



Increased Neutralizing Antibody Production and Interferon- γ Secretion in Response to Porcine Reproductive and Respiratory Syndrome Virus Immunization in Genetically Modified Pigs

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T cell-mediated immunity plays a prominent role in combating pathogens infection. Both the engagement of the T cell receptor with the peptide-bound major histocompatibility complex and a costimulatory signal are needed for the complete activation of the T cell. To determine whether host immune responses to vaccination could be improved by enhancing CD28-mediated costimulation and verify whether the boosted immune responses could protect the host against viral challenge, we produced a transgenic pig line expressing an extra copy of the CD28 gene controlled by its own promoter at the *Rosa26* locus. As expected, in response to porcine reproductive and respiratory syndrome virus (PRRSV) strain vaccination, CD4⁺ T cells was remarkably increased in CD28 transgenic pigs and a similar response in CD8⁺ T cells was elicited after challenge. Importantly, because of increased T cell frequencies, the virus-neutralizing antibody against JXA-1 (a highly pathogenic Chinese PRRSV strain), as well as interferon- γ secretion, were enhanced in transgenic pigs. These findings in our translational study provide a novel concept for farm animal breeding in disease resistance, in which we may use the transgenic technology to force overexpression of confirmed immunity-promoting molecules like CD28 and produce an animal with enhanced immune responses to vaccination and broad-spectrum resistance to infectious diseases.

Keywords: neutralizing antibody, interferon- γ , antiviral immune responses, CD28, transgenic pig

INTRODUCTION

There is growing evidence that T cells are central effectors for adaptive immune responses, which help protect the host against pathogens infection (1–3). In order to perform their function, T cells need to be activated, a process that requires two signals for their optimal function (4–6). One is an antigen-specific signal derived from the peptide-bound major histocompatibility complex (MHC)

interaction with the T cell receptor (2, 4, 5, 7, 8). The other signal is delivered by the binding of costimulatory molecules to their ligands (2, 6, 7). It is well known that signaling solely through the interaction of TCR with MHC–peptide complex results in anergy (9). Costimulatory molecules, playing a key role in the modulation of T cell responses, are becoming promising candidates for immunotherapy (10). Among them, CD28 provides a more robust costimulatory signal, which has been well documented in numerous studies (11–13).

CD28, a homodimeric stimulatory cell membrane receptor of the immunoglobulin superfamily, is expressed on almost all CD4⁺ T cells; on nearly half of CD8⁺ human T cells and on virtually all murine T cells (14–16). When engaged with its ligand B7 molecule, which is expressed on activated B cells, dendritic cells or macrophages, CD28-mediated costimulatory signals initiate activation of AKT, which subsequently results in activation of the downstream targets nuclear factor- κ B (NF- κ B), nuclear factor of activated T cells, and others (17). In addition, CD28 also induces activation of RAS and the downstream phosphorylation of AKT, c-Jun N-terminal kinases, and extracellular signal-regulated kinases (ERKs), enabling high-level production of IL-2 and effective T cell proliferation and differentiation (6, 18).

Previous studies have indicated that the primary T cell response can proceed in the absence of CD28 but requires a strong or long-lasting TCR signal (11). This suggests that CD28 costimulation is necessary for the amplification of TCR signaling. However, CD28 expression is not static, as its level increases following T cell activation while the inducible expression of cytotoxic T lymphocyte-associated antigen 4 on activated T cells can downregulate CD28 levels (6, 12). Hence, there are some controversies over whether CD28 is required for maintaining T cell responses following activation.

Recent studies have verified that CD28 costimulation substantially affects immune responses. For instance, CD28^{fllox/fllox}-OX40^{cre/+} mice in which CD28 is lost after T cell priming showed fewer Th1 cells and impaired formation and maintenance of T follicular helper cells after influenza virus infection (19). More importantly, for T cell memory responses, global removal of CD28-through blockade with antimouse CD28-specific antibodies or inducible deletion by tamoxifen treatment reveals its requirement for full clonal expansion and effector functions such as degranulation or interferon- γ (IFN- γ) production during the secondary immune response to *Listeria monocytogenes* (20). These findings show that CD28-mediated costimulation plays a critical role in the induction of effective T cell immune response. However, little is known about the effects of CD28 signaling on the enhancement of immune responses in pigs.

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically devastating diseases that severely threaten swine production worldwide, leading to reproductive failures in pregnant sows and respiratory problems that persistently infect offspring piglets (21–23). The causative agent of this disease, PRRS virus (PRRSV), first identified in Europe and the United States in the early 1990s, has been spreading and threatening the global swine farming (24). To date, although commercial vaccines against PRRSV are available, they fail to provide efficient protection against the virus variable strains, in particular against

highly pathogenic PRRSV (HP-PRRSV) strains (25). However, the weak T cell response to PRRSV is a particular feature of this host–virus interaction (26). Therefore, enhancement of T cell function would be an ideal measure not only for disease control but also for improving vaccine efficacy.

In the current study, to determine whether enhancing the porcine CD28 signaling could increase the host immune response to PRRSV infection, we first produced transgenic pigs expressing an extra copy of the porcine CD28 gene and then analyzed the protective effect of the immune response elicited by vaccination against the PRRSV challenge.

MATERIALS AND METHODS

Ethics Statements

All animal experiments were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of China and were approved by the Institutional Animal Care and Use Committee of China Agricultural University (certificate of the Beijing Laboratory Animal employee, ID: 18086). All efforts were made to minimize animal suffering.

Animals and PRRSV Strain

F0 generation CD28 transgenic landrace pigs were produced by somatic cell nuclear transfer (SCNT). Briefly, primary porcine fetal fibroblasts isolated from a 30-day fetus were cotransfected with 4 μ g of linearized pCDOCNDR and 1 μ g of pCas9-sgRNA. After a 10-day selection with G418 (in concentration ranges of 500 to 1,000 μ g/mL) and DTA negative selection, the cell clones were subjected to PCR analysis for the integration of CD28 at the *Rosa26* locus. Positive clones were used as donor cells and SCNT was performed as described previously (27). JXA1-R, a modified live Chinese HP-PRRSV vaccine, was a kind gift from China Animal Husbandry Industry Co. Ltd., Beijing, China. JXA-1 (GenBank ID: EF112445.1), one of the earliest Chinese HP-PRRSV strains, was the parental strain of JXA1-R, and propagated and titrated on porcine alveolar macrophages as previously described (28).

Southern Blot

For the detection of the correct integration of CD28 into the *Rosa26* locus, 10 μ g of genomic DNA were extracted from the tail samples of transgenic (Tg) and wild-type (WT) pigs, digested with *NotI* overnight and then resolved by 1% agarose gel electrophoresis. DNA samples were transferred onto a nylon membrane and then hybridized with a digoxigenin (DIG)-labeled probe amplified with the primers P1 and P2. Then they were incubated with anti-DIG AP (Cat#1093274, Roche), and the location of the probe was detected by chemiluminescent methods as described previously (29).

