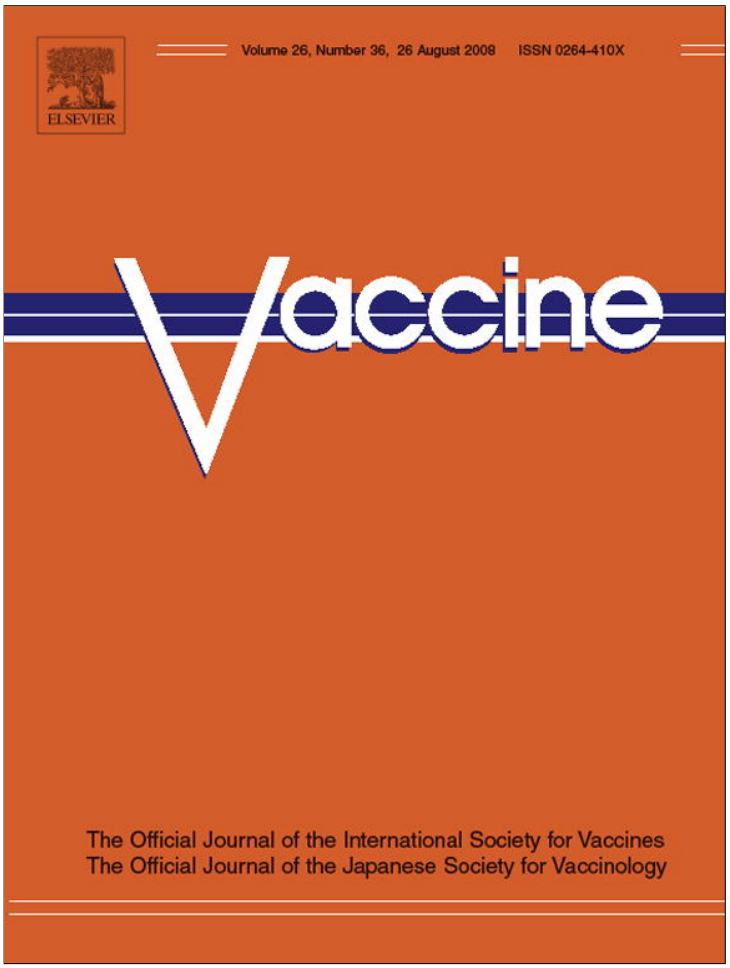

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Identification of immunodominant T-cell epitopes present in glycoprotein 5 of the North American genotype of porcine reproductive and respiratory syndrome virus

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ABSTRACT

Ninty-six pentadecapeptides spanning glycoprotein 5 (GP5) of porcine reproductive and respiratory virus (PRRSV) were screened for their ability to elicit a recall interferon-γ response from peripheral blood mononuclear cells isolated from 22 pigs infected with up to two genetically divergent PRRSV strains. Two distinct regions (amino acid residues 117–131, LAALICFVIRLAKNC, and 149–163, KGRLYRWRSPVII/VEK) appeared to contain immunodominant T-cell epitopes based on their ability to stimulate above average numbers of interferon-γ secreting cells as compared to other GP5 peptides. A survey of PRRSV isolates indicated that these two sites are relatively conserved with at most a two amino acid variation and thus should be considered for incorporation into a multi-valent vaccine against PRRS.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped RNA virus that has had a devastating economic impact on the swine industry [1]. Its considerable genetic and antigenic heterogeneity [2–4] has so far impeded the development of an effective vaccine against this pathogen [3]. When addressing this issue, it should be noted that cell-mediated immunity is a critical component of the host response against invaders such as PRRSV that replicate intracellularly [5]. Accordingly, some studies have focused on identifying T-cell epitopes and have been facilitated by the use of peptide libraries [6,7]. Using this approach, analyses designed to detect T-cell epitopes present in viruses that affect domestic animals have been accomplished [8,9]. This is feasible because MHC molecules on the surface of lymphocytes can be loaded with peptide ligands [10]. For instance, overlapping 15 amino acid peptide libraries have been used in conjunction with cytokine assays to identify epitopes recognized by antigen-specific $CD4^+$ and $CD8^+$ T-cells [6,8,9]. Further, archived samples can be used since the $CD4^+$ and $CD8^+$ subsets of human peripheral blood mononuclear cells (PBMC) have been shown to retain full functionality in cytokine assays such as the ELIspot [11] after recovery from cryopreservation.

