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ORIGINAL PAPER

Comparison of the effect of recombinant bovine wild and mutant lipopolysaccharide-binding protein in lipopolysaccharide-challenged bovine mammary epithelial cells

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Abstract Lipopolysaccharide (LPS)-binding protein (LBP) plays a crucial role in the recognition of bacterial components, such as LPS that causes an immune response. The aim of this study was to compare the different effects of recombinant bovine wild LBP and mutant LBP (67 Ala → Thr) on the LPS-induced inflammatory response of bovine mammary epithelial cells (BMECs). When BMECs were treated with various concentrations of recombinant bovine lipopolysaccharide-binding protein (RBLBP) (1, 5, 10, and 15 μg/mL) for 12 h, RBLBP of 5 μg/mL increased the apoptosis of BMECs induced by LPS without cytotoxicity, and mutant LBP resulted in a higher cell apoptosis than wild LBP did. By gene-chip microarray and bioinformatics, the data identified 2306 differentially expressed genes that were changed significantly between the LPS-induced inflamed BMECs treated with 5 μg/mL of mutant LBP and the BMECs only treated with 10 μg/mL of LPS (fold change ≥2). Meanwhile, 1585 genes were differently expressed between the inflamed BMECs treated with 5 μg/mL of wild LBP and 10 μg/mL of LPS-treated BMECs. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses showed that these differentially expressed genes were involved in different pathways that regulate the inflammation response. It predicted that carriers of this mutation increase the risk for a more severe inflammatory response. Our study provides an overview of the gene expression profile between wild LBP and mutant LBP on the LPS-induced inflammatory response of BMECs, which will lead to further understanding of the potential effects of LBP mutations on bovine mammary glands.

Keywords Bovine mammary epithelial cells · Lipopolysaccharide · Bovine recombinant lipopolysaccharide-binding protein · Microarray analysis

Introduction

Bovine mastitis, caused by a wide array of microorganisms, including Gram-negative pathogen such as *Escherichia coli* (*E. coli*), is one of the most costly diseases in the dairy industry due to its ability to reduce milk yield and quality (Hu et al. 2016). Lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, has been identified as a vital virulence factor for mastitis (Ibeagha-Awemu et al. 2008). Lipopolysaccharide-binding protein (LBP), as an acute-phase protein, was involved in host response to Gram-negative and Gram-positive pathogens (Rahman et al. 2010). LBP is a 50-kDa polypeptide synthesized mainly by hepatocytes and released into the bloodstream as a 60–65-kDa glycosylated protein (Rahman et al. 2010). It has a dual role depending on its relatively low or high concentration, and it augments or downregulates the innate host defense accordingly (Ding and Jin 2014). Low concentrations of LBP enhance the LPS-induced immune response, whereas high concentrations of LBP can inhibit LPS bioactivity in vitro and in vivo (Gutsmann et al. 2001; Kitchens and Thompson 2005). LBP levels increased in the blood and milk of mid-lactating Holstein cows when challenged with LPS. Basal levels of plasma and milk LBP were 38 and 6 μg/ml, respectively. Plasma and milk LBP levels increased and reached maximal levels of 138 μg/ml and 34 μg/ml by 24 h following LPS challenge (Bannerman et al. 2003). The concentrations of

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LBP were greater in the milk and blood of cows with naturally occurring mastitis than in those with healthy quarters (Zeng et al. 2009). Serum concentrations of LBP have been shown to be significantly correlated with serum levels of proinflammatory cytokines (Gonzalez-Quintela et al. 2013). One clinical study found that genetic variations within the LBP promoter leading to higher LBP concentrations correlated with susceptibility to Gram-negative infections and worse outcome (Chien et al. 2008). These data demonstrate that it is meaningful to investigate the utility of LBP, including wild and mutant LBP, as a biomarker to indicate activation of innate immune responses to microbial products in mastitis.

LBP binds to the amphipathic lipid A moiety of LPS with high affinity and presents the LPS to pattern-recognition receptors CD14, initiating signal transduction pathways leading to the activation of the inflammatory host response (Idoate et al. 2015). A polypeptide with a threonine (T)-to-methionine (M) mutation in amino acid 287 of LBP had high anti-endotoxin activity in vitro and in vivo, which suggested that amino acid 287 in the C-terminus of LBP may play an important role in LBP binding with CD14 (Fang et al. 2014). A synthetic peptide containing the N-terminal tip region of LBP has LPS-blocking activity, suggesting that the LBP binding site for LPS is located in the N-terminus of LBP (Lamping et al. 1996). The amino acid exchange at position 333 of LBP leading to a reduced ability to recognize pathogenic bacteria via LPS or lipopeptides results in a diminished inflammatory response after bacterial challenge (Eckert et al. 2013). Therefore, new findings on natural genetic variations of LBP leading to functional consequences may help in further elucidating the mechanism of LBP and its role in innate immunity and mastitis.

The 3D structure of LBP possesses an unusual *boomerang* shape including two apolar pockets, and 67Ala → Thr was in the apolar pocket near the interface of the barrels and the central β-sheet. The motif analysis showed that the mutation of 67Ala → Thr formed a new protein kinase C (PKC) phosphorylation site, which may be involved in phosphorylation binding. Both 43Ile → Thr and 67Ala → Thr made hydrophobic amino acids turn into hydrophilic amino acids (Bartel 2004). Therefore, the mutation of LBP in 67Ala → Thr may affect the structure of LBP. It is an interesting analysis for the influences of variations in the LBP gene, having a better understanding of the effects of LBP mutations on cows' susceptibility to clinical mastitis. We hypothesized that genetic variation in the LBP gene (67Ala → Thr) may disturb the structure of LBP and further influence the risk for immune responses.

LBP plays a vital role in modulating the innate immune response against bacteria, which is a major cause of bovine clinical mastitis, by two manners: concentration and structure, however, little is known about the effects of LBP mutation (67Ala → Thr) on cows' susceptibility to clinical mastitis. The aim of this study was to investigate the effects of the recombinant wild LBP and mutant LBP at the same concentrations against LPS-induced inflammatory injury of the bovine mammary epithelial cells (BMECs), and to clarify the possible mechanisms.

Material and methods

Cell culture

The study was approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University and performed in accordance with the Guidelines for Experimental Animals of the Ministry of Science and Technology (Beijing, China). Mammary tissue was collected from healthy, uninfected Chinese Holstein cows from a local slaughterhouse. In vitro cultures of BMECs were prepared in accordance with the established methods by Dairy Science Institute of Nanjing Agricultural University (Zhao et al. 2010). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 mg/ml glucose and supplemented with 10 % fetal bovine serum (Life Technologies, Gaithersburg, MD, USA) and 1 % antibiotic-antimycotic solution (Sigma-Aldrich, St. Louis, MO, USA) in a humidified incubator, with 5 % CO_2 at 37 °C. After reaching 80 % confluence, the cells were removed with 0.25 % trypsin and 0.15 % trypsin plus 0.02 % EDTA.

Recombinant lipopolysaccharide-binding protein treatment of cultured cells

Recombinant lipopolysaccharide-binding protein (RBLBP), both wild and mutant LBP, has been overexpressed on an eukaryotic system by Sangon Biotech Co. Ltd. Wild and mutant LBP genes was synthesized and then cloned to eukaryotic expression vector pcDNA3.1 (+). The cloning site is Hind III/Xho I. The recombinant plasmid pcDNA3.1 was transfected into HEK293 cells and the target proteins were produced by transient expression. RBLBP was expressed in mammalian cells, HEK293 cells, which ensures the proper folding, glycosylation, gamma-carboxylation, and other post-translational modification. Therefore, the bioactivity of RBLBP is as much as possible similar to the native LBP. Flag as protein purification tag is added to the C-terminus of LBP. The recombinant proteins are purified to above 95 % purity, suitable for in vitro experiments (Sun et al. 2015). The recombinant proteins were purified and then verified by SDS-PAGE (Fig. 1a) and western blotting (Fig. 1b). Endotoxin in the purified proteins after toxin removal was less than 0.1 EU/mg.

