Validation of RNA isolated from milk fat globules to profile mammary epithelial cell expression during lactation and transcriptional response to a bacterial infection

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ABSTRACT

Mastitis, an inflammation of the mammary gland, is the most costly infectious disease of dairy ruminants worldwide. Although it receives considerable attention, the early steps of the host response remain poorly defined. Here, we report a noninvasive method using milk fat globules (MFG) as a source of mammary RNA to follow the dynamics of the global transcriptional response of mammary epithelial cells (MEC) during the course of a bacterial infection. We first assessed that RNA isolated from MFG were representative of MEC RNA; we then evaluated whether MFG RNA could be used to monitor the MEC response to infection. Sufficiently high yields of good-quality RNA (RNA integrity numbers ranging between 6.7 and 8.7) were obtained from goat MFG for subsequent analyses. Contamination of MFG by macrophages and neutrophils, which can be trapped during creaming, was assessed and when using quantitative real-time PCR for cell-type specific markers, was shown to be weak enough (<8%) to affect MFG gene expression profiling. Using microarrays, we showed that RNA extracted from MFG and from mammary alveolar parenchyma shared approximately 90% of the highlighted probes corresponding in particular to genes encoding milk proteins (*CSN*, *BLG*, *LALBA*) and enzymes involved in milk fat synthesis and secretion (*FASN*, *XDH*, *ADRP*, *SCD*, and *DGAT1*). In addition, a gene involved in the acute-phase reaction, coding for the serum amyloid A3 (SAA3) protein, was found within the first 50 most highly expressed genes in a noninfectious context in both mammary alveolar parenchyma and MFG, strongly suggesting that *SAA3* is expressed in MEC. We took advantage of this noninvasive RNA sampling to follow the early proinflammatory response of MEC during the course of a bacterial infection and showed that the levels of mRNA encoding

SAA3 sharply increased at 24 h postinfection. Taken together, our results demonstrate that MFG represent a unique source of MEC RNA to noninvasively sample sufficient amounts of high-quality RNA to assess the dynamics of MEC gene expression in vivo, especially during the first steps of infection, thereby paving the way for the discovery of early biomarkers for the control of intramammary infections. Furthermore, this noninvasive technique could be used to provide mammary transcriptomic data on a large scale, thus filling the gap between genomic and phenotypic data.

Key words: gene expression profiling, milk fat globule, mastitis, serum amyloid A3

INTRODUCTION

Mastitis, which is an inflammation of the mammary gland (MG) commonly caused by bacterial infection, is the most costly infectious disease of dairy ruminants worldwide because of the reduced milk yield, milk discarded after treatment, and cost of veterinary services (Sordillo and Streicher, 2002). Although mastitis receives considerable attention, the early steps of the host response remain poorly defined and understood. Indeed, it is challenging to obtain genetic material from the MG. Usually, surgical sampling techniques from mammary biopsies or from tissue samples taken at slaughter provide large amounts of tissue (Farr et al., 1996; Schmitz et al., 2004). Unfortunately, multiple postinvasive complications can arise, and because of the great heterogeneity of the mammary tissue, these samples may not be representative of the milk-synthesizing tissue and mammary biology. In addition, they do not provide opportunities for multiple sampling, to perform longitudinal studies and follow-up on local epithelial changes at the early stages of infection. An alternative is to use endoscopic techniques for the collection of fresh MG biopsies (Vangroenweghe et al., 2006; VanKlompberg et al., 2012). However, with such techniques, it is difficult to collect biopsy samples from the alveolar tissue in the upper part of the MG

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rich in secretory parenchyma. Therefore, noninvasive techniques using milk components could overcome such limitations.

Milk contains somatic cells, a heterogeneous population of cells including epithelial cells exfoliated from the mammary epithelium. Indeed, during lactation, mammary epithelial cells (**MEC**) are normally subject to an important turnover and are continually replaced by new cells (Capuco et al., 2001). These exfoliated cells are discharged into the milk and form a small part of milk somatic cells. Using RNA extracted from this part of somatic cells could be a significant improvement in assessing the gene activity of the mammary secretory epithelium and could allow easy and repetitive sampling without damaging mammary tissue (Boutinaud and Jammes, 2002; Boutinaud et al., 2002). Nevertheless, most of the epithelial cells present in milk are dead cells that have reached the end of their secretory life. Consequently, the information contained in such cells probably does not reflect the true metabolic situation of MEC (Krappmann et al., 2012).

Another alternative was proposed by Maningat et al. (2007, 2009), who reported the use of milk fat globules (**MFG**) as a source of mammary RNA in humans. Indeed, cytoplasmic crescents can be included in MFG, as first described by Wooding et al. (1970) in the goat and human. This mode of secretion with the loss of secretory cell material was considered specific to fat and is termed the apocrine mechanism. In fact, cytoplasmic lipid droplets, first originating from the endoplasmic reticulum, bulge against and gradually become enveloped in the apical plasma membrane, finally pinching off from the cell as MFG (McManaman et al., 2007). Intracellular components (membranes and organelles) of MEC become trapped within the MFG, which therefore contain cytoplasmic material other than the nucleus (Huston and Patton, 1990; Heid and Keenan, 2005). In addition, this apocrine secretion process is not exclusive to humans and goats. Crescents of cytoplasmic material have been observed in several species, such as the cow, rat, pig, sheep, and rabbit (Wooding, 1971; Janssen and Walstra, 1982). Taking advantage of this feature, Maningat et al. (2009) claimed that MFG represent potential material from which it is easy to obtain repeated samples of the cytoplasm of MEC. However, given the difficulty of sampling healthy mammary tissue from the human breast, no demonstration existed that RNA extracted from MFG were really representative of the genome expressed in MEC.

In this study, we used the RNA sampling method proposed by Maningat et al. (2007) for the goat species, with the aim of checking whether RNA exported by MFG, given the fat secretion process, come exclusively from MEC and reflect its transcriptional activity. To

address this issue, we compared gene expression patterns of the mammary alveolar parenchyma (**MAP**), which are essentially composed of MEC, and of MFG isolated from milk of the same goat by using microarray technology. In addition, quantitative real-time PCR (**qPCR**), which is characterized by a wide dynamic quantification range, was used to determine the expression of some specific genes. We report here that such a noninvasive RNA sampling procedure provides a unique opportunity to obtain a sufficient amount of good-quality RNA. We also demonstrate that this material, which showed a good correlation with the gene expression profile of MAP, is representative of the MEC transcriptome in the goat species. Furthermore, we successfully applied this approach to follow the response of MEC early in the infection process of the goat MG by using the acute-phase serum amyloid A3 protein (**SAA3**), which is known to be a specific marker of inflammation.