Currently, unlike for some pathogens, limited information regarding T-cell epitopes in PRRSV proteins is available [12]. Thus, the goal of this study was to identify the site(s) of immunodominant T-cell epitopes for this virus's glycoprotein 5 (GP5) by using the methodology described above. In this case, the ability of individual, overlapping pentadecamer peptides designed to represent the entire PRRSV GP5 to induce interferon (IFN)-γ production (cell-mediated immune response) from PBMC obtained from PRRSV-infected pigs was assessed. GP5 was selected because it is the major transmembrane glycoprotein, has been well-characterized, is very antigenic and antigenically variable, and contains known B-cell epitopes [13–18]. To evaluate the importance of known viral genetic variation in GP5, two PRRSV strains, NADC-9 and NVSL-14, whose GP5 primary sequences differ by 10%, were included in the study. While pigs were initially vaccinated with attenuated versions of each strain, they were subjected to either autogenous or heterogenous challenge with one of the wild-type parental strains. Based on the extent of the measured, *in vitro* cell-mediated immune response of PBMC derived from pigs exposed to one or both virus strains to the peptides, two distinct regions of GP5 were identified as T-cell epitopes. Although strongly conserved between the two examined virus strains, variability within these two sites was noted when additional PRRSV isolates were considered.

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2. Materials and methods

2.1. Animals and housing facility

Twenty-two Yorkshire × Landrace crossed and out-bred, age-matched, recently weaned PRRS-naïve pigs randomly assigned from 6 litters, purchased from an established PRRS-negative university source herd were used in this study. These animals constituted part of a larger research project conducted by our laboratories. All pigs were housed in one of four units at the Edward R. Madigan bio-containment facility, University of Illinois. Each unit was equipped with separate ventilation, water, and waste disposal systems designed to avoid cross-contamination. All procedures were performed in compliance with the University of Illinois IACUC under protocol #05190.

2.2. Virus strains

NADC-9 and NVSL-14 attenuated (att) and wild-type (wt) strains of PRRSV were used for vaccination and challenge in this study. First described by Mengeling et al. [19], these four viruses are well characterized, have been utilized by other researchers, and exhibit low or moderate pathogenicity in swine [20–22]. Attenuation of both wt strains was achieved during serial passage in cultured cells [19]. The extent of identity between the primary structures of the viruses' GP5 is shown in Table 1. Virus inocula for animal injection were kindly provided by Dr. K. Lager and A. Vorwald (NADC, Ames, IA). PRRSV VR-2332, used as an immunogen in some of the ELIspot assays, was propagated and titrated in MARC 145 cells in our laboratory.

2.3. Experimental design and sample collection/preservation

Pigs were randomly assigned to one of four, four or six member groups (Table 2). All animals were vaccinated at 4 weeks of age, and then challenged 6 weeks later by intramuscular injection with 2×10^6 $TCID_{50}$ of the indicated wt PRRSV strain. The selected dosage and route of inoculation were based on methods used in previous studies [4,19]. All animals were bled at 10 weeks of age (prior to virus challenge) and 2 weeks later (prior to euthanasia). PBMC were separated from the blood samples by density gradient separation using Ficoll as described previously [23]. Purified PBMC were cryopreserved at a concentration of 3×10^7 viable cells/ml and later thawed according to conditions described by Disis et al. [24] and in protocols provided by Dr. Holden Maecker's laboratory (http://maeckerlab.typepad.com/maeckerlab_weblog/files/012505Freezethaw.pdf).

2.4. PRRSV GP5 peptide design

As described by Anthony and Lehmann [25] for T-cell epitope mapping, pentadecamer peptides with overlaps of 11 amino acids starting at position 5 of the att and wt NADC-9 and NVSL-14 GP5 were designed to span the entire protein. Due to inter-strain variation, the four viruses' GP5 amino acid sequences were aligned using MEGA version 3.1 [26] and the peptide sequences parsed using the computer program peptide chopper, kindly provided by Dr. Smita Ghanekar (BD Biosciences, San Jose, CA). Of the resultant, unique 100 peptides, 96 were successfully synthesized using 9-fluoronylmethoxycarbonyl (Fmoc) chemistry and verified to be ≥80% purity by using HPLC and mass spectrometry (Sigma Genosys, St. Louis, MO). Peptides were synthesized using an automated robotic platform in a 96-well format with 8 channels (columns A-H) and 12 rows. The alphanumerical designation of the peptides corresponds to the location of a given peptide in the 96-well synthesis plate. For example, the peptide representing the amino acid sequence at GP5 positions 117–131 happened to be in the G column and 9th row of the synthesis plate and was thus called G9. As can be seen in Table 6 there was no inter-strain variation in the amino acid sequence of this particular section of GP5 and thus just one peptide (G9) was needed to represent this sequence. In contrast, there was one amino acid difference between NADC-9 and NVSL-14 in the peptide representing the amino acid sequence at GP5 positions 149–163 of these two viruses. Thus, two peptides were produced to cover this variable sequence and their location in the synthesis plate was G10 and H10, respectively (see Table 6). After the initial screening of the peptide pools and identification of immunodominant epitopes, the G9, G10 and H10 peptides were selected for further investigation and synthesized at ≥95% purity. Peptide stock solutions (20 mg/ml) were prepared by dissolving freeze-dried peptides in DMSO (Sigma–Aldrich, St. Louis, MO). Working solutions of peptides at a final concentration, in the well, of 10 μg/ml individually or in pools (see below) were prepared in RPMI medium supplemented with 5% fetal calf serum. Titration experiments indicated that 10-μg/ml of peptide gave a maximal response.