RBLBP and LPS (*E. coli* serotype O55:B5, Sigma-Aldrich) were diluted in DMEM (1 mg/mL). Prior to LPS or RBLBP treatment, the BMECs were cultured with serum-free medium for 24 h. Control cells were incubated in serum-free DMEM. LPS cells were stimulated with 10 μg/mL LPS.

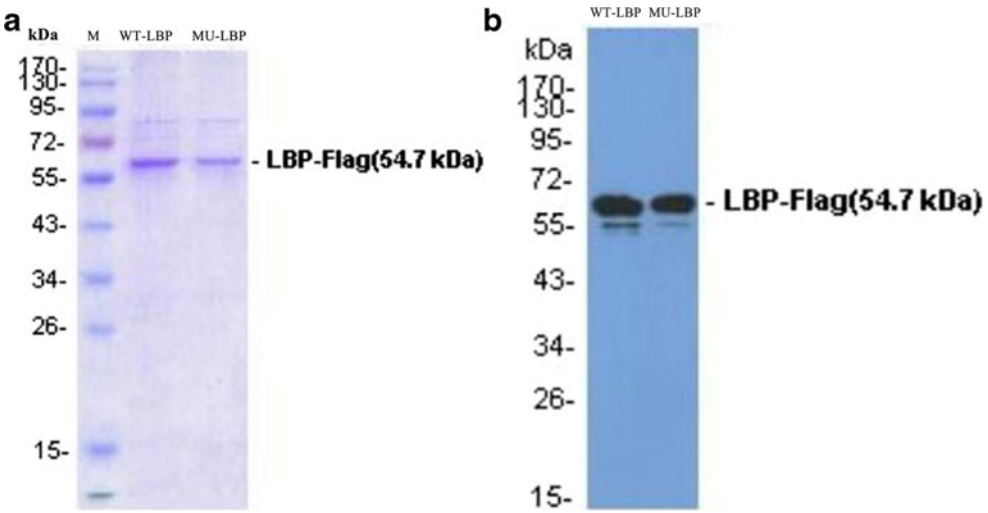

Fig. 1 The recombinant proteins were purified and then verified by SDS-PAGE (**a**) and western blotting (**b**)

LPS + WT-LBP cells were treated first with 10 μg/mL LPS followed immediately with increasing concentrations (1, 5, 10, and 15 μg/mL) of wild LBP. LPS + MU-LBP cells were treated first with 10 μg/mL LPS followed immediately with increasing concentrations (1, 5, 10, and 15 μg/mL) of mutant LBP. Then, cells were incubated at 37 °C in 5 % CO2 for 12 h and than samples were collected.

Identification of bovine mammary epithelial cell

Positive staining of cytokeratin-18 was conducted in the epithelial cells by the immunocytochemical staining method (Fig. 2). BMECs grew on a sterile microscope coverslip held in a six-well tissue-culture plate. After reaching 70–80 % confluence, the cells were fixed at room temperature (RT) for 30 min in phosphate-buffered saline (PBS, pH 7.5) containing 4 % paraformaldehyde (*w*/*v*) and then transferred to a permeabilization solution (1 % Triton X-100, [*v*/*v*] in PBS) for 10 min at 37 °C. After blocking for 1 h in PBS supplemented with 2 % BSA (*w*/*v*), the cells were incubated for 4 h at RT with anti-cytokeratin 18 antibody (1:200; Boster Biotechnologies, Wuhan, China). The cells were then washed three times in washing buffer (0.1 % Tween 20 in PBS, *v*/*v*) and incubated for 1 h at RT with goat anti-rabbit IgG-FITC (1:100; Santa Cruz Biotechnologies, CA, USA). The cells were then stained with DAPI (Nanjing KeyGEN Biotech. Co., Nanjing, China) for 10 min, washed three times in washing buffer, mounted onto slides, and then examined with a Zeiss LSM700 META confocal laser scanning microscope (Zeiss, Jena, Germany).

Cell viability assay

After the cells were treated with different concentrations of RBLBP (1, 5, 10, 15 μg/ml) for 12 h, the medium was removed and 200 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) medium (0.5 mg/ml MTT reagent in fresh medium) was added to each well. After incubation at 37 °C for 4 h, the MTT reagent was removed and 150 μl of dimethyl sulfoxide (Jiancheng Bioengineering Institute, Nanjing, China) was added to each well, followed by 10 min of gentle shaking. Cell viability was assessed by a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide trypan blue exclusion assay as the proportion of absorbance values to the control. The absorbance was read at a wavelength of 570 nm in a Multiskan MK3 (Thermo Electron Corporation, USA) as the value expressing the entity of proliferation.

Analysis of cell apoptosis with flow cytometry

The cells were treated with LPS or RBLBP based on the experimental requirements. Each treatment was repeated three times. The cells in each group were collected, and apoptosis was assayed by an annexin V/PI double staining kit (BD Pharmingen, CAT: 556547).

RNA isolation

Total RNA was extracted from the BMECs using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The absorbance values at 260 and 280 nm were obtained to assess RNA concentration and purity, respectively, in the samples. RNA integrity was assessed by electrophoresis on 2 % agarose gels (*m*/*v*).

Microarray assay

Gene-chip analysis of the Bovine Genome Array was performed by an outside service provider (Shanghai

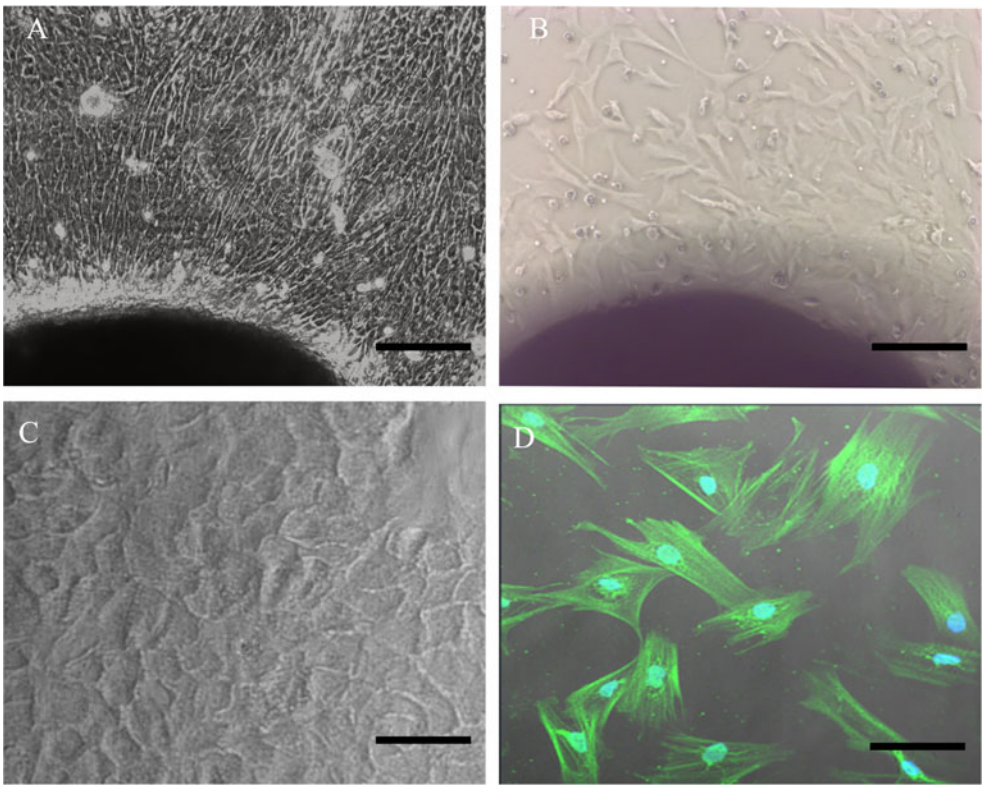

Fig. 2 The isolation and identification of BMECs. **a** Fibroblast emerging from the tissue margins. **b** Fibroblasts and colony epithelial cells. **c** Purified epithelial cells obtained over three passages. **d** Immunofluorescence of cytokeratin 18. *Scale bar* in **a** 200 μm, **b** 100 μm, **c** 50 μm, and **d** 20 μm

