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Cytotoxic T lymphocyte epitopes identified from a contemporary strain of porcine reproductive and respiratory syndrome virus enhance CD4 + CD8 + T, CD8 + T, and γδ T cell responses

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ABSTRACT

Immuno-stimulatory class I-restricted cytotoxic T lymphocytes (CTL) epitopes of porcine reproductive and respiratory syndrome virus (PRRSV) are important for vaccine development. In this study we first determined the expression frequency of swine leukocyte antigen (SLA) class I alleles in commercial pigs in the United States. The SLA genotyping result allowed us to predict potential CTL epitopes from a contemporary strain of PRRSV (RFLP 1-7-4) by using bioinformatic tools. The predicted epitopes were then evaluated in an *ex vivo* stimulation assay with peripheral blood mononuclear cells isolated from pigs experimentally-infected with PRRSV. Using flow-cytometry analysis, we identified a number of immuno-stimulatory CTL epitopes, including two peptides from GP3 and two from Nsp9 that significantly improved both degranulation marker CD107a and IFN-γ production in cytotoxic CD4 + CD8 + T cells, CD8 + T cells, and γδ T cells, and two peptides that inhibited IFN-γ production. These CTL epitopes will aid future vaccine development against PRRSV.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) causes significant economic burdens to the swine industry worldwide (Lunney et al., 2010). Its genomic RNA is capped at its 5′ end and polyadenylated at the 3′ end, is approximately 15 kb in length, and contains at least 10 open reading frames (ORFs): ORF1a, ORF1b, ORF2a, ORF2b, ORFs3-5, ORF5a, and ORFs6-7 (Dea et al., 2000; Firth et al., 2011; Johnson et al., 2011). ORF1a and ORF1b, composing approximately 75% of the viral genome at the 5′ proximal region, can be translated into at least 14 nonstructural proteins (Nsps) (Fang and Snijder, 2010). Nsp2TF is a recently-discovered non-structural protein encoded in Nsp2 gene sequence via a unique −2 programmed ribosomal frameshifting (PRF) mechanism (Fang et al., 2012). The 3' portion of the viral genome contains at least 8 ORFs, which encode structural proteins including N-glycosylated proteins GP2a, GP3, GP4, GP5a and GP5, non-glycosylated minor E protein and Matrix (M) protein, and nucleocapsid (N) protein. PRRSV continuously evolves into genetically and antigenically diverse field strains which pose significant hurdles to develop effective vaccines (Nilubol et al., 2004). As a result, current commercial modified live-attenuated vaccines (MLVs) are not broadly cross-protective against all heterologous PRRSV strains (Han et al., 2011; Martelli et al., 2009; Murtaugh and Genzow, 2011). Previous studies indicated that cytotoxic T lymphocyte (CTL) responses against PRRSV were generally impaired, mostly seen as dampened M1 response of monocyte-derived dendritic cells (Miller et al., 2017; Wang et al., 2007), delayed activation and poor protective cytotoxicity of T cells (Costers et al., 2009; Lamontagne et al., 2003). In order to mount effective protective immune responses, an ideal vaccine needs to not only reverse the interferon suppression (Ke and Yoo, 2017), but contain the protective epitopes derived from diverse virus strains. Besides the neutralizing B cell epitopes, T cell epitopes that induce CTL responses are important for clearance of PRRSV and enhancing protective vaccine efficacy.

A few previous studies have attempted to identify protective epitopes from PRRSV strains, but mostly focused on neutralizing B cell

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epitopes. A main neutralizing epitope was found in GP5 of both PRRSV-1 and PRRSV-2 genotypes, with another immunodominant decoy epitope also identified in GP5 (Ostrowski et al., 2002; Plagemann, 2004; Wissink et al., 2003). Potential T cell epitopes were reported in GP4, GP5, and N of PRRSV-1 (Diaz et al., 2009). For PRRSV-2, T cell epitopes reportedly reside in Nsp9 and Nsp10 (Parida et al., 2012), M (Wang et al., 2011), and GP5 (Vashisht et al., 2008). However, these potential T-cell epitopes reported in PRRSV were mostly generated by overlapping peptide libraries covering the designated sequence, and then tested by IFN-γ ELISpot assay. A major drawback with this approach is that IFN-γ secretion was measured from total PBMCs, with little information on which cell type is activated. More specifically, there is no direct correlation between CTL activity and the IFN-γ response detected. Moreover, currently a contemporary strain of PRRSV-2 (referred to as “RFLP 1-7-4”) is highly prevalent in the United States. This contemporary PRRSV strain with the pattern 1-7-4 based on restriction fragment length polymorphism (RFLP) of the ORF5 gene belongs to lineage 1 according to the ORF5 gene sequence (Cai et al., 2002; Ramirez et al., 2019; Shi et al., 2010; Tian et al., 2017; van Geelen et al., 2018). Its genomic information was largely unknown, nor were any potential epitopes reported. Therefore, this highly prevalent contemporary strain of PRRSV RFLP 1-7-4 was the focus in the present study, aiming to identify novel CTL epitopes for improving the current MLVs.

Only those infected cells presenting complexes of viral epitopes in association with class I molecules can be lysed by cytotoxic T lymphocytes (CTLs). Indeed, swine leukocyte antigen (SLA) class I alleles are highly polymorphic, but certain haplotypes and alleles were more frequently identified in pigs worldwide, especially from commercial pig populations (Gao et al., 2017; Ho et al., 2010; Pedersen et al., 2014; Schwartz et al., 2018; Xia et al., 2014). Since genotyping of SLA class I alleles facilitates the rational identification of T cell epitopes from PRRSV, in this study we first determined the most frequent SLA class I alleles at the SLA-1 and SLA-2 loci in commercial outbred pig farms in the United States by using a direct sequencing method. Subsequently we proposed an efficient pipeline to identify CTL epitopes in a contemporary strain of PRRSV RFLP 1-7-4 that are restricted to SLA class I alleles. A set of rational bioinformatic approaches were performed for genome-wide epitope screening for strong binders to the aforementioned most frequent SLA alleles identified in this study. These *in silico* results were further evaluated in *ex vivo* experiments with peripheral blood mononuclear cells (PBMCs) isolated from pigs experimentally-infected with the contemporary strain of PRRSV RFLP1-7-4. By using flow cytometry analyses, we successfully identified several epitopes from PRRSV RFLP 1-7-4 that magnified antiviral T cell responses including IFN-γ production and degranulating activity. We also identified some epitopes particularly from −2 frameshifting product in ORF1a that dampened the IFN-γ response of CTLs. This latter finding further adds to the evidence that PRRSV hampers the cell-mediated antiviral response as one of their immune-evasive strategies.

2. Materials and methods

2.1. Origin and collection of blood samples from outbred pigs in the United States for SLA genotyping

To determine the expression frequency of swine SLA-1 and SLA-2 in commercial pigs in the United States, heparinized swine blood samples were obtained from pigs originating from two swine herds in Virginia and two other herds in Minnesota (Table 1) as part of routine sample collection. The sampled pigs were at various ages and from different breeds including Landrace/Yorkshire, Duroc/Yorkshire, and Duroc/Berkshire, although the genetic source companies of these four farms are unknown.

