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Microfluidic high-throughput RT-qPCR measurements of the immune response of primary bovine mammary epithelial cells cultured from milk to mastitis pathogens

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Bovine mastitis, the inflammation of the udder, is a major problem for the dairy industry and for the welfare of the animals. To better understand this disease, and to implement two special techniques for studying mammary gland immunity in vitro, *we measured the innate immune response of primary bovine mammary epithelial cells (pbMEC) from six Brown Swiss cows after stimulation with the heat-inactivated mastitis pathogens,* Escherichia coli *1303 and* Staphylococcus aureus *1027. The cells were extracted and cultivated from milk instead of udder tissue, which is usually done. The advantages of this technique are non-invasiveness and less contamination by fibroblasts. For the first time, pbMEC gene expression (GE) was measured with a microfluidic high-throughput real-time reverse transcription-quantitative PCR platform, the BioMark HD™ system from Fluidigm. In addition to the physiological analysis, the precision and suitability of this method was evaluated in a large data set. The mean coefficient of variance (± s.e.) between repeated chips was 4.3 ± 0.4% for highly expressed and 3.3 ± 0.4% for lowly expressed genes. Quantitative PCR (qPCR) replicate deviations were smaller than the cell culture replicate deviations, indicating that biological and cell culture differences could be distinguished from the background noise. Twenty-two genes (complement system, chemokines, inflammatory cytokines, antimicrobial peptides, acute phase response and toll-like receptor signalling) were differentially expressed (P < 0.05) with* E. coli. *The most upregulated gene was the acute phase protein serum amyloid A3 with 618-time fold.* S. aureus *slightly induced* CCL5, IL10, TLR4 *and* S100A12 *expression and failed to elicit a distinct overall innate immune response. We showed that, with this milk-derived pbMEC culture and the high-throughput qPCR technique, it is possible to obtain similar results in pbMEC expression as with conventional PCR and with satisfactory precision so that it can be applied in future GE studies in pbMEC.*

Keywords: bovine mastitis, gene expression profiling, microfluidic qPCR, primary bovine mammary epithelial cells, innate immune response

Implications

We show that a time- and cost-efficient high-throughput quantitative PCR (qPCR) system, applied on primary bovine mammary epithelial cells (pbMEC) cultured from milk, is a convenient alternative to the two major standard procedures in measuring gene expression. We obtained similar results as studies with pbMEC from udder tissue and measurements on DNA microarrays or conventional qPCR. We suggest that the milk-derived pbMEC culture and the microfluidic high-throughput qPCR system could be applied in future experiments with pbMEC.

Introduction

Bovine mastitis, the inflammation of udder tissue, is one of the most frequent and most costly diseases in dairy cows. Bacteria are by far the most common cause of mastitis. *Escherichia coli* induces predominantly acute and severe mastitis, whereas *Staphylococcus aureus* often leads to mild and chronic mastitis (Petzl *et al.*, 2008). In order to better understand this disease, primary bovine mammary epithelial cells (pbMEC) have been intensively studied *in vitro*. They synthesize

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and secrete milk, but they also have immune capacity: on recognition of pathogens via toll-like receptors (TLRs), they produce inflammatory cytokines and chemokines to attract immune cells. They also secrete antimicrobial peptides and acute phase proteins as a first defence (Rainard and Riollet, 2006). pbMECs are generally extracted from udder tissue of slaughtered cows via enzymatic digest; however, we used exfoliated cells isolated from milk. The advantages of this method are its non-invasiveness and repeatability and non-contamination by fibroblasts (Buehring, 1990). A high-throughput gene expression (GE) instrument, the BioMark HD™ real-time reverse transcription-quantitative PCR (RT-qPCR) platform (Fluidigm, San Francisco, CA, USA), was chosen to determine the relative expression of 45 genes of the innate immune response of milk-derived pbMEC after *E. coli* and *S. aureus* stimulation. Spurgeon *et al.* (2008) describe the function and the advantages of this novel technique in detail. Briefly, with the applied microfluidic technology to manipulate nanolitre scales of samples and reagents in an automated manner, it is possible to measure the expression of up to 96 genes in up to 96 samples in one run. The system has successfully been used by other researchers. Jang *et al.* (2011) measured the expression of microRNA and found that the sensitivity of the measurement increased compared with conventional singleplex RT-qPCR. They also measured higher fold changes than with an Affymetrix microarray. Furthermore, they reported that the sample and reagent consumption was 50 to 100 times lower and the throughput was 5 to 20 times higher than in conventional RT-qPCR. These attributes of the system make it especially attractive when only small amounts of sample, such as in primary cell culture, are available, and when whole pathways and functional groups of genes are screened.