Biotechnology Corporation). Total RNA was extracted using Trizol Reagent (Cat#15596-018, Life technologies, Carlsbad, CA, US) following the manufacturer's instructions and checked for a RIN number to inspect RNA integration by an Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, US). Quantified total RNA was further purified by RNeasy micro kit (Cat#74004, QIAGEN, GmBH, Germany) and RNase-Free DNase Set (Cat#79254,QIAGEN, GmBH, Germany). Total RNA was amplified and labeled by Low Input Quick Amp Labeling Kit, One-Color (Cat#5190-2305, Agilent technologies, Santa Clara, CA, US), following the manufacturer's instructions. Briefly, in the first-strand complementary DNA (cDNA) synthesis reaction, 10 μg total of RNA was used for reverse transcription using a T7-oligo (dT) promoter primer. Then, the double-stranded cDNA was synthesized from the first-strand cDNA using RNase H. Labeled cRNA were purified by RNeasy mini kit (Cat#74106, QIAGEN, GmBH, Germany). Each Slide was hybridized with 1.65 μg Cy3-labeled cRNA using Gene Expression Hybridization Kit (Cat#5188-5242, Agilent technologies, Santa Clara, CA, US) in Hybridization Oven (Cat#G2545A, Agilent technologies, Santa Clara, CA, USA), according to the manufacturer's instructions. After 17 h hybridization, slides were washed in staining dishes (Cat#121,Thermo Shandon,Waltham, MA, US) with Gene Expression Wash Buffer Kit (Cat#5188-5327, Agilent technologies, Santa Clara, CA, US), following the manufacturer's instructions. Slides were scanned by Agilent Microarray Scanner (Cat#G2565CA, Agilent technologies, Santa Clara, CA, US) with default settings, Dye channel: Green, Scan resolution = 5 μm, PMT 100 %, 10 %, 16bit. Data were extracted with Feature Extraction software 10.7 (Agilent technologies, Santa Clara, CA, US). Raw data were normalized by Quantile algorithm, Gene Spring Software 11.0 (Agilent technologies, Santa Clara, CA, US). The gene expression level that had a ≥2-fold difference between BMECS treated with wild LBP and mutant LBP was checked and further analyzed. The Molecule Annotation System (http://david.abcc.ncifcrf.gov) was used to analyze the differentially expressed genes using the KEGG public pathway resource and the GO consortium.

Real-time reverse transcription-polymerase chain reaction

Reverse transcription-polymerase chain reaction (RT-PCR) was performed to confirm the microarray results. Total RNA was extracted from wild LBP- and mutant LBP-treated BMECs stimulated with LPS, as described above, and total RNA was reverse-transcribed using a Reverse Transcription Levels kit (Takara, Dalian, China) according to the manufacturer's protocol. The expression levels were checked for ten genes. The housekeeping gene 18S ribosomal RNA (rRNA) was used as the invariant control as it is stable in all cases and has low regulation by external influences (Wu et al. 2015), so the background is more stable in RT-PCR. Primers were

designed using Premier 5.0 and shown in Table 1. RT-PCR was performed with SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd., Japan). The reaction solution was prepared on ice, and consisted of 10 μL of 2× SYBR Premix Ex Taq, 0.8 μL of PCR forward primer (10 μM), 0.8 μL of PCR reverse primer (10 μM), 0.4 mL of 50× ROX reference dye, 2 μL of cDNA (100 ng μL^{-1}) and dH_2O to a final volume of 20 L. The reaction mixtures were incubated in a 96-well plate at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 60 °C for 30 s and 72 °C for 30 s. All reactions were performed in triplicate. The gene expression levels were analyzed with the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

All data were obtained from one independent experiment carried out in triplicate. The fold changes of genes between wild LBP and mutant LBP BMECs were calculated using fold change $=2^{-\Delta\Delta Ct(WT\text{-}LBP\text{-}MU\text{-}LBP)}=2^{-(\Delta\ Ct(WT\text{-}LBP)\ -\ \Delta\ Ct(MU\text{-}LBP))}$ (Livak and Schmittgen 2001). The main and interactive effects were analyzed by the independent-samples *t* test using SPSS 16.0 software. $P<0.05$ was considered statistically significant.

Results

Cytotoxicity of RBLBP on BMECs

The results of MTT showed that different concentrations (1, 5, 10, and 15 μg/mL) of RBLBP had no effect on BMEC viability after 12 h and 24 h in vitro treatment (Fig. 3). This showed that RBLBP has no cytotoxic effect on BMEC survival in vitro.

RBLBP and LPS-induced inflammatory injury

MTT assays and flow cytometric analysis showed that the cell viability was reduced and apoptosis was increased after 10 μg/mL of LPS stimulation for 12 h on BMEC. Therefore, LPS-induced the inflammatory injury of BMECs. MTT assays showed that the addition of 1 or 5 μg/mL of RBLBP reduced the total cell viability of inflammatory BMECs stimulated by LPS and cell viability was increased at 10 or 15 μg/mL of RBLBP (Fig. 4a, b). Flow cytometric analysis showed that 1 or 5 μg /mL of RBLBP increased the prevalence of apoptosis among inflammatory BMECs stimulated by LPS. In contrast, 10 or 15 μg/mL of RBLBP diminished the prevalence of apoptosis among inflammatory BMECs (Fig. 5a, b). Therefore, RBLBP regulates the LPS-induced inflammatory injury of BMECs.

In a comparison to the results of MTT and flow cytometry, respectively, when inflammatory cells were treated with wild LBP and mutant LBP at 5 μg/mL, the mutant LBP resulted in lower viability and higher apoptosis of the cells (Fig. 6a, b), which may suggest that mutant LBP induced a stronger inflammatory response than wild LBP.

Differential gene expression

The global gene expression profiles in BMECs samples representing mutant LBP-treated and wild LBP-treated cells were identified with the microarray technique. The total number of significantly differentially expressed genes when comparing LPS + WT-LBP cells with LPS cells was 1585 ($P<0.05$), of which 862 genes were highly expressed and 723 were significantly under-expressed (≥2-fold). In contrast, comparison of LPS + MU-LBP cells with LPS cells revealed that a total of 2,306 genes were significantly regulated ($P<0.05$), of which, 1,285 genes were upregulated and 1,021 were downregulated (≥2-fold). Both up- and downregulated genes were showed in Table 2. The global characteristics of LBP-specific transcriptome profiling showed that the number of differentially expressed genes was higher for inflammatory cells after the challenge with mutant LBP than with wild LBP. The genes significantly differentially expressed between LPS-induced inflammation BMECs treated with wild LBP and mutant LBP were showed in Table 3. The genes important for inflammatory response, such as cytokin, transcription regulators, cytokine receptors, pattern-recognition receptors, and transcriptional regulators and so on, were regulated differently on inflammatory BMECs treated with wild or mutant LBP.

