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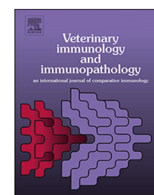

Research paper

Assessment of Poly(ADP-ribose) Polymerase1 (PARP1) expression and activity in cells purified from blood and milk of dairy cattle

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A R T I C L E I N F O

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A B S T R A C T

Poly(ADP-ribosyl)ation (PAR) is a post-translational protein modification catalysed by enzyme member of the poly(ADP-ribose) polymerases (PARPs) family. The activation of several PARPs is triggered by DNA strand breakage and the main PARP enzyme involved in this process is PARP1. Besides its involvement in DNA repair, PARP1 is involved in several cellular processes including transcription, epigenetics, chromatin re-modelling as well as in the maintenance of genomic stability. Moreover, several studies in human and animal models showed PARP1 activation in various inflammatory disorders. The aims of the study were (1) to characterize PARP1 expression in bovine peripheral blood mononuclear cells (PBMC) and (2) to evaluate PAR levels as a potential inflammatory marker in cells isolated from blood and milk samples following different types of infection, including mastitis. Our results show that (i) bovine PBMC express PARP1; (ii) lymphocytes exhibit higher expression of PARP1 than monocytes; (iii) PARP1 and PAR levels were higher in circulating PBMCs of infected cows; (iv) PAR levels were higher in cells isolated from milk with higher Somatic Cell Counts (SCC > 100,000 cells/mL) than in cells from milk with low SCCs. In conclusion, these findings suggest that PARP1 is activated during mastitis, which may prove to be a useful biomarker of mastitis.

1. Introduction

Poly(ADP-ribosyl)ation is a post-translational modification of cellular proteins highly induced after DNA damage. The reaction is catalysed by poly(ADP-ribose) polymerases (PARPs) (EC 2.4.2.30), an enzyme family using NAD^+ as substrate to form polymers of ADP-ribose (PAR). Poly(ADP-ribose) polymerase1 (PARP1) is the best studied family member which is responsible for approximately 90% of cellular PAR formation after genotoxic stress (Bürkle et al., 2004). PARP1 is a 116 kDa nuclear protein that is well conserved during evolution (Uchida and Miwa, 1994) with a 95% overall amino acid sequence similarity among mammalian species (Beneke et al., 1997). Bovine PARP1 shares 98% similarity with the human enzyme and the major caspase cleavage sites are conserved (Saito et al., 1980). Besides DNA repair, transient PAR formation has also been involved in several other cellular processes including control of epigenetic profile (Reale et al., 2005), transcription, chromatin re-modelling (Posavec Marjanovic et al., 2017; Sultanov et al., 2017), maintenance of genomic stability (Bai, 2015) and apoptosis (Valenzuela et al. 2002). In cases of inflammation resulting in extensive tissue damage, PARP1-mediated PAR synthesis may be excessive and lead to the cellular depletion of its substrate NAD^+ (Berger, 1985) as well as to cell death signaling (D'Amours et al., 2001). Several types of cellular stress, including genotoxic (DNA damaging) agents such as peroxynitrite formed in inflammatory processes from nitric oxide (NO), trigger a programmed cell death pathway, called apoptosis (Brüne, 2005). Apoptosis involves the activation of caspases to cleave proteins during programmed cell death. PARP1 cleavage by Caspase-3 has been regarded as a reliable marker of apoptosis in cellular research for many years (Lazebnik et al., 1994). In this study we have focused on changes in PARP1 activity associated with inflammatory responses of the bovine udder.

Mastitis, the inflammation of mammary gland, is a serious disease that causes huge economic losses to the dairy industry (Rollin et al., 2015) and has become one of the main research topic in the field of

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dairy science due to the financial losses generated and food safety concerns over antimicrobial use. Mastitis usually occurs as an immune response to bacterial invasion of the teat canal by a variety of bacterial sources present on farms. Following pathogen invasion, the resident leukocytes together with epithelial cells initiate the inflammatory response required to eliminate the invading bacteria (Paape et al., 2003; Rainard and Riollet, 2006). These cells release chemoattractants for the rapid recruitment of polymorphonuclear leukocytes (PMN) to the site of infection. The PMN eliminate pathogens by phagocytosis during which reactive oxygen species are generated (Paape et al., 2003).

Our study investigated whether the immune cells, involved in protecting the mammary glands from infection, exhibited increased PARP1 activity. Moreover, we evaluated whether PAR levels increased in cells isolated from milk particularly during an udder inflammation in dairy cattle. A few recent studies have reported data on PARP1 in cattle (Demeyere et al., 2013; Saccà et al., 2016) yet to our knowledge this is the first study evaluating PARP1 expression and its activity in cells purified from the blood and milk of dairy cattle. The aims of the study were (1) to characterize PARP1 expression in bovine peripheral blood mononuclear cells (PBMC) and (2) to evaluate PAR levels as a potential marker of inflammation in cells isolated from blood and milk samples following different types of infection, including mastitis.

