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Research paper

Generation of porcine monoclonal antibodies based on single cell technologies

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ABSTRACT

The development of a rapid and efficient system to generate porcine monoclonal antibodies (mAbs) is an important step toward the discovery of critical neutralizing targets for designing rational vaccines against porcine viruses. In this study, we established a platform for producing porcine mAbs based on single cell technologies. First, we singled out an optimal donor from 507 pigs based on serum antibody neutralizing activity against porcine reproductive and respiratory syndrome virus (PRRSV). After identifying the contribution of IgG to the neutralizing activity, single $CD45R^+IgG^+Ag^+$ B cells were sorted from peripheral blood mononuclear cells (PBMCs). Single B cell RT-PCR was performed using primers designed to cover the germline repertoire of the porcine VH/VL gene segments. Paired VH/VLs were cloned into a eukaryotic expression vector and transfected into 293T cells. We demonstrate that full-length porcine mAbs were produced, and antigen-specific mAbs were obtained after further validation. The approach reported in this study can be applied to generate porcine mAbs against any given antigen and may help with the screening of neutralizing antibodies against porcine pathogens.

1. Introduction

The swine industry is an important component of agricultural production worldwide (Rodríguez et al., 2013). However, porcine viruses, such as porcine reproductive and respiratory syndrome virus (PRRSV) (Nathues et al., 2017), foot-and-mouth disease virus (FMDV) (Jamal and Belsham, 2018), and African swine fever virus (ASFV) (Sanchez-Cordon et al., 2018), have become global concerns in this industry, causing enormous economic losses. Vaccination is the most effective and low-cost method for preventing viral infection, yet the development of efficient vaccines against many severe porcine viruses have had limited success. Antibodies are one of the most pivotal molecules in the immune system responding to vaccines. In general, the overall serum binding activity and/or neutralization titers of antibodies to vaccines or targeted viruses are measured; however, the overall serum antibody titer provides very little information about the critical components of the host humoral immune system that defend against viral infection. Nonetheless, for some viruses, such as PRRSV, antigen-binding antibodies can even contribute to viral infection through antibody-dependent enhancement (ADE) (Yoon et al., 1997), and the exact mechanism of ADE cannot be elucidated at the serum antibody or polyclonal antibody level. Therefore, to facilitate efficient vaccine design, it is essential to reveal crucial aspects of the porcine immunological response, such as key antigen epitopes for virus binding activity, neutralizing or even broadly neutralizing activity, as well as ADE, at the monoclonal antibody level. To achieve this goal, effective methods for recovering natural porcine monoclonal antibodies *in vitro* must be developed.

Thus far, the generation of porcine antibodies has been accomplished mainly by employing phage display (Li and Aitken, 2004; Muraoka et al., 2014) or producing chimeric antibodies (Chen et al., 2018), though a few porcine antibodies against PEDV were recently produced based on single cell RT-PCR (Fu et al., 2017). Compared with the slow development of porcine antibodies, significant progress on human monoclonal antibodies has been mainly due to substantial advances in single B cell antibody technologies (Tiller, 2011; Tiller et al., 2008). Many potent human monoclonal antibodies, including broadly neutralizing antibodies (Dey et al., 2009; Evans et al., 2015; Ouisse

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et al., 2017), have been discovered. Thus, it is important to learn from human antibody technologies and utilize existing data regarding porcine immunoglobulins (Igs) to fuel porcine monoclonal antibody production.

As reported previously, porcine Ig heavy (H) chain variable domains are randomly assembled from the following: approximately 25 variable (V) gene segments (Sun et al., 1994) belonging to only one gene family (Eguchi-Ogawa et al., 2010); 4 diversity (D) gene segments, of which one-half (IGHD1 and IGHD2) are functional; and 5 joining (J) gene segments, among which only one (IGHJ5) is functional (Butler et al., 1996; Sun and Butler, 1996). For light (L) chain genes, the porcine kappa (κ) locus contains 14 Vκ genes grouped into 5 IGKV families, and only 9 Vκ genes belonging to 2 IGKV families (IGKV1 and IGKV2) appear to be expressed (Schwartz et al., 2012a). The lambda (λ) locus contains 23 Vλ genes belonging to multiple IGLV families (Schwartz et al., 2012b), among which expression is dominated by only 2 families, IGLV3 and IGLV8 (Wertz et al., 2013). Additionally, 4 IGLJ genes exist, of which only 2 are functional (Schwartz et al., 2012b); there are 5 IGKJ genes (Butler et al., 2004). Although such a basic understanding of the genomic and expressed porcine antibody repertoire is indispensable, sequence analysis alone does not allow for predictions of antibody reactivity. To date, antigen-specific human single B cells have been efficiently isolated by flow cytometry based on rationally designed probes, such as the computer-assisted designed probe pair of RSC3 and ΔRSC for sorting functional HIV-specific antibody-expressing B cells (Wu et al., 2010). Many antibodies recovered from these cells have been demonstrated to bind specifically to corresponding antigens, and some even show broadly neutralizing activities, such as antibodies BG18 and IOMA (Freund et al., 2017; Gristick et al., 2016). Theoretically, single cell-based antibody-generating technologies are species independent. Recently, a PEDV-S-specific antibody was isolated from a single B cell from an immunized pig (Fu et al., 2017). However, no systematic protocol has been developed for porcine monoclonal antibody production based on single B cell technologies.

In this study, we established a platform for porcine antibody production including processes from optimal pig donor screening to functional antibody identification. We compared five staining strategies for single porcine B cell sorting and designed and validated two rounds of RT-PCR primers for Ig gene cloning from single porcine B cells. Finally, we verified that the recovered porcine monoclonal antibodies indeed show specific antigen-binding activities.

2. Materials and methods

2.1. Serum sample source

Serum samples were collected from commercial pigs (205 Landrace pigs, 61 Yorkshire pigs, and 241 crossbreds of Landrace and Yorkshire pigs) at XINDAMUYE Company (Luoyang, Henan, China) and NINGHEHUIKANG Company (Tianjin, China). To obtain functional sera, especially those with neutralizing or even broadly neutralizing antibodies, relatively long-living sows (mainly 1–2 years old when sampled) were selected for serum collection because broadly neutralizing antibodies are commonly induced in patients infected with HIV for a relatively long time (generally 2–4 years) (Klein et al., 2013; Mikell et al., 2011; Sather et al., 2009).

2.2. Cells and viruses

Marc145 cells, a monkey kidney cell line that is highly susceptible and permissive to PRRSV-2 infection (Kim et al., 1993), were obtained from Prof. Wenhai Feng's laboratory. The 293 T cell line was preserved in our laboratory. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin and cultured in a humidified incubator at 5% CO_2 and 37 °C.

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood harvested from porcine auricular veins by density gradient centrifugation with Lymphocyte Separation Medium, as described (Solarbio, Beijing, China), and then frozen in FBS supplemented with 10% dimethyl sulfoxide (DMSO) (Sigma, St. Louis, Missouri, United States).

Five PRRSV-2 strains were used: 1) CH-1a (GenBank accession no. AY032626), the first type 2 PRRSV strain isolated in China; 2) HV (GenBank accession no. JX317648); 3) JXA1 (GenBank accession no. EF1122445); 4) WUH3 (GenBank accession no. HM853673.2); and 5) XH-GD (GenBank accession no. EU624117.1). Both CH-1a and HV were gifts from Prof. Wenhai Feng of China Agriculture University; JXA1 was a gift from Prof. Kegong Tian of the National Research Center for Veterinary Medicine; WUH3 was a gift from Prof. Shujun Zhang of Huazhong Agriculture University; and XH-GD was a gift from Prof. Guihong Zhang of South China Agricultural University. The PRRSV strains were propagated in Marc145 cells and preserved at −80 °C. Viral titers were determined and denoted as the 50% cell culture infective dose ($TCID_{50}$)/mL, as calculated using the Reed-Muench method (Reed and Muench, 1938).

2.3. Virus neutralization (VN) assay

The measurement of virus neutralizing activity in serum (VN) was performed according to a previously described method, with slight modifications (Benfield et al., 1992). Briefly, PRRSV at approximately 200 $TCID_{50}$, diluted in 100 μL of tissue culture medium, was incubated with 100 μL of 1:2 serial dilutions of pig serum (with an initial dilution of 1:16) in a 96-well tissue culture plate. Following incubation at 37 °C and 5% CO_2 for 1 h, the samples were transferred to a 96-well tissue culture plate containing confluent Marc145 cells. The cells were incubated for 3 days and then examined for the cytopathic effect (CPE) by an indirect immunofluorescence assay. The results are reported as the VN titer, which was determined to be the reciprocal dilution ratio of the sample at which 50% inhibition in the PRRSV-induced immunofluorescence was observed.

2.4. Indirect immunofluorescence assay

An indirect immunofluorescence assay was performed to detect PRRSV infection in Marc145 cells. Cells were fixed with methanol for 10 min at 4 °C, washed with phosphate-buffered saline (PBS), and then blocked with 1% bovine albumin V in PBS for 30 min. The cells were then incubated with the anti-PRRSV N protein mAb SDOW17 (1:1,000, RTI, Alachua, Florida, United States) and washed with PBS. Next, the cells were incubated with an Alexa Fluor® 488-conjugated goat anti-mouse IgG (H + L) antibody (1:1,000, Beyotime, Shanghai, China) for 1 h at 37 °C, and N protein levels were assessed using a fluorescence microscope.