Material and methods

Cell culture

Fresh milk from six healthy Brown Swiss cows in mid and late lactation was taken after cleaning and disinfecting the teat surface. Mammary epithelial cells (MECs) were extracted by centrifugation and washed with Hank's Balanced Salt Solution containing antibiotics as described in a study by Danowski *et al.* (2012). Briefly, the cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) with a nutrient mixture F-12 HAM, 10% FBS (Life Technologies, Darmstadt, Germany), ITS liquid media supplement and antibiotics (Sigma-Aldrich, Munich, Germany) at 37°C and 5% CO_2. At reaching confluence, they were split using accutase (PAA, Pasching, Austria). After the second passage, a sample was reseeded at 10 000 cells per well in a 16-well chamber slide (Nunc, Langenselbold, Germany) for immunocytochemistry (IC). The rest were resuspended in freezing medium consisting of 70% DMEM, 20% FBS and 10% DMSO, and stored in liquid nitrogen until all cultures were collected. Cells from every animal were reseeded at 30 000 cells per well in a 12-well tissue culture plate and cultivated until confluence. The mean value of three counted wells was used to estimate the cell count in the other wells. Heat-inactivated *E. coli* 1303 and *S. aureus* 1027 (Petzl *et al.*, 2008) were added in a multiplicity of infection (MOI) of 30 colony forming units per cultured cell to ensure that every culture received the same bacterial load per cell. This MOI was chosen on review of the literature as a typical bacterial load often used in similar experiments (Gunther *et al.*, 2009; Danowski *et al.*, 2012). *E. coli* treated (6 and 30 h), *S. aureus* (30 and 78 h) and control wells (6, 30 and 78 h) were sampled in duplicates by washing the wells with phosphate buffered saline (PBS) and dissolving the cell layer in lysis buffer of the AllPrep RNA/Protein Kit (Qiagen, Hilden, Germany).

IC

IC was conducted with the method described in a study by Danowski *et al.* (2012). Briefly, after fixation of the chamber slides in methanol : acetone (1 : 1), washing, blocking of endogenous peroxidases in 1% H_2O_2 and reduction of background staining with goat serum (DAKO, Glostrup, Denmark), monoclonal mouse anti-cytokeratin pan antibody clone C-11 (1 : 400 in PBS-Tween, Sigma-Aldrich) was incubated over night at 4°C. After washing, horse radish peroxidase (HRP)-labelled goat anti-mouse-immunoglobulin (1 : 400 in PBS-Tween, DAKO) was incubated for 1 h at room temperature. HRP was visualized with diaminobenzidine and 0.01% H_2O_2. Nuclei were stained with Haemalaun after Mayer (Roth, Karlsruhe, Germany).

RNA extraction and reverse transcription

Total RNA and cell protein was extracted with the AllPrep RNA/Protein Kit (Qiagen) according to the manufacturer's protocol with an additional DNase treatment (RNAse-free DNase set, Qiagen). RNA concentration was measured with the Nanodrop ND-1000 spectrophotometer (Peqlab, Erlangen, Germany). RNA quality was analysed with RNA 6000 nano chips and kit on the 2100 Bioanalyzer (Agilent, Böblingen, Germany) and then stored at −80°C. For reverse transcription, 100 ng RNA and a master mix prepared from 5× buffer and 100 U M-MLV H(−) reverse transcriptase (Promega, Mannheim, Germany), 0.5 mM dNTPs and 0.5 μM Oligo-d(T) primer (Fermentas, St. Leon-Rot, Germany) and 2.5 μM random hexamer primers (Invitrogen Life Technologies, Darmstadt, Germany) were used in a total volume of 30 μl. A pooled RNA sample of all samples from each RNA extraction run was transcribed to cDNA with the same reaction mix without reverse transcriptase and included in the quantitative PCR (qPCR) measurements to check for contamination by genomic DNA. The incubation programme consisted of an annealing phase at 21°C for 10 min, transcription phase at 48°C for 50 min and degrading phase at 90°C for 2 min. cDNA was stored at −20°C.

