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A standardized method to study immune responses using porcine whole blood

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ABSTRACT

Background: 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.
Objectives: Herein, this study aimed to develop a porcine WBA for *in vitro* evaluation of immune responses.
Methods: 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. After 24 h, cells were stained for interferon (IFN)-γ secreting T-cells followed by flow cytometry, and the supernatant was analyzed for tumor necrosis factor (TNF)-α. In addition, diluted WB was stimulated by lipopolysaccharide (LPS) and polyinosinic:polycytidylic acid (poly I:C), reference strain KCTC3557 (RS), field isolate (FI), of heat-killed (HK) *Streptococcus suis*, and porcine reproductive and respiratory syndrome virus (PRRSV).
Results: The frequency of IFN-γ$^+$CD3$^+$ T-cells 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 interleukin (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 IFN-γ$^+$CD4$^-$CD8$^+$ cells, and IFN-γ was non-significantly induced in the supernatant of re-stimulated samples.
Conclusions: 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.

Keywords: Porcine whole blood assay; in vitro test; PRRSV; cytotoxic T lymphocytes; flow cytometry; ELISA

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Conflict of Interest
The authors declare no conflicts of interest.

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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 [1,2]. Similarly, porcine PBMCs have been used to evaluate immune responses in various studies, including pathogen challenge and vaccination [3,4], 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 [5,6]. The use of WB is an attractive strategy because it is simple, fast, and requires less blood volume. In addition, WB may retain 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 may occur within and across laboratories. The whole blood assay (WBA) is low-cost and well suited to measure monocyte cytokine production [7]. Further, purified cells provide low reproducibility when compared with WB [8].

The idea of using WB was first introduced by Eskola et al. [9] in 1975 to evaluate phytohemagglutinin and ConA-induced lymphocyte proliferation. Later, Digel et al. [10] independently used WB to study the cytokines induced by a wide variety of stimulants. Additionally, WB cultures have been used over the last three decades to assess various aspects of immune responses [11]. 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-γ after *in vitro* stimulation with *Mycobacteria tuberculosis* antigens [12]. Recently, Duffy et al. [11] 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. By contrast, porcine WB is used less frequently to assess immune response because it lacks a standardized method. Yancy et al. [13] showed that in WB, RNA expression was comparable or higher than PBMC culture systems. However, Segura et al. [14] observed very little or no difference at protein level. In addition, most previous studies examining porcine reproductive and respiratory syndrome virus (PRRSV) induced immune responses have been tested *in vitro* using PBMCs [4,15,16], but not in WB. Moreover, the suite of whole-blood syringe-based assays developed by Duffy et al. [11] 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 lesser volume of WB, and II) evaluation of the immune responses in WB following stimulation with TLR ligands, bacteria, and viruses.

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MATERIALS AND METHODS

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 µg/mL streptomycin, and 0.25 µg/mL Fungizone®.

Optimization of the standard method for WB stimulation

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

TM

In TM, 100 µL 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 (phorbol myristate acetate [PMA], 40.5 nM and ionomycin [ION], 670 nM) PMA/ION (cell stimulation cock tail; Life Technologies Corp., USA) or unstimulated for 24 h at 37 °C in a 5% CO_2 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× each; PMA, 81 nM and ION, 1340 nM) were added to all samples. Following incubation, the tubes were centrifuged at 1,200 rpm for 6 min, and the supernatant was collected and stored at −80°C until further analysis. The cell pellets were stained for flow cytometric analysis.

PM

In PM 30 µL non-diluted and 1/8 diluted WB (final volume, 270 µL) 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× each) were added to all samples. Samples were centrifuged at 1,200 rpm for 6 min, 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 µL of WB were diluted (1/8), respectively, and stimulated as stated in sections 2.2.1 and 2.2.2.

Comparison of WB and PBMCs

Thirty µl of WB was stimulated by PM as described in section 2.2.2. Alternatively, 10^6 PBMCs were isolated as per previous report [16] and were stimulated with pre-added 0.5X PMA/ION or not stimulated in 100 µL of complete RPMI (RPMI-1640 with 10% FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL Fungizone®). For the last 4 h of incubation, a cocktail of PMA/ION and BFA (1X each) were added to all samples.

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.

Bacterial strains and growth conditions

Two strains of *S. suis, RS (reference strain, KCTC3557) and FI (field isolate)* were used in this study.

