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The replicase protein nsp2 of Chinese highly pathogenic porcine reproductive and respiratory virus is involved in protective immunity by promoting viral clearance

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Abstract

The genome segment for replicase protein nsp2 represents the fastest evolving region of porcine reproductive and respiratory syndrome virus (PRRSV), and our previous studies have shown that the PRRSV nsp2 genetic variation contributes to poor cross-neutralization. By using in vitro antibody absorption assay, here we show that the papain-like protease 2 (PLP2) domain of nsp2 is a target of neutralizing antibodies. This was further verified by cross-neutralization assay with a series of inter-lineage chimeric mutants between the Chinese highly pathogenic PRRSV (HP-PRRSV) strain JXwn06 and the low virulent NADC30-like strain CHsx1401 (lineage 1). The role of nsp2 in protective immunity was subsequently tested in a one-month SPF piglet model by immunizing the piglets with CHsx1401 or its derivatives carrying JXwn06 structural protein region (SP) alone (CHsx1401-SP_{JX}) or in combination with PLP2 region (CHsx1401-$SPplp2_{JX}$), or the whole nsp2 region (CHsx1401-$SPnsp2_{JX}$), followed by challenge with JXwn06 at 42 days post immunization, a time point when the viremia was undetectable. All chimera groups were protected from the challenge by JXwn06, whereas the group CHsx1401 failed to provide beneficial protection. Interestingly, the group CHsx1401-$SPnsp2_{JX}$, but not CHsx1401-$SPplp2_{JX}$, showed the lowest lung microscopic lesions and viral tissue load. Significantly, the vaccine virus CHsx1401-$SPnsp2_{JX}$ was undetectable in the examined tissues, and so was for the challenge virus except for one piglet, highlighting an important role of HP-PRRSV nsp2 in promoting viral clearance. The findings provide insight into the mechanisms underlying the protective immunity against PRRSV and have important implications in PRRSV vaccine development.

Keywords PRRSV, nsp2, Protective immunity

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is currently a severe threat to the Chinese and worldwide swine industry. Reproductive failure in sows and respiratory diseases in all pigs are the two major clinical symptoms associated with PRRSV infections in the field, and these are often followed by secondary bacterial or viral infections, leading to colossal economic losses annually [1, 2]. Phylogenetically, PRRSV is an enveloped, positive-stranded RNA virus of about 15 kb that belongs

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to the family *Arteriviridae* in the order *Nidovirales* [3]. This agent harbors at least 8 open reading frames (ORFs) necessary for replication and virion morphogenesis [4]. Specifically, ORF1a and ORF1b encode viral replicase polyproteins pp1a and pp1ab that are further proteolytically processed into at least 12 nonstructural proteins (nsps), including nsp1α, nsp1β, nsp2-5, nsp6, nsp7α, nsp7β, nsp8, and nsp9–12 [5–7]. Meanwhile, ORFs2-7 code for viral structural proteins, including GP2a, E, GP3, GP4, GP5a, GP5, M, and the nucleocapsid protein (N) [8, 9]. Of them, GP2a, GP3 and GP4 are minor glycoproteins that form heterotrimers to mediate virus-cell membrane fusion [10, 11], whereas GP5 and M are major envelope proteins that form heterodimers involved in viral attachment [12, 13].

Vaccination is a critical strategy for PRRSV control. The first commercial vaccine Ingelvac® PRRS, a modified live virus (MLV), was launched in 1994 in the United States and has since been used widely [14–16]. The current MLV vaccines provide excellent protective efficacy against the genetically homologous viruses but fail to induce sterilizing immunity to clear both the vaccine virus and the incoming field viruses [15, 17, 18]. Limited cross-protection against the infections of heterologous PRRSV strains is also of great concern [15, 16, 19], as it allows the selection of immune escape mutants and the emergence of frequent recombinants. Not surprisingly, the past 30 years have seen a continuous emergence and expansion of genetically diverse PRRSV strains, including the most recent strains such as the Chinese highly pathogenic PRRSV (HP-PRRSV), Lena, NADC30, NADC34, and their derivatives [20–25]. Meanwhile, recombination events between HP-PRRSV strain/vaccines, NADC30, and NADC34 strains have increasingly been reported [26–31], striking a critical need for further understanding the mechanisms of PRRSV-induced immune protection.

Among PRRSV structural proteins, the major envelope proteins (GP5 and M) and minor glycoproteins (GP2, GP3 and GP4) are the well-known targets of neutralizing antibodies [32–38]. Interestingly, a recent report indicates that ORF1a contains a neutralization region [39], and our work further showed that the genetic variation of the replicase protein nsp2 contribute to poor cross-neutralization [40], raising the possibility that PRRSV nsp2 plays a role in protective immunity. In retrospect, PRRSV nsp2 represents the genetically most divergent and fastest evolving genomic region among PRRSV strains [41–43]. This protein contains an N-terminal papain-like cysteine protease 2 domain (PLP2), a highly variable middle region (HV) with a size of 300–500 amino acids, a C-terminal transmembrane domain (TMD) as well as a cytoplasmic tail (CT) [44, 45]. Insertions and most notably deletions, recombination, as well as extensive amino acid substitutions, are often seen within nsp2 [2, 41]. In addition, PRRSV nsp2 contains many B-cell epitopes that are highly immunogenic and can induce antibodies to a level comparable to that to N proteins [46, 47].

Our work in this report started with dissecting the region of nsp2 in induction of neutralizing antibodies by construction of chimeric viral mutants between genetically highly heterologous stains, namely HP-PRRSV strain JXwn06 (lineage 8) and NADC30-like strain CHsx1401 (lineage 1), that share nsp2 nucleotide identity only about 76%, and followed by animal experiments. Our findings showed that PRRSV nsp2 PLP2 contains targets of neutralizing antibodies, and that HP-PRRSV nsp2 is involved in protective immunity by promoting viral clearance. The details are described below.

Results

The PLP2 domain of PRRSV nsp2 is a target of neutralizing antibodies

Our previous analysis by reverse genetics revealed that genetic variation of the-nsp2 coding region contributes to poor cross-neutralization between the strain JXwn06 (lineage 8) and HB-1/3.9 (lineage 8) [40]. To determine the region within nsp2 as the target for neutralizing antibodies, we performed the antibody absorption assay. Specifically, the nsp2 extracellular domain of PRRSV stain JXwn06 and its truncation derivatives tagged with strepII epitope tag at the C-terminus were individually expressed in *E. coli* BL21 cells and purified by affinity chromatography and gel filtration (Fig. 1A and B), whereas the viral polymerase nsp9 served as a control. All proteins were expressed as soluble forms and purified to homogeneity. The purified proteins on strepII beads were then incubated with the hyperimmune swine serum against JXwn06 at 37 °C for 1 h to absorb the corresponding antibodies. After treatment, the titers of neutralizing antibodies (NA) of the serum against JXwn06 were titrated in MARC-145 cells. The nsp9 treatment did not affect to the neutralization ability as compared to the mock-untreated control, and a 8-to-16 fold dilution of the nsp9-treated serum could still completely neutralize the input virus. In contrast, the same treatment with nsp2 mutants lost the ability to neutralize the virus (Fig. 1C), suggesting that the antibodies to nsp2 are critical for neutralization. A difference between nsp2 and nsp9 treatment could still be discerned at a further 32-fold dilution (Fig. 1C), although not statistically significant. In addition, the treatment with nsp2(12–323) showed the similar effect to that by the other two derivatives (Fig. 1C), suggesting that the PRRSV PLP2 domain is the target of neutralizing antibodies.

