Genetic susceptibility to *S. aureus* mastitis in sheep: differential expression of mammary epithelial cells in response to live bacteria or supernatant

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Bonnefont CMD, Rainard P, Cunha P, Gilbert FB, Toufeer M, Aurel MR, Rupp R, Foucras G. Genetic susceptibility to *S. aureus* mastitis in sheep: differential expression of mammary epithelial cells in response to live bacteria or supernatant. *Physiol Genomics* 44: 403–416, 2012. First published February 14, 2012; doi:10.1152/physiolgenomics.00155.2011.— *Staphylococcus aureus* is a prevalent pathogen for mastitis in dairy ruminants and is responsible for both clinical and subclinical mastitis. Mammary epithelial cells (MEC) represent not only a physical barrier against bacterial invasion but are also active players of the innate immune response permitting infection clearance. To decipher their functions in general and in animals showing different levels of genetic predisposition to *Staphylococcus* in particular, MEC from ewes undergoing a divergent selection on milk somatic cell count were stimulated by *S. aureus*. MEC response was also studied according to the stimulation condition with live bacteria or culture supernatant. The early MEC response was studied during a 5 h time course by microarray to identify differentially expressed genes with regard to the host genetic background and as a function of the conditions of stimulation. In both conditions of stimulation, metabolic processes were altered, the apoptosis-associated pathways were considerably modified, and inflammatory and immune responses were enhanced with the upregulation of *il1a*, *il1b*, and *tnfa* and several chemokines known to enhance neutrophil (*cxcl8*) or mononuclear leukocyte (*ccl20*) recruitment. Genes associated with oxidative stress were increased after live bacteria stimulation, whereas immune response-related genes were higher after supernatant stimulation in the early phase. Only 20 genes were differentially expressed between *Staphylococcus spp*-mastitis resistant and susceptible animals without any clearly defined role on the control of infection. To conclude, this suggests that MEC may not represent the cell type at the origin of the difference of mastitis susceptibility, at least as demonstrated in our genetic model. Supernatant or heat-killed *S. aureus* produce biological effects that are essentially different from those induced by live bacteria.

mammary epithelial cell; *Staphylococcus aureus*; mastitis; genetic predisposition; microarray

MASTITIS IS CONSIDERED THE most costly disease of dairy cattle (4, 18, 59) and sheep (6). Infections are associated with huge changes in the defense system of the mammary gland (50) and cause a reduction in milk yield (59) and quality (6). Among mammary pathogens, *Staphylococci*, particularly *Staphylococcus aureus*, are the most prevalent bacteria for intramammary infections (IMI) in small ruminants (6); they often lead to chronic infections and a strong increase in the somatic cell count (SCC) (51). After infection, somatic cells, mainly polymorphonuclear neutrophils (36, 45, 53), are recruited to the mammary gland through the local synthesis of chemokines such as interleukin 8 (IL-8, CXCL8) (35). Mammary epithelial cells (MEC) line the lumen of the mammary gland and represent the first and most abundant cell type in contact with invading bacteria and their secreted products (27). MEC have the ability to synthesize CXCL8 (24) and proinflammatory cytokines in vitro; thus they may contribute actively to the early immune and inflammatory responses of the mammary gland (25, 29, 30, 35, 43).

S. aureus IMI have already been studied in small ruminants (8, 39). The complexity of the mammary defense and the dynamic cross talk between host cells and the pathogen (2) make the study of mastitis particularly difficult. To offset part of these issues, in vitro models of MEC cultures have been largely used to study the interaction of *Escherichia coli* or *S. aureus* with the host cells (11, 22, 25, 29, 52, 58). Although the interaction of MEC with live bacteria has already been studied in several reports (29, 31, 34), heat-killed bacteria (22–25) and pathogen-associated molecular patterns (PAMP), such as lipopolysaccharide (LPS) for *E. coli* or lipoteichoic acid (LTA) for *S. aureus* (1, 11, 13, 30, 37, 41, 58) or supernatant (Sp) (34), are more commonly used. The recourse to synthetic or inert stimuli avoids bacterial development in the culture medium and ensuing consumption of nutrients by the bacteria or release of highly toxic compounds. However, they do not cover the full array of bacteria-cell interactions, and the difference between both types of stimulus is poorly known (34).

At the host level, mastitis outcomes depend not only on the virulence of the bacteria, but also on the strength and timely development of the host's defenses. Thus, genetic improvement is considered to be an interesting option for diminishing mastitis frequency. In this context, we have developed a divergent selection protocol of Lacaune dairy sheep based on somatic cell score (SCS) extreme breeding values (47). Staphylococcal IMI and abscess development were more frequent in

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high SCS animals than in low SCS animals (47, 8), and bacterial count after experimental infection with *S. aureus* was significantly lower in the low SCS animals (8). Therefore, high SCS animals were associated with higher susceptibility to *Staphylococcus spp*-mastitis. To date, two experiments have been performed on these sheep lines to investigate associated gene mechanisms that operate in response to *Staphylococcus* infection in animals with a different degree of susceptibility. After intramammary inoculations of *S. epidermidis* or *S. aureus*, milk somatic cells (MSC) were collected for transcriptomic analysis (8). In vitro, dendritic cells (DC) were also analyzed after stimulation by *S. aureus* to describe gene reprogramming in cells of the monocytic lineage (54).

Until now, very few reports have addressed the question of mastitis resistance and measurement of gene expression in ruminants. Recently, another group has established two models in dairy cattle to address the same question. In the first approach, cows were chosen according to extreme breeding values for SCS (23), and in the second one, a quantitative trait locus (QTL) localized on BTA18 was used to distinguish two groups of cattle on their susceptibility to mastitis (14, 23, 28). MEC of cows with resistant or susceptible alleles at the BTA18 QTL were examined in vitro after they were stimulated by either heat-killed *S. aureus* or *E. coli* over a period of 24 h (14).

Thus, the model of divergent sheep lines represents an interesting opportunity to examine the response of MEC to *Staphylococcus* infection in relation to genetic predisposition to mastitis. Therefore, MEC were isolated from ewes in the two divergent lines and were stimulated in vitro with *S. aureus* to compare their response. We evaluated the MEC response to two different kinds of stimuli, either live bacteria or their soluble factors that are present within the culture supernatant. To look into the network of genes that coordinate the early immune and inflammatory response of MEC upon interaction with live *S. aureus* or its culture supernatant, gene expression profiling was done using an ovine-specific microarray.

MATERIAL AND METHODS

Animals and Sample Collection

MEC were obtained from primiparous dairy ewes at the onset of lactation. The animal model has been previously described (47). Briefly, animals were from a divergent selection of French Lacaune dairy ewes based on extreme breeding values for the SCS. Previous studies indicated that the low-SCS ewes (designated in this paper as "*Staphylococcus spp*-mastitis resistant") are characterized by enhanced mastitis resistance compared with the high-SCS ewes (designated as "*Staphylococcus spp*-mastitis susceptible") (47) and show a lower frequency of infection in natural conditions (47) and a lower bacteriological count after experimental infection (8). Here, individual MEC were isolated from two groups of six ewes without parental link from the *Staphylococcus spp*-mastitis-resistant and -susceptible lines, respectively.

Isolation and Culture of Ovine MEC

Mammary parenchyma was collected upon slaughter. The ewes were killed in the Institute's slaughterhouse as per established standards and in compliance with the policies of Institut National de la Recherche Agronomique's (INRA) Animal Care Committee. Ovine MEC were isolated from mammary secretory tissue as previously described for bovine MEC (29). After one passage, cell cultures that displayed the typical cobblestone morphology at confluence (Figure 1) were cryopreserved in liquid nitrogen. Then they were expanded and prepared for stimulation as previously described (12) and were used at their third passage. After thawing, MEC were multiplied in growth medium comprising Advanced DMEM/F12 medium (Gibco, Invitrogen, Carlsbad, CA), which contains albumin (0.4 mg/ml) supplemented with 2 mM L-glutamine, 10 ng/ml IGF-1 (Peprotech, Rocky Hill, NJ), 5 ng/ml fibroblast growth factor (Peprotech), 5 ng/ml human recombinant epidermal growth factor (Sigma-Aldrich, St. Louis, MO), 1 μg/ml hydrocortisone (Sigma), and 20 mM HEPES (Cambrex Biowhittaker, East Rutherford, NJ). Cells were seeded in six-well tissue culture plates (5×10^5 cells/well) and cultured until confluence. Then the growth medium was replaced with stimulation medium composed of Advanced DMEM/F12 medium with 2 mM L-glutamine, 20 mM HEPES, and 4 ng/ml hydrocortisone as additives 18 h before stimulation.

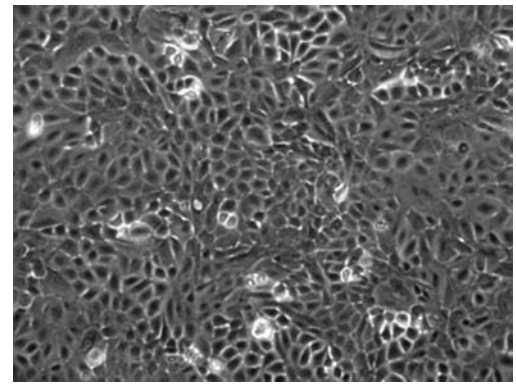

Fig. 1. Microphotograph of ovine mammary epithelial cells (MEC) cultured on a plastic surface. Cells were cultured in growth medium until confluence and were examined with an inverted microscope equipped with a camera. This image shows a cell monolayer exhibiting the typical cobblestone appearance of freshly isolated MEC.

Bacterial Strain, Culture Conditions, and Stimulation of Ovine MEC

The *S. aureus* strain Sa9A was isolated from the milk of a mastitic ewe. The isolate was typed by PCR and shown to be of the agr2 group (21) and to possess the genes for the hemolysins hla and hlb, and for the leukotoxins LukE/D and LukM/F′ but not for the Panton-Valentine leucocidin. It produced a supernatant after overnight culture in Brain Heart Infusion broth with a cytotoxic titer of 80 to bovine neutrophils (moderate activity), determined as described previously (40).

For the preparation of bacterial culture supernatant, Sa9A bacteria were cultured for 7 h in Advanced DMEM/F12 medium. When the culture was discontinued, the optical density (OD) at 660 nm was 0.513, approximately half of the OD value reached at the stationary phase. Following centrifugation at 4,000 *g* for 20 min at 4°C, the supernatant was sterile filtered (0.2 μm) and stored in aliquots at −70°C. Live bacteria were grown overnight in Advanced DMEM/F12, washed once in Dulbecco's phosphate-buffered saline, and adjusted to the concentration of 3×10^5 colony forming units (cfu)/ml in Advanced DMEM/F12 by reference to the absorbance value at 660 nm and with retrospective checking by means of cfu plate assays.

