



Marked up-regulation of anti inflammatory cytokine gene expression in the peripheral blood mononuclear cells of postpartum cows with endometritis

RAFIQUL ISLAM¹✉, HARENDRA KUMAR², SUKDEB NANDI² and M K PATRA²

ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh 243 122 India

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ABSTRACT

This study was designed to determine the mRNA expression profile of inflammatory and anti-inflammatory cytokines in peripheral blood mononuclear cells (PBMC) of endometritic and non-endometritic cows to find out a possible marker for diagnosis of endometritis. Cows (21) including 12 endometritic, and 9 normal (non-endometritic) that did not develop uterine diseases postpartum (pp) were selected. Expression profile (mRNA) of cytokines, viz. IL-1 β , TNF α , IL-8 and IL-4 in endometritic cows relative to non-endometritic was studied in PBMC isolated from the blood of the cows collected within 4 to 5 weeks pp. Fold difference (n-fold) in cytokine gene expression in the PBMC of endometritic cows relative to normal was calculated using relative quantification method ($2^{-\Delta\Delta C_t}$). The relative fold change in gene expression for IL-1 was significantly lower in endometritic animals (0.31) than in cows that did not develop endometritis. Significant up-regulation was noted for the proinflammatory cytokine TNF α (9.53 fold) and anti-inflammatory cytokine IL-4 (90.09) in cows suffering from endometritis relative to non-endometritic cows. Up-regulation of chemokine IL-8 gene (6.25 fold) was also observed in endometritic compared to non-endometritic cows. Highest and significant up-regulation of mRNA expression for IL-4 followed by TNF α in PBMC of endometritic cows recorded in the study may indicate the development of endometritis in postpartum cows.

Keywords: Endometritic cows, Inflammatory and anti-inflammatory cytokines, mRNA expression, Peripheral blood mononuclear cells

Incidence of postpartum clinical and subclinical endometritis (SCE) in dairy cattle is very high and prevalence decreases with increasing postpartum days (Gilbert *et al.* 2005, Sheldon *et al.* 2009, Plontzke *et al.* 2010). Cows diagnosed with SCE had prolonged days open and a reduced probability of conception at first AI (Kasimanickam *et al.* 2004, Gilbert *et al.* 2005, Barlund *et al.* 2008) and SCE delayed postpartum ovarian cyclicity in buffaloes as compared to those without SCE (Elsayed *et al.* 2020). In the recent past, impressive works have been conducted on the cytokine profile in the endometrial tissue of cattle (Herath *et al.* 2009, Chapwanya *et al.* 2009, Fischer *et al.* 2010, Galvao *et al.* 2011, Manimaran *et al.* 2019, Raliou *et al.* 2019) and in circulatory monocyte from periparturient cows (Galvao *et al.* 2012, Raliou *et al.* 2019). Significant increase of IL-10 in the serum of periparturient cattle showed a new direction and hope for prediction and diagnosis of postpartum uterine diseases (Islam *et al.* 2013). Leukocytes that have been mobilized into the bovine endometrium from the peripheral blood during parturition are also capable of synthesizing pro-inflammatory cytokines

(Saji *et al.* 2000). The process of transmigration into the uterine lumen also modulates PMN function. Interleukin 8 (IL-8) induced attraction of PMNs into the uterine lumen increased the generation of reactive oxygen species by these cells (Zerbe *et al.* 2003), which aids in clearance of invading microbes. These reports suggested important role of pro- and anti-inflammatory cytokines in resolving uterine infections. Perusal of available literature revealed scanty information on the expression profile of cytokine in peripheral blood in relation to postpartum reproductive health in cattle. Present study was undertaken with the objective to determine mRNA expression profile of pro- and anti-inflammatory cytokines in peripheral blood mononuclear cells (PBMC) to explore the possibility of finding a marker or tool for early identification of the postpartum reproductive diseases in cattle.

