generation of PRRSV-neutralizing antibodies and protective immunity [10–14]. GP5 is a glycosylated

W. Jiang · P. Jiang (✉) · X. Wang · Y. Li ·
X. Wang · Y. Du
Key Laboratory of Animal Disease Diagnostic and Immunology,
Ministry of Agriculture, College of Veterinary Medicine,
Nanjing Agricultural University, Nanjing 210095, China
e-mail: jiangp@njau.edu.cn

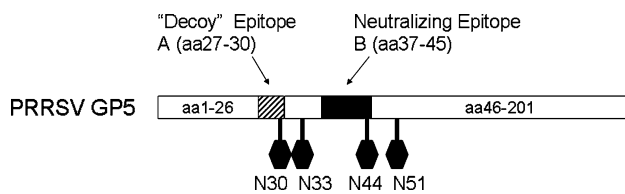


Fig. 1 Schematic diagrams of NGS (black hexagon) as well as neutralizing epitope (black box) and non-neutralizing epitope (hatched box) located in the amino terminal of the GP5 of PRRSV

transmembrane protein of approximately 25 kDa [15–17]. It has a putative N-terminal signal peptide and possesses two to four putative N-linked glycosylation sites (NGS) [6–18] (Fig. 1). It has been identified that non-neutralizing and neutralizing epitopes were located in its N-terminal region [19]. Delay in neutralizing antibody response has been postulated to be due to the presence of a nearby immunodominant decoy epitope (aa27–30), which evokes a robust, early, and non-protective immune response that masks and/or slows the response to the neutralizing epitope (aa37–45) for at least 3 weeks [19]. While this is a plausible explanation for the delay of PRRSV-neutralizing antibody response, it remains to be tested.

NGS of the GP5 ectodomain may be critical for proper functioning of the protein. N-linked glycosylation, in general, is important for correct folding, targeting, and biological activity of proteins [20–23]. In many enveloped viruses, the envelope proteins are modified by the addition of sugar moieties and the NGS of envelope protein plays diverse functions in viral glycoproteins, such as receptor binding, membrane fusion, penetration into cells, and virus budding [24, 25]. Recent studies have demonstrated the role of NGS of Hantaan virus glycoprotein in protein folding and intracellular trafficking [26], as well as in the biological activity and antigenicity of influenza virus hemagglutinin (HA) protein [27]. Furthermore, it has become evident that glycosylation of viral envelope proteins is a major mechanism for viral immune evasion and persistence used by several different enveloped viruses to escape, block, or minimize the virus-neutralizing antibody response. Examples of this effect have been reported for simian immunodeficiency virus [28] and human immunodeficiency virus type 1 [29], hepatitis B virus [30], and influenza virus [31] and more importantly, in the case of the arterivirus LDV [32]. In these cases, “glycan shielding” was postulated to be a primary mechanism to explain evasion from neutralizing immune response, ensuring *in vivo* persistence of virus.

Recently, the application of adenovirus expression system for expressing exogenous protein has been reported from several laboratories, including ours [14, 33, 34]. In order to examine the importance of NGS in the biological activity of GP5 of PRRSV in eliciting antibodies, especial

neutralizing antibodies *in vivo*, we constructed a series of mutant GP5 expressed by replication-defective recombinant adenoviruses in which the NGS have been mutated either individually or in various combinations. The resulting mutant proteins were examined for eliciting ELISA and neutralizing antibodies and lymphocyte proliferation against PRRSV in mice. It demonstrated that the level of neutralizing antibodies induced by all mutant GP5 expressed in recombinant adenoviruses were higher than that of wild-type GP5. Especially, our data from neutralizing antibody response indicated that glycans at residues N30 and N33 were critical for induction of neutralizing antibodies. It was also showed that lymphocyte proliferation responses elicited by wild type and NGS mutant GP5 were no significant differences.

Materials and methods

Cells and viruses

The 293 human embryo kidney cell line (ATCC CRL1573), which provides phenotypic complementation of the E1 and E3 genes, and MARC-145 cells was grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and maintained in a 37°C humidified chamber with 5% CO₂. The virulent PRRSV strain S1 (GenBank no. AF090173) was propagated in MARC-145 cells.

