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Development of a monoclonal antibody against swine leukocyte antigen (SLA)-DR α chain and evaluation of SLA-DR expression in bone marrow-derived dendritic cells after PRRSV infection

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ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS) is one of the most common diseases in the global swine industry. PRRSV infection is highly restricted to cells of the monocyte-macrophage lineage. However, the lack of antibodies to swine monocyte-macrophage lineage markers significantly hampers PRRSV research. In this study, we have developed a monoclonal antibody against the swine leukocyte antigen (SLA)-DRα chain and confirmed its reactivity with endogenous expressed SLA-DR in a variety of cell lines and primary swine antigen-presenting cells (PAMs, PBMC and BM-DCs). Moreover, the level of SLA-DR expression after PRRSV infection were evaluated by our homemade Mab and a commercial anti-SLA-DR antibody. Based on our result, the protein level of SLA-DRα expression is increased after PRRSV infection in DC, while the mRNA of both SLA-DRα and SLA-DRβ were significantly inhibited by PRRSV replication. In conclusion, we successfully developed a MAb reactive with endogenous SLA-DR in western blotting, and this MAb could be a useful tool for further research and analysis. Moreover, the inconsistency of SLA-DR expression between protein and mRNA levels may suggest a novel role of DC played during the immune response after PRRSV infection.

1. Introduction

Since its discovery, PRRSV has been recognized as one of the most notorious pathogens worldwide. PRRS is characterized by respiratory syndrome and delayed growth in piglets, along with reproductive disorders in sows, leading to enormous economic losses to the global swine industry (Chand et al., 2012). Current control of PRRS is inadequate despite substantial efforts to minimize its impact. The typical immune features of PRRSV infection in the host include persistent viremia, a strong inhibition of innate cytokines (interferon (IFN)-α/β, tumor necrosis factor (TNF)-α, and interleukin (IL)-1β), dysregulation of natural killer (NK) cell function, rapid induction of non-neutralizing antibodies, delayed appearance of neutralizing antibodies (Lunney et al., 2016), a late and low CD8+ T-cell response, and the induction of regulatory T cells (Lunney et al., 2016). However, understanding of the molecular mechanism and viral-host interaction for PRRSV remains incomplete.

One major obstacle to understanding the viral-host interaction between PRRSV and the host immune system is the lack of qualified antibodies for cell surface marker or other cytoplasm proteins with swine origin. Therefore, generation of qualified MAbs recognized host proteins of swine origin is urgently needed. In this study, we successfully obtained a hybridoma secreting MAb recognized endogenous SLA-DRα. We also used the MAb to evaluate the SLA-DR level in bone marrow derived dendritic cells (BM-DC) after PRRSV infection. This MAb will be a useful tool for future understanding of viral-host interaction for PRRSV.

2. Materials and methods

2.1. Cells, virus, and chemicals

The MARC-145, PK-15, 3D4/21 ATCC® CRL-2843, and HEK293 T

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cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37℃ and 5% CO_2. Porcine alveolar macrophages (PAMs) were prepared from bronchoalveolar lavage of 4-week-old PRRSV-negative piglets and maintained in Roswell Park Memorial Institute (RPMI) 1640 (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS (Thermo Fisher Scientific). Porcine peripheral blood mononuclear cell (PBMC) were prepared from fresh swine peripheral blood collected from 4-week-old PRRSV-negative piglets using Ficoll®-Paque (GE healthcare, Chicago, Illinois, USA). Mouse myeloma cell line Sp2/0-Ag14 (ATCC® CRL-1581™) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in RPMI 1640 (Gibco) supplemented with 10% FBS (Gibco) as well. Swine BM-DCs were obtained as previously described (Carrasco et al., 2001). Induction of BM-DC was conducted by stimulating bone marrow cells with porcine granulocyte–macrophage colony-stimulating factor (pGM-CSF, GenScript, Nanjing, Jiangsu, China) at the concentration of 40 ng/mL for 6 consecutive days, and only the suspended cells (with typical dendritic cells morphology) were collected.

PRRSV-SD16 (GenBank No. JX087437.1) and JXA1 (GenBank Accession No. EF112445.1) were used to inoculate MARC145 and BM-DC at 1 or 0.1 multiplicity of infection (MOI). Transfection of HEK293 T cells with mammalian expression plasmids was conducted using FuGENE HD transfection reagent (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions.