2.5. Expression and purification of PRRSV structural proteins

The DNA coding sequences of the PRRSV GP2, GP3, GP4, GP5, and M proteins were amplified by RT-PCR using PRRSV JXA1 cDNA as a template. In addition, the coding sequence of the decoy epitope (A epitope) in ORF5 was replaced with the SGSG coding sequence by overlap PCR to form the GP5(ΔA) coding sequence. Each protein-coding sequence was cloned into the prokaryotic expression vector pET-28(+) for expression with a 6× His tag fused to the C-terminus of each protein. Each protein was expressed in *E. coli* BL21 competent cells and purified using Ni-NTA agarose (Qiagen, Venlo, Netherlands). For antigenic staining of B cells, a portion of each purified protein was biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Thermo, Waltham, Massachusetts, United States).

2.6. Western blotting

Proteins (i.e., porcine antibodies or PRRSV structural proteins) were collected, and their concentrations were measured using the bicinchoninic acid (BCA) assay (Beyotime). Equal amounts of protein from each sample were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes, which were blocked with 5% skim milk in TBST (Tris-buffered saline, 0.1% Tween 20) at room temperature for 1 h. For porcine IgG detection, a goat anti-pig IgG (Fc):HRP (1:10,000, BIO-RAD, Hercules, California, United States) was used to detect the heavy chain. To detect the lambda light chain, a mouse anti-pig Ig lambda light chain Ab (1:5,000, BIO-RAD) was used as the primary antibody, and a goat anti-mouse IgG (H + L):HRP (1:10,000, ZSGB-BIO, Beijing, China) was used as the secondary antibody. For antigen-specific porcine IgG detection, the blocked membrane was incubated with the tested porcine IgG before the anti-pig IgG antibodies mentioned above were applied. Each incubation with antibody was for 1 h at room temperature, followed by washes with TBST before incubation with another antibody or visualization by chemiluminescence.

2.7. Flow cytometry

Porcine PBMCs were centrifuged to remove the culture medium and resuspended in 100 μL of FACS buffer (PBS containing 2% FBS). After washing twice with FACS buffer, the cells were suspended in 50 μL of FACS buffer and stained on ice with mouse anti-pig CD45RA:FITC (1:200, AbD Serotec, Raleigh, North Carolina, United States), mouse anti-pig CD45RA:RPE (1:100, BIO-RAD), goat anti-pig IgG (Fc):FITC (1:1,000, BIO-RAD), mouse anti-bovine CD21:RPE (1:50, AbD Serotec), and/or goat anti-pig IgM:FITC (1:50, AbD Serotec) for 30 min and/or with antigens conjugated to biotin in the first incubation. Two washes with FACS buffer were performed before staining with mouse anti-biotin:APC (1:200, Miltenyi Biotec, San Diego, California, United States) as the secondary antibody. These antibodies and/or antigens were combined according to five different staining strategies (Table 1). After antibody incubation, two additional FACS buffer washes were conducted, and the cells were suspended in 200 μL of sterile PBS for detection or sorting by flow cytometry.

2.8. Single porcine B cell RT-PCR and IgV gene amplification

Single cell RT-PCR was performed according to a previously reported method, with slight modifications (Tiller et al., 2008). For single cell RT-PCR, single cells were sorted according to the flow cytometry procedure described above into 96-well PCR plates containing 4 μL/well ice-cold 0.5 × PBS with 10 mM DTT (Invitrogen, Carlsbad, California, United States) and 8 U RNAsin (Promega, Sunnyvale, California, United States). The plates were sealed with film and immediately frozen on dry ice before storage at −80 °C.

Before performing RT-PCR, cDNA was synthesized in the original 96-well sorting plate. In addition to the 4 μL/well mentioned above, a reaction buffer containing nuclease-free water, 150 ng of random hexamer primer (pd(N) 6) (GE Healthcare, Chicago, Illinois, United States), 0.5 mM dNTP Mix (Sigma), 10 mM DTT (Invitrogen), 0.5% v/v Igepal CA-630 (Sigma), 8 U RNAsin (Promega), 50 U Superscript® IV reverse transcriptase (Invitrogen), and 1 × SSIV buffer was added to each well (10 μL/well). Reverse transcription (RT) of total RNA was carried out using the following protocol: 42 °C for 10 min, 25 °C for 10 min, 50 °C for 60 min, and 94 °C for 5 min. Afterwards, the cDNA was stored at −20 °C.

Heavy and light (κ or λ) chain V gene transcripts were amplified independently by nested PCR in 96-well plates in a total volume of 40 μL per well. The first round of PCR was performed using 3.5 μL of cDNA as the template; the second round of PCR was performed with 3.5 μL of the unpurified first PCR product as the template. In addition to the template, each well contained an extra 20 nM of each primer or primer mix, 300 nM of each dNTP (Sigma), 1.2 U HotStar® Taq DNA polymerase and the corresponding buffer (Qiagen); all of these components were mixed in nuclease-free water. Each round of PCR was performed for 50 cycles at 94 °C for 30 s, 58 °C for 30 s, 72 °C for 55 s (1^{st} PCR) or 45 s (2^{nd} PCR).

2.9. Porcine IgV gene analysis and recombinant antibody expression

The PCR products for porcine IgV genes were purified and cloned into pMD19-T for sequencing. The sequences of porcine IgV regions were analyzed for IGHV, IGKV and IGLV gene subfamily distribution and for their CDR3 length using IMGT/V-QUEST (http://www.imgt.org) from IMGT® (Brochet et al., 2008) and the international ImMunoGeneTics information system® (Lefranc et al., 2015).

To construct a porcine monoclonal antibody, genes encoding the full-length porcine CL (Cκ or Cλ) and CH of IgG1 were cloned and ligated seamlessly to VL (Cκ or Cλ) and VH. For porcine antibody expression, eukaryotic expression plasmids were constructed and transfected into 293 T cells, and cell culture supernatants were collected for detection.

2.10. Porcine antibody purification

Cell culture supernatants (for recombinant antibodies) or pig sera (for serum antibodies) were purified using Protein G magnetic beads (NEB, Ipswich, Massachusetts, United States) according to the manufacturer's instructions. Briefly, 20 mL of cell culture supernatant or 20 μL of pig serum (diluted to 1 mL with binding buffer before incubation with beads) was incubated with 25 μL of Protein G magnetic beads overnight at 4 °C with rotation. The supernatants were removed after the beads were pulled to the side of the tube using a magnetic field. After two rounds of washing with 1 mL of binding buffer, the antibodies were eluted with 50 μL aliquots of 0.25 M glycine (pH 2.5). Each elution fraction was collected and neutralized by the addition of 20 μL of 1 M Tris (pH 9.0). All elution fractions were stored at 4 °C.

2.11. Antigen-specific enzyme-linked immunosorbent assay (ELISA)

Briefly, 96-well plates were coated with purified PRRSV structural

Table 1
Five different staining strategies for flow cytometry.

Strategy	Antibody					
	Mouse anti-pig CD45RA (FITC)	Mouse anti-pig CD45RA (RPE)	Goat anti-pig IgG (Fc) (FITC)	Mouse anti-bovine CD21 (RPE)	Goat anti-pig IgM (FITC)	Mouse anti-biotin (APC)
$CD45R^+Ag^+$	+					+
IgG^+Ag^+			+			+
$CD45R^+IgG^+Ag^+$		+	+			+
$CD45R^+CD21^-Ag^+$	+			+		+
$CD21^-IgM^-Ag^+$				+	+	+

protein at 200 ng/well in a carbonate-bicarbonate coating buffer (pH 9.6) at 4 °C overnight. The plates were blocked at 37 °C for 1 h with PBST containing 1% bovine serum albumin (BSA). The porcine antibodies for testing were added to the well and incubated for 1 h at 37 °C. After washing, the wells were incubated with goat anti-pig IgG (Fc) Ab:HRP (1:10,000, BIO-RAD) for 1 h at 37 °C followed by washing before the reaction substrate TMB was added, followed by incubation in the dark at room temperature for 30 min; the reaction was stopped with 2 M H_2SO_4. The optical density of each well was measured at 450 nm (OD_{450}) using a microplate reader (Thermo). Statistical presentations were generated with GraphPad Prism 5 (San Diego, California, United States).

3. Results

3.1. Screening for optimal pig donors for B cell sorting

To select an optimal pig donor for PBMC sampling, we sought to screen pig sera with broadly neutralizing activity against PRRSV. The pig serum panel included 507 samples from mainly long-living sows from different pig farms. The PRRSV strains used for screening included one traditional strain, CH-1a, and four highly pathogenic strains, WUH3/XH-GD/JXA1/HV. The screening was performed in two rounds. First, all 507 serum samples were examined for neutralization against only the traditional PRRSV strain CH-1a. The neutralization results are summarized in Fig. 1.

To screen sera for potent neutralizing activity, only those that showed a 50% inhibitory concentration (IC_{50}) ≥ 32 (6.71% of all samples) were subjected to further screening for neutralization breadth using the WUH3/XH-GD/JXA1/HV PRRSV strains. The results of the second round of screening are summarized in Table 2. We defined sera that showed IC_{50} titers ≥100 to at least 4 different PRRSV strains tested in our study as "elite neutralizers". As shown in Table 2, 4 serum samples (0.79% of all samples), #44, #94, #309 and #417, were considered elite neutralizers, among which #94 showed the best neutralizing activity and was further investigated in-depth.

3.2. Attribution of serum antibody neutralizing titers to IgG

To isolate specific antibody-producing B cells, determination of the type of antibodies contributing to the broadly neutralizing activity against PRRSV is required. To confirm whether IgG, which is known to exert the main neutralizing activity in sera, was associated with neutralizing activity against PRRSV, IgG was isolated from the #94 serum sample (Fig. S1) and then subjected to a neutralization assay against CH-1a. The results showed that PRRSV infection was significantly inhibited following IgG treatments (Fig. 2), and the concentration of IgG achieving 50% neutralization against PRRSV strain CH-1a was 0.2 mg/mL.

