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Lipopolysaccharide Stimulation of Peripheral Blood Mononuclear Cells of Indigenous Pigs Causes Enhanced Expression of Pro-inflammatory Cytokines Over Exotic Pigs

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ABSTRACT

Background: Selective breeding in pigs based on a higher potential of disease resistance can be achieved by studying the association between immune responsiveness and susceptibility to infectious diseases. The early immune responsiveness of pigs assessed in a quantifiable scale for different determinant cytokine genes' expression involved in regulation of immune response can be a major determinant for evaluating the immune responsiveness.

Methods: In this study, we carried out the quantitative relative expression of candidate cytokine genes [Interleukin-1 beta-(IL1β), IL6, IL10 and tumor necrosis factor-alpha (TNFα) and toll-like receptor-4 (TLR4) gene expression in the lipopolysaccharide (LPS) stimulated peripheral blood mononuclear cells (PBMCs) of Zovawk (indigenous) and Large White Yorkshire (LWY-exotic) pig breeds using real-time PCR to understand the differences in swift immune responsiveness against gram-negative bacteria.

Results: Zovawk PBMCs' pro-inflammatory cytokines IL1β and IL6 expression increased rapidly with LPS stimulation up to 12 h, being significantly higher as early as 4 h compared to that of the LWY PBMCs. In Zovawk pigs, the pro-inflammatory cytokine genes' expression was found to be quick and high; whereas, in LWY pigs the response was slow and low. Interestingly, the expression of IL10 was not detected in LPS-stimulated PBMCs of Zovawk pigs indicating that the remarkably higher expressions of IL6 and IL1β in response to LPS are within the threshold of the Zovawk pigs which have evolved through generations. Our findings clearly indicate the sturdy and early immune responsiveness of indigenous pigs against gram-negative bacteria over the exotic pigs.

Key words: Cytokine, Gram-negative bacteria, Immune competence, Lipopolysaccharide, Porcine, Real-time PCR.

INTRODUCTION

Various infectious diseases caused by viral and bacterial pathogens adversely affect swine production and well-being which substantially limit production efficiency and cause huge economic losses to the farmers. Therefore, reducing the susceptibility to different infectious diseases in swine has become the top priority. Over the last decade, the focus of many researchers has been to produce more disease-resistant animals due to limitations in disease control strategies. To succeed in this direction, the genes and gene networks that determine the immune response in pigs that in turn are associated with disease-resistance phenotype need to be established (Bishop and Woolliams, 2014). Some non-genetic factors like environment, behavior, and management can also affect immune competence in pigs by influencing their genetic components. Hence, it is always important to evaluate the immune status of different pig breeds, which have evolved through many generations in specific regions, for effective swine breeding programs to attain superior immune competence apart from improving production traits.

Generally, the immune response in pigs is measured, either *in vivo* or *in vitro*, by determining the proliferative response of peripheral blood mononuclear cells (PBMCs) to mitogenic stimulation. PBMCs are key immune cells with a mixed population of T-lymphocytes, B-lymphocytes,

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natural killer cells, monocytes, macrophages, and dendritic cells. These cells have the ability to recognize different pathogen-associated molecular patterns (PAMPs) that are typical of microorganisms followed by the genetic expression of various cytokines leading to innate and adaptive immune and inflammatory responses. Lipopolysaccharide (LPS), also termed endotoxin, is a prominent feature of gram-negative bacteria and is responsible for the inflammatory response observed during endotoxic shock (Akira *et al*., 2006). LPS stimulates host cells by binding to toll-like receptor 4 (TLR4) and results in the expression and release of various pro-inflammatory cytokines. Therefore, the analysis of cytokine profiles and their immune regulation in pigs is also essential for a better understanding of the functional properties of immune cells like PBMCs in physiological and pathological conditions and will help in designing therapeutic and prophylactic interventions against infectious diseases. Moreover, the early responsiveness of adaptive immune mechanisms that can be assessed by the quantity of different signature cytokine genes expressed for regulation of immune response in a time scale is also a major determinant for evaluating the immune competence of a breed of animals. This knowledge will help develop pure lines of the breed with better disease resistance for the conservation of the germplasm and also can be used in crossbreeding for maintaining the optimal inheritance level for disease resistance. Although cytokine gene expression studies on PBMCs from some exotic breeds have been reported (Reddy and Wilkie, 2000; Duvigneau *et al*., 2007), the scientific reports in this regard in indigenous pig breeds in India are scanty. In the present study, the comparative dynamics of different immune regulating key cytokine genes' expression in the LPS-stimulated PBMCs of indigenous (Zovawk) and exotic (Large White Yorkshire-LWY) pig breeds was analyzed to characterize the immune responsiveness of these two breeds against gram-negative bacteria.