MATERIALS AND METHODS

Experimental animals were selected from Vrindavani cattle herd maintained at Cattle and Buffalo Farm, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh. Animals were maintained under uniform feeding and managerial conditions. Diagnosis of endometritis was based on clinical findings observed at per-rectal or per-vaginal examinations 21–28 days postpartum, i.e. presence

Present address: ¹ICAR-National Research Centre on Pig, Rani, Guwahati, Assam; ²ICAR-Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh. ✉Corresponding author email: rafiqvet@gmail.com

Table 1. Primers used for PCR amplification of various genes in peripheral blood mononuclear cells of endometritic and non-endometritic cows

Oligo name	Sequence (5'–3')	Accession No.	Product size (bp)	Annealing temp (°C)
IL-1 β (LP)	ACCAGCTCTACAACAAAAGC	AY514903	205	53°
IL-1 β (RP)	TTGCACTTTACTGACTGCAC			
IL-4 (LP)	TGCATTGTTAGCGTCTCCTG	M77120	196	57°
IL-4 (RP)	TCGTTGTGAGGATGTTTCAGC			
IL-8 (LP)	GCTCTCTGCAGCTCTGTGTG	NM_173925	189	55°
IL-8 (RP)	CAGACCTCGTTTCCATTGGT			
TNF- α (LP)	CACAAGGCTCTCCTGTCTCC	AY221122	162	55°
TNF- α (RP)	AGAAGAGGCTGAGGCACAAG			
β -actin (LP)	GTCCACCTTCCAGCAGATGT	NM_173979	249	55°
β -actin (RP)	GTCACCTTACCAGTTCAGT			

of purulent or mucopurulent discharge along with the gross uterine changes in the diameter (Sheldon *et al.* 2006, Islam *et al.* 2013). Clinical endometritis was diagnosed by the presence of purulent (>50% pus) uterine discharge detectable in the vagina 21 days or more after parturition, or mucopurulent (approximately 50% pus, 50% mucus) discharge detectable in the vagina after 26 days postpartum on collection of pus discharged through vagina in transparent 50 ml tubes (Sheldon *et al.* 2006).

Blood sampling was done after diagnosis of the animals as endometritic or non-endometritic. Diagnosis was made in the animals around 21–28 days postpartum. Peripheral blood sample was collected aseptically from jugular vein using heparin (20 IU/ml of blood) as anticoagulant into sterile tubes cows within 4 to 5 weeks postpartum. Expression profile (mRNA) of cytokines IL-1 β , TNF α , IL-8 and IL-4 was studied in PBMC isolated from the blood samples collected from 21 postpartum cows comprising of 12 endometritic and 9 non-endometritic cows.

Peripheral blood mononuclear cells (PBMCs) were separated immediately after collection as described by Boyum (1968) with some modifications using density gradient centrifugation method using Lymphocyte Separation Media (density 1.077 \pm 0.001, Eurobio) and cultured in RPMI 1640 media in 6 well microplate at final cell concentration of 2 \times 10⁶ cells/ml. The culture medium in each plate was supplemented with 20 μ l/ml (2%) heat inactivated fetal calf serum (Sigma Aldrich Co., St Louis, MO, USA), 100 IU/ml penicillin G and 100 μ g/ml streptomycin sulfate. The PBMCs in the culture plates were stimulated with LPS @ 1 μ g/ml (Lipopolysaccharides from *Escherichia coli*, Sigma Aldrich Co., St Louis, MO, USA) and incubated at 37°C in a humidified 5% CO₂ atmosphere for 24 h. After 48 h of incubation, the cells were harvested by centrifugation in a diethylpyrocarbonate (DEPC) treated micro-centrifuge tube at 3,000 rpm for 3 min.

Total RNA from PBMC was extracted using Trizol® (*mono-phasic solution of phenol and guanidine isothiocyanate*) reagent (Gibco-Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA) following standard protocol. Quantity and quality of total RNA was checked by taking OD of the RNA sample at 260 and 280

nm at Nanodrop spectrophotometer (ND-1000, Thermo Scientific, USA). The RNA samples having ratio of OD value (A260:A280) ranging between 1.8 and 2.0 indicating presence of more than 90% total RNA with least protein and DNA contaminations were used for cDNA synthesis.