Construction of transfer plasmid encoding the wild-type GP5 of PRRSV

GP5 of PRRSV was amplified by RT-PCR with primers Ad-GP5.1 (5′-GCC GGT ACC ACC ATG TTG GAG AAA TGC TTG AC-3′) and Ad-GP5.2 (5′-GCA CTC GAG CTA AGG ACG ACC CCA TTG TTC-3′), and cloned into the shuttle vector pShuttle-CMV (Qbiogene), as previously described [14], producing recombinant shuttle plasmids named pShuttle-CMV-ORF5. The sequence of GP5 was confirmed.

Construction of transfer plasmid encoding NGS mutant GP5 of PRRSV

The NGS mutant GP5 coding regions were generated by replacing the asparagine triplet (AAC) at positions N30, N33, N44 and AAT at position N51 of the GP5 cDNA with the serine triplet (AGC) or (AGT) using PCR method. Site-directed mutagenesis was performed as standard techniques [35] using PCR with PfuTurboTM DNA polymerase, glycosylation-site specific reverse primers designed as in Table 1. The mutant gene fragments were cloned into *Kpn*

Table 1 Primers used to generate GP5 glycosylation-site mutant proteins^a

N-glycan location	Primer	Nucleotide sequence (5'-3')
N44	P44	C ACA TAG CGT CAA <u>GCT</u> GTA AAT CAG
N51	P51	C TGT GCC <u>ACT</u> CAG CTC ACA TAG CG
N30	P30	T GTC <u>GCT</u> GCT GGC GTT GGC GAGC
N30/33	P30/33	T GTC <u>GCT</u> GCT GGC <u>GCT</u> GGC GAGC

^a The nucleotide change is underlined. The middle nucleotide of the triplet codons was change to exchange the asparagine triplet codon (AAC or AAT) with a serine triplet codon (AGC or AGT) to produce primers for the next PCR amplification

I/Xho I-digested pShuttle-CMV, producing shuttle plasmids named pShuttle-CMV-ORF5N44S (mutation of N44), pShuttle-CMV-ORF5N44/51S (mutation of N44 and N51), pShuttle-CMV-ORF5N30/44/51S (mutation of N30, N44 and N51), pShuttle-CMV-ORF5N30/33/44/51S (mutation of N30, N33, N44 and N51), and pShuttle-CMV-ORF5N30/33S (mutation of N30 and N33). The sequences of the mutant gene fragments were confirmed by DNA sequencing for the presence of site-directed mutagenesis.

Generation of recombinant adenoviruses

The recombinant adenoviruses were produced as previously described [14]. They were plaque-purified three times by growth in HEK-293 cells, named rAd-GP5, rAd-GP5N44S, rAd-GP5N44/51S, rAd-GP5N30/44/51S, rAd-GP5N30/33/44/51S, and rAd-GP5N30/33S, respectively.

Viral growth kinetics

HEK-293 cells were infected with recombinant adenoviruses expressing mutant or wt GP5 at an MOI of 0.5 TCID₅₀ per cell and incubated at 37°C in an incubator. At various time points post-infection, aliquots of culture supernatants from infected cells were collected and the virus titer in the supernatants was determined by an end-point limit dilution assay on 293 cells and expressed as 50% tissue culture infectious dose per ml (TCID₅₀/ml).

Indirect immunofluorescence assay

In order to determine the interest genes expression, HEK-293 cells were cultured in 96-well plates and infected with the recombinant adenoviruses at an MOI of 0.1. After incubation for 24 h at 37°C, cells were washed with PBS and fixed with cold ethanol for 45 min at 4°C. The cells were washed and incubated with monoclonal antibody

against GP5 (produced from BALB/c immunized with eukaryotic plasmid pcDNA3-GP5) for 1 h at 37°C. After washed with PBS-T (PBS containing 0.05% Tween-20), the cells were incubated with goat anti-mouse IgG conjugated with fluorescein (1:50 in PBS-T) for 1 h at 37°C. After rinsing by six times, the colored cells were observed under fluorescent microscope.

