Interleukin-1 receptor antagonist: an early immunomodulatory cytokine induced by porcine reproductive and respiratory syndrome virus

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) infection poorly induces pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) and type I IFN production during the early phase of infection. Our microarray analysis indicated strong upregulation of the IL1RA gene in type 2 PRRSV-infected monocyte-derived dendritic cells. Interleukin-1 receptor antagonist (IL-1Ra) is an early inhibitory cytokine that suppresses pro-inflammatory cytokines and T-lymphocyte responses. To investigate the induction of IL-1Ra by PRRSV, monocyte-derived dendritic cells were cultured with type 2 PRRSV or other swine viruses. PRRSV increased both IL1RA gene expression and IL-1Ra protein production in the culture. The enhanced production of IL-1Ra was further confirmed in PRRSV-cultured PBMC and PRRSV-exposed pigs by flow cytometry. Myeloid cell population appeared to be the major IL-1Ra producer both in vitro and in vivo. In contrast to the type 2 PRRSV, the highly pathogenic (HP)-PRRSV did not upregulate IL1RA gene expression in vitro. To determine the kinetics of PRRSV-induced IL1RA gene expression in relation to other pro-inflammatory cytokine genes, PRRSV-negative pigs were vaccinated with a commercially available type 2 modified-live PRRS vaccine or intranasally inoculated with HP-PRRSV. In modified-live PRRS vaccine pigs, upregulation of IL1RA, but not IL1B and IFNA, gene expression was observed from 2 days post-vaccination. Consistent with the in vitro findings, upregulation of IL1RA gene expression was not observed in the HP-PRRSV-infected pigs throughout the experiment. This study identified IL-1Ra as an early immunomodulatory mediator that could be involved in the immunopathogenesis of PRRSV infections.

INTRODUCTION

Innate immunity is critical for induction of virus-specific immune responses [1]. Pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) and antiviral cytokines [type I interferon (IFN)] are innate cytokines, which help promote leukocyte recruitment and inhibition of viral replication [2–6]. Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the major swine pathogens that causes serious economic losses in the swine production industry worldwide [7]. PRRSV can be classified into two genotypes: type 1 (EU) and type 2 (US) strains [8]. PRRSV infection usually induces poor innate and adaptive immune responses [9, 10]. In addition, PRRSV infection usually enhances secondary infections in the lungs, leading to porcine reproductive and respiratory disease complex [11] or vaccination failure [12–15]. The findings indicated that PRRSV infection could affect overall immunocompetency of the infected pigs. Recently, the emergence of the variant PRRSV strain, known as a highly pathogenic (HP)-PRRSV, has been reported [16]. The clinical manifestation of HP-PRRSV is different from that of the typical PRRSV strains, causing severe clinical outcomes and high mortality rate among the
infected pigs [17, 18]. In contrast to the typical PRRSV, infection of HP-PRRSV resulted in strong pro-inflammatory cytokine production in the infected host [19, 20]. However, the underlying mechanisms of the differences in the pathogenicity of the two viruses are not fully understood.

PRRSV primarily infects monocytes/macrophages and dendritic cells, resulting in inhibited expression of MHC and co-stimulatory molecules in the infected cells [21–23]. Moreover, PRRSV infection promotes apoptosis of the infected cells [24, 25]. In the early phase of infection, PRRSV induces delayed and weak pro-inflammatory cytokines, including IL-1, IL-6 and TNF-α and type I IFN production [26–29]. It has also been reported that PRRSV suppressed the production of IL-1β, IL-8 and IFN-α in serum during the first 14 days post-infection (p.i.) [30]. In the infected lungs, decreased IL-1, IL-6 and TNF-α production was also observed during the first 10 days of PRRSV infection [28]. Some studies suggested that the non-structural proteins and nucleocapsid protein of PRRSV could modulate type I IFN production in vitro [31–33]. These findings clearly indicated that PRRSV infection significantly interferes with the innate immune functions. In addition, recent publications indicated that type 2 PRRSV infection could induce IL-10 production and viral-specific regulatory T cells (Treg) both in vitro and in vivo [10, 34–37], which could lead to the inefficient induction of viral-specific immune responses. Interestingly, some of the type 1 PRRSV strains did not induce IL-10 and Treg production, yet still exhibited clinical and immunological manifestations as do type 2 PRRSV [38, 39]. Thus, the immunosuppressive mechanism of PRRSV during the early phase of PRRSV infection remains incompletely understood.