The importance of PLP2 in induction of NA was further investigated by constructing a series of chimeric

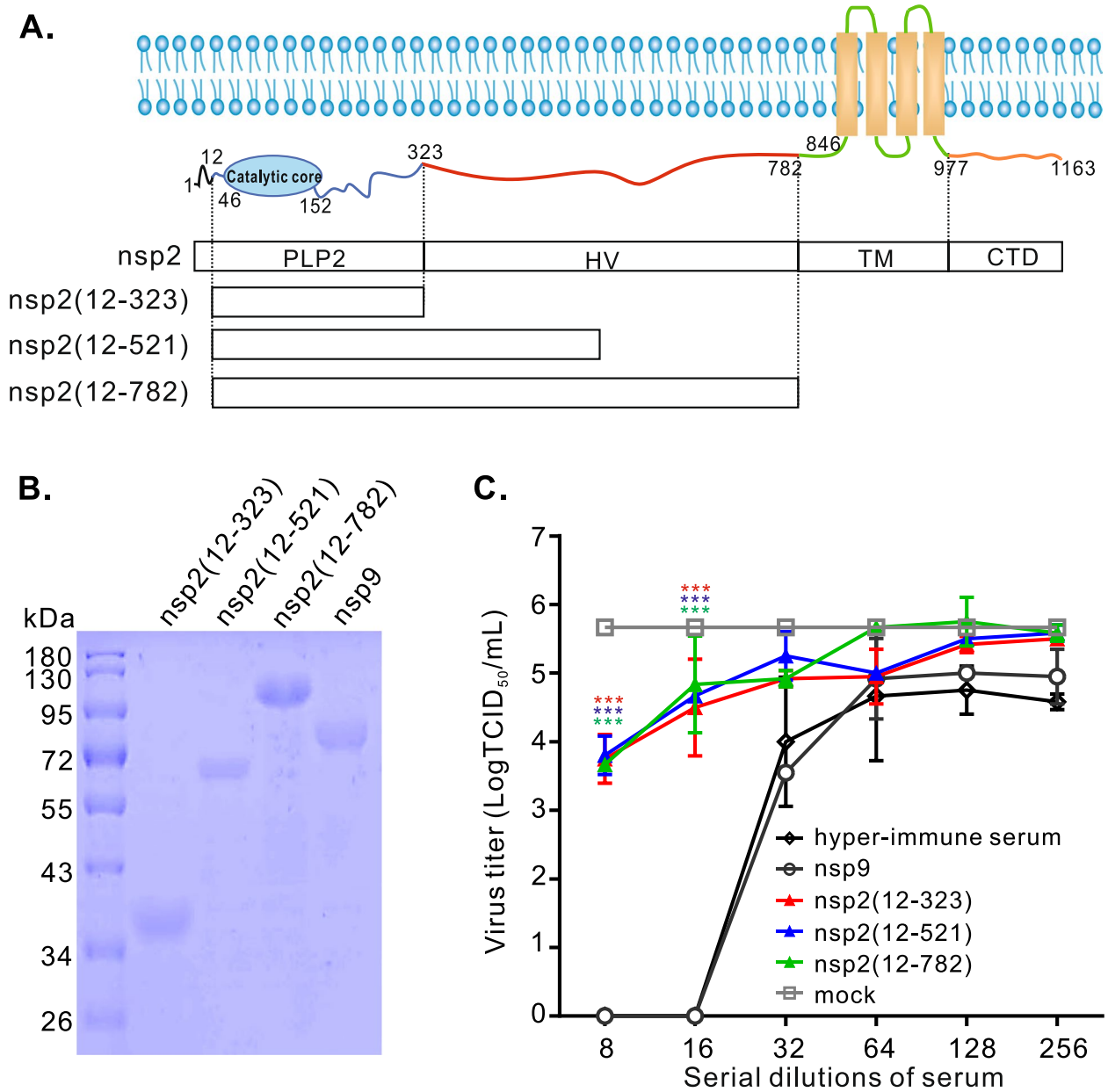


Fig. 1 The PLP2 domain is a target of neutralizing antibodies. **A** Construction strategies for prokaryotic expression of truncated nsp2 proteins. **B** SDS-PAGE analysis of the purified nsp2 proteins. **C** The antibody absorption assay. The hyper-immune serum was absorbed by nsp2 derivatives or by nsp9, followed by NA titration in MARC-145 cells. Error bars represent standard deviation (SD). Asterisks (*) mean the statistical difference (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$)

mutants between HP-PRRSV strain JXwn06 (lineage 8) and the low virulent NADC30-like strain CHsx1401 (lineage 1) by reciprocal substitution of the structural protein coding region (SP) or in combination with the region for PLP2 (Fig. 2A). All the mutants were successfully rescued and verified by immunofluorescence staining to N protein (Fig. 2B) and the full-length genome sequencing. The ability of JXwn06 hyperimmune serum to neutralize these chimeric mutants were tested in MARC-145 cells (Fig. 2C and D). As expected, the virus CHsx1401 is resistant to neutralization by the serum to JXwn06 (Fig. 2C, left); however, exchange of either JXwn06 PLP2 region (CHsx1401-$PLP2_{JX}$) or SP region (CHsx1401-SP_{JX}) enabled CHsx1401 to gain sensitivity to the serum (Fig. 2C). Simultaneous substitution of both SP and PLP2 increased the NA titer, but not to that

of WT (Fig. 2C). Thus, these results suggest that both PLP2 and SP regions are targets of neutralizing antibodies. In the reciprocal studies, substitution with either the SP or PLP2 region from CHsx1401 reduced the NA titers to JXwn06 (Fig. 2D). Together, these results suggest that PRRSV PLP2 region is a critical region for induction of neutralizing antibodies, and the genetic differences of PLP2 affect the cross-neutralizing activity among PRRSV different strains.

The SP region alone is sufficient to provide homologous immunity

To investigate the respective role of nsp2 and SP in induction of protective immunity, we performed immunization/challenge experiments in a one-month-old piglet model. 25 SPF piglets were randomly divided into 5 groups and immunized intranasally with the indicated viruses (CHsx1401-SP_{JX}, CHsx1401-$SPnsp2_{JX}$, CHsx1401-$SPplp2_{JX}$ and CHsx1401) at a dose of 2×10^5 $TCID_{50}$ or with Dulbecco's Modified Eagle's Medium (DMEM) as a mock control. Following immunization, CHsx1401 and the chimera-infected piglets showed transient fever, whereas the group CHsx1401-$SPnsp2_{JX}$ exhibited high fever remaining above 40 °C for a streak of 7 days, but all piglets survived (Fig. 3A and B). All groups completed clearance of viremia within 42 days but with different rates. Consistent with the previous report [48], the group CHsx1401-$SPnsp2_{JX}$ group showed much faster viremia clearance dynamics, two weeks earlier than CHsx1401 group (Fig. 3C). At 42 days post immunization, the piglets were challenged with JXwn06 at a dose of 2×10^6 $TCID_{50}$. The pig rectal temperatures and clinical symptoms were monitored in a daily manner. The DMEM and CHsx1401 groups contracted high fever over 41 °C for 5 days and above 40 °C for about two weeks. During the course, one pig in the DMEM group died. In contrast, the piglets infected with all chimeric mutants showed transient fever over 40 °C but with good appetite (Fig. 4B).

The other clinical symptoms and weight changes were also assessed. No significant clinical symptoms were observed for pigs immunized with the chimeric mutants, except for occasional sneezing and coughing. In contrast, the pigs immunized with CHsx1401 or DMEM displayed frequent coughing and sneezing, clear depression, and anorexia (Fig. 4C). Significantly, the piglets in the groups CHsx1401 and DMEM stopped to gain weight following challenge, whereas all the chimera groups maintained normal body weight gain (Fig. 4D).