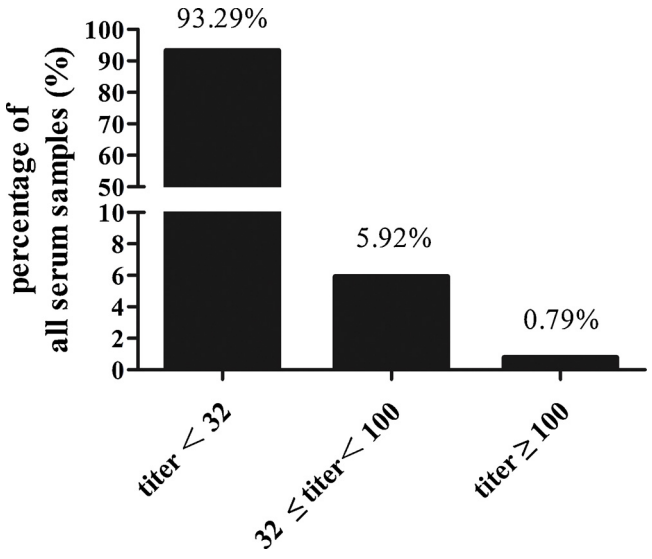

Fig. 1. Serum neutralization titer distribution of samples in the first round of screening. A total of 507 pig serum samples were subjected to a neutralization assay using the PRRSV strain CH-1a. Serum samples were divided into three sections by neutralization titers of 32 and 100. Serum samples with titers greater than 32 were subjected to further screening.

Table 2
Serum neutralization against different PRRSV strains.

Sample	PRRSV strain				
	CH-1a	WUH3	JXA1	XH-GD	HV
#41	43	32	> 256	< 32	40
#43	32	< 32	—[a]	—	89
#44	45	128	> 256	> 256	128
#46	56	< 32	—	—	32
#70	40	100	—	—	45
#76	50	< 32	—	—	< 32
#78	43	78	214	< 32	72
#94	214	> 256	> 256	> 256	128
#96	50	35	200	—	32
#111	45	128	—	—	< 32
#124	79	128	—	—	< 32
#154	45	< 32	—	—	—
#170	45	56	141	32	—
#213	128	50	107	< 32	—
#270	40	50	< 32	—	—
#279	64	128	256	45	—
#306	80	56	< 32	—	—
#309	128	128	158	100	—
#318	47	< 32	—	—	—
#336	45	50	79	< 32	—
#338	50	45	< 32	—	—
#343	95	< 32	—	—	—
#352	40	< 32	—	—	—
#417	128	256	> 256	256	—
#425	< 32	< 32	—	—	—
#431	32	64	> 256	< 32	—
#440	64	< 32	—	—	—
#443	40	< 32	—	—	—
#444	64	< 32	—	—	—
#477	< 32	< 32	—	—	—
#495	64	< 32	—	—	—
#496	64	89	162	117	—
#503	45	< 32	—	—	—

[a] —indicates that the serum sample was not subjected to a neutralization assay for the corresponding PRRSV strains, as it showed a titer of < 32 for at least one PRRSV strain.

To further examine whether the purified IgG exerts broadly neutralizing activity against PRRSV, two highly pathogenic PRRSV strains, WUH3 and JXA1, were used for the neutralization assay. The purified IgG achieved 50% neutralization against both WUH3 and JXA1 at concentrations of 0.12 mg/mL and 0.08 mg/mL, respectively, indicating that the broadly neutralizing activity of the #94 serum sample was indeed attributed to IgG.

To further examine the stability of the IgG-associated broadly neutralizing activity, sera from the #94 pig collected at four time points (February 2014, September 2014, May 2016, and September 2016) were analyzed. The neutralization results indicated a stable neutralizing activity during the study course (data not shown).

3.3. Correlation of neutralizing activities with the quantities of different antigen-specific IgGs

To isolate antigen-specific antibody-producing B cells, we first sought to identify specific viral proteins that correlated strongly with the broadly neutralizing activity. Therefore, we selected serum samples

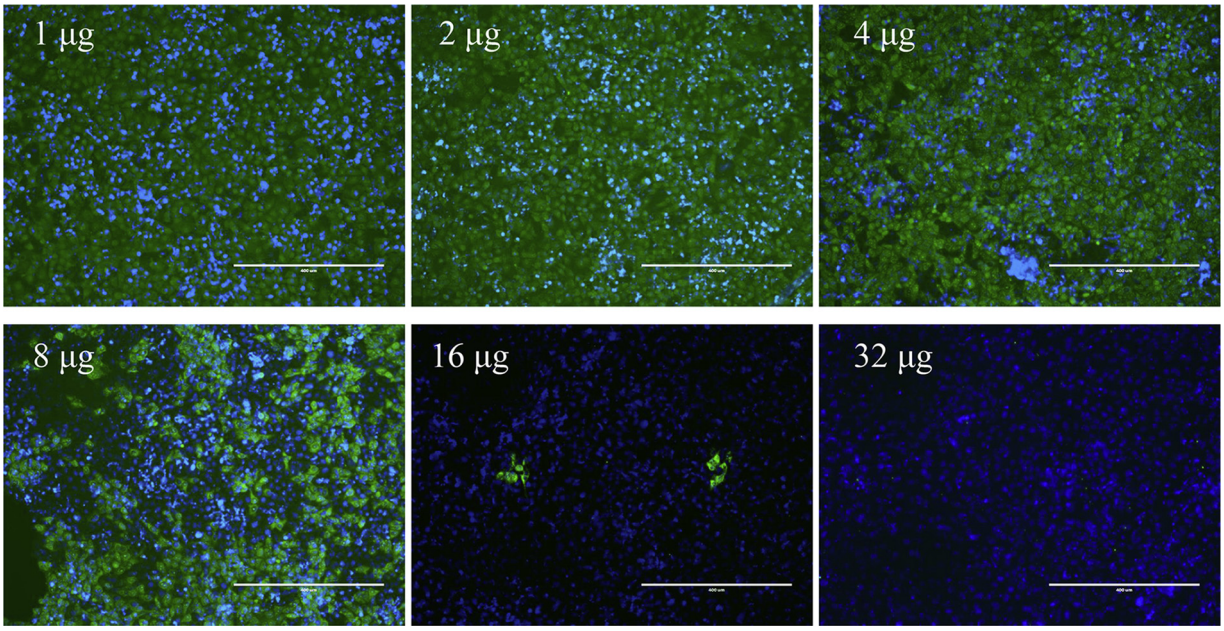

Fig. 2. Detection of purified porcine IgG neutralization of PRRSV. IgG was purified from the #94 serum sample using protein G magnetic beads and used for PRRSV neutralization assays. First, 1, 2, 4, 8, 16, or 32 μg of purified IgG was mixed with 200 $TCID_{50}$ of PRRSV at 37 °C for 1 h before being added to confluent Marc145 cells. Neutralization of three PRRSV strains, CH-1a, WUH3, and JXA1, was examined. A representative result of the neutralization assay for CH-1a is shown.

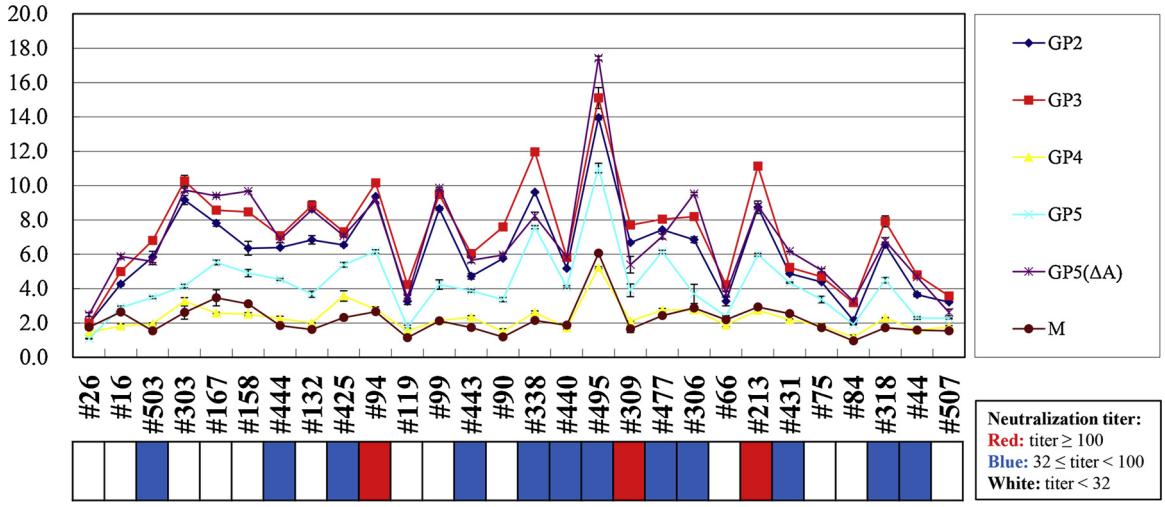

Fig. 3. Correlation of neutralizing activities and quantities of different antigen-specific IgGs. Serum samples, such as #26, #503, and #94, with different ranges of neutralization titers against PRRSV CH-1a were selected to specifically examine quantities of GP2, GP3, GP4, GP5, M, and GP5(ΔA)-specific IgG. In the graph, the X-axis indicates the tested serum samples, and the Y-axis indicates the OD_{450} values when the sera were detected at a dilution of 1:10,000. Under each serum sample, the corresponding boxes are filled with different colors, indicating different ranges of neutralization titers. The boxes filled with red, white, and blue indicate the corresponding sera with IC_{50} titers of ≥100, < 32, and 32–100 (not including 100), respectively (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