MATERIALS AND METHODS

Overall Strategy

The first part of the study, which was dedicated to comparing MFG RNA with MAP RNA, was performed in a noninfected context, starting from materials (milk fat and mammary tissue) collected from the same goat, by using both microarray and qPCR. The objective was to demonstrate that the MFG transcriptome reflects the MEC transcriptome. The second part, designed to validate the noninvasive RNA sampling protocol using MFG, was conducted on 8 goats to assess this sampling procedure and thereby to demonstrate by using qPCR that MFG-extracted material is useful in monitoring the MEC response to an infectious challenge by *Staphylococcus aureus*.

Animals and Milk Samples

Eight Alpine goats, at the early peak of lactation (30 to 40 DIM), at first parity and without IMI, were chosen and housed at the Centro Zootecnico Didattico Sperimentale of the Facolta di Medicina Veterinaria (Universita di Milano, Milan, Italy). Milk samples from the 8 healthy goats were collected by hand into sterile, RNase-free tubes (3 × 50-mL Falcon tubes), taking care to avoid any RNase contamination. The samples were immediately placed on ice before MFG collection.

Before intramammary challenge, both half-udders were milked by hand and emptied, and the teat ends were carefully disinfected with chlorhexidine. The left half-udder of each goat was infused with 1 mL (10³ cfu/mL) of inoculum of *Staph. aureus* strain DV137.

The right half-udder, used as a noninfected control, was infused with 1 mL of sterile pyrogen-free PBS. Subsequent milk sample collections were carried out at 6, 12, 18, 24, and 30 h postchallenge from both the right (uninfected) and left (infected) half-udder, taking care to prevent RNase contamination. At each time point, 150 mL of milk samples were collected for each half-udder of the 8 goats and transferred into sterile RNase-free tubes (3 × 50-mL Falcon tubes). Samples were immediately kept on ice before MFG preparation.

MFG Collection

Samples were centrifuged at $2,000 \times g$ for 10 min at 4°C to isolate milk fat. The supernatant fat layer was then transferred to a new 50-mL Falcon tube by using a sterile spatula. A 500-μL quantity of fat was then put into a 15-mL Falcon tube, and 1.5 mL of TRIzol LS solution (Invitrogen Life Technologies Inc., Carlsbad, CA) was added and the mix was vortexed vigorously before storage at -80°C. The entire process of milk sample collection and storage of MFG was completed within 2 h, and all procedures were carried out at 4°C.

MAP Collection

Goats were slaughtered at 30 h postinfection according to surgical and experimental procedures in compliance with the policy of the INRA Animal Care Committee after milk sampling. Tissue samples were collected aseptically from goats within 10 min after slaughter. A piece of deep alveolar parenchyma, without visible connective tissue, was removed from the left udder (infected) and right udder (noninfected), rapidly frozen in isopentane chilled over liquid nitrogen, and stored as original material at -80°C for further analyses.

Total RNA Isolation and Assessment of RNA Integrity

Total RNA was extracted from MFG and MAP by using a TRIzol LS solution (Invitrogen Life Technologies Inc.) following the original manufacturer's protocol, with slight modifications. Briefly, frozen samples (500 μL of milk fat + 1.5 mL of TRIzol LS) were placed at room temperature for 30 min or until thawing. A first centrifugation at $12,000 \times g$ was performed for 10 min at 4°C to remove lipids. A 400-μL quantity of chloroform was then added to the remaining solution and the mix was shaken vigorously. After centrifugation, the upper aqueous layer (approximately 60%), which contained RNA, was carefully pipetted, and RNA was precipitated by adding 750 μL of isopropanol/1 mL of TRIzol LS used for extraction. After centrifugation ($12,000 \times g$ for 10 min at 4°C), RNA pellets

were washed with 75% ethanol, dissolved in 30 μL of RNase-free water, and finally stored at -80°C until further processing. Before RNA quality control, a DNase treatment was carried out to remove any contaminating genomic DNA according to the manufacturer's protocol (Qiagen, Hilden, Germany).

RNA Quality Control and Single-Strand cDNA Synthesis

The purity, concentration, and integrity of total RNA intended for microarray analysis and qPCR were assessed using 2 independent techniques. Purity of the RNA was evaluated by absorbance (A) readings (ratio of A260/A230 and A260/A280) using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The fluorometric method and microcapillary electrophoresis device developed by Agilent Technologies (Palo Alto, CA) was used to determine RNA concentration and quality with an RNA 6000 Pico LabChip Kit in an Agilent Bioanalyzer 2100 system (Agilent Technologies). Quality was evaluated using the RNA integrity number value introduced by Schroeder et al. (2006). First-strand cDNA was synthesized from 5 to 10 ng of total RNA primed with oligo(dT)₂₀ and random primers (3:1, vol/vol) using Superscript III reverse transcriptase (Invitrogen Life Technologies Inc., Carlsbad, CA) according to the manufacturer's instructions. One microliter of 2 U/μL RNase H (Invitrogen Life Technologies) was then added and the reaction mix was incubated for 20 min at 37°C to remove RNA from the heteroduplexes. The single-strand cDNA thus obtained was stored at -20°C.

Microarray Processing and Data Analysis

Usually, this sensitive method is used to compare similar material. Because our purpose was to compare 2 materials from different origins (RNA from a heterogeneous tissue made up of different cell types vs. RNA from MFG, presumably arising from a unique cell type, MEC), we chose to perform technical replicates from 1 uninfected goat to overcome the individual variability and to test the repeatability of the method. Ribonucleic acid samples were labeled using T7 RNA polymerase, which simultaneously amplifies target material and incorporates cyanine 3 (**Cy3**)- or cyanine 5 (**Cy5**)-labeled cytidine triphosphate (Agilent Technologies), according to the manufacturer's instructions. Briefly, total RNA (120 ng) was first reverse transcribed and then converted to labeled cRNA with Cy3 or Cy5 dyes. Before hybridization, the quantity and quality of purified cRNA was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies). Given that goat microarrays were not