Western blot

The recombinant adenoviruses were propagated in HEK-cells were cultured for 48 h at 37°C, the virus were harvested from the infected cells by centrifugation, and lysis with 0.5 ml lysis buffer containing 50 mM Tris-Cl (pH7.4), 150 mM NaCl and 1% Triton X-100. HEK-293 cells infected with wild-type adenovirus (wtAd) were treated as above and used as control. Lysates were clarified by centrifugation for 20 min at 12,000 rpm. Supernatants were subjected to a 12% SDS-PAGE and transferred to nitrocellulose membrane. Following the transfer, membrane was blocked overnight at 4°C with 10% non-fat milk in PBS-T (0.01 M PBS, 0.05% Tween-20), washed three times with PBS-T and incubated for 2 h with monoclonal antibody against GP5. The membrane was washed for three times with PBS-T and incubated at room temperatures for 1.5 h with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (1:5000 dilution in PBS-T). Proteins were visualized by using enhanced chemiluminescence luminol reagents (SuperSignal West Pico Trial Kit, PIERCE).

Immunization and samples collection

A total of 192, 6–8 week-old female BALB/c mice were randomly divided into eight groups, including six groups were immunized subcutaneously (s.c.) at day 0 and boosted at day 14 with the recombinant adenoviruses, rAd-GP5, rAd-GP5N44S, rAd-GP5N44/51S, rAd-GP5N30/44/51S, rAd-GP5N30/33/44/51S, or rAd-GP5N30/33S, respectively. At each immunization, the mice received 10^{6.0} TCID₅₀ of each recombinant in 500 µl PBS. Two control groups received the Ad5 vector alone (wtAd) (kept in our lab) or PBS following the same immunization protocol.

Serum samples and lymphocytes were routinely obtained over the course of immunization. At 14, 28, 42 and 56 days post-immunization (dpi), the mice of each group were euthanized and the serum samples (*n* = 6) were obtained from mice for detection of specific antibody responses. Fresh lymphocytes were separated from the spleens of mice for detection of specific cell-mediated immune responses.

Indirect ELISA

During antibody detection using indirect ELISA, PRRSV antigen was prepared as previously described [14]. Firstly, cellular components were removed by centrifugation at $6,000 \times g$ for 30 min. Then the virus particles in the supernatant were precipitated by centrifugation at $50,000 \times g$ for 2 h and resuspended in PBS. Finally, the condensed viruses were precipitated by centrifugation at $50,000 \times g$ for 2 h in 30% sucrose solution and were subsequently solubilized by Triton X-100 at a final concentration of 0.2%. The solubilized preparation was then used as PRRSV ELISA antigen. Meanwhile, an antigen from uninfected cell culture lysate was cleared by centrifugation as above and used as negative control. The positive and the negative antigens were coated in 96-well plates overnight at 4°C at concentration of 2 ng per well in 50 mmol/l sodium carbonate buffer (pH 9.6). After washing with PBS-T, wells were blocked with 200 μ l of 5% dried milk powder in wash buffer for 2 h at 37°C. Mouse sera samples were diluted by 100-fold in PBST and incubated with the plates in quadruplicate: two wells for PRRSV antigen and two parallel wells for negative control antigen. Meanwhile, mouse serum antibody to PRRSV was used as positive control and mouse serum without antibody to PRRSV as negative control, and bound antibodies were detected with HRP-conjugated goat anti-mouse IgG (Boshide) (1:30,000 diluted in PBST). After 1 h incubation, plates were washed and bound peroxidase was detected with 100 μ l substrate solution TMB. Plates were incubated for 10 min at 37°C and 50 μ l of 2 M H₂SO₄ was added in each well to stop the reaction. The optical density (OD) was determined at 450 nm in an ELISA reader. The calibrated OD for each tested and control serum was calculated by subtraction of the mean OD of the wells containing negative antigen from that of the parallel wells containing PRRSV antigen.

