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Pulmonary Alveolar Macrophages Reveal Higher Basal Cytokine mRNA Expression than Peripheral Blood Leucocytes in Healthy Piglets

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ABSTRACT

The host immune system plays a vital role in protection from various harmful stimuli. Cytokines play a very important role in inflammation and immune response even at minimal concentrations. In this study, basal mRNA expression of various cytokines in porcine pulmonary alveolar macrophages (PAM) and peripheral blood leukocytes (PBL) of healthy piglets was assessed. Basal expression of all cytokines was higher in PAM as compared to PBL and the differences in normalized threshold cycle values ranged between 13.6% (IFN-α) and 53% (IFN- β). There was a positive correlation between the expression patterns in PAM and PBL, with a correlation coefficient of 0.76. Relative quantification of cytokine mRNA in PAM showed that there was wide variation in the degree of expression in terms of fold changes, which ranged from 3.5 (IFN-α) to 1130.6 (IFN-β). The results showed that exposure levels to pathogens can influence the basal cytokine expression, which depends on the microenvironment even in healthy piglets.

Keywords: Porcine alveolar macrophages, PBL, Cytokine, Pig, Realtime PCR

The immune system plays a vital role in protection of host from various harmful stimuli such as microbial pathogens, irritants, or toxic cellular components (Turner *et al.*, 2014). Primary response to stimuli is inflammation which is mainly orchestrated by various immune cells through different mediators including cytokines and chemokines. Cytokines play a very important role in inflammation and immune response even at minimal concentrations.

Cytokines have been classified based on their intended immune response (cytokines for adaptive immunity, pro-inflammatory cytokines and anti-inflammatory cytokines), their primary function, target cells and action (Interleukins, Tumour necrosis factor, Interferons, Colony stimulating factors), production profiles in T helper cells (Th1 and Th2 cytokines), etc. (Mossmann *et al.*, 1996; Romani *et al.*, 1997, Turner *et al.*, 2014). In general, Th1 cytokines are involved in cell mediated immunity whereas, Th2 cytokines are involved in adaptive immunity. Cytokines can function in an autocrine or paracrine manner.

Immune cells encounter external stimuli almost constantly in the body leading to expression of various cytokines and chemokines, even in apparently healthy animals. Pulmonary alveolar macrophages (PAM) are phagocytes that play important roles in homeostasis, host defence and tissue remodelling (Lambrecht, 2006). They are loosely attached in the pulmonary alveolar sacs and protect the lungs through phagocytosis, without affecting the air exchange process (Stafford *et al.*, 2008). Similarly, peripheral blood leukocytes (PBL) also play a pivotal role in the defence and elimination of foreign substances from the body. Due to their strategic locations and constant exposure to various microbes and other agents, these immune cells are expected to have high basal cytokine activity. Hence, the study of basal expression of cytokines

becomes imperative to assess the changes that may occur during disease conditions.

There have been several studies, most of them in vitro, related to cytokine responses to stimuli such as concanavalin A, lipopolysaccharide, microbes, toxins, etc., in various immune cells of porcine origin (Bailey 1994, Vezina, 1995). Although there are comparative studies on cytokine secretion by various cell types in disease conditions or upon stimulation, there has been no study to compare the expression profiles of cytokines in pulmonary alveolar macrophages and peripheral blood leukocytes in healthy pigs. In the present study, we report the comparative expression of some important cytokines in PAM and PBL of healthy pigs.

MATERIALS AND METHODS

Porcine alveolar macrophages and peripheral blood leukocytes collection

Three 7-week-old Large white Yorkshire-Non-descript crossbred healthy piglets were used for this study. Whole blood was collected from anterior venacava in a vacutainer containing EDTA (Becton Dickinson, USA). Blood was centrifuged at 1000 g for 10 min to remove plasma. RBC were lysed by incubating with equal volume of erythrocyte lysis buffer (Sigma, USA) for 10 min at room temperature, followed by centrifugation at 1000 g for 10 min. Supernatant was discarded and the resultant pellets containing PBL were washed once with sterile nuclease free PBS (pH 7.4; Sigma, USA). After centrifugation to remove the supernatant, the PBL pellet was resuspended in PBS for further use.

Pulmonary alveolar macrophages were harvested from bronchoalveolar lavage of porcine lungs as described previously (OIE manual, 2014) with minor modifications. Briefly, the lavage fluid was centrifuged at 400 g for 10 min to collect the cellular components. Isolated PAM were washed with sterile nuclease free PBS and centrifuged at 400 g for 10 min. The PAM pellet was resuspended to final concentration of 1×10^6 cells per ml before RNA extraction**.**

Due approval from the Institute Animal Ethics Committee (No. 83/IAEC/HSADL/14), and CPCSEA were obtained for the experiment involving pigs.