2. Materials and methods

2.1. Animals and sample collection

The management and care of the experimental animals was carried out in compliance with the 86/609EEC European Union directive guidelines. The animals were kept at the Research Centre for Animal Production and Aquaculture of CREA where blood samples from lactating cows were collected according to good veterinary practice. Two separate trials were performed to determine PARP1 and PAR levels in cells from blood and from milk samples. In the *Trial 1,* blood samples were taken from healthy cows in order to evaluate *PARP1* mRNA (n = 5 cows) and PARP1 protein (n = 2 cows) in purified PBMC, lymphocytes and monocytes. In addition, blood samples from 2 *Staphylococcus aureus* were taken from naturally infected cows in order to determine PARP1 and PAR levels in PBMCs isolated during infection. The status of *S. aureus* infection was established by the local Institute for animal public health (Istituto Zooprofilattico Sperimentale-IZSLT). In *Trial* 2, 36 single quarter milk samples were collected separately from 9 cows in order to determine PAR levels in purified milk somatic cells. The milk cells obtained from different udder quarters were separated using the Somatic Cell Count (SCC), a parameter used as indicator of udder health and milk quality, considering a threshold of 100,000 cells/mL to define a quarter as normal (Schwarz et al. 2011; Pilla et al., 2013).

2.2. Trial 1

2.2.1. Isolation of PBMC and separation of lymphocytes and monocytes

The blood samples (50 mL) were taken from the external jugular vein and placed in vacutainer tubes containing anticoagulant EDTA and PBMC were isolated by density gradient (Lymphoprep™-1.077 g/mL; AXIS-SHIELD) according to the manufacturer's instructions. Monocytes and lymphocytes were separated from purified bovine PBMC (4×10^7 cells) by positive selection of monocytes using magnetic CD14 MicroBeads (Miltenyi Biotech) according to the manufacturer's instructions and subsequently stored at −80 °C. Furthermore, after separation aliquots of cells were stained with the mouse anti-human CD14 (clone TÜK4, Miltenyi Biotech), fluorescein isothiocyanate (FITC) conjugated antibody, and analyzed using a CytoFLEX flow cytometer (Beckman Coulter Inc). Lymphocytes and monocytes were identified in a back-gate on FSC *versus* SSC dot plot and CD14-negative and CD14-positive cells respectively. Monocytes purity was approximately 98% and lymphocytes purity was 95%.

2.2.2. Whole cell extract preparation and Western blot analysis

Aliquots of PBMC, lymphocytes and monocytes were thawed on ice and lysed immediately in RIPA buffer (50 mM Tris−HCl at pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 1 mM EDTA), supplemented with sodium-orthovanadate (3 mM), NaF (100 mM) and a mix of protease inhibitors (Sigma-Aldrich). The lysates were kept on ice for 25 min and then centrifuged (16,000*g* for 30 min at 4 °C). The supernatants were collected and quantified by the Bradford protein assay reagent (Bio-Rad). The proteins were loaded and separated by SDS-PAGE and electro-blotted onto nitrocellulose membrane (Hybond™ECL™, GE Healthcare). The immunoblots were probed with a specific PARP1 antibody (clone C2-10, Enzo Life Sciences), which recognizes full length PARP1 (116 kDa) and apoptosis-induced 89 kDa cleavage fragments, and by a specific anti-PAR antibody (clone 10H Enzo Life Sciences).

Secondary antibodies, labelled with horseradish peroxidase (HRP), were used to develop the immunoblot detection using an enhanced chemoluminescence reaction (ECL Plus, BioRad). Protein levels of the full length PARP1 band (116 kDa) were quantified densitometrically using Quantity One Software (Bio-Rad).

In order to control the specificity of anti-PAR antibody, time- dependent PAR formation was assessed in Human colon carcinoma HCT116 cells treated with the DNA damaging agent doxorubicin (Doxo) (Sigma-Aldrich). HCT116 cells were grown in high glucose (4.5 g/l) Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. When the cells were approximately 70% confluent, they were treated with 1 μM Doxo at different times up to 2 h and when indicated with ABT-888 (Sigma-Aldrich). More specifically, HCT116 cells were pre-treated with 0,5 μM ABT-888 30 min prior to Doxo treatment and the inhibitor was maintained in the medium throughout the course of the damage (2 h).

Total cell extracts were obtained by lysis in RIPA buffer, as previously described. Equal amounts of protein were separated by SDS-PAGE, electroblotted onto nitrocellulose membrane and probed with the specific anti-PAR antibody.

2.2.3. Primer design, RNA extraction and RT-qPCR

In order to detect *PARP1* gene expression, primers were designed based on the *Bos taurus* sequence (Gen Bank Accession Number: NM_174751) to amplify a 158 bp segment spanning from exon 12 to exon 13 of the bovine *PARP1* gene. The ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*) and the mitochondrial ribosomal protein S15 (*MRPS15*) were tested for reference gene

Table 1
Characteristics of the primer pairs used and the efficiency of amplification.