PCR primer pairs

The mRNA sequences of the studied genes were taken from the National Center for Biotechnology Information (NCBI) Gene database (NCBI, National Library of Medicine, Bethesda, MD, USA). Primer pair oligos (Metabion, Martinsried, Germany)

were designed using HUSAR (DKFZ, German Cancer Research Center, Heidelberg, Germany) or PrimerBLAST (NCBI). Specificity of primer pairs was checked via melting curve analysis and gel electrophoresis of the amplified product (data not shown). PCR efficiencies of the assays were determined with a 5-point dilution series of two representative samples from the experiment and untreated bovine spleen tissue cDNA in qPCR triplicates with the calculation described in Bustin *et al.* (2009). Primer sequences and gene names are shown in Supplementary Table S1. The analysis was performed on a relative quantification of mRNA expression in treated samples *v.* control samples for each target gene separately.

RT-qPCR

4 μl cDNA was preamplified with the thermal protocol: 95°C for 3 min followed by 18 cycles of 95°C for 20 s, 55°C for 3 min and 72°C for 20 s. The reaction volume was 20 μl containing the iQ Supermix (Bio-Rad, Munich, Germany) and 25 nM of each primer pair. Preamplified cDNA was subsequently diluted with water 1 : 9. qPCR was conducted on the BioMark™ HD system. PCR efficiencies of the assays were measured on a gene expression (GE) Dynamic Array 48.48 chip (Fluidigm). The 84 preamplified cDNA samples from the stimulation experiment were measured together with 213 other preamplified cDNA samples, no reverse transcriptase (NoRT) control and no template control (NTC) from cultured pbMEC on four GE Dynamic Array 96.96 chips (Fluidigm). One 6 h *E. coli* treated pbMEC sample was measured repeatedly on all four 96.96 chips and used as between-chip calibrator. It was chosen as a representative and stable sample that expressed all genes of interest to provide similar reaction conditions and expression levels as in the other samples. 5 μl sample premix consisting of 2.5 μl SsoFast EvaGreen Supermix (Bio-Rad), 0.25 μl Sample loading reagent (Fluidigm), 0.1 μl ROX (diluted 1 : 3, Invitrogen), 1.25 μl preamplified and 1 : 9 diluted cDNA and water, as well as 5 μl assay premix consisting of 2 μl 10 μM primer pairs in the final concentration of 4 μM, 2.5 μl Assay loading reagent (Fluidigm) and water were prepared and transferred to the primed GE Dynamic Array 96.96. The samples and assays were mixed inside the chip using the Nanoflex IFC controller (Fluidigm). The final concentration of primers in the individual reaction was 400 nM. Thermocycling parameters included an initial phase of 98°C for 40 s followed by 40 cycles, consisting of 95°C for 10 s and 60°C for 40 s. After completion of the run, a melting curve of the amplified products was determined. Data were collected using BioMark Data Collection Software 2.1.1. built 20090519.0926 (Fluidigm) as the cycle of quantification (Cq), where the fluorescence signal of the amplified DNA intersected with the background noise.

