

# A standardized method to study immune responses using porcine whole blood

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## Research Article

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# Abstract

Peripheral blood mononuclear cells (PBMCs) are commonly used to assess *in vitro* immune responses. However, PBMC isolation is a time-consuming procedure, introduces technical variability, and requires a relatively large volume of blood. By contrast, whole blood assay (WBA) is faster, cheaper, maintains more physiological conditions, and requires less sample volume, laboratory training, and equipment. Herein, this study aimed to develop a porcine WBA for *in vitro* evaluation of immune responses. Heparinized whole blood (WB) was diluted (non-diluted, 1/2, 1/8, and 1/16) in RPMI-1640 media, followed by phorbol myristate acetate and ionomycin (PMA/ION) stimulation in humidified air at 37°C and 5% CO<sub>2</sub>. After 24 h, cells were stained for IFN-γ secreting T-cells followed by flow cytometry, and the supernatant was analyzed for TNF-α. In addition, diluted WB was stimulated by lipopolysaccharide (LPS) and polyinosinic:polycytidylic acid (poly I:C), field isolate (FI), reference strain KCTC3557 (RS) of heat-killed (HK) *Streptococcus suis*, and porcine reproductive and respiratory syndrome virus (PRRSV). Cell surface immune-fluorescent staining showed consistent results at all dilutions following stimulation of WB by PMA/ION, while in non-diluted WB, intracellular cytokine staining was obstinate. The frequency of cytotoxic T-cells (CTLs) and concentration of TNF-α in the supernatant of WB increased with increasing dilution factor and were optimal at 1/8. WB TNF-α and IL-10 cytokine levels increased significantly following stimulation with LPS or poly I: C. Further, FI and RS induced IL-10 production in WB. Additionally, PRRSV strains increased the frequency of CTLs, and IFN-γ was non-significantly induced in the supernatant of re-stimulated samples. We propose that the WBA is a rapid, reliable, and simple method to evaluate immune responses and WB should be diluted to trigger immune cells.

## 1. Introduction

There are limitations to obtaining tissue specimens from humans to study various immune parameters. Hence, peripheral blood is the most common source of human immune cells, and peripheral blood mononuclear cells (PBMCs) are broadly used for clinical and scientific purposes (Haller et al. 2000; Sela et al. 2018). Similarly, porcine PBMCs have been used to evaluate immune responses in various studies, including pathogen challenge and vaccination (Darwich et al., 2003; Gerner et al., 2008; Shabir, N. et al., 2018), because this avoids the need to sacrifice animals. However, PBMC isolation is a time-consuming multi-step procedure that requires laboratory training. In addition, this procedure may introduce technical variations and is difficult to standardize within laboratories. Furthermore, isolated PBMCs are normally devoid of various growth factors and other immune components that can alter the physiological functions of cells. Therefore, assays using whole blood (WB) have been developed for human clinical studies (Gaines et al. 1996; Leroux et al. 2003; Godoy-Ramirez et al. 2004). The use of WB is an attractive strategy because it is simple, fast, and requires less blood volume. In addition, WB retains the physiological condition of cells by maintaining all blood components. Intensive laboratory training and equipment are unnecessary to evaluate the immune responses using WB; therefore, fewer variations occur within and across laboratories. The whole blood assay (WBA) is low-cost and well suited to

measure monocyte cytokine production (Damsgaard et al. 2009). Further, purified cells are inaccurate and provide low reproducibility when compared with WB (Hodge et al. 2000).

The idea of using WB was first introduced by Ruuskanene and colleagues in 1975 (Eskola et al. 1975) to evaluate phytohemagglutinin and ConA-induced lymphocyte proliferation. Later, Kirchner and Digel independently used WB to study the cytokines induced by a wide variety of stimulants (Kirchner et al. 1982; Digel et al. 1983). Additionally, WB cultures have been used over the last three decades to assess various aspects of immune responses (Duffy et al. 2014). A notable improvement in WBA was the development of the QuantiFERON TB Gold In-Tube assay, which can diagnose latent tuberculosis infection by measuring INF- $\gamma$  after *in vitro* stimulation with *Mycobacteria tuberculosis* antigens (Santin et al. 2012). Recently, Duffy and co-workers developed a suite of whole-blood, syringe-based assay systems to evaluate immune responses induced by bacteria, fungi, viruses, agonists specific for defined host sensors, clinically employed cytokines, and activators of T-cell immunity (Duffy et al. 2014). By contrast, porcine WB is used less frequently to assess immune response because it lacks a standardized method. Yancy and colleagues have reported that WB RNA expression is comparable or higher when compared with PBMC culture systems; however, very little or no change is found in protein expression (Yancy et al. 2001; Segura et al. 2006). In addition, most previous studies examining porcine reproductive and respiratory syndrome virus (PRRSV) induced immune responses have been tested *in vitro* using PBMCs (Costers et al. 2009; Shabir et al. 2018b; Madapong et al. 2020; Nazki et al. 2020), but not in WB. Moreover, the suite of whole-blood syringe-based assays developed by Duffy and co-workers were established for human studies and are comparatively expensive to use in the veterinary field.

In the present scenario, a porcine WB *in vitro* culture assay is required to study the immunological properties of various immunostimulants. This system should be economical, efficient, mimic the natural environment, and less time-consuming. Therefore, we focused on two main aspects: I) the development of an *in vitro* WBA to study porcine immune responses using a minimal volume of WB, and II) evaluation of the immune responses in WB following stimulation with TLR ligands, bacteria, and viruses.

## **2. Materials And Methods**

### **2.1. Collection and dilution of WB**

WB from 4 PRRSV negative, 5-6-week-old cross bred (Yorkshire and Landrace) piglets was collected in sodium heparin-containing vacutainers (BD Biosciences) from external jugular vein and was used within 1 h of collection. WB was processed as non-diluted, 1/2, 1/8, and 1/16 dilutions (where 1/2 specifies 1 part of WB and 2 parts of RPMI, so on and so forth) in RPMI-1640 media containing antibiotic-antimycotic solution (Invitrogen) with a final concentration of 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin, and 0.25  $\mu$ g/ml Fungizone®.