Gene	Primer sequence (5' to 3')	Accession Number	bp	% Efficiency
PARP1	For-CATCGTCAAAGGGACCAACT Rew-AAATGCTCAATGGCATCCTC	NM_174751	158	100–104
ATP5B	For-TTTGGACTCCACGTCTCGCATC Rew-TCCTGGAGGGATTTGTAGTCCTG	NM_175796.2	108	97–102
MRPS15	For-GCAGCTTATGAGCAAGGTCGT Rew-GCTCATCAGCAGATAGCGCTT	NM_001192201.1	151	99–103

suitability based on the findings of previous studies (Bionaz and Loor, 2007; Crisà et al., 2013). Information on PCR primer sets is summarized in Table 1. Total RNA extraction from PBMC and sorted lymphocytes and monocytes was performed using the RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. RNA purity was measured by spectral photometry (NanoPhotometer® Pearl, IMPLEN). RNA integrity was assessed on Agilent 2100 Bioanalyzer (Agilent Technologies). All of the RNA samples used in this study had RIN values ranging between 8.8 to 9.8. Complementary DNA (cDNA) was synthesized from 1μg of total RNA using a Maxima First Strand cDNA Synthesis Kit (ThermoFisher Scientific). PCR for each sample was carried out in triplicate using an iCycler iQ System (BioRad). A six-point standard curve was generated for each gene using a 2-fold dilution of cDNA in order to determine amplification efficiency for each couple of primers. The PCR was performed in a 30 μL final volume containing 50 ng of cDNA, 15 μL of SYBR Premix Ex Taq™ (Takara) and 0.6 μL each of 10 μM forward and reverse primers.

All of the samples were analyzed in triplicate and each plate included the no-template and minus-reverse transcription samples as controls. Amplification conditions for *PARP1, MRPS15* and *ATP5B* were as follows: denaturation step at 95 °C for 30 s; 40 cycles of 95 °C for 5 s, 60 °C for 30 s A melting curve analysis (60–90 °C) with a heating rate of 0.5 °C/sec) was carried out to check the specificity of the amplification and to detect the presence of non-specific amplicon. The sequence of amplified product was confirmed by direct sequencing on the Genetic Analyzer (Applied Biosystems). The *PARP1* expression was normalized using both *MRPS15* and *ATP5B* according to $2^{-\Delta Ct}$ equation, considering that the threshold CT value of the 2 reference genes are comparable in the different population of cells (PBMC, monocytes and lymphocytes obtained from the same animal). The results, expressed as mean ± SD of three independent experiments carried out on five different cows, represent the relative expression level computed as the difference between the *PARP1* gene CT and both *MRPS15* and *ATP5B* CT values.

2.3. Trial 2

2.3.1. Purification of milk somatic cells

Milk samples (100 mL) were collected from nine cows and a total of 36 quarter milk samples were analyzed. The International Dairy Federation (IDF) currently recommends carrying out both SCC and bacteriological analysis as criteria for determining udder health. Milk somatic cells include macrophages, PMN and lymphocytes that exist initially in blood together with epithelial cells from the mammary gland.

The amount of somatic cells in each milk sample was measured with a DeLaval Cell counter DCC instrument (DeLaval International AB) and the experimental design was established to divide the quarter milk samples into two groups: Group 1 composed of 17 samples with SCC ≤ 100,000 cells/mL and Group 2 composed of 19 samples with SCC > 100,000 cells/mL. Immediately after collection, the milk samples were centrifuged at 800*g* for 15 min at 8 °C and the fat layer and supernatant were discarded. The cell pellets were washed twice with 40 mL of cold PBS and aliquots of $1.0*10^6$ cells were stored at −80 °C for later PAR quantification.

2.3.2. Assessment of PAR concentration in milk somatic cells

The HT PARP *in vivo* Pharmacodynamic Assay II (Trevigen), which measures the net concentration of PAR in cellular extracts, was used to determine the PAR level in milk somatic cells. The assay was run according to the manufacturer's instructions. PAR concentration was quantified as a chemiluminescent signal measurement (Victor X light instrument, Perkin Elmer Inc.) and the PAR concentration was reported as pg/mL per $1.0*10^6$ cells. Purified PAR polymers supplied with the kit were used to create a standard curve with trend line. The standard curve ranged from 10-1000 pg/mL. The R^2 value for the linear curve fit was 0.993. Jurkat cell lysates were used as standards to control assay.

2.3.3. Flow cytometric analysis of milk somatic cells

Out of 36 quarter milk samples, 12 were analyzed by flow cytometry to perform the determination of different types of immune cells in milk. A three-colour flow-cytometric analysis was performed to identify total leukocytes and to differentiate lymphocytes from phagocytes [PMN + monocytes/macrophages].