Data preprocessing and analysis

Fluidigm Melting Curve Analysis Software 1.1.0. (build 20100514.1234, Fluidigm) and Real-time PCR Analysis Software 2.1.1. (build 20090521.1135 (Fluidigm)) were used to determine the valid PCR reactions. Invalid reactions were not used for later analyses and treated as missing data. Raw Cq values were processed with Genex 5.3.2 (MultiD Analyses AB, Gothenburg, Sweden) using between-chip calibration and reference gene normalization. Six putative reference genes had been identified after review of the available literature. Stability of their expression was evaluated with the Normfinder tool in Genex 5.3.2 (MultiD Analyses AB). For the gene regulation analysis, the cut-off was set to 25 and higher values were replaced with 25. Cq values >30 were regarded as invalid and treated as missing data. The subtraction of reference gene Cq mean from target gene Cq value yielded the ΔCq value. Genex 5.3.2 was used for principal component analysis (PCA) on the auto-scaled ΔCq values. Distribution of within-chip deviation of the BioMark™ HD chips was calculated with gnuplot 4.4.0 (Sourceforge.net, Geeknet Inc., Fairfax, VA, USA). Genes with an overall Cq mean below 20 were termed as 'high expression', above 20 as 'low expression' genes for the within-chip and between-chip deviation analysis. Statistics and charts were produced with SigmaPlot 11 (SYSTAT, Chicago, IL, USA) or SPSS Statistics Standard 19.0 (IBM, Armonk, NY, USA). Genes were observed as differentially expressed for $P < 0.05$ in a paired *t*-test on ΔCq of control and treated samples at each time point. The fold change in expression was calculated with the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001) for every sample and then expressed as the mean of all these fold changes. It must be noted that no correction for multiple testing was imposed on the *P*-values, although we are aware that this increases the risk of false positive significances. This study is of explorative and descriptive character only, not of a diagnostic one. Such a correction would have been too stringent and masked many of the differences that we found between treatment and control. RT-qPCR was conducted following the MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines (Bustin *et al.*, 2009).

Results

Cell culture and IC

The extracted cells attached after 24 h and proliferated after a few days. A mean total cell count of 6.5 million cells per culture with a range between 1 and 19 million cells per culture was harvested after the second passage and stored in liquid nitrogen. The cells showed the typical cobblestone-like monolayer in cell culture with varying cell sizes (Supplementary Figure S2 (a)). The purity of all the cultures was estimated at nearly 100%. Thorough visual inspection with light microscopy detected 0% unstained nucleated cells after immunocytochemical staining against cytokeratins, whereas in the negative controls there were 0% stained cells visible (Supplementary Figure S2 (b)).

RT-qPCR

Supplementary Figure S3 shows the quantile–quantile (Q–Q) plot of the Cq values from chips one to three exemplarily to depict the correspondence of Cq variation between these chips.

The fourth chip contained only few samples from this experiment. The rest consisted of NTCs, negative RT controls and RT calibration samples. Those were not comparable with the samples of the other three chips in terms of Cq value range. With the remaining comparable samples, it was not possible to draw a valid Q–Q plot *v.* the other chips. The Cq values from two chips were each ranked according to their value in ascending order. Beginning with the lowest two values from both chips, they were paired to form coordinates of points. The resulting curve was a straight ascending diagonal line that showed that the ranked Cq values increased in the same rate up to cycle 25. From there the Cq values started to increase in inconsistent intervals, indicated by the bends in the curves. Supplementary Figure S4 shows the distributions of the Cq differences of qPCR replicates (qPCR) and the differences of the mean Cq values of cell culture replicates over all chips (within-chip deviation). The qPCR replicates of 25 high and 20 low expression genes had 83% and 59% of the values in the lowest deviation range between 0 and 0.5 cycles, respectively. The cell culture replicates for high and low expression genes had 49% and 33% of the values in that range, respectively.

The mean coefficient of variation (CV; ± s.e.) of the calibration sample over the four chips was 4.3 ± 0.4% for the high expression genes and 3.3 ± 0.4% for the low expression genes after Cq values over 30 had been cut-off and Cq values over 25 had been set to 25. A visualization of the Cq values is shown in Supplementary Figure S5.