### **2.2. Optimization of the standard method for WB stimulation**

Processed WB samples were further stimulated using the tube method (TM) and plate method (PM).

## 2.2.1. Tube method (TM)

In TM, 100  $\mu$ l non-diluted, 1/2, 1/8, and 1/16 diluted WB samples were stimulated or not stimulated in 2 ml microcentrifuge tubes with 0.5 X (PMA, 40.5 nM and ION, 670 nM) PMA/ION (cell stimulation cocktail Life Technologies Corp.) or unstimulated for 24 h at 37°C in a 5% CO<sub>2</sub> humidified chamber. The WB samples were mixed every 7 h by inverting the centrifuge tubes six times before adding BFA (brefeldin A, Life Technologies Corp). For the last 4 h of incubation, a cocktail of PMA/ION and BFA (1 $\times$  each; PMA, 81 nM and ION, 1340 nM) were added to all samples. Following incubation, the tubes were centrifuged at 1200 rpm for 6 minutes, and the supernatant was collected and stored at - 80°C until further analysis. The cell pellets were stained for flow cytometric analysis.

## 2.2.2. Plate method (PM)

In PM 30  $\mu$ l non-diluted and 1/8 diluted WB (final volume, 270  $\mu$ l) were stimulated or not stimulated by PMA/ION (0.5X) in a flat-bottom 96-well plate. WB samples were mixed (six times) every 7 h using a multichannel pipette before adding BFA. For the last 4 h of incubation, a cocktail of PMA/ION and BFA (1 $\times$  each) were added to all samples. Samples were centrifuged at 1200 rpm for 6 minutes, and the supernatant was collected and stored at - 80°C until further analysis. The cell pellets were stained for flow cytometry. Moreover, PM was compared with TM in which 30 and 100  $\mu$ l of WB were diluted (1/8), respectively, and stimulated as stated in sections 2.2.1 and 2.2.2.

## 2.2.3 Comparison of WB and PBMCs

Thirty  $\mu$ l of WB was stimulated by PM as described in section 2.2.2. Alternatively, 10<sup>6</sup> PBMCs were isolated as per previous reports (Nazki et al. 2020) and were stimulated with pre-added 0.5X PMA/ION or not stimulated in 100  $\mu$ l of complete RPMI (RPMI-1640 with 10% FBS, 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin, and 0.25  $\mu$ g/ml Fungizone®). For the last 4 h of incubation, a cocktail of PMA/ION and BFA (1X each) were added to all samples.

## 2.3. Stimulation of WB with TLR ligands and bacteria

After standardising the WB dilution, porcine WB (1/8 dilution) was stimulated with TLR ligands and bacteria.

### 2.3.1. Bacterial strains and growth conditions

Two strains of *S. suis*, RS (reference strain, KCTC3557) and FI (field isolate) were used in this study. FI was kindly provided by the Swine Disease Lab, Jeonbuk National University, South Korea. Briefly, bacteria were maintained as stock in 50% glycerol (USB Corporation) and stored at - 80°C. A loop full of bacteria was grown on sheep blood agar at 37°C overnight. Single colonies, used as inoculum for Tryptic soy broth (TSB) (Sigma-Aldrich), were incubated for 8 h at 37°C. Next, 1:1000 dilution of this culture was added to 30 ml of TSB for 24 h at 37°C. Bacteria were washed twice with PBS and re-suspended at 10<sup>10</sup>

cfu/ml in PBS. Finally, the bacteria were heat-killed at 100°C for 5 minutes in a water bath. HK bacteria were further diluted to working concentrations of  $10^8$  and  $10^6$ cfu/ml in RPMI.

## 2.3.2. Stimulation with TLR ligands and bacteria

Lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 (Sigma-Aldrich) and polyinosinic:polycytidylic acid (poly I:C, Sigma-Aldrich) were used at a final concentration of 50 and 10 µg/ml, respectively. WB (1/8) was stimulated by PM with LPS, poly I:C, RS, FI, or kept unstimulated in quadruplicate at 37°C in a 5% CO<sub>2</sub> humidified chamber for 24 h. After stimulation, WB supernatant was collected and stored at -80°C until further analysis.

## 2.4. Evaluation of cytotoxic T-cells response in PRRSV challenged pigs

WB was optimally stimulated by chemical and biological stimulants at 1/8 dilution; therefore, we further employed the WBA to evaluate the cytotoxic T-cell (CTLs) response in PRRSV infected pigs.

### 2.4.1. Cells and virus

The Meat Animal Research Center-145 (MARC-145) cell line was used to prepare the NA42 and NA45 PRRSV stocks. The PAM were used to prepare the PRRSV strain 10D415 stock. These viral strains were extracted from the clinical samples (lung and sera) of infected pigs at the diagnostic lab of the Animal and Plant Quarantine Agency, Korea. Cells were maintained in RPMI medium (Life Technologies) supplemented with heat-inactivated 10% fetal bovine serum (Life Technologies), 2 mM L-glutamine and antibiotic-antimycotic solution at 37°C in a 5% CO<sub>2</sub> humidified chamber. Viral strains were further concentrated using a Vivaspin 20 centrifugal concentrator (MWCO 300 kDa, Merck), according to the manufacturer's instructions.

### 2.4.2. Experimental procedure

Nine, 8-week-old crossbred piglets purchased from a PRRSV seronegative farm were arbitrarily assigned to 3 groups and housed in separate pig pens. After 3 days of acclimatization, pigs were infected intranasally with 2 mL of the NA42, NA45, and 10D415 PRRSV-strains ( $1 \times 10^3$  TCID<sub>50</sub>/mL) separately in different groups. For re-stimulation, a titer of  $1 \times 10^{4.5}$  TCID<sub>50</sub>/mL was used after concentrating the respective strains by the Vivaspin 20 centrifugal concentrator. Feed and water were provided *ad libitum* to all pigs. The animals showed usual signs of PRRSV disease. Animals were humanely euthanized by electrocution after intramuscular injection of 2 mL of azaperone (40 mg/mL, StressGuard®).