Table 1
Amplification of SLA-1/2 coding sequences from PBMCs of 100 outbred pigs located in four different commercial farms in the United States.

Farm ID	Location	No of pigs	Pig age
1	Minnesota	25	9 week old
2	Minnesota	30	> 1 year old (Sow)
3	Virginia	13	Newborns to finishers
4	Virginia	32	5 to 6 week old

2.2. Isolation of peripheral blood mononuclear cell (PBMC) for SLA typing

PBMCs were isolated from at least 15 mL heparinized blood by density gradient centrifugation (Ficoll-Paque, GE Healthcare, Uppsala, Sweden) as described previously (Cao et al., 2016; Subramaniam et al., 2014). The freshly isolated PBMCs were used to immediately extract total cellular RNAs, or cryopreserved in storage media (90%FBS, 10% DMSO) and kept in liquid nitrogen for total RNA extraction and flow cytometry at a later stage.

2.3. Amplification by RT-PCR and cloning of SLA-1 and SLA-2 genes

The total cellular RNAs from PBMCs were extracted using TRizol LS Reagent (Ambion, Carlsbad, CA) and the RNA Clean & Concentrator Kit (ZYMO RESEARCH, Irvine, CA) according to the manufacturer's instructions. The extracted total cellular RNAs were stored at −80 °C for later use. The full SLA-1 and SLA-2 gene coding sequences (approximately 1100 bp in length) were amplified directly from the total cellular RNAs extracted from PBMCs using the SuperScript III One-Step RT-PCR kit (Invitrogen, Carlsbad, CA) according to manufacturer's instruction. RT-PCR primers (forward primer of SLA-1: ATGGGGCCTGGAGCCCTCTTCCTG; forward primer of SLA-2: ATGCGGGTCAGAGGCCCTCAAGCCATCCTCATTC; reverse primer of SLA-1 and SLA-2: TCACACTCTAGGATCCTTGGTAAGGGAC) were designed to amplify the SLA-1 and SLA-2 genes together, and therefore the amplified RT-PCR products contained a mixture of SLA-1 and SLA-2 gene products. The amplified RT-PCR products were purified from agarose gel with NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel GmbH, Germany) and subsequently cloned into the pGEM-T Easy Vector System (Promega, Madison, WI).

2.4. Colony direct sequencing and sequence analyses of the SLA genes

The individual bacterial colonies containing the recombinant plasmids with SLA RT-PCR products were subjected to colony direct Sanger sequencing (MCLAB, San Francisco, CA, U.S). The T7 promoter primer present in the cloning vector was used to cover approximately 700 bp of SLA sequence including the exons 2 and 3 which are the primary determinants of allele group assignment (Lunney et al., 2009). For each pig blood sample, multiple bacterial colonies were selected and sequenced until a total of at least ten valid SLA sequences per pig sample were obtained. Each sequence was subsequently imported into the Immunopolymorphism Database IPD-MHC (https://www.ebi.ac.uk/ipd/mhc/blast) to perform a BLAST searches for matching swine alleles. The best match was selected as the representative SLA allele.

2.5. Experimental infection of pigs with a contemporary strain of PRRSV RFLP 1-7-4

PRRSV RFLP 1-7-4 strain ISU2014016404 was kindly provided by Dr. Jianqiang Zhang of the Iowa State University College of Veterinary Medicine. A total of 8, three-week-old, PRRSV-negative pigs were experimentally infected with PRRSV RFLP1-7-4 at $5 \times 10^{3.5}$ $TCID_{50}$ per pig intranasally (half) and intramuscularly (half). The pigs were similarly re-infected at 4 weeks post-infection (wpi). Serum and heparinized blood samples were collected weekly from each pig for a total of 11 weeks. The animal study was approved by the Virginia Tech Institutional Animal Care and Use Committee (IACUC) (protocol approval no. 16–127).

2.6. Computational prediction and synthesis of putative immunogenic CTL epitope peptides

A total of 86 nonamer or decamer peptides were predicted as immunogenic CTL epitopes based on the coding sequences covering all the structural proteins and 5 non-structural proteins (Nsp2, Nsp2TF, Nsp8, Nsp9, Nsp10) of PRRSV strain RFLP1-7-4 (Table 4). These epitope peptides were synthesized and confirmed to be above 95% purity by reversed-phase high pressure liquid chromatography (RP-HPLC) and mass spectrometry (GenScript, USA). Each of the 86 epitope peptides was subsequently dissolved in dH_2O or DMSO at a stock concentration of 1–2 mg/mL. Since Nsp2 and Nsp2TF share the same N-terminal region before the −2 frameshifting, a total of 18 peptides located in Nsp2, and additional 8 peptides located in the TF domain resulting from the −2 frameshifting were selected for synthesis.

2.7. Flow cytometry analyses

PBMCs were dispensed into 96-well U-bottom plates (BD Falcon, Bedford, MA) at 1.5 million cells per well, and individually incubated with each of the synthesized 9 or 10-mer epitope peptide at a concentration of 6 μg per 1.5 million cells. PBMCs were also separately infected with PRRSV strain RFLP 1-7-4 at a moi of 0.01. GolgiPlug and GolgiStop (Thermo Scientific, Waltham, MA) were added at a final concentration of 1:1000 at 15-h post-infection (hpi) for additional 5 h. Cells were then washed with FACS buffer as described previously (Cao et al., 2018). Briefly, they were first stained with Aqua live/dead (Thermo Scientific, Waltham, MA), and then stained with SPRD anti-porcine CD4 (clone 74-12-4, SouthernBiotech), FITC anti-porcine CD8 (clone 76-2-11, SouthernBiotech) and Pacific-blue anti-human CD107a (clone H4A3, Biolegend), mouse IgG2a anti-porcine CD3 (clone 8E6-8C8, Washington State University), and APC anti-pig γδ (clone MAC320, BD biosciense). After washing, cells were surface-stained again with PE-Cy7 anti IgG2a (Biolegend) for CD3. Subsequently, cells were fixed and permeabilized, and then incubated with PE anti-porcine IFN-γ (Clone P2G10, BD Bioscience) for 20 min. The fluorescence of cells was assessed with a BD FACSAria II. All the fluorophores were well separated and run by an experienced flow cytometry specialist at Virginia Tech. Samples below 40,000 live cells were discarded in order to ensure convincing gating. The stimulation assay and flow cytometry were performed at least twice or three times for each individual peptide in at least five different pigs. Data were pooled together for analysis.

2.8. Statistical analyses

The One-way ANOVA and its built-in Dunnett's multiple comparison were used to evaluate the data for statistical differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$) in GraphPad Prism (version 7.0).