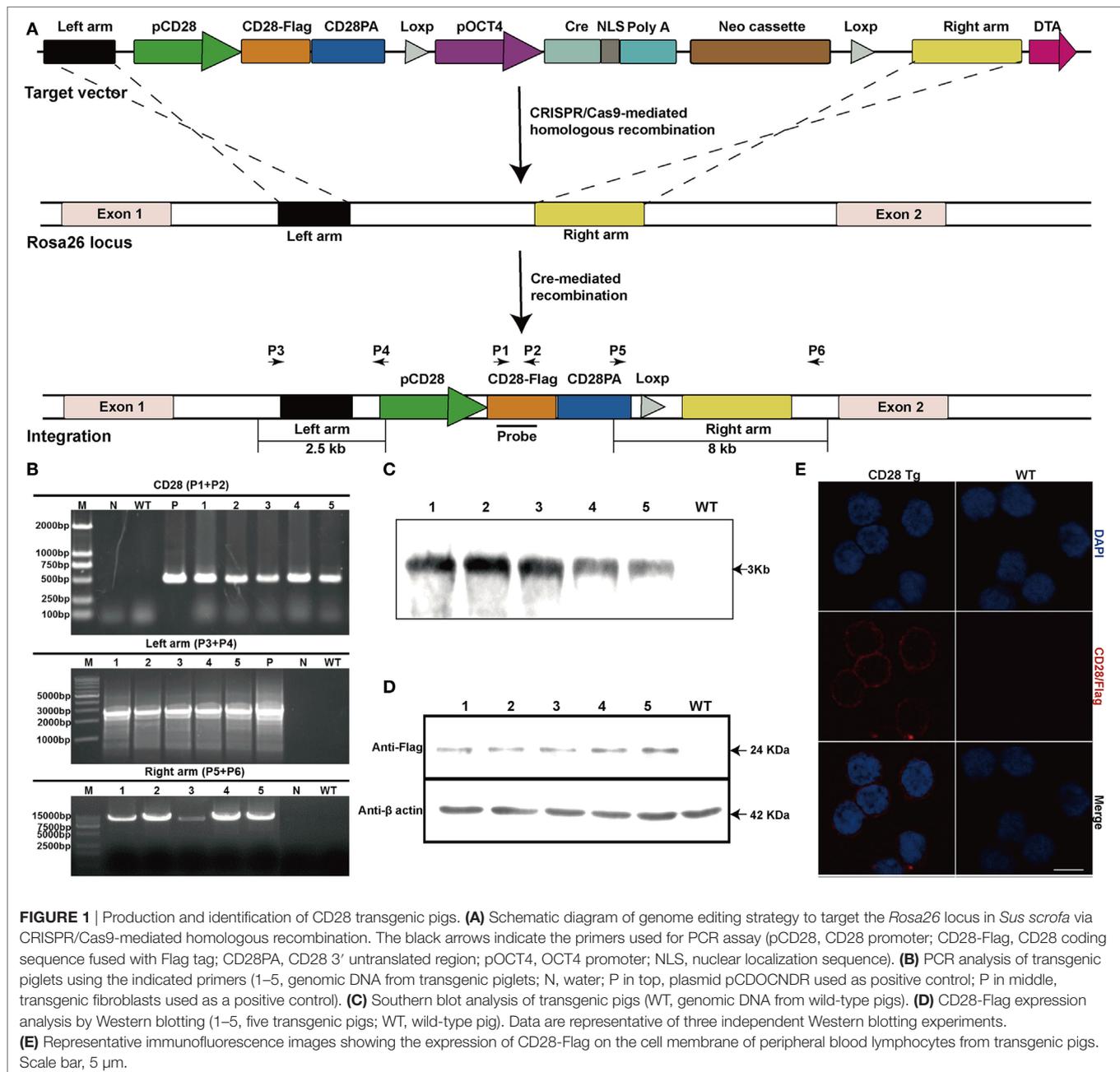
Western Blot

For the detection of CD28-Flag expression, peripheral blood lymphocytes (PBLs) were lysed with RIPA buffer (10 mM Tris,

TABLE 1 | Primer pairs used for conventional PCR and real-time PCR.

Primer	Sequences (5'–3')	Amplicon size (bp)
P1	ATGATCCTCGGGTTACTCCTG	501
P2	GCTATAGAAAGCTACGACTCC	
P3	CGCACCCCTTACCTTGCCCA	2,500
P4	GAAGGTGGGATGGAGGGTGA	
P5	ACGCTTCCTCGTGCTTTAC	8,000
P6	AGCTGCCTCCTGTGATTACC	
P7	AACAGTGACATTCTACCTCC	146
P8	CTGGACAATGTTTCTTTTCAC	
P9	ACTCTCTGCACTCACGGAAGGTG	127
P10	GGCGTTTCGCATCTTAAACGGC	

pH 7.4, 150 mM NaCl, 0.2% Triton X-100, 2 mM EDTA, 1 mM PMSE, and 1× protease inhibitor mixture) and then separated on 15% polyacrylamide gels. Separated proteins were transferred to nitrocellulose membranes (Amersham Pharmacia, UK), blocked with 5% skim milk in PBST at room temperature for 1 h, and then detected with anti-Flag mouse monoclonal antibody (Sigma-Aldrich F1804). Anti-β actin antibody was used as an internal reference. Next, proteins were incubated with goat antimouse IgG-HRP (ab97023; Abcam) for 1 h at room temperature and washed three times with in PBST. The membranes were subjected to luminol-based chemiluminescence with a commercial substrate (Millipore, USA) and Kodak film.



Since ERK1/2 mitogen-activated protein kinase pathway transduces extracellular signals into intracellular responses in mammalian somatic cells (30), we evaluated the expression of phosphorylated (p)-ERK1/2 in PBLs of WT and Tg pigs. We used anti-p-ERK1/2 (9101, Cell Signaling) and anti-total-ERK1/2 (4695, Cell Signaling) antibodies at 0, 30, 60, and 90 min after stimulation with anti-pig CD3 mAb (5 µg/mL, clone PPT3, Abcam) and soluble anti-pig CD28 (2 µg/mL, prepared by National Animal Protozoa Laboratory, Beijing, China). Anti-GAPDH antibody (14C10, Cell Signaling) was used as an internal reference.

Indirect Immunofluorescence Assay (IFA)

PBLs from Tg and WT pigs were subjected to IFA. Briefly, permeabilized and blocked cells were incubated with anti-FLAG monoclonal antibody (F1804, Sigma-Aldrich) and then with the goat antimouse IgG H&L (Cy3) secondary antibody (ab97035, Abcam, Cambridge, UK). Nuclei of cells were stained with 4',6-diamidino-2-phenylindole (Sigma-Aldrich), and then slides were observed under a fluorescence microscope (Leica TCS SP5, Leica Microsystems, Germany).

