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The non-structural protein Nsp2TF of porcine reproductive and respiratory syndrome virus down-regulates the expression of Swine Leukocyte Antigen class I

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) is arguably the most economically-important global swine pathogen. Here we demonstrated that PRRSV down-regulates Swine Leukocyte Antigen class I (SLA-I) expression in porcine alveolar macrophages, PK15-CD163 cells and monocyte-derived dendritic cells. To identify the viral protein(s) involved in SLA-I down-regulation, we tested all 22 PRRSV structural and non-structural proteins and identified that Nsp1α and Nsp2TF, and GP3 significantly down-regulated SLA-I expression with Nsp2TF showing the greatest effect. We further generated a panel of mutant viruses in which the Nsp2TF protein synthesis was abolished, and found that the two mutants with disrupted −2 ribosomal frameshifting elements and additional stop codons in the TF domain were unable to down-regulate SLA-I expression. Additionally we demonstrated that the last 68 amino acids of TF domain in Nsp2TF are critical for this function. Collectively, the results indicate a novel function of Nsp2TF in negative modulation of SLA-I expression.

Introduction

Since its appearance in 1987, porcine reproductive and respiratory syndrome (PRRS) has arguably been the most economically-important global swine disease causing severe late-term reproductive failure in sows and respiratory diseases in pigs of all ages, especially young pigs (Albina, 1997a; Holtkamp et al., 2013; Neumann et al., 2005; Rowland, 2010). Clinically it is often seen with complications due to secondary bacterial infections and/or co-infections with other viruses such as porcine circovirus type 2, porcine parvovirus and swine influenza virus (Albina, 1997b). The causative agent, PRRS virus (PRRSV), is an enveloped, positive-sense, single-stranded RNA virus that belongs to the family *Arteriviridae* in the order *Nidovirales* (Lunney et al., 2010a). Two genotypes of PRRSV, the European type (type 1) and North American type (type 2), were identified with extensive genetic variations as well as differences in pathogenicity and antigenicity (Meng et al., 1994, 1995a, 1995b; Morozov et al., 1995).

The genomic RNA of PRRSV of approximately 15 kb in size is capped at 5' end and polyadenylated at 3' end, and contains at least 10 open reading frames (ORF): ORF1a, ORF1b, ORF2a, ORF2b, ORFs 3–5, ORF5a and ORFs 6–7 (Dea et al., 2000; Firth et al., 2011; Johnson et al., 2011). Approximately 75% of the viral genome at 5' proximal region can translate into polyproteins 1a and 1ab, which are further co-translationally processed into at least 14 non-structural proteins (Nsps) by auto-proteolytic cleavages (Fang and Snijder, 2010; Li et al., 2012). These Nsps mainly function in viral replication and transcription, and some Nsps are also involved in host immune modulation. The Nsp2TF is a newly-discovered non-structural protein encoded within ORF1a and produced via a unique −2 programmed ribosomal shifting (−2 PRF) mechanism (Fang et al., 2012). Sequence analysis revealed that the Nsp2TF appears to be more conserved than the Nsp2. The biological function(s) of the Nsp2TF are largely unknown. The 3' portion of the viral genome contains at least 8 ORFs which encode structural proteins (SPs) including GP2a, E, GP3, GP4, GP5, GP5a, M and N, respectively, translated from a nested set of subgenomic mRNAs (Kappes and Faaberg, 2015; Kappes et al., 2013; Meng et al., 1994).

PRRSV infection in pigs typically induces an impaired immune response in the host including a weak innate immunity (Albina et al., 1998), a delayed neutralizing antibody response (Loemba et al., 1996; Yoon et al., 1994), and a gradual slow development of cell-mediated immune (CMI) responses (Loving et al., 2015; Meier et al., 2003). The same scenario also applies to the PRRSV modified live-attenuated vaccine (MLV)-induced immunity (Meier et al.,

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2003). Current commercially available vaccines include both inactivated vaccines with limited efficacies and MLVs (Huang and Meng, 2010). The MLVs are generally effective against homologous or closely-related strains but ineffective against heterologous strains. Antigenic variations and immune evasion of PRRSV are thought to be the major obstacles for developing a more efficacious vaccine against PRRSV (Huang and Meng, 2010; Meng, 2000).

Although CMI plays a critical role in the antiviral immunity against PRRSV, little is known about T cell and memory responses against PRRSV. It is frequently observed that PRRSV-specific IFNγ-secreting cells were not detected in pigs until 2−3 weeks either post-infection or post-vaccination, and their frequency in peripheral blood increased slowly afterwards and remained at low levels (Loving et al., 2015; Meier et al., 2003). It is also reported that PRRSV infection reduced the surface expression of Swine Leukocyte Antigen class I (SLA-I) in macrophages and dendritic cells (Park et al., 2008; Wang et al., 2007). SLA-I (swine MHC class I) molecules are expressed on the surface of all nucleated cells and present peptide fragments derived from the 'housekeeping protein' of cells or from viral antigens in the context of virus infection. Moreover, SLA-I molecules are essential components of antigen presentation for the subsequent activation CD8+ T cells, as well as for the detection of virally infected cells by cytotoxic T cells (Schmidt et al., 2013). As a result, it is not surprising that many viruses, especially those establishing persistent or chronic infections, utilized different strategies of interfering MHC class I pathway in order to evade the host immune surveillance. In most cases, the viral proteins responsible for such immune evasion have been identified and characterized, such as the adenovirus E3/19K, human cytomeglavirus (HCMV, herpesvirus) US2 and US11, and human immunodeficiency virus Nef protein (Hewitt, 2003). Since PRRSV also causes persistent infection (Lunney et al., 2010b), the inhibition of SLA molecules by PRRSV may be partly responsible for the impaired CMI responses against the virus in pigs.