3. Results

3.1. Identification of the ten most prevalent SLA-1 and SLA-2 alleles in pig farms in the United States

The SLA class I region is highly polymorphic, but the most highly expressed SLA class I loci are SLA-1 and SLA-2 (Frels et al., 1990; Ho et al., 2010; Lee et al., 2005; Lunney et al., 2009; Pedersen et al., 2014; Tennant et al., 2007). In this study we first determined the most frequently expressed SLA-1 and SLA-2 in commercial pig farms in the United States, in order to facilitate the bioinformatical prediction of T cell epitopes. Heparinized blood samples from pigs of various ages were collected from four different commercial pig herds located in Minnesota and Virginia (Table 1). Blood samples were collected from a total of 110 pigs, and sufficient amount of PBMCs for amplification of SLA-1/2 coding gene sequences with specific primers were obtained from 100 of these blood samples. Bacterial colonies containing the RT-PCR-amplified SLA-1/2 gene inserts from each pig sample were subjected to direct colony sequencing. Each sequence from an individual clone was subjected to a BLAST search in the SLA Database IPD-MHC to identify the best-matched SLA allele. Multiple bacterial clones from each sample were sequenced until at least ten valid sequences per pig matched one or more SLA alleles.

Table 2
The 25 SLA-1 alleles and their frequencies identified from 100 outbred pigs in four different commercial swine herds in the United States.

Allele	Colony number[a]	Pig frequency (%)
SLA-1*04:01:01	25	15
SLA-1*14:02	37	13
SLA-1*04:02	16	10
SLA-1*13:01	14	10
SLA-1*14:01	16	10
SLA-1*08:01	15	9
SLA-1*15:01	20	8
SLA-1*08:07	5	4
SLA-1*08:10	5	4
SLA-1*11:01:02	5	3
SLA-1*08:02	3	2
SLA-1*01:01	3	2
SLA-1*11:02	2	2
SLA-1*08:18	2	2
SLA-1*06:02	7	2
SLA-1*15:02	2	1
SLA-1*15:03	3	1
SLA-1*12:03	1	1
SLA-1*16:01	1	1
SLA-1*17:02	1	1
SLA-1*12:01	1	1
SLA-1*14:05	1	1
SLA-1*12:02	2	1
SLA-1*07:05	1	1
SLA-1*08:08	2	1

Note.
[a] Bacteria colonies that have the particular SLA allele sequence.

In general, the SLA-1 and SLA-2 loci showed high polymorphism, and in most pigs three or more different alleles were identified (data not shown). A total of 25 different SLA-1 alleles were identified from the 100 pigs that originated from 4 different commercial herds in the United States (Table 2). The top five most frequent SLA-1 alleles identified were SLA-1*04:01:01, SLA-1*14:02, SLA-1*04:02, SLA-1*13:01, and SLA-1*14:01. As for the SLA-2 locus, a total of 37 different alleles were identified in this study, and the top five most frequent SLA-2 alleles were SLA-2*05:02, SLA-2*04:01, SLA-2*06:02:01, SLA-2*10:01, and SLA-2*10:04 (Table 3).

3.2. In silico prediction of CD8 T cell epitopes from a contemporary PRRSV strain RFLP1-7-4 (ISU2014016404)

Since the genomic sequence of the PRRSV strain RFLP 1-7-4 had not been published, we isolated the viral genomic RNA from PRRSV-infected MARC-145 cells at passages 4, 5, and 6. By using RT-PCR amplification and sequencing, we were able to determine the coding sequences of all eight structural proteins of PRRSV RFLP1-7-4 from GP2a to N protein. We also determined the gene sequences of Nsp8, Nsp9, and Nsp10 that are relatively conserved among PRRSV strains, as well as the sequences of Nsp2 and Nsp2TF that are among the most variable regions of PRRSV. Protein sequences from these 13 regions of PRRSV strain RFLP1-7-4 were then used in the subsequent *in silico* prediction of

Table 3
The 37 SLA-2 alleles and their frequencies identified from 100 commercial outbred pigs in four different swine herds in the United States.

Allele	Colony number[a]	Pig frequency (%)
SLA-2*05:02	133	28
SLA-2*04:01	118	25
SLA-2*06:02:01	97	24
SLA-2*10:01	80	21
SLA-2*10:04	58	16
SLA-2*11:05	16	13
SLA-2*08:01	36	12
SLA-2*04:02:02	73	11
SLA-2*01:01	22	7
SLA-2*10:06	30	7
SLA-2*06:02:02	7	6
SLA-2*05:03	22	6
SLA-2*02:02	12	6
SLA-2*05:04	9	6
SLA-2*10:03	4	4
SLA-2*08:03	13	3
SLA-2*06:04	3	3
SLA-2*09:03	4	3
SLA-2*07:01	5	3
SLA-2*04:02:01	4	3
SLA-2*06:16	3	3
SLA-2*09:02	7	3
SLA-2*05:01	2	2
SLA-2*06:02	3	2
SLA-2*06:10	2	2
SLA-2*11:04	8	2
SLA-2*04:09	2	2
SLA-2*09:01	6	2
SLA-2*12:01	1	1
SLA-2*12:02	2	1
SLA-2*01:02	4	1
SLA-2*10:05	5	1
SLA-2*03:01	1	1
SLA-2*06:05	1	1
SLA-2*07:03	5	1
SLA-2*08:07	1	1
SLA-2*16:03	4	1

Note.

[a] Bacteria colonies that have the particular SLA allele sequence.

CTL epitopes.

It has been reported that, during viral infection, a number of 9-10mer viral peptides become the main source of CTL epitopes only when they are produced from proteasome cleavage and subsequently assembled with MHC class I molecules (Gronostajski et al., 1985; Koopmann et al., 1997; Monu and Trombetta, 2007). Therefore, in this study we first employed a well-established computational algorithm- PepCleave II [http://peptibase.cs.biu.ac.il/PepCleave_II/runCleavageScore.php] to predict 9-mer peptides showing high probability to be proteasomal cleavage products from each input protein sequence from the PRRSV strain RFLP 1-7-4 (Ginodi et al., 2008). The predicted peptides were further ranked by another well-established epitope bioinformatic tool - NetMHCpan *ver* 4.0 according to their binding affinity to SLA class I alleles. Since the genotyping of the 8 pigs that we used to experimentally infect with the PRRSV RFLP1-7-4 consistently revealed a similar profile of popular SLA-1 and SLA-2 alleles, the 7 most popular alleles were used in the NetMHCpan: SLA-1:0401, SLA-1*0801, SLA-2*0101, SLA-2:0401,SLA-2:0502, SLA-2:1001 and SLA-3:0401. We prioritized those strong binders with affinity to multiple alleles. In order to exclude repetitive epitopes that overlap each other or fall into the same region of the genome, only those binders containing the empirically validated binding motifs to SLA-1*0401, SLA-2*0401 and SLA-3*0401 were selected (Pedersen et al., 2011, 2013, 2016). In this way of thorough screening, we selected a total of 86 epitopes spanning the coding sequences in PRRSV RFLP1-7-4 within 0–0.5% percentile rank, which were indicated as the strongest binders to the given alleles (Table 4). The amino acid sequences of these 86 epitopes are included in the Supplemental Table S1.

