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D3.8

Identification of CD8+ T cell PRRSV protein targets

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EXECUTIVE SUMMARY

Background	PRRSV is a rapidly evolving and diversifying pathogen necessitating the development of vaccines with broad reactivity. Data suggest that the T cell mediated immunity plays a role in protection. Importantly, T cell response is generally broader than the neutralising antibody response. Indeed whereas neutralising antibodies are most often directed against outer structural determinants of viral particles, T cell response is also directed against internal or non-structural determinants. In addition T-cell receptor can tolerate a certain degree of epitope change permitting cross reactivity. Therefore PRRSV antigen targets of T cells shall be identified to develop optimal vector vaccines that induce broad immunity. Since the proposal preparation, a publication by Mokhtar et al identified several T cell antigens which appeared frequently recognized in UK Large White pigs infected with divergent strains (1). We thus focused on these conserved antigens in the present task.
Objectives	The goal of T3.8 is to identify PRRSV protein targets of CD8+ T cells in Large White pigs, in the case of the PRRSV Flanders 13
Methods	-Ten Large White pigs from the INRA breeding unit were rendered immune with PRRSV Flanders 13 by 2 consecutive intra-pulmonary infections. -Peripheral Blood Mononuclear Cells (PBMC) were collected and used fresh. Spleen cells were collected, and frozen in liquid nitrogen. -IFNγ ELISPOTS were performed on PBMC and spleen cells using overlapping peptides from several T-cell antigen candidates (GP4-GP5-M, N, NSP1b, RdRp) -IFNγ Intracellular staining (ICS) was performed on PBMCs using overlapping peptides from several antigen candidates (GP4-GP5-M, N, NSP1b, RdRp)
Results & implications	In PBMCs, T cells from PRRSV Flanders13 infected pigs reacted against NSP1b (9/10 pigs), N and GP4-GP5-M (5/10 pigs) and RdRp (3/10 pigs), as assessed by IFNg ELISPOTS. In spleen, T cells from PRRSV Flanders13 infected pigs reacted against NSP1b (9/10), GP4-GP5-M (8/10), N (3/10) and RdRp (6/10). Depletion of gamma delta T cell appears beneficial to detect IFNγ producing cells. In conclusion, NSP1b and GP4-GP5-M are frequent antigen targets of IFNγ producing T cells elicited by PRRSV Flanders13 infection in the Large White pigs from the INRA breeding unit. N and RdRp are more variably used as T cell antigens among pigs. All these antigens are to be selected in the vaccine design in WP10 and WP11 in order to maximise T cell antigen recognition. No T cell response could be detected with the ICS technique. The assay of CD107 translocation was not successful. Thus we cannot determine if the T cell response detected in ELISPOT is from CD8+ or CD4+ T cells. This does not have impact on other tasks.

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1. Introduction

PRRSV is a rapidly evolving and diversifying pathogen necessitating the development of vaccines with broad reactivity. Data suggest that the T cell mediated immunity plays a role in protection. Importantly, T cell response is generally broader than the neutralising antibody response. Indeed whereas neutralising antibodies are most often directed against outer structural determinants of viral particles, T cell response is also directed against structural internal and non-structural determinants. In addition T cell receptor can tolerate a certain degree of epitope change permitting cross reactivity. Therefore PRRSV antigen targets of T cells shall be identified to develop optimal vector vaccines that induce broad immunity.

Since the proposal preparation, a publication identified several T cell antigens which appeared quite frequently recognized in UK Large White pigs infected with divergent strains (1).This conserved reactivity led us to focus on these antigens in order to validate them in the case of the PRRSV Flanders13 and of the genetic make-up of the pigs from the INRA breeding unit. If validated, these antigens will be further selected for DNA vaccine development in WP10 and WP11.

2. Material and methods

1.1. Peptide design and antigen selection (INRA, EDI-IVI, UGENT)

The peptide design aimed at covering the entire sequence of the most promising T cell antigens to be used in the vaccines developed in WP10 and WP11. The design was done by INRA.