Table 1
Extent of identity (%) of amino acid sequence of GP5 of PRRSV strains used in this study

	NADC-9 att[a]	NADC-9 wt[b]	NVSL-14 att	NVSL-14 wt
NADC-9 att	100	95	90	90
NADC-9 wt	95	100	90	90
NVSL-14 att	90	90	100	99
NVSL-14 wt	90	90	99	100

[a] Attenuated.
[b] Wild-type.

Table 2
Description of treatment groups[a]

Group	Vaccine virus	Challenge virus	No. animals/group
1	NADC-9 att[b]	NADC-9 wt[c]	6
2	NADC-9 att	NVSL-14 wt	6
3	NVSL-14 att	NVSL-14 wt	4
4	NVSL-14 att	NADC-9 wt	6

[a] Animals vaccinated at 4 weeks of age and challenged at 10 weeks of age with the indicated virus.
[b] Attenuated.
[c] Wild-type.

2.5. IFN-γ ELIspot assay

The frequencies of antigen-specific IFN-γ-secreting cells per million PBMC were determined by using a previously described ELIspot assay [23,27]. This test was selected since it is sensitive and is a well-established technique to identify T-cell epitopes [8,25,28,29]. In this assay, 5×10^5 or 10×10^5 PBMC/well were incubated with either individual or pooled peptides, 10^4 $TCID_{50}$ of one of the four PRRSV strains used in the study or the North American prototype strain VR-2332, 20 μg/ml phytohemagglutinin (PHA, positive control to verify responsiveness of the cells), or culture medium. The frequency of antigen-specific IFN-γ-secreting cells in each PBMC population was determined as the average number of spots in duplicate PBMC cultures stimulated with virus or peptide minus the number of background spots in duplicate PBMC cultures exposed to diluent (culture medium) only. The data was expressed as the number of IFN-γ-secreting cells per one million PBMC.

Table 3
Matrix used to prepare peptide pools and to select peptides for evaluation[a]

	I	II	III	IV	V	VI	VII	VIII	IX	X
XI	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11
XII	A12	B1	B2	B3	B4	B5	B6	B7	B8	B9
XIII	B10	B11	B12	C1	C2	C3	C4	C5	C6	C7
XIV	C8	C9	C10	C11	C12	D2	D3	D4	D5	D6
XV	D7	D8	D9	D10	D11	D12	E1	E3	E4	E5
XVI	E6	E7	E8	E9	E10	E11	E12	F1	F2	F3
XVII	F4	F5	F6	F7	F8	F9	F10	F11	F12	G1
XVIII	G2	G3	G4	G5	G6	G7	G8	**G9**	**G10**	**G11**
XIX	G12	H1	H3	H4	H5	H6	H7	**H8**	**H9**	**H10**
XX	H11	H12	A13	B13	C13	D13				

[a] Peptide pools are indicated by Roman numerals. The individual 15 amino acid long peptides are identified by alphanumerical designation based on their location in the 96-well peptide synthesis plate. Pools VIII, IX, X, XVIII and XIX elicited IFN-γ responses greater than the averaged value in all selection criteria (see Table 4). Cross-matching of these positive pools in the matrix indicated six peptides (G9, G10, G11, H8, H9, H10; in bold) that could potentially have immunodominant epitopes and thus were selected for individual testing. Matrix design based on Hoffmeister et al. [30].

2.6. Analysis of the stimulation of PBMC with PRRSV GP5 peptides

Initially, all of the peptides were distributed into 20 pools (composed of 10 different members, except pools VII, VIII, IX, X and XX which contained a lesser number of peptides) based on a published matrix [11,25,30] (Table 3). Each pool was screened for its ability to stimulate an IFN-γ response in an IFN-γ ELIspot assay using fresh PBMC obtained from PRRSV-vaccinated animals at 2 weeks post-challenge (Table 4). Subsequently, individual peptides, identified as being potentially stimulatory based on analysis of the initial peptide pool data, were tested with cryopreserved PBMC originating from the PRRSV-challenged pigs. To control for non-specific stimulation by the peptides, two conserved peptides, H4 (LIYNLTLCELNGTDW) and H5 (ANEFDWAVECFVIFP), were selected from the PRRSV GP5 peptide library for use as individual negative controls. These choices was made after the initial peptide pool screen shown in Table 4 and was based on the subsequent established inability of peptides H4 and H5 to stimulate a detectable IFN-γ response in PBMC from highly PRRSV-responsive pigs.