2.2. RNA isolation, reverse transcription, plasmid construction and quantitative polymerase chain reaction (qPCR)

Total RNA was isolated from PAMs using RNAiso Plus (TaKaRa, Dalian, China) and reverse transcribed using the PrimeScript® RT reagent Kit (TaKaRa) according to the manufacturer's instructions. The cDNA sequence of SLA-DRα was cloned from cDNA of PAMs by Q5® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) and ligated to pET-28a prokaryotic expression vector and fused with 6XHis-tag at the N-terminal. The constructed pET-28a-6XHis-SLA-DRα vector was subjected to DNA sequencing and transformed into *Escherichia (E.) coli* strain BL21 (DE3) for protein expression. The primers used for cloning and their corresponding sequence are listed in Table 1.

The cDNA sequence of SLA-DRα was cloned to *EcoRI* and *XhoI* sites of pCAGEN expression vector (Addgene plasmid# 11,160) by Q5® High-Fidelity DNA Polymerase (New England Biolabs) as well. For qPCR analysis of relative gene expression, reverse transcription and qPCR were conducted using a PrimeScript RT reagent kit (TaKaRa, Dalian, China). Transcripts of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were also amplified and used to normalize the total RNA input. The primers and corresponding sequence used for qPCR amplification are also listed in Table 1. Relative quantification of target genes was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.3. Preparation of recombinant SLA-DRα

The pET-28a-SLA-DRα was transformed into the *E. coli* strain BL21 (DE3) and cultured in LB medium at 37 ˚C for1.5 to 2 h until the OD value of bacterial culture medium reach 0.6 to 0.8. Then the protein expression was induced with 0.5 mM isopropyl β-D-thiogalactoside (IPTG) at 25 ˚C. After IPTG induction and sonication, inclusion bodies containing recombinant 6XHis-SLA-DRα were washed by phosphate-buffered saline (PBS) and reconstituted with 8 M urea (Sigma-Aldrich) for Ni^+ affinity chromatography purification (Transgene, Beijing, China). Dialysis of recombinant 6XHis-SLA-DRα was conducted by gradient decreasing of urea concentration until buffer was completely replaced by PBS and quantified by BCA protein assay kit (Thermo Fisher Scientific).

2.4. Ethics statement, mice immunization, and cell fusion

The 6-week old *Balb/C* mice were obtained from Dashuo Biotech (Chengdu, Sichuan, China), and the animal protocol was reviewed and approved by the Animal Welfare Committee of Northwest A&F University. All mice were monitored at a daily basis for any clinical sign. All effort was made to minimize the suffering of mice, and mice were euthanized if a humane endpoint was reached according our protocol.

Mice were immunized with 100 μg of recombinant 6XHis-SLA-DRα (2 mg/mL) with an equal volume of adjuvant for a total of 3 times with an interval of two weeks. The Freund's Complete Adjuvant (Sigma-Aldrich) was used for the primary immunization, while Freund's Incomplete Adjuvant (Sigma-Aldrich) was used for the rest. Mice serum was collected on a weekly basis to monitor the serum antibody level by enzyme-linked immune immunosorbent assay (ELISA). Serum collected before immunization was included as the negative control. After the final immunization, 100 μg of recombinant protein without adjuvant was used to boost the mice 4 days before the spleen collection. Spleen from the mouse with the highest antibody titers were used for cell fusion. Cell fusion of spleen cells with S/p20 cell was conducted as previous described (Monoclonal et al., 1999). The selected positive clones of hybridoma were subjected to subcloning via limited dilution and antibody isotyping was conducted by Mouse Monoclonal Antibody Isotyping Reagents (Sigma-Aldrich) according to manufacturer's instruction.