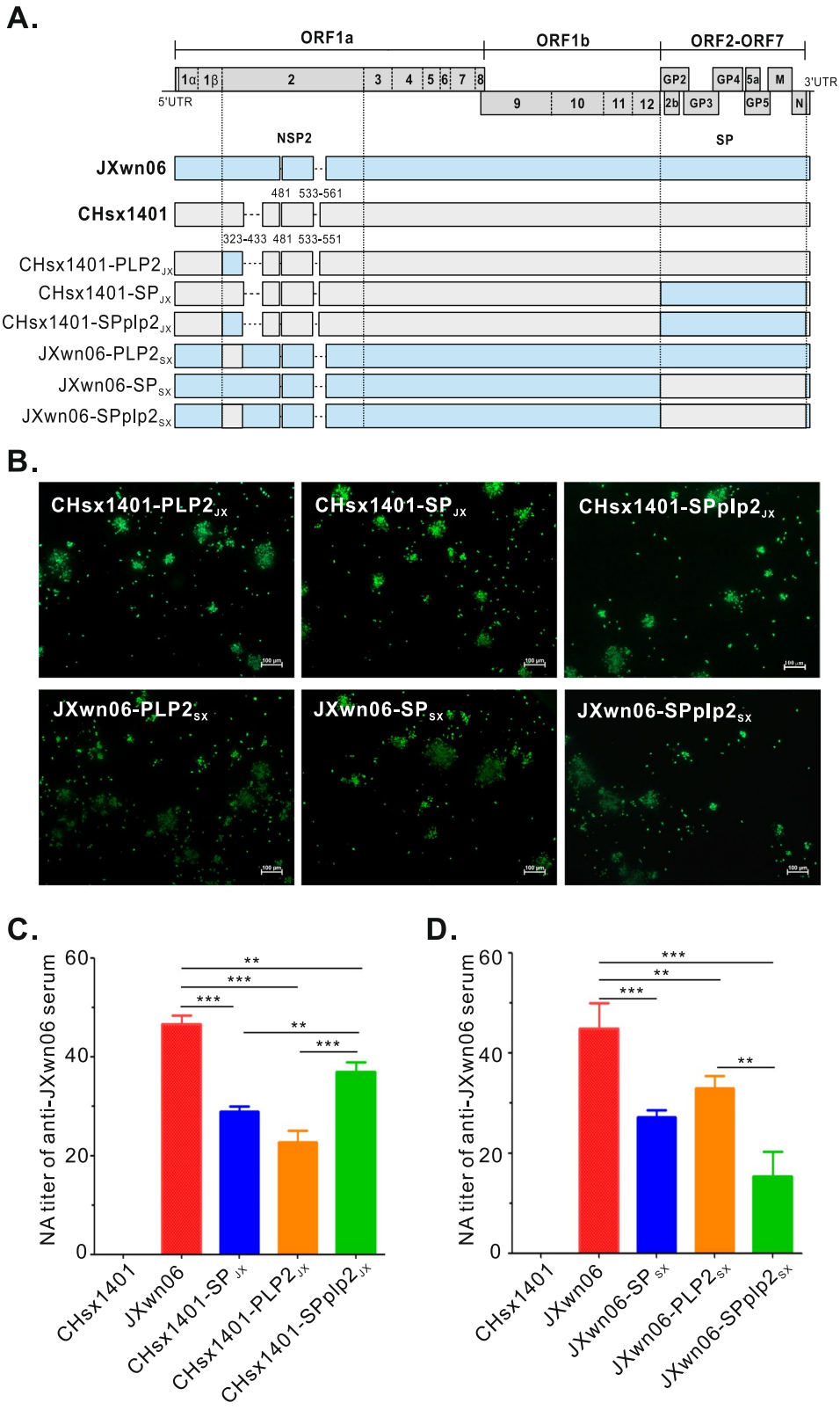
The piglets were euthanized and necropsied at 21 days post challenge. Examination of the lung gross lesions revealed that CHsx1401 and DMEM groups presented the typical HP-PRRSV-mediated pneumonia, including severe congestion, edema, and large areas of consolidation. The chimera groups showed only scattered consolidation and had much lower lung lesion scores (Fig. 5A and C, left). Similar trend was observed for the lung microscopic lesions as revealed H&E. The piglets from CHsx1401 and DMEM groups had severe lung injury, including pulmonary congestion, typical small intravascular hemorrhage, large numbers of inflammatory and necrotic cell infiltration, and interlobular septal thickening (Fig. 5B and C, right). In contrast, CHsx1401-SP_{JX} caused much less tissue damage, despite that small intravascular hemorrhage could still be observed for the group. Interesting, the intravascular hemorrhage was greatly reduced from the nsp2 chimera groups CHsx1401-$SPnsp2_{JX}$ and CHsx1401-$SPplp2_{JX}$. Thus, immunization with Chsx1401 did not provide observable beneficial protection (e.g., clinical sign, body weight gain, tissue injury, etc.) against the heterologous challenge by the lineage 8 strain JXwn06, and that SP region is sufficient to provide clinical protection against homologous challenge, while additional exchange of the nsp2 region appears to provide better protection against tissue injury.

HP-PRRSV nsp2 contributes to viremia clearance and reduction of viral tissue load

We investigated the turnover of the viruses in serum and various tissues. The piglets in the groups DMEM and CHsx1401 mounted a significant viremia following challenge by JXwn06 that peaked at around 10 days post challenge, and the viremia were not cleared at the termination of the experiment (21 days post challenge). In contrast, all three chimera groups had low level of viremia that were quickly cleared from the blood at 5 days post challenge. Specifically, the group CHsx1401-$SPnsp2_{JX}$ had the lowest viremia level, and only one pig showed measurable viremia, whereas the group CHs1401-$SPplp2_{JX}$ had two piglets showing viremia. In contract, all pigs from

(See figure on next page.)

Fig. 2 Genetic variation of the PLP2 domain contributes to weak cross-neutralizing responses between heterologous strains. **A** Genomic organization and construction of chimeric PRRSV mutants. **B** IFA analysis of PRRSV mutants**.** MARC-145 cells in six-well plates were infected with the indicated viruses at an MOI of 0.1, fixed at 24 h post-infection (hpi), and then stained with mouse monoclonal antibody against PRRSV N protein. **C–D** Determination of NA titer of anti-JXwn06 hyper-immune serum against PRRSV mutants in MARC-145 cells. The error bars indicate means standard deviation (SD). Asterisks (*) indicate mean the statistical difference (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$)



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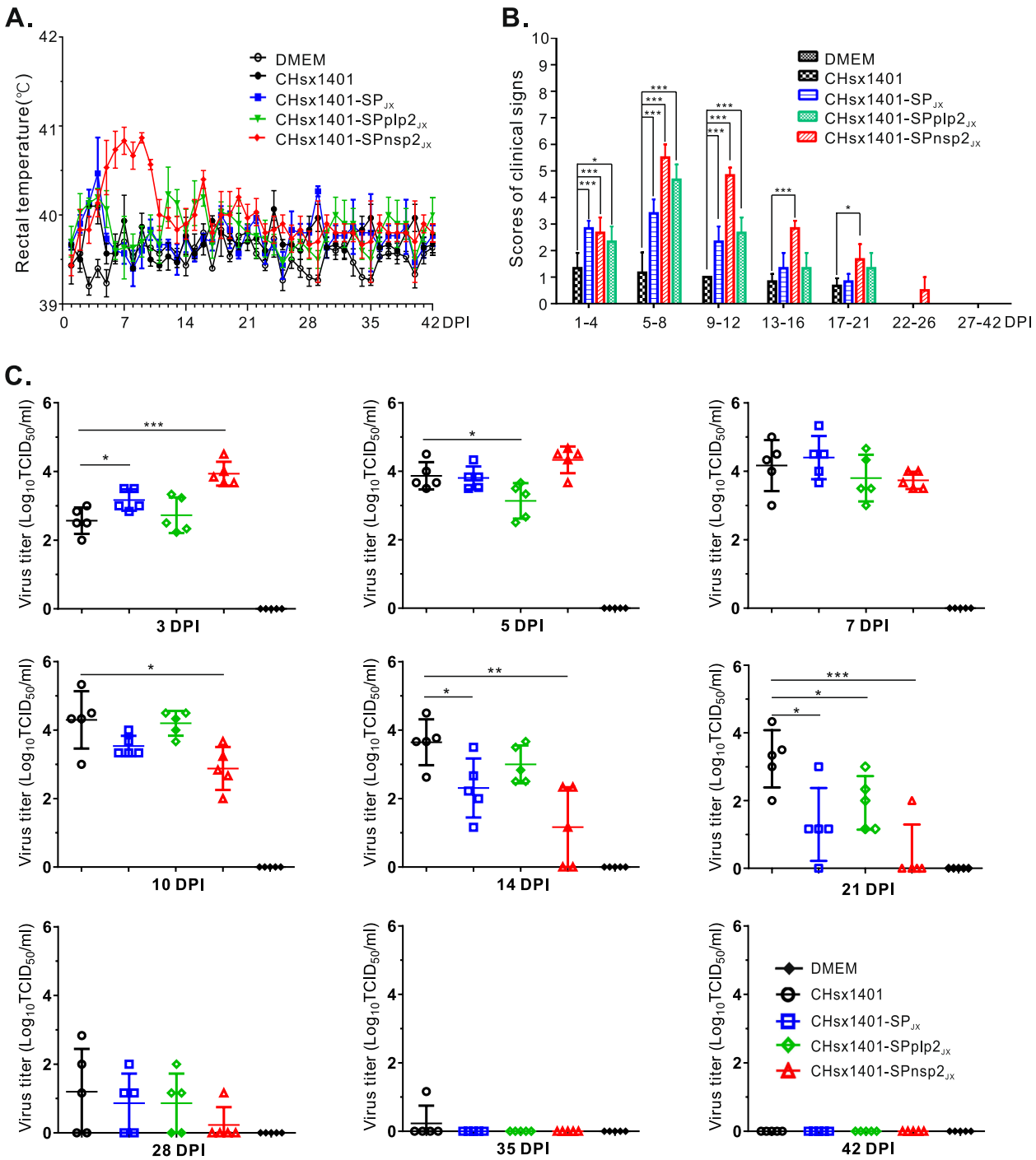

Fig. 3 The manifestations of piglets after immunization. **A** The rectal temperature curves of piglets after immunization and before challenge. **B** Clinical mental state scores of piglets inoculated with the rescued viruses. The total clinical scores were the sum of gross clinical score (GCS), respiratory clinical score (RCS), and nervous signs score (NSS). **C** The viremia of piglets inoculated with the rescued viruses after immunization and before challenge. Virus titers were determined by endpoint dilution assay in MARC-145 cells. Error bars represent standard deviation (SD). Asterisks mean the statistical difference between chimeric viruses and CHsx1401 (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$)

CHsx1401 group and DMEM group have much higher viral load, consistent with the previous report about JXwn06 [49, 50]. The viremia of JXwn06 reached peak on day 5, remained at plateau at day 7, and then decreased slowly; clearance was not completed on day 21 after challenge (Fig. 6A).