In short, 100 μL of the cell suspension at 1×10^6cells/mL were incubated on ice for 30 min in the dark, with 5 μL of PE-conjugated anti-bovine CD45 antibody (clone CC1, BioRad), 5 μL of FITC- conjugated anti-bovine CD11b (clone CD126, Bio-Rad) and 5 μL of APC- conjugated anti-human CD14 (clone TÜK4, Miltenyi Biotec). After incubation, the cells were washed twice with cold PBS and resuspended in 300 μL of PBS for the flow cytometric analysis. Stained samples were analyzed with FC500 Flow Cytometer Analyzer (Beckman Coulter Inc.) and the data were analyzed with Kaluza Analysis Software (Beckman Coulter Inc.). Besides debris exclusion, PMN and monocytes/macrophages were identified with the gating strategy as $CD45^+/CD11b^+/CD14^-$ cells and $CD45^+/CD11b^+/CD14^+$ cells respectively; lymphocytes were identified by size and granularity (FSC-H *vs* SSC-H) and as $CD45^+/CD11b^{-/low}/CD14^-$ cells.

2.4. Statistical analysis

Differences in PAR level, lymphocytes or phagocytes percentages between SCC classes (SCC ≤ 100,000 cells/mL and SCC > 100,000 cells/mL) were evaluated with the following ANOVA model:

$$y_{ij} = \mu + c_i + e_{ij}$$

Where:

y_{ij} = PAR levels (pg/mL), lymphocytes or phagocytes percentage;

c_i = fixed effect of the SCC classes: (i = 1, 2);

e_{ij} = random residual effect.

3. Results

3.1. Differential PARP1 expression in bovine PBMC (Trial 1)

PARP1 protein levels were detected in freshly isolated PBMC and in lymphocytes and monocytes. Western blot analyses (Fig. 1A), confirmed the presence of a protein of the expected molecular weight (116 kDa). The presence of the 89 kDa band in PBMC and lymphocytes, protein corresponding to the PARP1 fragment derived from the Caspase3-dependent apoptotic cleavage of the protein, suggest some apoptosis activity in these cells. Densitometric analysis of the 116 kDa band indicates that the full length protein was present at much higher levels in lymphocytes (3.4 fold) than in monocytes (Fig. 1B).

To verify whether the low level of PARP1 protein in monocytes resulted from low mRNA expression, we performed RT-qPCR analysis of the freshly sorted cells. Fig. 1 **(C, D)** shows the relative expression of *PARP1* mRNA in lymphocytes and monocytes compared to unsorted PBMC and normalized to *MRPS15* and *ATP5B*. These two candidate reference genes (Table 1) were selected through the analysis of literature survey on studies in bovine with RT-qPCR analysis (Bionaz and Loor, 2007; Crisà et al., 2013 and some still unpublished results). They can be considered good as reference genes for the comparison of *PARP1* expression in different cell types since their expression is stable with low coefficient of variation CV in different bovine samples tested in different experiments (data not shown) and there are no substantial differences in gene expression between PBMC, lymphocytes and monocytes, both for *MRPS15* and *ATP5B*. Results highlighted that *PARP1* expression was much higher in lymphocytes than in monocytes