2.7. Depletion of T-cell subsets from PBMC to define the phenotype of the peptide-responsive cells

$CD3^+$, $CD4^+$, $CD8\alpha^+$, and $CD8\beta^+$ T-cell subsets were separately depleted from PBMC populations obtained from 4 PRRSV-immunized and challenged pigs which had been identified as producing a strong IFN-γ response to GP5 epitopes and the intact virus. The selected animals had been vaccinated with either att NADC-9 or NADC-14 virus and then challenged with the respective, heterologous wt virus. Depletion was accomplished by using low-toxicity rabbit complement (Pel-Freez® Biologicals, Rogers, AR) and T-cell subset specific antibodies CD3 (8E6), CD4 (74-12-4), CD8α (76-2-11), and CD8β (PG164A) (VMRD, Inc., Pullman WA). [31] as previously described [23]. Flow cytometry was used to verify the composition of the depleted samples and was performed using a COULTER® EPICS® XL™ flow cytometer (Beckman Coulter, Inc., Miami, FL) at the Veterinary Medicine flow cytometry satellite facility of the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign (http://www.biotech.uiuc.edu/index.html). The depleted cell populations as well as a non-depleted sample exposed to medium or complement only were counted after the final post-depletion wash and used in ELIspot assays at 5×10^5 cells/well. Each population (medium treated, complement only treated, and monoclonal antibody plus complement treated)

Table 4
Selection of peptide pools likely to contain immunodominant PRRSV GP5 epitopes

Selection criteria	IFN-γ response and number of responding pigs to the indicated peptide pool[a]																		Mean ±S.D. of pools[b]
	I	II	III	IV	V	VI	VII	VIII[c]	IX[c]	X[c]	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII[c]	XIX[c]	XX	
Maximum response[d]	3	5	3	5	7	3	3	8	16	9	4	4	4	5	3	5	5	10	13	3	6 ± 3.6
Total response[e]	7	22	9	21	22	15	7	35	54	48	17	15	6	8	20	19	24	29	51	14	22 ± 14.6
Average response[f]	0.3	1.0	0.4	1.0	1.0	0.7	0.3	1.6	2.5	2.2	0.8	0.7	0.3	0.4	0.9	0.9	1.1	1.3	2.3	0.6	1 ± 0.7
No. of responsive pigs[g]	3	5	3	5	5	5	2	8	9	12	6	4	1	1	9	5	6	6	9	4	5.4 ± 2.8
Avg. responsive pigs[h]	1.8	3.7	1.8	2.1	2.4	1.9	1.8	3.9	5.4	3.4	1.7	2.1	2.0	2.0	1.8	1.7	2.4	3.6	4.6	1.4	3 ± 1.1

[a] Peripheral blood mononuclear cells (PBMC) were isolated from 22 pigs, which had been previously immunized and challenged with PRRSV as described in Section 2. PBMC samples were individually tested against the indicated peptide pool. Except for row 4 (see [g]), listed values represent the number of IFN-γ producing cells per million PBMC detected by ELIspot against the indicated peptide pool minus the background response detected in mock stimulated cell cultures. In all cases the background response to mock stimulation with diluent alone (culture medium) was ≤5 spots (range 0–5). Roman numerals indicate the identity of the peptide pool (Table 3) used to stimulate the PBMC cultures.

[b] Mean ± standard deviation of the values obtained for each of the 20 pools for each of the selection criteria. These averages were used to identify the peptide pools likely to contain peptide(s) with an immunodominant epitope.

[c] Peptide pools that that generated values greater than or equal to the mean value for all of the selection criteria.

[d] Maximum response: number of IFN-γ producing cells detected in PBMC from the highest responder pig among all 22 PBMC samples tested.

[e] Total response: sum of all of the IFN-γ producing cells (minus background) detected in the 22 individually tested PBMC samples.

[f] Average response: sum of all of the IFN-γ producing cells (minus background) detected in the 22 PBMC samples tested divided by 22, the number of pigs tested.

[g] Number of responsive pigs: number of pigs whose PBMC exhibited a peptide-specific IFN-γ response with an stimulation index ≥ 2. If background response was zero the response to the peptide had to be ≥2 to be considered positive.

[h] Average response of responsive pigs: Average of the peptide-specific IFN-γ response (minus background) of all the pigs exhibiting a response to the peptide pool.

was stimulated with medium only (spontaneous response), PHA, PRRSV VR-2332, or the indicated peptides.

2.8. Identity comparison of PRRSV GP5 peptides

The amino acid sequences of selected T-cell epitopes were aligned to the respective regions of GP5 of various PRRSV strains using the CLUSTAW program in Biology WorkBench 3.2 (http://workbench.sdsu.edu). The following PRRSV strains (GenBank accession numbers in parentheses) were used: the wt challenge and att vaccinating NADC-9 (AF396836 and AF396837, respectively) and NVSL-14 (AF396839 and AF396840, respectively) viruses used in this study; the ATP (EF442774), PrimePac (AF066384), and RespPRRS (AF066183) commercial vaccines; the FL-12 (NVSL 97-785, AY545985) and P129 (AF494042) infectious clones; and the prototype VR-2332 (U87392). In addition, a comparison to other PRRSV GP5 listed in GenBank was performed using the BLAST function (http://www.ncbi.nlm.nih.gov/BLAST/).