Interestingly, our preliminary microarray analysis demonstrated that type 2 PRRSV-infected monocyte-derived dendritic cells (MoDCs) exhibited strong upregulation of interleukin-1 receptor antagonist (IL-1Ra) gene expression (unpublished observation). IL-1Ra is an early anti-inflammatory cytokine that controls inflammatory responses during an early stage of immune activation [40]. IL-1Ra competitively binds to the interleukin-1 receptor and subsequently blocks the intracellular IL-1 signalling cascade [41]. IL-1Ra is produced by monocytes, macrophages or dendritic cells, which are known to be PRRSV target cells [22, 41, 42]. IL-1Ra can modulate the production of IL-1 and TNF-α [43] and type I IFN [44]. Therefore, early IL-1Ra production could affect induction of pro-inflammatory and antiviral cytokines during the early phase of PRRSV infection. In this study, we aimed to confirm and investigate the induction of IL-1Ra by type 2 PRRSV strains, including the variant HP-PRRSV, using both in vitro and in vivo infection models.

**RESULTS**

PRRSV upregulated *IL1RA* gene expression in cultured porcine MoDCs and PBMCs

To investigate the factors involved in virus-induced immunomodulation during the early phase of PRRSV infection, porcine MoDCs were cultured in the presence of type 2 PRRSV (01NP1) for 24 h, and the total RNA was subjected to microarray analysis using the Porcine_GXP_4x44K (two-colour microarray-based gene expression platform). The relative expression of the innate, T-helper polarizing and inhibitory cytokine genes is shown in Fig. 1(a) (upper panels). Strong upregulation of genes *IL10* and *IL1RA* was clearly evident. Upregulations of genes *IFNA*, *IFNB*, *IL1B* and *IL2* were also observed. The in vitro findings on upregulation of type I IFN gene were consistent with previous reports [45, 46]. Interestingly, while most of the type I IFN receptor downstream signalling genes were upregulated, consistent with the enhanced type I IFN gene expression, upregulation of the IL-1 receptor downstream signalling genes was not evident in the system (Fig. 1a, lower panels). The results indicated that the presence of PRRSV upregulated expression of *IL1RA* and *IL1B*, but not the IL-1 receptor downstream signalling genes, in the cultured MoDC.

To confirm the above findings, the levels of *IL1RA* gene expression in the MoDC cultured in the presence of type 2 PRRSV (01NP1) or other swine respiratory viruses were analysed using quantitative real-time PCR (qPCR). Classical swine fever virus (CSFV) and swine influenza virus, which had been reported to induce strong pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) and type I IFN production during the early phase of infection [47–49], were also included in the experiment. The levels of *IL1RA* gene expression determined by qPCR are shown in Fig. 1(b). The results demonstrated that the presence of PRRSV, but not other swine viruses, significantly enhanced *IL1RA* gene expression in the cultured MoDC. In addition, it should be pointed out that PRRSV-induced *IL1RA* gene expression was much stronger than that from the cells treated with lipopolysaccharide (LPS) (Fig. 1b). The kinetics of PRRSV-induced *IL1RA* gene expression in the cultured porcine PBMC were investigated and are shown in Fig. 1(c). The percentages of PRRSV cells in the myeloid subpopulation (SWC3 cells) after 3, 6, 12 and 24 h incubation were 12.2 ±1.6 %, 14.4±3.2 %, 24±2.6 % and 30.1±2.9 %, respectively. Upregulation of the *IL1RA* gene was evident from 3 h and peaked at 12 h following infection with type 2 PRRSV (Fig. 1c). Interestingly, the level of *IL1RA* gene expression in the PRRSV-infected PBMC remained higher than that from LPS treatment at 24 h (Fig. 1c). The data suggested that the presence of PRRSV enhanced and prolonged *IL1RA* gene expression in the culture system.