2.5. The enzyme-linked immunosorbent assay (ELISA)

The ELISA assay was conducted to evaluate the serum antibody level for recombinant 6XHis-SLA-DRα of immunized mice or screen for hybridoma supernatant obtained from surviving hybridoma after cell fusion. Briefly, purified 6XHis-SLA-DRα (400 ng) were used to coat 96-well polystyrene microplates (Corning Inc.) and further blocked with 5% skim milk in PBS containing 0.5% Tween 20 (Sigma-Aldrich). Diluted mouse serum or hybridoma supernatant was added to the wells and incubated for 1 h at 37 ˚C followed by washing with PBS containing 0.5% TritonX-100(Sigma-Aldrich). The binding of antibody to the corresponding antigen was detected using horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibodies (GenScript) and visualized by a TMB substrate kit (Tiangen Biotech, Beijing, China). The absorbance of each well was measured using a VictorX5™ Multilabel Plate Reader (Perkin Elmer, Waltham, MA, USA) at the wavelength of 450 nm.

Table 1
Primer list and corresponding sequence.

Target	Forward primer	Reverse primer	Application
SLA-DRα	cgGAATTCATGACCATACTTGGGGTCCC	gcCTCGAGTCAGTGGTGGTGGTGGTGGTGGTGCAGAGGCCCTCGGCGT	Cloning primers to pET vector
SLA-DRα	GCCCTGAAGCCACTCTAA	GGAAAGCCAGCACAAGAA	qPCR primers for SLA-DRα mRNA
SLA-DRβ	GAGGGCACGGTCTGAATC	AGGGCGTCCTTTCTGATT	qPCR primers for SLA-DRβ mRNA

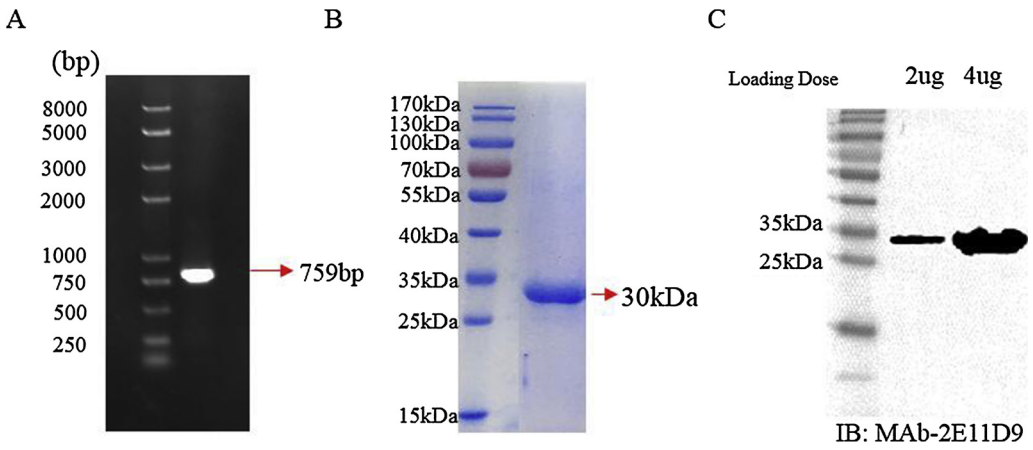

Fig. 1. Cloning and expression of SLA-DRα chain. A. Electrophoresis of SLA-DRα cDNA cloned from total RNA isolated from PAMs. B: Analysis of purity for recombinant SLA-DR expressed in *E. coli. C: WB analysis using home-made Mab-2E11D9 to verify the reactivity with recombinant SLA-DR.*