Results of GO and KEGG analyses

In order to clarify the different biological patterns of the two groups, genes differently expressed were individually analyzed using GO and the KEGG, with the criterion for significance set at $P<0.05$.

The GO analysis showed that the differently expressed genes in LPS + WT-LBP cells versus LPS cells (Fig. 7a) were mainly implicated in the acute-phase response, positive regulation of the I-κB kinase/NF-κB cascade, positive regulation of signal transduction, positive regulation of the cellular biosynthetic process, positive regulation of the response to stimulus, the cell cycle, the endomembrane system and enzyme activator activity. The differently expressed genes between LPS + MU-LBP cells and LPS cells (Fig. 7b) were largely associated with activation of the immune response, the MAPK kinase kinase (MAPKKK) cascade, regulation of protein kinase activity, the inflammatory response, regulation of the acute inflammatory response, the immune response-regulating cell surface receptor signalling pathway, immune response-activating signal transduction, the endomembrane system, unfolded protein binding, and enzyme activator activity.

Table 1 Primer sequences for RT-PCR

Gene	GenBank access no.	Product size (bp)	Primer sequence (5–3) sense/antisense
TNF	NM_173966	193	CATCCTGTCTGCCATCAAGA
			GGCGATGATCCCAAAGTAGA
STAT3	NM_001012671.2	84	CTGCAGCAGAAGGTTAGCTACAAA
			TTCTAAACAGCTCCACGATTCTCTC
TAB1	NM_001102057	134	GTTCTCTTACCCCACAGCCT
			ACCCGCATTTGGAGAAACAC
PARD6G	NM_001098100	288	TCCATCATCGACGTGGACAT
			GCTGTTGGCGATCATCATGT
CXCR5	XM_010812421	292	AGAACCAAGCCGAAACCAAC
			ATGGCCATGGAGAGATAGCC
PTK2B	NM_001102252	148	AGGGGTTACAAAGAGGCTCC
			AAGGACTAGCTTGGTTCCCC
ADCY9	XM_005224482.2	89	TCCTGGTATTCGCCCTGAC
			AGCCCGAGTATGATTGAAGTTGT
CSF2	NM_174027	206	ACTACGAGAAACACTGCCCA
			TGGCTCTTTGTGGGTAGGAG
TNFRSF9	NM_001035336	123	GAACATGGCATCTGTCGACC
			TGCACCTGGAGAGAAGTCAG
CXCR1	NM_174360	131	TCCCTGTGAGATAAGCACTGAGACA
			AGCGACCAATCCGGCTGTA

Gene expression changes that reached statistical significance were analyzed in KEGG. These regulated genes in LPS + WT-LBP cells versus LPS cells (Fig. 8a) are associated with the following signalling pathways: cytokine-cytokine receptor interaction, the GnRH signalling pathway, ECM-receptor interaction, the calcium signalling pathway, focal adhesion, glutathione metabolism, and glycine, serine, and threonine metabolism. In contrast, regulated genes between LPS + WT-LBP cells and LPS cells (Fig. 8b) are associated with the following signalling pathways: cytokine-cytokine receptor interaction, the GnRH signalling pathway, ECM-receptor interaction, the calcium signalling pathway, focal adhesion, the MAPK signalling pathway, the TLR signalling pathway, the chemokine signalling pathway and the phosphatidylinositol signalling system.

Validation of microarray results

Figure 9a shows the quantitative RT-PCR results for some of the genes which were significantly affected by mutant LBP treatment of BMECs. Three genes were associated with the TLR signalling pathway, including TNF, STAT1, and TAB1, and four genes were associated with the chemokine signalling pathway, including PARD6G, CXCR5, PTK2B, and ADCY9. Three other genes were associated with cytokine-cytokine receptor interactions, including CSF2, TNFSF9, and CXCR1 (Table 4). The RT-PCR results were in accordance with the gene-chip findings (Fig. 9b).

Discussion

Mastitis leads to immeasurable economic losses for farmers, which is an unsolved human challenge faced by all dairy farms (Hisaeda et al. 2011). The susceptibility to pathogenic bacteria among species or individuals is due to mutations of immune-function-related genes (Beutler and Poltorak 2000; Michel et al. 2003; Smirnova et al. 2000). The acute-phase protein LBP, as a general recognition molecule, plays

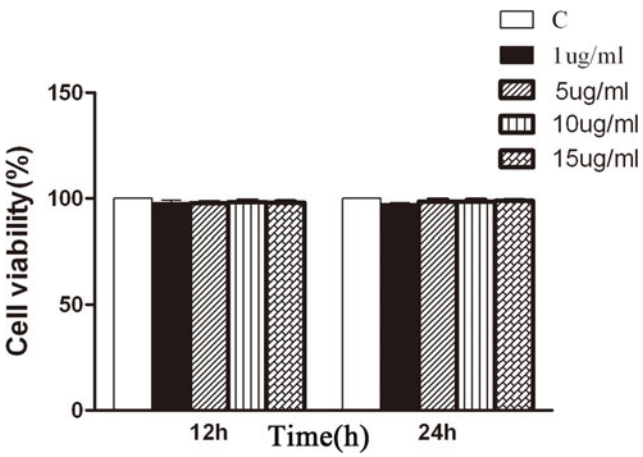

Fig. 3 Cytotoxicity of RBLBP to cells. Cell viability was measured using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) after treatment with various concentrations of RBLBP (1, 5, 10, and 15 μg/mL) for 12 and 24 h. The data are means ± SEM ($n = 3$). *$P < 0.05$ vs. control group; **$P < 0.01$ vs. control group. *Con* Control cells without any processing

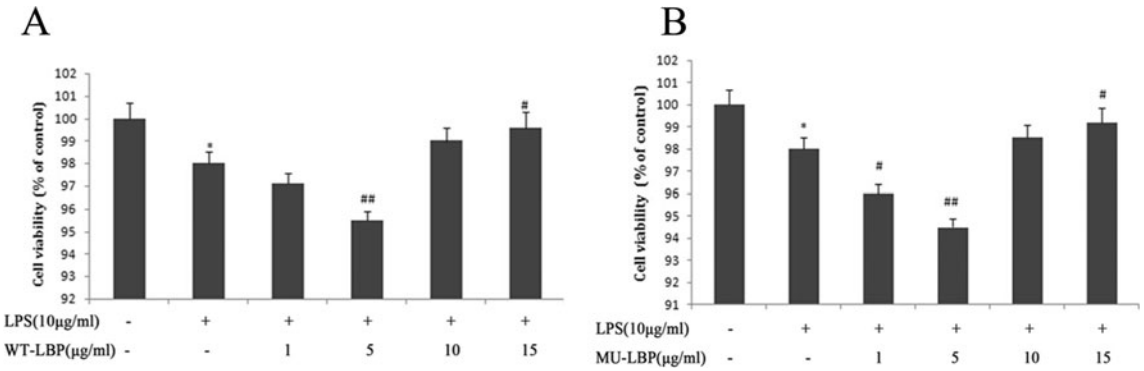

Fig. 4 Effects of RBLBP on cell viability induced by LPS. Cells were treated with the indicated concentrations (1, 5, 10, and 15 μg/mL) of wild LBP (**a**) or mutant LBP (**b**) and stimulated with LPS of 10 μg/mL for 12 h. Cell viability was estimated by MTT. The data are means ± SEM ($n = 3$). $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. group treated with LPS alone; $^{\#\#}P < 0.01$ vs. group treated with LPS alone

important roles in modulating the innate immune response against bacteria (Zweigner et al. 2006). LBP's concentration-dependent immunologic function and structural integrity must require precise genetic regulation of gene transcription, suggesting that genetic variation in the elements controlling LBP production or structure may affect an individual's immune