In order to detect the antibody to wild-type adenovirus in mice immunized with adenovirus recombinants, the wild-type adenovirus was purified, concentrated and used as ELISA antigen as described elsewhere [36]. Briefly, HEK-293 cells were incubated with wild-type adenovirus at an MOI of 1 until extensive CPE was observed and detached from the flask surface. The supernatant was cleared of cellular debris by centrifugation (10 min, 12,000 g) and a virus pellet was obtained by centrifugation (25,000 rpm for 2.5 h in a Sorval AH 629 rotor) and resuspended in PBS. The procedures for ELISA were the same as above. Antibody levels were estimated by measuring the OD₄₅₀.

Serum neutralization assay

The titers of PRRSV-neutralizing antibodies in serum samples were determined as described previously [14]. Prior to neutralization assay, sera from all animals in each immunization group were heat-inactivated for 30 min at 56°C. Serial two-fold dilutions of test sera were incubated for 60 min at 37°C in the presence of 200 TCID₅₀ of PRRSV-S1 in DMEM containing 2% FBS. The mixtures were added to 96-well microtitration plates containing confluent MARC-145 cells which had been seeded 48 h earlier. After incubation for 5 days at 37°C in a humidified atmosphere containing 5% CO₂, cells were examined for cytopathic effects (CPE). The titers of neutralizing antibodies to PRRSV were determined as the reciprocal of the highest serum dilution in which no CPE was observed. Meanwhile, the titer of anti-adenovirus neutralizing antibodies in the serum samples were also determined as described above, using 200 TCID₅₀ of wild-type adenovirus.

Lymphocyte proliferation assay

The lymphocyte proliferation assay was performed mainly as described previously [37, 38]. Briefly, the spleens were aseptically isolated at 14, 28, 42, and 56 dpi, and single cell suspensions were prepared by centrifugation over Ficoll-hypaque (Jinghua, Shanghai, China). Cells were suspended in RPMI-1640 supplemented with 10% fetal bovine serum, 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine and 100 μ g/ml gentamicin. Subsequently, these cells were cultured in triplicate at 5×10^5 cells per well in 96-well flat-bottomed tissue culture plates in 100 μ l volumes of RPMI-1640 complete medium. The lymphocyte proliferation assays were performed by stimulation of splenocytes with PRRSV (live purified PRRSV S1 virus at an M.O.I. of 1) and mock antigen preparation (purified mock culture fluid) in triplicates. As a control for the ability of T cells to proliferate, the mitogen Concanavalin A (Con A) at a concentration of 5 μ g/ml was included in each assay. Following 66 h incubation at 37°C, 5%CO₂, the proliferation responses were measured by a standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described. In brief, 20 μ l of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (5 mg/ml, Sigma) was added to each well and the plates were incubated for a further 6 h. The stimulation index (SI) was calculated as the ratio of average OD₅₇₀ value of wells containing PRRSV-stimulated cells to the average OD₅₇₀ value of wells containing mock antigen.

Statistical analysis

The differences in the level of ELISA, neutralizing antibodies, and lymphocyte proliferation among the different groups were determined by student's *t*-test. Differences were considered statistically significant when *P*-values < 0.05.

Results

Construction of recombinant shuttle vectors and generation of recombinant adenoviruses

The shuttle vectors encoding wild-type and five PRRSV GP5 NGS mutant proteins were constructed and DNA sequencing confirmed that the specific Asn were replaced with Ser for each of glycosylation-site mutant plasmids, as well as located in the proper reading frame.

The growth of recombinant adenoviruses was analyzed on 293 cells by using an MOI of 0.5 (Fig. 2). The growth of recombinant adenoviruses rAd-GP5N44/51S, rAd-GP5N30/44/51S, rAd-GP5N30/33/44/51S, and rAd-GP5N30/33S was similar and they were only slightly delayed compared to that of rAd-GP5 that expressed the wild-type GP5. However, the growth of rAd-GP5N44S was significantly delayed compared to that of rAd-GP5. The delayed growth of rAd-GP5N44S may be due to a lower replication rate or to reduced adenovirus budding. Nevertheless, the final yield of recombinant adenoviruses expressing NGS mutant GP5 were similar to that of rAd-GP5 (Fig. 2).