In this study, we first demonstrated that PRRSV indeed induced down-regulation of SLA-I expression in three different but relevant cell types including a susceptible porcine kidney epithelial cell line stably expressing CD163 (PK15-CD163). Subsequently, we aimed to investigate the specific viral protein(s) involved in the virus-induced down-regulation of SLA-I expression. We found that PRRSV Nsp1α, Nsp2TF and GP3 proteins significantly down-regulated the surface expression of SLA-I. Additionally, we further mapped that the TF domain especially the last 68 amino acids in Nsp2TF is critical for this novel function in modulating SLA-I expression. The results from this study may aid in designing an improved vaccine against this economically-important swine pathogen.

Results

PRRSV down-regulates SLA-I expression on cell surface of three different but relevant cell types

It has been reported that PRRSV can down-regulate SLA class I molecules *in vitro* (Park et al., 2008; Wang et al., 2007). In this study, three different cell types were used to further confirm this previous finding. The PK15-CD163 cells that stably express porcine CD163 were infected with a pathogenic strain of PRRSV VR2385 at 2 multiplicity of infection (MOI) and subsequently subjected to flow cytometry at 60 h post-infection (hpi). Both the intracellular viral N protein (Npr) and cell surface SLA-I molecules were stained (Fig. 1A and B) to ensure that the comparison was between the infected and mock-infected cells, and the gating procedure was shown in (Figs. S1 and S2). As summarized in Figs. 1C and S2, PRRSV infection reduced the surface SLA-I expression on cells by at least 50%. In addition, porcine alveolar macrophages (PAMs) and monocyte-derived DCs (MoDCs) were also infected with PRRSV VR2385 at 0.2 MOI, and the SLA-I surface expression was similarly evaluated at 24 hpi. Consistently, SLA-I expression was found to be down-regulated by PRRSV infection both in PAMs (Fig. 1D) and MoDCs (Fig. 1E). These findings collectively demonstrated that PRRSV down-regulates SLA-I expression in susceptible cells.

PRRSV nonstructural proteins Nsp1α and Nsp2TF and structural protein GP3 are responsible for down-regulating SLA class I expression

In order to determine the mechanisms of the virus-induced down-modulation of SLA-I, we first sought to identify the individual PRRSV protein(s) responsible for the observed down-regulation. First, we constructed a total of 22 mammalian expression constructs including all 14 PRRSV Nsps and all 8 PRRSV SPs. Their expressions in the PK15-CD163 cells following transient transfection of each individual expression construct were demonstrated by either western blot or IFA with anti-flag monoclonal antibody (Fig. S3).

At 20 h post-transfection with each individual expression construct, cells were subjected to flow cytometry with a dual-labeling analysis for simultaneous detection of both surface SLA-I expression and intracellular co-expressing flag-tagged individual viral protein. In each individual transfection, cells are divided into viral protein-expressing population and non-expressing population (Fig. 2C) and the SLA-I expression intensities were compared between the viral protein-expressing population and a separate group transfected with the empty vector (EV) pIHA-flag (Fig. 2A). PRRSV nonstructural proteins Nsp1α and Nsp2TF as well as PRRSV structural protein GP3 were found to significantly reduce the surface expression of SLA-I molecules when compared to other viral proteins as well as the empty vector control (Fig. 2B). Additionally, we also evaluated the SLA-I expression in each individual transfection group by comparing between viral protein-expressing population and non-expressing population (Fig. 2C). Similarly, we found that the same three viral proteins were able to significantly reduce SLA-I surface expression. The Nsp1α and GP3 displayed diminished SLA-I expressions, approximately 70–80% that of the cell only control, while Nsp2TF showed the greatest reduction to approximately 50% (Fig. 2D).

To further determine whether these viral proteins worked synergistically in down-regulating SLA-I expression, PK15-CD163 cells were co-transfected with the expression constructs in different combinations (Fig. S4), and the SLA-I surface expression of each group was subsequently measured by flow cytometry as described above. Even though co-transfections of these viral protein constructs generally reduce SLA-I surface expression, there was no significant difference among the co-transfection groups of different combinations. The Nsp5 was also included in this experiment, since it had a down-regulating effect on SLA-I expression to some extent but was not statistically significant. Single transfection with each individual Nsp1α, Nsp2TF or GP3 was used as positive controls and transfection with GP5 construct was used as a negative control.

Furthermore, to exclude the possibility that the decreased SLA-I expression was due to a general suppression of cellular gene expression by PRRSV infection, we evaluated another surface protein, sodium–potasium (Na^+–K^+) ATPase, in a similar dual-color flow cytometry analysis. The results showed that none of the viral proteins, especially the three proteins that down-regulate SLA-I expression, had an inhibitory effect on the Na^+–K^+ ATPases (Fig. 2E). These results indicated that PRRSV proteins Nsp1α, Nsp2TF and GP3 suppressed SLA-I presentation on the cell surface.