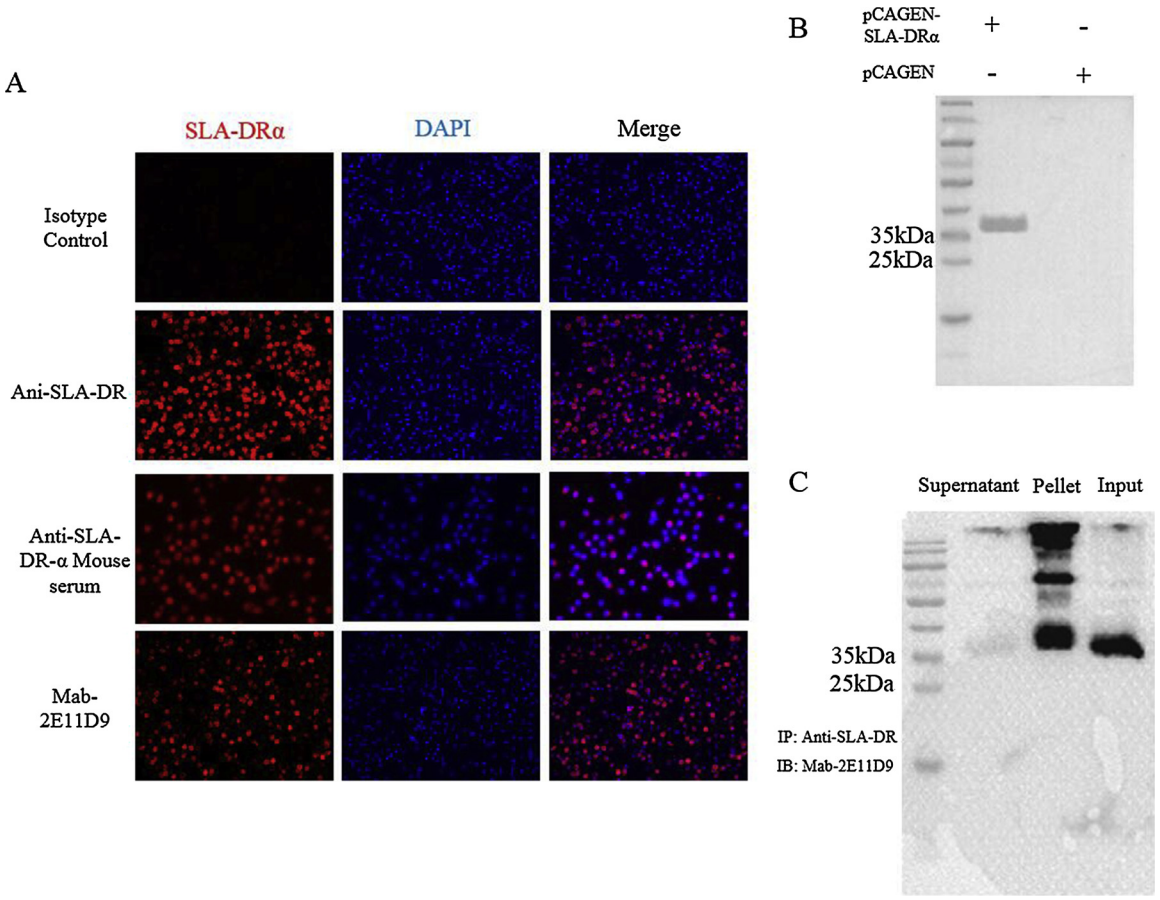

Fig. 2. Screening and characterization of MAbs recognizing endogenous SLA-DR from PAMs. A: Immunofluorescence assay for PAMs by different antibodies (isotype control antibody, commercial anti-SLA-DR antibody, anti-SLA-DR-α mouse serum, MAb-2E11D9); B: HEK293 T cells transfected with pCAGEN-SLA-DRα or empty vector were used to confirm the reactivity with SLA-DR by MAb-2E11D9; C: Immune precipitation of SLA-DR via commercial anti-SLA-DR antibody to test the specific reactivity of MAb-2E11D9.

2.6. Immunofluorescence assay (IFA) for hybridoma screening and flow cytometry

PAMs and CRL-2843 were fixed by 4% paraformaldehyde (Sigma-Aldrich). The anti-SLA-DRα hybridoma supernatant and a commercial anti-SLA-DR MAb (Clone No. 1053H2-18, BD Biosciences, San Jose, CA, USA) were used to probe PAMs or CRL-2843. Specific antibody-protein reactions were detected by Alexa Fluor 555 goat anti-mouse IgG conjugate (Thermo Fisher Scientific). The coverglass was mounted onto a slide using SlowFade Gold Antifade reagent containing 4′6′-diamidino-2-phenylinodole (DAPI) (Invitrogen) and observed by a Leica DM1000 fluorescence microscope. All images were captured and processed using Leica Application Suite X (Version 1.0. Leica Microsystems).