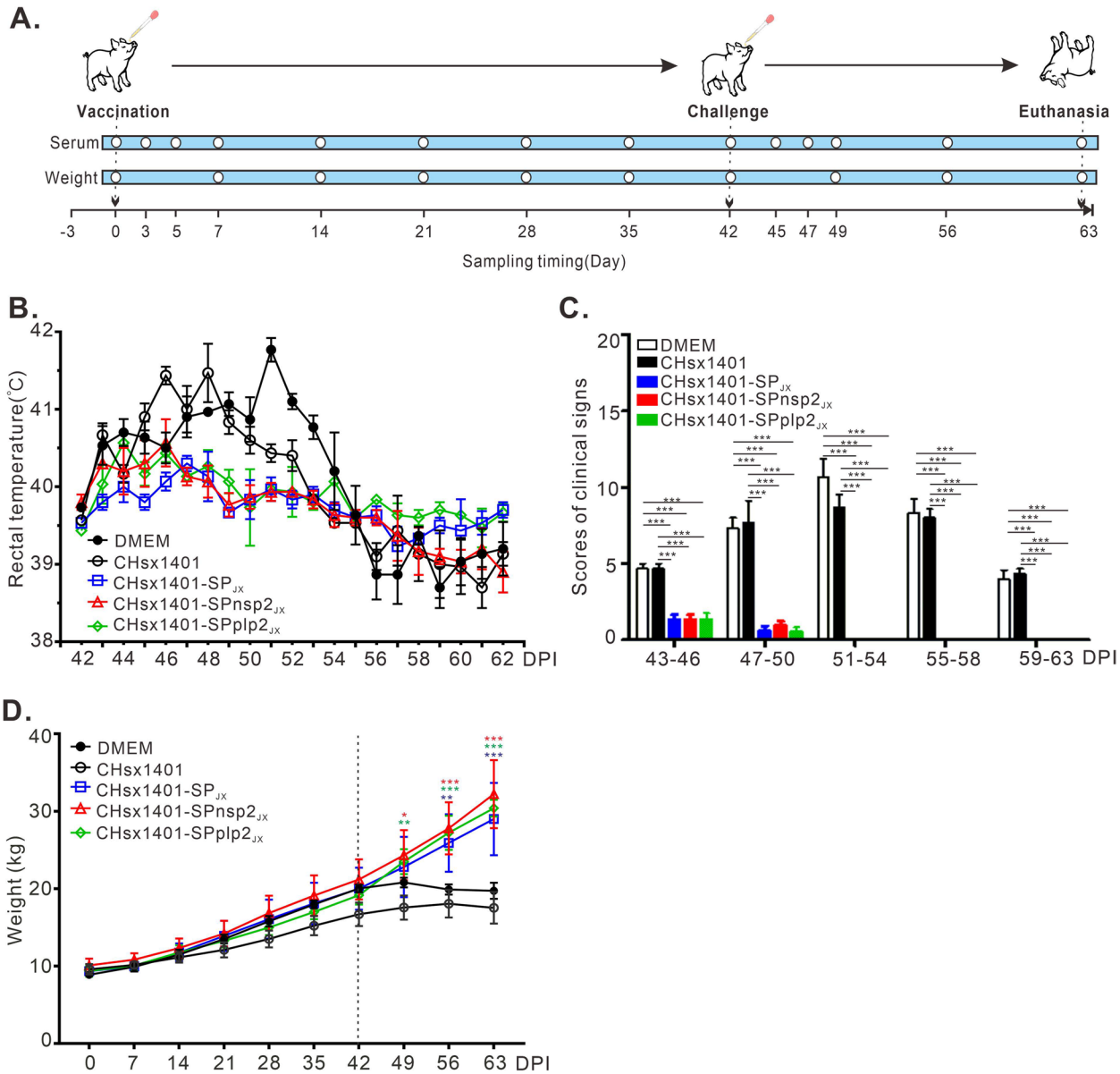

Fig. 4 The SP region alone is sufficient to provide homologous immunity. **A** Protocol of the animal trial. **B** The rectal temperature curves of piglets. **C** Clinical mental state scores of piglets of different groups following challenge. The total clinical scores for each piglet were the sum of gross clinical score (GCS), respiratory clinical score (RCS), and nervous signs score (NSS). **D** The weight curves of piglets after immunization. The starting point of DPI was on the first day after immunization. All piglets were challenged with JXwn06 at 42 DPI. Error bars represent standard deviation (SD). Asterisks mean the statistical difference (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$)

The tissues, such as lung, tonsil, inguinal lymph nodes (ILNs), and submandibular lymph nodes (SLNs), were collected and individually processed for quantitative analyses of viral tissue load by absolute real-time reverse transcription PCR (RT-qPCR). Two sets of discriminating primers were designed by targeting the nsp9-coding region to differentiate CHsx1401 or its derivatives from JXwn06. All chimera groups showed at least 2 logs lower viral tissue load for JXwn06 than the groups CHsx1401and DMEM. The lowest viral load was associated with the group CHsx1401-SPnsp2$_{JX}$, and only one pig was positive for the presence of JXwn06 RNA in SLNs and tonsil (Fig. 6B). Quite interestingly, the vaccine virus RNAs for the group CHsx1401-SPnsp2$_{JX}$ were not detectable, a stark contrast with the other two chimera groups and the group CHsx1401 (Fig. 6C). Thus, the full-length of nsp2, but not SP or PLP2, contributes to viral tissue