### 2.4.3. Stimulation of WB with different PRRSV strains

Heparinized WB, collected at 7- and 14-days post challenge (DPC) was diluted 1/8 in a 96-well plate and stimulated for 24 h by Media (mock), NA42 ( $10^{4.5}$  TCID<sub>50</sub>/ml), NA45 ( $10^{4.5}$  TCID<sub>50</sub>/ml), or 10D415 ( $10^{4.5}$

TCID<sub>50</sub>/ml) for 24 h at 37 °C in a 5% CO<sub>2</sub> humidified chamber. For last 4 h of incubation PMA/ION and BFA (1× each) were added to all samples.

## 2.5. Flow cytometry and ELISA

After stimulation or re-stimulation, samples were transferred to 96-well U bottom plate and were pelleted by centrifugation (1200 rpm at 4°C, for 5 min). Cell pellets were re-suspended in fluorescent antibody cell sorting (FACS) buffer and supernatant was saved at -80°C for further analysis by ELISA. Three different strategies of antibody combinations were used in the current study:

1. 2.5.1 While stimulating undiluted WB, cells were stained (cell surface staining, antibodies were suspended in FACS buffer) with CD3-FITC (Bio-Rad) and CD4 PE (BD-Pharmingen) in dark on ice for 30-minutes. Cells were washed 2-times with cold FACS buffer. Intracellular fixation was performed by cold IC fixation buffer (eBioscience) in dark for 30 min on ice, followed by 2 times washing with permeabilization buffer (eBioscience). After that RBC lysis cells was done by lysis buffer (Invitrogen) for 10 minutes at room temperature in dark, followed by two times washing with permeabilization buffer. Cells were further stained (intracellular staining, antibodies were suspended in permeabilization buffer) with IFN-γ-PerCP-cy<sup>TM</sup>5.5 (BD-Pharmingen) in dark for 30 min on ice.
2. 2.5.2 While standardizing the dilution of WB for optimal stimulation or comparison with PBMCs, WB or PBMCs samples were stained with CD3-FITC, as described in section 2.5.1 for surface staining. After intracellular fixation and RBC lysis, cells were stained with IFN-γ-PerCP-cy<sup>TM</sup>5.5, as described in section 2.5.1.
3. 2.5.2 In recall response experiment, WB from each animal was stained with CD4-PE. Further, cells were stained by CD8-FITC (BD-Pharmingen), as described in section 2.5.1 for cell surface staining. After intracellular fixation and RBC lysis, cells were stained with IFN-γ-PerCP-cy<sup>TM</sup>5.5, as described in section 2.5.1. Further details of the antibodies are provided in additional file 1.

Flow cytometry was performed using an Accuri C6 flow cytometer (BD Accuri<sup>TM</sup> C6 Plus, BD Biosciences). Phenotype data were collected for 100,000 events followed by a gating strategy using FLOWJO version 10.6.1 (LLC, 385 Williamson Way, Ashland, OR 97520), (see additional file 2).

The concentrations of IFN-γ, TNF-α, and IL-10 in supernatant were determined by using enzyme-linked immunosorbent assay (ELISA) (R&D Systems), according to the manufacturer's instructions.

## 2.6. Data analysis

Data are presented as mean ± SEM except for comparison of TM with PM for which values are displayed as mean ± SD. Statistical analyses were performed using Graph Pad Prism 5 (GraphPad Software, Inc., CA). The Mann-Whitney U test, or one-way or two-way ANOVA were used to compare groups. Differences were considered significant at p < 0.05.

## 3. Results

### 3.1. Un-diluted WB does not respond to stimulants

To test whether WB can be used directly to study immune responses without further processing, it was stimulated with PMA/ION or PRRSV for 24 h. As shown in Fig. 1A, undiluted WB samples stained well for cell surface molecules, including CD3 and CD4; however, the frequencies of IFN- $\gamma$  producing cells did not differ between the unstimulated and stimulated samples. Moreover, a high TNF- $\alpha$  background was detected in the non-stimulated samples (Fig. 1B). PMA/ION is a commonly used strong activator of PBMCs that induces robust cell proliferation and cytokine production; however, WB seemed refractory to PMA/ION treatment for a 24-h culture.

### 3.2. WB dilution is necessary to induce cell activation

Previous studies using human WB have suggested that dilution is helpful to minimize interference by RBC (Yancy et al. 2001) and other unknown factors (May et al. 2013). Therefore, we diluted porcine WB up to 1/16 and evaluated IFN- $\gamma$  producing T cells and TNF- $\alpha$  secretion by flow cytometry and ELISA, respectively. The frequencies of IFN- $\gamma^+$  cells in CD3 $^+$  T cells were increased as the dilution increased, even in unstimulated samples at relatively low levels (less than 3%) (Fig. 2A). Upon stimulation with PMA/ION, the percent frequencies at 1:8 and 1:16 dilutions were significantly higher and increased up 8% (Fig. 2B). Similarly, the concentration of TNF- $\alpha$  in the supernatant increased with higher dilution factors. At 1/2, 1/8 and 1/16, the concentration of TNF- $\alpha$  in the supernatant of WB was  $1712 \pm 733.15$ ,  $4251 \pm 2588.54$ , and  $7811.45 \pm 1847.16$  pg/ml (mean  $\pm$  SEM), respectively. Highest stimulation was obtained at 1/16 dilution, with no significant difference between 1/8 and 1/16 dilutions (Fig. 2C). Keeping in view the maximum volume a 96-well can accommodate, the 1/8 dilution was used to stimulate WB in the further experiments.