Total RNA extract was reverse transcribed to synthesize complimentary copy of DNA (cDNA) using RevertAID™ H Minus Reverse Transcriptase enzyme and oligo dT primer following manufacturer's guidelines (Fermentas Life Sciences). Both forward and reverse primers for each target genes (IL-1, IL-4, IL-8 and TNF- α) including house-keeping gene (β -actin) were designed using online primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) from the published complete or partial gene sequences available with the National Centre for Biotechnology Information. The oligonucleotide primer sets (20 base pair) were custom synthesized by Bioserve, Hyderabad, India.

Standardization of cyclic condition of a newly designed primer set is performed by trial and error method using different concentration of magnesium chloride and annealing temperatures in a Thermocycler (Applied Biosystem, USA). Initial denaturation was done at 94°C for 5 min followed by 30 cycles of cyclic denaturation at 94°C for 30 sec, annealing at temperature specified as in Table 1 for 30 sec and extension at 72°C for 30 sec followed by final extension at 72°C for 5 min. Amplified PCR product was verified by 1.5% (w/v) agarose gel electrophoresis using known 100 bp ready to use DNA ladder.

Realtime (RT) PCR was performed using the iQ™5 Multicolor RT PCR Detection system (iCycler, Bio Rad, USA) by using QuantiTect™ SYBR^R Green PCR Master mix. Reaction mixture was prepared as per the manufacturer's specification and the reaction was run following standard cyclic condition used in RT PCR. Threshold cycle value (Ct) for each target and house-keeping genes was recorded for evaluation of fold of expression. Dissociation curve for each amplified product of the target genes was analyzed to verify the specificity of product and rule out any false amplification due to primer-dimer.

Relative fold change in gene expression of each target

gene in PBMC of cows suffering from endometritis was calculated using the relative quantification method ($n\text{-fold} = 2^{-\Delta\Delta Ct}$) as described by Livak and Schmittgen (2001) taking β -actin as the endogenous control and normal (non-endometritic) as calibrator. Difference in threshold cycle value (Ct) of target genes and house-keeping gene (β -actin) for each sample was considered for calculation of fold expression and expressed as ΔCt . $\Delta\Delta Ct$ of target gene in endometritic cows was calculated by deducting the average ΔCt of target gene of healthy animal (normalized calibrator) from the ΔCt of target gene of endometritic cows. The fold of expression of a target gene in endometritic cows was finally estimated as $2^{-\Delta\Delta Ct}$.

Data obtained from this experiment were analyzed using SPSS-16.0. Test of normality of the data was performed using Shapiro-Wilk Test. Because n -fold was not normally distributed, the statistical analysis was performed on the $\Delta\Delta Ct$ values using independent t -test and then converted to $2^{-\Delta\Delta Ct}$ for data presentation of endometritic compared to non-endometritic cows. Level of significance was set at $P < 0.05$. The data obtained from real-time PCR for all target genes have been presented as their relative fold change (n fold) \pm SEM.

RESULTS AND DISCUSSION

Fold change ($2^{-\Delta\Delta Ct}$) in expression of various cytokines in peripheral blood mononuclear cells of endometritic relative to non-endometritic cows is given in Table 2. Relative fold change in gene expression for IL-1 was significantly ($P < 0.05$; $P = 0.010$) lower in endometritic animals (0.31) than in cows that did not develop endometritis. PBMC from a cow with severe persistent endometritis did not amplify. Endometrial IL-1 α and IL-1 β expression was reported to be decreased at 3–7 wk pp compared to 1 wk pp in both infertile and fertile animals (Herath *et al.* 2009). Lower expression of IL-1 gene has also been reported at 14 d and 35 d pp in stimulated blood monocytes of metritic cows compared to healthy cows (Galvao *et al.* 2012). Patra *et al.* (2014) reported higher expression of IL-1 in PBMC of endometritic buffaloes compared to healthy one, but fold change was low. Increased IL-1 expression was reported in the endometrial cells obtained from uterine tissues of endometritic compared to healthy cows (Gabler *et al.* 2009, Fischer *et al.* 2010, Galvao *et al.* 2011). Most of the endometrial tissue samples (90%) used for gene expression by Fischer *et al.* (2010) were having only non-immune cells (endometrial epithelial cells and no