For the stimulation experiments, ovine MEC were incubated with either live bacteria (Sa samples) or bacterial supernatant (Sp samples) for 1 (T1 samples) or 5 (T5 samples) h. Just before stimulation, the medium was removed from the confluent epithelial monolayers and replaced with fresh medium plus stimulus. The final concentration of bacteria was 3×10^5 cfu/ml, i.e., equivalent to an initial multiplicity of infection of 1 cfu per cell. The final concentration of the bacterial supernatant was 20% in Advanced DMEM/F12 during incubation with MEC. After 1 h and 5 h of incubation, the cell culture medium

was pipetted out, sterile filtered and stored at −70°C. The monolayers were washed twice with HBSS, and cells were harvested for RNA extraction. The control cell culture consisted of MEC that received fresh culture medium at *time 0* and were harvested 1 h later (Tref samples), at the same time as cells that had been incubated for 1 h with live Sa9A or bacterial supernatant.

Quantification of CXCL8 and CXCL3 by ELISA

The sandwich ELISA for CXCL3 (previously known as GROγ), the "constitutive/inducible" chemokine in milk of dairy ruminants, was carried out as previously described (42). Cell culture supernatants were tested undiluted. Concentrations of the chemokine CXCL8 were determined as for CXCL3 except that commercial antibodies were used to set up the sandwich ELISA. Microtiter plates (96-well, Nunc Immunoplate Maxisorp) were coated with a monoclonal antibody (clone 8M6, MCA1660; Serotec, Oxford, UK) at the concentration of 2 μg/ml in Na carbonate/bicarbonate buffer 0.1 M pH 9.6 overnight at 4°C. After blocking the plates with phosphate-buffered saline (PBSG) supplemented with 0.5% (wt/vol) gelatin (Gibco), samples diluted two- to 10-fold in PBSG were incubated for 2 h at 37°C. Recombinant bovine CXCL8 (42) was used to establish a standard curve. Rabbit anti-ovine CXCL8 (AHP425, Serotec) diluted 1/2,000 in PBSG was used as the detection antibody. After an 1-h incubation at 37°C and washes, anti-rabbit IgG-peroxidase conjugate (Jackson Immunoresearch) diluted 1/20,000 in PBSG was incubated for 30 min at 37°C. Finally, the ELISA was revealed by incubation with TMB ELISA substrate (Uptima; Interchim, Montluçon, France). After addition of stop solution, OD was read at 450 nm with a plate reader (Multiskan RC; Labsystems, Helsinki, Finland). The intra-assay variability was determined by testing replicates of a few samples and of standard dilutions of recombinant CXCL8. The intraplate and interplate coefficients of variations were <10 and 15%, respectively. Dilution of recombinant CXCL8 in MEC culture supernatant and recovery of the spiked chemokine indicated that there was no inhibition or matrix effect interfering with the ELISA results.

Microarray Analysis

RNA extraction, amplification, labeling, and RNA quality. Total RNA was extracted by a double extraction method using first TRIzol (Invitrogen) and then RNeasy columns (Qiagen). Quantification was performed using a spectrophotometer (NanoDrop Technologies) and RNA quality was assessed using an Agilent Bioanalyzer 2100. The RNA integrity number index was 8.6 ± 1.4 (mean ± SD). We converted 200 ng of RNA into double-stranded cDNA and amplified with the Quick Amp Labeling kit, two colors (ref.: 5190-0424, Agilent Technologies), and RNA was then labeled with Cy3 and Cy5.

Hybridization, scanning, and raw data storage. We hybridized 60 microarrays [2 sheep lines * 6 sheep * 5 time-stimulation points (Tref, T1Sa, T5Sa, T1Sp, and T5Sp)] using the ovine oligonucleotide slide (ref. 019921, Agilent Technologies) in a two-color dye-swap experimental design. On each microarray, two samples from the same experimental condition, one from a *Staphylococcus spp*-mastitis-resistant ewe and the other from a susceptible ewe were hybridized together (Fig. 2). Chips were hybridized at 65°C for 17 h and then washed according to the manufacturer's protocol. Intensity values were recorded with a 4000B Axon scanner. Two channel images were imported into the Agilent's Feature Extraction software for spot finding and alignment. One microarray was removed from the analysis due to poor hybridization quality.

Only 8,547 genes among the 15,008 different probes that are present on the ovine oligoarray, were annotated with the Human ortholog Gene Nomenclature Committee (HGNC) (http://www.sigenae.org/, sheep oligo annotation version 7 of 2011/03/02) (16). We obtained additional information on unannotated probes using the Basic Local Alignment Search Tool program on the National Center for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and using the ENSEMBL website (http://www.ensembl.org/index.html).

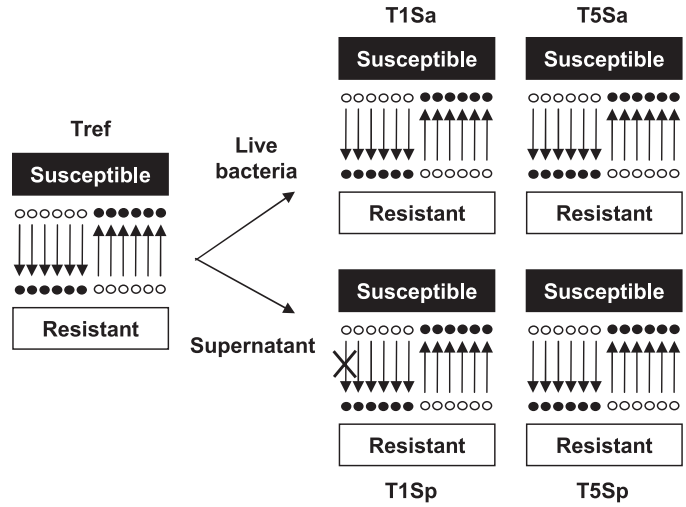

Fig. 2. Hybridization design of MEC samples on the 15K ovine Agilent microarray. Each dot represents a dyed sample (black dots for Cy3-dyed samples and white dots for Cy5-dyed samples). Each arrow stands for a microarray with 2 samples hybridized. This protocol enabled a direct comparison of *Staphylococcus spp*-mastitis-resistant and -susceptible animals. Both samples were stimulated simultaneously and for the same duration with the same preparation of *S. aureus*. Tref corresponds to samples after 1 h of culture without stimulation. The other samples were stimulated either by Sa and collected at 1 or 5 h poststimulation (T1Sa and T5Sa, respectively) or by Sp during the same duration (T1Sp and T5Sp, respectively). The hybridization design was a dye-swap design on 60 arrays, but 1 array was removed because of bad quality (the crossed arrow).

Statistical analysis of microarray data. Raw data from Agilent's Feature Extraction were imported in R (version 2.9.0) through the LIMMA package (49). For each sample, probes were flagged if their intensity was of good quality, unsaturated, and uniform. Flagged probes were conserved for further analysis. A set of 4,225 out of 15,008 probes were retained. Previously, the same probe filter was performed on other datasets, and it enabled us to retain many more genes in other cell types like DC and MSC. Lowess normalization without background correction was applied to raw intensity data to correct the dye effect. Then T-quantile normalization was applied considering the five class conditions (Tref, T1Sa, T5Sa, T1Sp, and T5Sp) to correct the array effect. A $\log_2$ transformation of data was applied, and then preprocessed data were imported in SAS (version 9.1). The $\log_2$-intensity of each dye was analyzed separately, since Bossers et al. (9) showed that this method enhanced the reproducibility and the sensitivity of the detection of differentially expressed gene (DEG) analysis compared with ratio-based analysis. Differences of expression in MEC were analyzed probe by probe by an analysis of variance (ANOVA) using the SAS MIXED procedure. First, MEC responses to infection were studied separately in the "live bacteria" condition (Tref, T1Sa, T5Sa) designated by Sa or "supernatant" condition (Tref, T1Sp, T5Sp) designated by Sp using an ANOVA model including the time effect and the color effect (M1.1). Then stimulation effect was analyzed within time effect (without data of Tref) to compare T1Sa vs. T1Sp and T5Sa vs. T5Sp considering the color effect (M1.2). Finally, the differences between *Staphylococcus spp*-mastitis-resistant and -susceptible animals were analyzed using two models: the first model (M2.1) included a global line effect, a combined time and stimulation effect (TimeStim), and the color effect; in the second model (M2.2) the line effect was fitted within the TimeStim effect:

M1.1: $\log_2 (i_{norm}) = \mu + \text{Time} + \text{Color}$

M1.2: $\log_2 (i_{norm}) = \mu + \text{Stimulation (Time)} + \text{Color}$

M2.1: $\log_2 (i_{norm}) = \mu$ + Line + TimeStim + Color

M2.2: $\log_2 (i_{norm}) = \mu$ + TimeStim + Line (TimeStim) + Color

$Log_2(i_{norm})$ was the normalized intensity of a probe; time was either Tref, T1Sa, and T5Sa or Tref, T1Sp, and T5Sp; stimulation was Sa or Sp; line was *Staphylococcus spp*-mastitis resistant or susceptible; TimeStim represented the five conditions: Tref, T1Sa, T5Sa, T1Sp, and T5Sp, and color stood for the dyed label: Cy3 or Cy5.

All *p* values were corrected with the Benjamini-Hochberg false discovery rate procedure (38). Fold-changes (FC) were computed as the ratio of the ANOVA-estimated-$\log_2$-intensity of samples from two conditions. For the time analysis, the numerator was the latest time and the denominator the earliest time, so a positive FC meant an upregulation during the time-course. For the stimulation analysis, the numerator was Sa and the denominator Sp. For the line effect analysis, the numerator was the *Staphylococcus spp*-mastitis-resistant ewes and the denominator was the *Staphylococcus spp*-mastitis-susceptible ewes. Probes were declared to be differentially expressed if their corrected *p* values were below the threshold of 0.05 and if their absolute FC was >0.585, which corresponds to a minimal 1.5-fold increase in gene expression in one of the conditions.

Furthermore, as few DEG between *Staphylococcus spp*-mastitis-resistant and -susceptible animals were identified, gene set enrichment analysis (GSEA) was performed. It was based on the list of genes found in the ANOVA (Model 2.1) with a Benjamini-Hochberg corrected *p* value <0.05 ($n = 596$ probes, with 537 HGNC annotated genes). Gene sets with at least 15 genes were retained for analysis. The *p* value was calculated by computing the enrichment score in 1,000 phenotype permutations. Gene sets with a *p* value <0.05 were declared as significantly enriched.

The experiment was deposited in the Gene Expression Omnibus (GEO) at the identifier number GSE30390 (BioArray Software Environment - version SIGENAE).