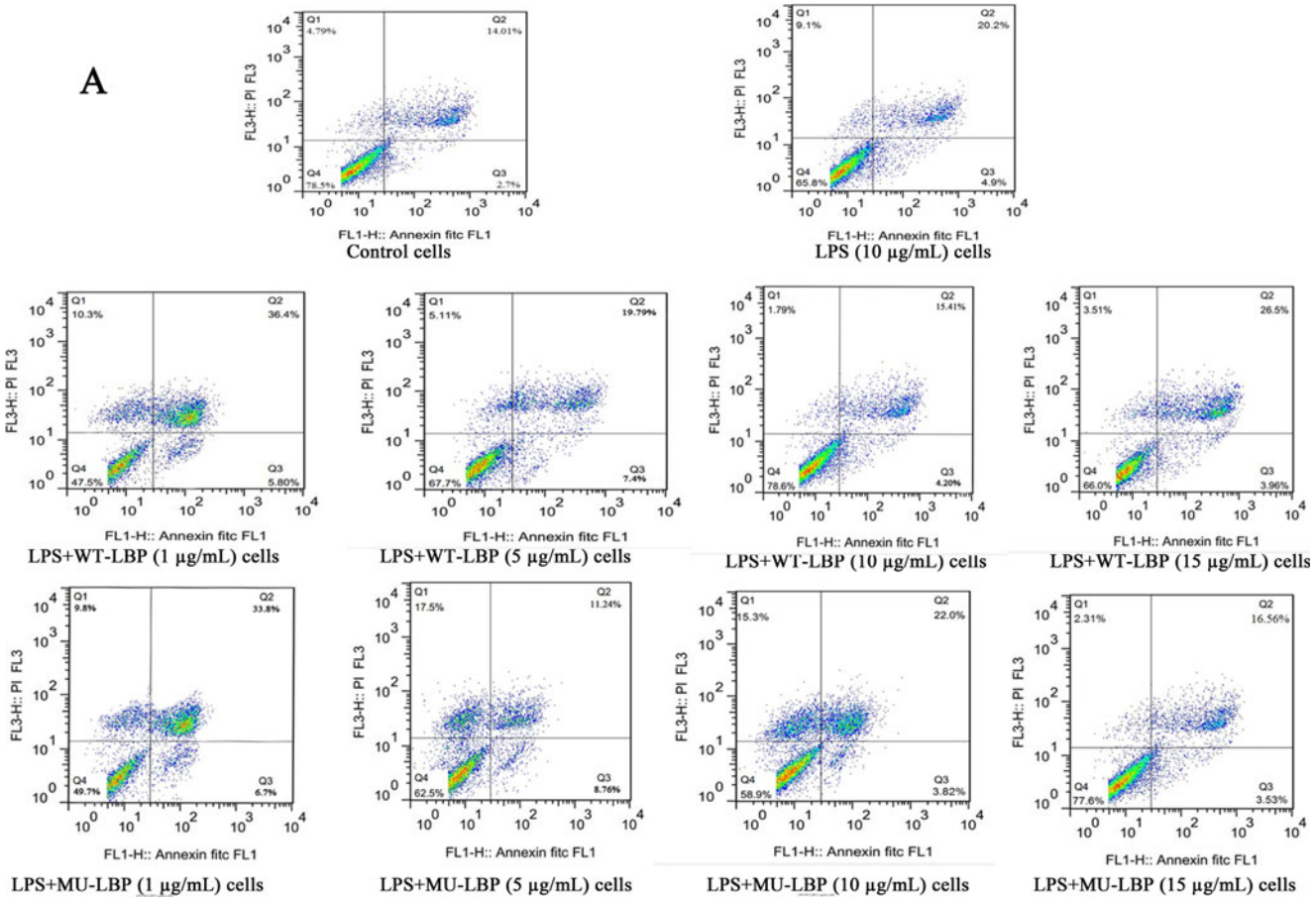

Fig. 5 Effects of RBLBP on cell apoptosis induced by LPS. **a** After cells were treated with RBLBP or LPS, the relative number of cells undergoing apoptosis was determined by flow cytometry after annexin V/propidium iodide (PI) staining. Control cells, incubated in serum-free DMEM. LPS cells, stimulated with 10 μg/mL LPS. LPS + WT-LBP (1 μg/mL) cells, LPS + WT-LBP (5 μg/mL) cells, LPS + WT-LBP (10 μg/mL) cells, and LPS + WT-LBP (15 μg/mL) cells, treated first with 10 μg/mL LPS followed immediately with increasing concentrations, as indicated, of wild type LBP. LPS + MU-LBP (1 μg/mL) cells, LPS + MU-LBP (5 μg/mL) cells, LPS + MU-LBP (10 μg/mL) cells, and LPS + MU-LBP (15 μg/mL) cells, treated first with 10 μg/mL LPS followed immediately with increasing concentrations, as indicated, of mutant LBP. **b**, **c** The analysis of the apoptosis rates of panel **a**. The data are means ± SEM ($n = 3$). $^{**}P < 0.01$ vs. control group; $^{\#}P < 0.05$ vs. group treated with LPS alone; $^{\#\#}P < 0.01$ vs. group treated with LPS alone

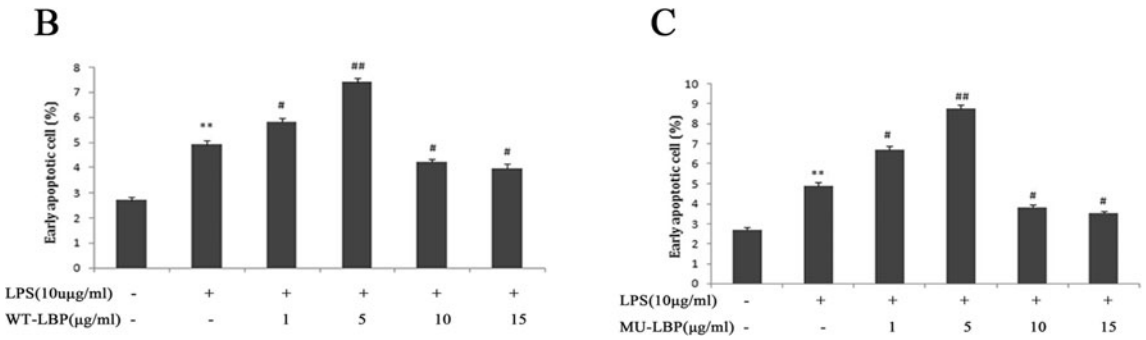

Fig. 5 (continued)

response to LPS and gram-negative bacteria. This idea is supported by a previous detailed study of truncation mutation experiments of the LBP promoter region, which indicate that a region of the LBP promoter is responsible for regulating the efficiency of gene transcription (Nehammer et al. 2015). Genetic variation in the promoter region of the LBP gene is associated with the blood level of LBP and with the risk of developing gram-negative bacteremia and gram-negative bacteremia-related death after hematopoietic cell transplantation (HCT) (Kumar et al. 2015). In addition, several alanine-substituted synthetic LBP-derived peptides inhibited LPS-LBP interaction (Reyes et al. 2002). A mutation affecting amino acid 98, which affects an exposed loop of LBP, has been described to confer a risk for several infectious and inflammatory diseases (Shukla et al. 2011). Therefore, LBP or mutant LBP may serve as a clinical marker in severe infections and may carry therapeutic potential.