Table 4
A total of 86 synthesized peptides (9-mer or 10-mer) were based on the amino acid sequences from partial ORF1a and ORF1b, and from ORF2 to ORF7.

ORF	PRRSV proteins	# of peptides	amino acid position of peptides
ORFs2-7	E	3	6, 15, 25,
	GP2	7	23, 41, 50, 62, 73, 134, 153
	GP3	9	43, 65, 110, 118, 128, 150, 161, 171, 243
	GP4	7	1, 7, 70, 90, 110, 126, 156
	GP5	9	35, 52, 68, 79, 92, 104, 126, 144, 184
	GP5a	2	7, 20
	M	7	12, 24, 39, 67, 84, 108, 161
	Npr	3	58, 96, 104
ORF1b	Nsp8	1	29
	Nsp9	10	29, 198, 252, 279, 364, 405, 455, 477, 502, 583
	Nsp10	5	82, 161, 204, 298, 369
ORF1a	Nsp2	18	96, 124, 185, 196, 317, 498, 567, 585, 717, 731, 764, 818, 833, 845, 889, 980, 1000, 1020
	TF domain	6	4, 17, 31, 54, 104, 129

Note.

1. The amino acid position refers to that of the first aa of the peptide on its corresponsive protein.
2. TF domain of RFLP 1-7-4 contains 169 amino acid (aa) starting with the amino acid sequence LTLFPI. Additional 8 aa was put prior to the start of TF domain in PepCleave II Algorithum. Therefore the 1st aa position of TF domain here refers to the elongated 177 aa sequence, rather than the original 169aa.
3. One peptide from GP3 (GP3-3) failed in the synthesis.

3.3. PRRSV GP3 and GP4 contained four main epitopes that greatly stimulated CD4+CD8+ T and CD8+ T cell responses

To evaluate immuno-stimulatory efficiency of the synthesized epitope peptides, we first conducted an experimental PRRSV infection study in 3-week old specific-pathogen-free pigs. A total of 8 pigs were experimentally-infected with PRRSV strain RFLsP1-7-4 ($5X10^{3.5}$ $TCID_{50}$/mL) via a combination of intranasal (2 mL) and intramuscular (3 mL) routes of inoculation. PBMCs were isolated every week for 11 weeks for co-incubation with individual synthesized peptides. We found that, during the early stage of PRRSV infection from 3-6 weeks post-infection (wpi), all pigs had generally low frequencies of T cell subpopulations, with approximately 9–12% CD8+ T cells, 1–3% CD4+CD8+ (double positive, DP) T cells and 7–11% CD4+ T cells (data not shown). Possibly due to this reason, the IFN-γ and CD107a (a degranulation marker used to indicate T cell cytotoxicity) were expressed too scarcely in T cells to be compared among groups stimulated with different epitope peptides. Although anti-PRRSV T-cell response (TCRαβ) was previously reported to be strongest at 4–6 weeks post-infection (Talker et al., 2013), we did not find a strong T response at 4–6 wpi in this present study. In comparison, PBMCs isolated during the later stages of the infection after 8 wpi had at least twice as many of these three T cell subpopulations, and also had much more prominent expressions in IFN-γ and CD107a (Fig. 1). Therefore, in this study we mainly used PBMCs collected from experimentally-infected pigs from 8-11 wpi for use to incubate with each individual epitope peptide.

In the subsequent flow cytometry analyses of antiviral T cell responses, we found that the frequencies of total T cells and each T cell subsets were similar, either among different epitope peptides incubation within each individual pig sample of PBMCs, or compared with a separate group re-stimulated by PRRSV RFLP1-7-4 infection at 0.01 multiplicity of infection (m.o.i) (Fig. 1). These observations indicated that the contemporary PRRSV RFLP1-7-4 strain, like most of the pathogenic PRRS viruses, was not potent in stimulating anti-PRRSV T cell response. In spite of this, however, when compared to the non-stimulated group, we identified 2 epitope peptides from GP3 [GP3-1 (FSFELLVNY) and GP3-7 (ITAVYQTYY)], and another 2 epitope peptides