To confirm the gene expression experiments, the levels of IL-1Ra protein in the cultured supernatants were determined by ELISA. Consistent with the aforementioned results, the presence of PRRSV enhanced IL-1Ra production in the cultured MoDC (Fig. 2a). The levels of IL-1Ra protein detected in the supernatants correlated well with those of *IL1RA* gene expression (r²=0.7664, P<0.001) (Fig. 2b).
The myeloid population was responsible for IL-1Ra production in the PRRSV infection model

To determine the porcine leukocyte subpopulation responsible for IL-1Ra production in this model, various porcine leukocyte subpopulations were cultured in vitro with type 2 PRRSV for 24 h. The presence of type 2 PRRSV resulted in significant upregulation of IL1RA gene expression in the cultured MoDC and PBMC, but not in the peripheral blood lymphocyte (PBL) subpopulation (Fig. 3a). The IL-1Ra protein production by porcine leukocytes was further confirmed by flow cytometry. The cultured PBMCs were initially gated into myeloid and lymphocyte subpopulations based on the forward and side scatter profiles. The cells were further identified by the expression of SWC3 (myeloid), CD3 (T-lymphocyte) and surface immunoglobulin (sIg) (B-lymphocyte) markers. The identified lymphocyte subpopulations consisted of the cells that highly expressed CD3 (T lymphocytes) and sIg (B lymphocytes), while the myeloid subpopulation primarily expressed the SWC3, but not CD3 and sIg, marker (Fig. 3b). The numbers of myeloid cells obtained from the cells treated with PRRSV and mock-infected PBMC were insignificant (data not shown). The results indicated that the presence of type 2 PRRSV significantly enhanced IL-1Ra production in the myeloid, but not lymphocyte subpopulations (Fig. 3c, d). Together, the results indicated that porcine myeloid population is the major IL-1Ra producer in this culture system, and the presence of PRRSV significantly enhanced IL-1Ra production from this cellular population.

The myeloid population was responsible for IL-1Ra production in the PRRSV infection model
PRRSV infection increased numbers of IL-1Ra producing cells in infected pigs

To confirm the induction of IL-1Ra by PRRSV in vivo, PRRSV-seronegative pigs were vaccinated with the commercially available type 2 modified-live PRRS vaccine (MLV), which has previously been shown to possess immunomodulatory properties similar to the field strain PRRSV [50, 51]. On days 0, 4, 7 and 14 post-vaccination (p.v.), PBMCs were collected from the immunized pigs to assess the numbers of IL-1Ra-producing cells by flow cytometry. The myeloid and lymphocyte subpopulations were gated and identified, based on the expression of SWC3, CD3 and sIg surface markers (see above) (Fig. 4a). The percentages of the myeloid subpopulation obtained from the MLV and PBS control groups were comparable (data not shown). Consistent with the in vitro findings, immunization with type 2 PRRS-MLV significantly enhanced IL-1Ra production in the myeloid subpopulation (Fig. 4b, c). Viral genomic RNA was detected from 4 days p.v. and remained high throughout the observation period, confirming ongoing PRRSV infection in the vaccinated pigs (Fig. 4d). The kinetics of IL-1Ra production between the experimental groups
are shown in Fig. 4(e). The numbers of IL-1Ra-producing cells in the myeloid subpopulation significantly increased from 4 to 7 days p.v. (Fig. 4e). Therefore, consistent with the in vitro findings, type 2 PRRSV enhanced IL-1Ra production in the myeloid subpopulation of the infected pigs.

**HP-PRRSV induced different patterns of IL1RA and pro-inflammatory cytokine gene expression compared to type 2 PRRSV**

HP-PRRSV exhibits different clinical outcomes and patterns of inflammatory cytokine responses compared with the typical type 2 PRRSV strain [52, 53]. In this study, the kinetics of IL1RA and other innate cytokine gene expression in the presence of type 2 PRRSV (01NP1) and HP-PRRSV (10PL01) were compared. In contrast to type 2 PRRSV, HP-PRRSV did not enhance IL1RA gene expression in the cultured MoDC (Fig. 5a). To confirm the in vitro findings, PRRSV-seronegative pigs were vaccinated with type 2 PRRSV-MLV or PBS at 0 days p.i. Subsequently, serum and heparinized whole blood samples were collected at 0, 4, 7 and 14 days p.i. *** and ** indicate significant difference at $P<0.0001$ and $P<0.001$, respectively.