RNA Extraction and Quantitative Real-time PCR

For the detection of the transcription levels of CD28, total RNA from PBLs of Tg or WT pigs was extracted using TRIzol reagent® according to the instructions of the manufacturer (Invitrogen, Carlsbad, CA, USA). All RNA samples were DNase-treated prior to cDNA synthesis. Total RNA was reverse-transcribed using an EasyScript First-Strand cDNA Synthesis SuperMix (Transgen, Beijing, China). Primers for real-time PCR are shown in **Table 1**. For PCR amplifications, 1 µL of cDNA was added to a mixture containing 10 µL of 2 × SYBR green master mix (Takara, Dalian, China), 0.4 µL of ROX reference dye, and 0.4 µL of each primer P7 and P8 (50 pmol/µL). All samples were tested in duplicate. At the end of the amplifications, cycle threshold (Ct) values were obtained. Transcripts of porcine GAPDH were used as internal reference. Relative transcript levels were quantified by the $2^{-\Delta\Delta CT}$ method as described previously (31).

For the evaluation of the viremia of PRRSV-infected WT and Tg pigs, viral RNA was extracted from serum samples collected at day 10 postchallenge with TRIzol® LS reagent (Invitrogen, Carlsbad, CA, USA). The plasmid containing the non-structural protein gene of PRRSV was used to construct the standard curve. The levels of viral load in sera of Tg and WT pigs were determined with primers P9 and P10 by using absolute quantitative real-time PCR as previously described (32).

Enzyme-Linked Immunosorbent Assay (ELISA)

Serum was separated from clotted blood and preserved at -80°C until use. PRRSV antibody levels in serum were measured using the IDEXX PRRS X3 ELISA Kit (IDEXX Laboratories HerdCheck Switzerland AG) following manufacturer's instructions. The presence or absence of antibody to PRRSV was determined by calculating the sample to positive (S/P) ratio for each sample. An S/P ratio of 0.40 or greater was considered seropositive (26).

Anti-IFN- γ antibody in serum samples was determined using Pig IFN gamma ELISA Kit (ab113353, Abcam). All ELISA procedures were performed as per the manufacturer's instructions. Each sample was assayed in triplicate.

Flow Cytometry Analysis

Swine peripheral blood mononuclear cells (PBMCs) were cultured in 24-well round bottom plates (1×10^6 cells/well) and stimulated for 12 h with PRRSV antigen, recovered by centrifugation and resuspended in 100 µL of PBS containing 10% porcine serum. After washing twice with a cell-staining buffer (BD Biosciences), the cells were suspended in 50 µL of staining buffer and stained with PE-Cy™ 5.5 anti-pig CD3 ϵ (clone BB23-8E6-8C8, BD Biosciences), APC anti-pig CD4 (clone 74-12-4, BD Biosciences) and FITC anti-pig CD8 (clone 76-2-11, BD Biosciences) at 4°C for 30 min. Following another two washes, the cells were suspended in 200 µL of sterile PBS and analyzed using a BD Accuri™ C6 flow cytometer.

Virus Neutralization (VN) Assay

The PRRSV neutralizing antibody response in serum was measured using a modified fluorescent focus neutralization assay (33). Briefly, twofold serial dilutions (1:4 to 1:256) of heat-inactivated serum samples were prepared in 96-well plates using Minimum Essential Medium (MEM, GIBCO® brand; Life Technology, Grand Island, NY, USA) supplemented with 10% FBS. An equal volume (50 µL) of PRRSV (HP-PRRSV strain) at a concentration of 2×10^2 TCID₅₀/mL was added to each sample, incubated for 1 h at 37°C and transferred to a 96-well plate containing confluent pulmonary alveolar macrophages (PAMs). After 24 h, the plates were washed, fixed in ice-cold 80% acetone and stained with mouse anti-PRRSV nucleocapsid monoclonal antibody diluted 1:5,000 in PBS. Cells were incubated with FITC-conjugated goat

TABLE 2 | Serum hematological and biochemical parameters of wild-type and CD28 transgenic pigs.

Items	WT	CD28 Tg	Significant (P value)
RBC ($10^{12}/\text{L}$)	7.35 ± 0.13	7.42 ± 0.09	0.70
HGB (g/L)	123.00 ± 3.06	117.30 ± 3.18	0.27
HCT (%)	39.43 ± 0.66	37.83 ± 0.78	0.19
PLT ($10^9/\text{L}$)	333.00 ± 13.58	312.00 ± 16.56	0.38
WBC ($10^9/\text{L}$)	19.30 ± 2.46	21.40 ± 1.42	0.50
MCH (pg)	16.70 ± 0.15	16.13 ± 0.18	0.07
MCHC (g/L)	311.70 ± 2.67	310.30 ± 3.53	0.78
GLU (mmol/L)	4.82 ± 0.08	4.65 ± 0.07	0.17
TP (g/L)	71.22 ± 2.37	73.68 ± 2.08	0.48
ALT (U/L)	56.45 ± 2.39	60.50 ± 7.32	0.63
AST (U/L)	35.57 ± 2.79	33.99 ± 1.85	0.66
UN (mmol/L)	6.13 ± 0.14	6.15 ± 0.15	0.91
Ca (mmol/L)	2.64 ± 0.03	2.63 ± 0.03	0.76
P (mmol/L)	2.65 ± 0.06	2.58 ± 0.06	0.41

P < 0.05 was considered statistically significant.

RBC, red blood cell; HGB, hemoglobin; HCT, red blood cell-specific volume; PLT, blood platelet; WBC, white blood cell; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; GLU, glucose; TP, total protein; ALT, alanine aminotransferase; AST, aspartate transaminase; UN, urea nitrogen; Ca, calcium; P, phosphorus.

antimouse IgG-HRP (ab97023; Abcam) for 1 h at room temperature. The neutralizing activity of each serum was reported as the reciprocal of the highest dilution that caused a 90% or greater reduction in the number of fluorescent foci (34). Triple independent repeats were performed in this study.

