Vaccine 35 (2017) 782–788

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Cross reactivity of immune responses to porcine reproductive and respiratory syndrome virus infection

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ARTICLE INFO

Article history:
Received 26 October 2016
Received in revised form 16 December 2016
Accepted 18 December 2016
Available online 3 January 2017

Keywords:
Porcine reproductive and respiratory syndrome virus (PRRSV)
Cell-mediated immunity
Interferon-γ
T cell cross reactivity
Heterologous immunity

ABSTRACT

Because porcine reproductive and respiratory syndrome virus (PRRSV) exhibits extensive genetic variation among field isolates, characterizing the extent of cross reactivity of immune responses, and most importantly cell-mediated immunity (CMI), could help in the development of broadly cross-protective vaccines. We infected 12 PRRSV-naïve pigs with PRRSV strain FL12 and determined the number of interferon (IFN)-γ secreting cells (SC) by ELISpot assay using ten type 2 and one type 1 PRRSV isolates as recall antigens. The number of IFN-γ SC was extremely variable among animals, and with exceptions, late to appear. Cross reactivity of IFN-γ SC among type 2 isolates was broad, and we found no evidence of an association between increased genetic distance among isolates and the intensity of the CMI response. Comparable to IFN-γ SC, total antibodies evaluated by indirect immunofluorescence assay (IFA) were cross reactive, however, neutralizing antibody titers could only be detected against the strain used for infection. Finally, we observed a moderate association between homologous IFN-γ SC and neutralizing antibodies.

1. Introduction

PRRSV is the etiological agent of the most important infectious disease of swine worldwide, causing late-term reproductive failure in sows, sperm abnormalities in boars, and respiratory illness in young pigs [1,2]. PRRS causes substantial financial losses to swine producers and in the United States alone, PRRSV-associated losses were estimated to be at least $664 million [3].

Classified within the family *Arteriviridae*, order *Nidovirales*, PRRSV is an enveloped, positive sense, single stranded RNA virus [4]. PRRSV can be further classified into type 1 (European) and type 2 (North American) genotypes that share ~65% genomic sequence identity [5,6].

Although PRSSV viremia can last up to one month post infection (PI) or more, and persistent continuous low levels of viral replication in lymphoid tissues can be detected up to 150 days post-infection (DPI) or more, the virus is eventually cleared by the host [7]. This reveals that later in the course of infection the pig immune response is competent in removing the virus from the animal, demonstrating that an appropriate adaptive immune response has been mounted [8]. The humoral immune response against PRRSV can be detected as early as 7 DPI, when abundant non-neutralizing antibodies appear. These antibodies are cross reactive against heterologous PRRSV isolates [9]. Serum neutralizing antibodies only appear on or after 28 DPI [9–11], and have been shown to provide full protection against homologous challenge when such antibodies attained appropriate concentrations (i.e. titer) in the circulation, however, titers of cross-neutralizing antibodies are meager and frequently rare [12–14]. The PRRSV-specific T cell response has been shown to be variable over time and among individual pigs, appearing as early as two weeks' post infection, but showing a fairly low initial onset [11,15]. Previous studies have evaluated the importance of the IFN-γ T cell response and its correlation to protective immunity, and several authors have concluded that an adequate correlation exists between the IFN-γ T cell response and protective immunity [16–19]. With exceptions, only heterologous strains have been used to evaluate CMI responses, and a comprehensive evaluation of the cross reactivity of T cell responses against PRRSV is still lacking.

PRRSV genetic heterogeneity has been thoroughly documented [20] and it has been hypothesized that antigenic relatedness of the strains or isolates used for immunization and challenge plays a major role in determining their immunogenic effectiveness, thus constituting a central factor towards the development of more broadly protective vaccines [21]. Because a combination of neutralizing antibody (NAb) and T cell responses seem to be responsible

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http://dx.doi.org/10.1016/j.vaccine.2016.12.040
0264-410X/

for protective immunity against PRRSV, we were interested in evaluating how genetic diversity, and hence antigenic diversity, played into the cross reactivity of cell-mediated and humoral immune responses.