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**Fig. 4.** PRRSV enhanced IL-1Ra-producing cells in pigs receiving PRRS-MLV. (a) Characteristics of the myeloid and lymphocyte subpopulations in the PBMC of the pigs receiving PRRS-MLV. (b) Mean fluorescent intensity (MFI) and (c) Percentage of IL-1Ra-producing cells in the indicated leukocyte subpopulations. (d) PRRSV genomic copy numbers detected in the sera of the experimental pigs and (e) percentages of IL-1Ra-producing cells in the myeloid subpopulation during the observation period. PRRSV-seronegative pigs (eight pigs per group) were vaccinated with type 2 PRRSV-MLV or PBS at 0 days p.i. Subsequently, serum and heparinized whole blood samples were collected at 0, 4, 7 and 14 days p.i. *** and ** indicate significant difference at $P<0.0001$ and $P<0.001$, respectively.
pigs from 2 days p.i. to the end of the observation period (14 days p.i.) (Fig. 5b). In the pigs receiving MLV, upregulation of IL1RA gene expression was clearly observed (Fig. 5c), while upregulation of IL1B and IFNA gene expression was not evident during the same period (Fig. 5d, e). Upregulation of IL10 gene expression was observed later than that of IL1RA at 4 days p.v. (Fig. 5f). Consistent with the in vitro findings, HP-PRRSV infection did not upregulate IL1RA gene expression in the infected pigs throughout the experiment (Fig. 5c). Infection with HP-PRRSV significantly enhanced IL1B and IFNA gene expression in the infected pigs, from 2 days p.i. (Fig. 5d, e), while upregulation of IL10 gene expression was observed from 7 days p.i. Together, our findings indicated that type 2 PRRSV and HP-PRRSV induced different patterns of IL1RA and other innate cytokine gene expression.

**DISCUSSION**

To investigate the immunomodulatory mechanism of early PRRSV infection, we identified IL-1Ra as a potential negative immunomodulatory mediator. The effects of PRRSV on induction of IL-1Ra were demonstrated, both in vitro and in vivo. Notably, both type 2 PRRSV (strain 01NP1) and type 2 PRRS-MLV were able to induce IL-1Ra production in this study. The findings supported previous opinions, indicating that the immunomodulatory properties of PRRS-MLV are similar to those of field PRRSV [50, 51]. PRRSV primarily
infected the myeloid cell population, including macrophages and dendritic cells [22, 42], which appear to be responsible for IL-1Ra production. Consistent with the findings in this study, the cells of myeloid lineage, including monocytes/macrophages, dendritic cells and neutrophils, have been reported as the major IL-1Ra producers in humans [56–59]. It should be noted that the production of IL-1Ra was observed at the very early stage following PRRSV infection, concurrent with the absence of pro-inflammatory cytokines and type I IFN gene expression, while enhanced IL-10 gene expression was observed at the later time point (Fig. 5). Although it has been indicated that PRRSV-induced IL-10 can negatively modulate the host immune responses [35, 60], the results from this study suggested that PRRSV-induced IL-1Ra production, rather than IL-10, was likely responsible for the decrease in pro-inflammatory cytokine and type I IFN production during the early phase of PRRSV infection. The findings on different patterns of IL-1Ra production following type 2 PRRSV and HP-PRRSV infections further highlighted the potential regulatory role of IL-1Ra, which could affect the clinical outcome following PRRSV infections.

IL-1Ra is known as an early inhibitory cytokine that plays an important regulatory role in acute inflammatory response [56]. One previous report demonstrated that IL-1Ra could modulate the production of pro-inflammatory cytokines and type I IFN; IL-1Ra suppressed the production of IL-1α-induced IL-6 and IL-8 in the epithelial cells [61]. Exogenous IL-1Ra inhibited the production of IL-1β and TNF-α in LPS-stimulated monocytes [43]. In rheumatoid arthritis patients, treatment with synthetic IL-1Ra peptide drastically decreased the production of IL-1β, IL-6 and TNF-α in the affected joints [62]. In addition, it has been shown that IL-1Ra modulated IFN-α and IFN-β production in a mouse model [44, 63]. IL-1Ra also modulated the adaptive immune functions by reduction of antigen-specific T-cell activation, proliferation and polarization [64–66]. Furthermore, lack of an IL-1 receptor signalling cascade inhibited the polarization of T-helper type 2 and production of antigen-specific antibodies [66–68]. Together, the above information indicates that IL-1Ra production influences host immunocompetency.

