

Identification of cross-reacting T-cell epitopes in structural and non-structural proteins of swine and pandemic H1N1 influenza A virus strains in pigs

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Abstract

Heterologous protection against swine influenza viruses (SwIVs) of different lineages is an important concern for the pig industry. Cross-protection between 'avian-like' H1N1 and 2009 pandemic H1N1 lineages has been observed previously, indicating the involvement of cross-reacting T-cells. Here, reverse vaccinology was applied to identify cross-reacting MHC class I T-cell epitopes from two different SwIV H1 lineages in pigs. *In silico* prediction followed by *in vitro* and *in vivo* testing was used to identify SLA-1*0702 T-cell epitopes in heterologous SwIV-infected pigs. Following viral infection, tetramer specific T-cell populations were identified. The majority of the identified T-cell epitopes were conserved between the examined lineages, suggesting that targeting cross-reactive T-cell epitopes could be used to improve vaccines against SwIV in SLA-1*0702-positive pigs.

Swine influenza virus (SwIV) is considered to be an important pathogen in pig herds. Different subtypes and lineages are circulating in pigs [1] and the involvement of both arms of the immune system is considered to be necessary for an effective immune response [2]. Although commercially available vaccines were designed to confer homologous protection, the control of heterologous infections is of high importance for controlling influenza in pig herds. The identification of protective cross-reacting antigens thus remains an open challenge.

Some previous studies have shown that infection with SwIVs of European 'avian-like' H1N1 lineage can induce complete protection against the influenza A virus of the 2009 pandemic H1N1 lineage [3, 4]. Such cross-protection was conferred in the absence of cross-reactive antibodies capable of inhibiting hemagglutination or virus neutralization, suggesting that other factors, like cross-reacting T-cell responses, might have been involved.

Reverse vaccinology tries to overcome problems related to the empirical identification of antigens. Previously, a combination of different bioinformatics prediction methods and *in vitro* testing has been used to identify T-cell epitopes in pigs

[5]. In addition, recent approaches that use immunoinformatic tools have been used to identify MHC class I and class II T-cell epitopes that are highly conserved in SwIVs circulating in the US swine population [6]. In the present study, reverse vaccinology technologies were extended to identify cross-reactive T-cell epitopes to two different H1N1 influenza A virus (IV) strains during the infection of pigs expressing SLA-1*0702 allele. Strain A/swine/Spain/SF11131/2007 (SpH1N1) is an 'avian-like' H1N1 SwIV [7], whereas strain A/swine/Denmark/101310-1/2011 (pdmH1N1) is an H1N1 strain that circulated in pigs, but belongs to the 2009 pandemic lineage.

Firstly, T-cell epitopes residing in SpH1N1 and pdmH1N1 were predicted for SLA-1*0702 binding using a previously described strategy that combines two methods for *in silico* prediction [8]. Briefly, the neural network NetMHCpan v. 2.8 prediction tool (www.cbs.dtu.dk) and a positional scanning combinatorial peptide library (PSCPL) for the SLA-1*0702 binding motif were used to predict T-cell epitopes. The experimental strategy of PSCPL has been described previously for both HLA [9] and SLA [10] proteins.

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Abbreviations: d.p.b., days post boost; d.p.i., days post primary infection; IV, influenza virus; PSCPL, positional scanning combinatorial peptide library; SwIV, swine influenza virus.

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Secondly, the peptide sequences that were consistently predicted by these two methods in combination were further analysed for actual MHC binding affinities (K_d less than 1000 nM) to recombinant SLA-1*0702 (Table 1) by an *in vitro* immunosorbent assay [11]. Following these criteria, 11 sequences were predicted to be T-cell epitopes for the selected SLA allele and seven of them were conserved between both SwIV strains (Table 1). These sequences happened to be located within HA, NA and M1 proteins. Finally, tetramers were generated, as previously described [10, 12], using the 11 peptides identified *in vitro* and a specifically designed peptide as a negative control. Theoretically, the detection of T-cell populations by recognizing specific peptides presented on SLA-1*0702 would indicate the recognition of viral T-cell epitopes.