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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, USA), were incubated for 8 h at 37°C. Next, 1:1,000 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^{10} CFU/mL in PBS. Finally, the bacteria were heat-killed at 100°C for 5 min in a water bath. HK bacteria were further diluted to working concentrations of 10^8 and 10^6 CFU/mL in RPMI.

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_2 humidified chamber for 24 h. After stimulation, WB supernatant was collected and stored at −80°C until further analysis.

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 frequency of interferon (IFN)-$\gamma^+CD4^-CD8^+$ cells in PRRSV infected pigs.

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 Corp.) supplemented with heat-inactivated 10% fetal bovine serum (Life Technologies Corp.), 2 mM L-glutamine and antibiotic-antimycotic solution at 37°C in a 5% CO_2 humidified chamber. Viral strains were further concentrated using a Vivaspin 20 centrifugal concentrator (MWCO 300 kDa; Merck, USA), according to the manufacturer's instructions.

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[INSERT FIGURE 001] × [INSERT FIGURE 002]10^3 $TCID_{50}$/mL) separately in different groups. For re-stimulation, a titer of $1 \times 10^{4.5}$ $TCID_{50}$/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 after intramuscular injection of azaperone (40 mg/mL, StressGuard®).

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 mock (supernatant of freeze thawed cells, MARC-145 and PAM for NA42, NA45, and 10D415 challenged pigs, respectively), treated for 24 h by NA42 ($10^{4.5}$ $TCID_{50}$/mL), NA45 ($10^{4.5}$ $TCID_{50}$/mL), or 10D415 ($10^{4.5}$ $TCID_{50}$/mL) for 24 h at 37ºC in a 5% CO_2 humidified chamber. For last 4 h of incubation PMA/ION and BFA (1× each) were added to all samples.

Flow cytometry

After stimulation or re-stimulation, samples were transferred to 96-well U bottom plate and were pelleted by centrifugation (1,200 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. Two different strategies of antibody combinations were used in the current study:

First, 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, Thermo Fisher Scientific, USA) 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 min 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™5.5 (BD-Pharmingen) in dark for 30 min on ice.

Second, while standardizing the dilution of WB for optimal stimulation or comparison with PBMCs, WB or PBMCs samples were stained with CD3-FITC. After intracellular fixation and RBC lysis, cells were stained with IFN-γ-PerCP-cy™5.5.3) In recall response experiment, WB from each animal was stained with CD4-PE. Further, cells were stained by CD8-FITC (BD-Pharmingen). After intracellular fixation and RBC lysis, cells were stained with IFN-γ-PerCP-cy™5.5. Further details of the antibodies are provided in **Supplementary Table 1**.

Flow cytometry was performed using an Accuri C6 flow cytometer (BD Accuri™ C6 Plus, BD Biosciences). Phenotype data were collected for 100,000 events followed by a gating strategy using FLOWJO version 10.6.1 (LLC, USA) (see **Supplementary Fig. 1**).

Enzyme-linked immunosorbent assay (ELISA)

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

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., USA). The Mann-Whitney *U* test, or one-way or two-way ANOVA were used to compare groups. Differences were considered significant at $p < 0.05$.

Ethics approval

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

RESULTS

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**,

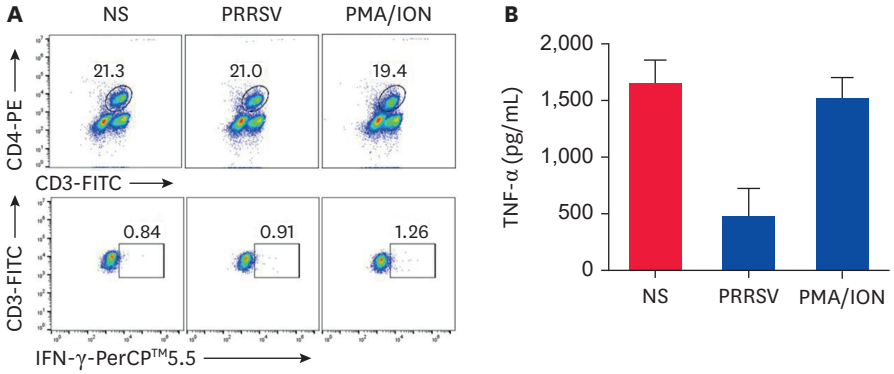

Fig. 1. Undiluted WB is refractory to PRRSV or PMA/ION stimulation: Heparinized whole blood (100 μL) was stimulated for 24 h by PRRSV or PMA/ION (n = 33). (A) Representative flow cytometry scatter dot plots, cell surface immunofluorescent staining showed consistent results (upper panel), intracellular immunofluorescent staining (IFN-γ) was obstinate (bottom panel). (B) TNF-α (pg/mL) in supernatant of WB.
NS, non-stimulated; PRRSV, porcine reproductive and respiratory syndrome virus; PMA, phorbol myristate acetate; ION, ionomycin; IFN, interferon; TNF, tumor necrosis factor; WB, whole blood.