Mammary epithelial cells (MECs) are poised to respond quickly to bacterial intrusion through the activation of several pattern-recognition receptors (PRRs), notably the well-analyzed family of Toll-like receptors (TLRs) (Kumar et al. 2009), by the so-called microbe-associated molecular patterns (MAMPs) (Chen et al. 2014; Wu et al. 2013). Bovine MECs (BMECs) in vitro culture are able to sense bacteria or bacterial products, and to react by upregulating the expression of several genes involved in the innate immune response (Chen et al. 2014; Wang et al. 2015). BMEC cultures were used as a model for udder tissue to profile the kinetics and extents of global changes in the transcriptome of BMEC after challenging them with heat-inactivated preparations of *E. coli* or *S. aureus* pathogens (Gunther et al. 2011). An inflammatory cell model has been established with 10 μg/mL of LPS stimulation on BMEC for 12 h, and the mechanism of RBLBP was studied using this model (Sun et al. 2015). Therefore, BMECs stimulated with 10 μg/mL of LPS for 12 h were used as an inflammatory model for monitoring the change of gene expression modifications to study mastitis in our experiment (Figs. 4, 5, and 6).

The aim of our study was to compare the different effects of recombinant bovine wild LBP and mutant LBP (67 Ala → Thr) on the LPS-induced inflammatory response of BMECs. The concentration of RBLBP that enhances the proinflammatory activity of cells would be needed. According to the previous studies in our laboratory (Sun et al. 2015), various concentrations (1, 5, 10, and 15 μg/mL) of RBLBP were selected for cell viability assay and cell apoptosis with flow cytometry. The results showed that the cells stimulated with LPS had a lower viability and a higher apoptosis at 5 μg/mL of RBLBP (Figs. 4 and 5) and that mutant LBP resulted in a lower

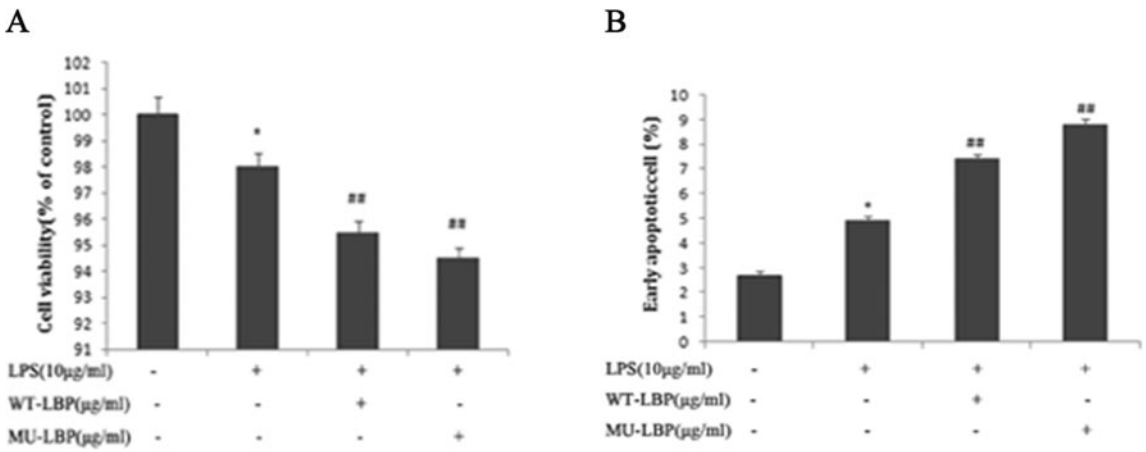

Fig. 6 The comparison of wild LBP and mutant LBP on cell viability and apoptosis. **a**, **b** Cells stimulated with LPS of 10 μg/mL for 12 h were challenged with the indicated RBLBP concentration (5 μg/mL). The data are means ± SEM ($n = 3$). $^{*}P < 0.05$ vs. control group; $^{\#\#}P < 0.01$ vs. group treated with LPS alone

Table 2 The number of differentially expressed genes (DEG)

DEG	(LPS + WT-LBP) vs LPS	(LPS + MU-LBP) vs LPS
Total	1585	2306
Upregulated	862	1285
Downregulated	723	1021

viability and a higher cell apoptosis than wild LBP did (Fig. 6a, b). This indicated that 5 μg/mL of RBLBP enhanced the proinflammatory activity of cells. Therefore, 5 μg/mL of RBLBP was selected for further microarray analyses.

Gene-chip microarrays showed that mutant LBP (67Ala → Thr) triggered a more dramatic and complex program of altering gene expression in inflamed BMECs than wild LBP did when the two RBLBPs were all at the same concentration of 5 μg/mL. It was in accordance with the results that the mutant LBP has a lower viability and a higher apoptosis on the cells than wild LBP (Fig. 6a and b). The GO and KEGG results (Figs. 7 and 8) revealed that the differently expressed genes were related to the biological process category of the terms *inflammatory response*, *defence response* and *immune response* and the molecular function terms *cytokine activity* and *chemokine activity*. GO biological process analysis revealed that GO terms related to inflammatory response, signal transduction, and regulation of response to stimulus gathered in high levels (Fig. 7a and b). However, the level in mutant LBP-treated BMEC was higher than in wild LBP-treated BMEC. The KEGG results revealed that genes related to cytokine-cytokine receptor interaction, ECM-receptor interaction, calcium signalling pathway and GnRH signalling pathway were highly expressed (Fig. 8a, b) and that the level in mutant LBP-treated BMEC was higher than in wild LBP-treated BMEC. These data suggest that mutant LBP leads to a stronger inflammatory response than wild LBP on BMECs

Table 3 The genes significantly differentially expressed between LPS-challenged cells treated with wild LBP and mutant LBP

Symbol	Gene description	Gene bank	Fold change	
			(LPS + WT-LBP) vs LPS	(LPS+ MU-LBP) vs LPS
CXCL2	Chemokine (C-X-C motif) ligand 2	XM_005208062	1.74	2.13
CXCL3	Chemokine (C-X-C motif) ligand 3	NM_001046513	1.06	1.98
CCL16	Chemokine (C-C motif) ligand 16	XM_002695627	2.07	2.56
IL1B	Interleukin 1, beta	NM_174093	2.71	3.05
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	NM_001045868	3.16	3.97
ZNFX1	Zinc finger, NFX1-type containing 1	NM_001205716	1.83	2.75
FAS	Fas cell surface death receptor	NM_174662	2.91	3.52
IL6ST	Interleukin 6 signal transducer	XM_010816768	2.74	3.45
TLR4	Toll-like receptor 4	NM_174198	3.07	3.87
NF-κB	Nuclear factor kappa light chain enhancer of activated B-cells	XM_005226181.1	4.01	4.85
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NM_001076409	2.68	3.93
CEBPD	CCAAT/enhancer binding protein (C/EBP), delta	NM_174267	3.98	4.51
BCL3	B-cell CLL/lymphoma 3	NM_001205993	2.36	3.85
NOS3	Nitric oxide synthase 3 (endothelial cell)	NM_181037	−1.67	3.57
SOD1	Superoxide dismutase 1, soluble	NM_174615	1.62	4.89
KNG1	Kininogen 1	XM_005201452	−1.48	3.50
MAPK11	Mitogen-activated protein kinase 11	NM_001080335	2.08	3.87
AKT1	v-akt murine thymoma viral oncogene homolog 1	NM_173986	3.91	6.29
TGFBR3	Late endosomal/lysosomal adaptor, MAPK and MTOR activator 3	NM_001075982	−1.36	−1.02
LAMTO3	Transforming growth factor (TGF)-beta type III receptor	XM_005204338	1.23	2.62
KRT80	Keratin 80	NM_001077952	−1.44	−1.66
CLDND1	Claudin domain containing 1	NM_001206357	−1.67	−1.04
VCL	Vinculin	BC120206	−1.01	−1.41
GJB6	Gap-junction protein6	NM_001015546	−1.17	−1.46
BCCIP	BRCA2 and CDKN1A interacting protein	NM_001046122	−1.59	−1.71