3. Results

3.1. Identification of PRRSV GP5 T-cell epitopes

Previously, a stimulation index (frequency of IFN-γ secreting cells in a PBMC population responding to an antigen divided by that value obtained with a negative control) has been used to evaluate the intensity of an elicited, *in vitro* cell-mediated immune response. In that case, a quotient of 2 or more is considered to be biologically significant ([32,33], and H. Maecker, personal communication). In addition, for this study immunodominant peptides were identified based on the following, additional criteria: (1) the maximum response, namely the number of IFN-γ producing cells detected in PBMC from the highest responder pig among the 22 animals tested; (2) the total response, namely the sum of all of the IFN-γ producing cells (minus background) detected in the 22 individually tested PBMC samples; (3) the average response, namely, the sum of all of the IFN-γ producing cells (minus background) detected in the 22 PBMC samples divided by 22, the number of pigs tested; (4) the number of peptide-responsive pigs, namely the number of pigs whose PBMC exhibited a peptide-specific IFN-γ response with an stimulation index ≥2. For instance, if the background response was zero then the response to the peptide had to be ≥2 IFN-γ producing cells to be considered positive; (5) the average response of peptide-responsive pigs, namely, the average of the peptide-specific IFN-γ response (minus background) of all the pigs exhibiting a response to the peptide. Using these criteria, the overall immune responses of PBMC from the PRRSV-challenged pigs to peptide pools VIII, IX, X, XVIII and XIX (Table 3) were greater than or equal to the average responses noted for all 20 pools combined (Table 4). By cross-matching the members of these 5 pools, six peptides, G9, G10, G11, H8, H9, and H10 were identified as potentially having immunodominant epitopes (Table 3). It should be noted that PBMC isolated from the naïve animals (i.e., before PRRS vaccination) did not exhibit a PRRSV-specific IFN-γ response to any of the peptide pools or the six selected peptides (data not shown). Thus, we preliminarily concluded that virus-specific T-cells capable of secreting IFN-γ upon stimulation with their specific peptide recall antigen were being detected in the PBMC from PRRSV-immune pigs. Subsequently, a secondary round of screening using cryopreserved PBMC from the same PRRSV-vaccinated and challenged animals, indicated that peptides G9, G10, and H10 elicited comparatively stronger IFN-γ responses than the other three peptides G11, H8 and H10 (Table 5). As before with the peptide pools deemed to contain T-cell epitopes, the individual effects of peptides G9, G10 and H10 were greater in all five of the selection criteria than the average values determined using the six selected peptides and thus these three peptides were considered to contain immunodominant epitopes. During the analysis of the individual peptides we identified several peptides that did not stimulate a recall IFN-γ response in PBMC populations, which were highly responsive to peptides G9, G10 and H10. The sequence of two such peptides (H4 and H5) is listed in Section 2. The lack of reactivity to peptides H4 and H5 by PBMC that otherwise were highly responsive to peptides G9, G10 and H10, together with the lack of reactivity to the latter peptides by PBMC isolated from non-immune pigs, indicates that the IFN-γ response against these three peptides is a recall response mediated by PRRSV-specific memory T-cells.

To verify the reactivity of these three peptides, they were re-synthesized to a higher purity (≥95% versus ≥80%) and then evaluated in an ELIspot assay with cryopreserved PBMC from the 22 PRRSV-vaccinated pigs. In each case, the response was approximately 80–90% of that observed with the original peptides (data not shown). Thus, the strong reactivity to these three peptides was not due to unknown contaminating peptides and did indeed repre-

Table 5
Identification of peptides containing an immunodominant PRRSV GP5 epitope

Selection criteria	IFN-γ response to the indicated peptide[a]						Mean ± S.D. of peptides[b]
	G9[c]	G10[c]	G11	H8	H9	H10[c]	
Maximum response[d]	23	42	8	12	5	41	21.8 ± 16
Total response[e]	128	173	26	29	9	144	84.8 ± 21
Avg. response of all pigs[f]	5.8	7.9	1.2	1.3	0.4	6.5	4.0 ± 3
No. of responsive pigs[g]	9	11	5	4	2	10	6.8 ± 3
Avg. responsive pigs[h]	10.7	14.4	4.0	3.0	3.0	13.1	8.0 ± 5

[a] Peripheral blood mononuclear cells (PBMCs) were isolated from 22 pigs, which had been previously immunized and challenged with PRRSV as described in Section 2. These PBMC samples were individually tested against the indicated peptide. Listed values represent the number of IFN-γ producing cells per million PBMC detected by ELIspot against the indicated peptide minus the background response detected in mock stimulated cell cultures. In all cases the background response to mock stimulation with diluent alone (culture medium) was ≤6 spots (range 0–6). In addition, stimulation with the negative control peptides H4 and H5 of four different PBMC populations highly responsive to peptides G9, G10 and H10, did not elicit a detectable IFN-γ response.

[b] Mean ± standard deviation of the values shown for each of the six candidate peptides for each of the selection criteria. These values were used to establish the presence of an immunodominant epitopes.

[c] Peptides that generated values greater than or equal to the mean value for all of the selection criteria were concluded to contain an immunodominant peptide.

[d] Maximum response: number of IFN-γ producing cell detected in PBMC from the highest responder pig among all of the 22 PBMC samples tested.

[e] Total response: Sum of all of the IFN-γ producing cells (minus background) detected in the 22 individually tested PBMC samples.