MATERIALS AND METHODS

Animals, blood collection and PBMCs separation

Six Zovawk and six LWY apparently healthy pigs of 6 to 10 weeks of age were selected from the Livestock Farm Complex and AICRP farm of the College of Veterinary Sciences and Animal Husbandry, Aizawl, Mizoram. Blood samples (5 ml) were collected in Lithium Heparin coated Vacutainer® tubes (AcCuvet-premium) by puncturing the vena cava and transported immediately to the laboratory. All the animal experiments were approved by the Institute Animals Ethics Committee of the college. From the whole blood, PBMCs were separated using Histopaque® 1077 medium (Sigma-Aldrich, USA). Cells were counted using a hemocytometer and viability was determined by the trypan blue exclusion method.

Culturing of PBMCs and LPS treatment

The PBMCs separated from the whole blood of Zovawk and LWY pigs were resuspended at a concentration of 1×10^6 live cells/ml in Roswell Park Memorial Institute (RPMI)-1640 culture medium containing 10% fetal bovine serum, 1% antibiotic-antimycotic solution and L-glutamine (2 mM). The lymphocyte proliferation was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoltetrazolium bromide (MTT) assay as described by Kirthika *et al.* (2017) using LPS (From *Salmonella enterica* serotype typhimurium; Sigma-Aldrich, USA), which is a gram-negative bacteria specific PAMP. The lymphocyte proliferation assay was conducted at different concentrations of LPS *i.e.* 0, 5, 10 and 20 µg/ml to determine the optimum concentration of LPS at which the proliferation indices are maximum. Briefly, PBMC suspensions were pipetted in triplicate (100 µl) into 96-well U-bottom cell culture plates followed by the LPS at different concentrations. The plates were incubated for 48 h (37°C, 95% air: 5% CO_2) after which 10 µl of MTT reagent (5 mg/ml; Sigma-Aldrich, USA) was added. After an additional 4 h of incubation, 100 µl of SDS-solubilisation buffer was added. The next day, the optical density (O.D.) at 570 nm and 690 nm (background) was measured using a microplate reader. Proliferation data were expressed as a proliferation index.

$$(PI) = \frac{\text{(O.D. in the presence of mitogen)}}{\text{(O.D. in the absence of mitogen)}}$$

Time course studies of cytokine gene expression by real time quantitative PCR

Time course experiments were carried out to define and compare the kinetics of TLR4 and cytokine genes (TNFα, IL1α, IL6 and IL10) expression in the PBMCs of Zovawk and LWY pigs following *in vitro* stimulation at optimum LPS concentration based on lymphocyte proliferation index. For this, aliquots of PBMCs were cultured along with LPS for 4, 12 and 24 h (0 h served as the control without LPS) in the same way as for the proliferation assay. Cultures were centrifuged after various stimulation times and cells were collected for RNA isolation. Total RNA was extracted from aliquots of PBMCs using a GeneJET RNA purification kit (Thermo Fisher Scientific). Total RNA from each group was reverse transcribed into cDNA using QuantiTect reverse transcription kit (Qiagen). The relative expression of the target genes was quantified and compared at each time point *i.e.* 0 (unstimulated), 4, 12 and 24 h after LPS-stimulation by Rotor Gene-Q real-time PCR instrument (Qiagen). Beta-2 microglobulin (B2M) was used as a housekeeping gene and each sample was tested in triplicate. The list of primers used for qPCR is mentioned in Table 1. The reaction mixture (10 µl total volume) comprised 2 µl cDNA, 5 µl 2× SYBR green master mix (Thermo Fisher Scientific), 0.5 µM primer pairs and 2 µl PCR-grade water. The thermocycling conditions employed for all the genes were: preincubation at 95°C for 5 min

followed by 40 cycles of 20 s at 95°C, 20 s at 60°C and 20 s at 72°C. The cycle threshold (Ct) values were recorded and used for the calculation of mean fold change. Relative quantification of a target gene was done by comparing the expression level of reference gene beta-2 microglobulin using the 2-ΔΔCT method (Livak and Schmittgen, 2001).