For the flow cytometry analysis of endogenous SLA-DR expression of BM-DCs or with commercial anti-SLA-DR MAb or homemade MAbs, 1×10^6 BM-DC infected with PRRSV-SD16 with indicated time points and MOI was fixed by 4% paraformaldehyde and Permeabilized using BD FACS™ Permeabilizing Solution (BD Bioscience). Then, BM-DC were

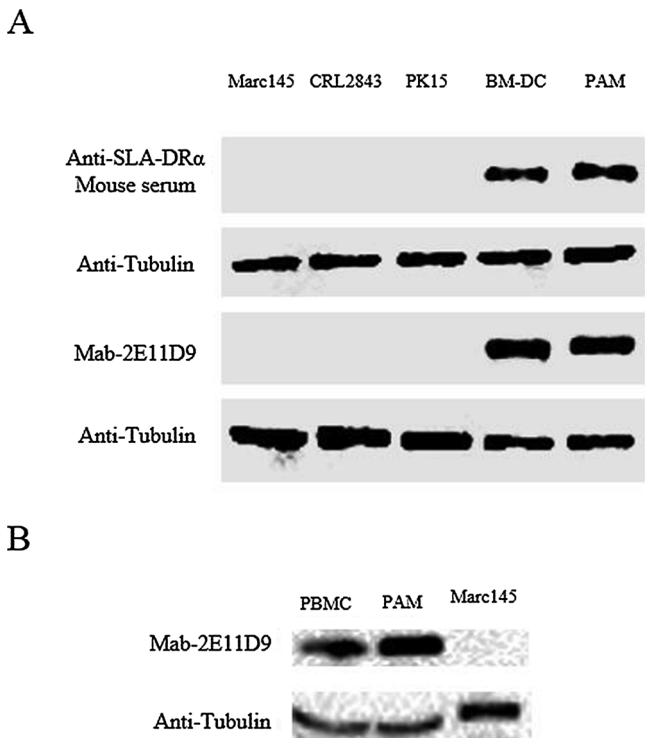

Fig. 3. Evaluation of SLA-DR expression from different cells. A: Two immortalized cell lines with swine origin PK15 (pig kidney) and CRL2843 (alveolar macrophage), along with two primary swine cells (PAMs and BM-DCs) were used to evaluate the reactivity of MAb-2E11D9 to endogenous expressed SLA-DRα expression. The MACR145 cells from African Green Monkey kidneys were used as negative control cells. B: Evaluation of SLA-DR expression in swine PBMC cells, SLA-DR positive PAMs and negative MARC-145 cell were included as controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

stained with above Mabs at a concentration of 10ug/mL in PBS and PRRSV convalescent pig serum with a 1 to 50 dilution in PBS at 4℃ for 30 min. Specific antibody-antigen interaction was detected by fluorescein isothiocyanate (FITC)-conjugated goat anti-swine IgG (Jackson ImmunoResearch, West Grove, PA, USA) and APC-conjugated goat anti-mouse Cross-Adsorbed Secondary Antibody (Thermo Fisher Scientific) at a dilution folds of 1 to 100 in PBS at 4℃ for another 30 min. To compare the Mab-2E11D9 with anti-SLA-DR from BD Bioscience, PAMs with or without permeabilization were stained by Mab-2E11D9 and anti-SLA-DR(BD Bioscience) following the same protocol above, and stained PAMs were subjected to flow cytometry analysis. The flow cytometry analysis was conducted in a BD FACS Aria™ III cell sorter (BD Biosciences) and analyzed using FlowJo software version 10.0 (FlowJo, LLC, Ashland, Oregon, USA).

2.7. Western blot (WB) analysis and Immunoprecipitation (IP)

Whole cell lysates of PAMs, PBMC, BM-DCs, HEK293 T and other cell lines used in this study were harvested by Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA, USA) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for WB as previously described (Patel et al., 2008). Antibodies against SLA-DRα, β-tubulin (Sigma-Aldrich), and a home-made anti-PRRSV-N monoclonal antibody (Clone No. 6D10) were used for blotting as previously described (Li et al., 2018). The chemiluminescence signal was recorded digitally using a ChemiDoc MP imaging system (Bio-Rad Laboratories). Digital signal acquisition and densitometry analyses were conducted with the ImageLab Program, Version 5.1 (Bio-Rad Laboratories).