### 3.3. Comparison of the TM and PM for stimulation

To make the stimulation process less laborious and time consuming, WB samples were stimulated by TM and PM and the results were compared. Briefly, 30 and 100  $\mu$ l of WB was diluted (1/8) and stimulated in a flat bottom 96-well plate and microcentrifuge tubes for the PM and TM, respectively. The frequencies of IFN- $\gamma^+$  cells in CD3 $^+$  cells were perceived as  $12.58 \pm 4.08$  and  $14.47 \pm 1.04$  (mean  $\pm$  standard deviation) in the TM and PM (Fig. 3A), respectively. Further, TNF- $\alpha$  displayed a SD of 157.0 by TM and 130.9 by PM (Fig. 3B). This suggested that the PM displayed uniformity in results with a lower SD; therefore, WB was stimulated using the PM in further experiments due to its ease of use and lower variation.

### 3.4 Comparison of WB and PBMCs stimulation

To compare WB and PBMCs, respective samples were stimulated. The frequencies of IFN- $\gamma^+$  cells in CD3 $^+$  cells were perceived as  $1.18 \pm 0.32$  and  $2.07 \pm 0.70$  (mean  $\pm$  standard deviation) in non-stimulated WB and PBMCs samples, respectively and  $6.11 \pm 1.8$  and  $8.14 \pm 1.72$  in stimulated samples, respectively (Fig. 4).

### 3.5. Cytokine release by WB, induced on stimulating with TLR ligands, LPS and poly I:C, and *S. suis*

PBMCs are widely used to study immune responses in pigs and other animals. However, there are limited reports on *in vitro* stimulation of WB in pigs, especially for longer incubation times. We stimulated 1/8 diluted WB with LPS (TLR4 ligand), poly I:C (TLR3 ligand), and with the RS and FI strains (HK) of *S. suis* using the PM. The highest concentration of TNF- $\alpha$  was detected in LPS-stimulated samples, followed by poly I:C and RS ( $10^8$  cfu/ml):  $1584 \pm 124.1$ ,  $1103 \pm 177.7$ ,  $762.7 \pm 42.72$  pg/ml, respectively (Fig. 5A). Both FI and RS were unable to induce TNF- $\alpha$  response in WB at  $10^6$  cfu/ml. (Fig. 5A) Further, IL-10 expression was highest in the LPS-stimulated samples, followed by FI ( $10^8$  cfu/ml), RS ( $10^8$  cfu/ml), FI ( $10^6$  cfu/ml), RS ( $10^6$  cfu/ml) and poly I:C ( $434.4 \pm 138.3$ ,  $309.5 \pm 33.91$ ,  $230.6 \pm 29.61$ ,  $100.2 \pm 14.30$ , and  $57.11 \pm 10.16$  pg/ml, respectively; Fig. 5B). FI induced IL-10 but there was no significant effect on the induction of TNF- $\alpha$  in porcine WB (Fig. 5A and 5B).

## 3.5. Re-stimulation response after PRRSV challenge

### 3.5.1 CTLs response in PRRSV infected pigs at 7 and 14 DPC on re-stimulation with respective strains, *in vitro*.

The CTLs (IFN- $\gamma^+$  in CD4 $^-$ CD8 $^+$ ) response was measured by analysing the T-cell populations in the WB of pigs at 7 and 14 DPC after re-stimulating 1/8 diluted WB with respective homologous PRRSV strains for 24 h (Fig. 6A). CTLs response was significantly induced which was higher ( $p < 0.05$ ) at 14 DPC in NA42 challenged pigs. On the other hand, there was no significant upregulation in the concentration IFN- $\gamma$  in the supernatant of re-stimulated samples treated with homologous strains when compared with mock stimulation *in vitro* (Fig. 6B).

## 4. Discussion

PBMC isolation is time consuming and requires a relatively large volume of blood. The isolation procedure removes polymorphonuclear cells that may play a role in intracellular communication for the synthesis and release of cytokines. In contrast to PBMCs, WB contains granulocytes which can be subdivided into neutrophils, eosinophils, and basophils. Moreover, the membrane properties and activation state of PBMCs may be influenced by centrifugation (Damsgaard et al. 2009). Often, PBMCs require the addition of fetal calf serum, which contains various nutrients and hormones that may alter the immunological responsiveness of the cells (Albers et al. 2005). Furthermore, cells in isolated PBMCs undergo a significantly high level of apoptosis when compared with WB cultures (Hodge et al. 2000), and stained cell populations clearly separate in WB with better clarity (Appay and Rowland-Jones 2002). Therefore, the current study aimed to develop a time-saving method to assess porcine immune responses using a smaller volume of WB.

Undiluted WB stimulated for 6 h with PMA/ION displayed a rapid increase of TNF- $\alpha$  and IFN- $\gamma$  (Ai et al. 2013). However, on stimulation of WB with PMA/ION for 24-h we were unable to detect any significant

stimulation of T-cell, as evaluated by flow cytometry and ELISA. This may be due to the poor stability of undiluted WB when incubated for a longer time. Further, there are certain unknown factors in WB (May et al. 2013) that might interfere with the stimulation process, and the presence of RBCs in close proximity of lymphocytes may reduce the space available for stimulants to interact with immune cell receptors. Diluting WB with PMA/ION using the TM resulted in an increase in stimulation with increasing dilution as evaluated by flow cytometry and TNF- $\alpha$  ELISA. We observed better stimulation of IFN- $\gamma$  secreting T-cells in 1:8 and 1:16 diluted WB. In contrast to IFN- $\gamma$  secreting CD3<sup>+</sup> T-cells, the TNF- $\alpha$  response started to increase from 1:2 dilution of WB, which might be due to the fact that TNF- $\alpha$  is one of the most abundant cytokines (Mascher et al. 1999) and is produced by a variety of cells, including monocytes (Olsnes et al. 2007), activated lymphocytes (Baran et al. 2001) and granulocytes (Dubravec et al. 1990). Our results are consistent with previous studies; 1:5 or 1:10 diluted WB is optimal to obtain measurable and accurate IFN- $\gamma$  response (Godoy-Ramirez et al. 2004) and cell-mediated immune responses (Leroux et al. 1985; Weir et al. 1994; Gaines et al. 1996) in a long term culture.