available, we used a microarray developed for a closely related species (*Ovis aries*) to profile gene expression. The microarray used in this study was a Sheep Gene Expression Microarray, 8×15K (Agilent Technologies). The hybridization was performed according to the manufacturer's instructions. Briefly, each microarray was cohybridized with 2 fluorescent-labeled (Cy3 and Cy5) cRNA samples. Three hundred nanograms of each labeled cRNA was fragmented for 30 min at 60°C in 25× fragmentation buffer. The fragmented and labeled cRNA samples were then diluted in 2× GEX Hybridization Buffer Hi-RPM (Agilent Technologies) and hybridized on the 15K ovine microarray for 17 h at 60°C in a rotating hybridization oven. After hybridization, microarrays were washed twice with Wash Buffer 1 (Agilent Technologies) at room temperature for 1 min and then with Wash Buffer 2 (Agilent Technologies) at 37°C for 1 min. Microarrays were scanned on an Agilent G2565BA scanner (Agilent Technologies) using the extended dynamic range scan mode. Data were processed (including Lowess normalization) and extracted with Feature Extraction software, version 10.5 (Agilent Technologies). This software quantified mean and median signals for each sample and for background estimation according to the GE2_NonAgilent_91 extraction protocol. Extracted data were analyzed using a qualitative method to determine which genes were expressed, considering the false positives. In this process, we used an intensity-based analysis, in which the mean intensity of the dye-normalized intensities in the 2 channels were kept separate, instead of a ratio-based analysis, considering that 1 cohybridized target does not influence the hybridization of the other ('t Hoen et al., 2004). Indeed, the distribution of mean signal intensity of each cyanine was similar from a log₂ (Lowess-normalized mean signal) >5. Therefore, the intensity of each spot (for both the Cy3 and the Cy5 channels) was initially filtered for good quality, based on the default parameters provided by the Feature Extraction Software using R statistical software (<http://www.r-project.org/>). Briefly, a spot was defined as *good* if it was not flagged as a saturation and nonuniformity outlier and was considered as being found [according to the local background and the spot diameter (Agilent Feature Extraction Software version 10.5 User Guide, Agilent Technologies)] in a single channel. A gene was defined as *present* if it was represented by a *good* spot in both the Cy3 and Cy5 channels, and the mean intensity signal was positive and significantly above background according to a 2-sided *t*-test ($P = 0.01$) and was not in the negative control range. Otherwise, a gene was considered *absent*. Probes considered *present* for each material, MAP and MFG, were then compared using a Venn diagram to obtain a common list between MFG

and MAP and a specific list for MFG and for MAP. Each set of genes was referenced to biological functions with Ingenuity Pathway Analysis software version 6.0 (IPA; Ingenuity Systems, Redwood City, CA). The contribution of genes to the identified function was explained with an associated *P*-value, calculated with a right-tailed Fisher exact test.

Primer Design and qPCR

The RNA isolated from MFG was used to determine, for each goat, the expression of selected genes of interest by qPCR, as described by Bevilacqua et al. (2006), by using a SYBR Green PCR Master Mix and an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Life Technologies, Carlsbad, CA). The primers used were described previously (Bevilacqua et al., 2006) except for CD15 (Hisano et al., 2001), CD18, GPR97 (Robbins et al., 2008; Crozat et al., 2010), MPO, and M-SAA3 (this study). The Bovine Genome Database (<http://BovineGenome.org>) was queried for reference mRNA sequences. Expressed sequence tags of caprine sequences found by a nucleotide Basic Local Alignment Search Tool search and reference were aligned to create consensus. Only exon-exon junctions, with great homology between sequences, were chosen for primer design with Primer Express Software version 2.0 (Applied Biosystems, Life Technologies) and were purchased from MWG Biotech (Ebersberg Germany; Table 1).

To estimate the representativeness of MFG as a starting material to analyze gene expression in MEC, 3 MEC-specific markers, namely, *CSN2*, *CSN3*, and *LALBA* transcripts, were quantified after normalization by 2 internal control genes, S24 ribosomal protein (*RPS24*) and cyclophilin (*PPIA*), which were validated by the GeNorm Visual Basic application (Microsoft Corp. Redmond, WA). Furthermore, qPCR systems were designed to quantify specific markers for mammary myoepithelial cells (*Krt14*), lymphocytes (*CD3e*), neutrophils (*MPO*, *CD15*, *GPR97*, and *CD18*), and macrophages (*CD68* and *CD18*). We also quantified transcripts from fatty acid synthase (*FASN*), which is expressed in several mammary cell types, including MEC and adipocytes.

Primer efficiency was first validated with a standard curve of 4 serial dilution points (ranging between 1 ng and 1 pg of reverse-transcribed total RNA) and a no-template control. Regarding the specificity of neutrophil markers, we validated primers using a standard curve of RNA extracted from a polymorphonuclear neutrophil (PMN) population isolated from the blood of a healthy goat. The qPCR amplification mixture (20 µL) contained 5 µL of single-strand cDNA template,

Table 1. Primers used for quantitative real-time PCR analysis and sizes of amplicons¹

Gene	Primer	Sequence, 5' > 3'	Amplicon size (nt)
α S2-CN (<i>CSN1S2</i>) ²	Forward	AAC ATG CTG GTT GTA TGA AGT AAA GTG	76
	Reverse	CTG GTT ATG GTT GGA CTG GAAAA	
κ -CN (<i>CSN3</i>) ²	Forward	AGG TGC AAT GAT GAA GAG TTT TTT C	66
	Reverse	CCC AAA AAT GGC AGG GTT AA	
Cluster of differentiation 3 epsilon (<i>CD3e</i>) ³	Forward	ACG CTG TAC CTG AAA GCA AGA	138
	Reverse	AAT ACA CCA GCA GCA GCA AG	
Cluster of differentiation 68 (<i>CD68</i>) ³	Forward	GAT CTG CTC TCC CTG AAG CTAC A	78
	Reverse	CAT TGG GAC AAG AGA AAC TTG GT	
Cluster of differentiation 18 (<i>CD18</i>)	Forward	AGC GAC CTC AGG GAG TAC CA	65
	Reverse	TTA TCG TTG TTC CAC TGG GAC TT	
Cluster of differentiation 15 (<i>CD15</i>)	Forward	CAA GTT CTA CCT GGC CTT CGA	248
	Reverse	GCC ACA GCT TCT CGG TGA TAT AA	
Cyclophilin A (<i>PPA1</i>) ²	Forward	TGA CTT CAC ACG CCA TAA TGG T	62
	Reverse	CAT CAT CAA ATT TCT CGC CAT AGA	
Fatty acid synthase (<i>FAS</i>) ²	Forward	ACA GCC TCT TCC TGT TTG ACG	225
	Reverse	CTC TGC ACG ATC AGC TCG AC	
G protein-coupled receptor 97 (<i>GPR97</i>)	Forward	GAG ATC ACC TTC TCC CAC CAG	204
	Reverse	TGT GGA GCA GCC CAA GGA	
Myeloperoxidase (<i>MPO</i>)	Forward	CTC TAT CAG GAA GCC CGA AAG A	64
	Reverse	GGT AAT CCC GGT AAG TGA TGA TCT	
Keratin 14 (<i>KRT14</i>) ²	Forward	CCC AGC TCA GCA TGA AAG C	57
	Reverse	AGC GGC CTT TGG TCT CTT C	
Serum amyloid A3 (<i>SAA3</i>)	Forward	CTG GGC TGC TAA AGT GAT CAG TAA C	69
	Reverse	CCC TTG AGC AGA GGG TCT GTG ATT	
S24 ribosomal protein (<i>RPS24</i>) ²	Forward	TGG TGG TGG CAA GAC AAC TG	66
	Reverse	TTC TTC GCG TAA TCC AAG GAA	