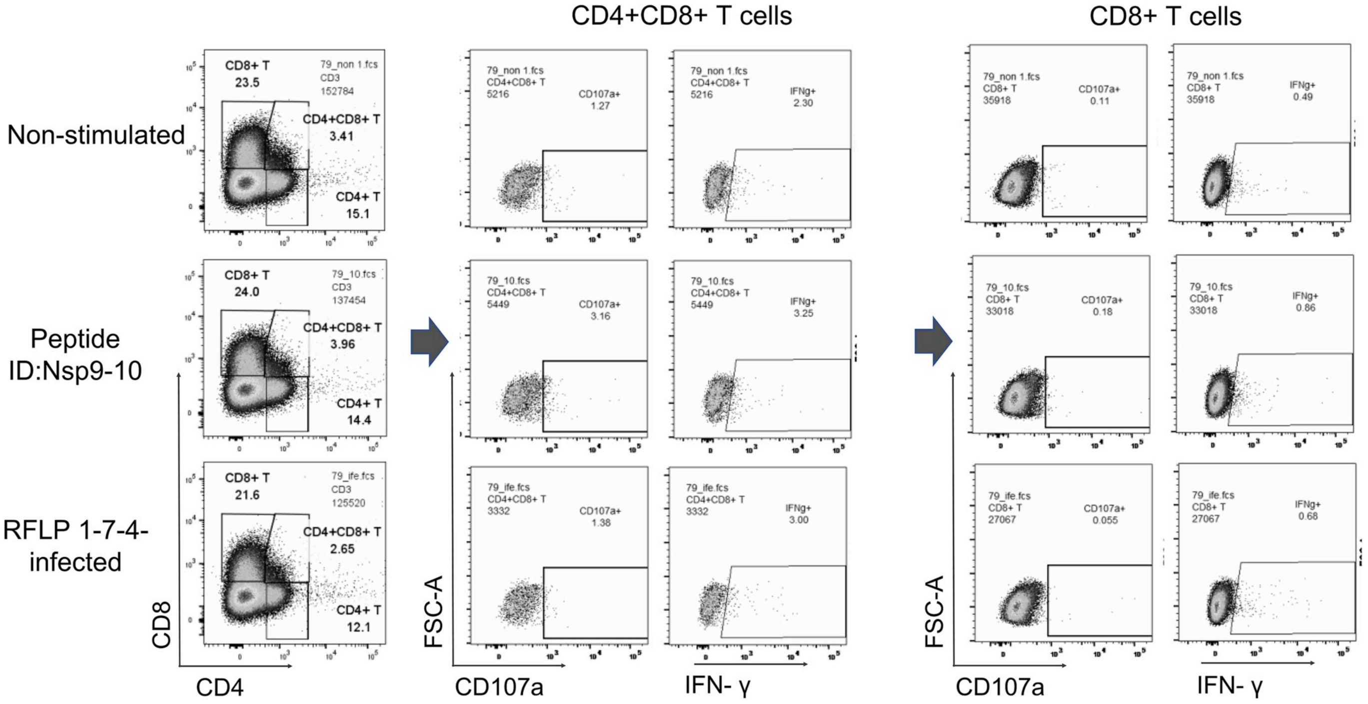

Fig. 1. Schematic diagrams of T cell gating strategy in the flow cytometry analyses. At 8–11 weeks post-infection (wpi), peripheral blood mononuclear cells (PBMCs) isolated from each of the 8 pigs experimentally-infected with PRRSV RFLP 1-7-4 were individually unstimulated (top lane) or stimulated with each of the 86 synthesized epitope peptides at a concentration of 6 μg per 1.5 million cells (middle lane). A separate group of PBMCs was infected with PRRSV RFLP 1-7-4 at a multiplicity of infection (m.o.i.) of 0.01 (bottom lane). In the flow cytometry analyses, only viable CD3 + T cells were further gated into CD4 + CD8 + DP T cell, CD8 + T cells, and CD4 + T cells. Each subpopulation of T cells was further analyzed for surface expression of degranulation marker CD107a and intracellular IFN-γ. Representative data were shown here with frequencies of each cell population noted at the upper right or left corner of each flow figure. The stimulation assay and flow cytometry were performed at least two or three times for each individual peptide tested in at least 5 different individual pigs. Data from repetitive experiments were pooled together for analysis. Samples with fewer than 40,000 live cells (by Aqua live/dead stain) were discarded to filter out false-high frequencies in gating.

from GP4 [GP4-1 (MAASFLFLL) and GP4-3 (RTAIGTPVY)] that significantly promoted DP T cell responses of both IFN-γ and CD107a in at least 5 of the 8 experimentally-infected pigs (Fig. 2A). At the same time, the same 2 epitope peptides from GP3 also enhance IFN-γ in CD8 + T cells, of which the extent seemed to be lower than the DP T cell response (Fig. 2B). Furthermore, we found that additional epitope peptides, GP5-5 (GLITVSAAGY) and M-4 (STNKVALTM), were able to stimulate IFN-γ response from DP T or CD8 + T cells, respectively (Fig. 2A-B). We did not further show CD107a expression in CD8 + T cells here, mainly because only PRRSV RFLP 1-7-4 infection, but not the epitope peptides, seemed to enhance CD107a + CD8 + T cells to some extent. It is also because that the actual cell counts of CD107a + CD8 + T cells were generally 3–5 times fewer than the CD107a + DP T cells, giving even smaller number of cell percentage (most < 0.09%) that prevented us from comparison (Fig. 1).

3.4. RNA-dependent RNA polymerase (RdRp) of PRRSV RFLP1-7-4 contained two CTL epitopes

In addition to identifying potential CTL epitopes from structural proteins, we also investigated whether the PRRSV non-structural region also harbors CTL epitopes. In the relatively conversed ORF1b encoding Nsp8, Nsp9, and Nsp10, we found that 2 epitope peptides in Nsp9 [Nsp9-9 (YASAAAILM) and Nsp9-10 (YSFPGPPFF)] consistently enhanced the IFN-γ response in DP T cells and CD8 + T cells (Fig. 3A). These 2 epitope peptides also increased degranulation in DP T cells (Fig. 3B). Furthermore, in Nsp2, the largest protein produced from ORF1a, several epitope peptides [Nsp2-1 (QTLKLPAAL), Nsp2-7 (SIFQAPFTL), and Nsp2-13(VVGPVGLGL)] were also found to slightly improve DP T cell responses (Fig. 4A) either in fewer numbers of pigs, or to a lesser extent of the improvement. Such T cell responses stimulated by the epitope peptides derived from the nonstructural proteins appeared to be less robust than those by epitope peptides from the structural proteins.

3.5. CTL epitopes identified in PRRSV RFLP 1-7-4 GP3 and RdRP also enhance the γδ T response while the TF domain and N contained two epitopes that diminish IFN-γ T responses

γδ T cells are able to produce IFN-γ as well as perform degranulation. In both young and older pigs, porcine γδ T cells present as a majority T cell subset with unknown roles in protection against PRRSV (Talker et al., 2013). Since we previously demonstrated that γδ T cells were responsive to PRRSV infection *in vivo* (Cao et al., 2018), in this study we further examined the γδ T cell response after incubation with synthetic epitope peptides (Fig. 5A). Three aforementioned CTLs epitope peptides (GP3-1, GP3-7, Nsp9-9) located in PRRSV GP3 and Nsp9 are found to profoundly stimulate both CD107a and IFN-γ production in γδ T cells, with the fourth epitope peptide Nsp9-10 (YSFPGPPFF) only effective in enhancing IFN -γ + γδ T cells (Fig. 5B). The same effects were also observed in another epitope peptide located in Nsp2 (Nsp2-3: ALAEMSGNF).

Interestingly, one peptide in −2 frameshifting product TF domain [TF-6 (SVLPFLVGY)] (Fig. 4B), another peptide in ORF1b [Nsp10-5 (FVYDPHRQL)] (Fig. 3A), and a third epitope peptide from ORF7 [N-2 (GRISYTVEF)] (Fig. 2B) are found to readily inhibit IFN-γ response in CD8 + T or DP T cells. Two of these three epitope peptides, TF-6 and N-2, were also able to downregulate IFN-γ response in γδ T cells (Fig. 5B. TF-6: $p = 0.53$).

In summary, a number of immuno-stimulatory CTL epitopes were successfully identified through the computational prediction-flow cytometry evaluation pipeline described in this study. Among them, 2