For the IP assay, the 1×10^7 PAMs was harvested by 200 μL RIPA buffer (Thermo Fisher Scientific) with supplementary of protease inhibitor cocktail (Sigma-Aldrich), 3 μg commercial SLA-DR monoclonal antibody (BD Bioscience) was added to cell lysate and incubated under 4℃ for 3 h, then 25 μL protein G agarose beads (GenScript) was added in to cell lysate and incubated for another 1 h to pull-down immune complex. The IP supernatant was removed by centrifugation with 10,000 *g* under 4℃, then the IP pellet was further washed by RIPA buffer for 3 times before harvesting the beads with Laemmli Sample Buffer for western blot analysis.

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). Differences in indicators between groups and controls were assessed using Student's *t*-test. A two-tailed *P*-value of less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Cloning and expression of recombinant SLA-DRα

To obtain the cDNA sequence of SLA-DRα, PAMs was used for RNA purification and subjected to reverse transcription via SLA-DRα specific primer. The cDNA of SLA-DRα was cloned by both forward primer and backward primer. Agarose gel electrophoresis indicated that the obtained PCR product matched the expected size of 759 bp (Fig. 1A). After the DNA sequencing of the PCR product, the cDNA sequence was ligated to pET-28a vector for recombinant expression and protein purification. The final expression product of recombinant SLA-DRα was subjected to SDS-PAGE to probe the purity before immunization of mice (Fig. 1B). Moreover, the reactivity of recombinant SLA-DRα with our home made anti-SLA-DRα monoclonal antibody was verified as well (Fig. 1C).

3.2. Mice immunization, cell fusion, and screening for positive hybridoma clones

After the fourth immunization, the mouse with highest antibody titers is euthanized and the spleen cells were collected for cell fusion (data not shown). One week after cell fusion, all supernatant from fused cells was tested for the existence of anti-SLA-DRα antibody via ELISA methods. Among all fused hybridomas, more than 100 clones demonstrated reactivity during ELISA screening, and these ELISA-positive clones were further screened via IFA. However, most hybridoma clones (more than 95%) positive for ELISA do not show any reaction with endogenous SLA-DR expressed in PAMs during IFA (data not shown). Only 6 hybridoma clones demonstrated positive reactions with PAMs compared with commercial anti-SLA-DR MAb.

Among all 6 IFA-positive clones, the MAb-2E11D9 demonstrated the strongest reactivity with PAMs in IFA assay (Fig. 2A). Antibody isotyping suggested MAb-2E11D9 is an IgG2b (Figure.S1). To ensure that MAb-2E11D9 was an SLA-DRα-specific MAb, HEK293 T cell lysate transfected with pCAGEN empty vector or pCAGEN-SLA-DRα was used to probe this MAb. Only cells transfected with pCAGEN-SLA-DRα could be recognized by MAb-2E11D9 with the expected protein size (Fig. 2B). Moreover, anti-SLA-DR MAb from BD Bioscience recognizing a conformational epitope was used to pull down the SLA-DR from PAMs in IP assay, and MAb-2E11D9 was used to probe the existence of SLA-DR in pellets. According to the result, SLA-DRα could be detected by MAb-2E11D9 in input and pellet, but was much weaker in the supernatant after the IP (Fig. 3C).