In general, typical PRRSV infection induced weak innate cytokine production and delayed cell-mediated and humoral immunity in infected pigs [29]. It is likely that early induced and prolonged IL-1Ra production will influence the induction of innate and, subsequently, adaptive immune responses resulting in the unique immunological profiles observed following PRRSV infection. Supporting this notion, enhanced IL-1Ra production played a crucial role in suppression of IL-1/IL-8-induced inflammatory responses during an early phase of *Yersinia pestis* infection. It is believed that induction of IL-1Ra accommodated bacterial survival within the infected lungs [69]. The induction of IL-1Ra by *Aspergillus fumigatus* correlated with increased fungal burden and suppressed inflammation in the infected lungs [70]. Overexpression of IL-1Ra enhanced *Listeria monocytogenes* replication and interfered with the macrophage maturation process in infected mice [71]. In HIV-infected patients, levels of serum IL-1Ra correlated with decreased CD8 activation and enhanced viral load during the acute phase of HIV infection [72]. The biological relevance of PRRSV-induced IL-1Ra on the functions of porcine innate and adaptive immune functions is currently under investigation.

In contrast to typical PRRSV, HP-PRRSV induces strong innate cytokine production during the early phase of infection and causes severe clinical disease in infected pigs [17, 19, 20]. Our findings that HP-PRRSV enhanced high IL1B and IFNA gene expression (Fig. 5d, e) are consistent with the above reports. To date, the reasons for variation in the pathogenesis of HP-PRRSV are not clearly understood [73, 74]. In contrast to the typical type 2 PRRSV, HP-PRRSV did not induce IL-1Ra, either in vitro or in vivo. Our findings suggest that inability to induce IL-1Ra production could be one of the underlined mechanisms, resulting in uncontrolled production of innate cytokines and severe clinical outcome following HP-PRRSV infection. Interestingly, mice lacking IL-1Ra exhibited enhanced production of pro-inflammatory cytokines, including IL-1, IL-6 and IL-17, and increased mortality in the subsequent Ebola virus infection [75]. The role of IL-1Ra in the immunopathogenesis of HP-PRRSV should be further explored.

It should be pointed out that different PRRSV strains possess different immunomodulatory properties – in particular, induction of IL-10 production and viral-specific Treg [39, 76, 77]. In this study, the potential of other PRRSV strains, including type 1 (EU) PRRSV, and other type 2 PRRSV strains or other PRRSV-MLV, to induce IL-1Ra production was not explored due to limited availability of the viruses. It would be interesting to further investigate whether the ability to induce IL-1Ra production is conserved among the PRRSV strains. In addition, the precise molecular mechanism responsible for PRRSV-induced IL-1Ra production is not known. This information will be crucial for better understanding of the immunopathogenesis of PRRSV.

In conclusion, this study identified IL-1Ra as a negative immunomodulatory mediator during the early phase of PRRSV infection. In addition, type 2 PRRSV and HP-PRRSV exhibited variation in their capability in regard to induction of IL-1Ra. These findings could partly explain the differences in immunopathogenesis and clinical outcomes following PRRSV infection.

**METHODS**

**Viruses and cells**

Type 2 PRRSV, strain 01NP1 [78], HP-PRRSV, strain 10PL01 [79], classical swine fever virus, swine influenza virus H1N1 (AT/swine/Thailand/CU-P573/2010) and H3N2 (A/swine/Thailand/CU-CB8.4/2007) were kindly provided by the Chulalongkorn University Veterinary
Diagnostic Laboratory (CU-VDL; Bangkok, Thailand). PRRSV was propagated and titrated in the MARC-145 cell line (CU-VDL) at 10^6 TCID_{50} ml^{-1}. CSFV and influenza virus were propagated to 10^6 TCID_{50} ml^{-1} in the SK-6 (CU-VDL) and MDCK cell lines (CU-VDL), respectively. Mock-infected cell lysates were prepared from MARC-145 (for PRRSV), SK-6 (for CSFV) and MDCK (for influenza virus) cell lines. All viruses and mock-infected cell lysates were stored at −80 °C until needed.

**Antibodies and secondary conjugates**

Anti-swine CD3-FITC mAb (BB23-8E6, IgG2b) conjugate and biotinylated anti-swine SWC3 mAb (74-22-15, IgG1) were purchased from SouthernBiotec. Anti-swine slg (H +L)-PE polyclonal Ab (15H6, IgG1) was purchased from AbSerotec. Anti-swine IL-1Ra mAb (114801, IgG2a) and the isotype controls were purchased from R&D Systems. Streptavidin-APC and goat anti-mouse IgG2a-PE/cy7 were purchased from BioLegend. Anti-PRRSV mAb (SDOW-17, RTI, SD, US) was provided by CU-VDL.

