Major histocompatibility complex I of swine respiratory cells presents conserved regions of influenza proteins

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Abstract

Influenza A virus in swine (IAV-S) is a prevalent respiratory pathogen in pigs that has deleterious consequences to animal and human health. Pigs represent an important reservoir for influenza and potential mixing vessel for novel influenza gene reassortments. Despite the central role of pigs in recent influenza outbreaks, much remains unknown about the impact of swine immunity on IAV-S transmission, pathogenesis, and evolution. An incomplete understanding of interactions between the porcine immune system and IAV-S has hindered development of new diagnostic tools and vaccines. In order to address this gap in knowledge, we identified swine leukocyte antigen (SLA) restricted IAV-S peptides presented by porcine airway epithelial cells using an immunoproteomics approach. The majority of MHC-associated peptides belonged to matrix 1, nucleoprotein and nonstructural 1 proteins. Future investigation of the potential cross-reactive nature of these peptides is needed to confirm antigen recognition by cytotoxic T lymphocytes and their utility as vaccine candidates.

The 2009 H1N1 pandemic and 2012 H3N2v outbreak have solidified the pig as an important reservoir for influenza maintenance and potential mixing vessel for novel influenza gene reassortments [1, 2]. These recent cases of swine influenza virus (IAV-S) have also highlighted knowledge gaps in influenza transmission, pathogenesis and immunity in swine. Understanding protective immune responses in swine against influenza represents one of the greatest challenges in the IAV-S field. This knowledge would foster development of new diagnostics and rationale for improved vaccine design.

Mucosal and systemic adaptive immune responses are categorized into two basic components: humoral (antibody) and cell-mediated responses. The humoral or B-lymphocyte response is necessary to neutralize extracellular viruses and prevent viral attachment to the host cell. The humoral response is predominantly directed against viral surface components such as the influenza glycoproteins, haemagglutinin (HA) and neuraminidase (NA) [3, 4]. Thus, both HA and NA are under strong antibody pressure, which drives their highly variable structure. Although the humoral response is one of the mechanisms that contribute to protection, it is virus strain-specific and delivers effective homosubtypic protection. The cell-mediated response is primarily directed by T-lymphocyte recognition of conserved internal viral proteins, such as nucleoprotein (NP) and matrix (M1) [5] that are presented on host major histocompatibility complex (MHC) molecules. The highly conserved nature of these internal proteins suggests that mutations within these regions are deleterious to viral fitness and replication [6].

Due to its unparalleled ability to reduce the severity and clinical signs of illness, accelerate viral clearance, promote recovery after natural infection and achieve cross-protective, heterosubtypic protection, cytotoxic T lymphocytes (CTLs) are under intense investigation to facilitate the design of a universal influenza vaccine [7, 8]. Viral elimination through host cell apoptosis by CTLs is achieved through recognition of viral determinants or epitopes displayed on the host cell’s MHC class I molecules. The T-cell response against influenza remains poorly characterized in pigs. Although pigs are the natural host for IAV-S and are widely available, the majority of immunological data concerning the T-cell response against IAV-S have been obtained from experiments performed in laboratory mice. While the commercial accessibility of reagents, cost effectiveness and genetic tractability of mice make this model desirable, findings obtained from mice may not translate to...
swine health. For example, CD4+ and CD8+ double-positive T cells are commonly found outside the thymus in pigs; however, extrathymic CD4+CD8+ lymphocytes are rare events in mice [9]. Differences between the mouse and pig immune systems also extend to the number of γδ T cells and natural killer cell receptors [10]. Furthermore, the heterogeneous pig population displays a greater range of MHC diversity that is not observed in inbred laboratory mice strains [11–14]. MHC polymorphisms impact the 3D structure of the peptide binding groove (PBG), and thus the types of epitopes displayed must contain motifs compatible to these PBG pockets. Recently, binding of the 2009 pandemic swine H1N1 strain with crystallized pSLA-3*hs0202 from Chinese Heishan pigs showed two distinct conformations in these PBG pockets. Recently, binding of the 2009 pandemic swine H1N1 strain with crystallized pSLA-3*hs0202 from Chinese Heishan pigs showed two distinct conformations in the PBG [15]. These areas of immune divergence likely influence disease manifestation and progression.