ELISPOT

Interferon- γ ELISPOT was performed as previously described (26). Briefly, 96 wells of ELISPOT plates were coated with antiporcine IFN- γ monoclonal antibody (5 μ g/mL) (Mabtech, Mariemont, OH, USA). 5×10^5 PBLs from PRRSV-vaccinated Tg and WT pigs in 100 μ L RPMI 1640 complete medium were added to each well. Cells were stimulated with CD4⁺ (M6_{aa33-47}: ALKVSRRGLLGLLHL) and CD8⁺ (Nsp5_{aa149-167}: LHNMLVGDGSFSSAFFLRY) T cell epitopes of PRRSV (prepared by China Animal Husbandry Industry Co., Ltd., Beijing, China) (35). The wells received an equal amount of bovine serum albumin-coated beads or 2 μ g/mL concanavalin A (ConA), which were used as negative and positive controls, respectively. Cells were cultured for 24 h at 37°C in 5% CO₂. The plates were then washed five times with PBS (200 μ L/well). Captured IFN- γ was detected with biotinylated antiporcine IFN- γ antibody P2C11 and streptavidin-ALP. All procedures were conducted according

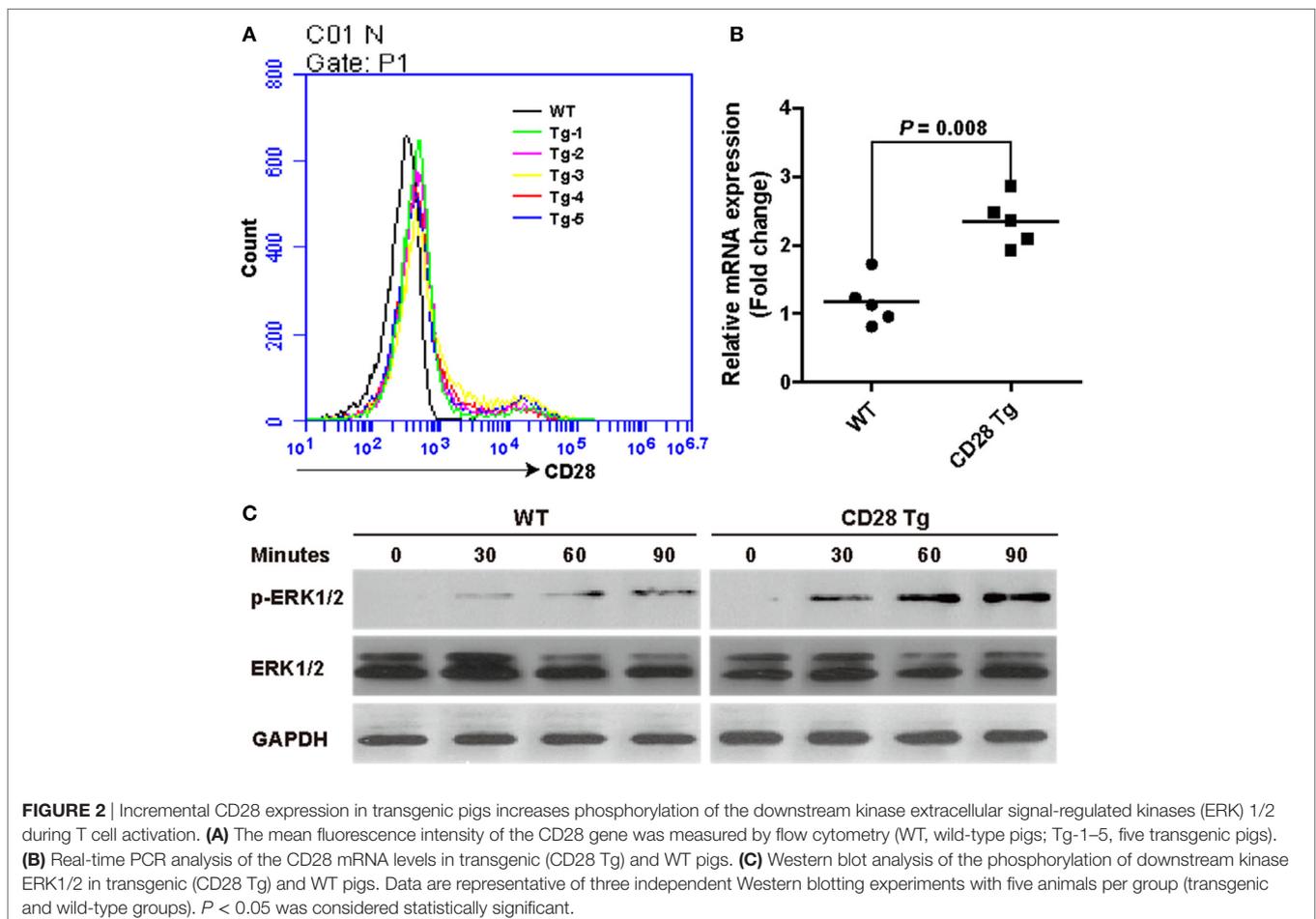
to the instructions of the manufacturer (Mabtech, Mariemont, OH, USA).

Experimental Design

To test whether the upregulated CD28 costimulation could protect the piglets from the PRRSV challenge, five male Tg and five male WT pigs 4 weeks old were treated and housed in separate pens. All pigs were confirmed sera-negative for anti-PRRSV antibodies by ELISA and PRRSV-free in blood by real-time qPCR as described above. On day 0, all pigs were immunized intramuscularly (IM) with a single dose of JXA1-R vaccine. On day 28 postvaccination, all pigs in each group were challenged with 50-time doses of JXA1-R vaccine, and monitored daily for rectal temperature during the first seven days after challenge. Body weight was measured on days 0 and 28 postvaccination and day 10 postchallenge. Blood samples were collected on days 0, 14, and 28 postvaccination and day 10 postchallenge.

Statistical Analysis

SPSS 19.0 software was used for statistical analysis. Data were expressed as mean \pm SEM. Statistical significance was determined by Student's *t*-test and *P* values <0.05 were considered significantly different.