2. Materials & methods

2.1. Cells, antibodies and PRSSV isolates

MARC-145 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C with 5% CO_2.

The PRRSV-specific monoclonal anti-N protein (clone SDOW17 [22]) was used for IFA, Alexa Fluor 488-conjugated goat anti-mouse antibody was purchased from Invitrogen (Eugene, OR), and mouse anti-pig IgG antibody was purchased from BD Biosciences (San Jose, CA).

The PRRSV isolate FL12 used in this study was derived from isolate NVLS 97-7895 and recovered from a full-length infectious cDNA clone [23]. Other isolates used in this study include: 1692-98, 21599-00, 46517-00, 16244B [6], 3805-00, 43807-00, 18565-01, 18066-04, and MN184C, the latter kindly provided by Dr. Faaberg at the National Animal Disease Center [24]. The type 1 strain SD01-08 was recovered from a cDNA clone kindly provided by Dr. Fang at the Kansas State University [25]. The list of PRRSV isolates with GenBank accession numbers is presented in Table 1.

All isolates were grown to approximately 80% cytopathic effect, released from cells by one freeze-thaw cycle, and clarified by centrifugation. The titers of the viruses were determined and viruses were frozen at −80 °C.

The PRRSV full-genome coding sequences were aligned using MUSCLE 3.8 [26]. The calculated pairwise nucleotide distances between FL12 and the remaining isolates are presented in Table 1.

2.2. Animals and experimental design

24 four-week-old PRRSV-seronegative pigs were randomly divided into four groups of six pigs each. Each group was housed in a separate room at the University of Nebraska-Lincoln Life Sciences Annex facilities (Lincoln, NE). Two groups were inoculated intramuscularly with $10^{5.0}$ $TCID_{50}$ of PRRSV strain FL12 while two groups were left uninfected as controls. Blood samples were collected periodically. All work was approved by the University of Nebraska-Lincoln (UNL) Institutional Animal Care and Use Committee protocol number 930.

2.3. Isolation of PBMC

PBMC were isolated as previously described [11,27].

Table 1
Strain/isolate GenBank accession number and calculated pairwise nucleotide distance to FL12.

Strain/isolate	GenBank accession no.	Distance to FL12
FL12	AY545985	–
1692-98	KY348847	5.02%
21599-00	KY348850	5.02%
46517-00	KY348852	6.36%
16244B	AF046869	9.66%
3805-00	KY348853	9.71%
43507-00	KY348851	10.11%
18565-01	KY348849	12.65%
MN184C	EF488739	14.32%
18066-04	KY348848	14.48%
SD01-08	DQ489311	36.72%

2.4. ELISpot assay

The ELISpot assay was performed as previously described [11,27]. Two replicates of 5×10^5 PBMC/well were stimulated with 5×10^4 $TCID_{50}$ of PRRSV. Spots were counted and analyzed using a CTL ImmunoSpot counter (Cellular Technology Limited, Shaker Heights, OH).

2.5. IFA

IFA was performed as previously described. A titer $\geqslant$1:20 was considered to be positive [28].

2.6. Serum viral neutralization (SVN) assay

The SVN assay was performed as previously described [29]. The end point titer was expressed as the reciprocal of the highest serum dilution that neutralized PRRSV in 2 replicate wells. Absence of NAb titers was considered as 0 for statistical analysis.

3. Results

3.1. Variability of T cell responses

FL12-specific IFN-γ SC were detected as early as 14 DPI in some animals, with most animals showing a highly variable number of IFN-γ SC at 28 and 42 DPI, after which variability could still be observed, but was reduced. We observed fundamentally distinct IFN-γ SC kinetics among the FL12-infected animals (Fig. 1A).