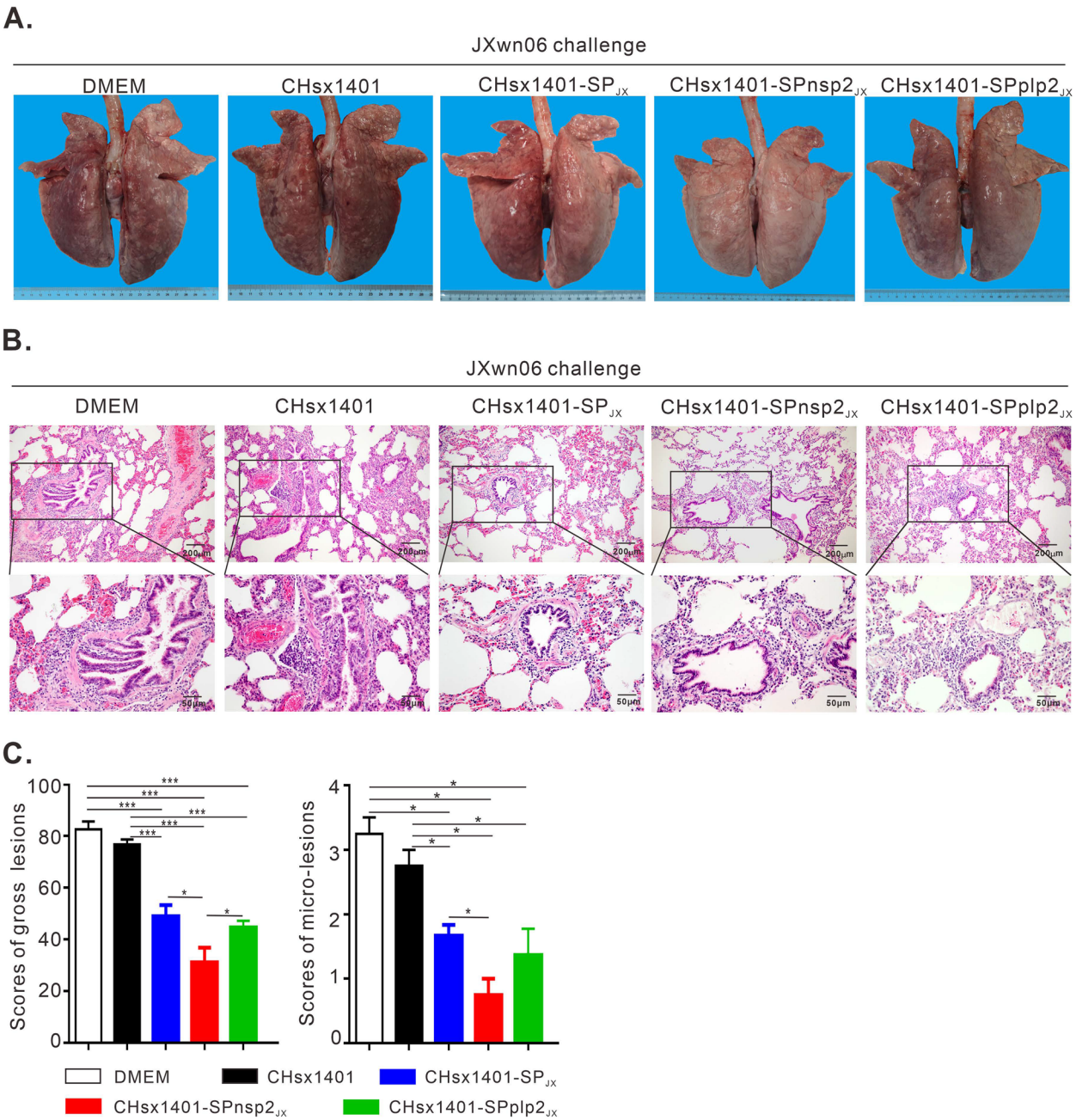

Fig. 5 Evaluation of lung lesions after JXwn06 challenge. **A** Representative gross lung lesions at 21 day post challenge. **B** Histopathological examination of lung lesions at 21 day post challenge. Representative images are showed. **C** The mean scores of gross (left) or microscopic (right) lung lesions of each group based on the severity of interstitial pneumonia. Error bars represent standard deviation (SD). Asterisks mean the statistical difference (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$)

load reduction. These results suggest that the full-length nsp2 of HP-PRRSV can synergize with SP in homologous protection toward inducing sterilizing immunity for viral clearance.

The effect of SP and nsp2 on humoral immunity

To investigate the status of humoral immunity induced by the chimeric viruses, we collected serum from the piglets at indicated time points for analyzing the level of PRRSV

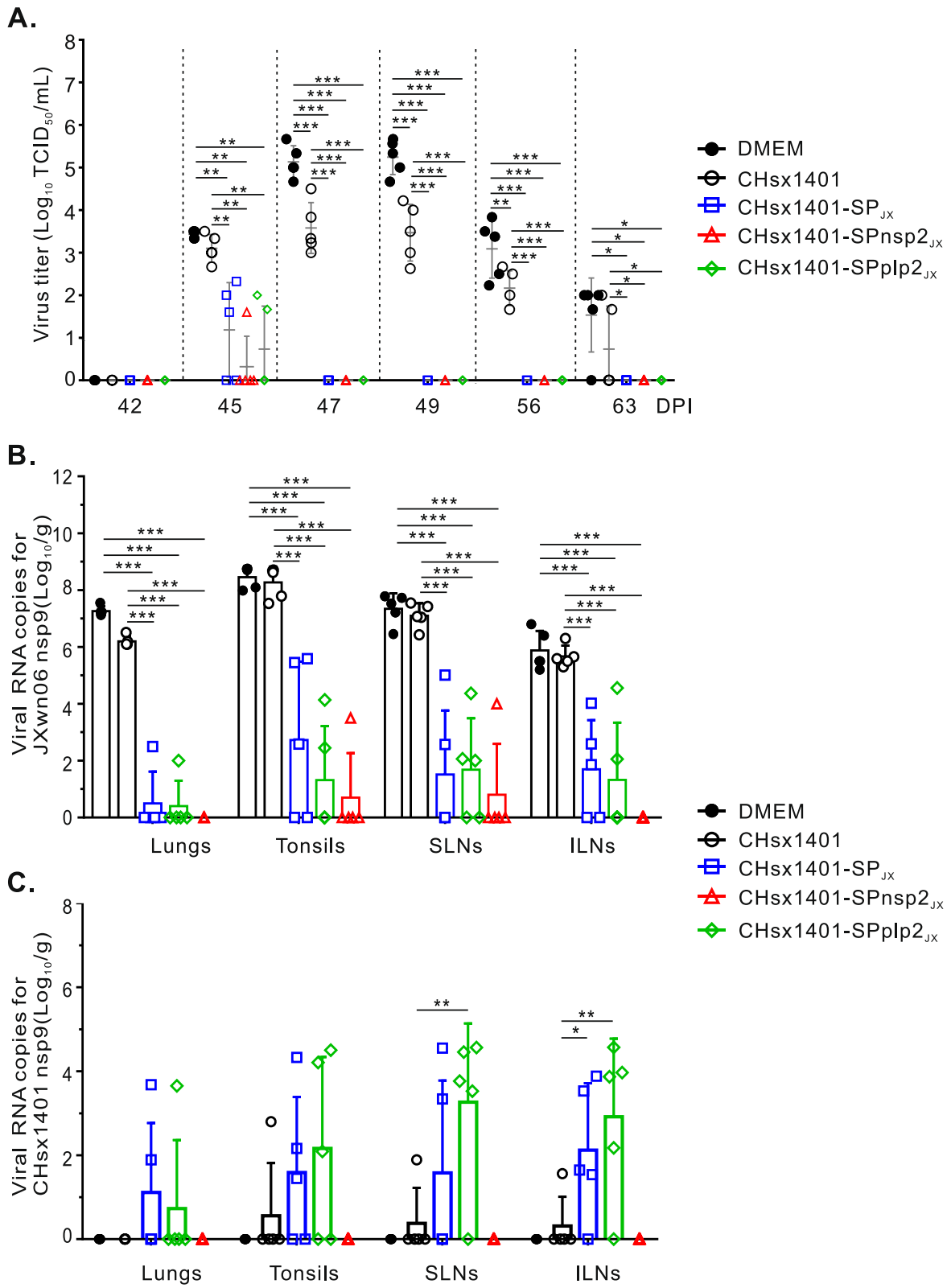

Fig. 6 HP-PRRSV nsp2 promotes viral clearance in vivo. **A** The blood viral load at indicated time points post-infection determined by endpoint dilution assay in MARC-145 cells. **B**, **C** Viral load in lungs and secondary lymphoid tissues by absolute qPCR at 21 d post-challenge. The abundance of viral genome RNA in tissues were determined with the primers targeting the nsp9 coding region. Error bars represent standard deviation (SD). Asterisks mean the statistical difference (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$)

antibodies by IDEXX HerdChek PRRS X3 ELISA kit. The PRRSV antibodies of all piglets became positive at day 10 after immunization (Fig. 7A), while a relatively delayed, low level of antibodies to PRRSV could be discerned for the group CHsx1401. In contrast, the SP exchange significantly improved the antibodies responses (Fig. 7A), suggesting antigenic difference of structural proteins between the two lineages. Interestingly, additional swapping of nsp2 or PLP2 did not change the antibody responses; the CHsx1401-SP_{JX} group showed comparable antibody level as to CHsx1401-$SPnsp2_{JX}$, suggesting that the SP region is the major determinant for the antibody responses. Following challenge, all groups of piglets showed a quick rise of antibodies, despite that the group CHsx1401-$SPnsp2_{JX}$ mounted an only limited viremia.

We next tested the induction of neutralizing antibodies (NA) at 42 and 63 days post infection, respectively. At 42 days post infection, all chimera induced a low level of NA to JXwn06 (Fig. 7B, right panel), but not to CHsx1401 (Fig. 7B, left), whereas CHsx1401 developed low level, specific NA to itself (Fig. 7B, left), but not to JXwn06 (Fig. 7B, right), suggest a significant antigenic difference between CHsx1401 and JXwn06. At 63 DPI (following challenge), the NA level increased a little bit, but the overall trend did not change (Fig. 7C). Overall, the mutant CHsx1401-$SPnsp2_{JX}$ that mounted only limited viremia induced higher level of NA, but not statistically significant from the other two chimera groups. Considering the overall low level of NA, it is likely that the cell-mediated immunity plays a much more important role against the challenge by JXwn06. On the other hand, the fact that the level of antibody and NA to JXwn06 were further improved after JXwn06 challenge suggest that the low level of viremia of pigs immunized with the chimeric viruses is not due to the failed challenge, but rather attributed to the immune clearance.