Table 1 *Differentially expressed genes (P < 0.05) between treatment and control in pbMECs from six Brown Swiss cows after 6 and 30 h stimulation with heat-inactivated* Escherichia coli *1303*

	x-time fold change[1]			
	E. coli 6 h		*E. coli* 30 h	
	Mean	s.e.	Mean	s.e.
Complement system				
C3	4.4	0.9	8.6	1.8
Chemokines				
CCL2	28.8	12.3	43.9	13.1
CCL5	ns[2]	ns	51.6	33.3
CCL20	39.4	17.1	50.2	11.1
CXCL5	8.1	2.1	7.8	1.3
CXCL8	20.1	7.7	21.9	7.5
Inflammatory cytokines				
IL6	3.6	1.1	5.3	1.2
IL10	18.8	11.0	53.1	35.1
TNF	21.9	7.0	21.5	4.1
Antimicrobial peptides				
LAP	2.8	0.6	19.5	8.0
LF	3.2	0.6	8.6	2.3
LPO	ns	ns	4.2	1.2
LYZ1	ns	ns	40.2	27.6
TAP	54.8	32.8	105.7	57.9
Acute phase response				
SAA3	ns	ns	618.2	272.4
TLR pathway				
CASP8	1.3	0.1	1.4	0.1
CD14	ns	ns	1.8	0.3
TLR2	ns	ns	5.0	2.1
Others				
MX1	ns	ns	4.4	1.4
MX2	11.6	7.6	8.6	2.3
S100A9	3.9	0.8	12.0	4.1
S100A12	ns	ns	4.2	0.9

pbMEC = primary bovine mammary epithelial cells; CCL = (C-C motif) ligand; CXCL = (C-X-C motif) ligand; IL = interleukin; TNF = tumour necrosis factor; LAP = lingual antimicrobial peptide; LF = lactoferrin; LPO = lactoperoxidase; LYZ = lysozyme; TAP = tracheal antimicrobial peptide; SAA3 = serum amyloid A3; TLR = toll-like receptor; CASP8 = caspase 8.
[1]Calculated with the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001).
[2]Not significant ($P > 0.05$).

Immune response of the pbMEC

With the Normfinder tool within Genex 5.3.2 (MultID) *ACGT1*, *KRT8* and *H3F3A* were identified as stably expressed over all samples and all conditions and thus being suitable reference genes. They were used for normalization of the target gene Cq values, resulting in the ΔCq value. Of the 39 target genes, 28 were successfully quantified. *C1QA*, *C3aR1*, *C5aR1*, *CASP1*, *CD163*, *IL1B*, *HP*, *IRF3*, *NLRP1*, *NRLP3* and *RELA* were found to have too many invalid PCR reactions to be subjected to processing. Differentially expressed genes ($P < 0.05$) between treatment and control are shown in Tables 1 and 2. Twenty-two genes were differentially expressed with the *E. coli* stimulation, but only four with the *S. aureus* stimulation. *E. coli* strongly activated complement component 3 (*C3*), chemokines and inflammatory cytokines after 6 and 30 h, as well as antimicrobial peptides after 30 h. The two myxovirus resistance genes (myxovirus (influenza virus) resistance 1, interferon inducible protein p78 (mouse) (*MX1*), myxovirus (influenza virus) resistance 2 (mouse) (*MX2*)) and the two S100 calcium-binding genes (S100 calcium-binding protein A9 (*S100A9*), S100 calcium-binding protein A12 (*S100A12*)) were also similarly upregulated after 30 h *E. coli.* The most induced gene was serum amyloid A3

Table 2 *Differentially expressed genes between treatment and control in pbMECs from six Brown Swiss cows after 30 and 78 h stimulation with heat-inactivated* Staphylococcus aureus *1027*

	x-time fold change[1]	
S. aureus	Mean	s.e.
30 h		
TLR pathway		
TLR4	1.3	0.2
78 h		
Chemokines		
CCL5	1.5	0.2
Inflammatory cytokines		
IL10	1.5	0.2
Others		
S100A12	1.3	0.1

TLR = Toll-like receptor; *CCL5* = (C-C motif) ligand 5; *IL10* = interleukin 10.
[1]Calculated with the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001).

(*SAA3*) with 618-time fold after 30 h exposure to *E. coli*. With *S. aureus*, the most induced genes were the chemokine (C-C motif) ligand 5 (*CCL5*) and the anti-inflammatory cytokine interleukin 10 (*IL10*) after 78 h.

The PCA on the relative expression of the target genes is shown in Supplementary Figure S6. *E. coli* samples form a distinct subgroup only slightly overlapping with the other samples. No separation between *S. aureus* and control samples is visible.