¹Each pair of primers amplifies the target cDNA [amplicon sizes ranging between 57 and 248 nucleotides (nt)] in its 3' untranslated region. Primer pairs were designed with Primer Express Software version 2.0 (Applied Biosystems, Foster City, CA) except for S24 ribosomal protein primers, which were manually designed.

²Primer pairs were from Bevilacqua et al. (2006).

³Primer pairs were from Bevilacqua et al. (2010).

10 μ L of 2 $\times$ Power SYBR Green PCR Master Mix buffer (Applied Biosystems, Life Technologies), and 1.2 μ L of forward and reverse primers (5 μ M) to reach a final primer concentration of 300 nM. After optimization of the qPCR systems (efficiency ranging between -3.30 and -3.45), amplification reactions were run (in triplicate). The results generated by the Sequence Detection Software (version 2.3; Applied Biosystems, Life Technologies) were exported as tab-delimited text files and imported into Excel (Microsoft Corp.) for further analyses. Relative quantification analysis was performed using the free software program qBase (Hellems et al., 2007), in which relative expression levels were normalized with respect to the selected housekeeping genes (*RPS24* and *PPIA*), following the MIQE (minimum information for publication of qPCR experiments) guidelines (Bustin et al., 2009).

Statistical Analysis

The reliability of reference genes (*RPS24* and *PPIA*) was evaluated with the GeNorm Visual Basic application for Excel (Microsoft Corp.) as described by Vandesompele et al. (2002). All the statistical analy-

ses were performed with the Rcmdr package (<http://cran.r-project.org/web/packages/Rcmdr/index.html>), using R software. A nonparametric Mann-Whitney *U*-test was used to analyze the differences in relative expression of target genes between MFG and MAP.

RESULTS AND DISCUSSION

As an alternative to the commonly used techniques of sampling RNA from MEC (biopsies or somatic cells), we evaluated the use of material isolated from MFG, as first proposed for humans by Maningat et al. (2007). Such an approach could, in fact, be a convenient strategy to access the genome expressed by MEC noninvasively, provided that MFG RNA proved to be representative of the MEC transcriptome. Therefore, our first objective in this study was to demonstrate that this RNA comes essentially, if not exclusively, from MEC, which are by far the main cell type represented in the MAP. The second part of this study was designed as a validation test, to show that MFG-extracted material is pertinent for following the MEC response accurately during the time course of an experimental infection.

MFG, a Unique Source of RNA from MEC

Total RNA Isolation and Assessment of Quantity and Quality. Ribonucleic acids from MFG and MAP were isolated according to the complete workflow given in Figure 1. Quantities of total RNA extracted from the MFG and MAP of goats ($n = 8$) were 78 ± 20 ng/mg of cream layer (2.5 mL of milk) and 1.2 ± 0.5 μ g/mg of mammary tissue, respectively. Total RNA were then assessed for their purity and shown to be free of protein contamination, with absorbance ratio (A260/A280) values ranging between 1.94 and 2.11. In addition, the integrity of RNA was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies), which calculates RNA integrity number values ranging between 6.7 and 8.7. These results underline the fact that RNA extracted from MFG was of comparable quality with RNA obtained from MAP.

Potential Contamination by Immune Cells Present in Milk. Before MFG and MAP transcriptomes were compared by microarray and qPCR, it was crucial to evaluate the possible contamination of RNA from MFG by RNA from macrophages and PMN, which are the main leukocyte cell types in milk from healthy goat udders: 63.0% PMN, 16.2% macrophages, 7.2% lymphocytes, and 13.0% remaining cells (Rota et al., 1993). In addition, these immune cells are well known to have a strong affinity for the MFG, directed toward cell membranes. Therefore, they can be trapped in the cream layer during the centrifugal preparation of MFG (Lee et al., 1980; Sarikaya, 2006), and RNA extracted from MFG can be contaminated by RNA from macrophages or PMN. To evaluate the level of RNA contamination, qPCR analysis was carried out by using specific markers of immune cells reported in the literature: *CD68* for macrophages and *CD3e* for lymphocytes (Hisano et al., 2001). Concerning PMN markers, expression levels of 4 genes (*MPO*, *CD15*, *GPR97*, and *CD18*) were tested on RNA extracted from a PMN population isolated from goat blood, with a standard curve diluted in 10-fold increments to cover a quantitative range of 4 magnitudes (1,000 to 1 pg). Only 2 markers (*CD18* and *GPR97*) were accepted, given their calculated efficiency (95%) and their expression levels. A marker for mammary myoepithelial cells (*Krt14*) was also searched as a negative control. The results showed that *Krt14* and *CD3e* messengers were absent from the MFG, and markers for macrophages and PMN (*CD68*, *CD18* and *GPR97*) were present at very low amounts relative to MAP (Figure 2). These weak values for *CD68* [relative quantity (RQ) mean *CD68* = 0.11], *GPR97* (RQ mean *GPR97* = 0.07), and *CD18* (RQ mean *CD18* = 0.18) reflect a slight but nonsignificant contamination by the corresponding immune cells during MFG preparation.

This contamination remained 20-fold lower, as compared with MAP. Therefore, our results demonstrate that we obtained a sufficient amount of good-quality RNA from MFG, which was slightly contaminated by immune cells, starting from milk collected from a healthy udder.