The TM is a time-consuming process because all samples need to be treated separately. One alternative to this was to use a 96-well plate instead of microcentrifuge tubes. The 96-well plate cannot accommodate a large volume of diluted WB (100  $\mu$ l WB + 800  $\mu$ l RPMI = 900  $\mu$ l); therefore, we sought to reduce the volume of WB for stimulation. To test our hypothesis, we diluted (1:8) 30  $\mu$ l and 100  $\mu$ l of WB and stimulated by the TM and PM and compared their stimulation efficiency. The PM revealed a higher mean with reduced standard deviation when compared with the TM for IFN- $\gamma$  producing CD3<sup>+</sup> cells and TNF- $\alpha$  response. This is consistent with previous reports of reduced intra-subject precision values using the TM when compared with PM (Russell et al. 2018). Using a smaller volume in a 96-well plate enables the simultaneous processing of a high number of samples with a reduced amount of WB. Additionally, reagent use is reduced, and the harvesting of samples is simple and time saving. For further experiments in this study, PM was used because it was easier and produced lower inter-individual variation.

Depending on the method employed, human PBMCs and/or WBA techniques provide fine conditions for the aim proposed and WB cultures are well-suited proxy-measures (Silva et al. 2013). On stimulating 1:8 diluted WB and 10<sup>6</sup> PBMCs, the results were at least comparable with no significant difference in the frequency of IFN- $\gamma$  secreting CD3<sup>+</sup> T-cells.

WB was optimally stimulated with PMA/ION at 1:8 dilution; therefore, we next assessed our assay with a wide array of immune stimuli, including TLR agonists (LPS and poly I:C), bacteria (*S. suis*), and virus (PRRSV). LPS induces human PBMCs to synthesize pro-inflammatory cytokines, including TNF- $\alpha$  (Janský et al. 2003; Ngkelo et al. 2012; Ai et al. 2013; Irtegun et al. 2016) and anti-inflammatory cytokines, including IL-10 (Janský et al. 2003). Stimulating WB with LPS produces a higher concentration of cytokines when compared with PBMCs (Chen et al. 2010). WB contains the LPS binding protein (LBP), which plays a vital role in eliciting an immune response by enhancing the transfer of LPS aggregates from an aqueous solution to the CD14-TLR4 receptor complex (Tobias 1986; Wright et al. 1990). Therefore, WB provides a more natural environment for measuring the inflammatory response. Moreover, poly I:C induces the synthesis of pro-inflammatory cytokines, including TNF- $\alpha$  (Dorresteijn et al. 2010;

Duffy et al. 2014; Pjanova et al. 2019), and anti-inflammatory cytokines, including IL-10 (Dorresteijn et al. 2010), in human PBMCs or WB. Porcine PBMCs stimulated with poly I:C elicit differential gene expression, similar to those induced by RNA viruses (Wang et al. 2014). Our study indicates that both LPS and poly I:C induced immune cells in porcine WB to produce TNF- $\alpha$  and IL-10.

*S. suis* stimulates the release of pro-inflammatory cytokines in mice (Segura et al. 1999) and human mononuclear cells (Vadeboncoeur et al. 2003) *in vitro*. Stimulation of 1:3 diluted porcine WB with HK *S. suis* increases cytokine mRNA expression; however, a low level of protein secretion is displayed (Segura et al. 2006). The present study provides evidence that HK *S. suis* stimulates WB to secrete pro- and anti-inflammatory cytokines (TNF- $\alpha$  and IL-10, respectively). In this study, HK RS induced TNF- $\alpha$  (non-significantly) and IL-10 in WB at  $10^8$  cfu/ml, while FI induced the secretion of IL-10 alone. The discrepancy with Segura and colleagues might be because of differences in the pathogenicity of *S. suis* strains used and WB dilution. Interestingly, lower cfu/ml ( $10^6$ ) in both strains stimulated IL-10 but there was no significant difference in TNF- $\alpha$  concentration.

Next, we extended our assay to analyze the induction of CTLs response by PRRSV strains: NA42, NA45, and 10D415. We analyzed the response at 7 and 14 DPC. The CTLs response was higher in samples re-stimulated with homologous strains when compared with mock treated samples. The PRRSV strain, NA42, significantly induced a higher ( $p < 0.05$ ) CTLs response at 14 DPC, while there was no significant response upon re-stimulation with NA45 and 10D415. Our study is consistent with a previous report showing that CD3<sup>+</sup>CD8<sup>high</sup>, designated as CTLs, are induced at 14 DPC (Costers et al. 2009). This might be due to strain differences; pigs respond differently to different PRRSV strains (Shabir et al. 2018b). Additionally, the cellular immune response depends on the viral isolate used *in vitro* stimulation, which can interact differently with different immune cells (Díaz et al. 2006; Correias et al. 2017; Madapong et al. 2020). More importantly, the role of CTLs in combating PRRSV infection in hosts requires further investigation, which is out of the scope of the present study. The kinetics of neutrophils in blood changes after infection with PRRSV (Liu et al. 2013). An advantage of WBA over PBMCs is that WB can be used to evaluate lymphocytes, monocytes, and granulocytes all at once with a small volume of blood. In conclusion, our study defines porcine WBA as a model to study the cellular responses that are induced by different PRRSV strains.