RESULTS

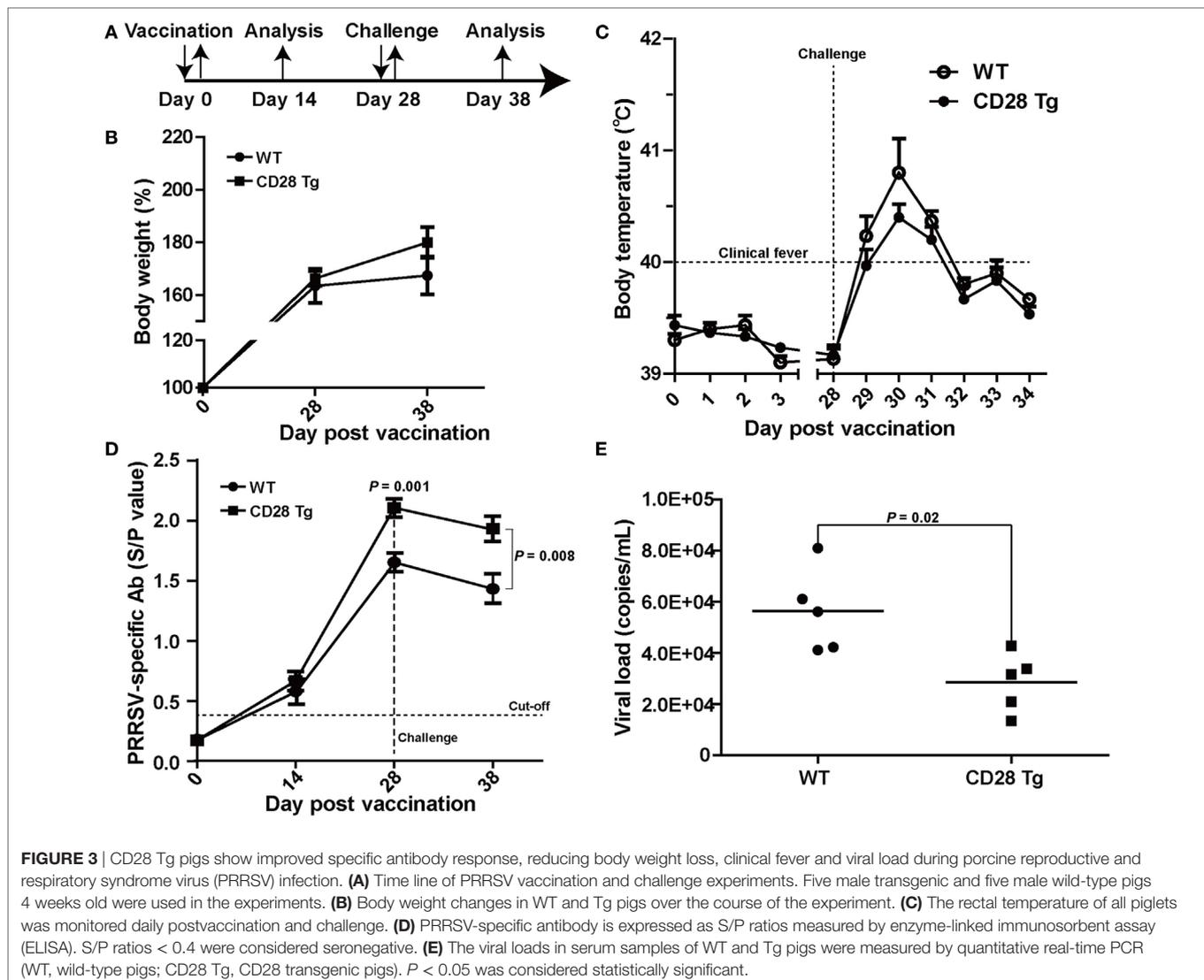
Generation and Identification of CD28 Transgenic Pigs

A correctly modified porcine fetal fibroblast cell clone obtained from the G418-resistant cell clone was used as a donor cell line for SCNT. Two out of four surrogate mothers became pregnant, and five cloned piglets were born 114 days later. PCR analysis showed that the sizes of amplicons were 0.5 kb with primers P1 and P2, 2.5 kb with primers P3 and P4 and 8 kb with primers P5 and P6 (Figures 1A,B), revealing that another copy of the CD28 gene was integrated into the *Rosa26* locus via CRISPR/Cas9-mediated site-specific homology recombination. Site-specific insertion of the CD28 gene was also confirmed by Southern blot analysis, and a single 3 kb DNA band indicated correct targeting (Figure 1C). CD28-Flag was expressed on PBLs from all the cloned piglets (Figures 1D,E). Notably, all Tg piglets developed normally and no significant differences were shown in complete

blood count and blood routine parameters between Tg and WT piglets (Table 2).

Increased CD28 Expression Enhances Phosphorylation of the Downstream Kinase during T Cell Activation

To further verify the efficient expression of CD28 at the *Rosa26* locus, mean fluorescence intensity of CD28 was analyzed between WT and Tg pigs. Remarkably, transgenic T cells showed a nearly one-fold increase in CD28's mean fluorescence intensity (956 ± 39 in WT vs. $1,655 \pm 73$ in Tg), consistent with CD28 mRNA expression levels (Figures 2A,B). Additionally, to determine the effect of CD28 overexpression on T cell activation, a downstream target kinase, ERK1/2, which is involved in TCR/CD28-mediated T cell activation, was analyzed by Western blotting. The results show that joint ligation of CD3 and CD28 with anti-CD3 plus anti-CD28 mAbs strongly synergized to



phosphorylate the ERK1/2 kinase (Figure 2C), demonstrating that overexpression of CD28 contributed to the increase of phosphorylation events during T cell activation.

CD28 Tg Pigs Show Improved Specific Antibody Response and a Reduction in Body Weight Loss, Clinical Fever, and Viral Load during PRRSV Infection

To test whether CD28 overexpression could protect the piglets from PRRSV infection, piglets in both WT and Tg groups were vaccinated IM with a modified live PRRSV vaccine (JXA1-R, China Animal Husbandry Industry Co., Ltd., Beijing, China) and challenged with a 50-fold dose of the same strain at day 28 (Figure 3A). First, we observed that CD28 overexpression enhanced host global responses to PRRSV vaccination. All piglets in both groups exhibited a similar increase in body weight after PRRSV vaccination (Figure 3B). Moreover, a specific antibody response to PRRSV vaccine was detected in the serum until

14 days after inoculation. Compared to pigs in the WT group, Tg pigs showed a significant increase in PRRSV-specific antibody in serum at day 28 postvaccination ($P = 0.001$, Figure 3D), indicating that CD28 overexpression amplified the vaccine efficacy for eliciting a robust viral-specific antibody response.

We assessed whether the boosted immune response through CD28 signaling could protect the vaccinated pigs from challenge with a 50-fold dose of PRRSV vaccine strain. After challenge, all piglets showed a poor growth performance along with clinical fever ($>40^{\circ}\text{C}$). It was noted that WT pigs had more growth retardation and higher fever than pigs in the Tg group (Figures 3B,C). The clinical fever gradually subsided in the following 4 days in both groups. Strikingly, a significant amount of antibody was still present in both Tg and WT pigs 10 days after challenge ($P = 0.008$, Figure 3D). Moreover, virus levels in blood of Tg pigs were lower than those in WT at day 10 postchallenge (Figure 3E). Overall, CD28 overexpression improved PRRSV-specific antibody response and reduced body weight loss, clinical fever and viral load during PRRSV challenge.