Statistical analysis

Results were expressed as the means±SEM. Data were analyzed by ANOVA and statistical differences between various treatment group means were determined by DMRT using the SPSS software (v17; SPSS Inc., USA).

RESULTS AND DISCUSSION

Proliferation index of PBMCs

Culturing of PBMCs with LPS followed by incubation with MTT, formazan crystals formation was observed in MTT assay (Fig 1a and 1b). The proliferative response in LPS-stimulated PBMCs of Zovawk represented a mean value of 1.31±0.02, 1.327±0.03 and 1.239±0.022 at concentrations of 5, 10 and 20 µg/ml respectively, whereas, the PBMCs of LWY represented mean values of 1.089±0.02, 1.133±0.019 and 1.059±0.026. No significant difference ($p>0.05$) in proliferation was observed between different doses of LPS stimulation in LWY, whereas, a significant difference ($p<0.05$) was observed between 20 µg/ml LPS and 5 and 10 µg/ml LPS in Zovawk pigs. As the proliferation index was found to be higher at 10 µg/ml LPS in both the breeds, the kinetics of cytokine genes' expression in PBMCs was studied at the same optimal LPS concentration *i.e.* 10 µg/ml.

Time course expression of cytokine transcripts:

The relative expression of cytokine transcripts is reported in terms of fold change at different time intervals compared to unstimulated PBMCs (Table 2). The expression of beta-2 microglobulin did not vary significantly ($p>0.05$) in PBMCs between different time periods of LPS stimulation either in Zovawk or LWY pigs, therefore, it was aptly served as the reference gene in our real time PCR assay. Time course expression studies revealed that the expression of TLR4, host cell receptor for LPS, increased continuously up to 12 h in the PBMCs of both the pig breeds, although slightly higher in Zovawk and was maintained up to 24 h (Table 2). Its expression was found to be significantly different ($p<0.05$) between unstimulated and stimulated PBMCs of all time periods in both breeds (Table 2). The TNFα expression in Zovawk PBMCs increased up to 4 h of LPS stimulation and then slightly declined at later time intervals (Table 2). On the other hand, TNFα expression in LWY was significantly decreased ($p<0.05$) at all-time intervals compared to unstimulated PBMCs. In Zovawk PBMCs, IL1α expression was increased rapidly with LPS stimulation up to 12 h and maintained up to 24 h (Table 2; ~93-fold increase over unstimulated PBMCs). However, IL1α expression in LWY PBMCs steadily increased from 0 to 24 h and was found to be significantly different ($p<0.05$) between all the time intervals. The IL6 expression in Zovawk PBMCs was increased very rapidly with LPS stimulation and peaked at 12 h (Table 2; ~163-fold increase over unstimulated PBMCs) and then decreased by 24 h which resulted in a bell-shaped expression profile. On the other hand, IL6 gene expression in LWY PBMCs gradually increased from 0 to 24 h and was found to be significantly different ($p<0.05$) between all the time intervals. The relative expression of IL10 in PBMCs of LWY significantly increased ($p<0.05$) up to 12 h and its expression was maintained up to 24 h. Interestingly, IL10 expression was not observed in LPS-stimulated Zovawk PBMCs.

LPS is an immensely studied immunostimulatory component that can induce innate immune and systemic inflammatory responses. It consists of a polysaccharide component and the lipid A moiety. The O-chain of polysaccharide component is principally responsible for antigenicity, whereas the lipid A moiety activates innate immune cells. LPS mimics bacterial infection by stimulating mammalian cells through interactions with PRRs like TLR4, LPS binding protein, CD14 and MD-2 (Lu *et al*., 2008).

Table 1: Primer sequences and amplicon size of each gene target analyzed in peripheral blood mononuclear cells of pigs by quantitative real time PCR.