Biological interpretation of the focus genes. Two hierarchical clustering of the DEG in the time course based on the Pearson-centered distance metric and centroid linkage rule were performed with GeneSpring. Samples were averaged to present mean values for Tref, T1, and T5. Using the heat map, we sorted genes into clusters that showed different time profiles, and these clusters were interpreted with the available biological information.

Significant canonical pathways of the DEG datasets were identified using Ingenuity Pathways Analysis (IPA, Ingenuity Systems, http://www.ingenuity.com). As all Agilent ovine microarray spots were not annotated, the whole slide cannot be used as a reference database for the canonical or function analysis. Human databases were used as references. The significance of the association between the dataset and the canonical pathways was measured in two ways: *1*) a ratio of the number of molecules from the dataset that mapped to the pathway over the total number of molecules that map to the canonical pathway; and *2*) Fisher's exact test was used to calculate a *p* value determining the probability that the association between the genes in the dataset and the canonical pathway could be explained by chance alone. Only the canonical pathways with a ratio $>10\%$ and a *p* value <0.05 were kept in this study. About network analysis a score is calculated for the significance of the network. The higher the score is, the more significant it is.

Gene Ontology (GO) analyses were performed using DAVID (Database for Annotation, Visualization and Integrated Discovery) (http://david.abcc.ncifcrf.gov/). The singular enrichment analysis and the modular enrichment analysis tested the enrichment of each annotation term in a linear model and led to establishing relationships between genes with similar biological functions. Thereafter, we considered that a gene list was enriched for an annotation term if this term was represented by at least three genes and had an EASE score <0.05. The gene lists and the biological functions of the DEG in the time course after stimulation by Sa or Sp were compared by generating Venn diagrams. To further interpret these data, the sense of the regulation (up or down) was considered.

Overrepresented transcription factors that are known to bind to the promoter regions of the listed focus genes were identified with InnateDB (http://www.innatedb.com). This analysis was performed to all DEG during the time course following stimulation by Sa or Sp. First, two lists were created by pooling the genes from the downregulated (C0 and C1 from the Sa experiment, and CA and CD from the Sp experiment) and upregulated (C4 and C5 from the *Sa* experiment, and CB and CC from the *Sp* experiment) clusters. Then the genes were annotated by ENSEMBL, and the files with *p* values and annotations were imported in InnateDB. The hypergeometric algorithm was applied to the data, and the *p* values were corrected by Benjamini-Hochberg at 5%.

Reverse Transcription

cDNA was generated from 300 ng of total RNA using the Superscript III First Strand Synthesis System Kit (Invitrogen) and random hexamer primers and following the manufacturer's instructions. The relative mRNA expression levels were verified for Tref, T5Sa, T1Sp, and T5Sp samples. The gene expression of T1Sa samples was measured only for *cxcl3* and *cxcl8*; for the other genes it was not measured because too few differences were observed when compared with the Tref samples after microarray analysis.

For quantification of mRNA transcripts, primer pairs were designed using Primer3 (46) based on the relevant ovine mRNA sequences and synthesized commercially (Eurogentec). The specificity of designed primers was checked with BLAST (http://blast.ncbi.nlm.nih.gov/) and Primer Express v2.0. For some genes for which ovine sequence was not available, a comparative gene alignment of bovine, human, rat, and mouse sequences was performed, and primers were then designed on the most conserved regions between these species. Optimal annealing temperatures were determined for each primer pair, and the primers were checked for the absence of primer dimers and efficiency before use. The primers used in RT-qPCR experiments are listed in Table 1.

RT-qPCR was performed following two different protocols depending on the number of genes to be tested. When measuring expression of a small number of genes, we used an Applied Biosystems 7300 Real time PCR system. All assays were carried out in duplicate, and each reaction contained 5 μl of diluted cDNA with 2.5 μl (0.5 μM) of each forward and reverse primer along with 12.5 μl of Power SYBR Green PCR Master Mix (Applied Biosystems).

The high-throughput microfluidic BioMark qPCR platform (Fluidigm) was used for qPCR analysis running the 48.48 dynamic array. Methods were essentially as described before (54). Briefly, cDNA from all samples was preamplified using a pool of forward and reverse primers. After priming the empty dynamic array, we then loaded 5 μl of sample reaction mixtures into the sample wells carefully avoiding any bubbles, 5 μl of primer reaction mixtures were loaded into the assay wells, and the NanoFlex 4-IFC Controller was used for loading and mixing. The loaded dynamic array was then transferred onto the BioMark HD system. For both approaches, the qPCR cycling program was 10 min at 95°C for activation of the hot-start enzyme, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 1 min, and elongation at 72°C for 20 s. Melting curve analysis was performed after completion of the qPCR by collecting fluorescence intensities between 60 and 95°C.

RT-qPCR Data Analysis and Normalization

The stability of six HKG (*gapdh, hprt, rp19, rp26, sdh,* and *tyr),* previously mentioned in the literature, was checked in our 48 samples, and data obtained were analyzed by GeNorm (http://medgen.ugent.be/~jvdesomp/genorm/) (55), a software package freely available for research use. Hence, RT-qPCR data were normalized against the four most stable HKG (*rp19, rp26, tyr,* and *gapdh*) as determined by GeNorm analysis. Specific amplification of each targeted cDNA was confirmed by melt curve analysis. Measured Ct values were exported

Table 1. *Sequences of the couples of primers used in RT-qPCR*

HGNC	Accession Number	PRIMER_F1_SEQ	PRIMER_R1_SEQ	Description
AXL	DY498294	atatccgggagtggagaaca	acatcagggcatacagtcca	AXL receptor tyrosine kinase
CASP8	EE784040	gggtgaagacattctgaccatc	aagtaggttgcggcatctgt	caspase 8, apoptosis-related cysteine peptidase
CAT	EE850070	tctgccctggaacataggac	tagaaatcccgcacctgagt	catalase
CD14	EE818529 NM_001077209	gacccttcatggcttctttg	ttggttgatgtcctgggtct	CD14 molecule
CTGF	EE757904	agtccatcaaccccagacac	tctactggcctcctcccact	connective tissue growth factor
CXCL2	DY496264	ccgaagtcatagccactctt	ttggtgctgcccttgtttag	chemokine (C-X-C motif) ligand 2
CXCL5	FE033277	tgcttgttcttgcagctttc	cagcattccagtcaaattcc	chemokine (C-X-C motif) ligand 5
CYR61	DQ239628	tcggaggtggagttaacgag	ctgggaccacgaagttgttt	cysteine-rich, angiogenic inducer, 61
DDIT4	EE826574	gtacgcacttgtcccaacag	caaggacgaggacgaagaag	DNA-damage-inducible transcript 4
EGR1	EE838247	actgcccgactgtactcaac	aaggcaccaagacgtgaaac	early growth response 1
GSTM3	DQ223552	taagaacaggctcacccaga	tgtccactcgaatcctttcc	glutathione transferase M3
IL1a	NM_001009808	gatgacctggaagccattgc	atgtgctgatctgggcttgat	interleukin 1, alpha
IL1b	NM_001009465	tgaagagctgcacccaacac	gagaaatctgcagctggatgttt	interleukin 1, beta
IL8	NM_001009401	cattgccacaaagcaagaaa	gcacaatcaaggctatttttcc	interleukin 8
LAMA3	EE782590	gaccgtctcaatctcaccaa	tgaatgaccacaggagacca	laminin, alpha 3
MAN1A1	EE789683	cctggcagtttacagagcttc	gcacaaacaaagagcaatgg	mannosyl-oligosaccharide alpha-1,2-mannosidase
MYC	NM_001009426	gacacggaggagaatgacaa	gatctggtcacgaagagcaa	v-myc myelocytomatosis viral oncogene homolog (avian)
NFIL3	EE756836	gaacaagctctgggccttta	cagccttgcgatggactatt	nuclear factor, interleukin 3 regulated
PLAT	EE784649	gaagatggacaaacgccagt	tcgtgtgcatagaggaggaa	plasminogen activator, tissue
SAA3	EU366476 EE866720	aggctcttcagggaatcaca	gtcagccttcgtgtcctctc	serum amyloid A3
SLC2A3	NM_001009770	atgtcgcaggagaagcaagt	ctggagcatgatggagatga	solute carrier family 2 (facilitated glucose transporter), member 3
SOD2	EE760845	gtttctgagcgtggcttgtt	cgattgaccaggatggaaag	superoxide dismutase 2, mitochondrial
TNFa	NM_001024860	gagcaccaaaagcatgatcc	ggcgatgatcccaaagtaga	tumor necrosis factor alfa
VEGFA	EE772615	cgatgtcctcagaccattga	tgggttaaccactcacacacac	vascular endothelial growth factor A
GAPDH	HKG*	tgacaaagtggtcgttgagg	tggagaaacctgccaagtatg	glyceraldehyde-3-phosphate dehydrogenase
HPRT	HKG†	tcctcatggactaattat	ccacccatctccttcatcac	hypoxanthine phosphorybosyl transferase
RPL19	HKG*	aagggcaggcatatgggtatag	gttaccttctcgggcattcg	ribosomal protein 19
RPL26	HKG*	gttgtgccattcacagcaag	gtctaaatcggggtggaggt	ribosomal protein 26
SDHA	HKG**	gtcaagactggggaggtca	gtcggtctcgttcaaagtcc	succinate dehydrogenase complex, subunit A
TYR	HKG*	gtcatcttggagggtcgtct	tcgagccatctgctgttttt	tyrosinase

*HKG, housekeeping genes used for normalizing the other gene expression. †HKG tested but not used for normalization.

from the BioMark software to Excel for data analysis. qPCR technical replicates of samples were averaged, and expression ratios were calculated by the delta delta Ct method normalized to the multiple HKG (55). A Wilcoxon test was performed to assess the effect of line or time using the NPAR1WAY procedure of SAS (v. 9.1). The values were expressed as a ratio of the sample with the reference made of susceptible animal samples at Tref.

RESULTS

MEC Response to Staphylococcal Stimulations

MEC were cultivated in vitro and stimulated with Sa or Sp. They were collected without any stimulation (Tref) or at 1 or 5 h poststimulation (T1, T5). To ascertain that MEC responded to the stimulations, the expressions of five genes were measured by RT-qPCR (*ccl20, cxcl3, cxcl8, il1b,* and *tnfa*) at Tref and T5 (Fig. 3). The genes *cxcl8, ccl20, il1b,* and *tnfa* were significantly upregulated at T5 with both Sa and Sp compared with Tref (p value < 0.01, except for *il1b* but p value < 0.05). The amplitude of gene expression was weak for *il1b* with an FC between T5Sa vs. Tref and T5Sp vs. Tref near to 2, whereas it was high for *ccl20* and *tnfa* after Sa stimulation with an FC (T5Sa vs. Tref) ~15 and 100, respectively. Neither comparison (between lines or stimulations) was significantly different.