Four pigs bearing the allele SLA-1*0702 and a mismatched pig (not bearing the selected SLA allele) were selected following genomic SLA-I amplification by high-resolution sequence-specific primers [13] and infected with SpH1N1. All animals but one (L12) were intranasally infected with 3 ml, 9.04 logTCID₅₀ of SpH1N strain. L12, the SLA-I-matched pig, was intratracheally infected with the same dose. At 139 days post-primary infection (d.p.i.), animals were intranasally infected with 6 ml, 5.58 logTCID₅₀ of the Danish pdmH1N1 strain. The study was carried out in accordance with Danish legislation on animal experimentation and EU regulations on the use of laboratory animals for research (protocol number 2012-15-293–00682). All animals were euthanized 14 days post-second infection (boost; d.p.b.), corresponding to 153 d.p.i. Serum samples were collected to monitor antibody levels against both SwIVs by hemagglutination inhibition assay [14] and influenza A Ab ELISA (IDEXX). PBMCs were isolated from animals at different days post-infection. Biotinylated tetramers

(Table 1) were used to stain the PBMCs and were visualized with a streptavidin-BV₄₂₁ fluorophore. PE-conjugated MAb against porcine CD8 α (clone 76-2-11, BD Pharmingen) and FITC-conjugated MAb against porcine CD3 ϵ (clone PPT3, Southern Biologend) were additionally used to characterize cells that stained positive with the tetramers. CD3⁺CD8^{high} cells contain a CD8⁺ $\alpha\beta$ T-cell subpopulation which recognises class-I epitopes [5, 15]. Tetramers showing frequencies two times higher compared to background (negative control or tetramer 831) and two times higher compared to the mismatched pig staining were regarded as positive.

None of the pigs developed clinical signs due to infection. Humoral SpH1N1- and pdmH1N1-specific responses were generated against each virus after infection (data not shown). Tetramer-positive T-cell subpopulations were below detection levels at 4, 7 and 9 d.p.b. (data not shown). At 14 d.p.b. specific T-cell responses were detected within the CD3⁺CD8^{high} subpopulations (Tet⁺CD3⁺CD8^{high}) as compared with the negative controls (Fig. 1, Table 2). The frequency of Tet⁺CD3⁺CD8^{high} ranged from 0.4 to 2.6 %, depending on individual peptides and animals (Table 2). Nine peptides were identified as T-cell epitopes by tetramer staining; three located in the M1 protein (Tet62, Tet56 and Tet64), three in NA (Tet55, Tet60 and Tet59) and three in HA (Tet93, Tet57 and Tet66). These results highlighted the high efficiency of the discovery of class I restricted T-cell epitopes using this pipeline. The NA_{171–180} (CPIGEVPSPY) peptide was considered to be immunodominant, as it was consistently detected in all pigs with the highest frequency within CD3⁺CD8^{high} cells. Importantly, most of the other T-cell epitopes were only found in animal L6, in which NA_{171–180} was not dominant.

Table 1. SwIV peptides *in silico* predicted to bind with SLA-1*0702 with their respective *in vitro* binding results

Strong binding peptides with a K_d less than 1000 nM were used to generate tetramers.

Peptide sequence	Viral protein of origin (no.)	Position	Virus		Kd (nM)	Tetramer name
			pdmH ₁ N ₁	SpH ₁ N ₁		
NADTLCIGY	HA(1)	16–24	+		1 543	
SLSTASSWSY	HA(2)	86–95	+		35	57
TLYQNNHTY	HA(3)	207–215		+	6	66
YVSVGSSKY	HA(4)	215–223		+	987	93
SVKNGTYDY	HA(5)	295–303	+		20 000	
GMIDGWYGY	HA(6)	300–308		+	20 000	
EIGNGCFEFY	HA(7)	476–485	+	+	132	63
CPVSGWAIY	NA(1)	92–100	+	+	6	55
CPIGEVPSPY	NA(2)	171–180	+	+	37	60
GPSNGQASY	NA(3)	245–253	+	+	25	59
SVELNAPNY	NA(4)	266–274		+	20 000	
EMNAPNYHY	NA(5)	268–278	+		231	61
NMDRAVKLY	M1(1)	92–100	+	+	293	62
ALASCMGLIY	M1(2)	123–132	+	+	127	56
LASCMGLIY	M1(3)	124–132	+	+	9	64
VSAAAAAY	Negative control					831