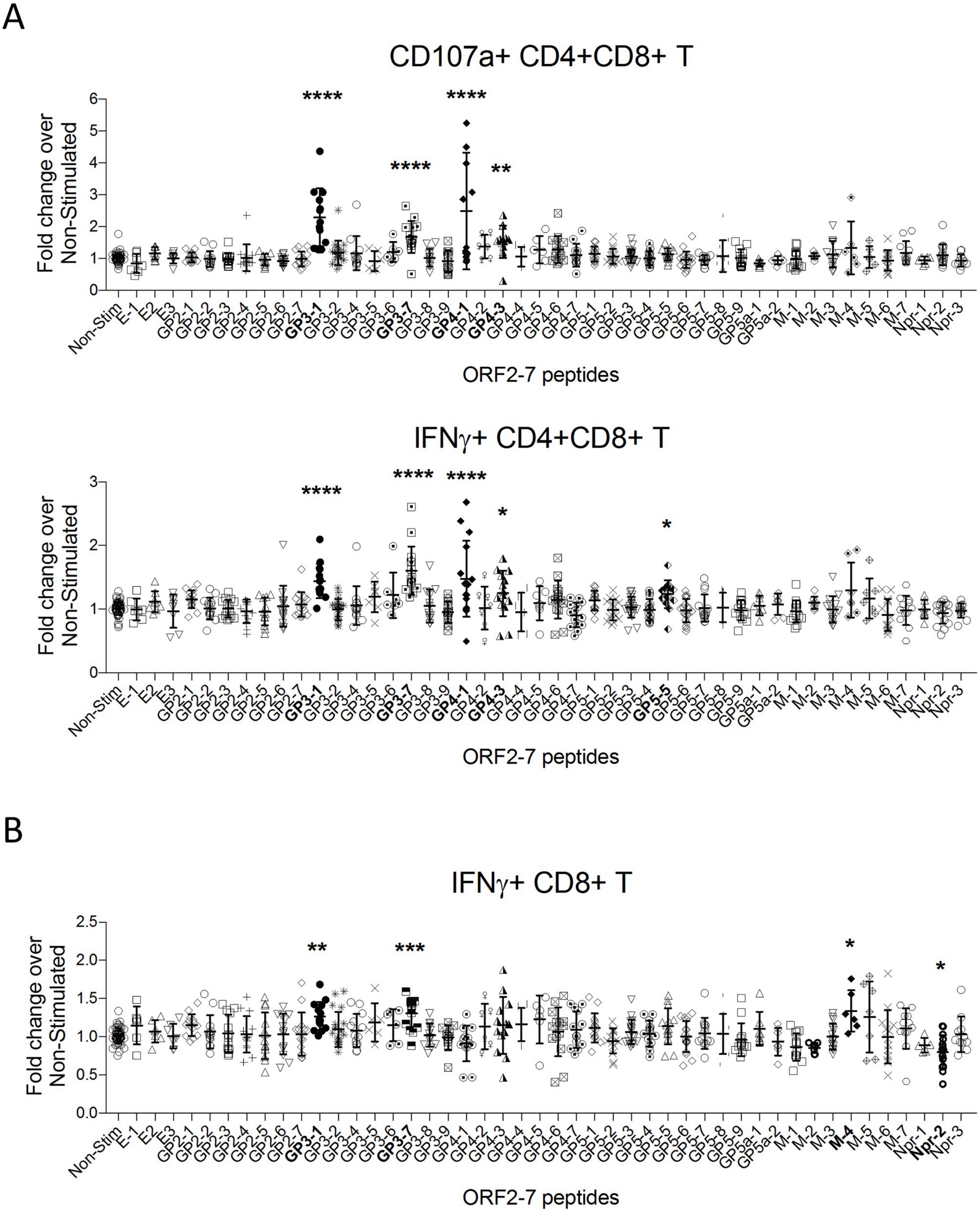

Fig. 2. CTL epitopes identified from PRRSV ORF2 to ORF7 enhance IFN-γ+ and CD107a+ DP and CD8+ T responses. For each epitope peptide-stimulated PBMC sample from each individual pig, the frequencies of IFN-γ+ and CD107a+DP T cells were normalized to that in an unstimulated sample from the same individual pig. The frequency of IFN-γ+ CD8+ T cells was normalized to the unstimulated control sample. The normalized value for each sample was referred to as fold change over non-stimulated on the y-axis. (**A**) shows CD107a+ and IFN-γ+ DP T cells in each peptide-stimulated sample. (**B**) shows of IFN-γ+ CD8 T cells in each sample. Data was analyzed by one-way ANOVA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

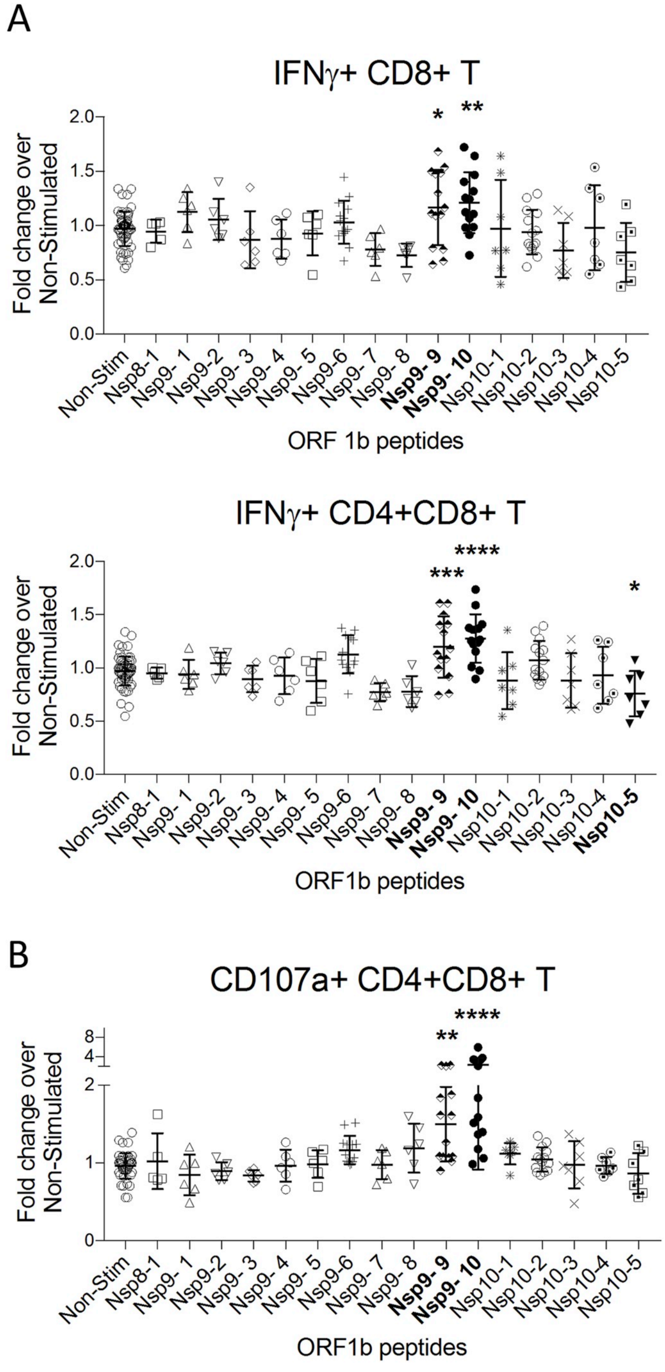

Fig. 3. PRRSV ORF1b contained CTL epitopes stimulating both DP T and CD8+ T cell response. PBMCs from each individual pig were similarly incubated with synthesized epitope peptides from Nsp8-10. The frequency of IFN-γ in CD8+ T cells and DP T cells (**A**), as well as the frequency of CD107a in DP T cells (**B**) in each sample were compared to that of unstimulated control sample from the same individual pig.