[f] Average response: Sum of all of the IFN-γ producing cells (minus background) detected in the 22 PBMC samples tested divided by 22, the number of pigs tested.

[g] Number of responsive pigs: number of pigs whose PBMC exhibited a peptide-specific IFN-γ response with an stimulation index ≥2. If background response was zero the response to the peptide had to be ≥2 to be considered positive.

[h] Average response of responsive pigs: average of the peptide-specific IFN-γ response (minus background) of all the pigs exhibiting a response to the peptide.

Table 6
Conservation of PRRSV GP5 T-cell epitopes

Peptide/PRRSV	Amino acid sequence at GP5 position	
	117-131	149-163
G9	LAALICFVIRLAKNC	
G10		KGRLYRWRSPVIVEK
H10		.....I..
NADC-9 (att)	.....	.....
NADC-9 (wt)	.....	.....
NVSL-14 (att)	.....	.....I..
NVSL-14 (wt)	.....	.....I..
ATP	....T.....	.....I..
PrimePac	.....	.....I..
RespPRRS	....T.....F....	..G.....I..
NVSL_97-785	.....	.....I..
P129	.....	.....I..
VR-2332	....T.....F....	.....I..
Consensus	****-*****:****	**-**********:**

[a] att: attenuated. [b] wt: wild-type. [c] For consensus, * = complete identity; : = strongly conserved; _ = non-conservative substitution. Dot (.) or letter indicates amino acid sequence identity or deviation, respectively, from peptide G9 or H10.

sent PRRSV GP5 epitopes. Interestingly, when the IFN-γ response of the PBMC from individual, PRRSV-vaccinated animals to peptides G10 and H10, which have a single amino acid difference, were compared, differences in the intensity of reactivity were at most approximately twofold and did not correspond to identical peptide and virus GP5 amino acid composition (data not shown). Therefore, replacement of the valine with isoleucine (a conservative change) at position 13 of the G10 and H10 peptides (Table 6), respectively, did not appear to influence recognition of this epitope by T-cells.

3.2. Phenotype of peptide-responsive PBMC

To identify the phenotype of the PBMC of a PRRSV-infected pig reacting to either intact virus or the immunodominant GP5 peptides, the IFN-γ response of PBMC populations depleted of specific subsets of cells by complement-mediated lysis was examined. Depletion of the targeted cell subsets (CD3, CD4, CD8α, and CD8β) appeared to be efficient since flow cytometry analysis of the treated PBMC indicated that 68–93% of cells having the respective cell-surface marker had been eliminated (data not shown). As shown in Table 7, most of the cells responding to intact PRRSV VR-2332 were removed by treatment with antibody against CD3 or CD4 protein. In contrast, separate exposures to anti-CD8β or anti-CD8α antibodies resulted in a progressively less depletion of the PRRSV-responsive cells. Moreover, only approximately 59–66% of the cells recognizing the immunodominant GP5 peptides were removed by treatment with antibody against either the T-cell-specific CD3 or the lymphocyte CD4 protein. In this case, depletion with anti-CD8α or anti-CD8β antibody resulted in a decrease in the frequency of GP5 specific IFN-γ responding cells similar to or slightly less than, respectively, that observed when intact PRRSV VR-2332 was the mitogen. Comparable reductions were observed for PBMC isolated from a pig inoculated with PRRSV in the converse manner, i.e., immunized with att NADC-9 and challenged with wt NVSL-14 (data not shown). Thus, the IFN-γ producing cells responding specifically to intact PRRSV VR-2332 appear to be primarily $CD3^+CD4^+$ T-cells with a minority expressing CD8α or CD8β. This $CD3^+CD4^+$ T-cell phenotype also represents 59–66% of the GP5 peptide-specific IFN-γ producing cells with very few of them being in the $CD8\alpha^+$ or $CD8\beta^+$ subsets.

Table 7
IFN-γ response of T-cell subset depleted PBMC populations to PRRSV and peptides

Stimulus	IFN-γ response after cell subset depletion (%)[a]			
	C′ + anti-CD3	C′ + anti-CD4	C′ + anti-$CD8\alpha^+$	C′ + anti-$CD8\beta^+$
VR-2332	14	14	89	64
Peptide G9	40	40	79	79
Peptide G10	41	42	93	83
Peptide H10	34	34	77	86

[a] PBMC were treated with medium alone, rabbit complement (C′) alone, or the indicated antibody plus C′. The cell number was adjusted after depletion and each population plated at 5×10^5 cells per well. Data represents the percentage of the response remaining after treatment with the indicated mAb and C′. The response of the PBMC treated with C′ alone for each stimulus is considered 100% response. This response ranged from 12 to 23 spots per 5×10^5 cells. The response of cell cultures stimulated with medium alone ranged from 0 to 1 spots per 5×10^5 cells. The experiments were performed with cryopreserved PBMC from 4 pigs vaccinated with NVSL-14 and challenged 2 weeks later with NADC-9. These cell samples were selected for this assay because they had previously given a high response to virus and the selected peptides.