Expression of CXCL3 and CXCL8 was also confirmed by ELISA at the protein level. As indicated by CXCL8 concentrations (median value, first and third quartiles) in cell culture supernatants, MEC had reacted to Sp as soon as 1 h poststimulation (1.08 ng/ml; Q1-Q3, 0.19–0.46) compared with Tref (0.37 ng/ml, 0.19–0.46) (p value < 0.001), whereas with Sa, CXCL8 concentration (0.42 ng/ml, 0.34–0.65) was not significantly different from Tref (test of Kruskal-Wallis followed by multiple comparison with Bonferroni's correction). At 5 h poststimulation, both stimuli induced CXCL8 production by MEC (T5Sp 6.69 ng/ml, T5Sa 3.63 ng/ml). The delayed reaction of MEC to live staphylococci applied also to CXCL3 secretion (Tref 0.25 ng/ml, T1Sp 1.32 ng/ml, T1Sa 0.32 ng/ml, T5Sp 4.5 ng/ml, T5Sa 2.05 ng/ml).

MEC stimulation with live bacteria. When stimulated with Sa, a total of 531 probes, corresponding to 378 genes, were differentially expressed in MEC during the time course (model M1.1, Additional File 1).[1] The expression of four downregulated genes (*ctgf, cyr61, egr1,* and *myc*) and seven upregulated genes (*ddit4, il1a, cxcl8, nfil3, saa3, slc2a3,* and *vegfa*) was measured by RT-qPCR. The expression changes of these 11 genes were indeed confirmed ($P < 0.015$) (Table 2). For the global gene list obtained from the microarray analysis, the canonical pathways "IGF1 signaling" ($P < 0.001$, with the genes *ywhaq, pxn, ctgf, ywhab, csnk2a1, igfbp3, rac1, prkaca, pik3r2,* and *cyr61*) and "DNA damage checkpoint regulation pathways" ($P < 0.001$, with the genes *ywhab, ywhaq, cks2, ccnb3, cdkn1a, mdm2*) belonged to the list of pathways that were significantly affected after Sa stimulation.

[1] The online version of this article contains supplemental material.

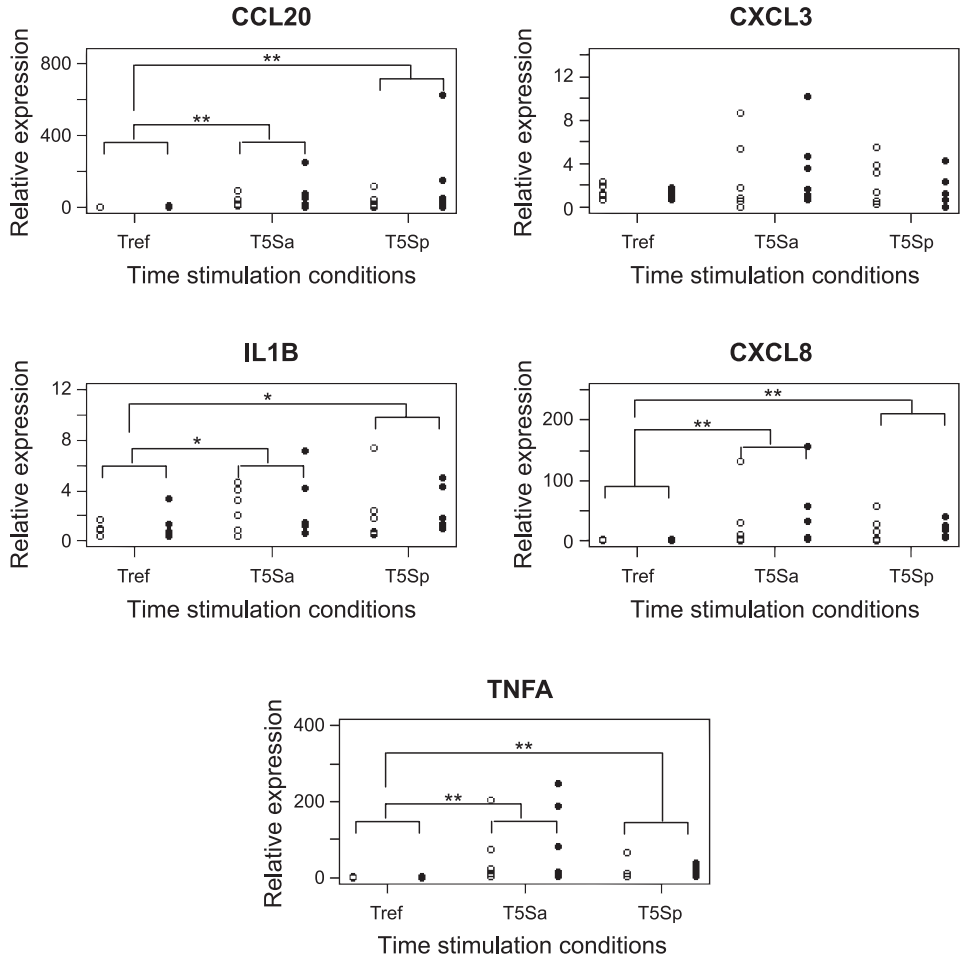

Fig. 3. RT-qPCR of representative genes. RNA quantification of *cxcl3*, *cxcl8*, *ccl20*, *tnfa*, and *il1b* by RTq-PCR at Tref or 5 h poststimulation by Sa (T5Sa) or Sp (T5Sp) are presented. Opened and closed symbols are respectively for *Staphylococcus* spp-mastitis-susceptible and -resistant sheep, respectively.

Furthermore, hierarchical clustering of the DEG in the time course produced six clusters (Fig. 4*A*). First, the expression of 361 probes was downregulated in the time course (*clusters 0* and *1*). A first cluster of 154 probes (*cluster 0*) showed no change in expression levels between Tref and 1 h of stimulation and then a diminution between 1 and 5 h. The GO analysis of the *cluster 0* revealed an enrichment in genes involved in the regulation of cell cycle ("cell cycle," "cell cycle process," "regulation of cell growth," "mitosis"..., $P < 0.001$), induction of apoptosis ("apoptosis," "programmed cell death," "regulation of apoptosis," ..., $P = 0.003$), catabolic processes ("regulation of caspase activity" $P = 0.023$, "regulation of macromolecule metabolic process" $P = 0.025$, "cyclin catabolic process" $P = 0.030$...), and the "p53 signaling pathway" ($P = 0.002$). The expression of 207 probes (*cluster 1*) dropped after the stimulation; the GO analysis of this cluster showed that whereas a few of these genes were involved in cell growth ("regulation of growth" $P = 0.011$, "cell cycle process" $P = 0.018$...) or in "regulation of apoptosis" ($P = 0.006$), most of them were involved in response to stress ("cellular response to stress" $P = 0.019$, "cellular response to oxidative stress" $P = 0.031$...), in development ("muscle and tissue development" $P < 0.001$, "tissue morphogenesis" $P < 0.001$, "morphogenesis of an epithelial sheet" $P = 0.002$) and in regulation of biosynthetic process ($P = 0.008$). A third cluster of 67 probes (*cluster 2*) comprised the genes that were the most expressed at T1 after which their expression came back to the previous levels at T5 (Fig. 4*A*). The GO analysis of the *cluster 2* showed enrichment in genes involved in cell cycle ("cell cycle" $P < 0.001$, "positive regulation of cell cycle" $P = 0.014$, "mitosis" $P = 0.034$...) and in cellular respiration ("cellular respiration" $P = 0.040$, "electron transport chain" $P = 0.006$, "ATP synthesis-coupled electron transport" $P = 0.014$...). Another group of 33 probes was on the contrary downregulated at T1, whereas their expression was higher at T5 (cluster 3, Fig. 4*A*) and was involved in the "negative regulation of molecular function" ($P = 0.018$) and in the "regulation of apoptosis" ($P = 0.044$). Two more clusters included 70 probes that were upregulated during the time course (*clusters 4* and *5*). The expression of 47 probes increased lately at T5 only (*cluster 4*), and these genes were involved in metabolic processes ("hexose metabolic process" $P = 0.001$, "protein polymerization" $P = 0.007$, "regulation of homeostatic process" $P = 0.033$...), apoptosis ("regulation of apoptosis" $P < 0.001$, programmed cell death $P < 0.001$...), and immune and inflammatory responses ("inflammatory response" $P = 0.009$, "immune response" $P = 0.028$, "response to oxygen level" $P = 0.005$, "induction of positive chemotaxis $P = 0.025$). Finally 23 probes were upregulated early after stimulation (at both T1 and T5) (*cluster 5*) and were involved in "protein N-terminus binding" ($P < 0.001$).

MEC stimulation with S. aureus supernatant. After Sp stimulation, a total of 687 probes (486 genes) were found to be differentially expressed over time (model M1.1, Additional

Table 2. *RT-qPCR of 17 DEG over time*

Genes	Time, *P* Value				Stimulation				
	Tref-T5Sa	Tref-T1Sp	Tref-T5Sp	T1Sp-T5Sp	*P* Value T5Sa-T5Sp	Tref	T1Sp	T5Sa	T5Sp
CD14	0.20	0.18	0.01	0.00	0.01	1.18 ± 0.56	0.95 ± 0.57	1.01 ± 0.52	2.65 ± 1.65
CTGF	0.01	0.47	0.00	0.00	0.14	1.44 ± 1.17	1.65 ± 1.49	0.55 ± 0.41	0.42 ± 0.29
CXCL2	0.11	0.05	0.02	0.46	0.51	1.26 ± 0.43	3.21 ± 2.79	2.92 ± 2.52	2.58 ± 1.57
CXCL5	0.42	0.43	0.01	0.00	0.02	1.24 ± 0.68	1.12 ± 0.57	1.48 ± 1.07	3.95 ± 3.39
CYR61	0.00	0.24	0.00	0.00	0.40	1.22 ± 0.62	1.10 ± 0.63	0.39 ± 0.24	0.39 ± 0.12
DDIT4	0.00	0.48	0.00	0.00	0.01	1.86 ± 1.53	1.40 ± 0.67	10.61 ± 3.35	6.57 ± 2.89
EGR1	0.00	0.17	0.00	0.00	0.14	1.02 ± 0.49	1.46 ± 0.84	0.19 ± 0.14	0.24 ± 0.16
IL1a	0.01	0.02	0.08	0.46	0.49	1.11 ± 0.63	1.78 ± 0.8	1.65 ± 0.52	1.82 ± 1.2
IL8	0.00	0.00	0.01	0.18	0.43	1.76 ± 2.55	5.29 ± 4.44	12.11 ± 13.34	11.33 ± 13.22
MAN1A1	0.20	0.24	0.10	0.02	0.03	0.83 ± 0.36	0.73 ± 0.49	0.69 ± 0.38	1.62 ± 1.09
MYC	0.00	0.03	0.04	0.30	0.00	1.20 ± 0.36	0.91 ± 0.34	0.43 ± 0.17	0.95 ± 0.31
NFIL3	0.00	0.47	0.00	0.00	0.12	1.24 ± 0.56	1.15 ± 0.39	3.23 ± 0.91	2.86 ± 1.4
PLAT	0.40	0.47	0.08	0.04	0.13	1.22 ± 1.09	1.21 ± 1.26	1.61 ± 1.71	4.73 ± 6.25
SAA3	0.00	0.12	0.00	0.01	0.28	3.80 ± 8.27	3.28 ± 2.8	16.91 ± 17.73	50.44 ± 85.53
SLC2A3	0.00	0.42	0.01	0.00	0.10	1.18 ± 1.35	0.79 ± 0.58	5.56 ± 4.31	3.27 ± 2.05
SOD2	0.33	0.07	0.07	0.00	0.14	1.49 ± 1.13	0.56 ± 0.4	1.96 ± 2.08	4.29 ± 4.97
VEGFA	0.01	0.04	0.00	0.00	0.11	1.07 ± 0.43	0.82 ± 0.5	1.98 ± 1.04	2.64 ± 1.61