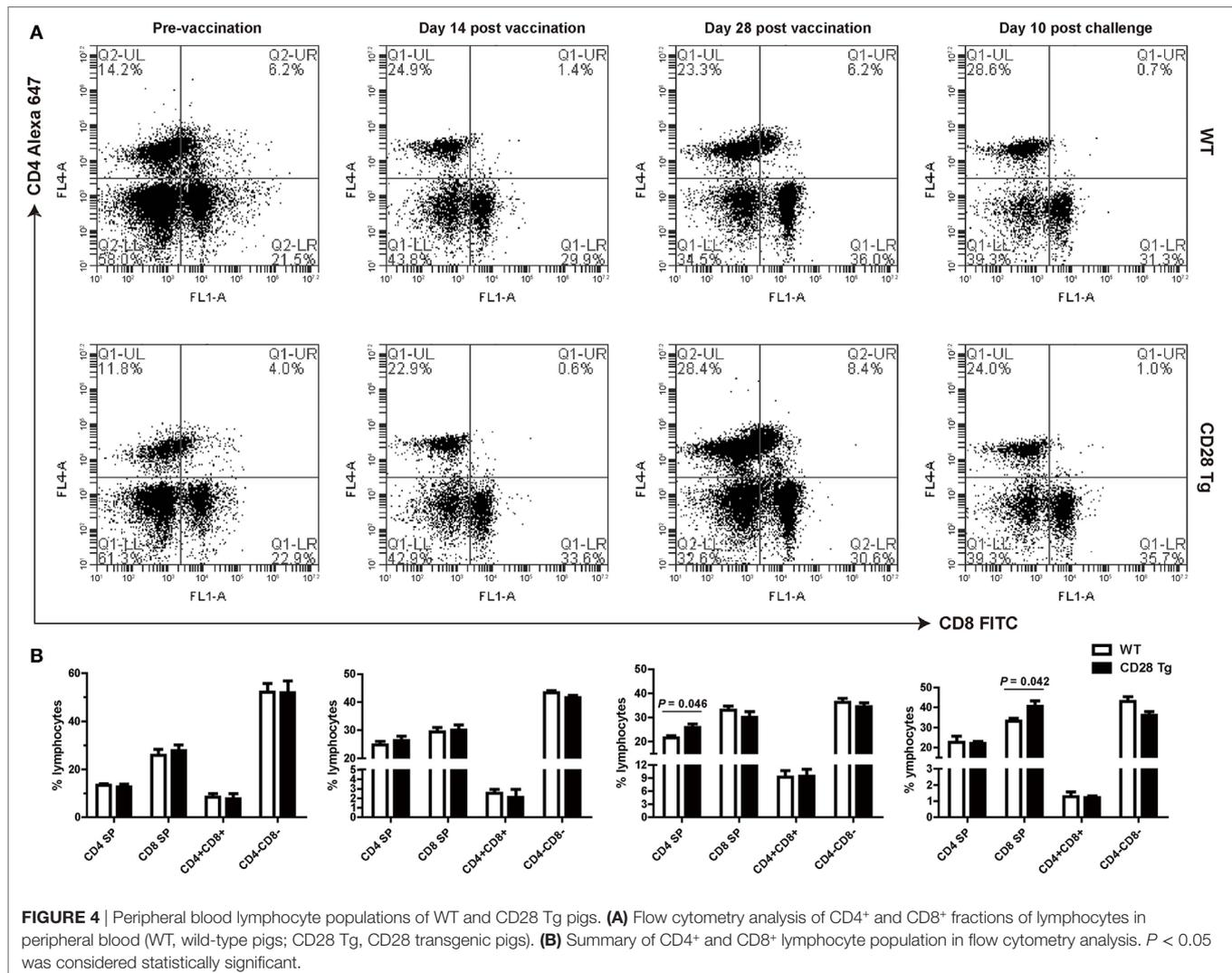
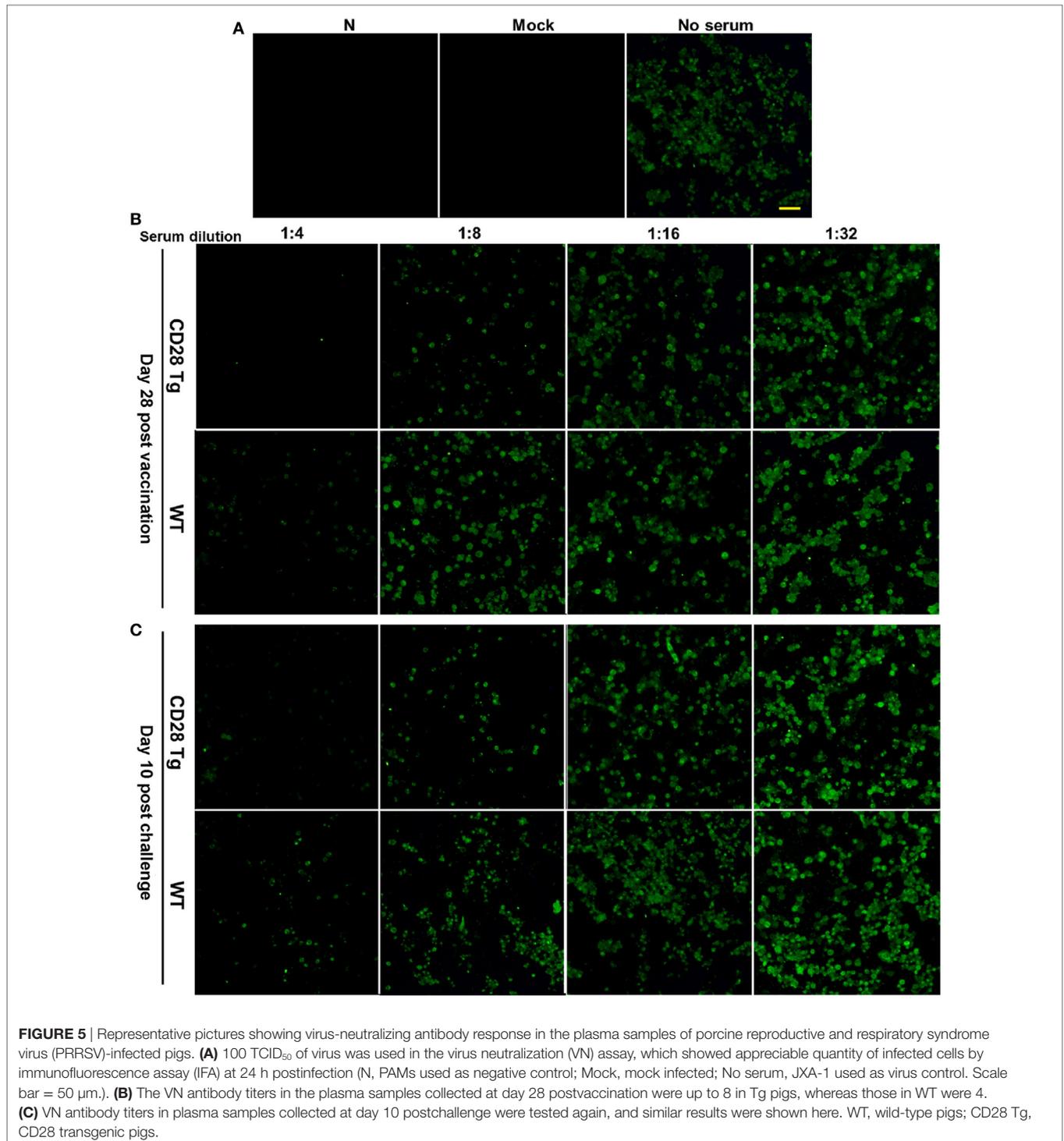


FIGURE 4 | Peripheral blood lymphocyte populations of WT and CD28 Tg pigs. **(A)** Flow cytometry analysis of CD4⁺ and CD8⁺ fractions of lymphocytes in peripheral blood (WT, wild-type pigs; CD28 Tg, CD28 transgenic pigs). **(B)** Summary of CD4⁺ and CD8⁺ lymphocyte population in flow cytometry analysis. $P < 0.05$ was considered statistically significant.