RNA extraction

Total RNA was extracted from 1×10^6 cells each of porcine PAM and PBL using one ml of TriReagent solution (Sigma, USA) following manufacturer's recommendations. Briefly, RNA from aqueous phase was recovered and precipitated by adding 500 µl of isopropanol. The RNA pellet obtained after final ethanol wash was air dried, resuspended in 50 µl of nuclease free water (Promega, USA). Total RNA was treated with RNase-free DNase (Thermo scientific, USA) for 30 min at 37ºC. DNase was inactivated by 5 mM EDTA at 65ºC for 10 min. The resultant product was directly used for first strand cDNA synthesis.

First strand complementary DNA (cDNA) synthesis

Reverse transcription of total RNA was carried out using Revert Aid H-minus first strand cDNA synthesis kit (Thermo Scientific, USA) following manufacturer's protocol using random hexamer primers and MMLV-RT. The cDNA was stored at -20ºC until further use.

Amplification of porcine cytokine genes

Prior to carrying out SYBR green based real time PCR for cytokine mRNA estimation, conventional PCR was optimized for each cytokine studied (IL-1β, IL-6, TNF-α, HMGB-1, IL-8, IL-2, IL-4, Interferons α and β). Amplification of porcine cytokines was done by conventional PCR using Platinum™ PCR super mix (Invitrogen, USA). Each 25 µl reaction consisted of 1x reaction mix, 12 pmol each of forward and reverse primers, 1.5 µl of cDNA template. The list of primers used in this study is given in Table 1. Thermal cycling condition optimized for a single annealing temperature is as follows: initial denaturation at 95°C for 2 minutes followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 55ºC for 30 s, extension at 72ºC for 30 s and a final extension step of 72ºC for 10 minutes. PCR products were resolved by electrophoresis on 1.5% agarose gels to confirm the expected size. The bands of expected size were gel purified using QiaQuick gel extraction kit (Qiagen, Germany) for further confirmation of respective cytokine genes by sequencing.

Cloning and sequencing of cytokine genes

Purified PCR products were ligated in a TA cloning

Table 1: List of oligonucleotide primers used

Sl. No.	Primer Name	Sequence (5'→ 3')	Expected product size	Reference
1	IL 1β F	AAAGGGGACTTGAAGAGAG	286	Darwich *et al.*, 2003
	IL 1β R	CTGCTTGAGAGGTGCTGATGT		
2	IL6 F	CTGGCAGAAAACAACCTGAACC	94	Sipos *et al.*, 2005
	Il6 R	TGATTCTCATCAAGCAGGTCTCC		
3	IL8 F	GGGTGGAAAGGTGTGGAATG	75	Vaithilingam *et al.*, 2013
	IL8 R	GGCTGCAGTTCTGGCAAGAG		
4	TNF α F	CACCACGCTCTTCTGCCTAC	132	Bao *et al.*, 2013
	TNF α R	ACGGGCTTATCTGAGGTTTGAG		
6	IFN α F	TGGTGCATGAGATGCTCCA	55	Duvigneau *et al.* 2005
	IFN α R	GCCGAGCCCTCTGTGCT		
7	IFNβ F	TGCAACCACCACAATTCC	80	Bao *et al.*, 2013
	IFNβ R	CTGAGAATGCCGAAGATCTG		
8	IL4 F	GCCGGGCCTCGACTGT	68	Dawson *et al*., 2005
	IL4 R	TCCGCTCAGGAGGCTCTTC		
9	IL2 F	GAGCCATTGCTGCTGGATTT	111	Lühken *et al.*, 2000
	IL2 R	GTAGCCTGCTTGGGCATGTAA		
10	HMGB1 F	TTGCCGGGAGGAGCATAAGAAGAA	136	Wang *et al.*, 2013
	HMGB1 R	GTCCGCCTTTGCCATGTCTTCAAA		
12	18s rRNA F	CCCCAACTTCTTAGAGGGACAA	70	Zhang *et al.*, 2013
	18s rRNA R	GGGCATCACAGACCTGTTATTG		

vector pTZ57R/T vector (Thermo Scientific, USA) and transformed in to competent JM 109 strain of *E.coli* cells (Promega, USA) using InsT/Aclone PCR cloning kit (Fermentas, USA) as per manufacturer's protocol. Plasmid DNA were isolated from overnight cultures of recombinant colonies using Pureyield plasmid miniprep system (Promega, USA). Inserts in the plasmids were confirmed by restriction enzyme digestion using *ECoRI* and *PstI* (NEB, USA) enzymes and by nucleotide sequencing using Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) and ABI 3130 automatic DNA sequencer (ABI, USA), employing M13 forward and M13 reverse sequencing primers (Promega, USA). The online tool BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to confirm the cytokine genes sequenced.