A recent study conducted by Talker et al. [16] characterized T-cell populations in pigs infected with H1N2. Flow cytometric analysis of swine lung lymphocytes showed influenza-specific CD4+ and CD8+ gamma-interferon-producing cells by four days post-infection (p. i.) followed by an increase in CD8+, Perforin+, CD27+ and memory CD4+ and CD8+ T cells by six days and six weeks p. i., respectively. Isolated CD4+ and CD8+ memory T cells displayed cross-reactivity against heterologous influenza strains [16]. Similarly, Khatri et al. have also identified that CTL infiltrates were detected within the lungs of IAV-S infected pigs as early as 6 days p. i. [17]. Together these data suggest that a robust, multi-functional T-cell response develops early during IAV-S infection, which is capable of cross-protection against heterologous influenza strains in swine. It is unknown which epitopes are presented by the various MHC I molecules, herein referred to as swine leukocyte antigen (SLA), to T-cell receptors in swine. Three classical SLA I loci, SLA-1, SLA-2 and SLA-3, have been identified in pigs [18]. Polymorphisms within SLA genes influence the structure of the PBG and the diversity of viral motifs displayed on the host cell. A select number of studies have identified SLA-specific influenza T-cell epitopes using immuno-informatic tools, such as PigMatrix [19] and NetMHCpan [20], coupled with ex vivo validation using pig T-cell functional assays. The scope of these reverse immunology approaches is limited as they can only identify predicted dominant epitopes recognized by antigen-experienced pigs rather than those viral peptides that are presented, which could be recognized by CTLs. In other words, our understanding of the full repertoire of influenza epitopes presented by SLA I and its variants remains incomplete.

In the present study, mass spectrometry acquisition and analytical methods were used to directly characterize the IAV-S variant H3N2 [A/swine/Ohio/12TOSU268/2012 (H3N2)] [21] epitope repertoire presented by two SLA I haplotypes in primary porcine airway epithelial cells (pAECs). We show that early infection of pAECs is dominated by M1 and NP presentation by both haplotypes. While coverage throughout the entire length of the M1 protein is well represented, the greatest number of spectral hits occurred within M1229–242. The conserved region of M1229–242 across multiple influenza strains may be incorporated into new vaccine formulations against IAV-S.

For complete details on methods used in this short communication please refer to File S1, available in the online version of this article. All animal work performed was in concordance with the institutional guidelines and approved animal care and use protocols at the University of Minnesota. Briefly, pAECs [22] isolated from neonatal pigs were previously haplotyped using the methods described by Ho et al. [23]. Two SLA I haplotypes were identified as SLA 1*0101 and SLA 1*0401. PAECs from individual pigs were infected with variant H3N2 (H3N2v) swine influenza virus (A/swine/Ohio/12TOSU268/2012[H3N2]) (Centers for Disease Control and Prevention) at a multiplicity of infection (MOI) of 0.01 in DMEM/F12 medium containing 10% FBS, penicillin G (100 U ml−1), streptomycin (100 µg ml−1), gentamycin (100 µg ml−1), 25 mM HEPES, 1X non-essential amino acids, amphotericin B (5 µg ml−1), 1% epidermal growth factor (EGF) and recombinant human insulin (5.5 µg ml−1), and trypsin (0.5 µg ml−1) for 1 h at 37 °C in a humidified chamber containing 5% CO2. PAECs were washed thrice with Hank’s buffered salt solution and the virus was allowed to propagate for a further 24 h. Post-infection pAECs were detached and lysed, and membrane extractions were isolated using phase separation. SLA I complexes were immunoprecipitated by incubating membrane fractions with protein A/G magnetic beads (Crosslink Magnetic IP/Co-IP kit; Pierce, Thermo Scientific, Rockford, IL) cross-linked to a SLA I-specific monoclonal antibody (PT85-A; Washington State University Monoclonal Antibody Center, Pullman, WA). SLA I peptides were eluted and sent to the Medical Genome Facility Proteomics Core at Mayo Clinic (Rochester, MN) for peptide ion exchange chromatography and tandem mass spectrometry. Strong anion exchange (SAX) fractionation was performed on samples, followed by LC-MS/MS analysis on the Q-Exactive Orbitrap mass spectrometer (Thermo Scientific, Rockford, IL). Peptide identification was completed using the Uniprot protein database (uniprot.org, version 2013_09) against human, porcine, bovine and H3N2v strain influenza reference proteomes. A custom decoy database was used to validate Uniprot identifications. Data were analysed in Scaffold. The mass spectrometry proteomics raw data have been deposited to the ProteomeXchange Consortium via the Proteomics Identifications Database (PRIDE; www.ebi.ac.uk/pride/archive) [24] partner repository with the dataset identifier PXD007866 and 10.6019/PXD007866.