In the Mokhtar et al paper (1), NSP1, RdRp, GP5, M, NSP5 were the most frequently recognised antigens in pigs infected with PRRSV of the serotype 1 and 3. As NSP5 is a protein with many transmembrane domains, we excluded it because transmembrane domains preclude the capacity to be secreted, a property mandatory for the development of dendritic cell targeted vaccines in WP10. Finally we selected a chimeric sequence made of large fragments of GP5 and M fused to GP4 which lack the native transmembrane domains and was successfully used in a recent paper to induce T cell responses in pigs upon dendritic cell targeting with anti-DC-SIGN antibodies (2).

PRRSV Flanders 13 had been initially sequenced by UGENT using Next Generation Sequencing on an initial viral stock. In order to confirm the sequence of the new viral stock which will be used at INRA and EDI-IVI for the whole project, RT-PCR fragments were sequenced by EDI-IVI. Few non conservative mutations were found (4) and were taken in account in the overlapping peptide design.

Thus in total, INRA designed overlapping peptides (20 mers, Offset 8) covering the entire sequence of NSP1, RdRp, N and of the chimera GP4-GP5-M to restimulate T cells from pigs infected with Flanders13 PRRSV. The NSP1, RdRp, N and of the chimera GP4-GP5-M sequences are provided in Annex1. The synthetic peptide synthesis was done by Mimotopes http://www.mimotopes.com. Upon reception the peptides were diluted in H20:acetonitril (50:50 vol) at a 5 mg/ml concentration and grouped as pools of peptides not exceeding 25 peptides: pool N (15 peptides), pool Gp4-1 (from peptide1 to 20 of the GP4-GP5-M chimera), pool Gp4-2 (from peptide 21 to 40 of the GP4-GP5-M chimera), pool NSP1-1 (from peptide 1 to 22), pool NSP1-2 (from peptide 23 to 47), pool RdRp-1 (peptide 1 to 20), pool RdRp-2 (peptide 21 to 40), pool RdRp-3 (peptide 41 to 60), pool RdRp-4 (peptide 61 to 80).

1.2. Experimental infection (INRA, UGENT, ING)

Ten five week-old PRRSV piglets were tested PRRSV negative by ELISA (Ingezim PRRSV2, ING). They infected via the intra-tracheal route with 10^5 TCID PRRSV Flanders13. The original inoculum (passage 2) was provided by UGENT and amplified on primary pig pulmonary macrophages by INRA. In order to recall immunity, 4 weeks later, pigs were re-inoculated via the same route with 10^6 TCID PRRSV

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Flanders13. The experimental infection and cell collection was done at INRA-PFIE-Nouzilly. Clinical signs were monitored every day.

PRRSV Flanders 13 was chosen instead of PRRSV Flanders 08, because this is a recent strain available in the consortium and it is pathogenic in pigs under experimental conditions (UGENT) whereas PRRSV Flanders 08 is an older strain and is attenuated strain, so not suitable for challenge experiments. For these reasons, PRRSV Flanders 13 will be used for vaccine design by most of the consortium.

1.3. Viral detection (INRA)

PRRSV titers were assessed as recommended by the OIE. Alveolar macrophages are seeded overnight in 96 wells plates and then infected with the infected serum at different dilutions (from 1/10 to 1/10,000) in 5 replicates. The cytopathic effect is observed 6 to 7 days later. Immunostaining of positive wells using an anti-N monoclonal antibody followed by an anti-IgG2b-Ax555 secondary antibody allowed us to confirm the presence of PRRSV virus. TCID50 was then calculated.

1.4. Isolation of peripheral blood mononuclear cells (PBMCs) and spleen cells (INRA)

PMBCs were collected 25 days after the second infection and prepared fresh from 100 ml blood collections in 13mM citrate as described in (3). Spleen were collected 34 days after the second infection, and cells were isolated at described in (3). Over 400 x 10^6 cells were collected per 5 cm diameter spleen fragments. Five frozen vials (70 x 10^6 per vials) were prepared, frozen with progressively downscaling temperature and stored in liquid nitrogen for later use.