3.3. Conservation of GP5 epitopes in North American PRRSV isolates

An alignment of the two GP5 epitopes with the corresponding regions in this glycoprotein present in the two wild-type and attenuated strains used in this study and six other PRRSV North American genotype representatives demonstrated a great degree of identity at each site (Table 6). For peptide G9, seven viruses were autogenous while there were one or two amino acid differences at this location in GP5 for the other three. One of the alterations was a conservative replacement at amino acid position #11 (phenylalanine for leucine) while the other at position #5 was a non-conservative alteration (threonine for isoleucine). Conservative variation was also noted for the second epitope, in this case at amino acid position #13 (valine/isoleucine) as exemplified by peptides G10 and H10. In addition, a unique non-conserved replacement of arginine for glycine at amino acid position #3 was predicted for the GP5 of RespPRRS vaccine.

4. Discussion

Previous analyses of the host response to the GP5 of the North American genotype of PRRSV have revealed the presence of three B-cell epitopes. The first is highly immunodominant, variable, non-neutralizing, and located just downstream of the predicted leader cleavage site at amino acid positions #27–30 [13]. The second is also present within this protein's ectodomain at amino acid positions #37–45 [13,15]. This one is not immunodominant but is more conserved than the first one and also is neutralizing. The more recently described third one [34] resides within the terminal portion of the GP5 endodomain at amino acid positions #187–200. In contrast to their locales, we have found that two immunodominant T-cell epitopes for GP5 are sited primarily within the putative transmembrane domain (G9 peptide, amino acid positions #117–131) or in the endodomain (G10-H10 peptide, amino acid positions #149–163) upstream of the third B-cell epitope. Unfortunately, none of the B and T-cell epitopes in GP5 overlap as is the case for a site in VP1 of foot-and-mouth-disease virus [35] or in glycoprotein E2 of classical

swine fever virus [36]. Otherwise, use of such a combined region might potentiate its value as a synthetic peptide vaccine. Thus, in order to elicit both humoral and cellular immune responses, a peptide-based vaccine based on PRRSV GP5 will have to include separate peptide sequences representing immunodominant B- and T-cell epitopes.

Overall as illustrated in Table 6, the identified GP5 epitopes appear to be relatively conserved among PRRSV isolates of the North American genotype. In fact, a more exhaustive search of relevant GenBank entries revealed for individual isolates at most a deviation of two amino acids, albeit at various locations, from their respective primary composition. Whether such a degree of disparity would influence recognition of a heterologous PRRSV strain by a previously primed, host cell-mediated immune system is unknown. However, it should be noted that there was not an obvious bias toward peptide G10 (KGRLYRWRSPVIVEK) or H10 (KGRLYRWRSPVIIEK), derived from the GP5 of PRRSV NADC-9 or NVSL-14, respectively, when PBMC from pigs only vaccinated with either virus were used in the ELIspot assay (data not shown). Thus, a single amino acid substitution in this epitope did not impact its identification by T-cells. However, the similarity between the exchanged isoleucine and valine residues in the two peptides may have prevented their discrimination. Of more concern is the fact that there are viruses, i.e., VR-2332 and the ATP and RespPRRS vaccines, whose GP5 have a non-conservative amino acid replacement within at least one of the epitopes. At present, it is unknown whether this change would affect the antigenicity of peptides G9, G10, and H10. Thus future studies will be directed towards accessing whether peptides designed to mimic the respective sites in the GP5 of variant viruses evoke a response from PBMC of NADC-9 or NVSL virus-infected pigs and if PBMC isolated from animals infected with such variant viruses respond to the above mentioned peptides.

Although in this study outbred pigs were used in addition to two genetically divergent PRRSV strains to increase the probability that the principal PRRSV GP5 epitopes would be identified, the peptide screening was still limited by the genetic makeup (e.g. swine leukocyte antigen haplotype) of the available animals. Thus, other pigs that express different swine leukocyte antigen alleles could conceivably generate an immune response against other regions of GP5. Moreover, it is possible that there is a temporal development of the T-cell response to GP5 as is the case with B-cell recognition of this protein [13]. Accordingly, additional GP5 epitopes could become immunodominant at some point beyond our final point of examination, namely 8 weeks from virus vaccination of the pigs.