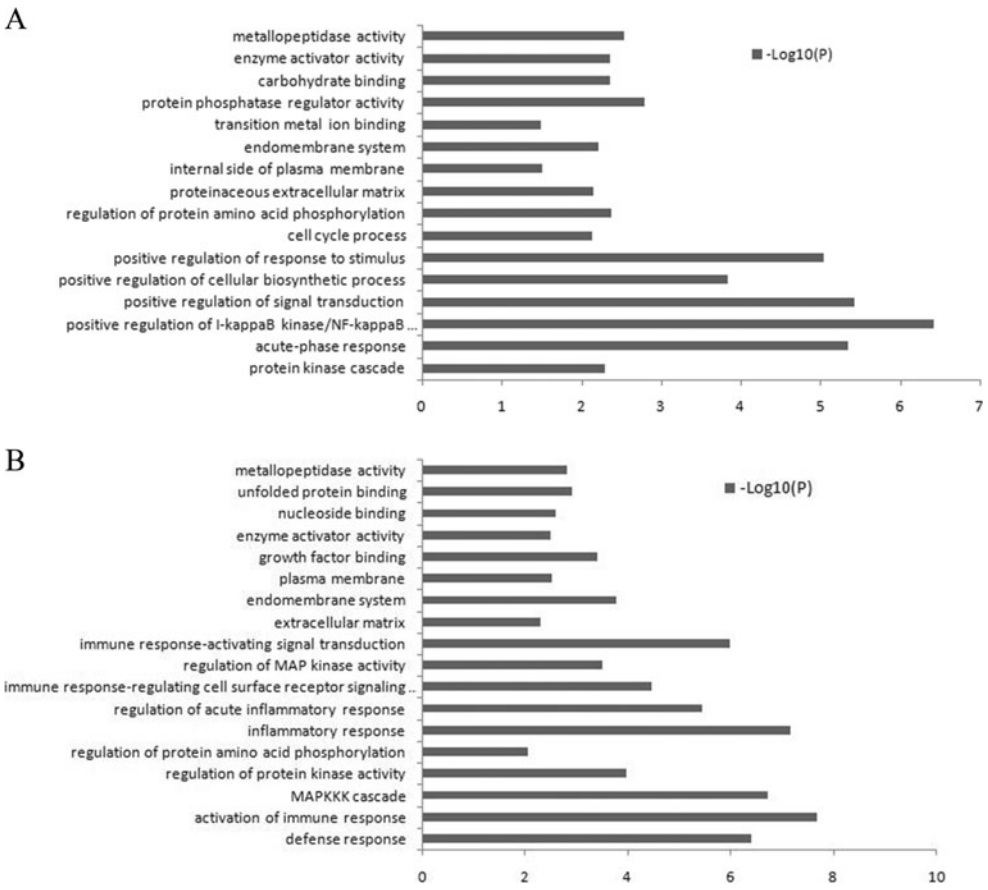

Fig. 7 Biological process analysis of genes that changed ≥2-fold in cells treated with wild LBP (**a**) and mutant LBP (**b**) by gene ontology analysis (*P* values < 0.05)

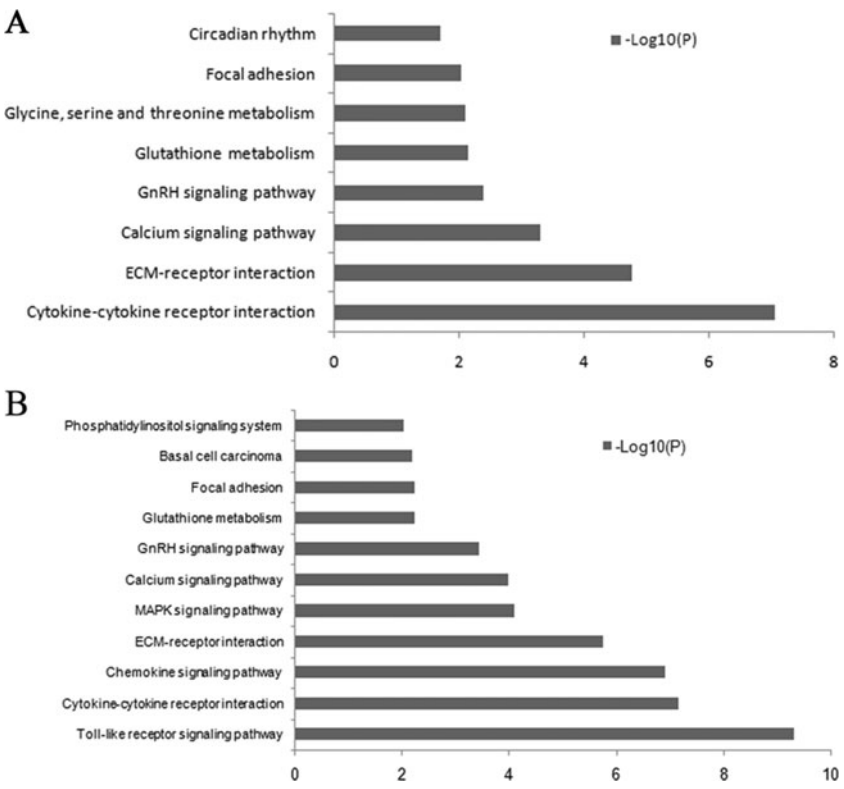

Fig. 8 Biological process analysis of genes that changed ≥2-fold in cells treated with wild LBP (**a**) and mutant LBP (**b**) by gene KEGG analysis (*P* values < 0.05)

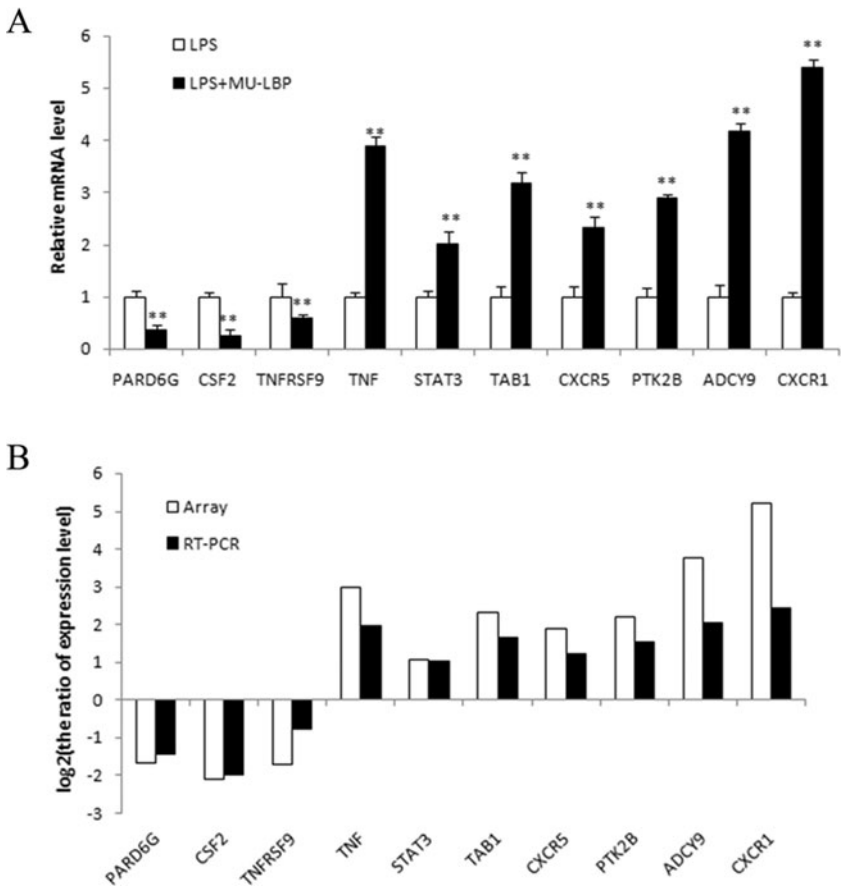

Fig. 9 Microarray results confirmed by RT-PCR. **a** RT-PCR results of genes selected. *q*RT-PCR values were determined from the $^{\Delta\Delta}$ Ct for the target genes relative to 18S. **b** Comparison of RT-PCR findings to microarray results by fold change of ten-selected genes. Note: *double asterisks* indicate a statistical difference ($P < 0.01$)

stimulated with LPS, resulting in enhanced defense during infection.