with a wide range of neutralization titers against CH-1a PRRSV and measured antigen-specific IgG to examine whether the neutralization activities of different pig sera correlate with the quantity of a particular antigen-specific IgG. First, GP2, GP3, GP4, GP5, M, and GP5(ΔA) (GP5 with epitope A substituted by an irrelevant SGSG motif (Ostrowski et al., 2002)) of PRRSV, all of which have been reported to contribute to the induction of neutralizing antibodies against PRRSV (Ansari et al., 2006; Das et al., 2011; Jiang et al., 2007; Kim and Yoon, 2008; Li and Murtaugh, 2012; Plagemann, 2006), were subjected to prokaryotic expression and purification. The purified proteins were then utilized as antigens for measuring antigen-specific IgGs in selected pig sera (Fig. 3). The results revealed that few antigen-specific IgGs were detected in some sera (such as #26, #119, #66, #84, and #507) with hardly any neutralizing activity. However, no particular antigen-specific IgG was noted for #94, #213, and #309, which showed the highest neutralization titers. In the case of #94, GP2, GP3, and GP5(ΔA)-specific IgGs were detected at higher quantities than GP4, GP5, and M-specific IgGs. Most of the other sera tested showed a similar pattern. Thus, no obvious correlation between serum neutralizing activities and specific antigen-specific IgGs was observed, suggesting that the PRRSV CH-1a-neutralizing activities of serum samples did not depend on the quantity of IgG specific to a particular antigen.

3.4. Isolation of antigen-specific porcine B cells

To determine an optimal strategy for sorting porcine antibody-

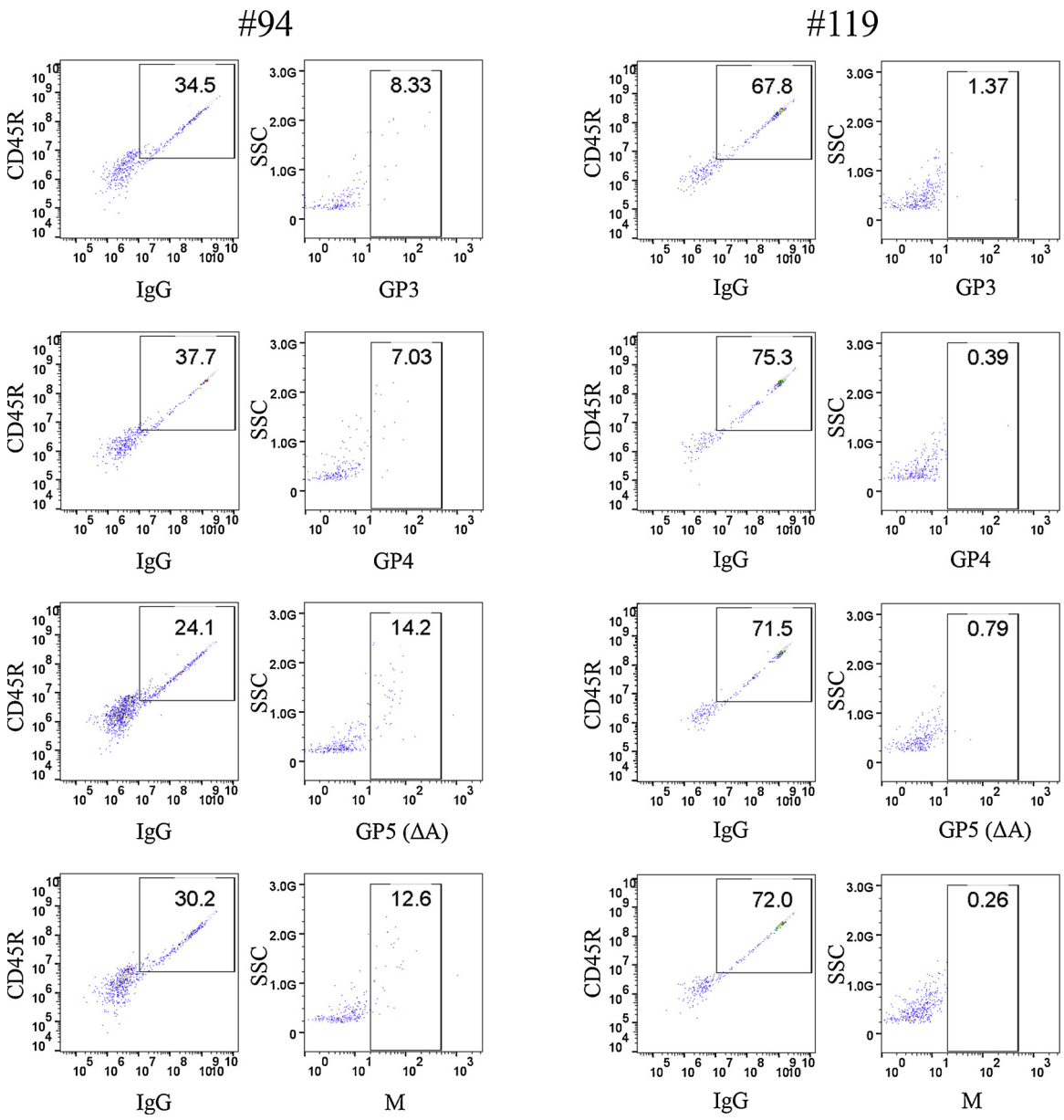

Fig. 4. Detection and isolation of antigen-specific IgG-producing B cells by flow cytometry. #119 PBMCs were used as negative controls to set the gate, and then CDR45$^+$IgG$^+$Ag$^+$ B cells were detected and sorted from #94 PBMCs under the same gating condition. Ag denotes antigen, including PRRSV GP3, GP4, GP5(ΔA), and M proteins conjugated with biotin. SSC, side scatter.

producing B cells, antibodies against porcine IgM, IgG and two surface markers (CD45R, CD21) were selected. For antigen-specific staining, the antigens were first biotinylated, and an anti-biotin antibody conjugated with APC was then utilized. Nevertheless, as there are few available antibodies conjugated with suitable dyes for porcine B cells, we first examined different combinations of the antibodies available for antigen-specific porcine cell staining. The results, shown in Fig. S2, suggested that by employing each of the staining strategies, i.e., CD45R$^+$Ag$^+$, IgG$^+$Ag$^+$, CD45R$^+$IgG$^+$Ag$^+$, CD45R$^+$CD21$^-$Ag$^+$, and CD21$^-$IgM$^-$Ag$^+$, we succeeded in detecting some positive cells among pig PBMCs. Considering that the broadly neutralizing activity was mainly attributed to IgG, we selected the sorting strategy CD45R$^+$IgG$^+$Ag$^+$ for further study.

To isolate antigen-specific antibody-producing B cells from #94 PBMCs, we utilized PBMCs from pig #119 as a negative control to set the gate because very few antigen-specific antibodies were detected for all six tested PRRSV antigens (Fig. 3). The two groups of PBMCs were subjected to flow cytometry for cell sorting using PRRSV GP3, GP4, GP5(ΔA), and M as antigens, which were first biotinylated and then incubated together with PBMCs. Next, the cells were simultaneously stained with anti-pig CD45RA:RPE, anti-pig IgG (Fc):FITC, and anti-biotin:APC antibodies. According to the flow cytometry results, CDR45$^+$IgG$^+$Ag$^+$ B cells were detected and sorted from #94 PBMCs, whereas hardly any positive cells were detected among #119 PBMCs (Fig. 4).

3.5. Primer design and validation

To design primers for cloning porcine IgG-derived variable (V) regions with full coverage, all of the previously reported functional pig V gene families, leader sequences, and IgG constant (C) regions were analyzed. Ultimately, a total of 2 heavy (H) chain primers, 5 kappa (κ) chain primers, and 8 lambda (λ) chain primers were designed for the first-round PCR (Table 3). The first-round PCR forward primers were designed based on leader sequences, including the L-PART1 and L-PART2 segments; the reverse primers were based on porcine IgG H

Table 3
First-round PCR primer list for all functional pig variable genes.

Primer name	Gene family/ family member	Sequence
IGHV		
HV-F1 (L1L2)	IGHV1	5'-TTACAAGGTGTCCAGGGT-3'
HV-R1		5'-GCTCGGGGAAGTAGCTTGAG-3'
IGKV		
KV-F1 (L1L2)	IGKV1	5'-CTGCTCTGGCTCCCAGGTGCCAG-3'
KV-F2 (L1L2)	IGKV2	5'-CCTGCTCTGGGTCCCAGG-3'
KV-F3 (L1L2)	IGKV3	5'-CATCCTGCTGCTCAGTGTCACAG-3'
KV-F4 (L1L2	IGKV5	5'-CTTCCTGCTGCTCTGTGTCTCTG-3'
KC-R1		5'-CGATGGCGGGAAGATGAAGA-3'
IGLV		
LV-F1 (L1L2)	IGLV8	5'-GATCGGGCTCCTCGCTGTC-3'
LV-F2 (L1L2)	IGLV3 (except IGLV3-2)	5'-GGCCTGGACCCCTCTCCTGCT-3'
LV-F3 (L1L2)	IGLV2	5'-CCCGGGCTTGGCTCCTTGTCA-3'
LV-F4 (L1L2)	IGLV3-2	5'-TCCCTCTCCTGCTCGGCCTCC-3'
LV-F5 (L1L2)	IGLV5	5'-CTGCTGATCGTGCGCCTCTGT-3'
LV-F6 (L1L2)	IGLV7-7	5'-GGGCTCCTCTCCTGCTCACAC-3'
LV-F7 (L1L2)	IGLV7-9	5'-TCCTTCTCCTCACTCACTGCC-3'
LC-R1		5'-TCGAGGGCTTGGTGGTCTCC-3'

Table 4
Second-round PCR primer list for all functional pig variable genes.