Discussion

PRRSV is notoriously known for its rapid evolution and genetic heterogeneity, leading to limited cross-protection against heterologous strains [15, 51, 52]. The replicase nsp2 represents the fastest evolving region of PRRSV genome, but whether it has a role in induction of protective immunity have remained unclear. Our work here showed that the nsp2 contributes to induction of NA via the PLP2 domain and its genetic variation can reduce the capacity of NAs. In addition, the SP region is sufficient to provide clinical protection, whereas HP-PRRSV nsp2 could corroborate this effect by reducing the lung injury and viral tissue load of both vaccine and the incoming challenge virus. The findings provide important insight into understanding the mechanism of PRRSV protective immunity and vaccine development. The relative significance and insight of these findings are discussed below.

One critical challenge facing the PRRSV scientific society is to develop an efficacious vaccine against heterologous strains. The prerequisite for this goal is to understand the basic components necessary for inducing a good protective immunity against a homologous strain. By construction of chimeric mutants, the past studies have identified minor structural proteins (GP2-4) and major structural proteins (GP5 and M) as important protective antigens [35, 38, 53, 54]. In this study, we used the similar approach but with two genetically more distant viruses (lineage 8 and lineage 1) to address this question. The fact that immunization with a lineage 1 virus did not provide observable beneficial protection (e.g., clinical sign, body weight gain, tissue injury, etc.) against the challenge by the lineage 8 strain JXwn06 highlights the true heterologous nature of the two strains. Interestingly, exchange of the SP region alone is sufficient to confer clinical protection against the challenge by JXwn06, suggesting that SP is a critical determinant for induction of homologous protective immunity and that the SP genetic variation is critical for evasion of a preexisting immunity conferred by a NADC30-like strain (CHsx1401). Future studies may be directed to dissect the individual role of the major envelope proteins and the minor glycoproteins in induction of protective immunity.

Our studies also unexpectedly revealed a role for HP-PRRSV nsp2 in protective immunity. Swapping of the nsp2 region led to faster viremia clearance, significant reduction of viral tissue load as well as the lung lesions. This result is consistent with our recent finding that HP-PRRSV nsp2 is a stronger inducer or activator of inflammation and host immune responses [48]. The faster viral clearance correlates with a better activated host immunity. Quite amazingly, the vaccine virus CHsx1401-$SPnsp2_{JX}$ was barely detectable in lung and secondary lymphoid tissues such as tonsil, SLN, and ILN. So was for the challenge virus JXwn06. This result is on the way toward a sterilizing immunity. Thus, our results establish nsp2 as a potentially critical component of the mosaic of the protective viral antigens and provide great insights into future vaccine design by activating sterilizing immunity. However, whether nsp2 acts merely on its own or work in coordination with SP requires intensive investigations in the future. Another salient finding of this report is the discovery of nsp2 PLP2 domain as the target neutralizing antibodies. This finding expands the targets of neutralizing antibodies to 6 viral proteins, including GP2, GP3, GP4, GP5, M and nsp2. Although the PLP2 domain is relatively conserved, especially about its catalytic residues, across various PRRSV strains, the difference is quite substantial; JXwn06 and CHsx1401

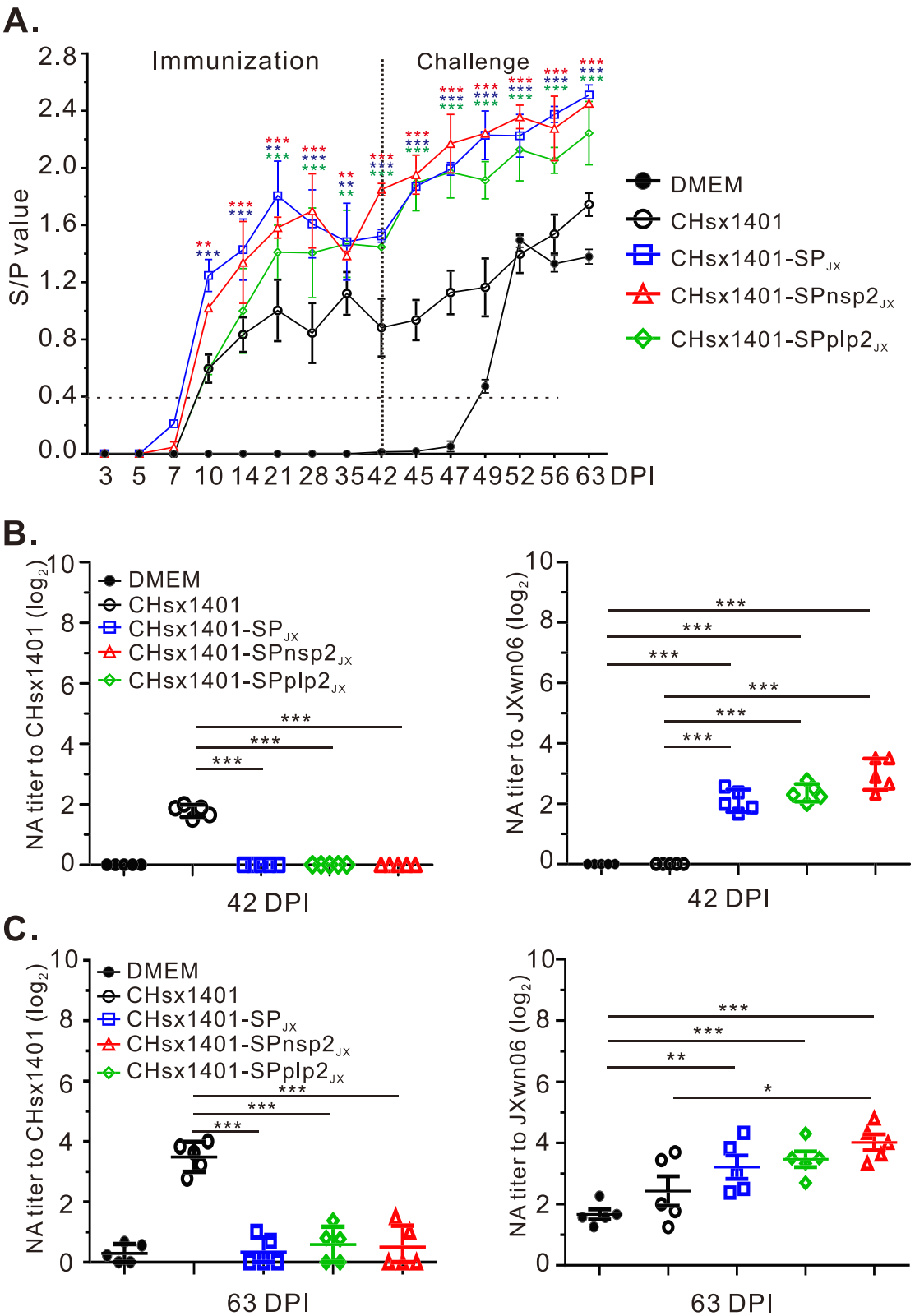

Fig. 7 The effect of exchange of SP and nsp2 on induction of humoral immunity. **A** The serum antibody dynamics from infected piglets by an IDEXX HerdChek PRRS X3 ELISA kit. S/P ≥ 0.4 was considered seropositive. **B** The serum NA titer at 42 DPI titrated in MARC-145 cells. **C** The serum NA titer at 63 DPI titrated in MARC-145 cells. Error bars represent standard deviation (SD). Asterisks mean the statistical difference between infected groups (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$)

share only about 71.5% identity at amino acid level within the PLP2 domain. Currently, it is not clear whether the protease activity of PLP2 can exert an effect on neutralizing antibodies by proteolytic cleavage that awaits future investigation. The fact that the PLP2 domain exchange alone renders the heterologous virus susceptible to the antisera to a donor strain suggests genetic variation of PLP2 region exerts an effect on the immune escape. Interestingly, in the backbone of CHsx1401-SP_{JX}, additional exchange of PLP2 (CHsx1401-$SPplp2_{JX}$) only did not achieve the same effect as to that by substitution with full-length nsp2 (CHsx1401-$SPnsp2_{JX}$) in terms of reducing viral tissue load. These results suggest that other regions, particularly the middle hypervariable region, likely play an important role in regulating nsp2 function. Previously, PRRSV nsp2 has been found to be incorporated into viral particles [55], although the specific isoform was not defined. In any case, the findings regarding PRRSV nsp2 in NA induction and inflammation and host immune response regulation may partly explain the selective immune pressure on nsp2-coding region.