Does the MFG Transcriptome Reflect the MAP Transcriptome? To delve further into the description of RNA obtained from MFG, we used microarray technology to create a global picture of the genome-wide profile of MFG. We chose to compare the set of genes present in MFG with the set of genes present in MAP, an easy-to-sample material composed of more than 90% MEC (Faucon et al., 2009). Among the 15,208 probe sets analyzed on the Sheep Gene Expression Microarray, 9,152 probes in MAP and 9,645 probes in MFG had a mean signal significantly greater than that of the background ($P < 0.01$). These results highlighted genes for which messengers were considered present in MAP and MFG, respectively. Given the cell composition of MAP, one would expect to see more genes present in MAP than in MFG. This unexpected result was, in fact, due to default parameters calculated by the Feature Extraction software, which flagged a higher number of hits in MAP, reducing the noise generated by this tissue. These 2 sets of probes corresponded to 8,789 genes in MAP and 9,241 genes in MFG, with 8,220 genes in common between MFG and MAP. In other words, MFG and MAP expression profiles shared between 89 and 93.5% of the genes highlighted (Figure 3), with a good gene expression correlation for all genes commonly expressed in MFG and MAP (Figure 4). Within this large set in common, we found, as previously reported in both mice and humans (Rudolph et al., 2003; Maningat et al., 2009), the most highly expressed mammary genes, which were mainly those encoding milk proteins (*LALBA*, *CSN*, and *BLG*) and those involved in protein synthesis (*RPL* and *RPS*; Table 2). It was surprising that *FTH1* (ferritin heavy polypeptide 1), the major intracellular iron-storage protein, was found within this set of highly expressed genes. The overexpression of *FTH1* is associated with a reduction in cellular labile iron, oxidative stress, and inhibition of apoptosis (Epsztejn et al., 1999; Berberat et al., 2003). Lee et al. (1996), however, observed that ferritin heavy chain was highly expressed during involution of the mouse MG. Perturbations in its expression levels have been reported as being associated with the progression of breast cancer (Shpyleva et al., 2011). Among these common genes, we found highly expressed genes involved in fatty acid and triglyceride synthesis and secretion (*FASN*, *XDH*, *ADRP*, *SCD*, and *DGAT1*) but also genes involved in lactose synthesis, such as uridine diphosphate-galactosyltransferase (*B4GALT1*).

Figure 1. Ribonucleic acid extraction workflow, including RNA yield and quality assessment. The workflow was composed of 3 steps. First, milk was skimmed by centrifugation, the milk fat was rapidly put in TRIzol LS solution (Invitrogen Life Technologies Inc., Carlsbad, CA), and the mammary alveolar parenchyma (MAP) were collected from the slaughtered animal (step 1). Next, RNA was extracted from MAP and from milk fat globules (MFG) of the same goat according to the manufacturer's recommendations (step 2). Finally, the quantity and quality of each sample was assessed using both a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and a Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA; step 3). Typical examples of Bioanalyzer RNA profiles, showing the 2 peaks of ribosomal RNA (28S and 18S), are given for each type of biological material and for the corresponding cDNA profile (in the box) obtained after reverse transcription. RIN = RNA integrity number; FU = fluorescence units.

Finally, a comparison of this set with the lactation literature gene set listed by Lemay et al. (2007) showed a similarity of more than 60%, providing further validation of the microarray results (Table 3). In addition, to gain a global understanding of the gene list shared between MFG and MAP, we further analyzed data according to the networks and canonical pathways determined by Ingenuity Pathway Analysis software. From this list, 2,982 different genes with known identifiers

were mapped to their corresponding gene objects in the Ingenuity Knowledge Base. The most significant canonical pathways obtained were the ubiquitination and PI3K/Akt signaling pathways, which belong to the top canonical pathways also found to be significantly overrepresented in lactating mice (Lemay et al., 2007; Rudolph et al., 2007). Indeed, these signaling pathways play a critical role in controlling the cellular growth and proliferation of MEC (Rossi and Loda, 2003) and

Figure 2. Contamination of milk fat globule (MFG) RNA by immune cell RNA, assessed by quantitative real-time PCR. To estimate the contamination of MFG by immune cells [macrophages, polymorphonuclear neutrophils (PMN), and lymphocytes], mRNA levels of cell-specific markers were assessed by measuring the relative amounts of the relevant genes between MFG and mammary alveolar parenchyma (MAP). Relative quantities (RQ) of specific markers for mammary myoepithelial cells (*Krt14*), macrophages (*CD18* and *CD68*), lymphocytes (*CD3e*), and PMN (*CD18* and *GPR97*) were assessed ($n = 8$ goats) after normalization using *PPAI* and *RPS24*. Mean RQ values are given for each gene. A more important signal for *CD18* was obtained, consistent with the fact that *CD18* targets both macrophages and PMN. Mean RQ $\pm$ SEM are given for each gene. An asterisk (*) indicates ratios are significantly different between MFG and MAP ($P < 0.001$).

in mammary differentiation and synthesis of milk components (Rudolph et al., 2003; Green and Streuli, 2004; Naylor et al., 2005). When such a global compara-

tive analysis is used, it appears that MFG and MAP transcriptomes share strong similarities correlating to the lactation function, as would be expected. In this respect, our results are consistent with recent studies carried out using RNA sequencing to perform a comprehensive profiling of genes expressed in cow's milk somatic cells, along the course of lactation (Medrano et al., 2010; Wickramasinghe et al., 2012).

Furthermore, the microarray analysis provides 2 distinct lists with an intensity value lower than 0.1% of the maximum intensity ($\log < 9$) because of an incomplete superimposition of the MFG and MAP gene lists. The first one consisted of 1,021 genes that were specifically present in MFG, and the second one consisted of 569 genes found only in MAP (Figure 3). This asymmetry could be explained by the fact that even if the whole goat mammary tissue is mainly composed of MEC (Faucon et al., 2009), other cell types, such as mammary myoepithelial cells, adipocytes, and endothelial and immune cells (see the Results section above), are part of this tissue. Thus, as expected, when genes from the MAP specific list were overlaid onto a global molecular network built from information contained in the IPA database, significant cell signaling and metabolic pathways were associated with cell types other than MEC (P -value approximately $5E-03$). For instance, analysis emphasized the granzyme B signaling, which characterizes a serine protease, an important granule-associated enzyme exclusively secreted by cytotoxic T lymphocytes (Bots and Medema, 2006). Likewise, the

Figure 3. Venn diagram depicting the distribution of transcripts commonly and differentially detected between milk fat globules (MFG) and mammary alveolar parenchyma (MAP). The diagram shows the number of positive probes, which differs significantly between MFG (light gray circle) and MAP (black circle). This diagram also gives the number of probes in common between MFG and MAP (gray area). Each set contains genes expressed significantly above the background by using a t -test ($P < 0.01$). Then, in each set, genes are proportionally divided up according to their $\log_2$ (signal intensity mean), represented by different levels of gray (4 classes). Percentages correspond to the proportion of transcripts in each $\log_2$ (signal intensity mean) class. The Venn diagram was built with VENNY software (Oliveros, 2007).