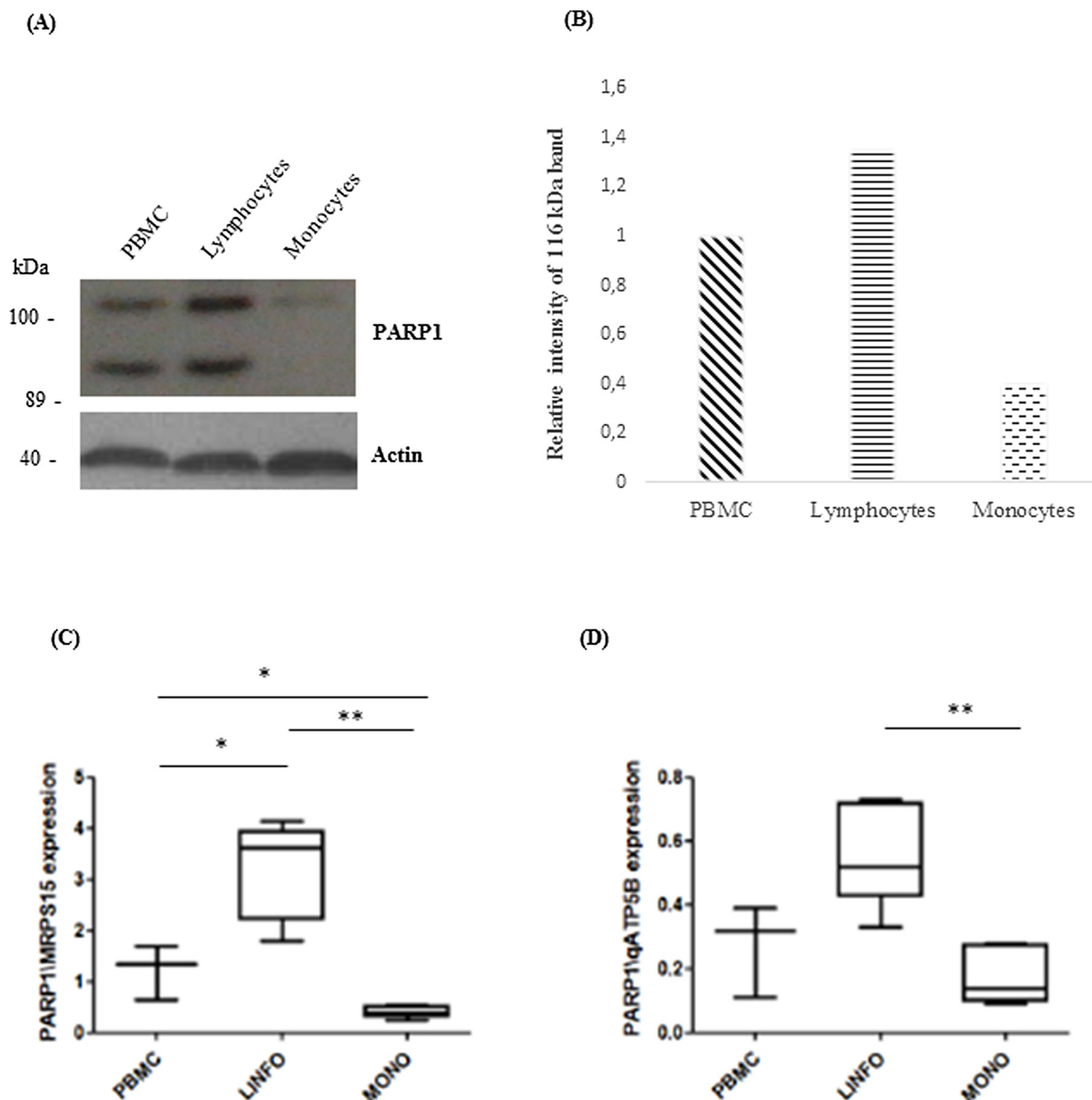

Fig. 1. Differential PARP1 expression in PBMC.
A) Representative Western blot of PARP1 detected with the monoclonal antibody C2-10 (which detects the full length 116 kDa PARP1, and the apoptosis-related 89 kDa fragment) in freshly isolated PBMC, in lymphocytes and monocytes isolated from PBMC by magnetic beads cell sorting using an anti-CD14 antibody. B) Densitometric analysis of the PARP1 higher band (116 kDa). PARP1 protein levels were normalized to β-actin levels and indicated as a ratio with respect to PBMC cells, setting protein levels on PBMC to 1. C) Real-time PCR measurements of *PARP1* mRNA levels in PBMC andisolated lymphocytes and monocytes by magnetic beads cell sorting using an anti-CD14 antibody. Box plots showing *PARP1* mRNA levels normalized to *MRPS15* and to *ATP5B*. The results are expressed as mean ± SD of three independent experiments performed in five cows. Significant differences between PBMC, lymphocytes and monocytes are indicated (* = *p-value* ≤0.05; ** = *p-value* < 0.01 by Student's t-test).

(3 fold higher normalizing with *ATP5B* - 7.5- fold normalizing with *MRPS15*), confirming the results of the protein analyses shown in Fig. 1**B**.

3.2. PARP1 and PAR levels are increased in bovine PBMC of animals with bacterial infection

In order to analyze PARP activity, we evaluated the presence of PAR which is an indirect measurement of PARP activity. As preliminary control, Doxo was added to HCT116 cell line and the resulting increase in PAR was measured by immunoblotting analysis. HCT116 cell line was used as control because the concentration of Doxo and incubation time was previously standardized to produce a non-apoptotic damage with a time-dependent PAR formation (Bacalini et al., 2011). In the presence of Doxo a clear increase in PAR level was detectable as early as 30 min after the treatment and PAR increased in a time-dependent manner until 2 h (Fig. 2A). Treatment with ABT-888, a specific inhibitor of PARP activity, induced a clear inhibition of PAR synthesis in Doxo-treated cells, confirming the specificity of PAR detection.

Immunoblotting analysis was performed to detect PARP1 and PAR levels in PBMC of infected *versus* non infected dairy cows (Fig. 2B). Purified PBMC of cow naturally infected by *S. aureus* exhibited higher levels of 116 kDa full-length PARP1 protein compared to the healthy control. The presence of 89 kDa PARP1 fragment in the sample from the infected cow suggested apoptosis activity. In addition, the concomitant formation of PAR polymers suggested PARP1 activity.