Primer name	Gene family/ family member	Sequence
IGHV		
HVFR1-F1	IGHV (except IGHV1-1 and IGHV1S6)	5'-GAGG**W**GAAGCTGGTGGAGT-3'
HVFR1-F2	IGHV1-1	5'-GAGGGGCAGCTGGTGGAGTCTG-3'
HVFR1-F3	IGHV1S6	5'-CAGGAGAAGCTGGTGGAGTCTG-3'
HJ-R1	IGHJ1 and IGHJ2	5'-TGAGGAGACGGTGACCAGGA-3'
HJ-R2	IGHJ3	5'-TGAGGAGACGGTGACCTCG-3'
HJ-R3	IGHJ4	5'-CGAGGCGTCGTAGACTAGG-3'
HJ-R4	IGHJ5	5'-TGAGGACACGACGACTTCAAC-3'
IGKV		
KVFR1-F1	IGKV1	5'-GCCATCCAGCTGACCCAGTCTC-3'
KVFR1-F2	IGKV2	5'- GCCAT**Y**GTGCTGACCCAGA-3'
KVFR1-F3	IGKV3	5'-GAAATTGTGCTGACCCAGTC-3'
KVFR1-F4	IGKV5	5'-GAAACAACAGTCACTCAATC-3'
KJ-R1	IGKJ2	5'-TTTGAGCTCCAGCTTGGTCC-3'
KJ-R2	IGKJ4	5'-TTTGATTTCCAGCTT**R**GTCC-3'
KJ-R3	IGKJ1	5'-TTTGAGTTCCAGCTTGGTTCC-3'
KJ-R4	IGKJ3	5'-TTTGGGCTCCACTTTGGTCC-3'
KJ-R5	IGKJ5	5'-TTCAATCTCCACGGATGTCC-3'
IGLV		
LVFR1-F1	IGLV3 (except IGLV3-2 and IGLV3-3)	5'-TCTTCTAAGCTGACTCAGCCC-3'
LVFR1-F2	IGLV8	5'-TCTCAGACTGTGATCCAGGAGC-3'
LVFR1-F3	IGLV2	5'-CAGTCTGCCCTGACTCAGCCC-3'
LVFR1-F4	IGLV3-2	5'-TCCTATGAGGTGACTCAGCC-3'
LVFR1-F5	IGLV3-3	5'-TCCTATGAGCTGACCCAGCCG-3'
LVFR1-F6	IGLV5	5'-CAGGCTGTGCTGACGCAGCCG-3'
LVFR1-F7	IGLV7-7	5'-TCCCAGATGGTGACTCAGG-3'
LVFR1-F8	IGLV7-9	5'-CCAAGCTGTGTGACTCAGGAAC-3'
LJ-R1	IGLJ2 and IGLJ3	5'-GAGGACGGTCAGATGGGTCC-3'
LJ-R2	IGLJ4	5'-GAGGACACTTAGACGGGTCC-3'

R = A + G, **W** = A + T, **Y** = C + T.

chain C regions (CH) or porcine L chain C regions (CL) (Fig. S3). For the second-round nested PCR, we designed 7 H chain primers, 9 κ chain primers, and 10 λ chain primers (Table 4), among which the forward primers were based on the 5′ end sequences of framework region 1 (FR1) of V regions and the reverse primers on the 3' end sequences of the joining (J) regions of V regions (Fig. S3). For both sets of primers, ensuring full coverage over all of the functional V regions was the first design principle, as fewer primers are better for avoiding amplification bias toward specific primers in the primer mix. For example, for amplifying IGHV, one forward primer was sufficient for the first-round PCR because the primer sequence is highly conserved among all of the leader regions from IGHV gene family members; in contrast, more primers were needed for amplifying IGKV and IGLV with multiple gene families to ensure full coverage (Table 3). For the second-round PCR primers, we needed three forward primers to cover all of the IGHV gene family members due to their sequence diversity at the very 5′ end (Table 4). Such considerations were also followed for other V gene family members (Tables 3 and 4).

To validate the primers, we utilized cDNA isolated from pig PBMCs to amplify V regions following two rounds of nested PCR. The V regions amplified by second-round PCR were sequenced, and the results were analyzed using IMGT/V-QUEST (http://www.imgt.org) from IMGT® (Brochet et al., 2008), the international ImMunoGeneTics information system® (Lefranc et al., 2015). As shown in Fig. 5, many family members of the pig H chain V regions (VH) were detected (Fig. 5a); for L chain V regions (VL), only IGKV1 and IGKV2 gene subfamilies (Fig. 5b) and IGLV3 and IGLV8 gene subfamilies (Fig. 5c) were successfully amplified. Furthermore, the lengths of the third complementarity-determining regions (CDR3s) were subjected to statistical analysis (Fig. 5d), revealing that the CDR3 of VH (HCDR3) varies from 4 to 24 amino acids (AA) in length, with an average of 12.6 ± 5.09 AA. The ranges of the detected CDR3 lengths for the κ and λ chain V regions (Vκ and Vλ) were 8–9 and 8–12 AA, respectively, averaging 8.7 ± 0.46 and 9.9 ± 0.84 AA, respectively. These results suggested that our primers were efficient at amplifying pig IgG V regions, though some VLs were not detected, perhaps due to their rare usage.

3.6. Amplification and characterization of V genes from antigen-specific IgG-producing single B cells

To clone V regions from antigen-specific IgG-producing single B cells, we sorted cells from #94 PBMCs into 96-well plates, and a total of 32, 50, 64, or 48 single B cells were isolated for PRRSV GP3, GP4, GP5(ΔA), or M, respectively. Following cDNA synthesis, two rounds of PCR were performed with the primers shown in Tables 2 and 3. For VH and VL (Vκ or Vλ), the products were ˜350 bp, as expected (Fig. S4). Of the 32 wells with GP3-specific single B cells, only 2 showed neither VH nor VL bands, suggesting a positive amplification efficiency of 93.8%. Notably, among the 30 positive cells, only some were detected to contain a pair of VH and VL (Vκ or Vλ). For VH, only 27 wells (84.4% of all 32 wells) showed positive signals; only VL signals were observed for the other 3 wells. For VL, the positive amplification efficiency was low, at only 68.8%. Interestingly, one well showed VH together with both Vκ and Vλ signals, indicating the possibility that a single B cell can simultaneously contain two different types of VLs.

To directly identify cells containing VH and VL pairs, we first amplified VH, and only wells showing positive VH signals were subjected to further amplification for VL. After cloning, 10 colonies for each product from the second-round PCR were chosen for DNA sequencing. For GP3-specific single B cells, 26 (96.3%) of the 27 wells showing positive VH signals also contained pig VH regions. Notably, only 15 clones (55.6%) contained a single VH, 2 of which carried stop codons; 9 clones (33.3%) contained two different VHs, and 2 clones even contained three different VHs. For in-depth analysis, we selected only clones containing 1–2 VHs to analyze VL sequences, and the same analysis was applied to other antigen-specific single B cells. Of all clones containing 1–2 VHs, 80.0% contained a single VH, among which only 6.3% had stop codons; 20.0% contained two different VHs. Additionally, of all the clones containing VL, 90.7% contained a single VL (Vκ or Vλ), among which 7.7% had stop codons; 9.3% contained two different VLs: two different Vκs or one Vκ and one Vλ.