RNA was quantified from samples of all animals at Tref, T5Sa, T1Sp, and T5Sp samples with Biomark Fluidigm technology. The values were normalized by HKG (*rp19*, *rp26*, *tyr*, and *gapdh*) with the Vandesompele method (55). For each gene at each time point, the values of the samples of *Staphylococcus spp*-mastitis-resistant and -susceptible animals were brought together and expressed as means ± SD, presented on the *right* side of the table. The statistical analyses were performed using Wilcoxon nonexact tests for each gene. *P* values of Tref vs. T5Sa, Tref vs. T1Sp, Tref vs. T5Sp, and T1Sp vs. T5SP and between stimulation conditions at T5 (T5Sa vs. T5Sp) are presented on the *left*. DEG, differentially expressed gene.

File 2). The expression of 16 of these genes was measured by RT-qPCR: three of them were downregulated (*ctgf*, *cyr61*, and egr1), and 13 were upregulated *(cd14*, *cxcl2*, *cxcl5*, *ddit4*, *il1a*, *cxcl8*, *man1a1*, *nfil3*, *plat*, *saa3*, *slc2a3*, *sod2*, and *vegfa*). The differences in gene expression over time were confirmed for all genes ($P < 0.02$) (Table 2). The main canonical pathways determined for the global list of DEG involved "interleukin signaling" (IL-1, -3, -6, -10, -17 $P < 0.001$, except for IL-22, $P = 0.036$), "TLR pathway" ($P < 0.001$), "CD40 pathway" ($P < 0.001$), "TNFR1 pathway" ($P = 0.002$), and "TNFR2 pathway" ($P = 0.001$), "NF-κB activation" ($P < 0.001$), and "Myc-mediated apoptosis signaling" ($P < 0.001$).

Hierarchical clustering of the DEG as a function of their expression over time produced four clusters (Fig. 4*B*). The first two clusters comprised 205 probes that were downregulated after stimulation (*clusters A* and *D*). The expression of 73 probes was not affected 1 h after stimulation but dropped at 5 h (*cluster A*) (Fig. 4*B*). The genes of this cluster were mainly involved in lipid and protein metabolic processes ("sterol biosynthetic process" $P < 0.001$, "cholesterol metabolic process" $P = 0.001$, "fatty acid biosynthetic process" $P = 0.036$, "positive regulation of protein metabolic process" $P = 0.013$, "proteasomal protein catabolic process" $P = 0.007$...) and cell cycle ("cell cycle" $P < 0.001$, "mitotic cell cycle" $P < 0.001$, "cell proliferation" $P = 0.006$). The expression of the other 132 probes were downregulated at T1 and then either remained at low levels at T5 or resumed normal levels (*cluster D*, Fig. 4*B*). The genes of this cluster were involved in tissue development ("muscle tissue development" $P = 0.001$, "tissue morphogenesis" $P = 0.006$...), lipid metabolism ("sterol biosynthetic process" $P = 0.001$, "cholesterol biosynthetic process" $P = 0.012$...), cell cycle ("cell cycle process" $P = 0.027$, "growth" $P = 0.029$...), and apoptosis ("induction of apoptosis" $P = 0.004$, "regulation of apoptosis" $P = 0.005$, "cell death" $P = 0.016$). A third cluster comprised 79 probes that were downregulated at T1 and then upregulated at T5 (*cluster B*) (Fig. 4*B*). These genes were involved in the cell cycle ("regulation of cell cycle" $P = 0.003$, "cell proliferation" $P = 0.012$) and apoptosis ("regulation of apoptosis" $P = 0.023$, "cell death" $P = 0.036$...) and in metabolic processes ("negative regulation of metabolic process" $P = 0.048$). The final cluster included 403 probes that were upregulated at T5 (*cluster C*) (Fig. 4*B*). These genes were mainly involved in the response to stress ("response to reactive oxygen species" $P = 0.018$, "response to oxidative stress" $P = 0.019$, "response to osmotic stress" $P = 0.026$, "response to extracellular stimulus" $P = 0.041$...), cell movement ("cell motion" $P = 0.002$, "cell motility" $P = 0.023$, "cell migration" $P = 0.025$), cell communication ("positive regulation of cell communication" $P = 0.017$ and "cell junction organization" $P = 0.028$), apoptosis ("regulation of apoptosis" $P < 0.001$, "apoptosis" $P = 0.006$...), metabolic processes ("positive regulation of biosynthetic process" $P = 0.002$, "protein transport" $P = 0.012$, "glycolysis" $P = 0.015$, "response to lipopolysaccharide" $P = 0.020$...), and in "mammary gland development" ($P = 0.009$).

Comparison of the response to live bacteria or the supernatant. MEC stimulation with Sa or Sp resulted in different gene expression profiles. The results of hierarchical clustering based on the individual expression values of the samples stimulated either by Sa or Sp actually revealed that MEC responded weakly to Sa at 1 h, whereas the response was much stronger with Sp at the same time point (data not shown).

In addition, Venn diagram comparison of the two lists of probes differentially expressed in the time course after exposure to Sa or Sp, shows that 286 probes were in common (Fig. 5*A*). Indeed, both responses shared common pathways but differed also largely.

To enhance insight into the regulation of MEC response to Sa and Sp, the evolution of the main biological functions with time through identification of significant GO functions (EASE score < 0.05, DAVID analysis) was compared. Some genes involved in "muscle and tissue development" were downregu-

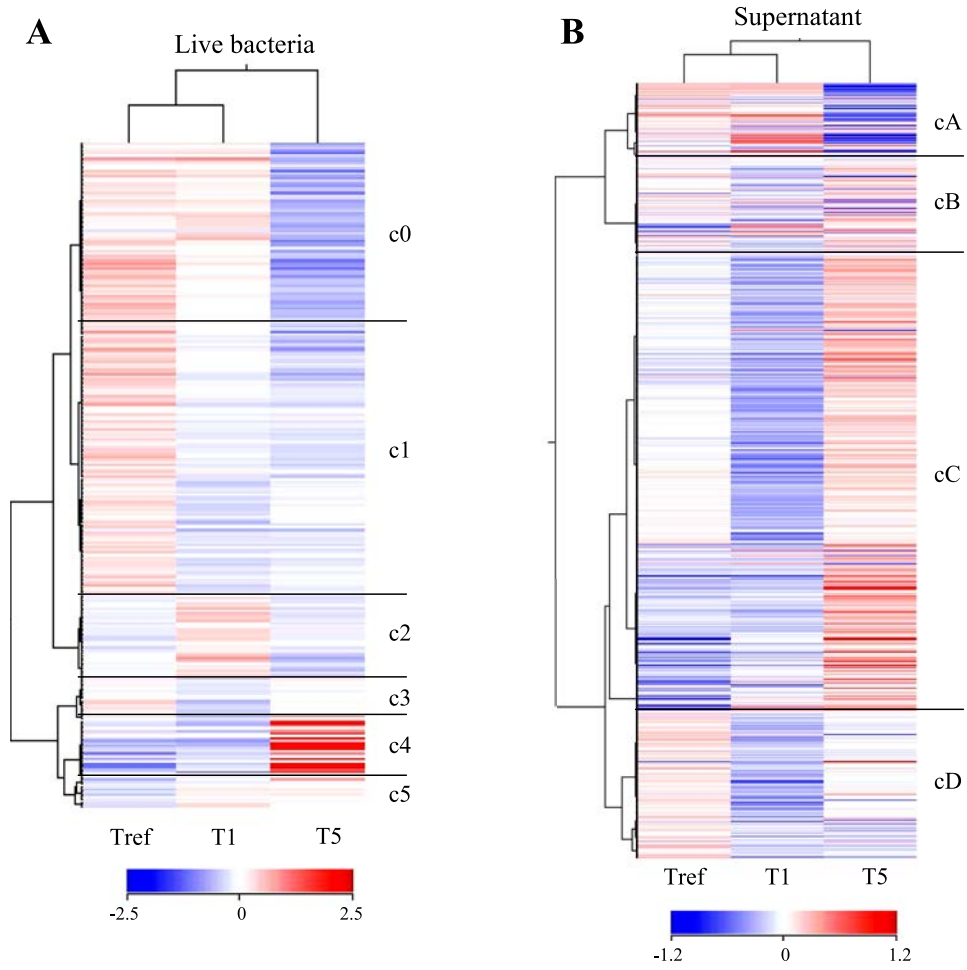

Fig. 4. Hierarchical clustering of the differentially expressed genes (DEG) in the time course following stimulation with Sa (*A*, n = 531 genes) or Sp (*B*, n = 687 genes). Each line corresponds to 1 gene, and each column is the mean value of all samples after 0, 1, or 5 h of stimulation. The clustering algorithm was based on Pearson correlation and was computed with GeneSpring. According to the tree on the graph's *left*, 6 clusters were discriminated for Sa data (named c0–c5, *A*) and 4 clusters for Sp data (named cA, cB, cC, and cD; *B*).

lated when MEC were stimulated with *S. aureus* (*clusters 1* and *D*), except for some of these genes that were also involved in "blood vessel morphogenesis" and were upregulated at T5 in both cases (*clusters 4* and *C*). Genes involved in "mammary gland development" were upregulated at T5 only with Sp (*cluster C*). In addition, "mitosis" and "cell proliferation" were affected in both conditions (*clusters 0, 1, 2, A,* and *D*).