Two animals (301 and 314) showed an increase of PRRSV-specific IFN-γ SC peaking at 42 DPI in very high numbers, after which the numbers slowly declined. Another group of animals (299, 304, 323 and 358) showed a weaker biphasic response, with a first peak occurring at 28 DPI and a second peak at 63 DPI. A third subset of animals (317, 321, 325, 330) demonstrated an even weaker response, in most cases only present in very small numbers at 28 and 42 DPI, but with a steady climb, peaking at 77 DPI. The CMI kinetics of two animals (333 and 315) did not fit any of the aforementioned descriptions. With the exception of animal 301, and regardless of the observed kinetics of the CMI, all animals at 77 DPI appeared to reach a similar number of IFN-γ SC. Similar variability of T cell responses could also be observed when we evaluated the IFN-γ SC the response using nine other type 2 isolates as recall antigen, but not with the type 1 strain SD01-08 (Suppl. Fig. 1). We did not detect any PRRSV-specific IFN-γ SC in uninfected animals regardless of recall antigen throughout the course of our study.

To further evaluate the overall variability over the extent the experiment, we calculated the area under the curve (AUC) for the number of IFN-γ SC from 0 to 77 DPI using the trapezoidal rule for each individual animal (Fig. 1B). The two animals with the fastest and highest responses (301 and 314) had, in turn, the highest calculated AUC, while the four animals with the slowest and weakest kinetics had the smallest calculated AUC. The remaining animals, including those representative of the biphasic response, had a calculated AUC in between the two aforesaid groups.

3.2. Cross reactivity of T cell responses

To evaluate the cross reactivity of T cell responses against PRRSV we used the homologous strain FL12, nine other type 2 isolates of varying genetic distance and one type 1 isolate (Table 1). A two-way ANOVA was performed to determine the effect of PRRSV isolate used as recall antigen on the number of IFN-γ SC

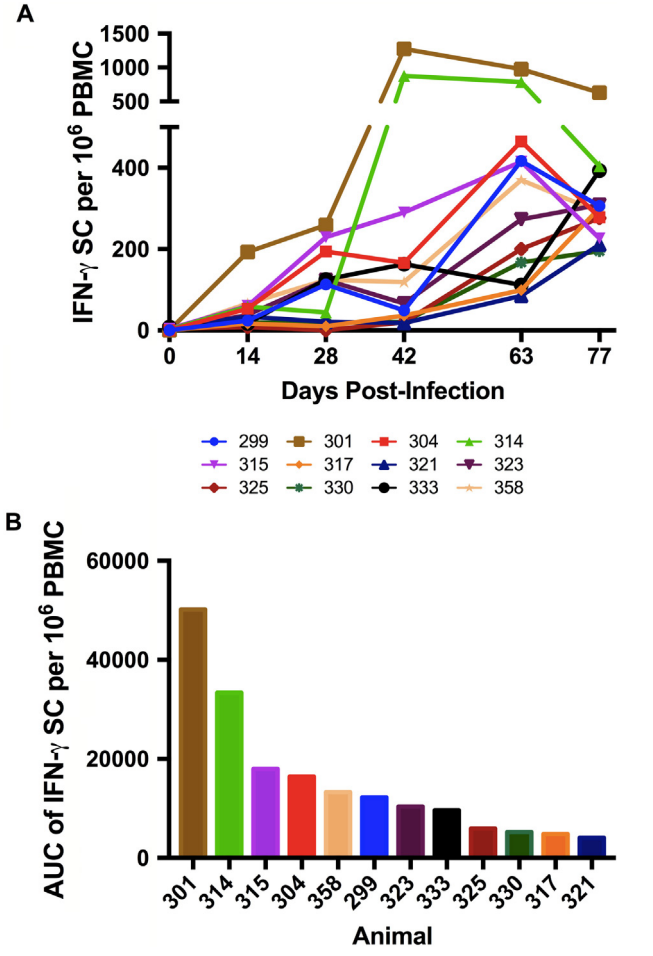

Fig. 1. Kinetics of IFN-γ SC responses against PRRSV strain FL12 in individual animals. (A) 12 four-week old pigs were inoculated intramuscularly with $10^{5.0}$ $TCID_{50}$ of PRRSV strain FL12. FL12-specific IFN-γ SC were detected in PBMC by ELISpot assay at the indicated times. (B) Area under the curve (AUC) of IFN-γ SC was determined using the trapezoidal rule.