Expression of the wild and NGS mutant GP5 by recombinant adenoviruses

Expression of the GP5 was evaluated using indirect immunofluorescence assay and western blot. The 293 cells were infected at an MOI of 0.1 TCID₅₀ per cell with each

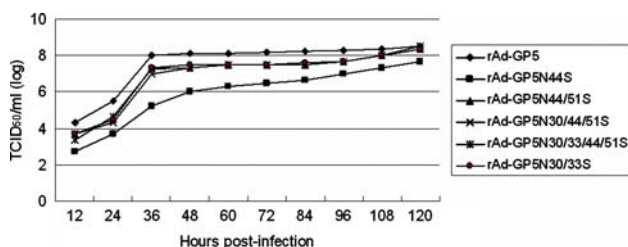


Fig. 2 Growth kinetics of recombinant adenoviruses on HEK-293 cells. Cells were infected with recombinant adenoviruses expressing wild-type and NGS mutant GP5 at an MOI of 0.5. At each time point, cells were collected, and cell-associated virus titers were determined by using the TCID₅₀ method

recombinant adenovirus and incubated at 37°C. Specific staining was detected in the 293 cells infected with recombinant adenovirus expressing the NGS mutant PRRSV GP5. The staining intensity of cells infected with recombinant adenovirus was comparable to cells infected with wild-type GP5. No staining was observed in the negative control HEK-293 cells that infected with wild-type adenovirus.

Cell lysates were collected from 293 cells infected with recombinant adenoviruses and analyzed by Western blotting. As shown in Fig. 3, wild-type GP5 migrated as ~24.5 kDa protein species (lane 2), which was equal to the GP5 from lysates of MARC-145 cells infected with PRRSV (lane 1) in molecular weight. Mutant GP5 carrying single mutation (N44S) migrated as ~23.0 kDa protein species (lane 3), and the double mutants (N44/51S and N30/33S) produced protein species that migrated close to ~20.5 kDa (lane 4 and 7). The triple mutant (N30/44/51S) generated a protein that migrated as ~19.0 kDa protein (lane 5), and the quadruple mutant (N30/33/44/51S) produced protein species that migrated close to ~17.0 kDa (lane 6). There no GP5 specific protein band was found in wtAd-infected 293 cells (lane 8). It indicated that the NGS mutant and wild-type GP5 were expressed by corresponding adenovirus recombinants.

ELISA antibody response of BALB/c mice following recombinant adenoviruses inoculation

As shown in Fig. 4, the antibody responses in the recombinants-inoculated groups were increased at 14 dpi. During the period of 28–56 dpi, the levels of GP5-specific antibody induced by recombinant adenoviruses expressing

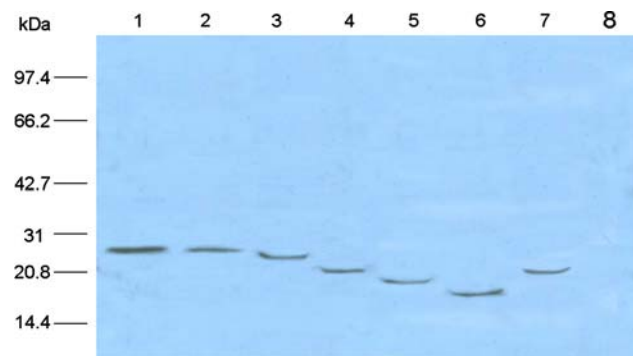
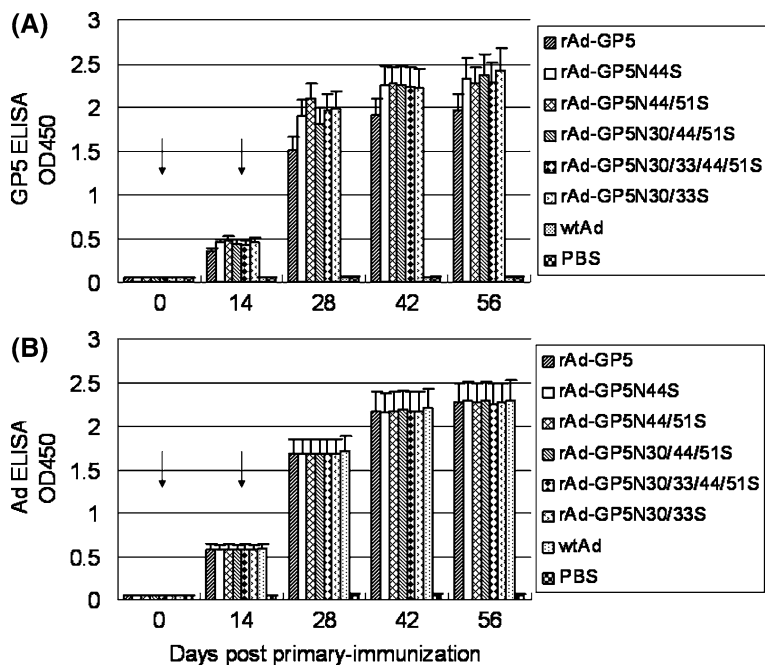