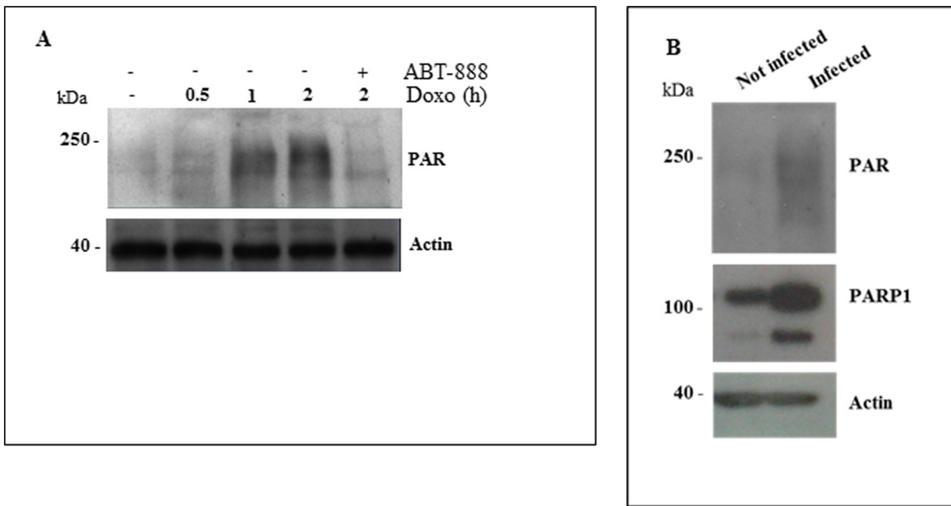

Fig. 2. PARP1 and PAR levels in PBMC from cows naturally affected by bacterial infection.
A) Time course of PAR formation in HCT116 cells after treatment with 1 μM Doxorubicin (Doxo) in the presence or absence of ABT-888 (30 min pre-treatment). Western blotting was performed on total cell extracts using antibodies against PAR and β-actin. B) Representative Western blot of PARP1 protein and PAR formation in PBMC purified from blood of infected and non-infected cows. Data shown are representative of two independent experiments.

3.3. High PAR levels in milk quarter samples with high somatic cell count (Trail 2)

To verify the presence of PAR in milk cells, samples from 17 quarters from Group 1 (SCC < 100,000 cells/mL) and 19 quarters from Group 2 (SCC > 100,000 cells/mL) were collected. The average of SCC in Group 1 (46,882 ± 23,545 cells/mL) and in Group 2 (319,707 ± 294,100 cells/mL) differed significantly (*p-value* = 0.002).

We measured intracellular PAR amounts with the HT PARP *in vivo* Pharmacodynamics assay.

The results indicate that PAR level is significantly higher in Group 2 (308.74 ± 209.92 pg/mL/1.0 × 10^6 cells) as compared to Group 1 (131.85 ± 97.05 pg/mL/1.0 × 10^6 cells) with a *p-value* = 0.003 (Fig. 3 and Table 2).

Table 2
PAR level in purified milk cells coming from udder quarters with differential SCC.

	SCC ≤ 100,000 cells/mL (n = 17)		SCC > 100,000 cells/mL (n = 19)		*P-value*
	Mean	SE	Mean	SE	
PAR (pg/mL/ 10^6 cells)	131,85	40,41	308,74	38,23	0.003

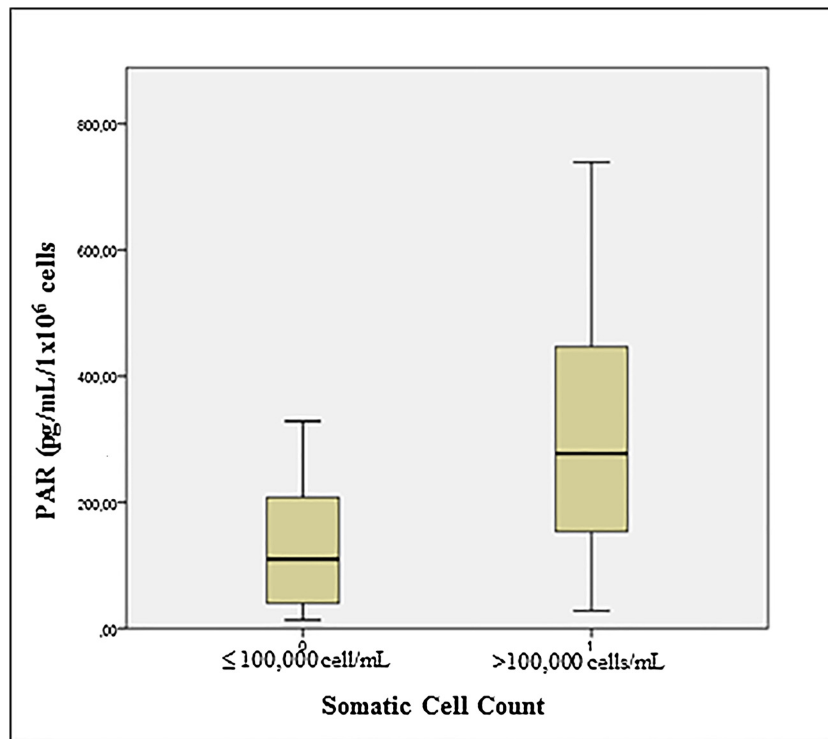

Fig. 3. PAR levels in cells isolated from quarter milk samples.
Box plot showing PAR level (pg/ml/1 * 10^6 cells) detected in milk samples coming from two different categories of bovine udder quarters: quarters with SCC ≤ 100,000 cells/mL and quarters with SCC > 100,000 cells/mL.