Discussion

Precision of the BioMark™ HD system

The Q–Q plot (Supplementary Figure S3) shows that the correspondence of the distribution of Cq values between the chips was very good, as the points formed almost the ideal diagonal line. However, values over 25 were not evenly distributed and deviated from the line. This is a confirmation of the need to set a cut-off at 25 when processing the data from this system. The within-chip deviations of the qPCR replicates were found to be smaller than those of the cell culture replicates. This is very important as small biological differences could be masked by the noise in measurement and may not be detected with this method. It is understandable that the replicate deviations were higher in low expression genes. In diluted nucleic acid samples with low target concentrations, the Poisson distribution occurs as a natural effect. It predicts large variations in target quantities in aliquots from the same sample (Rutledge and Stewart, 2010). This should be kept in mind when deciding how many qPCR replicates of an assay are to be carried out. It is recommended to run more replicates for genes that are known to be less expressed to cover this variation and increase the precision of the measurement. The mean between-chip CV was acceptable. The CV of the low expression genes was surprisingly lower than the CV of the high expression genes. However, this must be interpreted with caution, as it is likely that the low expression gene CV does not reflect the true variability of the data because of the cut-off at Cq 30. It only reflects the variability of the remaining data after preprocessing and cut-off.

General considerations

So far the expression of the immune response in pbMEC has either been measured by conventional RT-qPCR or on DNA microarrays. To our knowledge, this is the first time that a high-throughput RT-qPCR technique was applied to study a large set of genes in pbMEC cultured from milk. So far large sets of GE data are only available from pbMEC extracted from udder tissue in microarray studies (Gunther *et al.*, 2009). Gunther *et al.* (2009) also reported that the immune response to *E. coli* was much faster and stronger than to *S. aureus*; however, the authors were still able to identify several significantly upregulated genes by *S. aureus*. Generally, they identified higher fold changes in the regulated genes, but this could be because of the microarray technique or to different cell culture conditions. In their study, *SAA3* was also the most up regulated gene by *E. coli* (Gunther *et al.* (2009)), followed by the chemokine *CCL5*, lingual antimicrobial peptide (LAP*)* and *MX2* (Gunther *et al.*, 2009), which were also highly upregulated in our study. Our cells proved to be able to express a similar set of inflammatory cytokines (*IL6*, *IL10* and tumour necrosis factor (*TNF*)) and chemokines (chemokine *CCL2*, *CCL5*, chemokine *CCL20*, chemokine (C-X-C motif) ligand 5 (*CXCL5*), and chemokine *CXCL8*) compared with the study by Gunther *et al.* (2009). Lutzow *et al.* (2008) measured the intra-mammary immune response of dairy cows to *S. aureus in vivo* and found upregulated inflammatory cytokines and chemokines, as well as defence proteins. Both were measured on a DNA microarray and validated with RT-qPCR. However, two important innate immune genes, *TNF*α and *CD14*, were identified as differentially expressed by the RT-qPCR, but not on the microarray. Swanson *et al.* (2009) infected heifers with *Streptococcus uberis* and measured the transcriptional changes in the mammary tissue on a DNA microarray. Of the regulated genes, they validated 11 innate immune genes with RT-qPCR. Three of these showed a different direction of regulation or no regulation in the validation. These findings underline the need to carefully interpret microarray results and validate them with qPCR.