Spleen cells instead of lymph nodes (mentioned in the DoA) were used because we found that lymph node cells were much more fragile than spleen cells upon freezing.

1.5. Assessment of T cell response using IFNg ELISPOT (INRA)

PBMC or spleen cells were suspended at 5 × 10^6 cells per ml in X-VIVO-20 medium supplemented with 2% FBS, 100 U/ml penicillin and 1 µg/ml streptomycin (culture medium) and stimulated with peptides at 5µg/ml or ConA at 25µg/ml for 18 or 36 hours, in duplicate wells. Control wells with H2O :acetonitril were done. IFN-γ secreting cells were enumerated by ELISPOT assay essentially as previously described (1). In order to potentially improve the analysis of the antigen specific responses, spleen cells were depleted of γ/δ T cells using the 86D and PGBL22A mAb (4µg/ml) followed by anti-mouse IgG microbeads and LD column (Miltenyi Biotec).

1.6. Assessment of CD4+ and CD8+ T cell response using IFNg IntraCellular Staining (ICS)

PMBC were suspended at 15 × 10^6 cells per ml in culture medium in two 96-well plates and stimulated with peptides at 5µg/ml or ConA at 25µg/ml for 18 jours. Brefeldin at 5µg/ml was added for the last 4 hours. Cells were pooled from the 2 wells, labeled with 2µg/ml anti-pigCD3 (E86, IgG1), anti-pigCD8β (PG220, IgG2a) and anti-pigCD4 (74-12-4, IgG2b) and anti-isotype-specific fluorophore-conjugated antibodies. These primary antibodies were from WSU, USA. Zombie-aqua (1/100, Biolegend) was added to the fluorophore-conjugated secondary antibodies to identify dead cells. Cell were then fixed and permeabilised with the cytoFixcytoPerm kit following the manufacturer's instructions (Becton-Dickinson). Detection of IFNγ was performed using 5 µg/ml anti-pigIFNγ PE-conjugated antibody compared isotype control PE-conjugated antibody (both from Becton-Dickinson). FACS analysis was conducted on a minimum of 10^5 live $CD3^+$ T cells.

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1.7. CD107 translocation

PMBC or spleen cells were suspended at 7.5 10^6 per ml in 200µl culture medium, with ConA at 25 µg/ml or with PMA 10 ng/ml and Ionomycine 1µg/ml, in 96-well plates. After 2 hours culture, they were incubated for 4 hours with Monensin 5µg/ml and A647-conjugated anti-pigCD107 or isotype control (5µg/ml) (Serotec). The culture was stopped and cells placed at 4°C for labeling with anti-CD3 and anti-CD8β mAb followed by anti-isotype specific conjugated antibodies for FACS analysis.

3. Results

1.8. PRRSV Flanders 13 infection, symptoms and virology (INRA)

The PRRSV infected pigs developed mild symptoms upon the first infection, mainly sneezing and temperatures superior to 40,5°C during one to five days, with a peak at day 2 post-infection. All pigs displayed infectious PRRSV in the serum as attested by titration on pig alveolar macrophages, with a serum titer at day 10 post infection of $10^{3,4}$ to $10^{6,7}$ TCID50/ml depending of the animal.

1.9. T cell reactivity against PPRSV antigen candidates (INRA)

1.9.1. ELISPOT with fresh PBMC

PBMC were collected at day 25 after the second infection, following a similar infection scheme as published in (1) for an optimal comparison of the T cell response with (1). The results are presented below in Figure 1.