undiluted WB samples stained well for cell surface molecules, including CD3 and CD4; however, the frequencies of IFN-γ producing cells did not differ between the unstimulated and stimulated samples. Moreover, a high TNF-α 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, undiluted WB seemed refractory to PMA/ION treatment for a 24-h culture.

WB dilution is necessary to induce cell activation

Previous studies using human WB have suggested that dilution is helpful to minimize interference by RBC [13] and other unknown factors [18]. Therefore, we diluted porcine WB up to 1/16 and evaluated IFN-γ producing T cells and TNF-α secretion by flow cytometry and ELISA, respectively. The frequencies of IFN-γ^+ 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 ($p = 0.03$) and increased up 8% (**Fig. 2B**). Similarly, the concentration of TNF-α in the supernatant increased with higher dilution factors. At 1/2, 1/8 and 1/16, the concentration of TNF-α in the supernatant of WB was 1712 ± 733.15, 4,251 ± 2,588.54, and 7,811.45 ± 1,847.16 pg/mL (mean ± 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.

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 μL 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-γ^+ cells in $CD3^+$ cells were perceived as 12.58 ± 4.08 and 14.47 ± 1.04 (mean ± standard deviation) in the TM and PM (**Fig. 3A**), respectively. Further, TNF-α 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.

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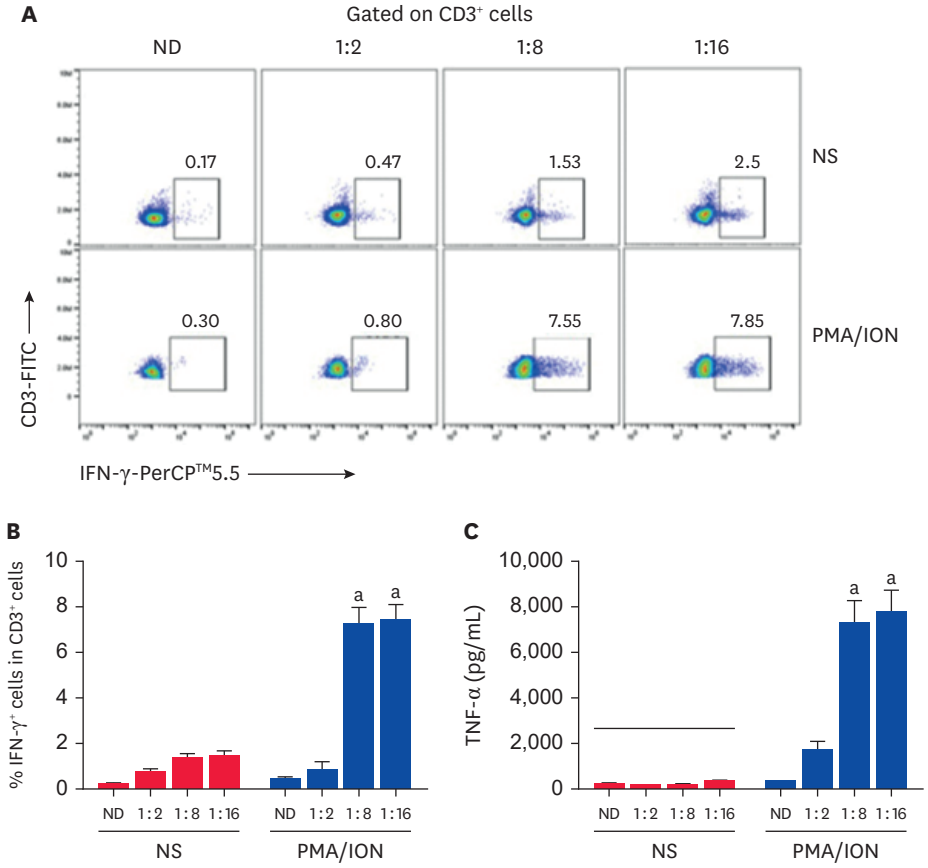