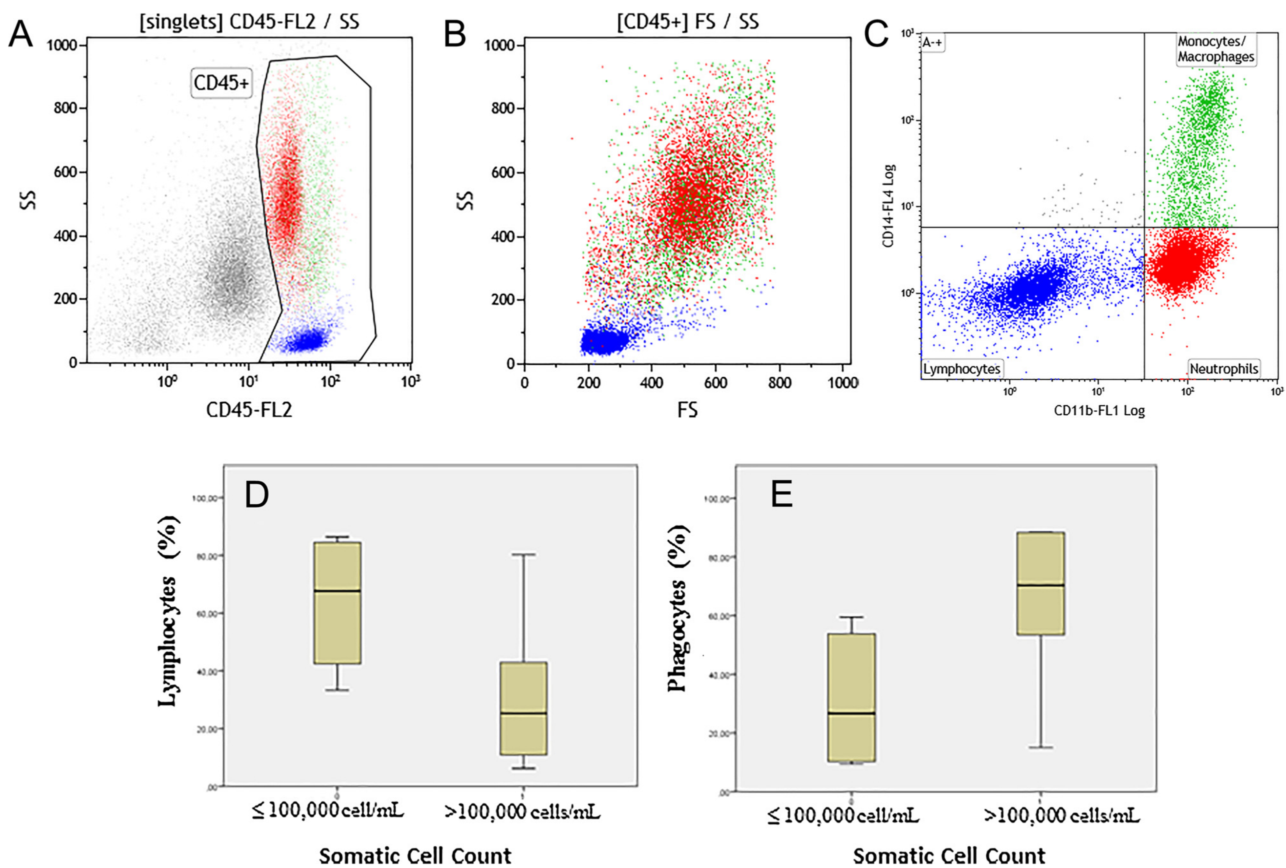

Fig. 4. Identification of main leukocyte populations in milk samples by flow cytometric analysis.
A) The anti-bovine CD45 antibody was used for the detection of milk leukocytes and a gate around $CD45^+$cells was drawn. Fourteen thousand $CD45^{high}$ cells from each sample were acquired for the analysis. B) Dot plot showing the back gate of $CD45^+$ cells in a forward scatter (FSC) *vs* side scatter (SSC); C) Dot plot showing percentages of lymphocytes, monocytes/macrophages and PMN leukocytes. Lymphocytes were identified by size and granularity (FSC-H *vs* SSC-H) and as $CD45^+$/ $CD11b^{low/-}$/CD14- cells. Phagocytes were calculate as the sum of PMN leukocytes and monocytes/macrophages originated from the analysis. D) and E) Boxplots showing the percentages of lymphocytes and phagocytes detected in two SCC categories of quarter milk samples, SCC ≤ 100,000 cells/mL and SCC > 100,000 cells/mL. Results shown in Table 3 are reported as mean ± SD.

Besides the determination of SCC, we analyzed the cellular composition of the leukocyte population in milk from udder quarters of cows in healthy (SCC ≤ 100,000 cells/mL) and potential inflammatory conditions (SCC > 100,000 cells/mL).