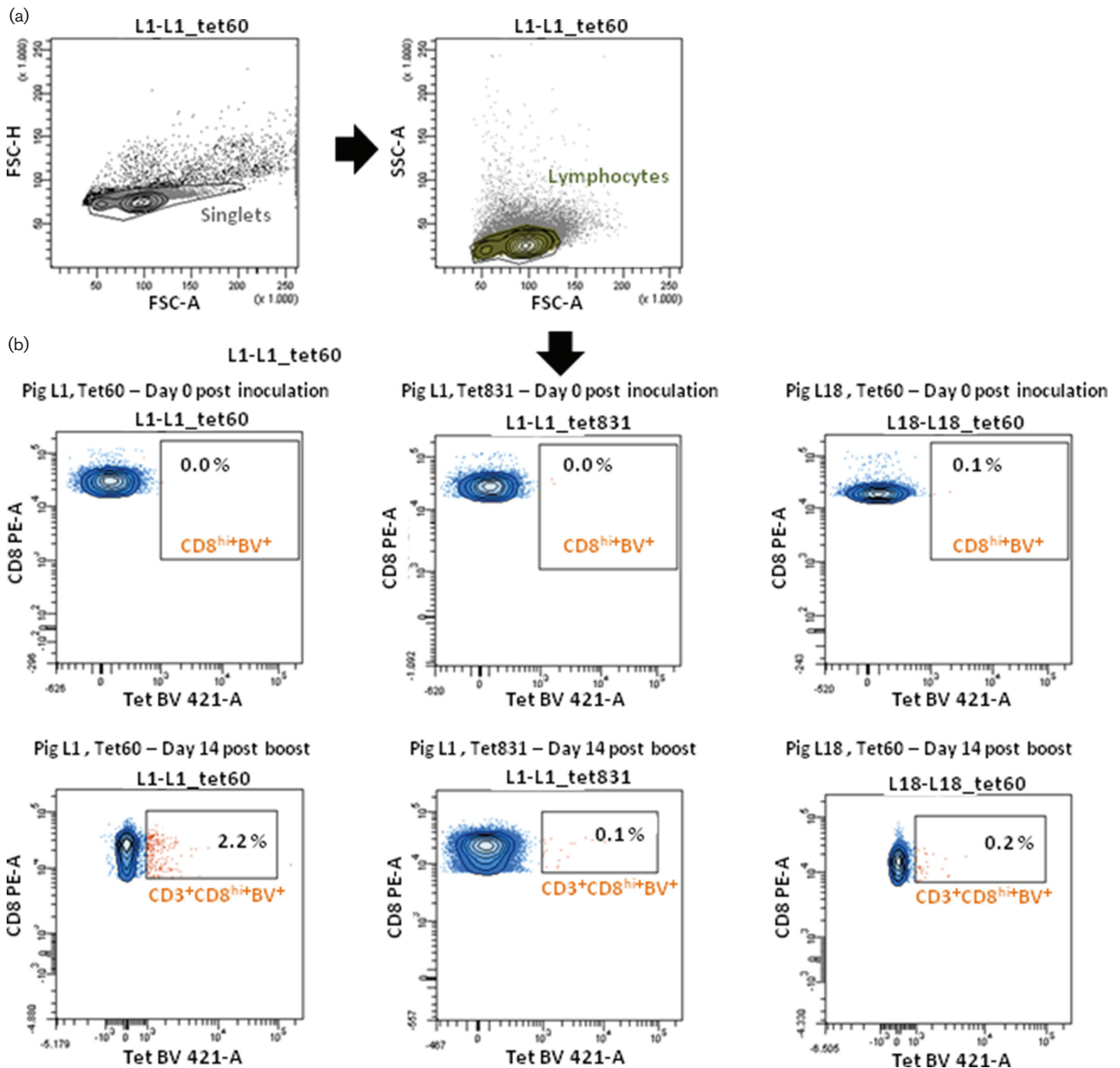


Fig. 1. Tetramer staining of PBMCs of infected pigs. Example of gating strategy and staining of cells from pig L1 and pig L18 (mismatched pig) at days 0 and 153 post-infection (14 d.p.b.). (a) Singlet and lymphocyte analysis. (b) CD3⁺CD8^{high} cells are shown. Tetramer 60 (Tet 60) is shown to specifically stain PBMCs from pig L1 at 14 d.p.b. The same tetramer and negative control tetramer (Tet 381) do not stain PBMCs at 0 d.p.i. or PBMCs from the mismatched pig (L18) at any time point.