Fig. 2. Optimal stimulation of WB is obtained at 1/8 dilution: Heparinized whole blood (100 μL) was diluted (ND, 1/2, 1/8, 1/16 and stimulated by tube method for 24 h by PMA/ION (n = 4). (A) Representative flow cytometry scatter dot plots for IFN- γ+ cells in CD3+ (upper panel = non-stimulated, lower panel = PMA/ION stimulated) (B) Frequency of CD3+ IFN-γ+ cells in CD3+cells. (C) Concentration of TNF-α (pg/mL) in supernatant of WB. The data are presented as the mean ± SEM. Experiments were done two times and data from single experiment is shown. Statistical analysis was done by Mann-Whitney test.
ND, no dilution; NS, non-stimulated; PMA, phorbol myristate acetate; ION, ionomycin; IFN, interferon; TNF, tumor necrosis factor; WB, whole blood.
[a]$p < 0.001$.

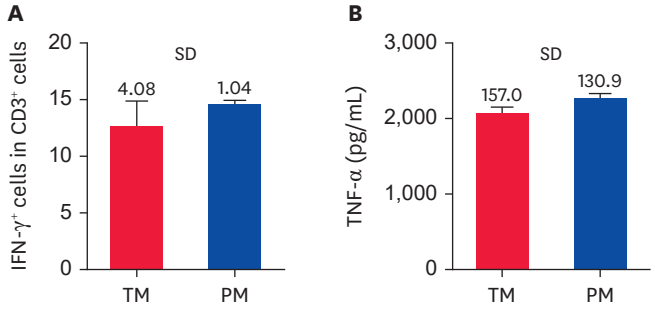

Fig. 3. Comparison of TM and PM for stimulation: WB was diluted 1/8 in 96 well plate (PM) or microcentrifuge tubes (TM) by PMA/ION for 24 h. (A) SD of IFN-γ+ cells in CD3+ cells. (B) SD of TNF-α (pg/mL) in supernatant of WB. The data are presented as the mean ± SD of 4 individual porcine WB samples.
IFN, interferon; TM, tube method; PM, plate method; TNF, tumor necrosis factor; WB, whole blood; PMA, phorbol myristate acetate; ION, ionomycin.

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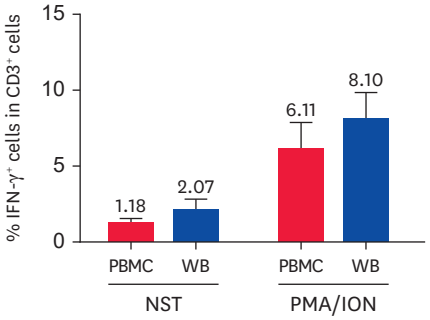

Fig. 4. Comparison of WB and PBMCs stimulation: WB or PBMCs were stimulated by PMA/ION for 24 h. Mean of % IFN-γ$^+$ cells in CD3$^+$ cells The data are presented as the mean ± SEM of 4 individual porcine WB samples.
IFN, interferon; WB, whole blood; PBMC, peripheral blood mononuclear cell; PMA, phorbol myristate acetate; ION, ionomycin.

Comparison of WB and PBMCs stimulation

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

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-α was detected in LPS-stimulated samples, followed by poly I:C and RS (10^8 CFU/mL): 1,584 ± 124.1, 1,103 ± 177.7, 762.7 ± 42.72 pg/mL, respectively (**Fig. 5A**). Both FI and RS were unable to induce TNF-α 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 ± 138.3, 309.5 ± 33.91, 230.6 ± 29.61, 100.2 ± 14.30, and 57.11 ± 10.16 pg/mL, respectively; **Fig. 5B**). FI induced IL-10 but there was no significant effect on the induction of TNF-α in porcine WB (**Fig. 5A and B**).