Fig. 3 Western blot analysis of cell lysates infected with recombinant adenoviruses with GP5-specific monoclonal antibody (α GP5). 293 cells were infected with rAd-GP5 (lane 2), rAd-GP5N44S (lane 3), rAd-GP5N44/51S (lane 4), rAd-GP5N30/44/51S (lane 5), rAd-GP5N30/33/44/51S (lane 6), and rAd-GP5N30/33S (lane 7). MARC-145 cells infection with PRRSV (lane 1) and 293 cells infected with wild-type adenovirus (wtAd) (lane 8) were used as controls. Proteins standards are showed on left side of panel

Fig. 4 ELISA for PRRSV (A) or Ad (B) specific antibodies of sera from mice immunized with recombinant adenoviruses. It was illustrated for the mice immunized with recombinant adenoviruses compared to control mice immunized with wtAd or PBS. Serum samples ($n = 6$) were collected at various time-points and antibodies to PRRSV or wild-type adenovirus were detected using a single dilution (1:100) ELISA. Data were shown as mean \pm standard error. Arrows (\downarrow) indicate time of primary immunization and boost; wtAd means wild-type adenovirus



mutant GP5 were significantly higher than that of rAd-GP5 ($P < 0.05$). But there were no significant differences between GP5-specific antibody levels induced by recombinant adenoviruses expressing mutant GP5. Meanwhile, no antibody against GP5 was detected over 56 days in groups immunized with wtAd and PBS. In order to evaluate the comparability of humoral immune responses among different groups, the ELISA antibody against adenovirus antigen was also measured. There were no significant differences among all the adenovirus-immunized groups ($P > 0.05$).

Neutralizing antibodies titers

The ability of sera from rAds-inoculated mice was also analyzed for virus neutralizing antibodies against PRRSV

in vitro. Mice inoculated with recombinant adenoviruses expressing NGS mutant GP5 proteins developed neutralizing antibodies to PRRSV at 56 dpi (Fig. 5). As early as 28 dpi, mice immunized with recombinant adenoviruses expressing NGS mutant GP5 developed neutralizing antibodies, however, mice inoculated with rAd-GP5 only developed detectable neutralizing antibody at 56 dpi and the titers were very low. With the addition of NGS mutation, from N44S, N44/51S, N30/44/51S to N30/33/44/51S, the neutralizing antibody titers were also increased correspondingly. The titers of neutralizing antibody developed by mice inoculated with rAd-GP5N30/33S were significantly higher than that elicited by rAd-GP5N44S, rAd-GP5N44/51S, and rAd-GP5N30/44/51S, but obviously lower than that of rAd-GP5N30/33/44/51S ($P < 0.05$). No neutralizing antibodies against