We conducted this study to standardize the dilution of porcine WB for optimal stimulation to evaluate immune responses with chemical and biological stimulants. We observed that the dilution of WB is necessary for stimulation. This assay can be used to study the immune responses that are induced by a wide variety of stimulants, including chemical and biological stimulants. Furthermore, the PRRSV strain, NA42, significantly induced CTLs at 14 DPC. Taken together, we have shown that this assay provides reproducible results and is better suited to study immune responses in swine because it maintains immune cells in more natural conditions.

## Declarations

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Not applicable

### *Statements & Declarations*

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### *Competing interests*

The authors declare that they have no competing interests.

### *Authors' contributions*

S.S.M designed and performed experiments, analyzed data, and wrote the original manuscript. W.I.K. and S.M.L. were the principal investigators and contributed to the study design and edited the manuscript. R.P.A., S.C.K., C.G.J., and S.N. helped in performing the experiments.

### *Data availability*

The datasets used and/or analyzed during the current study available on reasonable request.

### *Ethics approval*

The current study protocol was approved by Institutional Animal Care and Use committee of JBNU (Approval number: 2016-0041).

### *Consent to participate*

Not applicable.

### *Consent for publication*

Not applicable.

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## Figures

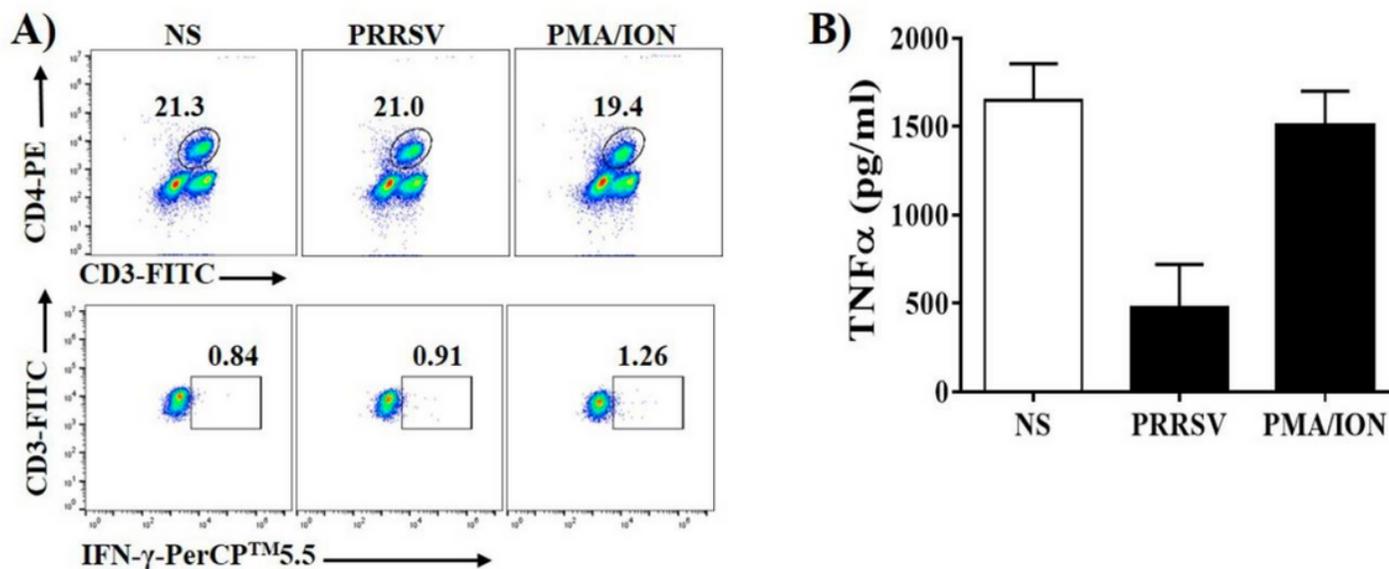
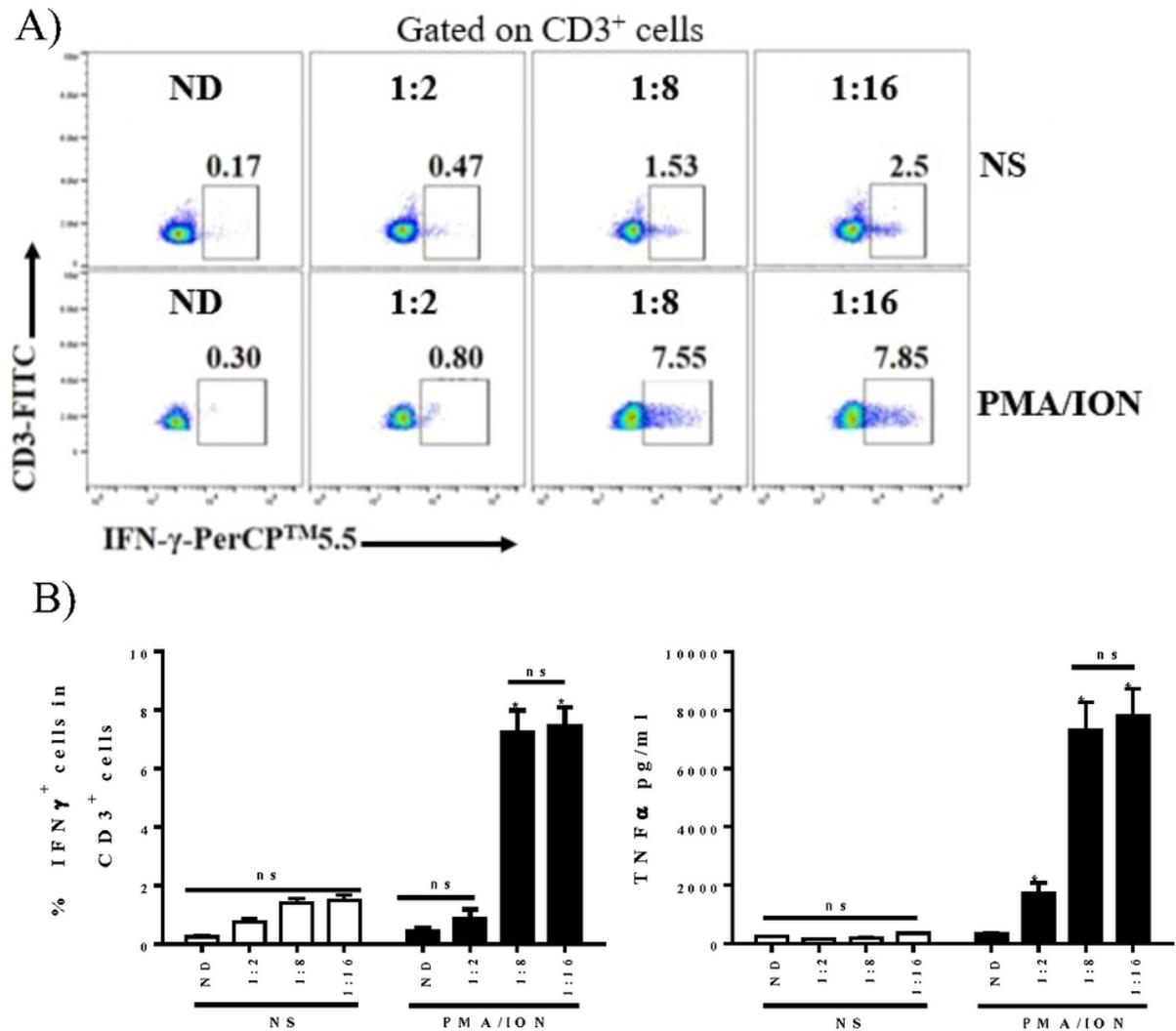