Several factors can account for the different immunodominance hierarchy among animals [16]. For example, the pigs in this study were not inbred; therefore, different levels of SLA-1*0702 might have been expressed on pig cells, depending on whether these animals were homozygous or heterozygous for this allele.

All T-cell epitopes identified in NA and M1 proteins were conserved between both IVs. By contrast, the T-cell epitopes

identified in HA were not conserved. However, it is noteworthy that SpH1N1-HA-specific T-cells (Tets 66 and 93) were detected after pdmH1N1 infection, indicating that a population of T-cells was primed after the second infection, which cross-reacted. A previous study in pigs suggested that there is flexibility in T-cell receptor recognition of class I epitopes and thus they can recognize non-homologous peptides [5]. It could also be speculated that HA-SpH1N1-specific T-cells, generated during the first infection, were

Table 2. Frequency of Tet⁺CD3⁺CD8⁺ cells at 14 d.p.b. Numbers in bold show values two times higher than the background (negative control or tetramer 831)

Those values that were also two times higher than the respective peptide in the mismatched pig (L18) are underlined.

Pig	Frequency of tetramers											
	831	93	55	56	57	59	60	61	62	63	64	66
L1	0.1	0.1	0.1	0.7	<u>1.9</u>	0.2	<u>2.2</u>	0.0	0.0	0.1	<u>0.7</u>	0.0
L6	0.1	<u>0.4</u>	<u>1.7</u>	<u>1.2</u>	1.2	<u>2.1</u>	<u>1.0</u>	0.4	<u>0.7</u>	0.1	0.1	<u>1.0</u>
L11	0.7	0.1	0.2	0.0	1.2	1.2	<u>2.6</u>	0.6	1.3	0.1	0.8	0.7
L12	0.3	0.1	0.2	0.1	0.4	0.2	<u>0.7</u>	0.1	0.4	0.6	0.3	0.5
L18	0.1	0.1	0.4	0.5	0.8	0.7	0.2	0.2	0.1	0.1	0.1	0.3

re-activated by pdmH1N1 in a non-specific or bystander way, as has been described for other systems [17].

A recent study identified a number of SLA-I epitopes by using another immunoinformatic tool in pigs with a different SLA-I [6]. Interestingly, seven of the eleven selected epitopes in this study (HA1, HA2, HA5, HA6, NA5, M1-1 and M1-3) were identical to or had just one amino acid change compared to those reported by Gutiérrez *et al.* [6] using outbred pigs expressing SLA-I alleles other than SLA-1*0702. The combination of these data suggests that these epitopes potentially have cross-reactive properties and may be relevant in a broad range of pigs. Out of these seven sequences, HA2 (Tet 57), NA5 (Tet 61), M1-1 (Tet 62) and M1-3 (Tet 64) were selected for the detection of epitope-specific T-cells (CD3⁺CD8⁺). All of them depicted specific T-cell subpopulations in some of the pigs, although they were not dominant. Remarkably, the majority of the predicted epitopes in this study and in Gutiérrez *et al.* [6] had tyrosine in position 9, suggesting that an amino acid with a hydrophobic side chain might be the preferable option for SLA-I antigen presentation in pigs.

In conclusion, the data in this work further extend the accuracy and versatility of our pipeline for SLA class I T-cell epitope identification and confirm the epitope predictions generated using other technologies. Several cross-reacting T-cell epitopes were identified and specific subpopulations were detected following heterologous SwIV infection. The functionality of these epitopes and their involvement in cross-protection will require further assessment in the future with the aim of applying these technologies to reverse vaccinology for SwIV in pigs.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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