Peptides mapped to porcine, human and bovine proteomes were removed from further analysis. One hundred and eighty-four and 367 peptides matched the H3N2v influenza proteome for SLA 1*0101 and SLA 1*0401 haplotypes, respectively (Fig. 1 and File S2). As expected, the majority of peptides presented by SLA I from both haplotypes were influenza's internal proteins. The number of mass spectral hits
corresponding to influenza proteins for each haplotype (SLA 1 *0101, SLA 1 *0401) were as follows: matrix 1 (106, 160) [GenBank: AFR61066.1], matrix 2 (0, 15) [GenBank: AFR61065.2], non-structural protein (59, 71) [GenBank: AFR61068.1], nucleoprotein (8, 73) [GenBank: AFR61063.1], haemagglutinin (5, 24) [GenBank: AFR61062.1], neuraminidase (2, 16) [GeneBank: AFR61064.1], polymerase (PA) (4, 5) [GenBank: AFR61061.1] and nuclear export protein (0, 3) [GenBank: AFR61067.1] (Fig. 1). SLA 1 *0401 consistently showed more mass spectral hits compared to SLA 1 *0101, suggesting that the *0401 haplotype is capable of presenting a greater range of influenza epitopes. For peptide coverage of individual IAV-S proteins, please refer to File S3.

The matrix 1 (M1) protein of influenza plays an integral role in budding and release of the influenza virus and its progeny [25]. M1 mutations result in morphological changes at the bud site and in viral progeny [26, 27]. Peptides from M1 dominated both haplotypes. This observation is consistent with other studies performed on mice [28] and human CTLs, which have shown that M1 epitopes are immunodominant. While the entire length of M1 was well represented, mass spectra showed the greatest number of hits in the 229–242 region (Fig. 2 and File S2). M1229–242 is conserved across H1N1, H3N2, H6N2 and H3N8 influenza strains. The previously identified HLA-B*2705 minor epitope M1128–135 was found within the identified M1128–145 region displayed by SLA 1 *0401 only. Furthermore, M1 epitopes at regions 2–28 and 244–252 were exclusively presented by SLA 1 *0401. Multiple studies performed in transgenic mice have demonstrated that immunization with M1 epitopes led to protection from subsequent influenza challenge [29, 30]. Given its high conservation across influenza strains and immunodominance, M1 epitopes identified in this study may be a rich source for IAV-S vaccine development.

The non-structural protein (NS1) of influenza performs a multitude of functions, including antagonism of host immune responses [31–33], control and regulation of viral mRNA synthesis [34] and translation [35] and viral particle morphogenesis [36]. NS1 represents the second and third most abundant peptides presented by SLA 1 *0101 and SLA 1 *0401, respectively (Fig. 1). The greatest number of mass spectral hits in NS1 for both haplotypes correlated with NS177–89 (Fig. 2 and File S2), which encompasses a linker region critical for viral and host protein interaction and potential counter-immune responses [37].