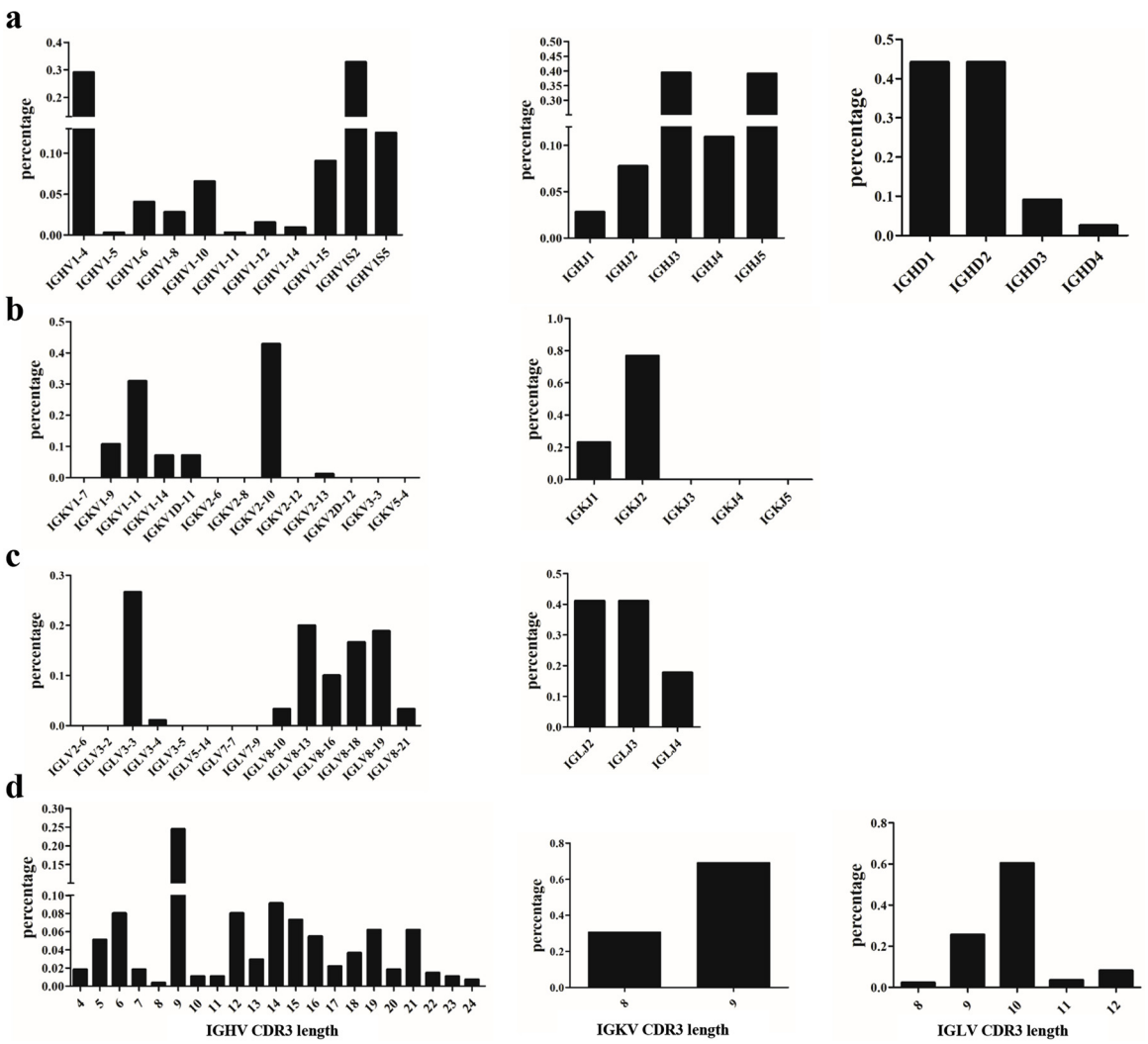

Fig. 5. Analysis of porcine V genes from PBMCs. (a) IGHV, IGHJ, and IGHD gene family members or subfamily distribution. (b) IGKV and IGKJ gene subfamily and/or family member distribution. (c) IGLV and IGLJ gene subfamily and/or family member distribution. (d) CDR3 AA length distributions of VH, Vκ, and Vλ.

3.7. Expression and binding activity verification of porcine monoclonal antibodies

To produce a full-length porcine monoclonal antibody, we first cloned the full-length porcine CL (Cκ or Cλ) and CH of IgG1, which were then ligated seamlessly to VL (Cκ or Cλ) and VH from single B cells. We performed overlapping PCR for seamless ligation of V and C regions. First, each V region was amplified using corresponding primers shown in Table 5, including forward primers containing the same sequence shown in Table 4 and an extra endonuclease site, Kozak sequence and leader (L) sequence at the 5′ end and reverse primers containing a constant sequence at the 5′ end. The PCR products were purified and used as templates coupled with purified CL or CH of IgG1 for overlapping PCR using the corresponding forward primers from Table 5 and reverse primers from Table 4. Both the full-length H and L chains were inserted into the eukaryotic expression vector pBudCE4.1 (Fig. S5a), which was then transfected into 293 T cells for expression. To examine whether full-length porcine monoclonal antibodies were successfully assembled and secreted into the supernatant, one of the expressed antibodies was purified from the supernatant and detected by Coomassie brilliant blue staining and Western blotting (Fig. S5b and c). The results suggested that the porcine monoclonal antibody produced was indeed intact.

To confirm antigen-binding specificity, six porcine monoclonal antibodies were selected due to their high levels in culture supernatants following transient transfection and identified by ELISA as binding to the antigens tested (Fig. 6a and b). Notably, four antibodies, Ab4-33 L, Ab5-5, Ab5-17, and Ab5-32, were found to bind to both GP4 and GP5(ΔA). To further verify this bispecificity, Ab5-32 was chosen for purification and Western blotting analysis for antigen-specific porcine IgG detection, which showed that Ab5-32 did bind to GP4 and GP5(ΔA) but not to BSA (Fig. 6c), suggesting that the observed binding activity was specific.

4. Discussion

In this study, we successfully established a systematic platform for generating porcine monoclonal antibodies based on single cell technologies. The protocol includes 6 major steps: 1) optimal pig donor screening; 2) antigen-specific antibody-producing single B cell sorting from the optimal donor; 3) efficient full-coverage primer design for pig IgV region gene amplification; 4) pig IgV region gene cloning from single B cells; 5) porcine IgG eukaryotic expression vector construction with paired VH and VL regions; and 6) porcine monoclonal antibody expression and function verification. This platform is an important step forward for rapidly producing porcine monoclonal antibodies against porcine viruses, such as PRRSV.

As a long-term goal of discovering broadly neutralizing PRRSV

Table 5
First-round overlapping PCR primers for V regions.

	Primer name	Sequence
Forward primers	**EF1α-L-**	5'-ataagaatGCGGCCGCGCCACCatggagtttcggctgaactgggtggtcttgtttgctctcttacaaggtgtccagggt-
	HVFR1-F1	-GAGG**W**GAAGCTGGTGGAGT-3'
	HVFR1-F2	-GAGGGGCAGCTGGTGGAGTCTG-3'
	HVFR1-F3	-CAGGAGAAGCTGGTGGAGTCTG-3'
Reverse primers	**IGHG1-**	5'-ccgatggggccgtcttggg-
	HJ-R1	-TGAGGAGACGGTGACCAGGA-3'
	HJ-R2	-TGAGGAGACGGTGACCTCG-3'
	HJ-R3	-CGAGGCGTCGTAGACTAGG-3'
	HJ-R4	-TGAGGACACGACGACTTCAAC-3'
Forward primers	**CMV-L-**	5'-gcGTCGACGCCACCatggacatgagggcccccgtgcagctcctcggcctcctgctgctctggctcccaggtgccaggagt-
	KVFR1-F1	-GCCATCCAGCTGACCCAGTCTC-3'
	KVFR1-F2	5'- GCCAT**Y**GTGCTGACCCAGA-3'
	KVFR1-F3	5'-GAAATTGTGCTGACCCAGTC-3'
	KVFR1-F4	5'-GAAACAACAGTCACTCAATC-3'
Reverse primers	**IGKC-**	5'-gacggatggcttggcatcagc-
	KJ-R1	-TTTGAGCTCCAGCTTGGTCC-3'
	KJ-R2	-TTTGATTTCCAGCTT**R**GTCC-3'
	KJ-R3	-TTTGAGTTCCAGCTTGGTTCC-3'
	KJ-R4	-TTTGGGCTCCACTTTGGTCC-3'
	KJ-R5	-TTCAATCTCCACGGATGTCC-3'
Forward primers	**CMV-L-**	5'-gcGTCGACGCCACCatggcctggacggtgcttctgatcgggctcctcgctgtcggctcaggggtggat-
	LVFR1-F1	-TCTTCTAAGCTGACTCAGCCC-3'
	LVFR1-F2	-TCTCAGACTGTGATCCAGGAGC-3'
	LVFR1-F3	-CAGTCTGCCCTGACTCAGCCC-3'
	LVFR1-F4	-TCCTATGAGGTGACTCAGCC-3'
	LVFR1-F5	-TCCTATGAGCTGACCCAGCCG-3'
	LVFR1-F6	-CAGGCTGTGCTGACGCAGCCG-3'
	LVFR1-F7	-TCCCAGATGGTGACTCAGG-3'
	LVFR1-F8	-CCAAGCTGTGTGACTCAGGAAC-3'
Reverse primers	**IGLC-**	5'-cgtgggagcggccttgggctg-
	LJ-R1	-GAGGACGGTCAGATGGGTCC-3'
	LJ-R2	-GAGGACACTTAGACGGGTCC-3'

R = A + G, **W** = A + T, **Y** = C + T.

antibodies, we selected commercial pigs that have been infected with wild PRRSV strains for screening, because they were more likely to develop potent neutralizing antibodies after a long period of virus-antibody mutually evolution. In our study, we screened a total of 507 pigs, 6.71% of which showed neutralization titers of ≥ 32 to PRRSV strain CH-1a, and 0.79% were elite neutralizers. The results indicate that broadly neutralizing antibodies are indeed present in commercial pigs.

When sorting suitable single B cells for functional antibody screening, memory B cells and antibody-secreting cells (ASCs, i.e., plasmablasts and plasma cells) are the two main targets because they bear somatically mutated B cell antigen receptors (BCRs) with high affinities toward antigens. However, there are no specific porcine clusters of differentiation (CD) markers on memory B cells or ASCs known to date (Sinkora and Butler, 2016). Recently acquired evidence indicates that the $CD2^+CD21^-$ or $CD2^-CD21^-$ population most likely represents antibody-producing B cells. Therefore, it is difficult to precisely sort memory B cells or ASCs directly using defined CD markers by flow cytometry. In addition, there are a limited number of commercial porcine antibodies suitable for flow cytometry. Accordingly, after comparing five staining strategies (Fig. S2), we isolated $CDR45^+IgG^+Ag^+$ B cells, mainly for assessing antigen-specific and IgG-producing characteristics.

To screen for antibodies with desired functions, such as broadly neutralizing activity, random B cell (i.e., memory B cell or ASC) sorting followed by single cell activation and functional screening may be a better approach. However, as mentioned above, we had difficulty sorting either porcine memory B cells or ASCs. Furthermore, few studies on porcine memory B cells or ASC culture and activation have been performed thus far, indicating that substantially more time will be required to optimize related culture strategies. Thus, we selected a method based on antigen-specific cell sorting, despite the requirement of well-defined neutralizing or binding epitopes in a pathogen. In the case of PRRSV, several structural proteins, GP2a, GP3, GP4, GP5, and M, have been reported to induce neutralizing antibodies (Ansari et al., 2006; Das et al., 2011; Jiang et al., 2007; Kim and Yoon, 2008; Li and Murtaugh, 2012; Plagemann, 2006). However, epitope A of GP5 reportedly serves as a decoy epitope to induce ADE-related non-neutralizing antibodies (Ostrowski et al., 2002). Therefore, we selected all of these potential neutralizing targets for single cell sorting in an effort to screen for functional antibodies, such as broadly neutralizing antibodies, against PRRSV. Overall, antibodies targeting these antigens can provide new insight into crucial epitopes for efficient vaccine design.