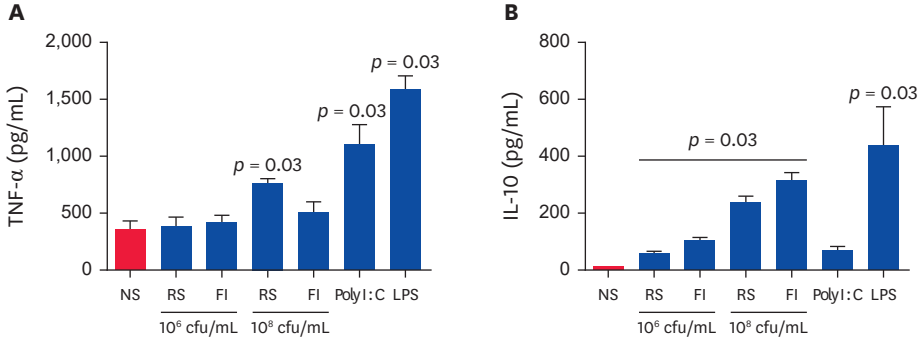

Fig. 5. Cytokine upregulation in porcine WB on stimulation with heat killed bacteria, TLR4, and TLR3 ligands: Heparinized WB was diluted 1/8 in 96 well plate and stimulated for 24 h by RS, FI, poly I:C, LPS for 24 h. (A) TNF-α (pg/mL) in supernatant of WB. (B) IL-10 (pg/mL) in supernatant of WB. The data are presented as the mean ± SEM of four individual porcine WB samples.
TNF, tumor necrosis factor; NS, non-stimulated; RS, reference strain (KCTC3557) of *S. suis*; FI, field isolate of *S. suis*; poly I:C, polyinosinic:polycytidylic acid; LPS, lipopolysaccharide; IL, interleukin.
Statistical analysis was done by Mann-Whitney test and *p* value is included in graphs

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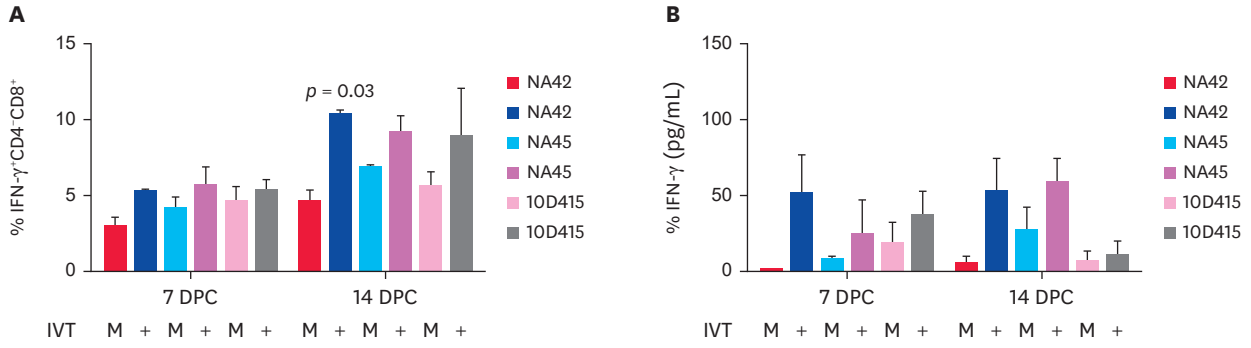

Fig. 6. Re-stimulation response after PRRSV challenge: Frequency of IFN-γ$^+$CD4$^-$CD8$^+$ at 7 DPC and 14 DPC in pigs challenged with PRRSV (n = 3 each group). Heparinized whole blood was diluted 1/8 in 96 well plate and stimulated for 24 hours by supernatant of freeze thawed PAM cells (mock), NA42 (104.5 TCID50/mL), NA45 (104.5 TCID50/mL) or 10 D415 (104.5 TCID50/mL) at 7 and 14 DPC. (A) Frequency of IFN-γ$^+$CD4$^-$CD8$^+$ in WB after re-stimulation. (B) Concentration of IFN-γ in supernatant of re-stimulated WB. The data are presented as the mean ± SEM. Statistical analysis was done by Mann-Whitney test. IVT stands for *in-vitro* treatment. IFN, interferon; PRRSV, porcine reproductive and respiratory syndrome virus; M, mock stimulated, + represents stimulation with homologous strain.

Re-stimulation response after PRRSV challenge

IFN-γ$^+$CD4$^-$CD8$^+$ cell response in PRRSV infected pigs at 7 and 14 DPC on re-stimulation with respective strains, *in vitro*.

The frequency of IFN-γ$^+$CD4$^-$CD8$^+$ cells was measured at 7 and 14 DPC after re-stimulating 1/8 diluted WB with respective homologous PRRSV strains for 24 h (**Fig. 6A**). IFN-γ$^+$CD4$^-$CD8$^+$ response was significantly induced at 14 DPC in NA42 challenged pigs. On the other hand, there was non-significant upregulation of IFN-γ in the supernatant of re-stimulated samples treated with homologous strains when compared with mock stimulation *in vitro* (**Fig. 6B**).