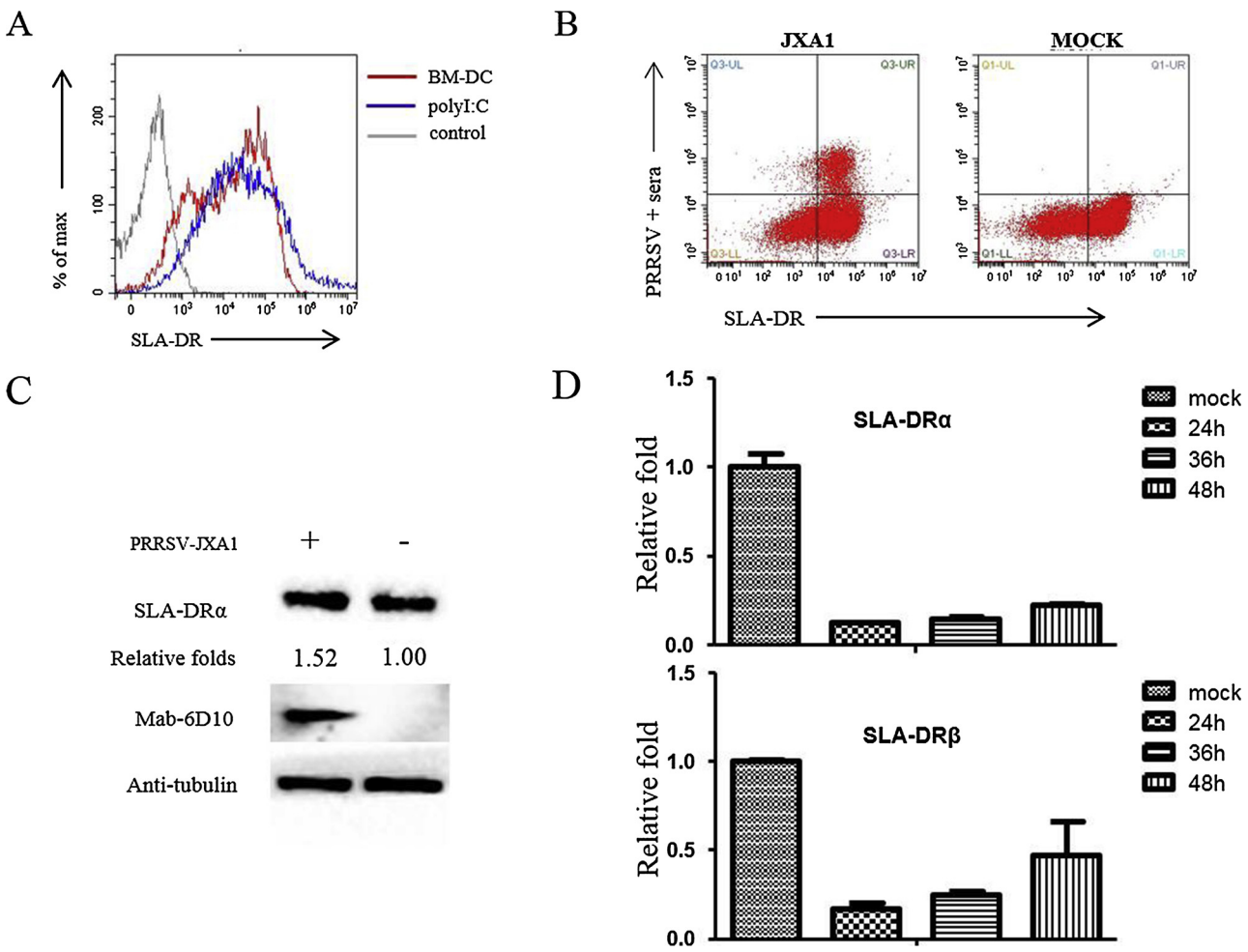

Fig. 4. Evaluation of SLA-DR expression of BM-DCs infected with PRRSV. A: Evaluation of SLA-DR expression via commercial SLA-DR antibody in flow cytometry, non-stained cells and polyI:C-stimulated cells served as negative and positive controls, respectively. B: Evaluation of SLA-DR expression via commercial SLA-DR antibody in flow cytometry after PRRSV infection. PRRSV-positive cells were stained by a PRRSV-SD16 strain of convalescent pig serum (Y-axis). C: Evaluation of SLA-DRα expression by homemade MAb-2E11D9 after PRRSV infection. PRRSV infection was confirmed by a PRRSV-N antigen-specific MAb-6D10; D: examination of SLA-DRα and SLA-DRβ expression in BM-DCs at the mRNA level via qPCR after PRRSV infection at different time points (24 h, 36 h, 48 h). Non-infected BM-DCs served as controls (MOCK).

3.3. Homemade anti-SLA-DRα recognized endogenously expressed SLA-DR from PAMs, BM-DCs and PBMC

As MHC class II molecules, SLA-DRs were mainly expressed in antigen-presenting cells, such as macrophages, dendritical cells, and B cells (Smith et al., 2005); therefore, we used our homemade MAb-2E11D9 to investigate SLA-DR expression in several swine originating cells line as well as primary cells, such as PAMs, PBMC and BM-DCs. A PRRSV permissive cell line, MARC145, served as a negative control cell line. Based on our WB result for MAb-2E11D9 and anti-SLA-DRα mouse serum, BM-DCs, PBMC and PAMs have significant levels of SLA-DRα expression(Fig. 3A and B), while there is no detectable expression of SLA-DRα in PK15 cells (a pig kidney derived cell line). However, it is notable that the immortalized alveolar macrophage cell line CRL2843 does not express detectable levels of SLA-DRα (Fig. 3A, Figure. S2). It is possible that the expression of SLA class II was lost during the immortalization of CRL2843. Therefore, our results demonstrated that this homemade MAb-2E11D9 can be used to detect the endogenous expression of SLA-DR in antigen-presenting cells (APCs) with swine origin.