Figure 4. Correlation in gene expression for the 8,220 genes commonly expressed between milk fat globules (MFG) and mammary alveolar parenchyma (MAP).

integrin signaling pathway, known to play an important role in organization of tissue structure and cell adhesion to the extracellular matrix (Schatzmann et al., 2003), was underlined. Consequently, this specific MAP list characterizes the biology of the whole mammary tissue. By contrast, regarding the MFG list, the statistical analysis enabled genes to be highlighted that should be slightly expressed in MEC and were therefore likely diluted among other genes in MAP. These genes were present in MFG, although they were ignored because of the default parameters applied for the whole-tissue analysis. Thus, among the 1,021 genes on this list, 582 with known identifiers were mapped in the Ingenuity Knowledge Base. It is interesting that significant canonical pathway patterns were associated with MEC biology (P -value approximately $2E-04$). First, the analysis underlined a marginally significant pathway (LXR/RXR activation). This pathway is composed of proteins involved in lipid metabolism (*APOA1*, *SCD*, and *PTGS2*) that are known to be active in MEC and can therefore enter milk via cytoplasmic crescents conveyed by MFG (Lemay et al., 2009). Furthermore, our results indicate that the MFG specific list is significantly associated with a specific cytokine signature of T-helper (Th) cell differentiation. It has been demonstrated that this paradigm also applies to MEC, which undergoes a switch from Th1 to Th2 cytokine production upon the induction of differentiation (Khaled et al., 2007). Consequently, this is compelling evidence that RNA obtained from MFG represent a source of RNA originating in MEC.

To validate the microarray results and accurately assess whether MFG RNA are quantitatively representative of the transcriptional information contained in MEC, we performed qPCR experiments on material isolated from the 8 uninfected goats. Selected genes,

known to be expressed specifically in MEC and found in common between MFG and MAP in the microarrays, were analyzed. Relative quantities of specific MEC markers (*LALBA*, *CSN2*, and *CSN3*) were assessed after normalization using *PPAI* and *RPS24* as reference genes. Levels of *LALBA* (RQ mean = 0.93), *CSN2* (RQ mean = 1.00), and *CSN3* (RQ mean = 0.93) were significantly unchanged between MFG and MAP, suggesting that these messengers are equally represented in both RNA extracts (Figure 5). Likewise, *FASN*, a gene expressed in a large panel of tissues (Kusakabe et al., 2000), including cell types with a high lipid metabolism (e.g., adipocytes and hepatocytes, but also MEC), was unchanged (RQ mean = 0.94). Taken together, these results strongly support the notion that the RNA content of MFG reflects the RNA content of MEC.

However, to have precise knowledge of the extent to which the transcriptome of MFG matches the transcriptome of MEC, it would have been necessary to have an MEC reference, such as microdissected cells, and to quantify all genes a priori. To go further in this direction, a study using RNA-seq has been undertaken with the aim of comparing RNA from MAP, microdissected MEC, milk somatic cells, and MFG. Given that this strategy requires the availability of a complete genome, the study was initiated in cattle (manuscript in preparation). This ongoing project will undoubtedly provide a more precise description of the real MEC transcriptome and shed light on the basic issue of what the most relevant material is to profile MEC gene expression during lactation. Nevertheless, this report provides a description of RNA extracted from MFG, and it can be concluded that MFG is a unique source of RNA that satisfactorily reflects the biological status of the MEC in its physiological context. In addition, we showed that this apocrine secretion mode, leading to cytoplasmic crescent formation in MFG, which is not exclusive to humans, makes this noninvasive sampling possible in goats, probably one of the first species in which such an apocrine secretion has been reported (Wooding et al., 1970). It also works in other species, such as mares (Cebo et al., 2012), camels [B. Saadaoui (INRA, UMR1313, Jouy-en-Josas, France), C. Cebo, and P. Martin, unpublished data], and, to a lesser extent, cows (Janssen and Walstra, 1982). Consequently, RNA from MFG is a promising material to easily study MEC functions in several species, which potentiates the use of milk as a biological fluid reflecting the pathophysiological status of mammals. As proof of this concept, we used this material to demonstrate that this approach can be used to follow the MEC response during the course of a bacterial infection in the goat species.

Table 2. Forty transcripts found in common between milk fat globules (MFG) and mammary alveolar parenchyma (MAP) among the first 50 genes with the highest signal intensity in each set¹