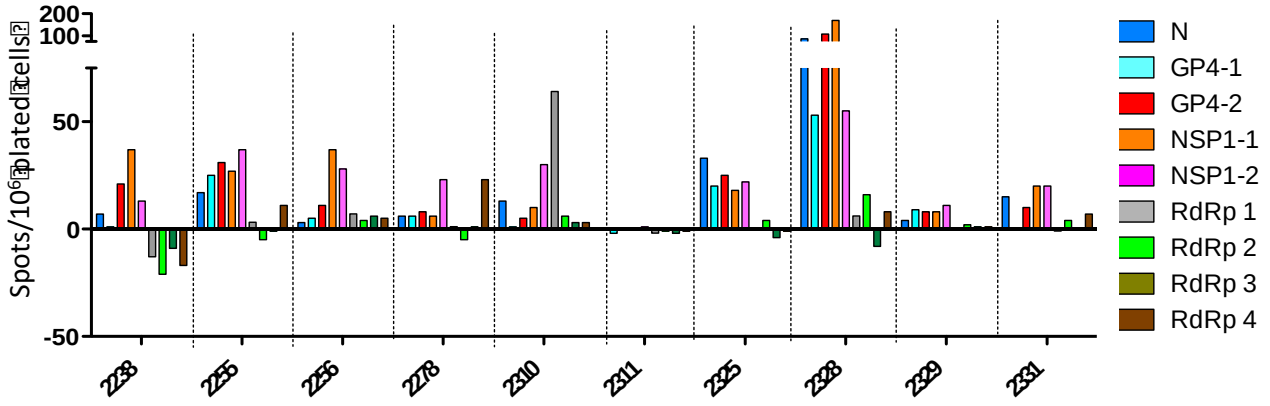

Figure 1: IFNγ T cell response in PBMCs from PRRSV Flanders13 infected pigs: 5 x 10^5 PBMCs from the 10 infected pigs (pig numbers on the X axis) were restimulated for 36 hours with the pool of peptides described in the M&M section (N, Gp-1, Gp-2, NSP1-1, NSP1-2, RdRp-1, 2, 3, 4, at 5µg/ml), see legends for color symbols).

We also run an IFNγ ELISA on the supernatant of similarly restimulated pig PBMCs but the signals were weak.

IFNγ ELISPOT on PBMC show IFNγ T cell responses against NSP1b (9/10 pigs), N and GP4-GP5-M (5/10 pigs) and RdRp (3/10 pigs), confirming the results from (1) for PRRSV Flanders13 in INRA Large White pigs.

1.9.2. ELISPOT with frozen spleen cells

Spleen cells were thawed from frozen stocks prepared 34 days after the second infection. Dead cells were removed by optiprep. Depletion of γ/δ in 2 pig samples increased the number of peptide-specific spots. We thus systematically depleted γ/δ T cells. The results are presented below in Figure 2.

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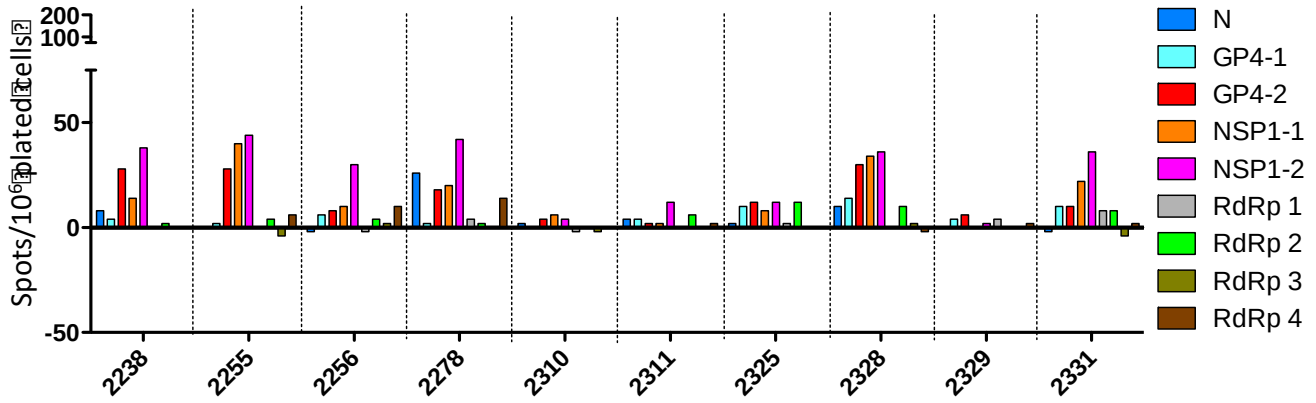