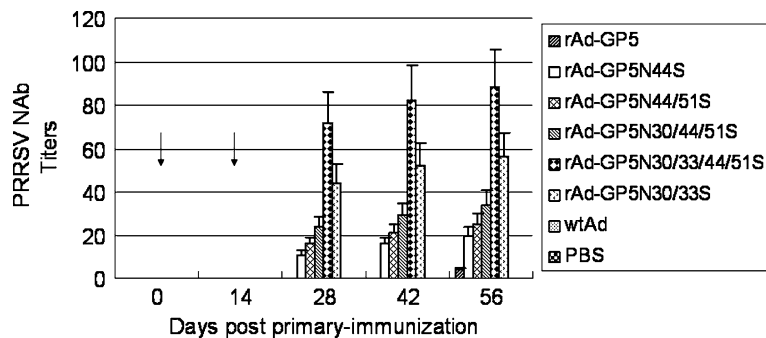


Fig. 5 Neutralizing antibodies against PRRSV were determined in sera of mice immunized with recombinant adenoviruses. Serum samples ($n = 6$) were collected at various time-points and detected with two-fold serial dilution virus neutralization assay. The titers

neutralizing antibodies were expressed as the reciprocal of the highest serum dilution in which no CPE was observed. Data were shown as mean \pm standard error. Arrows (\downarrow) indicate time of primary immunization and boost; wtAd means wild-type adenovirus

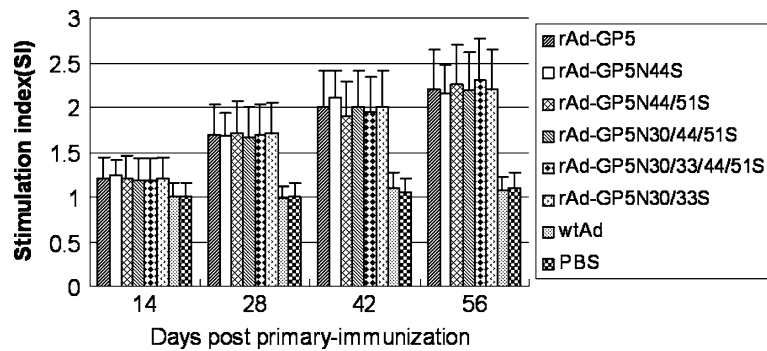


Fig. 6 Lymphocyte proliferative responses in mice immunized with different recombinant adenoviruses. Splenocytes samples ($n = 6$) were collected at various time-points and were stimulated with PRRSV at an MOI of 1 in triplicate. After 66 h of stimulation, MTS

was added and the OD₅₇₀ values were determined after a further 6 h of inoculation. The ConA control sample showed a stimulation index of 4–6. Error bars represent group standard deviations

PRRSV could be detected in mice inoculated with wtAd or PBS.

Lymphocyte proliferation response by recombinant adenoviruses

We have previously reported that GP5 expressed by recombinant adenovirus could induce a lymphocyte proliferation response [14]. Here, we determined, whether elimination of the N-glycans in the GP5 interfere with the T-cell proliferation response to the GP5. As shown in Fig. 6, proliferative responses were detected, as early as 14 dpi. These responses were significantly higher than the response of control groups ($P < 0.05$). However, there was no significant difference in T-lymphocyte proliferation response between the NGS mutant and wild-type GP5 groups.

Discussion

The aim of the present study was to explore the possibility to improve the immunogenicity of GP5 of PRRSV by elimination of N-linked glycans known to be engaged in shielding of neutralizing epitope. GP5 contains two to four sites for N-linked glycosylation and elimination of some of them could improve the ability of a PRRSV constructed by reverse genetic systems to induce high titers of neutralizing antibody [39]. Deletion of upstream NGS of VR2332 and/or other associated amino acid differences in GP5 ectodomain of the natural N-glycosylation mutant (HV-1 strain) enhanced the immunogenicity of the GP5 neutralization epitope and resulted in the formation of neutralizing antibodies that were specific for the mutant HV-1 [40].

In order to characterize the effects of the single or the combination of the four mutated N-glycans of PRRSV GP5 on the immunogenicity in BALB/c mice, recombinant

adenoviruses encoding GP5 protein with mutants were inoculated into BALB/c mice to evaluate their potential to induce immune responses. To our knowledge, this is the first report, comparing the effects of multiple NGS mutations of the PRRSV GP5 on immune responses including induction of neutralizing antibodies and lymphocyte proliferation response using recombinant adenovirus expression system.