Pathogen differences

The remarkable pathogen differences in immune response have been noted before (Griesbeck-Zilch *et al.*, 2008; Petzl *et al.*, 2008). However, the total failure of *S. aureus* to stimulate the innate immune defence in our study is remarkable. A direct comparison can be made with the study by Danowski *et al.* (2012) where milk-derived pbMECs were stimulated with the same strains of pathogens as in our study. There the PCA showed no distinct separation of *S. aureus* samples from *E. coli* and control, similar to our PCA. Therefore, it can be assumed that the weak *S. aureus* effect in our study is a reproducible physiological effect. Possibly, the dose of inoculation was insufficient. It could have been too low, taking into account that there is a dose-dependent immune response of pbMEC to lipopolysaccharide (LPS) and *S. aureus* (Wellnitz and Kerr, 2004) and a study by Swanson *et al.* (2009) with pbMEC from tissue showed an upregulation in four of nine measured immune genes to *S. aureus* with a much higher MOI of 1000. Another possibility is that we missed the proper time frame of the immune response: one study showed an early immune response of MECs to *S. aureus* that decreased to resting levels after 8 to 16 h (Strandberg *et al.*, 2005). Our bacteria had been isolated from a clinical case of mastitis and were shown to have elicited weak but measurable symptoms of mastitis when administered *in vivo* intra-mammary (Petzl *et al.*, 2008); thus, the question remains whether this strain exhibits sufficient virulence only in a live, but not in a heat-inactivated form. The fact that udder infections with *S. aureus* often remain subclinical and become chronic could be explained by this lack of a strong immune response of the MECs.

Pathogen recognition

E. coli should be recognized by TLR4 and its cofactor CD14, which binds to conserved LPS patterns of gram-negative bacteria (Lu *et al.*, 2008). Of this complex, only *CD14* has been regulated by *E. coli* in our study. In contradiction to the statement that MECs do not express *CD14* (Rainard and Riollet, 2006), we were able to measure an expressed and weakly regulated *CD14*. Apoptosis inducer caspase 8 (*CASP8*) is activated by the gram-positive bacteria recognizing *TLR2* (Aliprantis *et al.*, 2000). *CASP8* and *TLR2* were upregulated by *E. coli*. It has been shown in human cells that *TLR2* is able to respond to gram-negative bacteria when expressed in combination with the cofactor lymphocyte antigen 96 (*LY96*, also called *MD2*; Dziarski *et al.*, 2001). This cofactor was expressed but not regulated by *E. coli* in our cells, as well as other members of the pathway (LPS-binding protein (*LBP*), *TLR4* and myeloid differentiation primary response gene 88 (*MYD88*)). *TLR4*, on the other hand, was weakly upregulated by *S. aureus*. Although based on mRNA expression only, nothing can be said about the actual interaction of the pathogen components with the TLRs; both *TLR2* and *TLR4* mRNAs were present in all control and treated samples, and changes in expressions of *TLR2* and *TLR4* were pathogen specific in our study. The whole TLR signalling pathway here seemed to be less influenced than in other studies. It seems that it is not necessary to strongly upregulate the TLR pathway components for an efficient immune response. Strandberg *et al.* (2005) found a similar weak TLR activation in bovine MECs upon LPS stimulation and still came to the conclusion that a functioning and locally effective immune system was present.

Inflammatory cytokines and chemokines

These signalling and modulating molecules were highly influenced by *E. coli*. This is consistent with most other studies mentioned already and confirming that pbMECs exert a major sentinel function to trigger the immune response. However, in contrast to another study (Lahouassa *et al.*, 2007), we detected a modulation of the immune response by upregulation of the anti-inflammatory cytokine *IL10*, suggesting an instant self-regulation to avoid damage to the tissue.

Antimicrobial peptides and acute phase response

LAP and tracheal antimicrobial peptide (*TAP*) belong to the β-defensins, and together with lactoperoxidase (*LPO*), lysozyme (*LYZ1*) and lactoferrin (*LF*) they are antimicrobial peptides, able to inhibit and damage bacteria directly. All five studied antimicrobial peptides were differentially expressed after *E. coli* stimulation, with *TAP* being by far the most influenced one. This is a confirmation of the findings of Lopez-Meza *et al.* (2009) that MECs are the source for *TAP* found in the udder and in milk. Not many data are available for antimicrobial peptide expression of cultured pbMEC. Although it has been reported that repeated subcultivation of pbMECs lowered their ability to express *LAP* with and without stimulation (Gunther *et al.*, 2009), all of our three passage cultures expressed *LAP* and responded to the stimulation. The acute phase gene *SAA3* was the most upregulated one, same as in the study by Gunther *et al.* (2009). The antibacterial SAA protein is an opsonin for gram-negative bacteria (Shah *et al.*, 2006), and because of its massive increase during mastitis it has been suggested as a biomarker for this disease (Larsen *et al.*, 2010).