CD28 Tg Pigs Showed Increased Frequencies of CD4⁺ T Cells after Vaccination and of CD8⁺ T Cells after Challenge

We compared frequencies of CD4⁺ single-positive (SP), CD8⁺ SP, and CD4⁺CD8⁺ double-positive (DP) T cells in peripheral

blood of both Tg and WT pigs at day 0 (prevaccination), days 14 and 28 postvaccination, and day 10 postchallenge by flow cytometry (Figures 4A,B). It is notable that there was no significant difference neither in CD4 nor in CD8 lymphocytes between WT and Tg groups until day 28 postvaccination (Figures 4A,B). A remarkably increased population of CD4⁺ SP cells was present in Tg pigs, responding to PRRSV vaccination



at day 28 ($P = 0.046$, mean 28% of gated lymphocytes). CD8⁺ cell populations were not affected on either group during PRRSV vaccination (Figures 4A,B). By contrast, after challenge, Tg pigs had a significantly higher proportion of CD8⁺ SP cells than WT pigs ($P = 0.042$, Figures 4A,B). This means that the CD4⁺ T cell population was boosted in Tg pigs resulting from the PRRSV vaccination, and that the CD8⁺ T cell response was dominant during challenge. The population of CD4⁺CD8⁺ DP T cells did not change in response to vaccination, but decreased after challenge.

Virus Neutralization Antibody Response to Homologous PRRSV Strains Is Strong in CD28 Tg Pigs

Virus neutralization antibody titers against PRRSV in infected serum samples were quantified by IFA. For this assay, 100 TCID₅₀ of PRRSV in each well were used showing a substantial amount of infected cells 24 h postinfection (Figure 5A). Compared to WT pigs, Tg pigs exhibited a stronger anamnestic neutralizing antibody response to JXA-1 with a titer of 8 (Figures 5B,C).

Interferon- γ Secretion by CD8⁺ T Cells Was Augmented in CD28 Tg Pigs

After observing the increase in the CD8⁺ T cell population responding to PRRSV challenge, we measured the frequency of PRRSV-specific IFN- γ secreting cells in the PBLs, which has been used as indicator of cell-mediated immunity (31). PBLs collected from Tg and WT pigs at day 38 were restimulated *in vitro* with purified ConA, CD4⁺ T cell immunodominant epitope and CD8⁺ T cell immunodominant epitope. IFN- γ response to CD8 epitope in Tg pigs was significantly higher ($P = 0.04$) than in WT pigs: between 91 and 106 spots/ 5×10^5 cells in Tg pigs vs. 84–91 spots/ 5×10^5 cells found in WT pigs (Figures 6A,B). There was a similar IFN- γ response to the stimulation with CD4 epitope in both groups (Figures 6A,B). The secretion of IFN- γ was also confirmed in sera by ELISA. We also found that Tg pigs had a significantly higher concentration of IFN- γ in serum samples than WT pigs ($P = 0.048$, Figure 6C). This means that PRRSV-specific CD8⁺ T cell-mediated IFN- γ secretion during PRRSV challenge was significantly enhanced in Tg pigs.