Following stimulation, function of cytoskeleton and membrane remodeling changed. The "endocytosis" pathway was activated after stimulation by Sp (*cluster C*) but inhibited after stimulation by Sa (*cluster 1*). Cell communication was negatively regulated when cells were stimulated with Sa ("negative regulation of cell communication," *cluster 4*) but was promoted when cells were stimulated by Sp ("positive regulation of cell communication", *cluster C*) with an upregulation of genes involved in "membrane organization," "vesicle-mediated transport," "cell-matrix adhesion," and "cell junction assembly." The cellular metabolism was also affected by the stimulations: with Sa, the catabolism was enhanced with "regulation of caspase activity," and biosynthetic processes were decreased with "negative regulation of macromolecule metabolic process" (*clusters 0* and *1*); cellular respiration was at first activated and then inhibited ("respiratory electron chain transport," *cluster 2*). After stimulation by Sp, the lipid biosynthetic metabolism was highly affected ("lipid biosynthetic process," *clusters A* and *D*). The genes involved in "hexose catabolism process" were upregulated (*clusters 4* and *C*). Genes involved in the regulation of gene expression were downregulated after stimulation by Sa ("negative regulation of gene expression" in *cluster 0* and "positive regulation of gene

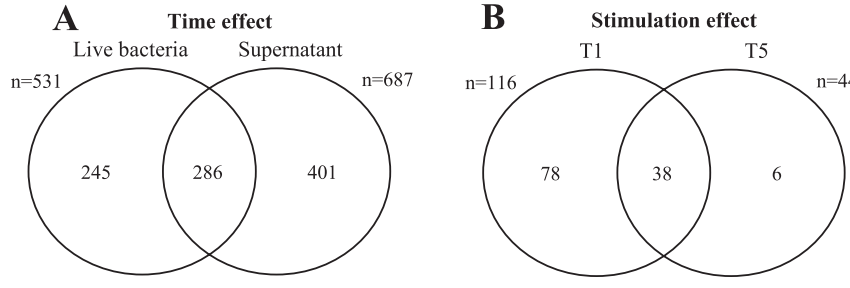

Fig. 5. Comparison of probe lists with Venn diagrams. In *A* "time effect" the lists of DEG in the time course experiment after "live bacteria" stimulation (n = 531 probes) and "supernatant" stimulation (n = 687 probes) were compared; a set of 286 probes belonged to both lists. In *B* "stimulation effect," the lists of DEG between Sa and Sp at T1 (n = 116) and T5 (n = 44) were compared; a set of 38 probes belonged to both lists.

Table 3. *List of the DEG between Staphylococcus spp-mastitis-resistant and susceptible animals*

Probe Name	HGNC	Description	*P* Value Corrected by BH5	Log Ratio	SE
A_70_P016556	GSTM3	glutathione S-transferase Mu 5	0.0001	−1.36	0.21
A_70_P016557	GSTM3	glutathione S-transferase Mu 5	0.0001	−1.34	0.20
A_70_P053411	PLAT	plasminogen activator, tissue	0.0004	−1.27	0.22
A_70_P051106	SLC2A3	solute carrier family 2, facilitated glucose transporter member 3	0.0000	−0.88	0.11
A_70_P003541	SAA3	serum amyloid A protein	0.0048	−0.85	0.27
A_70_P039662	IL8	interleukin-8	0.0049	−0.83	0.26
A_70_P041692	VIM	vimentin	0.0016	−0.76	0.17
A_70_P035536	ANKRD1	ankyrin repeat domain-containing protein 1	0.0052	−0.74	0.24
A_70_P016606	PRDX1	peroxiredoxin 1	0.0006	−0.74	0.14
A_70_P051107	SLC2A3	solute carrier family 2, facilitated glucose transporter member 3	0.0000	−0.74	0.09
A_70_P039661	IL8	interleukin-8	0.0051	−0.72	0.23
A_70_P026901	PLEKHB1	pleckstrin homology domain containing, family B member 1	0.0034	−0.71	0.20
A_70_P035621	CRIP1	cysteine-rich protein 1	0.0017	−0.70	0.16
A_70_P065771	CYP1B1	similar to cytochrome P450, family 1, subfamily B, polypeptide 1	0.0002	−0.70	0.11
A_70_P018256	S100A4	protein S100-A4 OS=Bos taurus GN=S100A4	0.0035	−0.69	0.20
A_70_P019151	LAMA3	laminin, alpha 3	0.0000	−0.69	0.08
A_70_P053606	MAN1A1	mannosyl-oligosaccharide alpha-1,2-mannosidase	0.0015	−0.65	0.15
A_70_P051921	PPP1R15A	protein phosphatase 1 regulatory subunit 15A	0.0001	−0.62	0.09
A_70_P003061	OVUBQ-L40	ubiquitin A-52 residue ribosomal protein fusion product 1	0.0013	−0.60	0.13
A_70_P003062	FTH1	ferritin heavy chain	0.0001	−0.59	0.09
A_70_P052621	PPA1	inorganic pyrophosphatase	0.0012	0.60	0.13
A_70_P065551	PLET1	placenta-expressed transcript 1 protein	0.0018	0.62	0.14
A_70_P006016	CAT	catalase	0.0002	1.03	0.16

The selected genes have a Benjamini-Hochberg corrected *P* value $< 5\%$ (BH5) and a log ratio between *Staphylococcus spp*-mastitis-resistant and -susceptible animals >0.58, i.e., a fold-change of 1.5 ($n = 20$ genes).

expression" in *cluster 1*) and upregulated after stimulation by Sp ("positive regulation of gene expression", *cluster C*). Furthermore, MEC expressed genes that revealed responses to extracellular stimuli. "Immune response" and "inflammatory response" with induction of "chemotaxis" were activated only following stimulation by Sa (*cluster 4*), whereas after stimulation with Sp, the main functions pointed toward a "cellular response to oxidative stress" (*cluster C*). Expression of apoptosis-related genes was either upregulated (*clusters 3, 4, b,* and *C*) or downregulated (*clusters 0, 1,* and *D*) depending on the condition.

In addition, InnateDB analysis showed that four transcription factors (TFDP1, HIF-1, AHR, and NRF1; $P < 0.001$) can bind 72, 57, 90, and 58 promoters of genes that were downregulated after stimulation by Sa or Sp (*clusters C0, C1, CA,* and *CD*). No transcription factor was found to significantly bind the promoters of the upregulated genes (*clusters C4, C5, CB,* and *CC*).

Furthermore, a direct comparison of MEC response to Sa and Sp was also performed within time stimulation (model M1.2). Indeed, 116 and 44 probes (corresponding to 104 and 42 genes) were identified as differentially expressed between Sa and Sp, at 1 and 5 h poststimulation, respectively (Additional Files 3, A and B). A set of 38 probes belonged to both lists (Fig. 5*B*). Two sets of 91 and 93% of the DEG were more expressed in Sa than Sp condition at 1 and 5 h poststimulation, respectively. On the contrary, the genes *cxcl8, egr1, tnfaip3, cxcl2, areg, fos, ptgs2, dusp5,* and *trib1* at 1 h, and the genes *areg, dusp5,* and *trib1* at T5 were expressed at higher levels in Sp than Sa condition.

At T1, the genes that were more highly expressed in Sp were enriched for functions related to the "inflammatory response" ($P = 0.015$) and in "learning or memory" ($P = 0.002$). On the contrary, the genes that were more highly expressed after stimulation by Sa were involved in "cellular response to oxidative stress" ($P = 0.018$), "positive regulation of I-kappaB kinase/NF-kappaB cascade" ($P = 0.027$), "cellular macromolecule organization" ($P = 0.001$), "intracellular transport" ($P = 0.002$), "negative regulation of translation" ($P = 0.018$), in "positive regulation of cell communication" ($P = 0.004$) and "cell proliferation" ($P = 0.049$), and "cell death" ($P = 0.011$). The list of DEG with higher expression after Sa than Sp stimulation at T5 was enriched for the same GO functions as the one at T1, except for "cell death." No GO function was found to be enriched for the list of the DEG with higher expression after Sp stimulation at T5.

Comparison of MEC Response Between Genetic Lines

When comparing the MEC of *Staphylococcus spp*-mastitis-resistant and -susceptible ewes on the overall experiment, after correcting for the other effects (model M2.1), we found only 23 probes (20 genes) to be differentially expressed (Table 3). Of these 20 genes, three were expressed at higher levels in the MEC of *Staphylococcus spp*-mastitis-resistant animals (*cat, plet1,* and *ppa1*). The expression of eight genes was measured by RT-qPCR in the samples collected at Tref, T1Sp, T5Sp, and T5Sa. The differences of expression were confirmed for six of the eight genes, but *saa3* and *cxcl8* were not found to be differentially expressed between the lines by RT-qPCR (Table 4). In keeping with the RT-qPCR result, the ELISA for CXCL8 at T5 did not indicate a significant difference between the cells of the two lines, whatever the stimulus: with Sp, CXCL8 concentration (median value, first and third quartiles) was 6.7 (6.0, 15.8) ng/ml for cells of *Staphylococcus spp*-mastitis-resistant animals vs. 6.7 (6.5, 13.5) ng/ml for cells of *Staphylococcus spp*-mastitis-susceptible animals, and with Sa, concentrations were 4.3 (3.3, 6.8) ng/ml and 3.6 (2.4, 4.3) ng/ml for *Staphylococcus spp*-mastitis-resistant and -susceptible animals, respectively.

Table 4. *RT-qPCR of 8 DEG between Staphylococcus spp-mastitis-resistant and -susceptible animals*

Gene	*P* Value Wilcoxon	Resistant Mean	Resistant SD	Susceptible Mean	Susceptible SD
CAT	0.00024	2.21	± 1.17	1.02	± 0.73
GSTM3	0.0045	0.31	± 0.14	1.18	± 0.96
IL8	0.12423	7.49	± 11.89	7.58	± 8.49
LAMA3	0.00404	0.8	± 0.41	1.22	± 0.51
MAN1A1	0.00007	0.63	± 0.53	1.3	± 0.74
PLAT	0.02985	1.9	± 3.48	2.37	± 3.47
SAA3	0.20784	19.93	± 56.46	15.71	± 29.8
SLC2A3	0.02671	1.86	± 1.86	3.56	± 3.87

RNA was quantified from samples of all animals at Tref, T5Sa, T1Sp, and T5Sp samples using Biomark Fluidigm technology. The values were normalized by HKG (*rp19, rp26, tyr* and *gapdh*) with the Vandesompele method (55). For each gene and for either resistant or susceptible animals, the values of the samples at Tref, T5Sa, T1Sp, and T5Sp were brought together and expressed as means and SE and are presented on the *right* side of the table. The statistical analyses were performed using Wilcoxon nonexact tests for each gene. *P* values of resistant vs. susceptible animals are presented on the *left* side.