Materials and methods

Ethics statements

The animal experiments were carried out according to *The Guidelines for the Care of Laboratory Animals,* Ministry of Science and Technology of People's Republic of China, and Laboratory Animal-Requirements of Environment and Housing Facilities (GB 14925–2010, National Laboratory Animal Standardization Technical Committee). The license number associated with this research was AW11402202-2–1 that was approved by the Laboratory Animal Ethical Committee of China Agricultural University.

Cells, plasmids, antisera and viruses

MARC-145 cells (African green monkey kidney epithelial cells, ATCC CRL-12231) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, #12491015) containing 10% FBS (Gibco, #16140071) and penicillin (50 U/mL) & streptomycin (50 μg/mL) at 37 °C with 5% CO_2. The full-length infectious cDNA clone plasmids pWSK-JXwn06 and pWSK-CHsx1401, containing a CMV promoter, hepatitis delta virus ribozyme, SV40 poly(A) signal, and the corresponding viral genome, derived from the Chinese highly pathogenic PRRSV strain JXwn06 (Gen Bank accession no: EF641008) and the NADC30-like PRRSV strain CHsx1401 (Gen Bank accession no: KP861625), have been constructed as previously reported [50]. Wild type JXwn06 F8 were used to for the animal challenge in this study. Preparation of antisera against JXwn06 have been reported previously [40].

Construction of chimeric cDNA clones

The DNA-launched PRRSV full-length infectious clone plasmids pWSK-JXwn06, pWSK-CHsx1401, pWSK-CHsx1401-SP_{JX}, and pWSK-CHsx1401-$SPnsp2_{JX}$ have been described previously [48]. To generate the plasmids pWSK-CHsx1401-$plp2_{JX}$ and pWSK-CHsx1401-$SPplp2_{JX}$, the JXwn06 PLP2 region and the up- and downstream flanking sequences of the CHsx1401 PLP2 region were individually amplified using KOD DNA polymerase (TOYOBO, #KFX-101). By overlapping PCR, the purified products were amplified to generate the JXwn06 PLP2 fragment flanked by the respective up- and downstream sequence of the corresponding CHsx1401 PLP2. The homologous recombination strategy with the ClonExpress MultiS one-step cloning kit (Vazyme, C113-02) was then used to then the infectious plasmids pWSK-CHsx1401-$plp2_{JX}$ and pWSK-CHsx1401-$SPplp2_{JX}$ by inserting back the generated fragment into the backbone plasmids (pWSK-CHsx1401 and pWSK-CHsx1401-SP_{JX}) that were digested with the restriction enzymes *Sgra*I and *Nhel*I. Aimilar strategy was employed to swap the SP regions. Briefly, the PCR product of the CHsx1401 SP region flanked by the corresponding up- and downstream sequence of JXwn06 was cloned into the backbone plasmid pWSK-JXwn06 digested with *Asc*I and *Pac*I to make the plasmid pWSK-JXwn06-SP_{SX}. Likewise, the CHsx1401 PLP2 region was swapped while JXwn06 served as the donor. In this case, the restriction enzymes *Swa*I and *BstB*I were used, and this resulted in the plasmids pWSK-JXwn06-$plp2_{SX}$ and pWSK-JXwn06-$SPplp2_{SX}$. The primers used for making the chimeric plasmids are shown in Table 1.

Recovery and identification of chimeric viruses

The PRRSV infectious clone plasmids were extracted using PureYield™ Plasmid Midiprep System (Promega, #A2492). To rescue the chimeric viruses, MARC-145 cells were transfected with infectious plasmids at a density of approximately 80% using the LTX (Invitrogen, #15338100) in 6-well plates. The viral cytopathic effect was assesed daily, and the whole cell culture was collected at 3–5 days post transfection. The recovered viruses were confirmed by indirect immunofluorescence assay (IFA) using PRRSV N monoclonal antibody (McAb) SDOW17 (Rural Technologies, Inc., Brookings, SD, USA). Finally, the RNAs of chimeric viruses passaged three times were extracted, reversed transcribed, and subjected to PCR and sequencing.

Animal experiments

The piglets of 4 weeks old, were purchased from Beijing Center for SPF Swine Breeding & Management. All

Table 1 Primers used for construction of infectious cDNA clones and viral RNA quantification

Primer name	Sequence (5′-3′)	Usage
UP-PLP2$_{SX}$-1F	AGCTGGTTTAGT**ATTTAAAT**ACCGTCTATGACGTATAGGTGTTG **(*Swa*I)**	Construction of chimeric cDNA clones CHsx1401-PLP2$_{JX}$
UP-PLP2$_{SX}$-1R	TGCTCTCTTTCCGGCACCATACCACTTATGGACGCCAAA	
PLP2$_{JX}$-2F	CATAAGTGGTATGGTGCCGGAAAGAGAGCAAGGAAACCA	
PLP2$_{JX}$-2R	TCGAGACTGAAATTCGCCCAAAGGCTCTTGAGTCACGGG	
Down-PLP2$_{SX}$-3F	CAAGAGCCTTTGGGCGAATTTCAGTCTCGAAAAGCGGAG	
Down-PLP2$_{SX}$-3R	CTACAGAGAG**CACCGGTG**TGTGGGGCATCATCAC **(*SgrA*I)**	
UP-PLP2$_{JX}$-1F	CAGAGCTGGTTTAGT**ATTTAAAT**ACCGTCATGACGTATAGGTGT **(*Swa*I)**	Construction of chimeric cDNA clones JXwn06-PLP2$_{SX}$
UP-PLP2$_{JX}$-1R	ACCGTACCACTTATGACTGCCAAATGTTCTCTTCCCAGC	
PLP2$_{SX}$-2F	CATAAGTGGTACGGTGCTGGGAAGAGAACAAGAAAAGCA	
PLP2$_{SX}$-2R	AGGGACCGAGTCCTTCGGAGACCTTTGGGCCACGAC	
Down-PLP2$_{JX}$-3F	CAAAGGTCTCCGCCGAAGGACTCGGTCCCTCTGACCGCC	
Down-PLP2$_{JX}$-3R	GCCTCCCCCTGAAGGC**TTCGAA**ATTTGCCTGATCTTTAGTCCATT **(*Bst*I)**	
UP-SP$_{JX}$-1F	TTACAATGATGCGTTTCG**GGCGCGC**CAGAAAGGGAAAAT **(*Asc*I)**	Construction of chimeric cDNA clones JXwn06-SP$_{SX}$
UP-SP$_{JX}$-1R	TGGGCTGGCATTCTTTGGCACCTC	
SP$_{SX}$-2F	TAGGCCTGAATTGAAATGAAATGGGGGCTTTGCAGAGCA	
SP$_{SX}$-2R	AAGAATGCCAGCCCATCATGCTGGGGGTGACGTCGTGAC	
Down-SP$_{JX}$-3F	GTCACCCCCAGCATGATGGGCTGGCATTCTTTGGCACCTC	
Down-SP$_{JX}$-3R	GGACCATGCCGGCCTTAA**TTAATTT**TTTTTTTTTTTT **(*Pac*I)**	
UP-SP$_{SX}$-1F	CAATGAAGCGTTTCG**TGCGCGCC**AGAAAGGGAAGATTTATAGAGCCA **(*Asc*I)**	Construction of chimeric cDNA clones CHsx1401-SP$_{JX}$
UP-SP$_{SX}$ -1R	TAGACCCCATTTCATTTCAGTTCAAGCCTAAAGTTGGTT	
SP$_{JX}$-2F	TAGGCTTGAACTGAAATGAAATGGGGTCTATGCAAAGCC	
SP$_{JX}$-2R	AAGAATGCCAGCTCATCATGCTGAGGGTGATGCTGTGGC	
Down-SP$_{SX}$-3F	TCACCCTCAGCATGATGAGCTGGCATTCTTGAGACATCC	
Down-SP$_{SX}$-3R	GGGACCATGCCGGCC**TTAATTAA**TTTTTTTTTTTTTTTTTTTTTT **(*Pac*I)**	
Nsp9-CHsx1401-qPCR-F	TTCTGCGGTTCCTTCGCTTG	amplification of CHsx1401 nsp9
Nsp9-CHsx1401-qPCR-R	TCCTCTTTGGTGGCTTCGGA	
Nsp9-CHsx1401-probe	FAM-CCCAAGTTACTTGCCCGC-MGB	
nsp9-JXwn06-F	CTGCGATCGATCCACACCTG	amplification of JXwn06 nsp9
nsp9-JXwn06-R	GCGTGACCAGTAAGTCGTGG	
nsp9-JXwn06-probe	VIC-TTTGCCGCCAATCTTCTT-MGB	