The efficacy of expression of GP5 in cells infected with recombinant adenoviruses might affect the induction of immune responses. Analyses of expression of the NGS mutant proteins in infected 293 cells by indirect immunofluorescence assay and western blot analysis demonstrated that the protein expression among these recombinants was comparable and similar to that of the wild-type protein. It indicated that the wild type or NGS mutant GP5 was expressed by corresponding adenovirus recombinants at similar expression efficacy.

Antibody responses of mice inoculated with recombinant adenoviruses expressing the wild-type or NGS mutant GP5 were analyzed using an indirect ELISA and virus neutralizing assay. The results showed that removal of PRRSV GP5 N-glycans resulted in increased neutralizing antibody responses against PRRSV. With the addition of NGS mutation, from N44S, N44/51S, N30/44/51S to N30/33/44/51S, the neutralizing antibody titers were also increased correspondingly. Moreover, even though the antibody to the decoy epitope was not detected here, the titer of neutralizing antibody elicited by rAd-GP5N30/33S was significantly higher than those elicited by rAd-GP5N44S, rAd-GP5N44/51S, and rAd-GP5N30/44/51S, but lower than that of rAd-GP5N30/33/44/51S. It indicated that the N-glycans at positions N30 and N33 might play a more important role in induction of neutralizing antibodies. Removal of N-glycans at positions N30 and N33, which are near the decoy epitope, may have inhibited the folding and exposure of this epitope, preventing and/or decreasing the

immune response to it. Removal of N-glycans at positions N44 and N51 which are near the neutralizing epitope may have facilitated the exposure of the neutralizing epitope and transport of the protein to the cell surface, improve exposure of the protein to antigen presenting cells (APCs). Therefore, it can be deduced that the glycans at N30, N33, N44 in the ectodomain of GP5 are real shielding glycans only reducing immunogenicity of NE and removal of N30 and N33 may open the space on the surface of APCs for neutralizing antibody accession to NE.

In previous study of Ansari et al. [39], they focused on the influence of N-linked glycosylation of PRRSV GP5 on virus infectivity, antigenicity, and ability to induce neutralizing antibodies by reverse genetic systems. They found that viruses carrying mutations at N34, N51, and N34/51 grew to lower titers than wt PRRSV, and mutations involving residue N44 did not result in infectious progeny production. Moreover, viruses carrying mutations at N34, N51, and N34/51 induced significantly higher levels of neutralizing antibodies against wt PRRSV. In our study, we also found that recombinant adenoviruses carrying mutations at N44/51, N30/44/51, N30/33/44/51, and N30/33 grew slowly than that expressing wild-type GP5 and recombinant adenovirus carrying only mutation at N44 grew more slowly. Mice inoculated with any of the five NGS mutant adenovirus recombinants resulted in increased neutralizing antibody responses against PRRSV.

The significance of eliminating N-glycans in immunogens for the T-cell response to viral glycoproteins is poorly investigated but ought to be considered when evaluating new vaccine constructs deficient in defined N-glycans. Though all the NGS mutant and wild-type GP5 could induce a proliferative T-cell response to the GP5, there were no significant differences in T-lymphocyte proliferation response among all these groups. It suggested that removal of any of the four N-linked glycans in adenovirus-based immunogens do not interfere with the T-cell response to the GP5.

In summary, glycosylation affects the ability of PRRSV GP5 in eliciting a humoral immune response, but not in cellular immune response. GP5 is the most important glycoprotein of PRRSV involved in the generation of PRRSV-neutralizing antibodies and protective immunity. Our results revealed that the absence of glycans at residues N30, N33, N44, or N51, enhances the immunogenicity of the nearby neutralizing epitope. The N-glycans at positions N30 and N33 may play a more important role in induction of neutralizing antibodies than those at positions N44 and N51. Continued studies using PRRSV GP5 NGS mutant proteins in the natural host to characterize the effects of glycosylation on immunogenicity may lead to a new approach for generation of PRRSV vaccines.

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