In the case of single B cell RT-PCR, primer coverage is a key factor determining the efficiency of Ig gene amplification. Based on data from IMGT®, all of the known functional V region sequences were summarized and utilized to design full-coverage primers (Tables 2 and 3). However, not all V gene family members were detected when the two sets of primers were utilized to perform RT-PCR with PBMC cDNA serving as the template (Fig. 5). One reason for the skewed amplification is that biased usage of V genes, such as using only IGKV1 and IGKV2, IGLV3 and IGLV8, occurred in the tested pigs, which is consistent with previous data (Schwartz et al., 2012a; Wertz et al., 2013). As we employed mixed primers, another possibility is that some undetected V genes were not well amplified because of the amplification bias of dominant genes coupled with their rare usage.

When using our designed primers (Tables 2 and 3) to perform RT-PCR on single B cells, we noticed that the amplification efficiency was approximately 84.4% and 68.8% for VH and VL, respectively. Notably, not every single B cell contained a VH and VL pair. In addition, a few single B cells contained more than 1 VH and/or 1 VL. Similar observations have also been reported for human single B cells (Wang et al., 2016), suggesting that a single B cell may produce more than one

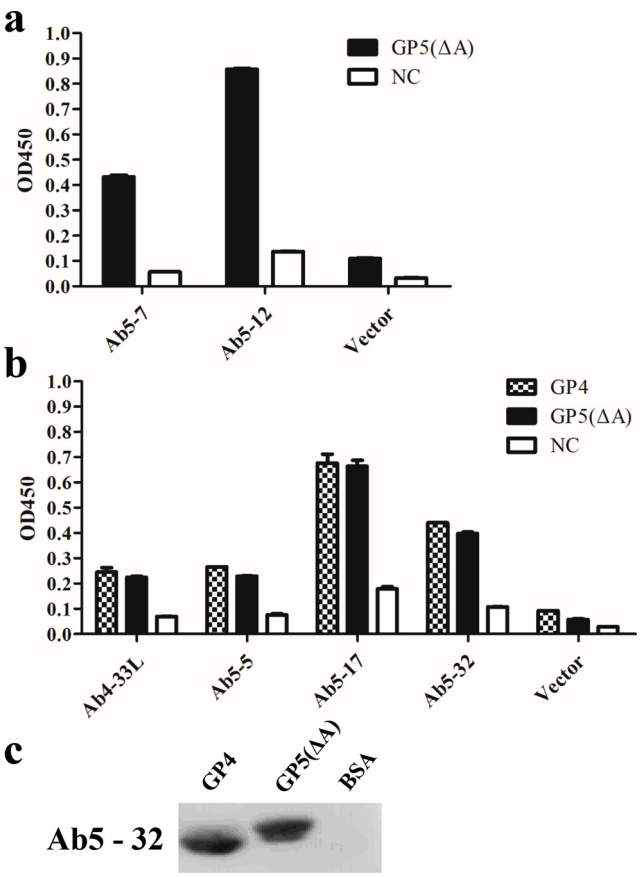

Fig. 6. Detection of antigen-binding activities of porcine monoclonal antibodies. (a–b) Detection of antigen-binding activities of porcine monoclonal antibodies by ELISA. GP4 or GP5(ΔA) was coated individually at a concentration of 200 ng/well in 96-well plates. NC indicates PRRSV-irrelevant protein coating at the same concentration as the tested antigens. Six supernatants individually containing porcine monoclonal antibodies were used as primary antibodies. The vector indicates the supernatant of cells transfected with the original pBudCE4.1. The X-axis indicates the tested samples, and the Y-axis indicates the OD_{450} values. (c) Detection of antigen-binding activities of the porcine monoclonal antibody Ab5-32 by Western blotting, where 3 μg of GP4, GP5(ΔA), or BSA (negative control for tested antigens) was subjected to SDS-PAGE and then transferred onto a PVDF membrane for detection of the antigen-specific binding activity of Ab5-32, which had been purified from the supernatant.

type of functional antibody, assuming that cell contamination, such as two or more cells per well, was completely avoided during the single cell isolation step. Furthermore, in RT-PCR, PCR template contamination might result in multiple IgV genes. If two or more cells per well were sorted, two or more VHs coupled with two or more VLs should have been observed. However, some single cells contained only one VH coupled with two VLs or two VHs coupled with only one VL, suggesting that they were indeed single cells. Thus, we speculate that at the single cell level, one B cell is capable of naturally producing more than one type of antibody or even one bispecific antibody bearing two different VHs or VLs.

In particular, four antibodies showed bispecificity toward GP4 and GP5(ΔA); the two antigens shared low DNA (48.0%) and amino acid sequence (14.3%) homology, suggesting a low possibility of sharing an epitope. Further analysis revealed that the VK sequences of Ab5-5, Ab5-17, and Ab5-32 were the same as those from several GP4-specific single B cells and that the VL from Ab4-33 L was the same as that from some GP5(ΔA)-specific single B cells. Accordingly, we speculate that these antibodies might simultaneously bind to GP4 and GP5(ΔA) through heavy and light chains that bear different antigenic specificities.

In addition, considering that the pig farms were far from our laboratory and the commercial pig donors could be weeded out at any time, we chose to cryopreserve the PBMCs isolated for further sorting of single B cells, which might have led to a decrease in the antibody-producing cell sorting ratio and even in the single B cell cloning efficiency in comparison to those of cells directly sorted from fresh PBMCs (Meng et al., 2015). However, despite the possible impact on sorting and cloning efficiency, cryopreservation is still a feasible and suitable method for collecting PBMCs, especially from pigs located at a distance from the laboratory.

This study reports a platform for generating porcine monoclonal antibodies. Theoretically, the protocol is applicable for screening porcine monoclonal antibodies against any porcine virus, providing a tool to uncover antibody-antigen interactions at the single cell level and facilitate the discovery and understanding of porcine monoclonal antibodies, which may eventually contribute to more efficient vaccine design.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2019.109913.

References

Ansari, I.H., Kwon, B., Osorio, F.A., Pattnaik, A.K., 2006. Influence of N-linked glycosylation of porcine reproductive and respiratory syndrome virus GP5 on virus infectivity, antigenicity, and ability to induce neutralizing antibodies. J. Virol. 80, 3994–4004.

Benfield, D.A., Nelson, E., Collins, J.E., Harris, L., Goyal, S.M., Robison, D., Christianson, W.T., Morrison, R.B., Gorcyca, D., Chladek, D., 1992. Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). J. Vet. Diagn. Invest. 4, 127–133.

Brochet, X., Lefranc, M.P., Giudicelli, V., 2008. IMGT/V-QUEST: the highly customized and integrated system for IG and TR standardized V-J and V-D-J sequence analysis. Nucleic Acids Res. 36, W503–508.

Butler, J.E., Sun, J., Navarro, P., 1996. The swine Ig heavy chain locus has a single JH and no identifiable IgD. Int. Immunol. 8, 1897–1904.

Butler, J.E., Wertz, N., Sun, J., Wang, H., Chardon, P., Piumi, F., Wells, K., 2004. Antibody repertoire development in fetal and neonatal pigs. VII. Characterization of the pre-immune kappa light chain repertoire. J. Immunol. 173, 6794–6805.

Chen, S., Li, S., Sun, H., Li, Y., Ji, S., Song, K., Zhang, L., Luo, Y., Sun, Y., Ma, J., Liu, P., Qiu, H.J., 2018. Expression and characterization of a recombinant porcinized antibody against the E2 protein of classical swine fever virus. Appl. Microbiol. Biotechnol. 102, 961–970.

Das, P.B., Vu, H.L., Dinh, P.X., Cooney, J.L., Kwon, B., Osorio, F.A., Pattnaik, A.K., 2011. Glycosylation of minor envelope glycoproteins of porcine reproductive and respiratory syndrome virus in infectious virus recovery, receptor interaction, and immune response. Virology 410, 385–394.

Dey, B., Svehla, K., Xu, L., Wycuff, D., Zhou, T., Voss, G., Phogat, A., Chakrabarti, B.K., Li, Y., Shaw, G., Kwong, P.D., Nabel, G.J., Mascola, J.R., Wyatt, R.T., 2009. Structure-based stabilization of HIV-1 gp120 enhances humoral immune responses to the induced co-receptor binding site. PLoS Pathog. 5, e1000445.