According to the role of LBP in inflammation of the cow udder, the highly expressed genes in LBP-stimulated BMECs included several molecules involved in cytokine-encoding genes (e.g., CXCL2, CXCL3, CCL16, and IL1B), transcription regulators (e.g., NFKBIA and ZNFX1), cytokine receptors (e.g., FAS, IL1RAP, and IL6ST), pattern-recognition receptors (TLR2 and TLR4) and transcriptional regulators. The latter include the NF-κB and C/EBP families of factors (e.g.,

Table 4 Selected genes involved in the inflammatory response regulated by mutant LBP

Symbol	Gene description	GenBank	Fold change
TNF	Tumor necrosis factor	NM_173966	1.002
STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)	NM_001012671	1.069
TAB1	TGF-beta activated kinase 1/MAP3K7 binding protein 1	NM_001102057	2.336
PARD6G	par-6 family cell polarity regulator gamma	NM_001098100	−1.654
CXCR5	Chemokine (C-X-C motif) receptor 5	XM_010812421	1.907
PTK2B	Protein tyrosine kinase 2 beta	NM_001102252	2.220
ADCY9	Adenylate cyclase 9	XM_005224482-.2	3.769
CSF2	Colony stimulating factor 2 (granulocyte-macrophage)	NM_174027	−2.114
TNFRSF9	Tumor necrosis factor receptor superfamily, member 9	NM_001035336	−1.725
CXCR1	Chemokine (C-X-C motif) receptor 1	NM_174360	5.227

RELB, NFKB1, BCL3, and CEBPD). The regulation of this battery of transcription factors highlights that mutant LBP provoked significant changes in the signal transduction machinery of the cells. In addition, the mRNA encoding factors important for host defense (e.g., NOS3, SOD1, and KNG1) were also maximally increased after the inflammatory cell model was stimulated with mutant LBP. Furthermore, the inflammatory response genes' relevant signalling cascades were significantly differentially expressed, including MAPK11, v-akt murine thymoma viral oncogene homolog 1 (AKT1) and transforming growth factor beta receptor 3 (TGFBR3) (Table 3). Recently, a study found that S. aureus induces apoptosis in BMECs through a Fas-FADD (Fas-associated death domain) death receptor-linked caspase-8 signalling pathway (Hu et al. 2014). Meanwhile, the genes coding MAPK11 (p38β) and LAMTOR3 (MP1) were upregulated by mutant LBP (Table 3). P38β upregulated the expression and secretion of monocyte chemotactic protein-1 (MCP-1) in breast cancer cells, and that upregulated MCP-1 activates osteoclast differentiation and activity (He et al. 2014). Endogenous MCP-1/CCL2, which is a key chemokine that can recruit monocytes, macrophages and lymphocytes by a mechanism dependent on its binding to the CCR2 receptor (Balamayooran et al. 2011), protects mice from sepsis by regulating proinflammatory and anti-inflammatory cytokine production, which shows that it promotes the balance between anti-inflammatory and proinflammatory responses to infection (Gomes et al. 2006, 2013). MP1, which with LAMTOR2 (p14) forms a heterodimer as part of the larger regulator complex that includes LAMTOR3 (MP1), LAMTOR2 (p14), LAMTOR1 (p18), LAMTOR4 (c7 or f59) and LAMTOR5 (HBXIP), is required for MAPK and mTOR1 signalling from late endosomes/lysosomes (de Araujo et al. 2013). Some results have revealed an unsuspected mechanism of mitogenic signalling activation via LAMTOR3, a scaffolding protein for ERK and MEK (Jun et al. 2013).

Aside from those genes relevant to inflammation, both mutant and wild LBP caused downregulation of genes encoding structural molecules (e.g. keratin 80 was −1.441- and −1.663-fold regulated, respectively) and the tight-junction protein complex (e.g., the claudins CLDND1, vinculin, and gap-junction protein 6, with a range of changes of expressional regulation, from −2-fold to −1-fold). Moreover, BRCA2 and CDKN1A (p21, CIP1)-interacting protein (BCCIP), which is an evolutionary conserved protein implicated in the maintenance of genome stability and cell cycle progression (Wyler et al. 2014), were downregulated after the wild or mutant LBP challenges (−1.59-fold and −1.71-fold, respectively), and the difference of fold change was not obvious (Table 3).

The Toll-like receptor (TLR) signalling pathway, one of the best-studied and well-characterized pathways to initiate host immune defense mechanisms against invading pathogens (Singh and Kumar 2015), are involved in regulated genes (Fig. 8b). The family of TLRs cause innate immune responses that include the production of inflammatory cytokines, chemokines, and interferons. The signal transduction is initiated from the Toll/interleukin-1 receptor (TIR) domain of TLRs after pathogen recognition. Almost all TLRs use a TIR-containing adapter, myeloid differentiation factor 88 (MyD88), to activate a common signalling pathway that results in the activation of NF-κB to express cytokine genes relevant to inflammation (Lin et al. 2006). MyD88, the best-characterized adaptor molecule, which mediates signalling by all TLRs except for TLR3, also mediates signalling by interleukin (IL)-1R and IL-18R (Pearlman et al. 2008). Three further TIR-containing adapters are Mal/TIRAP, which recruits MyD88 to TLR2 and TLR4; TRIF (TICAM1), which is a critical protein that mediates the MyD88-independent pathway in TLR3 and TLR4 signalling; and TRAM (TICAM2, TIRP), which specifically mediates the MyD88-independent pathway in TLR4 signalling but not in TLR3 signalling (Lin et al. 2006).

In this study, a genetic variation at position 67 of LBP, possibly altering its structure, led to an altered cytokine response, so it predicted that carriers of this mutation have an increased risk for a more severe course of clinical infection. One explanation for LBP mutation causing an increased inflammatory response after LPS challenge is that the amino acid exchange at position 67 may be causing additional structural rearrangements driven by the unfavorable exposure of hydrophobic regions, which leads to a stronger ability to recognize and bind to the LPS or the lipopeptides of pathogenic bacteria. This might lead to an increased cytokine response in the host, resulting in enhanced defense during infection. Gene-chip microarrays showed that the effects of gene expression between mutant LBP and wild LBP treatment on inflamed BMECs were differential, which could offer an improved understanding of the important immunomodulatory role of mutant LBP in gram-negative mastitis. The results also suggest that genetic testing may be helpful for the identification of cows with an unfavorable response to gram-negative infections.

Taken together, these data suggest that the common polymorphisms in the LBP gene in Chinese Holstein cattle are possibly associated with an increased risk for the development of mastitis and, furthermore, may be linked to serious outcomes. These findings might have consequences for risk assessments of individual cattle, and they support the concept of LBP as a key mediator in the inflammatory response. In mature LBP protein, five mutations were found: g.3034G → A (36Asp → Asn), g.3040A → G (38Asn → Asp), g.3056 T → C (43Ile → Thr), g.4619G → A (67Ala → Thr), 19975G → A (282Val → Met) (Cheng et al. 2012). Future studies should focus on the effect of different genetic variation in LBP on mastitis-resistant cows and evaluating the predictive value of the genetic biomarker for clinical outcomes in various high-risk populations.

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