DISCUSSION

WB and PBMCs both can be used for evaluating immune responses. However, both the systems have advantages and disadvantages. For example, PBMCs can be stored at −80°C and stimulated and examined later. Furthermore, PBMCs saved from several study time points can be analyzed simultaneously and under similar conditions. In contrast, WB has RBCs which shall be lysed before storage. Therefore, depending on the need of hour, after stimulation with various stimulants RBCs can be lysed in WB and white blood cells can be and stored at −80°C for subsequent analysis. 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 [7]. Often, PBMCs require the addition of fetal calf serum, which contains various nutrients and hormones that may alter the immunological responsiveness of the cells [19]. Furthermore, cells in isolated PBMCs undergo a significantly high level of apoptosis when compared with WB cultures [8], and stained cell populations separate in WB with better clarity [20]. 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-α and IFN-γ [21]. 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

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due to the poor stability of undiluted WB when incubated for a longer time. Further, there are certain unknown factors in WB [18] 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-α ELISA. We observed better stimulation of IFN-γ secreting T-cells in 1/8 and 1/16 diluted WB. In contrast to IFN-γ secreting $CD3^+$ T-cells, the TNF-α response started to increase from 1/2 dilution of WB, which might be due to the fact that TNF-α is one of the most abundant cytokines [22] and is produced by a variety of cells, including monocytes [23], activated lymphocytes [24] and granulocytes [25]. Our results are consistent with previous studies; 1:5 or 1:10 diluted WB is optimal to obtain measurable and accurate IFN-γ response [6] and cell-mediated immune responses [5,26,27] 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 μL WB + 800 μL RPMI = 900 μL); therefore, we sought to reduce the volume of WB for stimulation. To test our hypothesis, we diluted (1/8) 30 μL and 100 μL 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-γ producing $CD3^+$ cells and TNF-α response. This is consistent with previous reports of reduced intra-subject precision values using the TM when compared with PM [28]. 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.

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-α [21,29,30] and anti-inflammatory cytokines, including IL-10 [30]. Stimulating WB with LPS produces a higher concentration of cytokines when compared with PBMCs [31]. WB contains the LPS binding protein, 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 [32,33]. Therefore, WB may provide a more natural environment for measuring the inflammatory response. Moreover, poly I:C induces the synthesis of pro-inflammatory cytokines, including TNF-α [11,34,35], and anti-inflammatory cytokines, including IL-10 [34], in human PBMCs or WB. Porcine PBMCs stimulated with poly I:C elicit differential gene expression, similar to those induced by RNA viruses [36]. Our study indicates that both LPS and poly I:C induced immune cells in porcine WB to produce TNF-α and IL-10.

S. suis stimulates the release of pro-inflammatory cytokines in mice [37]. Stimulation of 1:3 diluted porcine WB with HK *S. suis* increases cytokine mRNA expression; however, a low level of protein secretion is displayed [14]. The present study provides evidence that HK *S. suis* stimulates WB to secrete pro- and anti-inflammatory cytokines (TNF-α and IL-10, respectively). In this study, HK RS induced TNF-α (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-α concentration.

Next, we extended our assay to analyze the induction of IFN-γ$^+$CD4$^-$CD8$^+$ cells by PRRSV strains: NA42, NA45, and 10D415. We analyzed the response at 7 and 14 DPC. The frequency of IFN-γ$^+$CD4$^-$CD8$^+$ cells was higher in samples re-stimulated with homologous strains when compared with mock treated samples. The PRRSV strain, NA42, significantly induced a higher frequency of IFN-γ$^+$CD4$^-$CD8$^+$ cells at 14 DPC, while there was no significant response upon re-stimulation with NA45 and 10D415. This might be due to strain differences; pigs respond differently to different PRRSV strains [4]. The cellular immune response depends on the viral isolate used *in vitro* stimulation, which can interact differently with different immune cells [38,39]. More importantly, the role of IFN-γ$^+$CD4$^-$CD8$^+$ 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 [40]. 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 can serve 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 IFN-γ$^+$CD4$^-$CD8$^+$ cells at 14 DPC. Taken together, we have shown that this assay provides reproducible results and can be used to study immune responses in swine.

SUPPLEMENTARY MATERIALS

Supplementary Table 1

List of antibodies used in the staining for flow cytometry analysis

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Supplementary Fig. 1

(A) Gating strategy of IFN-γ$^+$ cells in CD3$^+$. Representative flow cytometry scatter dot plots for gating of IFN-γ$^+$ cells in CD3$^+$ (related to **Fig. 2**). (B) Gating strategy for IFN-γ$^+$ CD4$^-$CD8$^+$ (related to **Fig. 6**).

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