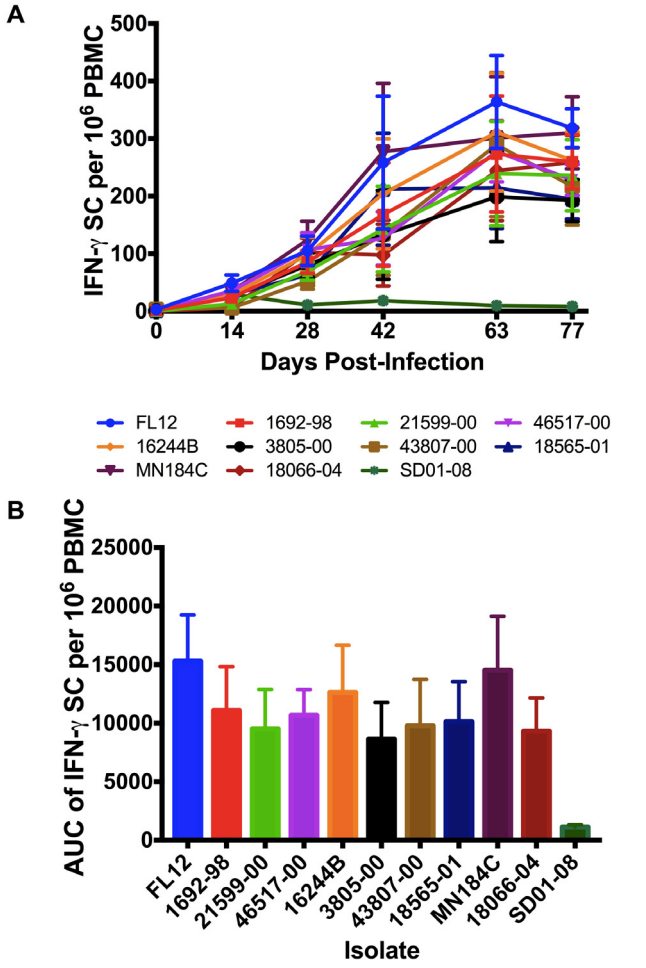

Fig. 2. Evaluation of T cell cross-reactivity in the context of PRRSV infection. (A) The mean and standard error IFN-γ SC number was calculated for each PRRSV isolate used as recall antigen and the kinetics analyzed by two-way ANOVA. (B) The IFN-γ SC AUC mean and standard error for each isolate was calculated and analyzed by one-way ANOVA.

(Fig. 2A). Although there was a statistically significant interaction between the effects of PRRSV isolate and the number of IFN-γ SC, simple main effects analysis showed that type 2 PRRSV isolates induced significantly stronger T cell responses than the type 1 strain SD01-08, but no significant differences were detected among type 2 PRRSV isolates. We observed similar results when examining the T cell responses of each individual animal (Suppl. Fig. 2).

Further analyses demonstrated no significant differences between SD01-08 and all type 2 isolates at 14 and 28 DPI (with the exception of MN184C at 28 DPI) and, starting at 28 DPI, and through every other time point, all type 2 isolates demonstrated a significantly higher number of IFN-γ SC than SD01-08. We were able to observe that FL12, MN184 and 16244B showed a number of IFN-γ SC significantly higher from other type 2 isolates at 42 and 63 DPI. Finally, FL12 and MN184C had higher numbers of IFN-γ SC than 3805-00 and 18565-01 at 77 DPI.