piglets were tested negative for PRRSV, pseudorabies virus (PCV2), porcine circovirus type 2 (PRV), classic swine fever virus (CSFV), and African swine fever virus (ASFV) by commercial ELISA kits and RT-PCR. The animals were accommodated in separate rooms within the animal facility at China Agricultural University (CAU) and given 3 days to acclimate to the surroundings prior to experiments. For the immunization/challenge experiment, pigs were randomly divided into five different groups with 5 pigs in each group (CHsx1401, CHsx1401-SP$_{JX}$, CHsx1401-SPnsp2$_{JX}$, CHsx1401-SPplp2$_{JX}$, and mock). The piglets were intranasally inoculated with the respective viruses at a dose of 2×10^5 $TCID_{50}$, while the mock group were inoculated with DMEM. All piglets were challenged at 42 DPI with JXwn06 at a dose of 2×10^6 $TCID_{50}$ and euthanized at 63 DPI. The animals were monitored daily for clinical signs and rectal temperatures. The comprehensive scoring system was carried out in accordance with the previously described method [56]. The piglets were also weighed to calculate the average daily gain (ADG) every week. The blood samples were collected at indicated time points to investigate viremia using a microtitration infectivity assay as described above [57]. The surviving animals were euthanized to perform gross pathological examinations of lungs. Meanwhile, lungs, tonsils, submandibular lymph nodes (SLNs), and inguinal lymph nodes (ILNs) were collected for histopathological examination by H&E staining. The standard

scoring system of overall level of gross lesions and microscopic pathology have been described elsewhere previously [48, 58]. Specifically, the gross lesions were assessed based on the score of 0 to 100 according to the lesion size of each lung lobe. Each accessory lobes were assigned for 5 points, whereas 27.5 points (15 for dorsal and 12.5 for ventral) were for each of the right and left caudal lobes. This brings up the total points to 100. The microscopic lung lesion scores were blindly evaluated from 0 to 4 according to the severity of the interstitial pneumonia (0, no microscopic lesions; 1, mild; 2, moderate multifocal interstitial pneumonia; 3, moderate diffusive interstitial pneumonia; 4, severe interstitial pneumonia). Meanwhile, the viral RNA of these tissues were quantitated by qPCR through discrimination primers targeted nsp9 between JXwn06 and CHsx1401 (Table 1).

Bacterial expression and purification of PLP2 and its derivatives

The prokaryotic plasmids expressing nsp2(12–323)-strepII, nsp2(12–521)-strepII, nsp2(12–782)-strepII, and nsp9-strepII were constructed by cloning the respective coding region from HP-PRRSV strain JXwn06 into the vector pET-28a (Novagen, Madison, WI, USA) at the enzyme restriction sites of *Nco*I and *Not*I with a strepII tag attached to C-terminus for purification purpose. The primers used for construction are listed in Table 2.

E. coli strain BL21 (DE3) containing the plasmid for nsp2 derivatives were cultured overnight at 37 °C and then inoculated at 1:200 into 500 ml of Luria–Bertani (LB) medium culture. When the bacterial density at 600 nm reached 0.6, protein expression was induced at 16 °C for 18 h by addition of Isopropyl-D-1-thiogalactopyranoside (IPTG) (Sigma, #I6758) at the final concentration 0.1 mM. The cells were harvested by centrifugation at 6000 rpm for 5 min and then resuspended in equilibration buffer (100 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, pH 8.0) with the protease inhibitor cocktail (Sigma, #P8340). The cells were sonicated to clarity at 4 °C. The cell lysates were centrifuged at 12,000 rpm for 30 min. The supernatants were purified by affinity chromatography columns (Smart, #SA053C15) at NGC system with equilibration buffer and elution buffer (100 mM Tris–HCl, 150 mM Nacl, 1 mM EDTA, 2.5 mM desthiobiotin, pH 8.0). The outflow was concentrated by 10 K centricon tubes (Millipore, #UFC9010), and the purity of the proteins was examined on 10% SDS-PAGE gel.

Antibody absorption assay

Purified proteins were incubated with StrepTactin sepharose (GE healthcare, #45002414) at 4 °C overnight on the rocker. Then 200 µL sepharose carrying viral protein were included with 1 mL anti-sera to JXwn06 at 37 °C for 1 h to absorb the antibodies to PRRSV nsp2, followed by centrifugation at 1500 rpm for 2 min to remove sepharoses. After treatment, the serum samples were collected for in vitro neutralization assay.

Serum neutralization assay

Heat inactivated serum samples were diluted at twofold series with DMEM and incubated with an equal volume (50 µL) of the respective virus at an amount of 100 $TCID_{50}$ at 37 °C for 1 h. Afterwards, the mixture was incubated with MARC-145 cells cultured in a 96-well plate for another 1 h at 37 °C. Then the cells were washed three times with PBS, followed by the addition of fresh DMEM media supplemented with 2% FBS. After 24 h, PRRSV-positive cells were detected by IFA with antibodies to PRRSV N protein. The titer of neutralizing antibodies (NA) was calculated using the Reed-Muench method [59].

Measurement of total antibody responses

The total antibody level to PRRSV in the swine sera was measured using an IDEXX HerdChek PRRS X3 ELISA kit, and expresssed as a ratio of sample/positive value (S/P). A ratio of more than 0.4 was considered seropositive.

Quantitative PCR (qPCR)

The viral RNAs were extracted by TRIzol (Thermo Fisher, #15596026) according to the manufacturer's instructions and then reverse transcribed into cDNA by FastKing RT

Table 2 Primers used for construction of plasmids coding for nsp2 derivatives and nsp9

Primer name	Sequence (5′-3′)
nsp2 (aa.12–323/521/782)$_{JX}$-F	ACTTTAGAGGAGATATACCATGGGCGCGACTACTATGGTCGCTCATCACGCT
nsp2 (aa.12–521)$_{JX}$-R	GAACTGCGGGTGGCTCCAGGTACCTGCCGACCCACTCAAAGGTGTCATCAG
nsp2 (aa.12–782)$_{JX}$-R	GAACTGCGGGTGGCTCCAGGTACCGCC GACACCACCTGTATCTGCGGGCGG
nsp2 (aa.12–323)$_{JX}$-R	GAACTGCGGGTGGCTCCAGGTACCGCCCAAAGGCTCTTGAGTCACGGG
nsp9$_{JX}$-F	ACTTTAGAGGAGATATACCATGGGCTTTAAACCTGCTAGCCGCCAGCGG
nsp9$_{JX}$-R	GAACTGCGGGTGGCTCCAGGTACCGCCCTCATGATTGGACCTGAGTTTTTC

kit (TIANGEN, #KR116). To measure the viral tissues load, one pair of discrimination primers and probe were designed by targeting nsp9 according to the sequence of JXwn06 and CHsx1401 for qPCR analysis. Standard curves were generated to quantify the abundance of nsp9 gene by plotting $\log_{10}$ copy number against the cycling threshold (CT) value. The quantitative PCR (qPCR) was carried out by Super Real PreMix (Probe) (TIANGEN, #FP206) in accordance with the guidelines provided by the manufacturer. The PCR reaction and the PCR parameter was set up as described previously [48].

Statistical analysis

The significance of the variability among the groups was determined by a two-tailed unpaired Student's t-test using GraphPad Prism version 7.0 (La Jolla, CA, USA). Data were expressed as means ± standard deviations (SD) and the asterisks indicate the statistical significance: NS, no significance; *, $P<0.05$; **, $P<0.01$ ***, $P<0.001$.

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Authors' contributions

C.K., D.L., Y.H. performed the investigation and data analyses; Y.Z., L.Z, X.G., and X.G. contributed reagents and guidance; C.H., P.G. and J.H. conceived the study, participated in its design and coordination, and drafted the manuscript. All authors read and approved the final manuscript; J.H. and H.Y. provided the funding.

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Availability of data and materials

The datasets in this study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Ethical approval was obtained from the Ethics Committee of the Laboratory Animal Ethical Committee of China Agricultural University (approval no: AW11402202-2–1).

Competing interests

The authors declare that they have no competing interests. Authors Jun Han and Lei Zhou are members of the Editorial Board for One Health Advances, and they were not involved in the journal's review or decisions of this manuscript.

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