Gene	Primer	Sequence ($5^{\circ}_{d} \rightarrow 3^{\circ}_{d}$)	Amplicon size
TLR4	Forward	TGCTTTCTCCGGGTCACTTC	144
	Reverse	TTAGGAACCACCTGCACGC	
TNFα	Forward	GGCCCAAGGACTCAGATCAT	82
	Reverse	CTGTCCCTCGGCTTTGACAT	
IL1α	Forward	ATTCAGGGACCCTACCCTCTC	164
	Reverse	GTGCAGCACTTCATCTCTTTGG	
IL6	Forward	GGCAAAAGGGAAAGAATCCAGAC	197
	Reverse	CATCAATCTCAGGTGCCCCA	
IL10	Forward	CGTAATGCCGAAGGCAGAGA	115
	Reverse	GGGCAGAAATTGATGACAGCG	
B2M	Forward	CAACCACTTTTCACACCGCTC	187
	Reverse	GGCGGATGGAACCCAGATAC	

In the present study, LPS was chosen as a mitogen as it has been a widely accepted stimulant for *in vitro* models to measure the expression of cytokines by PBMCs in different species (Poindexter *et al*., 2005). LPS induces the proliferation of immune cells of PBMCs *viz*. B lymphocytes which in turn results in the production of cytokines and other immune regulators to mediate inflammatory and immune responses (Venkataraman *et al*., 1999; Li *et al*., 2021; Lin *et al*., 2021). Keeping this in view, in this study, we observed LPS-induced proliferation of PBMCs in both breeds wherein, the proliferative response of Zovawk PBMCs was found to be higher compared to LWY PBMCs which signifies the better B-lymphocyte immune responsiveness in the former breed. The time points (0, 4, 12, and 24 h) for the study of cytokine expression was selected considering the different phases of the transcriptional program governing lymphocyte proliferation. Further, it has already been reported that the time points before 24 h are very important to interpret the onset of the response to stimulus as observed in different species (Ledger *et al*., 2004). Therefore, choosing time points up to 24 h in our study enabled us to observe the onset as well as the optimum expression level of the selected cytokines in response to the LPS stimuli.

LPS is recognized by a pattern recognition receptor, TLR4, present on the surface of phagocytic cells such as macrophages, dendritic cells, and neutrophils (Nijland *et al*., 2014). The recognition of LPS by TLR4 induces an inflammatory response through a signaling cascade which leads to the production of pro-inflammatory cytokines that include IL1α, TNFα, IL6, *etc*. (Rossol *et al*., 2012; Nijland *et al*., 2014). Thus, higher expression of these pro-inflammatory cytokines has been observed in both the pig breeds in our study. However, balanced production of these inflammatory mediators is required for adequate immune function as well as to avoid fatal consequences such as septic shock (Cohen, 2002). This may be the probable reason behind the observed down-regulation of TNFα gene expression in LWY PBMCs on LPS stimulation in our study (Lee *et al*., 2021). Further, IL10 expression was not observed in LPS-stimulated PBMCs of Zovawk pigs indicating that the remarkably higher expressions of IL6 and IL1α are within the threshold of the Zovawk pigs which have evolved through generations. Otherwise, had it been beyond the physiological range of expression of the above two cytokines, IL10 expression would have been obtained to down-regulate the said cytokines' expression. On the other hand, the basal expression of IL10 in LWY PBMCs might have resulted in the reduced expression of the pro-inflammatory cytokines IL1α, IL6, and TNFα compared to Zovawk pigs. Hohnstein *et al*. (2020) also observed a similar pattern of reduction in IL6 and TNFα levels following