epitope peptides from GP3 (GP3-1: FSFELLVNY and GP3-7: ITAVYQTYY) and 2 epitope peptides from Nsp9 (Nsp9-9: YASAAAILM and Nsp9-10: YSFPGPPFF) were most efficient in stimulating both CD107a and IFN-γ response in all possibly cytotoxic T cells including CD4+CD8+ T cells, CD8+ T cells, and γδ T cells. Additionally, 2 peptides in GP4 (GP4-1: MAASFLFLL and GP4-3: RTAIGTPVY) were demonstrated to effectively activate DP T cells. Other CTL epitopes identified in this study include GP5-5 (GLITVSAAGY) and M-4 (STNKVALTM), Nsp2-1 (QTLKLPAAL), Nsp2-7 (SIFQAPFTL), and Nsp2-13 (VVGPVGLGL), which were shown to facilitate mostly IFN-γ responses. It is worth mentioning that CTL epitopes from structural proteins seemed to be more potent in immune stimulation when compared to those from non-structural proteins. On the contrary, we also identified 2 epitope peptides, TF-6 (SVLPFLVGY) and Npr-2 (GRISYTVEF), which had inhibitory effects on IFN-γ production both in CD8+ T cells and γδ T cells. Because of the extensive heterogeneity among PRRSV strains, these CTL epitopes identified in PRRSV strain RFLP 1-7-4 were mostly not conserved, except for a few such as STNKVALTM in M, YASAAAILM in N, and YASAAAILM in Nsp9 which can also be found in several other prevalent PRRSV strains.

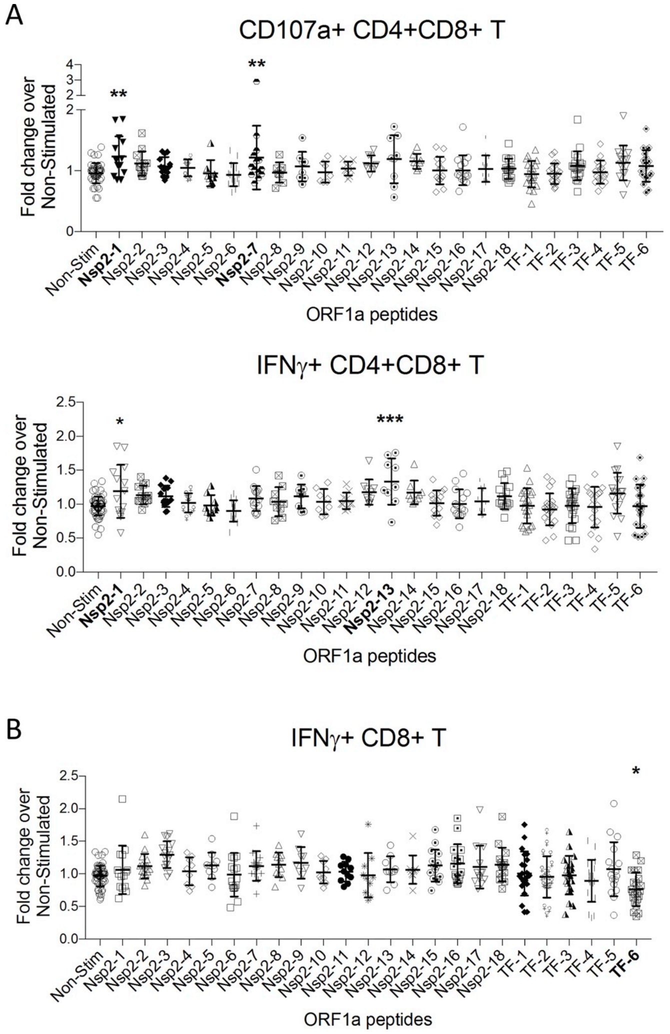

Fig. 4. Identification of CTL epitopes in PRRSV ORF1a. PBMCs from each individual pig were stimulated with synthesized epitope peptides from Nsp2 and Nsp2TF. The frequency of IFN-γ and CD107a in DP T cells (**A**), and the frequency of IFN-γ CD8+T cells (**B**) in each sample were compared to that of unstimulated control sample from the same individual pig.

4. Discussion

PRRSV emerged as the most economically important swine pathogen worldwide since the late 1980s. Current vaccines against PRRSV are not entirely satisfactory. The commercial modified live-attenuated vaccines (MLVs) are protective against homologous or closely-related strains but generally less effective against heterologous strains. The major barriers for MLVs to achieve effective heterologous cross-protection include antigenic variations in neutralizing epitopes, T-helper epitopes, and CTL epitopes, as well as negative modulation of host