The third dominant protein presented by SLA I was the nucleoprotein (NP). However, increased NP peptide presentation was shown in the SLA *0401 haplotype (Fig. 1). With few exceptions, SLA 1 *0401 presented overlapping peptides starting with amino acid position 48 (File S2). Sixteen of the total 40 peptides identified in our assay were also designated as CTL epitopes in silico by NetMHCpan. Immunoproteomic investigations by Wahl et al. have identified several HLA-B*0702 NP CTL epitopes, such as NP418–426 and 473–481 derived from three separate influenza strains [38]. NP417–426 was identified in both haplotypes (Fig. 2 and File S2). Furthermore, the NP417–426 peptide was predicted as a potential CD8+ T-cell epitope in SLA 1 *0101 and SLA 1 *0401 haplotypes (File S2). In addition to the HLA-B*0702 NP CTL epitope identified by Wahl et al. [38], NP417–426 overlaps with the HLA-A24 restricted peptide NP419–428 derived from 2009 pH1N1 virus [39]. Further investigations will need to determine whether NP417–426 is capable of cross-allele presentation among pigs and humans. In addition to M1229–242,
future epitope-based vaccines may include NP\textsubscript{417-426} and NS1\textsubscript{77-89}. It is tempting to speculate that the shared region between NP\textsubscript{417-426} and NP CTL epitopes characterized by Wahl et al. [38] and Liu et al. [39] may be suited for vaccine implementation in both pigs and humans. Furthermore, epitope-based vaccine formulations could be combined with non-neutralizing NP antibodies to achieve heterosubtypic protection [40].

In addition to the internal proteins of H3N2v, the immunoproteomic screen identified several peptides belonging to haemagglutinin and neuraminidase. These epitopes may aid in current diagnostics of IAV-S infection in swine to bolster current surveillance strategies. Furthermore, confirmation of CTL epitopes may provide a novel reagent to investigate influenza CTL populations in swine. Such reagents may include MHC I tetramers loaded with CTL epitopes to understand the swine immune response against influenza infection and influenza vaccine efficacy [41]. The development HA and NA epitopes as diagnostic tools may bolster surveillance strategies. Furthermore, epitopes that map to the HA stalk region, such as HA\textsubscript{125-135}, may also be further investigated for utility in a universal influenza vaccine [42].

The SLA 1 *0401 structure was recently characterized by Zhang et al. [43]. The SLA 1 *0401 PBG is composed of six pockets (A–F) that interact with viral peptides. Bound peptide residues in P1, P2, P3, P6 and P9 interact with pockets A, B, C and D, E and F, respectively, of the SLA 1 *0401 PBG. Pocket D contains a flexible Arg\textsubscript{156} residue that restricts the type of peptides able to bind to the PBG through a ‘one ballot veto’ mechanism. Viral peptides that do not have a small or negatively charged P3 residue will be repelled by Arg\textsubscript{156}. Pocket B accommodates neutral side chains, while pocket F binds to anchor residues that contain large aromatic rings. The P6 residue of viral peptides inserts into pocket E, and P7 and P8 residues project into the solvent, where they may contact TCRs. The well-defined SLA 1 *0401 binding motifs are represented in our peptide data: DILLENQAY (M1), QSVAVDVGDDGHF (M2), TIASVPTSR (NS1) and ASVPTSR (NS1) (File S2). Interestingly, Zhang et al. [43] found that SLA 1 *0401 and the human leukocyte antigen (HLA) class I, HLA-A *0101, are able to present the same peptides, albeit in different conformations. Peptides identified in this study containing the SLA 1 *0401 binding motif may be important in cross-species epitope presentation and may lend themselves to epitope vaccination in humans. Further structural and immunological studies are needed to confirm potential cross-species presentation of these PBG binding peptides.

In conclusion, we have identified SLA 1-associated influenza epitopes using a cell-based model of the natural host. Multiple peptides mapped to highly conserved internal influenza protein regions, which may serve as epitope based vaccines to achieve cross-protection against heterosubtypic influenza strains. Future studies by our group will seek to evaluate the potential for heterosubtypic protection of these epitopes in swine.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.
Ethical statement
All animal work performed was in accordance with the institutional guidelines and approved animal care and use protocols at the University of Minnesota.

References


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