**Animals and animal experiments**

Eight-week-old, crossbred, PRRSV-seronegative pigs (five pigs per group) were obtained from a commercial farm in Kanchanaburi Province, Thailand. The pigs were randomly grouped and housed throughout the experiment at the animal facility of the Faculty of Veterinary Medicine Kasetsart University, Kumpangsan campus the pigs were vaccinated in the SK-6 (CU-VDL) at 10^5 TCID_{50} ml^{-1}. CSFV and influenza virus cell lines. All viruses and mock-infected cell lysates were stored at −80 °C until needed.

**Isolation of porcine PBMCs and PBLs, generation of MoDCs and in vitro activation assays**

PBMCs were isolated from heparinized blood samples by gradient centrifugation, using LymphoSep™ separation medium (MP Biomedicals) according to the manufacturer’s protocol. For the generation of MoDC, the PBMCs were resuspended at 5 × 10^6 cells ml^{-1} in Iscove’s modified Dulbecco’s medium (Gibco) and plated on a 24-well plate (Corning-Costar) for 2 h. Non-adherent cells, containing 74.7±8.35 % CD3 and 16.1±3.76 % slg cells, referred to as PBLs, were collected and stored in liquid nitrogen until needed. The remaining adherent cells, referred to as monocytes, were cultured in 1 ml of RPMI medium, medium, for 7 days, with 50 % replacement with fresh DC medium every 2 days. For in vitro activation, the porcine leukocyte populations (PBMC, PBL and MoDC) were cultured with 0.1 m.o.i. of the indicated viruses or the relevant mock-infected cell lysates on a 24-well plate at 37 °C in a 5 % CO_2 incubator for 24 or 48 h, as indicated in the legends.

**Microarray analysis**

MoDCs were cultured with 0.1 m.o.i. of type 2 PRRSV (01NP1) or MARC-145 cell lysate on a 24-well plate at 37 °C in a 5 % CO_2 incubator for 24 h. Total mRNA was purified using a commercial RNA extraction kit (Qiagen) according to the manufacturer’s instructions. Samples were hybridized onto Porcine_GXP_4×44K (two-colour microarray-based gene expression analysis). The arrays were scanned with an Agilent scanner system, and data were collected using an Agilent Feature Extraction Software v10.7. Data were normalized using GeneSpring GX 11.5 software. The fold values were provided in terms of log base 2 when comparing the two groups: PRRSV (P) and mock-infected MoDC (M).

All microarray data have been approved and deposited in the Gene Expression Omnibus under accession number GSE86182.

**Quantitative reverse transcriptase PCR detection of PRRSV RNA**

Viral RNAs were extracted from the serum samples and lung tissues (approximately 1×1×0.5 cm) using an RNA extraction kit (NucleoSpin RNA virus kit; MACHEREY-NAGEL) according to the manufacturer’s protocol. Quantification of PRRSV RNA was performed using TaqMan probe-based real-time reverse transcriptase PCR as previously described [80]. The sequences of the probe and primers are as follows: US-PRRSV-specific probe (5’ FAM-TCC-CGG-TCC-CTT-GCC-TCT-GGA-TAMRA 3’), ORF7-US forward primer (5’ AAA-TGG-GGC-TTC-TCI-GGI-CTT-TTT 3’) and ORF7-US reverse primer (5’ AAA-TGI-GGC-TTC-TIC-GGT-TTT-TTT 3’). The amplification was carried out in a 25 µl reaction containing SuperScript III One-Step RT-PCR kit (Thermo Fisher Scientific) 1 × reaction mix, 0.4 mM of each primer and probe, 0.5 µl (100 U) of SuperScript III RT/Platinum Taq Mix and 0.5 µl (20 pmol) of viral RNA.