3.4. Evaluation of SLA-DR expression in BM-DCs after PRRSV infection

It has been previously reported that PRRSV infection leads to the down-regulation of SLA-I molecules in vitro (Cao et al., 2016; Du et al., 2016). Those observations were also consisted with delayed, low-level cytotoxic T lymphocyte (CTL) responses in pigs infected by PRRSV in vivo and are thought to contribute the persistent infections of PRRSV in swine herds (Du et al., 2016). Although PRRSV can infect a variety of APCs, such as macrophages, monocytes, and dendritic cells created in vitro from peritoneal cells (Chang et al., 2008; Chaudhuri et al., 2016; Duan et al., 1997a, b; Sur et al., 1996; Wang et al., 2016), the expression of SLA-II molecules after PRRSV infection has been less investigated, and available reports are conflicting with each other (Flores-Mendoza et al., 2008; Rodriguez-Gomez et al., 2015; Silva-Campa et al., 2010; Wang et al., 2007). A recent study demonstrated that monocyte derived dendritic cells (moDC) infected by either *PRRSV-1* or *PRRSV-2* demonstrated evaluated SLA-DR and CD80/86 expression in FCM analysis (Rodriguez-Gomez et al., 2015). Therefore, we examined the SLA-DR level in BM-DCs after PRRSV infection. Normally, BM-DCs demonstrate high levels of SLA-DR expression and can be upregulated by polyI:C, a double-stranded RNA analog recognized by Toll-like receptor 3 if analyzed by flow cytometry (Fig. 4A). However, it appears that MAb-2E11D9 demonstrated a very weaker reactivity to un-

permeabilized PAMs (Figure.S3 A) but react with intracellular SLA-DR similarly to commercial SLA-DR Mab from BD bioscience (Figure. S3B). Therefore, we mainly use our MAb-2E11D9 for WB analysis of total SLA-DR expression.

After PRRSV infection, most PRRSV-infected BM-DCs were found to be SLA-DR-positive, as only a small number of SLA-DR-negative BM-DCs could be infected by PRRSV (Fig. 4B). When analyzing SLA-DRα expression via WB with MAb-2E11D9, a moderate level of upregulation of SLA-DR was found (Fig. 4C). However, there is a significant reduction of mRNA in both chains (Fig. 4D), suggesting that PRRSV induced SLA-DR upregulation may occur at the post-translation level.

The biological significance of evaluated SLA-DR in PRRSV-infected BM-DCs or MoDCs remains elusive. It was observed very early that piglets infected with PRRSV developed a rapid but non-protective antibody response within the first week of infection (Lunney et al., 2016). Due to the nature of role played by SLA-II molecules in the development of humoral immunity against pathogens, SLA-II impairment (down-regulation or degradation) is unlikely occurred in PRRSV infection as impaired SLA-II function results in a delayed antibody response in the host regardless of whether the antibodies stimulated by infection are protective or not, which is inconsistent with previous observation of rapid induction for PRRSV-specific antibodies (but not neutralizing antibodies) in PRRSV-infected piglets.

In conclusion, a monoclonal antibody (Clone No. 2E11D9) recognized SLA-DRα was developed and confirmed using IFA and WB analysis for PAMs, BMDC and BM-DCs. Notably, MAb-2E11D9 recognized a linear epitope and could be used in for WB analysis of SLA-DR expression from APC with swine origins. Using this MAb, we discovered that PRRSV infection in BM-DCs caused a moderate upregulation of SLA-DR in protein levels but inhibited SLA-DR mRNA expression, suggesting that PRRSV infection causes post-translational modulation of SLA-DR by unidentified mechanism. This MAb will be a useful tool for further elucidating the mechanism of PRRSV regulation of SLA-DR expression in BM-DCs.

Conflict of interests

None.

Acknowledgements

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2019.04.001.

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