The gene list was enriched for the cellular response to "oxidation reduction" ($P = 0.031$) with the genes (*cat, prdx1,* and *cyp1b1* and *fth1*). Interestingly, 15 of the 20 DEG were involved in one IPA network that scored 39 (Fig. 6). These molecules were mainly linked to genes involved in the regulation of inflammation (*nfκb* and *myc*) and *tp53*. IPA of DEG between *Staphylococcus spp*-mastitis-resistant and -susceptible animals showed enrichment for "xenobiotic metabolism signaling" ($P = 0.004$; *gstm3, cat,* and *fth1*) and "NRF2-mediated oxidative stress response" ($P < 0.001$; *gstm3, cat, fth1,* and *prdx1*), whereas no canonical pathway was significant.

We showed above that the gene expression profile of MEC was strongly modified by the two stimulations (models M1.1 and M1.2). For that reason, MEC differences between *Staphylococcus spp*-mastitis-resistant and -susceptible animals were also analyzed within each combined time-stimulation condition (M2.2). In all conditions, three genes (*gstm3, slc2a3,* and *plat*) were more expressed in the MEC of the *Staphylococcus spp*-mastitis-susceptible animals, and conversely, *cat* was more expressed in MEC of the *Staphylococcus spp*-mastitis-resistant ones. Prior to stimulation (Tref), 10 DEG were identified between *Staphylococcus spp*-mastitis-resistant and -susceptible animals, eight of these had been identified above in the global analysis of the line effect (model M2.1), but *zak* and a nonclassical MHC class I gene were new. These 10 DEG were also differentially expressed in at least one another condition. The *MHC class I* gene was upregulated in MECs of the *Staphylococcus spp*-mastitis-resistant animals only after stimulation by Sa (T1Sa and T5Sa). The number of DEG between *Staphylococcus spp*-mastitis-resistant and -susceptible animals was higher at T5 than at T1 after stimulation by Sp (with 17 and 11 genes at T5 and T1, respectively).

As very few genes were identified by ANOVA, to further look for gene expression differences between the lines, GSEA was performed. Although 48 gene sets in the collections C2 (curated gene sets), C3 (motif gene sets), C4 (computational gene sets), and C5 (GO gene sets) were enriched (as they

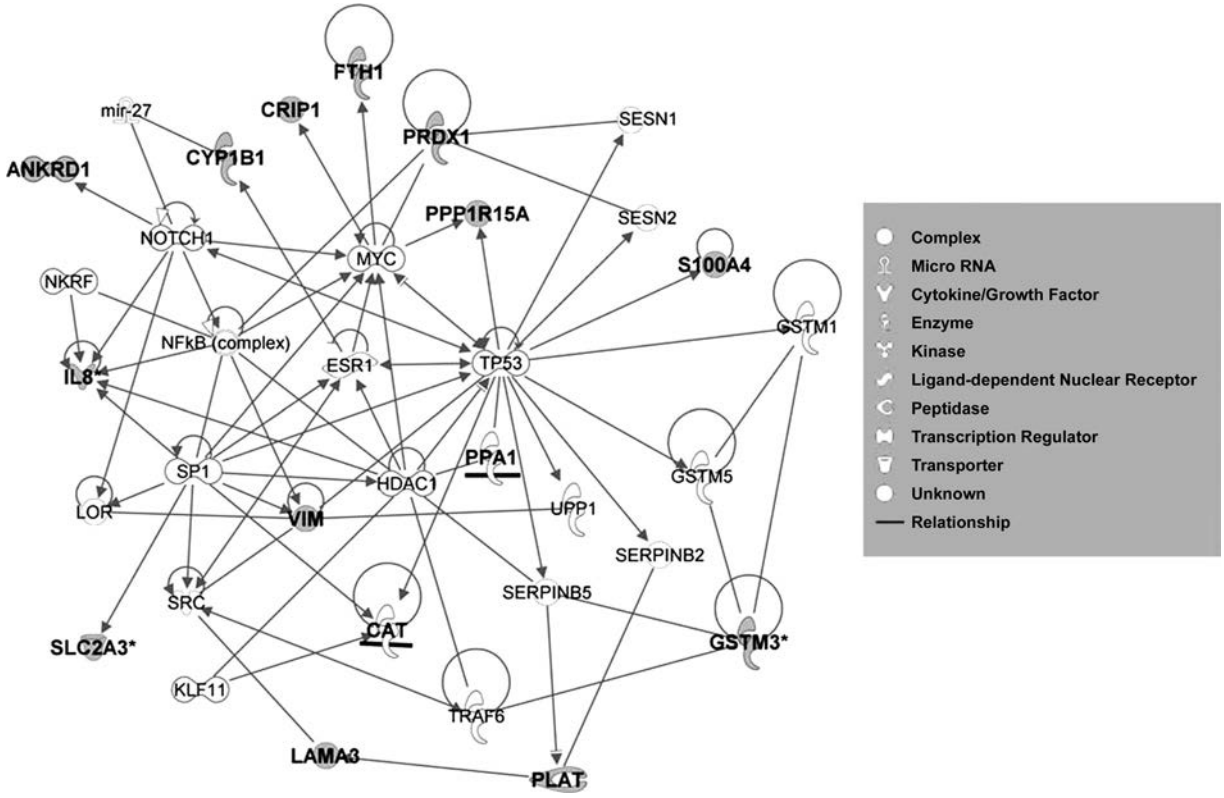

Fig. 6. Ingenuity networks of the DEG between MECs of *Staphylococcus spp*-mastitis-resistant and -susceptible animals. This network involves 15 of the 20 DE genes and scores 39 that reveal a high level of significance. The DEG are in boldface; the upregulated genes in the resistant animals are underlined; the upregulated genes in susceptible animals are in gray. The forms of the genes are explained in the legend. The main functions of this network were cancer, cellular movement, and gastrointestinal disease.

contained at least 15 focus genes). None of them was significant at the $P < 0.05$, indicating small differences of gene expression response between genetic backgrounds.

DISCUSSION

In the present study, we analyzed the response of an in vitro culture of MEC to the presence of *S. aureus* over a short time course. Using live bacteria, we focused on the early response of MEC to avoid the cytotoxic effects that result in RNA degradation and possibly, a difference of bacterial development between conditions. The expression of ~12,000 genes was analyzed using an ovine pangenomic microarray. Although there was a high variability in the individual responses to *S. aureus*, more differences were found between the two different conditions of stimulation than between genetic backgrounds with a difference of susceptibility to *Staphylococcus spp*-mastitis.

MEC Responses to Live S. aureus or Bacterial Supernatant

During natural IMI, MEC form a physical barrier that prevents bacteria to enter host tissues. *S. aureus* is known to secrete a number of proteins and to release a number of cell wall-associated molecules that have the potential to activate the innate immune system, under in vitro and in vivo conditions (19). Culture supernatant is a stimulus per se, representative of what happens during an infection, where live staphylococci release an array of molecules stimulating, luring, evading, or impairing the defenses of the host. Thus the two forms of stimulation used in this in vitro culture model are therefore both relevant but could, however, induce distinct biological effects.

Similarities between live bacteria and soluble factor stimulations. LOSS OF MEC FUNCTIONALITY. Our first analyses clearly showed a downregulation of the MEC genes involved in cell proliferation, metabolic processes, and transcription functions after interaction with *S. aureus*. Here, *S. aureus* induced the downregulation of genes involved in the cell cycle and mitosis, especially after stimulation by Sa. Upon contact with Sa, MEC mitosis probably arrested as previously highlighted by Matthews et al. (33). Sp also affects the expression of genes involved in the cell cycle. Conversely, changes in functions associated with cell proliferation were reported not to be altered in human airway epithelial cells following stimulation with Sp (34), although in the presence of streptococcal LTA, a major component of Sp, the proliferation of bovine MEC was shown to be inhibited in a dose-dependent manner (15).

Genes involved in cell metabolism were also highly affected upon MEC contact with bacteria or their soluble factors. Genes related to protein catabolism and lipid biosynthesis were inhibited after stimulation with both Sa and Sp, whereas the ones related to hexose metabolic processes were enhanced. Indeed, *S. aureus* seems to alter the genes that pertain to metabolic and secretory functions of MEC, and this is in accordance with the diminution of milk production during mammary infection (59). In addition, as shown by other authors (30, 34), the presence of Sa strongly modified the regulation of gene expression and in particular the presence of Sa supernatant-affected genes related to transcription or translation functions. In conclusion, MEC proliferation, metabolism, and transcription seem to be largely altered upon interaction with *S. aureus*.

MEC APOPTOSIS. Many of the MEC genes that were modulated in the time course experiment are involved in cell death and its regulation. The expression of such genes was either up (*tnfa, tnfaip3, tnfrsf6b* and interleukins *il1a, il1b,* and *cxcl8*)- or downregulated (*birc, bcl7c, casp3, stk17a,* and *tnfrsf12a*), depending on the nature of the stimulus and pointed toward a delay in MEC response to apoptosis. Regulation of apoptosis is a very complex phenomenon, and the analysis of the MEC transcriptome in the various conditions of stimulation tested in our study confirms this complexity. Moreilhon et al. (34) also reported the presence of antiapoptotic genes in airway cells stimulated by Sp, and to a lesser extent by Sa, whereas many other reports show proapoptotic mechanisms consecutive to an infection (5, 30, 48, 52). As a result we cannot make a clear conclusion about apoptosis; a large increase in MEC apoptosis might reveal degradation of the mammary gland tissue and so an alteration of its functioning, whereas enhanced apoptosis might be also a normal phenomenon with the cell turnover.

MEC IMMUNE AND INFLAMMATORY RESPONSES. The chemokines *ccl20, cxcl3* (*gro*γ), and *cxcl8* (*il8*) were upregulated at the RNA level. Overexpression of CXCL3 and CXCL8 was also detected at the protein levels. The upregulation of these chemokines was also demonstrated in MEC after LTA stimulation (13, 41) or after contact with Sa (29, 30, 34). These results support the idea that MEC attract neutrophils in the mammary gland in the presence of *S. aureus* (3, 17).