Figure 2: IFNγ T cell response in spleen cells from PRRSV Flanders13 infected pigs: 5 x 10^5 splenocytes from the 10 infected pigs (pig numbers on the X axis) were restimulated for 18 hours with the pool of peptides described in the M&M section (N, Gp-1, Gp-2, NSP1-1, NSP1-2, RdRp-1, 2, 3, 4, at 5µg/ml), see legends for color symbols).

IFNγ ELISPOT on γ/δ T depleted splenocytes show IFNγ T cell responses against NSP1b (9/10 pigs), GP4-GP5-M (8/10 pigs), RdRp (6/10) and N (3/10).

The IFNγ T cell response in spleen is coherent in magnitude and antigen specificity with the one in blood, especially for response to NSP1b and GP4-GP5-M restimulation. There is some slight discrepancies in the case of responses to RdRp and N.

1.9.3. IntraCellular Staining (ICS) and CD107 translocation

ICS allows to refine the ELISPOT results by determining the cell type responsible for IFNγ production ($CD4^+$ or $CD8^+$ T cells). However we could not detect significant staining for IFNγ expression in ICS on fresh PBMC, even upon activation with ConA. As the experiment was done on the same day as ELISPOT, it suggests that we encountered a technical problem not implicating the cell quality. It is also possible that the T cell response in the case of PRRSV Flanders13 infection is too low to be detected with this technique which is less sensitive than ELISPOT. In addition we could not detect CD107 translocation in 2 pilot experiments conducted with polyclonal activation (ConA and PMA+ionomycin). New attempts to detect intracellular IFNγ and CD107 translocation will be done using newly prepared reagents and polyclonal activation, before proceeding to analyse the spleen cells from frozen stocks and overlapping peptide stimulation. However the failure with these techniques has no impact on the other tasks as knowing whether the antigens are recognised by $CD4^+$ or $CD8^+$ T cells does not affect vaccine design.

Conclusions:

- our results show that NSP1β and GP4-GP5-M proteins from PRRSV Flanders13 are the main targets of IFNγ producing T cells in the genetic make-up of the INRA pigs, confirming the Mokhtar at al results (1). N and RdRp are also used as T cell targets, but with some variability across pigs, probably due to genetic polymorphism of the MHC and T cell receptors. Consequently all these antigens are to be selected for vaccine design.

-On the technical point of view we also conclude that ELISPOT is a sensitive, robust and convenient technique to analyse T cell response as compared to ELISA and ICS. This will thus be the technique of choice for use in WP11 and WP10.

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4. Glossary

PBMC: peripheral blood mononuclear cells

IFNγ; interferon gamma

GP4-GP5-M chimera: a fusion protein devoid of transmembrane domains

Gp-1 and 2: synthetic peptide pools from GP4-GP5-M chimera

NSP1-1 and NSP1-2: synthetic peptide pools from NSP1

RdRp-1, 2, 3, 4:synthetic peptide pools from RdRp

ISC: IntraCellular Staining

5. References

Mokhtar, H., et al., *Proteome-wide screening of the European porcine reproductive and respiratory syndrome virus reveals a broad range of T cell antigen reactivity.* Vaccine, 2014. **32**(50): p. 6828-37.

Subramaniam, S., et al., *In vivo targeting of porcine reproductive and respiratory syndrome virus antigen through porcine DC-SIGN to dendritic cells elicits antigen-specific CD4T cell immunity in pigs.* Vaccine, 2014. **32**(50): p. 6768-75.