Flow cytometry analysis **(**Fig. 4A–**C)** showed that quarters with SCC > 100,000 cell/mL mainly consisted of phagocytes (64.30 ± 27.50) while lymphocytes were lower (31.8 ± 27.01). On the other hand, in milk samples with SCC ≤ 100,000 cell/mL lymphocytes were the predominant cell type (63.65 ± 23.41) compared to the phagocytes population (31.04 ± 22.59). The difference between the percentage of lymphocytes and phagocytes was significant both in healthy (*p-value* = 0.05) and infected quarters (*p-value* = 0.05) (Fig. 4D**, E** and Table 3).

4. Discussion

This study provides for the first time insights on PARP1 expression and its activity in dairy cattle. Our results show that within PBMC population PARP1 is highly expressed in lymphocytes as compared to monocytes.

This result is in accordance with the findings that human monocytes show low expression of four DNA repair proteins, including PARP1, as reported by Bauer et al., (2012). Despite the low expression of PARP1 in monocytes, Aldinucci et al., (2007) reported that PARP1 activity plays a key role during human dendritic cell maturation and Hauschildt et al., (1997) showed that poly(ADP-ribosyl)ation is a crucial protein modification involved in intracellular processes leading to activation of monocytes/macrophages.

Over the last decade, a number of studies have verified the role of PARP activation in a wide range of pathophysiological conditions,

Table 3
Percentage of leukocyte populations in milk samples coming from udder quarters with differential SCC.

	SCC ≤ 100,000 cells/mL		SCC > 100,000 cells/mL		*P-value*
	(n = 6)		(n = 6)		
	Mean	SD	Mean	SD	
Lymphocytes (%)	63.65	23.41	31.8	27.01	0.05
Phagocytes (%)	31.07	22.59	64.30	27.50	0.05

including inflammation. Several studies on human inflammatory diseases have reported the involvement of poly(ADP-ribosyl)ation and PARP1, both locally and in circulating leukocytes (Murthy et al., 2004; Tempera et al., 2005; Grecchi et al., 2012). Furthermore, *in vivo* and *in vitro* studies demonstrated that PARP1 activation is involved in the cellular response to oxidative DNA damage in equine peripheral blood mononuclear cells, and suggest PARP inhibitors as potential therapeutic agents for intestinal ischemia-reperfusion injury (Douglas et al., 2015).

Taking into consideration the involvement of PARP1 in inflammatory conditions, we decided to investigate whether poly(ADP-ribosyl)ation increased during udder inflammation in dairy cattle both on PBMC and cells isolated from milk of potentially infected cows.

Cells isolated from quarter milk were divided based on the already described 100,000 cells/mL SCC threshold. The SCC is a well-established, robust and quantitative measurement in the diagnosis of mastitis, as SCC increases evidently during mastitis, and the composition of the leukocytes population changes significantly (Schwarz et al., 2011).

Interestingly, we can observe PAR accumulation in PBMC from diseased cows udder and significantly higher levels of PAR in quarters with higher SCC relative to quarters with lower SCC, supporting the conclusion that PARP1 activity increases significantly during mastitis (*p-value* < 0.03).

Characterization of milk cell population showed that quarters with high SCC exhibit a significant increase in phagocytes (combined proportion of PMN and monocytes/macrophages), which are cells involved in the inflammatory response. Phagocytes are the first line of defence at tissue level as they migrate from the blood to the peripheral tissues in response to infectious and inflammatory stimuli.

Ours findings show therefore that increased SCC is associated with PARP1 activation during bovine mastitis. Considering that monocytes are important players in the inflammatory response, the significant high PAR level detected in somatic cells purified from quarters with SCC $>$ 100,000 cells/mL could be ascribed also to PARP1 activation in the increased proportion of monocytes/macrophages in spite of their lower expression of the enzyme respect to lymphocytes. Thus, the high PAR level, resulting from involvement of both lymphocytes and monocytes in PARP activity, is associated with a change in cell composition in the high SCC group. The importance of the role played by PAR synthesis in inflammation is supported by a recent paper showing the presence of extracellular PAR derived from necrosis of macrophages with hyperactivated PARP1. Authors have provided evidence of a novel function for PAR as a signal for macrophages to engage the immune system at the site of infection (Krukenberg et al., 2015).

In summary, our results show that (i) bovine PBMC express PARP1; (ii) lymphocytes exhibit higher expression of PARP1 than monocytes; (iii) PARP1 and PAR levels are higher in circulating PBMC of infected cows and (iv) PAR levels are higher in cells isolated from milk with higher somatic cell count (SCC $>$ 100,000cells/mL).

Our data suggest an association between PARP1 activation and mastitis, although further investigation will be required to define the role of poly(ADP-ribosyl)ation in udder inflammation of dairy cattle. It would be of particular interest to verify if PAR level in milk somatic cells could have predictive value for diagnosing mastitis.

Competing interests

The authors declare that they have no competing interests.

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