PMNs or other immune cells). Lower expression of IL-1 β in PBMC of endometritic cows in the present study might be due to the fluctuating nature of expression of the cytokine with the increase in postpartum days as reported by Herath *et al.* (2009) and Galvao *et al.* (2012). Mette *et al.* (2010) showed significant up regulation of IL-1 β at 3 h post inoculation (pi), followed by marked depression at 12, 48 and 72 h pi of intrauterine (i.u.) infusion of *E. coli* in mares. IL-1 β expression in the endometrial cells was reported to be varied with time for endometritis in cattle (Herath *et al.* 2009, Galvao *et al.* 2011) and mare (Mette *et al.* 2010) and blood monocyte in cows (Galvao *et al.* 2012). Difference in expression is attributable to the source of sample, reproductive stage, species variation and also nature and course of infection in the animals. Thus, decreased IL-1 β expression was observed in PBMC of endometritic cows at 4–5 wk pp.

TNF- α expression in PBMC was significantly ($P < 0.01$; $P = 0$) higher in endometritic cows (9.53 fold) relative to non endometritic cows (Table 2). The cows suffering from moderate to severe endometritis showed higher expression of TNF α than cows with mild endometritis. Higher level of TNF- α expression in PBMC observed by Galvao *et al.* (2012) around 21 d post calving for both metritic and endometritic cows are in line with present findings with reference to TNF α expression in PBMC of endometritic cows. Increased TNF α expression has been reported in endometrial and stromal cells stimulated *in vitro* by LPS (Herath *et al.* 2006). In cattle suffering from post-partum endometritis failed to show induction of TNF α expression in the endometrium (Chapwanya *et al.* 2009). Fischer *et al.* (2010) observed increased expression of TNF α in endometrial tissue collected 21–27 d pp in cows. Increased expression of TNF α was reported by Mette *et al.* (2010) in the endometrial tissue of induced equine endometritis. Recently, increased expression of TNF α in the PBMC of endometritic buffalo has also been reported from our laboratory (Patra *et al.* 2014). Higher levels of TNF- α and IL-10 in uterine wash of repeat breeder animals with subclinical endometritis (SCE) has also been reported (El-Amrawi and El-Karim 2019). TNF α serves to promote synthesis of other cytokines, and early up-regulation might be necessary as a first line of defense against bacterial invasion (Herath *et al.* 2006). Increased expression of TNF α observed in PBMC of cows with all kind of endometritis (mild, moderate or severe) compared to non-endometritic cows indicates its association with the development of endometritis.

Higher IL-8 expression (6.25 fold, $P = .29$) recorded in present study is comparable with the report of IL-8 expression in circulating white blood cells of cattle (Galvao *et al.* 2012, Raliou *et al.* 2019) and buffalo (Patra *et al.* 2014) and endometrial tissue of cattle (Fischer *et al.* 2010, Manimaran *et al.* 2019) suffering from post partum endometritis. Cows showing moderate to severe endometritis for longer period with higher expression of TNF- α either revealed a very low or no IL-8 expression

Table 2. Fold change ($2^{-\Delta\Delta Ct}$) in expression of various cytokines in peripheral blood mononuclear cells of endometritic relative to non-endometritic cows

Cytokine	Fold change ($2^{-\Delta\Delta Ct}$)	SEM	P value
Interleukin 1*	0.31	0.17	0.010
Interleukin 4**	90.09	28.67	0.003
Interleukin 8	6.25	2.81	0.290
TNF- α **	9.53	2.30	0.000

* $P < 0.05$; ** $P < 0.01$.

and lower IL-1 expression. Higher cycle threshold (Ct) value for IL-1 and IL-8 expression in cows revealed no band pattern on gel electrophoresis. Decreased level of expression observed in the cows with moderate to severe persistent endometritis indicated disturbances in immune mechanism. In cows with mild uterine infection or spontaneously recovered from the uterine infection showed marked higher expression level leading to non-significantly higher expression in endometritic ($P=0.29$) compared to non-endometritic cows. Present finding is in accordance with Kimura *et al.* (2002) who reported a significantly lower concentration of IL-8 throughout the periparturient period in Holstein cows with ROP.