Eguchi-Ogawa, T., Wertz, N., Sun, X.Z., Piumi, F., Uenishi, H., Wells, K., Chardon, P.,

Tobin, G.J., Butler, J.E., 2010. Antibody repertoire development in fetal and neonatal piglets. XI. The relationship of variable heavy chain gene usage and the genomic organization of the variable heavy chain locus. J. Immunol. 184, 3734–3742.
Evans, K., Albanetti, T., Venkat, R., Schoner, R., Savery, J., Miro-Quesada, G., Rajan, B., Groves, C., 2015. Assurance of monoclonality in one round of cloning through cell sorting for single cell deposition coupled with high resolution cell imaging. Biotechnol. Prog. 31, 1172–1178.
Freund, N.T., Wang, H., Scharf, L., Nogueira, L., Horwitz, J.A., Bar-On, Y., Golijanin, J., Sievers, S.A., Sok, D., Cai, H., Cesar Lorenzi, J.C., Halper-Stromberg, A., Toth, I., Piechocka-Trocha, A., Gristick, H.B., van Gils, M.J., Sanders, R.W., Wang, L.X., Seaman, M.S., Burton, D.R., Gazumyan, A., Walker, B.D., West Jr, A.P., Bjorkman, P.J., Nussenzweig, M.C., 2017. Coexistence of potent HIV-1 broadly neutralizing antibodies and antibody-sensitive viruses in a viremic controller. Sci. Transl. Med. 9.
Fu, F., Li, L., Shan, L., Yang, B., Shi, H., Zhang, J., Wang, H., Feng, L., Liu, P., 2017. A spike-specific whole-porcine antibody isolated from a porcine B cell that neutralizes both genogroup 1 and 2 PEDV strains. Vet. Microbiol. 205, 99–105.
Gristick, H.B., von Boehmer, L., West Jr., A.P., Schamber, M., Gazumyan, A., Golijanin, J., Seaman, M.S., Fatkenheuer, G., Klein, F., Nussenzweig, M.C., Bjorkman, P.J., 2016. Natively glycosylated HIV-1 Env structure reveals new mode for antibody recognition of the CD4-binding site. Nat. Struct. Mol. Biol. 23, 906–915.
Jamal, S.M., Belsham, G.J., 2018. Molecular epidemiology, evolution and phylogeny of foot-and-mouth disease virus. Infect. Genet. Evol. 59, 84–98.
Jiang, Y., Fang, L., Xiao, S., Zhang, H., Pan, Y., Luo, R., Li, B., Chen, H., 2007. Immunogenicity and protective efficacy of recombinant pseudorabies virus expressing the two major membrane-associated proteins of porcine reproductive and respiratory syndrome virus. Vaccine 25, 547–560.
Kim, H.S., Kwang, J., Yoon, I.J., Joo, H.S., Frey, M.L., 1993. Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogeneous subpopulation of MA-104 cell line. Arch. Virol. 133, 477–483.
Kim, W.I., Yoon, K.J., 2008. Molecular assessment of the role of envelope-associated structural proteins in cross neutralization among different PRRS viruses. Virus Genes 37, 380–391.
Klein, F., Diskin, R., Scheid, J.F., Gaebler, C., Mouquet, H., Georgiev, I.S., Pancera, M., Zhou, T., Incesu, R.B., Fu, B.Z., Gnanapragasam, P.N., Oliveira, T.Y., Seaman, M.S., Kwong, P.D., Bjorkman, P.J., Nussenzweig, M.C., 2013. Somatic mutations of the immunoglobulin framework are generally required for broad and potent HIV-1 neutralization. Cell 153, 126–138.
Lefranc, M.P., Giudicelli, V., Duroux, P., Jabado-Michaloud, J., Folch, G., Aouinti, S., Carillon, E., Duvergey, H., Houles, A., Paysan-Lafosse, T., Hadi-Saljoqi, S., Sasorith, S., Lefranc, G., Kossida, S., 2015. IMGT(R), the international ImMunoGeneTics information system(R) 25 years on. Nucleic Acids Res. 43, D413–422.
Li, F., Aitken, R., 2004. Cloning of porcine scFv antibodies by phage display and expression in Escherichia coli. Vet. Immunol. Immunopathol. 97, 39–51.
Li, J., Murtaugh, M.P., 2012. Dissociation of porcine reproductive and respiratory syndrome virus neutralization from antibodies specific to major envelope protein surface epitopes. Virology 433, 367–376.
Meng, W., Li, L., Xiong, W., Fan, X., Deng, H., Bett, A.J., Chen, Z., Tang, A., Cox, K.S., Joyce, J.G., Freed, D.C., Thoryk, E., Fu, T.M., Casimiro, D.R., Zhang, N., A Vora, K., An, Z., 2015. Efficient generation of monoclonal antibodies from single rhesus macaque antibody secreting cells. MAbs 7, 707–718.
Mikell, I., Sather, D.N., Kalams, S.A., Altfeld, M., Alter, G., Stamatatos, L., 2011. Characteristics of the earliest cross-neutralizing antibody response to HIV-1. PLoS Pathog. 7, e1001251.
Muraoka, J., Ozawa, T., Enomoto, Y., Kiyose, N., Imamura, A., Arima, K., Nakayama, H., Ito, Y., 2014. Selection and characterization of human serum albumin-specific porcine scFv antibodies using a phage display library. Monoclon. Antib. Immunodiagn. Immunother. 33, 42–48.
Nathues, H., Alarcon, P., Rushton, J., Jolie, R., Fiebig, K., Jimenez, M., Geurts, V., Nathues, C., 2017. Cost of porcine reproductive and respiratory syndrome virus at individual farm level—an economic disease model. Prev. Vet. Med. 142, 16–29.
Ostrowski, M., Galeota, J.A., Jar, A.M., Platt, K.B., Osorio, F.A., Lopez, O.J., 2002. Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. J. Virol. 76, 4241–4250.
Ouisse, L.H., Gautreau-Rolland, L., Devilder, M.C., Osborn, M., Moyon, M., Visentin, J., Halary, F., Bruggemann, M., Buelow, R., Anegon, I., Saulquin, X., 2017. Antigen-specific single B cell sorting and expression-cloning from immunoglobulin humanized rats: a rapid and versatile method for the generation of high affinity and discriminative human monoclonal antibodies. BMC Biotechnol. 17, 3.
Plagemann, P.G., 2006. Neutralizing antibody formation in swine infected with seven strains of porcine reproductive and respiratory syndrome virus as measured by indirect ELISA with peptides containing the GP5 neutralization epitope. Viral Immunol. 19, 285–293.
Reed, L.J., Muench, H., 1938. A simple method of estimating fifty per cent endpoints. Am. J. Epidemiol. 27.
Rodríguez, S.V., Plà, L.M., Faulin, J., 2013. New opportunities in operations research to improve pork supply chain efficiency. Ann. Oper. Res. 219, 5–23.
Sanchez-Cordon, P.J., Montoya, M., Reis, A.L., Dixon, L.K., 2018. African swine fever: a re-emerging viral disease threatening the global pig industry. Vet. J. 233, 41–48.
Sather, D.N., Armann, J., Ching, L.K., Mavrantoni, A., Sellhorn, G., Caldwell, Z., Yu, X., Wood, B., Self, S., Kalams, S., Stamatatos, L., 2009. Factors associated with the development of cross-reactive neutralizing antibodies during human immunodeficiency virus type 1 infection. J. Virol. 83, 757–769.
Schwartz, J.C., Lefranc, M.P., Murtaugh, M.P., 2012a. Evolution of the porcine (Sus scrofa domestica) immunoglobulin kappa locus through germline gene conversion. Immunogenetics 64, 303–311.
Schwartz, J.C., Lefranc, M.P., Murtaugh, M.P., 2012b. Organization, complexity and allelic diversity of the porcine (Sus scrofa domestica) immunoglobulin lambda locus. Immunogenetics 64, 399–407.
Sinkora, M., Butler, J.E., 2016. Progress in the use of swine in developmental immunology of B and T lymphocytes. Dev. Comp. Immunol. 58, 1–17.
Sun, J., Butler, J.E., 1996. Molecular characterization of VDJ transcripts from a newborn piglet. Immunology 88, 331–339.
Sun, J., Kacskovics, I., Brown, W.R., Butler, J.E., 1994. Expressed swine VH genes belong to a small VH gene family homologous to human VHIII. J. Immunol. 153, 5618–5627.
Tiller, T., 2011. Single B cell antibody technologies. N. Biotechnol. 28, 453–457.
Tiller, T., Meffre, E., Yurasov, S., Tsuiji, M., Nussenzweig, M.C., Wardemann, H., 2008. Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning. J. Immunol. Methods 329, 112–124.
Wang, Q., Yang, H., Liu, X., Dai, L., Ma, T., Qi, J., Wong, G., Peng, R., Liu, S., Li, J., Li, S., Song, J., Liu, J., He, J., Yuan, H., Xiong, Y., Liao, Y., Li, J., Yang, J., Tong, Z., Griffin, B.D., Bi, Y., Liang, M., Xu, X., Qin, C., Cheng, G., Zhang, X., Wang, P., Qiu, X., Kobinger, G., Shi, Y., Yan, J., Gao, G.F., 2016. Molecular determinants of human neutralizing antibodies isolated from a patient infected with Zika virus. Sci. Transl. Med. 8, 369ra179.
Wertz, N., Vazquez, J., Wells, K., Sun, J., Butler, J.E., 2013. Antibody repertoire development in fetal and neonatal piglets. XII. Three IGLV genes comprise 70% of the preimmune repertoire and there is little junctional diversity. Mol. Immunol. 55, 319–328.
Wu, X., Yang, Z.Y., Li, Y., Hogerkorp, C.M., Schief, W.R., Seaman, M.S., Zhou, T., Schmidt, S.D., Wu, L., Xu, L., Longo, N.S., McKee, K., O'Dell, S., Louder, M.K., Wycuff, D.L., Feng, Y., Nason, M., Doria-Rose, N., Connors, M., Kwong, P.D., Roederer, M., Wyatt, R.T., Nabel, G.J., Mascola, J.R., 2010. Rational design of envelope identifies broadly neutralizing human monoclonal antibodies to HIV-1. Science 329, 856–861.
Yoon, K.J., Wu, L.L., Zimmerman, J.J., Platt, K.B., 1997. Field isolates of porcine reproductive and respiratory syndrome virus (PRRSV) vary in their susceptibility to antibody dependent enhancement (ADE) of infection. Vet. Microbiol. 55, 277–287.