Relative quantification of cytokine mRNA

SYBR Green based real time PCR was done using GoTaq qPCR master mix (Promega, USA) and cytokine specific primers in a real time PCR machine (LightCycler 480 II, Roche, Germany). Each reaction mixture consisted of 1X reaction master mix, 10 pmol each of cytokine specific forward and reverse primers, 1.5 µl of cDNA and nuclease free water to make up the volume to 15 µl. All reactions were carried out in duplicates and no template control (NTC) was included for each primer set. Cycling conditions involved initial denaturation at 95°C for 10 min, followed by 45 cycles of amplification (denaturation at 95°C for 15 sec, annealing and extension at 55 °C for 45 s). At the end of amplification cycles, melt curve analysis was carried out by gradual increment of temperature from 50°C to 95°C and melting temperature (Tm) of all amplicons were optically read with an increment of 0.3°C. Ability to generate a specific amplification curve along with a single specific Tm product was taken as the criteria to ascertain the specificity of cytokine mRNA amplification. Threshold cycle (C_T) values were normalized using 18s rRNA as the housekeeping gene. Relative quantification of cytokine mRNA expression was done by the comparative threshold method $2^{-\Delta\Delta Ct}$ as described previously (Pfaffl, 2004) by using Microsoft Excel 2010 software.

Percent difference in the C_T values for each cytokine in PBL and PAM was calculated using the formula [(Mean C_T of PBL - Mean C_T of PAM)/ Mean C_T of PBL] × 100.

RESULTS AND DISCUSSION

In this study, we assessed and compared the basal mRNA expression of various cytokines in PBL and PAM of normal healthy piglets. Peripheral blood mononuclear cells and leukocytes in the spleen have been known to take part in systemic defence immune reactions and alveolar macrophages as the local defence cells in the lung against invading pathogens (Pabst, 1996). Hence, we had selected PBL and PAM for the study so that two different microenvironments in the host body which play important roles in disease are represented. PBL are essential component of the host immune system designed to mount an effective systemic response to tackle disease through secretion of various chemical mediators including cytokines, alarmins, inflammasomes, etc (Darwich *et al.*, 2003). PAM cells in porcine lungs have receptors for several viruses like PRRSV (Nauwynck *et al.*, 1999), Influenza viruses (Chang *et al.*, 2015) etc., making them the target cells and are a major component of host immune response and disease pathogenesis. In addition, PAM cells are being extensively used for *in vitro* studies to understand the virus replication and disease mechanisms, where cytokine responses of infected cells are critical and are monitored. Hence, the study of basal cytokine profile in normal cells is important. This is the first report on comparative basal expression of cytokine mRNA in PBL and PAM of porcine origin.

All the cytokine genes amplified and sequenced were confirmed to be specific to the intended target genes. Normalized mean C_T values obtained for cytokine mRNA amplification in PBL and PAM are presented in Fig. 1.

In both PBL and PAM, mRNA levels of interferons (α and β) were the highest. It was followed by IL 8, TNF α, IL 4, IL6, HMGB-1, IL2 and IL-1 β in PBL and by IL 4, IL 8, TNF α, HMGB-1, IL-1 β, IL2 and IL6 in PAM, respectively. The results showed that PAM had higher basal expression of all cytokines studied as compared to PBL, with a correlation coefficient of 0.76. In terms of per cent difference between the groups, IFN- β had the highest (53%) values while IFN-α had the lowest (13.6%) (Table 2). Results of the comparative threshold analysis for relative quantification of cytokine mRNA in PAM as compared to PBL showed that there was wide variation in the degree of expression in terms of fold changes, which ranged from 3.5 (IFN-α) to 1130.6 (IFN-β) (Fig. 2).

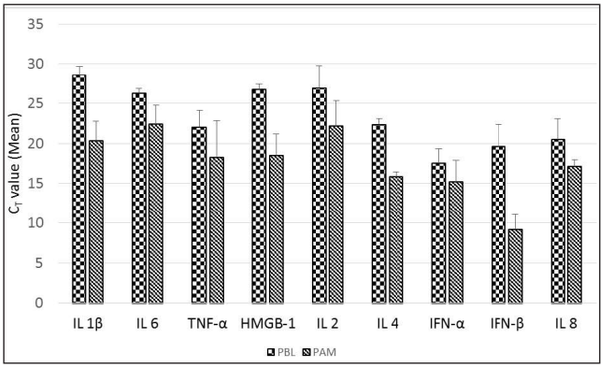

Fig. 1: Comparison of normalized C_T values obtained for cytokine mRNA amplification in PBL and PAM. 18s rRNA was used as the housekeeping gene for normalization.