To additionally evaluate T cell cross reactivity, we calculated the mean and standard error of the AUC for each isolate used as recall antigen (Fig. 2B) and performed a one-way ANOVA. There was a significant difference between PRRSV isolates used as recall antigens, however, there was no significant difference among type 2 isolates. Type 2 isolates had a significantly higher IFN-γ SC response than the type 1 strain SD01-08.

3.3. Isolate genetic distance and T cell responses

To study the relationship of isolate genetic distance and T cell responses we determined the mean IFN-γ SC number for each type 2 PRRSV isolate and fit a linear regression model against the previously calculated pairwise distance of said isolate to FL12 (Fig. 3A–E). Our results indicated that changes in genetic distance are not associated with changes in the mean number of isolate-specific IFN-γ SC at 14, 28, 42, 63 and 77 DPI.

To further evaluate this relationship over the entire course of the assay we determined the mean AUC of IFN-γ SC for each type 2 isolate and fitted a linear regression model with the calculated pairwise distance of said isolate to FL12 (Fig. 3F). No significant association could be found between the mean AUC of IFN-γ SC and pairwise distance of the isolate.

3.4. Total and NAb responses

Five of the ten type 2 PRRSV isolates were selected to evaluate the total and NAb response. Together with the homologous strain FL12, we included low distance isolates 1692-98 and 21599-00, medium distance isolate 16244B and high distance isolate 18565-01. The total antibody responses, evaluated by IFA, was deemed negative at 0 DPI for all animals (<1:20) and all isolates