Encoded protein	Gene symbol	Probe name	GenBank accession no.	Log ₂ (signal intensity mean)	
				MFG	MAP
α-LA	<i>LALBA</i>	A_70_P030266	NM_001009797	20.3	18.5
α-S1-CN	<i>CSN1S1</i>	A_70_P051341	NM_001009795	20.0	18.6
α-S2-CN	<i>CSN2S2</i>	A_70_P030311	NM_001009363	20.0	18.4
β-LG	<i>BLG</i>	A_70_P030361	NM_001009366	20.0	18.6
Brain protein 44-like	<i>BRP44L</i>	A_70_P054041	EE853414	17.9	18.2
CN β	<i>CSN2</i>	A_70_P039416	NM_001009373	20.4	18.9
CN κ	<i>CSN3</i>	A_70_P051196	NM_001009378	20.2	18.7
Eukaryotic translation elongation factor 1 α 1	<i>EEF1A1</i>	A_70_P024306	EE832800	18.0	18.3
Fatty acid synthase	<i>FASN</i>	A_70_P055631	EE852283	19.1	19.1
Ferritin, heavy polypeptide 1	<i>FTH1</i>	A_70_P039496	NM_001009786	18.4	18.2
Glypican 1	<i>GPC1</i>	A_70_P027526	DY509832	19.0	18.2
<i>Ovis aries</i> translationally controlled 1		A_70_P014676	EU370543	18.9	19.1
010914OSAB069080HT <i>Ovis aries</i> cDNA		A_70_P069091	EE842470	18.9	19.1
Brain protein 44-like	<i>BRP44L</i>	A_70_P054041	EE853414	17.9	18.2
Peptidylpropyl isomerase A (cyclophilin A)	<i>PPIA</i>	A_70_P012626	EE791245	18.4	18.5
Polyubiquitin		A_70_P045131	NM_001009202	18.2	18.1
Serum amyloid A3.2	<i>SAA3.2</i>	A_70_P003541	EU366476	19.6	18.1
Xanthine dehydrogenase	<i>XDH</i>	A_70_P051476	EF529448	18.3	18.1
Ribosomal protein L11	<i>RPL11</i>	A_70_P027341	CF115961	17.9	17.9
Ribosomal protein L17	<i>RPL17</i>	A_70_P018371	EE779016	17.9	18.1
Ribosomal protein L19	<i>RPL19</i>	A_70_P069131	EE780718	18.4	18.4
Ribosomal protein L23a-like	<i>RPL23A</i>	A_70_P001951	CD288091	18.0	18.1
Ribosomal protein L27a	<i>RPL27A</i>	A_70_P060102	EE820851	18.5	18.6
Ribosomal protein L36	<i>RPL36</i>	A_70_P001651	CD289189	18.3	18.3
Ribosomal protein L37	<i>RPL37</i>	A_70_P004951	EE847110	18.6	18.6
Ribosomal protein L5	<i>RPL5</i>	A_70_P003226	EE749547	18.1	18.2
Ribosomal protein L6	<i>RPL6</i>	A_70_P014776	EU366470	18.7	18.7
Ribosomal protein L7a	<i>RPL7a</i>	A_70_P068951	EE814708	18.4	18.4
Ribosomal protein P1-like	<i>RPP1</i>	A_70_P012906	EE843127	18.2	18.4
Ribosomal protein S10	<i>RPS10</i>	A_70_P006086	EE781464	18.6	18.7
Ribosomal protein S12	<i>RPS12</i>	A_70_P016536	DQ223559	18.7	19.0
Ribosomal protein S14-like	<i>RPS14</i>	A_70_P001976	EE761453	18.7	18.7
Ribosomal protein S16	<i>RPS16</i>	A_70_P006691	EE810328	18.3	18.2
Ribosomal protein S18	<i>RPS18</i>	A_70_P003171	EE813502	18.4	18.5
Ribosomal protein S2	<i>RPS2</i>	A_70_P050526	EE750624	18.6	18.7
Ribosomal protein S26	<i>RPS26</i>	A_70_P050961	NM_001009435	18.3	18.2
Ribosomal protein S27a	<i>RPS27a</i>	A_70_P045151	EU370542	18.2	18.4
Ribosomal protein S8	<i>RPS8</i>	A_70_P024511	EE853095	19.0	18.8
28S Ribosomal 1	<i>RN28S1</i>	A_70_P001791	EE768843	19.8	19.2
40S Ribosomal protein S20-like	<i>RPS20</i>	A_70_P001846	EU366496	18.3	18.3

¹Fluorescence values are the means of 8 technical replicates from a single goat, and data are presented in alphabetical order (ribosomal genes are given separately).

Table 3. Selected transcripts found in common with the lactation gene set listed by Lemay et al. (2007), specifically in milk fat globules (MFG) and mammary alveolar parenchyma (MAP) or shared between MFG and MAP, and classified according to the different classes of intensity given in the Venn diagram (Figure 3), using the same gray scale

Gray scale	Log ₂ (signal intensity mean)	MFG	MFG and MAP	MAP
Dark gray	>12		LALBA, CSN1S1, CSN2S2, CSN2, CSN3	
Medium-dark gray	12–9	OT-R, IGFII, fibroblast activation protein	TIMP-1, FAS, succinate dehydrogenase, IGFBP-2	
Medium-light gray	9–6	Matrilin, neuregulin 1, IGFBP-3, cytochrome P450, Ig lambda	Neuropeptide Y, prolactin receptor, leptin	EIF4A, INSR, POLA2, AA decarboxylase
Light gray	<6	ER, desmin, DGAT	Progesterone receptor, galanin receptor

Figure 5. Expression levels of specific markers in milk fat globule (MFG) RNA compared with mammary alveolar parenchymal (MAP) RNA, assessed by quantitative real-time PCR. Messenger RNA levels for cell-specific markers were assessed by measuring the relative expression of the relevant genes between MFG and MAP. Relative quantities of specific markers from mammary epithelial cells (MEC; *CSN2*, *CSN3*, *LALBA*) and a marker of a large panel of cell types, including adipocytes and MEC (*FASN*), were assessed after normalization (*PPAI* and *RPS24*). Mean relative quantity (RQ) values ($\pm$ SEM) are given for each gene ($n = 8$ goats).

MFG: A Promising Tool to Follow the MEC Response in the Early Stages of a Bacterial Infection

A better understanding of the natural ability of animal resistance could decrease the impact of mastitis causing economical losses and minimize the use of antibiotics. Up to now, different studies on mice (Zheng et al., 2006) and cows (Mitterhuemer et al., 2010; Rinaldi et al., 2010) have focused on the global gene expression of the whole infected mammary tissue. Nevertheless, several lines of evidence indicate a prominent role for MEC in the initiation of the innate immune response (Gray et al., 2005; Strandberg et al., 2005). Several studies have shown that MEC are able to generate a variety of inflammatory mediators, such as cytokines, chemokines, and host defense peptides (Rainard and Riollot, 2006). More recently CXCL3, a cytokine shown to be constitutively expressed by MEC, was identified as the major chemotactic factor for PMN in bovine milk, in the absence of inflammation (Rainard et al., 2008). However, global gene expression patterns of MEC in vivo are lacking because of the limitations in obtaining samples at close intervals during the early stages of mastitis. To assess whether MFG could be a promising tool to follow the MEC response, we studied the expression of the acute-phase protein SAA3 from MFG-extracted material during the course of an experimental infection.