**qPCR for detection of porcine cytokine gene expression**

Total mRNAs were extracted from the leukocyte subpopulations (PBMC, PBL and MoDC) using a commercial RNA extraction kit (Bioterabbit, Germany) according to the manufacturer’s instructions. The extracted mRNAs were quantified using NanoDrop (Thermo Scientific) and converted to cDNA using a commercial cDNA synthesis kit (Invitrogen). The levels of cytokine gene expression were determined using SYBR Green-based real-time PCR. The qPCR for detection of porcine IL1RA, IL1B, IFNA, IL10 and GAPDH gene expression was performed using
the primer sets described in Table 1. The qPCR was carried out in a 20 µl reaction and consisted of 2 µl (20 ng) of the cDNA template, 0.5 µl (20 pmol) of each specific primer, 7 µl of sterile water and 10 µl of SYBR Green master mix (Biotechrabbit). qPCR was carried out using a Rotor-Gene RG-3000 (Corbett Research). The amplification reaction consisted of initial denaturation at 95 °C for 15 min, followed by 40 cycles of denaturation at 94 °C for 20 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s. The specificity of the amplicon was verified by melting curve analyses. The C\textsubscript{t} values of gene expression were normalized against the housekeeping gene GAPDH. Differences in C\textsubscript{t} values between the treatment groups were analysed by the formula 2\(^{-\Delta\Delta C_{t}}\).

Detection of IL-1Ra in the cultured supernatants
Following in vitro activation, supernatants obtained from the cell culture were collected and stored at −80 °C until used. Determination of IL-1Ra protein was performed using a commercial ELISA kit (CUSABIO), according to the manufacturer’s protocol.

Immunofluorescent staining and flow cytometric analyses
The cells of interest were harvested, washed once with Ca-, Mg-free PBS (PBSA) and then resuspended in PBSA supplemented with 0.5 % BSA and 0.1 % sodium azide, referred to as the FACS buffer. The cells were distributed on a 96-well plate (Corning-Costar) at the concentration of 2\times10^6 cells per well and pelleted by centrifugation at 500 g for 5 min. Immunofluorescent staining of the surface molecules, CD3, SWC3 and slg, was performed by addition of 1 : 50 of anti-CD3-FITC, 1 : 100 of biotinylated anti-SWC3 and 1 : 100 of anti-slg (H+L)-PE antibodies, diluted in FACS buffer and followed by incubation at 4 °C in the dark for 40 min. The stained cells were then washed twice with PBSA. For secondary staining of SWC3, 1 : 500 of streptavidin-APC diluted in FACS buffer was added to the cells followed by incubation at 4 °C in the dark for 30 min.

For intracellular staining, the protein transport inhibitor monensin (GolgiStop; BD Biosciences), was added to the culture 12 h prior to harvesting. Following harvesting and staining of the surface molecules, the cells were then fixed and permeabilized with 100 µl per well of 50 % reagent A (Leucoperm, Serotec), then diluted in FACS buffer at room temperature in the dark for 30 min. For primary staining, 1 : 100 of anti-IL-1Ra (IgG2a), diluted in reagent B (Leucoperm), was added to the cells and further incubated at 4 °C in the dark for 45 min. For the secondary staining required for staining of IL-1Ra, 1 : 100 of goat anti-mouse IgG2a-PE/cy7, diluted in FACS buffer, was added to the cells followed by incubation at 4 °C in the dark for 30 min. As a final step, the stained cells were resuspended in 2 % formaldehyde in PBSA and kept at 4 °C until flow cytometric analyses were performed.

Similar cells stained with isotype controls were included and used as the background cut-off in this study. Fluorescent minus one staining samples were also performed during the establishment and validation of the assay. Flow cytometric analyses were performed using a Beckman Coulter FC 500 MPL.

Statistical analyses
Data were analysed using Student t-test or ANOVA, followed by Tukey’s multiple comparison tests all statistical analyses were performed using GraphPad Prism for Windows (GraphPad Software Incorporated).

<p>| Table 1. Oligonucleotide sequences of primers used for qPCR |
|----------------------------------|-----------------|-----------------|-----------------|</p>
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Conflicts of interest
The authors declare that there are no conflicts of interest.
Ethical statement
The animal care and use protocols for this study adhered to the Ethical Principles and Guidelines for the Use of Animals, National Research Council of Thailand, and the Guide for the Care and Use of Laboratory Animals, National Research Council, USA. All methods and animal studies were conducted under the approval of the Chulalongkorn University Animal Care and Use Committee, Chulalongkorn University (animal use protocol no. 1631029).

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