The results of the phenotypic characterization of the PBMC subsets which were responsible for the intact PRRSV VR-2332 specific IFN-γ response were as expected. The vast majority (86%) of the PBMC population responding to the virus were $CD3^+$ $CD4^+$ T-cells, with most of the remainder being classical $CD4^-/CD8\alpha\beta^+$ cytotoxic T-cells. Surprisingly, a contribution by CD4/CD8 double positive T-cells was not detected. This observation is in marked contrast to the results of one of our previous publications delineating nearly all of the PRRSV-specific IFN-γ-secreting cells as expressing a $CD4^+CD8\alpha^+$ phenotype [23]. We attribute this discrepancy to the fact that only a minor proportion of the PRRSV reactive cells obtained from the 3-month-old animals, at 8 weeks post-PRRSV vaccination with an inclusive 2-week period after challenge, exhibited this phenotype. In this regard, we have determined that the percent of the $CD4^+CD8\alpha^+$ subset within the PBMC populations of pigs used in this experiment did not exceed 2% (data not shown). It should also be noted that the sampling time of the current experiment is in stark contrast to the earlier study mentioned above, which utilized leucocytes from 7-month-old pigs that had been twice exposed to PRRSV at a 3-week interval, 5 months earlier [23]. Thus, it is very likely that the PBMC populations of these older pigs contained a greater frequency of memory T-cells defined as CD4/CD8 double positive cells [23,37–39], for the following reasons: (1) the 3 month differential in the age of the pigs before sampling would result in an enhancement of the frequency of memory cells with the $CD4^+CD8\alpha^+$ phenotype in the circulating lymphocyte population as this parameter increases temporally in young swine [39], and (2) the additional 3 months after PRRSV infection before sampling in the earlier study would be critical for allowing the development of a larger proportion of virus-specific CD4/CD8 double positive memory T-cells [23]. These results confirm our previous observation that in the pig virus-specific memory T-cells can reside in the CD4/CD8 double positive and the $CD4^+CD8\alpha^-$ cell populations [39].

Phenotypic characterization of the GP5 peptide-specific IFN-γ secreting cells demonstrated that this response was mainly (approximately 60%) mediated by T-cells which expressed CD3 and CD4. Since depletion of PBMC with anti-CD8α or CD8β antibodies had little effect on the IFN-γ response by the remaining cells, apparently there was minimal recognition of the GP5 peptides by cytotoxic T lymphocytes. It is possible in the pig that pentadecapeptides are more suited for MHC class II antigen presentation while octameric to undecameric peptides are better suited for MHC class I antigen presentation. This difference could explain the lack of a significant response from the porcine $CD8\beta^+$ T cytotoxic lymphocytes to the pentadecamer GP5 peptides in contrast to the reported reactivity of human [7] and cat [8] class I restricted cytotoxic T-cells to pentadecapeptides.

Unexpectedly, a significant number of peptide-specific IFN-γ-secreting cells remained after the depletion of PBMC with anti-CD3 or anti-CD4 antibody. Based on these results, it became apparent that the residual GP5 peptide-responsive population is apparently comprised of cells that do not express CD3, CD4, CD8α, or CD8β. We should point out that after each depletion the number of remaining cells was adjusted in order to deposit 5×10^5 cells in each test well. Essentially this resulted in an enrichment of the $CD3^-/CD4^-/CD8\alpha^-/CD8\beta^-$ cell subset. To clarify this issue a composite of non-depleted PBMC obtained from a group of five PRRSV-naïve animals, of the same age as the pigs in this study, was tested for its responsiveness to the immunodominant GP5 peptides G9, G10, and H10 in an IFN-γ ELIspot assay. Since negligible reactivity was recorded, the IFN-γ activity associated with the CD3 and CD4 depleted population apparently represented an adaptive immune response, i.e., requires the acquisition of immunological memory by immunization. Therefore, the $CD3^-/CD4^-/CD8\alpha^-/CD8\beta^-$ cell population responds to the GP5 peptides but not to the whole virus. Moreover, the remaining GP5 peptide specific IFN-γ reactivity may be over represented because of the inadvertent enrichment of non-depleted cells, especially after treatment with anti-CD3 antibody. Regardless, applying an estimated correction for such enrichment would not diminish the premise of a $CD3^-/CD4^-/CD8\alpha^-/CD8\beta^-$ subset, which does not recognize intact PRRSV VR-2332, but is still responsible for at least one-third of the specific IFN-γ response to the peptides containing the immunodominant GP5 epitopes. As of this time, we are unaware of a previous description of a subset of porcine IFN-γ-secreting cells that is responsive to recall peptide antigen exhibiting this phenotype. Clearly, additional experiments will be required to confirm the existence of this non-traditional IFN-γ-secreting cell, possibly by performing flow cytometric analysis of the peptide-responding cells.

In summary, we have identified pentadecapeptides that carry immunodominant PRRSV GP5 epitopes that are somewhat conserved in North American genotype isolates and elicit a significant IFN-γ response in almost half of the pigs tested in this study. Thus, pending *in vivo* verification of their immungenicity, one or both

should be considered for incorporation into a polyvalent, peptide-based vaccine against PRRS. In view of the inherent variability of the genome of PRRSV, other components could include novel GP5 epitopes recognized by pigs with different haplotypes as well as yet undiscovered T-cell epitopes present in other PRRSV proteins such as NSP2, which has been shown to contain a high frequency of immunodominant B-cell epitopes [34]. In addition, the identification of T-cell epitopes in other PRRSV structural proteins such as GP2 and the matrix (M) protein, which have are recognized by T-cells [12] need to be identified in order to develop a potentially effective peptide based vaccine expressing a multitude of conserved immunodominant B and T-cell epitopes which could be engineered into a more immunogenic biologic [40]. The use of immunomics tools [41], such as T and B-cell epitope mapping algorithms is likely to help in the development of an effective vaccine against PRRSV.

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