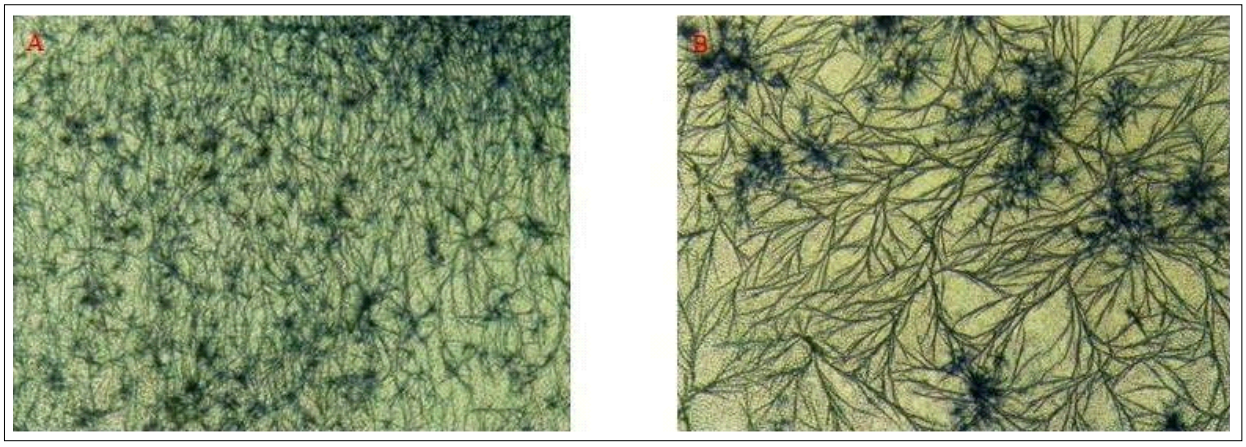

Fig. 1a and 1b: Peripheral blood mononuclear cells of LWY pigs after culturing with LPS showing formazan crystals (MTT metabolic product).

Table 2: Kinetics of Toll-like receptor and cytokines mRNA induction in LPS-stimulated PBMCs of zovawk and large white yorkshire pigs.

Gene	Breed	0 h	4 h	12 h	24 h
TLR4	Zovawk	1.01±0.11a	2.62±0.29b	3.92±0.69b	3.79±0.74b
	LWY	1.00±0.03a	2.17±0.37b	2.68±0.18b	2.59±0.17
TNFα	Zovawk	1.01±0.10	2.49±0.38	2.11±0.32	1.98±0.49
	LWY	1.01±0.12a	0.32±0.03b	0.46±0.04b	0.59±0.08b
IL1β	Zovawk	1.01±0.10a	15.96±2.48a	94.20±20.12b	93.49±11.32b
	LWY	1.03±0.20a	7.39±0.95b	14.13±2.29c	22.56±2.05d
IL6	Zovawk	1.01±0.07a	115.49±21.26bc	163.16±23.99c	87.82±19.35b
	LWY	1.02±0.16a	51.20±6.01b	95.26±8.25c	146.52±18.96d
IL10	LWY	1.01±0.12a	2.70±0.40b	2.95±0.38b	2.79±0.45b

in vitro Streptococcus suis stimulation of porcine PBMC in the presence of a neutralizing anti-porcine IL10 antibody. Moreover, it is well established that IL10 inhibits the production of pro-inflammatory cytokines such as TNFα and IL1α by activated monocytes/macrophages thereby protecting the host from overwhelming inflammatory responses (Moore *et al*., 1993). Interestingly, we also observed a low level of expression of all cytokine transcripts studied in unstimulated PBMCs. A similar observation was reported for IL6, IL10, and TNFα transcripts in unstimulated human PBMCs (Fan *et al*., 1998).

In the present study, a notable difference has been observed concerning the expression of pro-inflammatory cytokines between the two breeds of pigs. Though, the expression of TLR4 in LPS-stimulated PBMCs in both breeds showed a similar pattern, the expression level was slightly higher in Zovawk compared to LWY. However, for IL1α and IL6, the expressions peaked early (around 12 h) after stimulation in Zovawk PBMCs, whereas, a comparatively lower expression was observed after 12 h of stimulation in LWY. Altogether, in our present study we have observed that in Zovawk pigs the pro-inflammatory cytokine gene expression was found to be quick and high whereas in LWY pigs, the response was slow and low which indicates the ability of Zovawk in early adaptive immune responsiveness compared to LWY that may serve advantageous in fighting infectious diseases.

CONCLUSION

Our present study on the time course expression analysis of cytokine genes in LPS-stimulated PBMCs of Zovawk and LWY pigs revealed notable differences between the two pig breeds. Although both the breeds showed a several-fold increase in the expression of most cytokines on LPS stimulation, the Zovawk PBMCs showed a quicker and higher response than that of LWY which proves the superiority of this indigenous pig breed in terms of immune responsiveness against gram-negative bacterial infection over the exotic breed. On the other hand, coordinated regulations of cytokines were also observed in both the pig breeds to maintain immune function and homeostasis. Altogether, our study provides baseline information for further research in the direction of identification of proteins and protein networks responsible for immune competence against gram-negative bacterial infections in pigs to elucidate the key determinants/mechanisms for the sturdy and early immune responsiveness of the indigenous - Zovawk pigs over the exotic - LWY pigs.

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Conflict of intrest

The authors declare no conflicts of interest.

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