Complement system

The known lack of the classical pathway of the complement system in the mammary gland (Rainard and Riollet, 2006) was confirmed by the absence of complement component 1, q subcomponent, A chain (*C1QA*) expression. However, *C3* expression was induced showing that the alternative pathway was functional in our cells. C3 can opsonize bacteria and makes them available for phagocytosis, and it regulates the inflammatory response (Rainard and Riollet, 2006). In another study, it was also upregulated by *E. coli* and *S. aureus* in pbMEC (Griesbeck-Zilch *et al.*, 2008). These findings suggest that C3 in milk is at least partially synthesized by the epithelial cells and not just transported through the blood–udder barrier. Complement component 5a receptor 1 (*C5AR1*) and complement component 3a receptor 1 (*C3AR1*) were both found expressed in a part of the samples, and no statistical evaluation was done because of the missing data (data not shown). The expression of *C5AR1* in epithelial cells has been discussed controversially; however, one study found C5aR protein expression in the bovine MEC line MAC-T in a subpopulation of 10% to 12% of the cells (Nemali *et al.*, 2008). *C5AR1* encodes for the receptor of complement component 5a (C5a), which is mainly present on granulocytes, macrophages and some lymphocytes. C5a leads to cellular responses of the cells such as chemotaxis, phagocytosis and enzyme release (Rainard and Riollet, 2006). However, this author also mentions the stimulation of cytokine synthesis by C5a. This could be one possible function of pbMEC when recognizing C5a via the C5a receptor.

Others

S100A9 and *S100A12* encode for calgranulins, which are a group of mediator molecules with calcium-binding, proinflammatory, regulatory, anti-oxidant and protective properties. The S100-A12 protein has been shown to inhibit *E. coli* growth *in vitro* (Lutzow *et al.*, 2008). S100 genes are known to be upregulated in infected udder tissue and pbMEC (Gunther *et al.*, 2009). As in the pbMEC study by Gunther *et al.* (2009), *MX2* and *MX1* were induced by the *E. coli* treatment. MX proteins belong to the large GTPases family and have different antiviral capacities; their expression is stimulated by interferon and virus recognition (Lee and Vidal, 2002). Their potential effect on mastitis remains to be subject of further research.

Conclusions

For the first time, a high-throughput microfluidic RT-qPCR platform was applied to study a large set of genes in pbMEC cultured from milk. The sensitivity of the measurement was

found to be satisfactory for our purposes. We found it to be less time, sample, reagent and cost consuming than the conventional RT-qPCR, and unlike DNA microarrays, it does not require additional validation via conventional qPCR. With this technique and with cells cultivated from milk instead of tissue, we obtained similar results as other studies about the immune system in pbMEC. This confirms that our results are comparable with the results from conventional qPCR and tissue cultured pbMEC. With conventional qPCR, usually there are only few genes measured in each experiment. Therefore, it is necessary to assemble many different studies with different experimental conditions to achieve an overview of the immune response. We showed that, with microfluidic qPCR, it is now possible to do this in one experiment. The same holds true to other functions of these cells; cholesterol, fatty acid and milk protein metabolism are also important fields of study and could be screened in exactly the same way.

It is a subject of further research to analyse why *S. aureus* often fails to elicit a distinct immune response and what genes are exactly involved if there is a response. For that, microfluidic qPCR could be applied to screen a larger set of immune genes by omitting PCR replicates. The activation of antimicrobial peptides, the acute phase gene *SAA3*, *S100A12* and *S100A9* confirmed the diversified defence capability of pbMEC against *E. coli*. On the other hand, our pbMECs proved to be able to act as sentinel cells by expressing chemokines and inflammatory cytokines for the attraction and activation of immune cells. They were also able to express the anti-inflammatory gene *IL10* to modulate the immune response. However, many details and interactions of the immune response are still unclear and we suggest that this experimental set-up could be applied for further studies. Different pathogens and additional genes could be tested to broaden the picture as well as make it more detailed.

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Supplementary materials

For supplementary materials referred to in this article, please visit http://dx.doi.org/10.1017/S1751731112002315

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