Figure 1

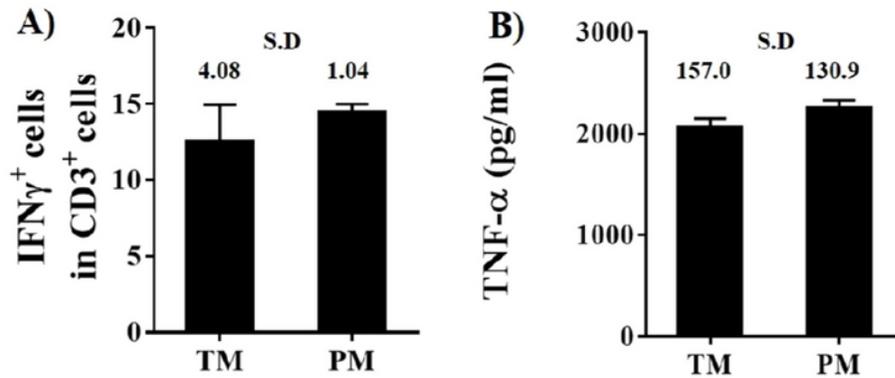
**Undiluted whole blood is refractory to PRRSV or PMA/ION stimulation:** Heparinized whole blood (100  $\mu$ l) was stimulated for 24 hours by PRRSV or PMA/ION (n = 33). A) Representative flow cytometry scatter dot plots, cell surface immunofluorescent staining showed consistent results (upper panel), intracellular immunofluorescent staining (IFN- $\gamma$ ) was obstinate (bottom panel) B) TNF- $\alpha$  (pg/ml) in supernatant of WB. NS= non-stimulated; PMA= phorbol myristate acetate; ION= Ionomycin.



**Figure 2**

**Optimal stimulation of WB is obtained at 1/8 dilution:** Heparinized whole blood (100  $\mu$ l) was diluted (ND,1:2, 1:8, 1:16 and stimulated by TM for 24 hours by PMA/ION (n = 4). A) Representative flow cytometry scatter dot plots for IFN- $\gamma$ <sup>+</sup> cells in CD3<sup>+</sup> (upper panel = non-stimulated, lower panel = PMA/ION stimulated) B) Frequency of CD3<sup>+</sup> IFN- $\gamma$ <sup>+</sup> cells in CD3<sup>+</sup> cells. C) Concentration of TNF- $\alpha$  (pg/ml) in supernatant of WB. Different letters on columns at same time point of treatment depict statistical

significance ( $p < 0.05$ ). The data are presented as the mean  $\pm$  S.E.M. Experiments were done two times and data from single experiment is shown. NS= non-stimulated; ND=non-diluted; PMA= phorbol myristate acetate; ION= Ionomycin. \* $P < 0.05$ ; ns = non-significant. Statistical analysis was done by two-way ANNOVA.



**Figure 3**

**Comparison of tube method (TM) and plate method (PM) for stimulation:** Whole blood was diluted 1/8 in 96 well plate (PM) or microcentrifuge tubes (TM) by PMA/ion for 24 hours A) S.D of IFN- $\gamma^+$  cells in CD3 $^+$  cells B) S.D of TNF- $\alpha$  (pg/ml) in supernatant of WB. The data are presented as the mean  $\pm$  S.D of 4 individual porcine WB samples.

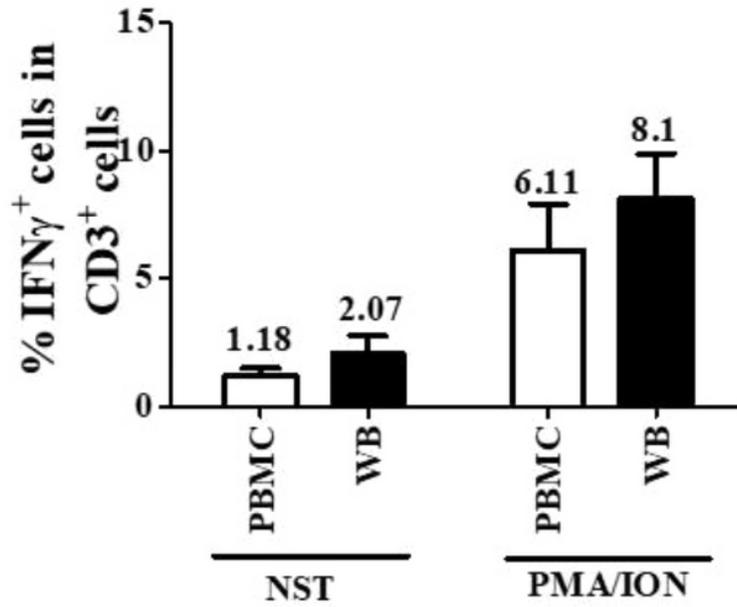


Figure 4

**Comparison of WB and PBMCs stimulation:** WB was stimulated by PM or 106 PBMCs were stimulated by PMA/ION for 24 hours. Mean of % IFN- $\gamma$ <sup>+</sup> cells in CD3<sup>+</sup> cells. The data are presented as the mean  $\pm$  S.E.M of 4 individual porcine WB samples.