Vu Manh, T.P., et al., *Defining Mononuclear Phagocyte Subset Homology Across Several Distant Warm-Blooded Vertebrates Through Comparative Transcriptomics.* Front Immunol, 2015. **6**: p. 299.

6. Annexes

T cell antigen candidates from Flanders13.

GP4-GP5-M:

MAATILFLLAGAQHFMVSEAFACKPCFSTHLSDIK-
TNTTAAAGFMVLQNINCPQFHRASTTSSSPLRKSPQCREAVGTPQYITIVANVTDESYL-
YNADLLMLSACLFYASEMSEKGFKVIFGNFADGNGDSSTYQYIYNLTICELNGTAWLSDKFY-
WAVAIRAAKNCMACRYARTRFTNFIVDDRGGVHRWKSPIVVEKLGKAEVGDALVTIKHV-
VIEGVKAQPLTRTTAEQWQAAGLDDFCYDSTAVQKLSRCRLCCLGRRYILAPAHHVESAA-
GLHPIPASGNQAYAVRKPGLTSVNGTLVPGLRGLVLGGKRAVKRGMVNLVKYGR*

N:

MAGKTQRQNRNKNPAPMGNGQSVNQLCQLLGSMLKSQRQQSRGGQVKKKKPEK-
PHFPLAAEDDVRHHLTQAERSLCLQSIQTAFNQGAG-
TASLSSSGKVGFQVEFMLPVTHTVRLIRVTSTSASQGVN*

RdRp:

MATGFKLLAASGLTRCGRGGLVVTETAVKIVKYHSRTFTLGPLDLKVTSEVEVKK-
STEQGHAVVANLCSGVVLMRPHPPSLVDVILKPGLDTTPGIQPGHGAGNMGVDGTIWDFETAPTRAE
LELSKQIIQACEIRRGDAPNLQLPYKLYPVRGDPERKEGRLINTRFGDLPYKTPQDTG-
SAIHAACCLNPNGAPVSDGKSVLGTTLQHGFELYVPTVPYSVMEYLDSRPDTPLMCTKHGTSRAATE
DLQKYDLSTQGFVLPGVLRLVRRFIFGHIGKAPPLFLPSTYPAKNSMAGINGQRF-
PTKDVQSIPEVDEMCARAVKENWQTVTPCTLKKQYCSKPKTRTILGTNNFIALAHRSALSGVTQAFMK
KAWRSPIALGKNKFKELHCTVAGRCLEADLASCDRSTPAIVRWFTAHLLY-
ELAGCEEYLPSYVLNCCHDLVATQDGAFTKRGGLSSGDPVTSVSNTVYSLIIYAQHMVLSALKMGHEI

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GLKFLEDQLKFEDLLEIQPMLVYSDDLVLYAEQPTFPNYHWWVEHLDLM-
LGFKTDPKKTVITDKPSFLGCKIEAGRQLVPNRDRILAALAYHMKAQNASEYYASAAAILMDSCACIDY
DPEWYEDLICGIARCARQDGYSFPGPPFFMSMWERLKSHNE*

NSP1b:

MSGTFSRCMCTPAARVFWNAGQVYCTRCLSARSLLPLELQDDDLGAIGLFHKPKDKLR-
WRVPVGIPLVECSPSGCCWLSAIFPLARMTSGNHNFLQRLVKVAEVLYRDGCLTPRHLREL-
QVYERGCDWYPITGPVPGMGMYANSMHVSDRPFPGATHVLTNS-
PLPQQACRQPFCPFEEAHSDVYKWKKFVIFTDSSPNGRSRMMWMPESGDSANLEEL-
PLELERQVEILVRSFPAHHPVDLA-
DWELTESPEHGFSFGTSHHCGYLAQHPYGFDGKCWLSCFLDLSTKVLRHEEYLAS-
AFGYQTRWGVPGKYLQRRLQINGVRAVVDPDGPIHVEALSCPQSWIR-
HLTLDDDATPGFVRLMSLRIIPNTEPTTLQIFRFGTHKWY

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