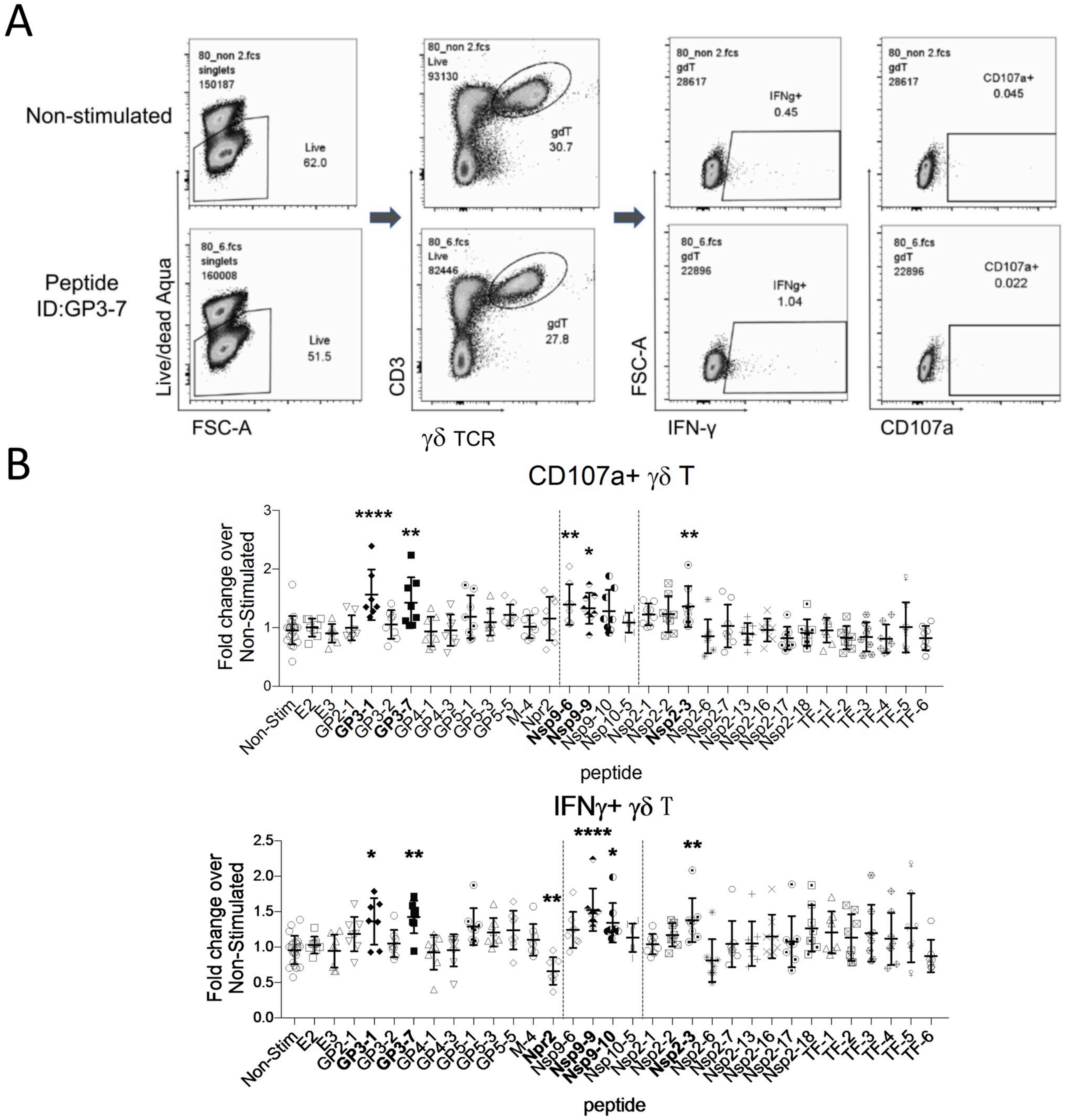

Fig. 5. **γδ T cell generated cytotoxic responses to epitope peptide stimulation by producing CD107a and IFN-γ.** (**A**) Gating strategy of γδ T cells. γδ T cells were designated as CD3+ γδ TCR+cells from live cell gating. They were further analyzed for surface CD107a and intracellular IFN-γ. Representative data are shown for non-stimulated (top) and one sample stimulated with a peptide from PRRSV GP3. (**B**) Frequencies of CD107a+ or IFN-γ+ γδ T cells from each sample. The vertical dashed line in the figure separates peptides from different ORFs of PRRSV.

immune responses by PRRSV.

A few studies have attempted to identify potential T cell epitopes in PRRSV-1 or PRRSV-2 by generating overlapping peptide libraries on a specific protein(s), which was costly and labor-consuming. Some also utilized well-trained computational algorithms to perform initial predictions, mostly according to the probable affinity of epitopes to class I alleles (Diaz et al., 2009; Pedersen et al., 2016; Welner et al., 2017). Since only the 8–10 mer viral peptides cleaved by proteasomes have a chance to evade further degradation and associate with class I alleles in order to eventually be CTL epitopes, in this present study we conducted an algorithm-based prediction of proteasomal cleavage products to enrich our epitope peptide pool. After proteasome cleavage, only class I/epitope complexes are eventually presented to and recognized by CTLs.

We first determined the most prevalent SLA class I alleles in commercial pig farms in the United States, since genotyping the frequency of the most prevalent class I alleles in commercial pigs in the United States is not only important for investigating CTL epitopes of PRRSV, but also of great value to the swine research community for rational design of veterinary vaccines against other important swine pathogens. It has been reported that the SLA-1 locus usually has a higher expression level compared to other classical class I SLA loci in inbred pigs

(Lunney et al., 2009; Tennant et al., 2007). However, in the present study, we found that SLA-2 alleles are more highly expressed than SLA-1 alleles in outbred commercial pigs in the United States. This may be due to the genetic diversity among different pig herds, or to the different SLA-typing methods used in different studies (Gao et al., 2017; Pedersen et al., 2014; Xia et al., 2014). The SLA-typing method used in this present study is a cloning-based direct Sanger sequencing approach, which is considered to be a direct and accurate method, but this approach also has its limitations due to the large numbers of colonies needed for adequate coverage (Lunney et al., 2009; Schwartz et al., 2018). A larger panel of pig samples with next-generation sequencing technology may yield more insight regarding the SLA expression profiles in outbred pigs in the United States (Sorensen et al., 2017).

This present study is the first of its type to use flow cytometry to evaluate PRRSV CTL epitope screening. By thorough analysis of different T cell populations, we were able to not only identify CTL epitopes (FSFELLVNY, ITAVYQTYY, YASAAAILM, YSFPGPPFF) efficiently stimulating CD8 +, DP, as well as γδ T cells, but also identified additional epitopes showing different efficiencies of activation or profiles on stimulated cells, such as MAASFLFLL and RTAIGTPVY in GP4, and GLITVSAAGY in GP5, STNKVALTM in M protein and another 4 epitopes in Nsp2. Additionally, two important epitopes FSFELLVNY and ITAVYQTYY, both located in the PRRSV GP3, are likely N-glycosylated when being translated, since they were flanked by Asparagine and/or Threonine. Therefore, glycosylation may have an impact on the processing and functions of T cell epitopes during PRRSV infection.

Recently studies have investigated host-virus interactions of the unique −2 PRF protein NspTF of PRRSV. Our previous work demonstrated that the knock-down of TF domain expression by disrupting the −2 frameshifting motif reversed the PRRSV-induced downregulation of class I molecules (Cao et al., 2016). Subsequently, another group showed that abolishing Nsp2TF expression by impairing the trans-activator of −2 PRF in PRRSV could increase IFN-α, IFN-γ production *in vivo* and enhanced NK cell function (Li et al., 2016). Interestingly, in the present study, we also identified one epitope peptide in the TF domain (SVLPFLVGY) that exhibited inhibitory effects on IFN-γ production both in CD8 + T cells and γδ T cells. These findings suggest that either disruption of −2 PRF or mutation of inhibitory epitopes in TF domain could serve as a potential strategy to improve PRRSV vaccines towards enhanced immune responses.

PRRSV infection, in general, leads to delayed and weak adaptive immune response. Consistently in this study, we found that PRRSV strain RFLP1-7-4, a prevalent contemporary pathogenic strain, was not able to activate the proliferation of T cells. Re-stimulation of PBMCs led to only slightly enhanced IFN-γ or CD107a expression in T cells. Nevertheless, this enhancement was not consistent in different experimentally-infected pigs (data not shown).

Additionally, from the results of flow cytometry analyses, the CD4 + CD8 + DP T cells appeared to be most active in expressing antiviral IFNγ and degranulation marker CD107a on surface. CD8 + T cells, on other hand, had much lower expression of CD107a upon stimulation. Likewise, it has been also reported that CD4-CD8 + CTL phenotype, although cytotoxic in most other animals, did not meditate anti-PRRSV CTL activities (Chung et al., 2018). As for IFN-γ production, fewer peptides were shown to significantly enhance IFN-γ + CD8 T cells when compared to CD4 + CD8 T cells. These observations were consistent with our previous report on T cell responses against cytokine-incorporating viruses as MLV candidates (Cao et al., 2018). Unfortunately, the exact mechanisms for these observations are unknown. Future studies are warranted to determine why DP T cells tend to be more responsive in anti-PRRSV T cell response when compared to CD8 + T cells. As there is a pressing need for detailed evaluation of PRRSV-specific T cell immunity, the results from this study provided further insight into the understanding of the dynamics and functions of antigen-specific T cells in PRRSV-infected pigs.

Declaration of interest

The co-authors Dennis Foss and Jay Calvert are employees of Zoetis Inc.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.09.006.

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