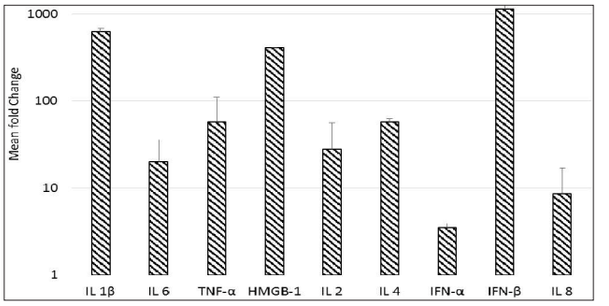

Fig. 2: Relative expression of cytokine mRNA in PAM as compared to PBL

Relative basal expression of proinflammatory cytokines was found to higher in porcine PAM. Hence, we decided to assess the High Mobility Group Box-1 (HMGB-1) mRNA in the present study. HMGB-1 is a nucleic acid binding protein with pro-inflammatory activity upon release from a cell, either by itself or by activating macrophages/ monocytes to express pro-inflammatory cytokines (IL-1 β, IL6 and TNF α), chemokines and adhesion molecules through RAGE, TLR 2 and TLR 4 receptors (Wang *et al.,* 2006). The results showed corresponding increase in basal HMGB-1 mRNA with that of proinflammatory cytokines. Although there is no study on constitutive HMGB-1 levels in unstimulated PAM, our findings were supported

by recent reports on HMGB-1 as a significant immune modulator in the host response to various stimuli (Bianchi, 2009; Kimura and Mori, 2014; Fraisier *et al.,* 2015).

Table 2: Differences in the normalized threshold cycle (C_T) values for cytokines studied in PBL and PAM

Cytokine	Mean C_T in PBL	Mean C_T in PAM	Difference	Percent difference
IL 1β	28.58	22.31	6.27	21.93
IL 6	26.28	20.50	5.78	21.99
TNF-α	22.04	18.26	3.78	17.15
HMGB-1	26.79	18.53	8.26	30.84
IL 2	26.93	21.27	5.66	21.02
IL 4	22.39	13.26	9.13	40.79
IFN-α	17.57	11.40	6.17	35.11
IFN-β	19.61	8.23	11.39	58.06
IL 8	20.53	14.12	6.41	31.21

Previous studies also have reported the basal expression of proinflammatory cytokine mRNA in unstimulated PBMC of various species including cats (IL- 10, IL- 12, IL-6, and TNF-α) (Rottman *et al.*,1995), cattle (IL-1 α, IL- 1 β, IL-6, IL- 10, and TNF-α, GM-CSF) (Covert and Splitter, 1995), and monkeys (IL-lβ, IL-10, and TNF-a) (Benveniste *et al.*, 1996). Vezina *et al.* (1995) reported constitutive IL-1, IL-6, and TNF α mRNA levels in unstimulated porcine alveolar macrophages.

Presence of higher basal levels of cytokine mRNA in PAM as compared to PBL in the present study may be justified by the differences in the microenvironment of the two categories studied. PAM cells are present in pulmonary alveolar sacs which are in constant exposure to the contaminants in external environment and are involved in immunosurveillance even in normal healthy animals. Due to the continuous stimulation, the expression of cytokines is always in 'switched on' condition leading to higher mRNA levels. Whereas, PBL are in a comparatively more secure microenvironment within the circulatory system with less direct exposure to the external stimuli. The fact that exposure levels to pathogens may influence the basal levels of cytokines in animals is supported by a previous study reporting low constitutive expression of mRNA for certain cytokines in freshly isolated, non-stimulated peripheral blood mononuclear cells of specific pathogen free cats (Rottman *et al.*, 1996).

Relative quantification of cytokine mRNA gives a preliminary idea about expression pattern that could be further correlated with protein secretion either by ELISA or Western blotting, if necessary. Although it has been contended that relative cytokine gene expression does not necessarily reflect the relative amounts of protein produced, cytokine mRNA levels have been shown to correlate with protein production in most cases. Post-transcriptional mechanisms prior to secretion as well as rapid utilization of cytokines by cells have been stated to be the reason for some of the differences observed between gene expression and protein detection in certain cases (Bailey *et al.*, 1994; Vezina *et al.*, 1995; Rottman *et al.*, 1996).

In conclusion, results of the present study shows that exposure levels to pathogens influence the basal cytokine expression, as evidenced by the presence of higher levels cytokine mRNA in PAM than in PBL of healthy pigs, indicating that expression of cytokines depends on the microenvironment even in healthy animal.

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