SAA3: A Good Marker to Follow the MEC Response to Infection. Acute-phase proteins are blood proteins primarily synthesized by hepatocytes as a part of the acute-phase response component of the early de-

fense. Proteins of the serum amyloid A (SAA) family belong to the acute-phase proteins. They are known to contain several differentially expressed apolipoproteins that can be divided into 2 main classes, acute-phase SAA and constitutive SAA, based on their concentration in plasma under normal conditions or after an acute-phase response (Kushner et al., 1989; Uhlir and Whitehead, 1999). The acute-phase SAA arising from different genes increase in concentration in plasma during the acute-phase response, whereas the constitutive SAA are found associated with high-density lipoprotein under normal conditions but are not significantly increased during the acute-phase response (Whitehead et al., 1992; Steel et al., 1996). Several animal species have been reported to show extrahepatic acute-phase SAA expression and production, particularly in the epithelial cells of several tissues (Urieli-Shoval et al., 1998; Upragarin et al., 2005). Thus, in cows, local production of SAA3, termed M-SAA3 for mammary SAA3, has been hypothesized, likely synthesized by MEC (McDonald et al., 2001; Larson et al., 2005; Weber et al., 2006; Molenaar et al., 2009). This protein has been described as an early specific marker of inflammation (Weber et al., 2006) because its level has been shown to increase dramatically in milk during mastitis (Grönlund et al., 2005; Eckersall et al., 2006). This extrahepatically produced acute-phase protein should play a critical role in protecting the mammary epithelium during mastitis and could be a potential marker to provide information about the inflammatory status of the udder (Weber et al., 2006). Recently, cDNA sequences encoding SAA3 were cloned and sequenced from milk, MG tissue, and the liver in goats (Domènech et al., 2012). We confirmed this expression by the presence of SAA3 messengers in MFG from healthy goats (Table 2). Using MFG RNA sampling, we then followed the evolution of M-SAA3 expression at close and early time points of an experimental infection by *Staph. aureus*. We observed a significant increase in SAA3 transcripts ($P < 0.01$) between 24 and 30 h postinfection (Figure 6). This sharp increase (2- to 3-fold) at 24 h postinfection is consistent with transcriptome analyses on cows (Eckersall et al., 2006; Mitterhuemer et al., 2010), further strengthening the validity of the method. Moreover, on the one hand, we were capable of monitoring the kinetics of SAA3 expression on the same animal, and on the other hand, we were able to use the same animal as a control by using the uninfected half-udder. Consequently, milk sampling appeared as the noninvasive method of choice, to obtain MFG and then RNA easily, to accurately evaluate the expression of genes specifically expressed in MEC during the kinetics of infection. However, given the possible contamination of MFG by immune cells, during the course of infection, we assessed the level of

Figure 6. Evolution of the relative expression of serum amyloid A3 (SAA3) in milk fat globules (MFG) during the kinetics of an experimental infection, assessed by quantitative real-time PCR. Milk samples were collected at 6, 12, 18, and 24 h after intramammary injection of *Staphylococcus aureus*. Data are presented as relative quantity (RQ) mean $\pm$ SEM expression ($n = 8$ goats). An asterisk (*) indicates a ratio significantly different from MFG at T₀ ($P < 0.01$).

contamination of MFG-extracted RNA by using RNA from inflammatory cells.

Possible Contamination of MFG by Immune Cells During Infection. During infection, the composition of milk cells is modified and the possible contamination of MFG by immune cells (macrophages, PMN) could have been a major drawback in performing the kinetic analysis. We showed that, in healthy goats, the contamination was negligible. However, during inflammation, PMN are massively recruited in the udder and they become the principal immune cell types in the mammary tissue and milk (Paape et al., 2000; Sordillo and Streicher, 2002; Zheng et al., 2006). Using qPCR for specific markers of macrophages and PMN (*CD18* and *CD68*), we showed that until 18 h, MFG remained very weakly contaminated by these immune cells ($P > 0.1$). In contrast, at 24 h postinfection, we observed a significant increase in the expression of specific markers for PMN, but not for macrophages ($P < 0.05$). Is this contamination by neutrophils responsible for the increase in SAA3 messengers observed in MFG? The answer is very likely no because, in a noninfected context, we did not observe any expression of the *SAA3* gene (RQ mean = 0.0004) by PMN isolated from goat blood. Consistent with this observation, Pisoni et al. (2010), who studied the expression profile of goat milk somatic cells, did not find an increase in SAA3 messengers at 24 h postinfection. In such infectious conditions, PMN account for 96%. Therefore, the gene expression profile recorded is mainly that of neutrophils, strongly suggesting that these immune cells did not express

SAA3 during the *Staph. aureus* challenge. Likewise, in a pulmonary infection context, the absence of SAA3 expression by neutrophils was observed (Wilson et al., 2005). These results strongly suggest that activated neutrophils exhibit a weak, if any, expression of SAA3, in an inflammation context. Accordingly, one may hypothesize that infiltrating PMN that contaminate MFG from infected goat milk do not contribute to the increase in SAA3 messengers in MFG.

Therefore, taken together, our results demonstrate that the RNA extracted from MFG at the early stages of infection is actually representative of the RNA present in lactating MEC. However, beyond 24 h postinfection, it is necessary to take into account the presence of PMN and their expression signature. Otherwise, expression analyses could lead to biased results, even though it is well known that the PMN gene expression profile is restricted to a weak number of genes. Indeed, a 3'-directed cDNA library from human peripheral granulocytes (90 to 95% of PMN) showed an expression profile of 748 different active genes (Itoh et al., 1998). A significant fraction of these genes consisted of nuclear proteins, such as DNA-binding proteins and cytokines, as well as proteins and receptors from the major histocompatibility complex. Nevertheless, it is still possible to adjust gene expression values for changes with cell contamination by applying a computational method for deconvoluting the gene expression profiles (Wang et al., 2006). A more precise reference expression pattern for the milk-activated PMN population, however, remains to be released.

CONCLUSIONS

To conclude, our results indicate that (1) it is possible to use MFG as a source of RNA representative of MEC RNA for subsequent gene expression studies; and (2) MEC, which form the majority of cells lining the MAP, undoubtedly play a crucial role in the innate immune response to bacterial infections. We succeeded in isolating good-quality RNA from goat milk fat in sufficient amounts to analyze the gene expression patterns of MEC, thus paving the way for a better understanding of functions of this cell type. Furthermore, this noninvasive technique enables genetic materials to be obtained from MEC in vivo, which opens up new avenues for performing longitudinal studies or even large-scale analyses, which have been limited and difficult to conceive to date. Thus, mammary transcriptional data that can be considered a kind of "expression typing," filling the gap between genomic and phenotypic data, could be rather easily accessible. Finally, we confirmed the expression of a gene coding for an SAA3 protein in

the MG and provided strong evidence that this local expression is ensured by MEC and very likely not only during the course of a bacterial IMI.

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