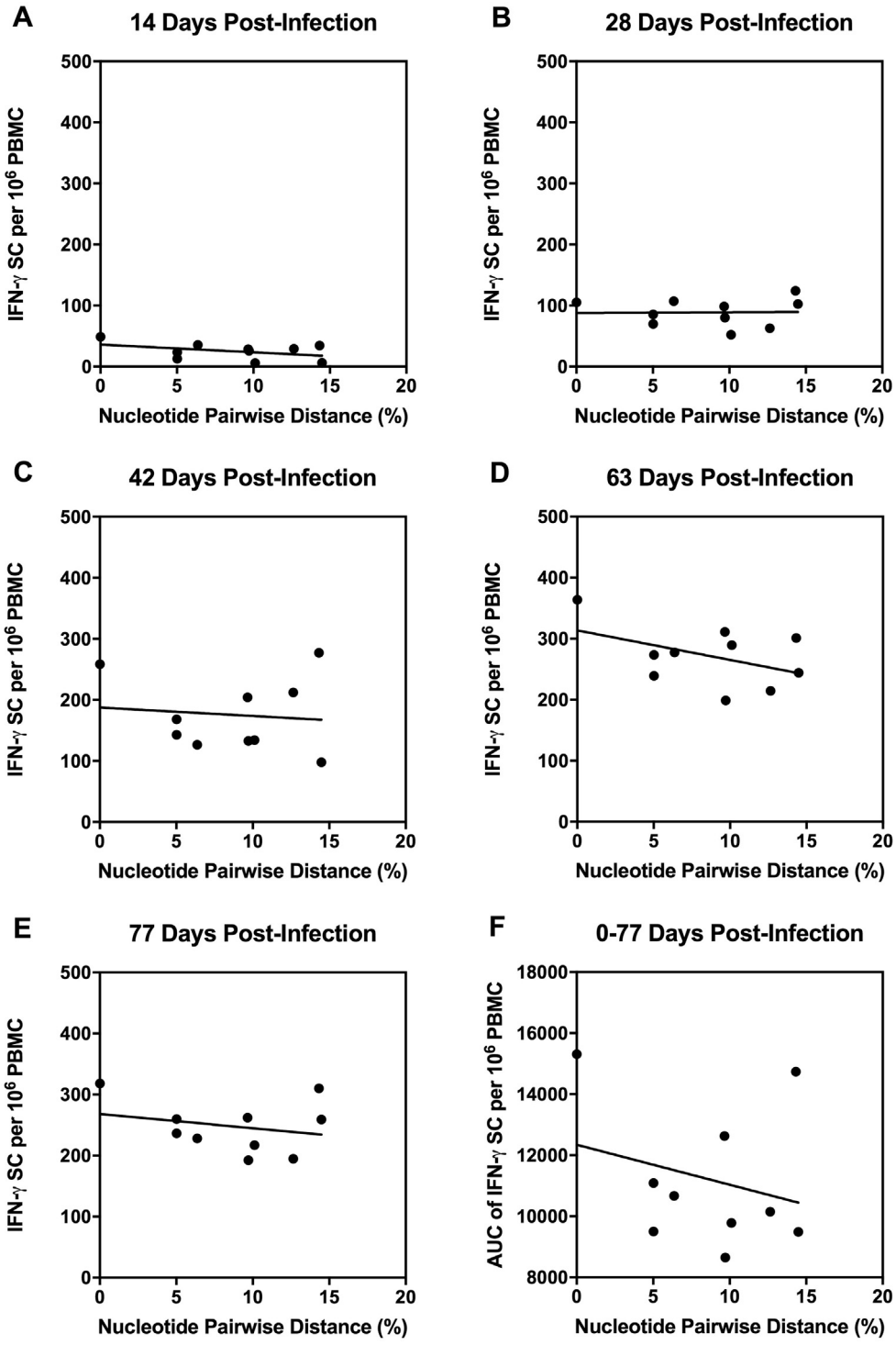

Fig. 3. Analysis of calculated nucleotide pairwise distance to FL12 and IFN-γ SC responses. (A–E) For every time point, the mean IFN-γ SC for each type 2 isolate was calculated and fitted a linear regression against the calculated nucleotide pairwise distance of said isolate to FL12. (F) A linear regression was fitted between the mean AUC for each type 2 isolate and its calculated nucleotide pairwise distance to FL12.

tested. Starting at 28 DPI, and at 63 and 77 DPI all animals were positive by IFA ($\geqslant$1:20) against all five isolates.

Neutralizing antibodies against isolate FL12 were determined by SVN and were undetectable at 0 and 28 DPI. Homologous titers could be detected starting at 63 DPI and continued to rise until 77 DPI (Fig. 4A). Subsequently, we sought to determine whether NAb titers could be obtained against the four heterologous isolates. We tested the sera obtained at 77 DPI and found that, with very few exceptions, no NAb titers could be detected against 1692-98, 21599-00, 16244B and 18565-01 (Fig. 4B).

3.5. Association between T cell and neutralizing antibody responses

To examine the association between NAb and IFN-γ SC at 63 and 77 DPI, we paired the homologous NAb titer against FL12 for each animal with its respective number of FL12-specific IFN-γ SC