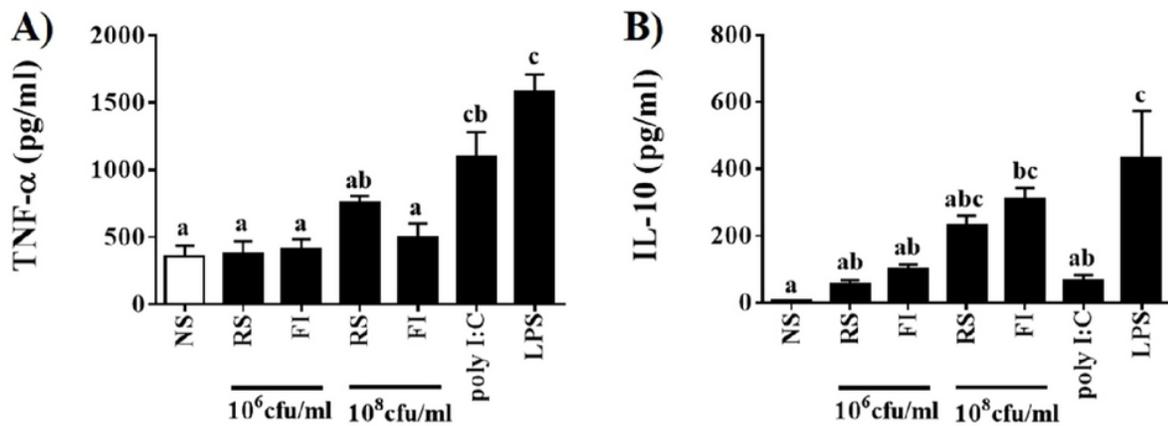
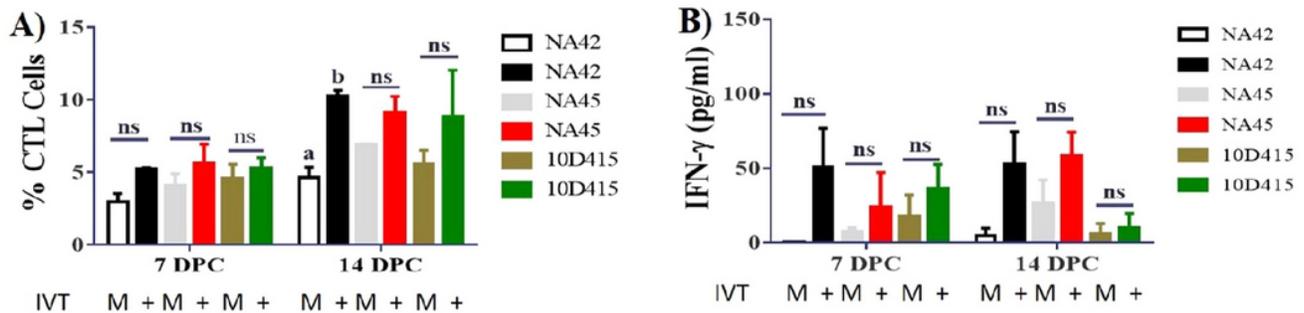


Figure 5

**Cytokine upregulation in porcine WB on stimulation with heat killed bacteria, TLR4, and TLR3 ligands:** Heparinized whole blood was diluted 1/8 in 96 well plate and stimulated for 24 hours by RS, FI, poly I:C, LPS for 24 hours. A) TNF- $\alpha$  (pg/ml) in supernatant of WB. B) IL-10 (pg/ml) in supernatant of WB. The data are presented as the mean  $\pm$  S.E.M of four individual porcine WB samples. Different letters on columns at same time point of treatment depict statistical significance ( $p < 0.05$ ). NS= non-stimulated; RS= reference strain (KCTC3557) of *S. suis*; FI=field isolate of *S. suis*; poly I:C=polyinosinic:polycytidylic acid; LPS= lipopolysaccharide. \* $P < 0.05$ ; ns = non-significant. Statistical analysis was done by one-way ANOVA.



**Figure 6**

**Re-stimulation response after PRRSV challenge:** Frequency of CTLs at 7 DPC and 14 DPC in pigs challenged with PRRSV ( $n = 3$  each group). Heparinized whole blood was diluted 1/8 in 96 well plate and stimulated for 24 hours by supernatant of freeze thawed PAM cells (mock), NA42 (104.5 TCID<sub>50</sub>/ml), NA45 (104.5 TCID<sub>50</sub>/ml) or 10 D415 (104.5 TCID<sub>50</sub>/ml) at 7 and 14 DPC. A) Frequency of CTLs in WB after re-stimulation B) Concentration of IFN- $\gamma$  in supernatant of re-stimulated WB. The data are presented as the mean  $\pm$  S.E.M. Statistical analysis was done by two-way ANNOVA. Different letters on columns at same time point of treatment depict statistical significance ( $p < 0.05$ ). Where ns means non-significant difference. IVT stands for in-vitro treatment: M= mock stimulated, + represents stimulation with homologous strain.

## Supplementary Files

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