IL-1 and IL-8 are important at the beginning of the bacterial contamination for promoting migration of PMN cells to the site of infection and effective PMN functions (Baggiolini *et al.* 1989, Sica *et al.* 1990, Roach *et al.* 2002). Failure of PMN function leads to the persistence of infection in the uterus resulting into development of endometritis. IL-1 and TNF α , the proinflammatory cytokines, stimulate the expression of IL-8 and adhesion molecules on vascular endothelial cells, leading to neutrophil and monocyte chemoattraction, and activate neutrophils and monocytes, promoting increased phagocytosis and bacterial killing (Sica *et al.* 1990, Roach *et al.* 2002). Significantly lower expression of IL-1 in the present study is indicative of the decreased IL-8 expression in cows with severe and persistent endometritis leading to the development of the diseases as reported for PBMC of cows with metritis (Galvao *et al.* 2012). An initial increase followed by decrease in IL-8 expression was also reported in induced endometritis in mare (Mette *et al.* 2010) and post partum endometritis in cattle (Herath *et al.* 2009). PMN influx by chemokines such as IL-8 plays a key role in the uterine immune response (Zerbe *et al.* 2003). IL-8 is important at the initial stage of the infection subsequent to the release of IL-1 and TNF- α . Time of sampling is important for level of gene expression as shown previously in endometrial cells from mare with induced endometritis (Mette *et al.* 2010), cattle with post partum endometritis (Herath *et al.* 2009, Galvao *et al.* 2011) and in PBMC of cattle with post-partum metritis (Galvao *et al.* 2012).

IL-4 being an anti-inflammatory cytokine indicated a significant role as the fold change in expression was very high compared to the other cytokines under study and was significantly ($P<0.01$) higher in endometritic (90.09 fold) than in normal cows. A significant up-regulated expression of the IL-10 along with the pro-inflammatory cytokines IL-1 β , TNF α and IL-8 was observed at 3 h pi of i.u. *E. coli* suggested a role of IL-10 for timely regulation of the pro inflammatory cytokines to return to the pre inoculation level within 12 h pi (Mette *et al.* 2010). Recently a higher serum level of Interleukin-10, an anti-inflammatory cytokine protein was reported in periparturient cows suffering from postpartum reproductive diseases and suggested a new direction for prediction of postpartum reproductive diseases based on estimation of IL-10 prepartum (Islam *et al.* 2013).

IL-4 promotes Th2 lymphocyte development and causes inhibition of LPS induced proinflammatory cytokine synthesis (Opal and DePalo 2000). The marked increased expression of IL-4 in the current study might lead to the marked decrease in the IL-1 expression in the PBMC. IL-4 and IL-10 are potent anti inflammatory cytokines that can inhibit the synthesis of IL-1 β (Dinarello 1997) and suppress other proinflammatory cytokines production by both monocyte or macrophages and neutrophils (Clarke *et al.* 1998, Opal and DePalo 2000). Increased expression of IL-4 (depending on timing relative to parturition and uterine disease) is indicative of impaired switching from Th2 to Th1 type environments in transition cows suffering from postpartum endometritis.

CD4⁺ lymphocytes synthesize IFN- γ and IL-4 in the peripheral blood cells increased in women patients with postpartum endometritis (Sukhikh *et al.* 2005) may also true for cows suffering from postpartum endometritis leading to the higher fold expression of IL-4 in PBMC in the present study. Higher expression of IL-4 in PBMC of endometritic cows relative to non-endometritic indicated higher level of preceding infection as reflected by the level of proinflammatory cytokine (Mette *et al.* 2010). IL-1 and IL-8 expression levels and partly TNF α varied with the degree of uterine inflammation in the present study. Increase in IL-4 expression followed by pro-inflammatory cytokine TNF α relative to non endometritic (normal) cows can be used as an indicator for diagnosis of postpartum endometritis in cows.

Based on the present findings, it can be concluded that highest and significant up regulation of mRNA expression observed for IL-4 followed by TNF α in peripheral blood mononuclear cells of endometritic cows indicate the development of endometritis in postpartum cows and thereby it can be helpful for diagnosis of endometritis in future.

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