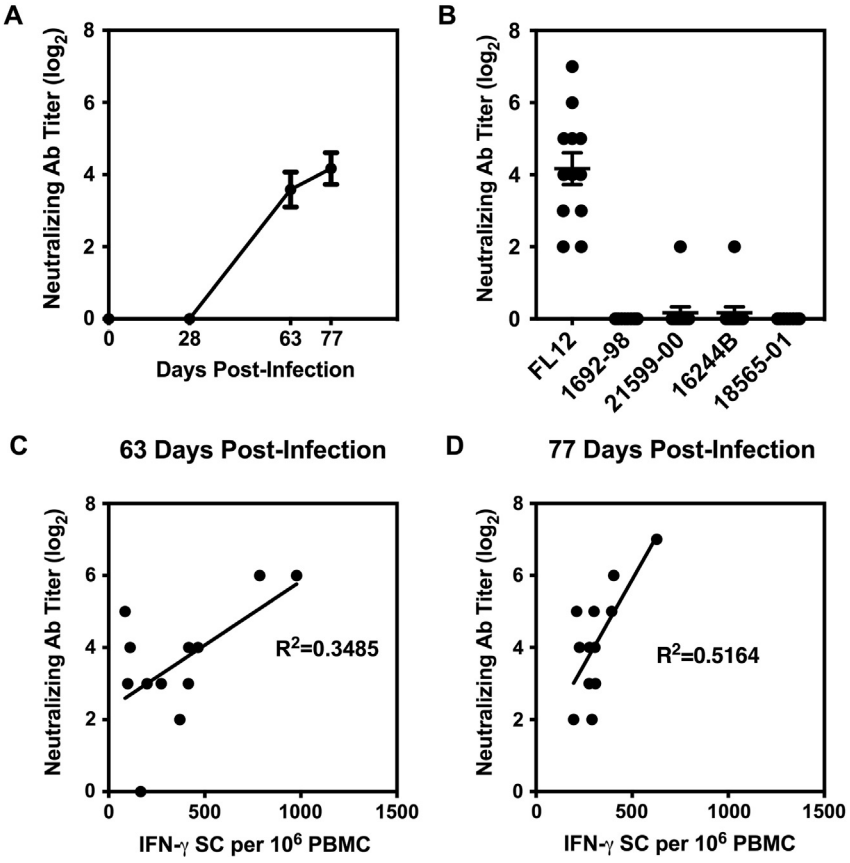

Fig. 4. Evaluation of humoral immune responses against PRRSV. (A) NAb titers against FL12 were determined by SVN and results are expressed as the $\log_2$ of the reciprocal of the largest dilution of serum that inhibited the development of virus in cell culture. (B) Neutralizing antibody titers against 1692-98, 21599-00, 16244B and 18565-00 were determined at 77 DPI. (C and D) A linear regression was fitted between the FL12-specific IFN-γ SC for each animal, and their respective neutralizing antibody titers at 63 and 77 DPI. Coefficient of determination (R^2) values are indicated.

and fitted a linear regression (Fig. 4C and D). Our results indicate that at 63 DPI 34.86% of the variation of NAb titers can be explained by the variation in the number of IFN-γ SC, while this value increases to 51.64% at 77 DPI.

4. Discussion

Since PRRSV was first reported in the late 1980s, our understanding of its pathogenesis and immunology have grown steadily, however, we are still lacking a broadly cross-protective PRRSV vaccine. The cross protection afforded by currently available commercial vaccine strains is at least that of the field isolates from which they were derived, but it has become clear that there is a great need for improvement in the breadth of this cross protection [30,31].

Because our understanding of CMI against PRRSV is inadequate, we were interested in determining how T cells cross reacted against other field isolates, and whether the isolate heterogeneity, determined by its genome-wide calculated nucleotide pairwise distance to the challenge isolate, could be linked to varying levels of cross reactivity. We have recently showed that minimizing the calculated nucleotide pairwise distance between immunization and challenge isolates provided an unprecedented level of cross-protection [32], hence we hypothesized that increased nucleotide pairwise distance as a determinant of antigenic variability and heterogeneity of field isolates could be negatively associated to CMI.

Our study demonstrates that an outbreed population of pigs infected with a type 2 PRRSV isolate shows a highly variable IFN-γ SC response among individual animals. Authors have described the PRRSV-specific T cell response as weak and slow, however, our findings reveal that individual animals can potentially achieve high numbers of PRRSV-specific T cell responses as early as 14 DPI. It was previously shown that inoculation with virulent PRRSV elicits a higher number of IFN-γ SC than inoculation with a modified live vaccine both in piglets and finisher pigs [33]. It could be hypothesized that the extreme variability observed among CMI responses in individual animals could be responsible for the variable protection observed against PRRSV challenge. The host and pathogen factors behind this variability are yet to be understood.