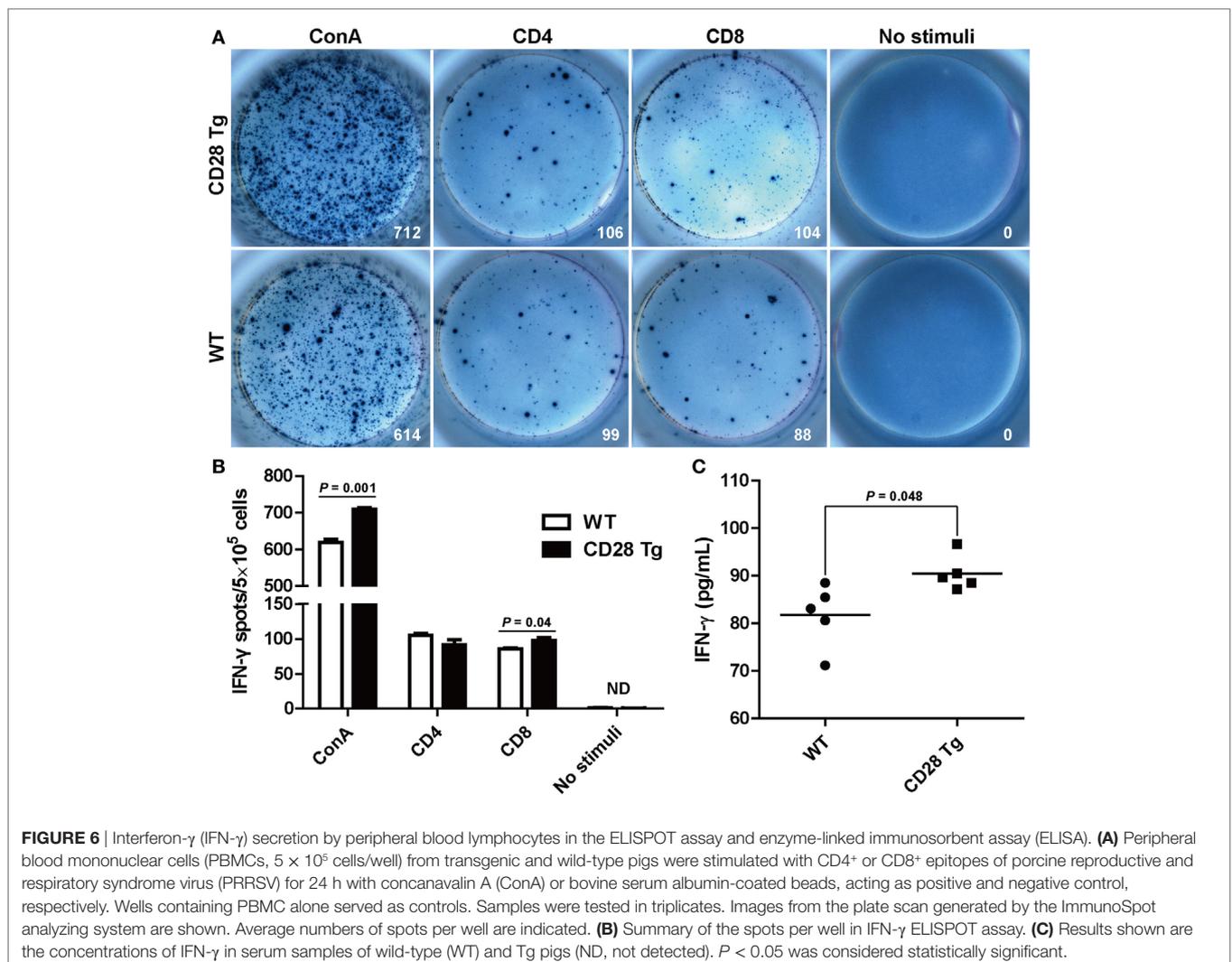


FIGURE 6 | Interferon- γ secretion by peripheral blood lymphocytes in the ELISPOT assay and enzyme-linked immunosorbent assay (ELISA). **(A)** Peripheral blood mononuclear cells (PBMCs, 5×10^5 cells/well) from transgenic and wild-type pigs were stimulated with CD4⁺ or CD8⁺ epitopes of porcine reproductive and respiratory syndrome virus (PRRSV) for 24 h with concanavalin A (ConA) or bovine serum albumin-coated beads, acting as positive and negative control, respectively. Wells containing PBMC alone served as controls. Samples were tested in triplicates. Images from the plate scan generated by the ImmunoSpot analyzing system are shown. Average numbers of spots per well are indicated. **(B)** Summary of the spots per well in IFN- γ ELISPOT assay. **(C)** Results shown are the concentrations of IFN- γ in serum samples of wild-type (WT) and Tg pigs (ND, not detected). $P < 0.05$ was considered statistically significant.

DISCUSSION

In this study, we generated Tg pigs expressing an extra copy of CD28 and evaluated the effect of the enhanced costimulatory signaling on T cell responses following PRRSV vaccination and challenge. We found that CD28 overexpression elicited an efficient immune response to PRRSV vaccination that can reduce the viral load in blood during challenge by improving the neutralizing antibody response and antiviral cytokine IFN- γ secretion. The role of CD28 on the enhancement of T cell-mediated immune responses suggests its potential for preventing infectious diseases.

Previous studies have raised safety concerns regarding genetic modification (36–38). Therefore, it is important to examine any potential risks derived from transgenesis. In this study, at least five founder lines of the CD28 Tg pigs were generated. The extra copy of the porcine CD28 gene was specifically integrated into the *Rosa26* locus with CRISPR/Cas9-mediated homologous recombination. In addition, the pigs had normal weights at birth and developed well under normal husbandry conditions, without any abnormalities.

Porcine reproductive and respiratory syndrome virus is still a global threat and will continue to pose challenges to pig farming because of its easy transmission, antigenic shift and drift and the limited efficacy of current vaccines and antiviral drugs (23, 39). Although deletion of CD163, a fusion receptor for PRRSV, has been previously described, resulting in a significant resistance to the virus infection, the pig could still be susceptible to other pathogens (22, 40). It is now clear that CD28 (a broad-spectrum, immunity-promoting molecule)-mediated costimulatory signaling has a crucial role in regulating T cell activation, subset differentiation, effector function, and survival (41). As expected, the forced overexpression of CD28 gene in genetically modified pigs did promote the PRRSV-specific neutralizing antibody production and IFN- γ secretion, as shown in this study.

Previous studies have shown that antibodies to PRRSV appear around 10 days after infection (26). We found that PRRSV-specific antibody in both Tg and WT groups was absent at day 7 (data not shown), but was detectable 14 days postvaccination. Increased neutralizing antibody levels were detected in CD28 Tg pig sera resulting from immunization with modified live strains of PRRSV (JXA1-R). Antibody neutralization was still evident in Tg pigs 10 days after challenge. These data indicate that CD28-mediated costimulation is necessary for eliciting a humoral immune response.

Cellular immune response, which is essential for the elimination of intracellular pathogens such as PRRSV, could protect swine from becoming infected and shedding viruses (42). Previous studies have shown that CD8⁺ T cell response is the dominant immune response to PRRSV challenge (26, 33, 43, 44). We found a remarkable increase in CD8⁺ T cell population in Tg pigs compared to WT pigs after challenge. We also found high levels of IFN- γ -secreting CD8⁺ T cells as well as high IFN- γ concentration in sera of Tg pigs. This means that CD28 overexpression considerably enhances CD8⁺ T cell-mediated cellular immune response contributing to the clearance of PRRSV in blood after challenge.

An excess of CD8 to CD4 T cells in blood is frequently observed in swine, in contrast to less CD8⁺ T cell population in human and mice (43, 45, 46). Notably, PRRSV does not alter the large majority of T cell subpopulations, including CD4⁺ SP, CD8⁺ SP, CD4⁺CD8⁺ DP, and CD4⁻CD8⁻ DN T cells (43, 47, 48). However, CD28 transgene showed a preponderance of CD4⁺ T cells in response to PRRSV vaccination, resulting in a strong VN antibody response. Moreover, PRRSV-specific IFN- γ -producing CD8⁺ T cells, another potential indicator of protective cellular immunity against PRRSV, also increased in CD28 Tg pigs. These increases indicate that CD28 plays a critical role in the induction of host humoral and cellular immune responses.

In conclusion, for the first time, we report the generation of CD28 Tg pigs, in which forced overexpression of porcine-derived CD28 gene induced protective immune responses against PRRSV infection. The role of CD28 costimulation in immune responses of transgenic pigs to a broad spectrum of pathogens such as bacteria and protists will also be examined in our future studies. Our findings reveal a successful translation of confirmed immunological findings into farm animal breeding: The broad-spectrum, immunity-promoting molecule CD28 combined with the technical use of transgene, produce a farm animal with improved immune responses to vaccination and pathogen infection.

ETHICS STATEMENT

All animal experiments were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of China and were approved by the Institutional Animal Care and Use Committee of China Agricultural University (certificate of the Beijing Laboratory Animal employee, ID: 18086). All efforts were made to minimize animal suffering.

AUTHOR CONTRIBUTIONS

The study was conceived and experiments designed by XS, GH, and XL. The experiments were performed by GH, LD, and XT. Data were analyzed and interpreted by XS, XL, GH, and LD. XS, QL, XH, WF, and LZ provided reagents, materials, or analysis tools. GH drafted the article and all authors revised the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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