In our study we evaluated CMI cross reactivity against an array of genetically diverse type 2 PRRSV isolates and one type 1 PRRSV strain. Our results indicate that T cells from FL12-infected animals are able to recognize other type 2 isolates and secrete IFN-γ in response to them. The overall kinetic of the T cell responses to all type 2 isolates were not significantly different between type 2 isolates, however, a significantly lower T cell response was observed when SD01-08, a type 1 strain, was used. Such cross reactivity is not surprising, as T cell cross reactivity has been documented for other pathogens. McMaster et al. [34] have demonstrated the existence of cross reactive T cells against influenza A virus: while these cross reactive T cells were shown to provide cross-protection against heterologous challenge, they did so in the presence of a limited NAb cross reactivity. Because the number of potential peptide antigens surpasses the number of T cell receptors available by many orders of magnitude, it can be argued that T cells are only able to provide comprehensive immune cover if each one of them is capable of recognizing many peptides [35]. In sharp

contrast, when a virulent type 1 PRRSV isolate was inoculated into pigs and PBMC isolated and stimulated in vitro with the homologous isolate and a heterologous isolate of calculated nucleotide pairwise distance of 12.5%, a significant reduction of IFN-γ SC was found to occur in the presence of the heterologous virus stimulation [36].

Our findings in regards to total antibody responses against PRRSV confirm what other authors have described, however, it is known that these antibodies do not mediate protection against infection [37]. At 63 and 77 DPI animals showed NAb titers against FL12, however, we were not able to detect NAb titers against heterologous PRRSV isolates. Our observation is different from what was reported by Martinez-Lobo et al. [38] where the authors describe the presence of cross reactive NAb titers against type 1 PRRSV isolates. This divergent observation might be due to the nature of the antisera used in the neutralization assay. Martinzez-Lobo et al. used hyperimmune antisera [38] obtained from pigs that were repeatedly immunized with PRRSV whereas we used the convalescent antisera obtained from pigs that were exposed only one time to PRRSV.

Because we evaluated the PRRSV CMI using the widely accepted IFN-γ ELISpot assay, our results are not without limitations. The techniques available in the field of swine immunology are not as comprehensive or precise as those available for other species, and there is a known necessity to develop a swine T cell biology toolkit [8]. Further characterization of T cell subsets and cytotoxicity in the context of PRRSV infection will require the usage of multi-color flow cytometry [39–43], in combination with MHC tetramer staining [44], and further understanding of the major histocompatibility molecules of swine, the swine leukocyte antigen (SLA), including their distribution within an outbred population.

Finally, we demonstrate a low to moderate association between the number of IFN-γ SC and the magnitude of the NAb response. This association could only be evaluated for the homologous strain FL12, as other isolates failed to elicit any NAb titers. However, our data suggests that between 30 and 50% of the variation of NAb titers can be explained by variation in IFN-γ SC. It is extensively documented that antigen-activated B cells establish interactions in the lymph node that allow them to receive helper signals from antigen-activated CD4+ T cells. Such interactions allow for class switch and affinity maturation [45,46]. However, studies conducted in measles-vaccinated patients demonstrated the independence between humoral and cellular immune responses [47,48].

Overall, our results could be interpreted in two distinct ways. On the one hand, it could be argued that due to the number of IFN-γ SC being not significantly different amongst type 2 PRRSV isolates, T cells play no major role in mediating cross protection. On the other hand, it could be hypothesized that, similarly to what has been described for influenza [49], T cells react against a broad spectrum of type 2 PRRSV isolates, contributing to partial levels of cross-protection against heterologous isolate infection. We prefer the latter hypothesis. Furthermore, we favor the idea that, very much like universal influenza vaccines, broadly protective PRRSV vaccines could rely on the concept of "heterosubtypic" immunity, in which T cell-mediated immune responses targeting conserved PRRSV epitopes would confer protection against infection and disease.

Competing interests

The authors declare no competing interests.

Acknowledgements

We would like to thank Dr. Laegreid at the University of Wyoming for sequence analysis of the PRRSV isolates and the staff of the UNL Life Sciences Annex and Veterinary Diagnostic Center for the care of animals. This research was supported by the Agriculture and Food Research Initiative competitive grants 2016-67015-24922 (to H.L.X.V.) and 2013-01035 (to F.A.O.) of the USDA National Institute for Food and Agriculture.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2016.12.040.

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