In parallel, the genes encoding the proinflammatory cytokines *il1a* and *tnfa* were upregulated in both conditions, whereas *il1r1* and *il1b* were upregulated only when cells were stimulated with Sa. Identical results were reported previously for *il1a* (22, 34), *tnfa* (22, 26, 29, 31, 56), and *il1b* (26, 29–31, 34, 52, 56). On the contrary, Günther et al. (24) showed an upregulation of *il6* but not of *il1a* and *tnfa* in MEC stimulated by heat-inactivated *S. aureus.* The genes encoding *nfkbia*, *nfkbiz*, and *rela* were upregulated in cells treated with Sa and Sp, respectively. Bottero et al. (10) have shown that the transcriptional level of *nfkbia* correlates well with the activation of the NF-κB protein complex. The genes encoding NFκB-REL proteins have previously been shown to be regulated after infections by diverse bacteria (7, 24, 30, 34, 60). The serum amyloid 3 (*saa3*), a marker of inflammation, was upregulated following stimulation by either Sa or Sp, as previously reported in bovine MEC stimulated with heat-killed *S. aureus* (52) or LTA (58). No upregulation of *tlr2* was observed in our study in contrast to several other reports (26, 34, 52).

As a result, the reduction of MEC metabolic functions, the regulation of apoptosis, and the enhanced production of immune mediators, which are all observed in an infected mammary gland, suggest that the in vitro MEC culture model may partially mimic the local in vivo responses to *S. aureus* infection.

Differences in MEC responses to bacterial cell and supernatant stimulations. Surprisingly the MEC gene expression profile was modified to a slightly greater extent in response to bacterial soluble factors than in response to Sa ($n = 687$ probes for Sp vs. $n = 531$ for Sa). Nevertheless, it seems that the difference observed between MEC responses to Sa and Sp is indeed considerable during the early phase of the stimulation, but greatly reduced later on. The more homogenous distribu-

tion of soluble factors within the culture medium and the more diffuse contact with the cell layer may partly explain this difference.

Some specific features were observed. Genes related to oxidative stress response were more highly expressed after stimulation by Sa than by Sp, which may be due to the bacterial cell internalization. On the contrary, genes involved in immune response were more highly expressed after stimulation by Sp than by Sa during the early phase of stimulation. In fact in accordance with Moreilhon et al. (34), the expression of *cxcl8, egr1, tnfaip3, cxcl2, areg, ptgs2,* and *fos* was higher at the 1-h time point after stimulation by Sp than by Sa, whereas at the 5-h time point only *areg* was upregulated in cells stimulated by Sp.

The comparison of early MEC responses as a function of the forms of *Staphylococcus* showed that stimulation with soluble components does not mimic exactly a bacterial infection.

Differences Between Staphylococcus Spp-Mastitis Resistant and Susceptible Animals

Although MEC stimulation by live *S. aureus* or its supernatant caused considerable gene expression remodeling and revealed some immune responsiveness, the information we obtained when *Staphylococcus spp*-mastitis resistant and -susceptible animals were compared is unclear. Very few genes were differentially expressed between the two groups of animals (20 genes, 23 probes), and no clear biological sense could be found from the list of identified genes. Only three genes were upregulated in our *Staphylococcus spp*-mastitis resistant group: *ppa1, plet1,* and *cat*. The protein encoded by *ppa1* is a member of the inorganic pyrophosphatase family that catalyzes the hydrolysis of pyrophosphate into inorganic phosphate. The gene *cat* encodes a catalase, a key antioxidant enzyme in cellular defense against oxidative stress that protects MEC against *S. aureus* virulent effects (20, 32). *Plet1* may participate in the wound response during the healing process and promotes wound repair.

Nevertheless network analysis enabled us to gather 15 of the 20 DEG between *Staphylococcus spp*-mastitis-resistant and -susceptible animals (Fig. 6). This suggests that there are biological links amongst these genes, and it is worth noticing that node genes of this network are *NFκB, MYC,* and *TP53*. The first two genes play major roles in inflammatory processes, whereas *TP53* was one of the most highly expressed genes in infected MSC of *Staphylococcus spp*-mastitis-resistant animals (8), suggesting that it may play a role in the differences between the genetic lines.

In the model of cows selected on BTA18 mastitis QTL, gene profiles in the time course of the experiment were characterized in resistant and in susceptible cows after heat-inactivated *S. aureus* stimulation, but there was no direct comparison of the two groups of cows in a global statistical model (14). Thus, both studies cannot be directly compared. Nevertheless, of the 20 DEG that we have identified between *Staphylococcus spp*-mastitis-resistant and -susceptible sheep, four genes were differentially expressed in the time course in resistant and susceptible cows (*cxcl8, saa3, slc2a3,* and *ppp1r15a*) (14). CXCL8 is highly involved in neutrophil recruitment (57), and SAA3 is an inflammation marker (58). In our study, expression of these four genes was higher in MEC of susceptible sheep according to the microarray results while we have not confirmed expression of *cxcl8* and *saa3* by RT-qPCR, and the production of CXCL8 was not different at the protein level (ELISA results). In the bovine study, the genes *slc2a3* and *cxcl8* were activated only in susceptible cows after *Sa* stimulation (14). Thus, sheep and bovine results seem to reveal a higher expression of *slc2a3* and *cxcl8* in mastitis-susceptible animals. However, the same group had shown a higher expression of *cxcl8* in MEC of resistant animals in a previous study (23), which gave confusion on the expression direction of CXCL8 and on its role. They also showed that the genes *saa3* and *ppp1r15a* were activated in the time course in resistant cows only (14), whereas we found that their expression was higher in MEC of susceptible sheep. About NF-κB subunits, they showed activation of *nfkb1*, *nfkb2*, *nfkbia*, and *nfkbie* in the course of the stimulation only in resistant cows, except for *nfkbia* that was also slightly activated in susceptible cows (14). Even if we did not identify *nfkb* as differentially expressed between *Staphylococcus spp*-mastitis-resistant and -susceptible sheep, its central situation in the network showed association with the DEG between the divergent lines. This observation highlights the fact that inflammatory processes are of main importance in MEC response to *S. aureus*. Although cows and sheep are phylogenetically close, to explain the differences between sheep and cow models we assume that *1*) their capacity to respond to pathogens might be slightly different, *2*) selections of cows on one QTL and selection of ewes on SCS EBV are probably not based on the same genetic mechanisms, and that probably explains the difference of results, and *3*) there may be some differences due to time of sampling as we focused on the early 5 h of stimulation and they studied a longer period of 24 h.

Previously, the low SCS line was shown to be less susceptible to *Staphylococcus* IMI (47) or experimental inoculations (8), but the gene expression of the MEC after in vitro stimulation by *S. aureus* did not reveal any straightforward contrasts that could explain the difference of resistance to IMI. Swanson et al. (52) have shown that in vitro MEC response to *Streptococcus uberis* was different to the in vivo response upon experimental inoculation of the mammary gland. We might thus hypothesize that the in vitro model using MEC, isolated from other immune cells, insufficiently accounts for the complex network of interactions that occur during pathogen invasion and thus is a limited representation of in vivo processes. Indeed, intestinal epithelial cell responses to in vitro stimulations with different kinds of bacteria have been shown to depend on the presence of other cell types such as DC (61). Intestinal epithelial cells can play a role in shaping DC functions, which then leads to either a tolerogenic or an inflammatory immune response depending on the local status of the intestine (44).

Also, the limited amount of information obtained from the comparison of MEC from the two lines of ewes may have resulted, at least partly, from technical constraints. On the one hand, the experiment was limited to a small number of animals per line. On the other hand, the array that we used does not contain more than one-third of the sheep genome and therefore might not include some of the focus genes that could explain the differences between *Staphylococcus spp*-mastitis-resistant and -susceptible animals. Our previous experiments are not in favor of these hypotheses. Indeed, the gene expression profiles

of *Staphylococcus spp*-mastitis-resistant and -susceptible animals have already been studied in two other cell types: MSC collected after *S. epidermidis* or *S. aureus* inoculations of the mammary gland (8) and *S. aureus*-stimulated DC (54) with two groups of four (54) or six (8) sheep. In both cases significant differences were detected.

In conclusion, we have shown that stimulation by bacteria soluble factors do partially mimic infection with live bacteria. MEC of the *Staphylococcus spp*-mastitis-resistant and -susceptible sheep express little differences in the early response to *S. aureus* stimulation even if inflammatory mechanisms were largely mobilized. In parallel, as interactions between cell types can considerably modify their gene transcription profiles, cocultures of several cell types at the same time with bacteria may be useful to reproduce the network of interactions that exist during an infection.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: C.M.B., P.R., P.C., F.G., M.T., and M.-R.A. performed experiments; C.M.B., P.R., R.R., and G.F. analyzed data; C.M.B., R.R., and G.F. interpreted results of experiments; C.M.B. prepared figures; C.M.B. drafted manuscript; P.R., M.T., R.R., and G.F. edited and revised manuscript; R.R. and G.F. conception and design of research; R.R. and G.F. approved final version of manuscript.

REFERENCES

1. **Aderem A, Ulevitch RJ.** Toll-like receptors in the induction of the innate immune response. *Nature* 406: 782–787, 2000.
2. **Van Amersfoort ES, Van Berkel TJC, Kuiper J.** Receptors, mediators, and mechanisms involved in bacterial sepsis and septic shock. *Clin Microbiol Rev* 16: 379–414, 2003.
3. **Barber MR, Pantschenko AG, Hinckley LS, Yang TJ.** Inducible and constitutive in vitro neutrophil chemokine expression by mammary epithelial and myoepithelial cells. *Clin Diagn Lab Immunol* 6: 791–798, 1999.
4. **Barkema HW, Green MJ, Bradley AJ, Zadoks RN.** Invited review: the role of contagious disease in udder health. *J Dairy Sci* 92: 4717–4729, 2009.
5. **Bayles KW, Wesson CA, Liou LE, Fox LK, Bohach GA, Trumble WR.** Intracellular Staphylococcus aureus escapes the endosome and induces apoptosis in epithelial cells. *Infect Immun* 66: 336–342, 1998.
6. **Bergonier D, de Crémoux R, Rupp R, Lagriffoul G, Berthelot X.** Mastitis of dairy small ruminants. *Vet Res* 34: 689–716, 2003.
7. **Boldrick JC, Alizadeh AA, Diehn M, Dudoit S, Liu CL, Belcher CE, Botstein D, Staudt LM, Brown PO, Relman DA.** Stereotyped and specific gene expression programs in human innate immune responses to bacteria. *Proc Natl Acad Sci USA* 99: 972–977, 2002.
8. **Bonnefont C, Toufeer M, Caubet C, Foulon E, Tasca C, Aurel MR, Bergonier D, Boullier S, Robert-Granie C, Foucras G, Rupp R.** Transcriptomic analysis of milk somatic cells in mastitis resistant and susceptible sheep upon challenge with Staphylococcus epidermidis and Staphylococcus aureus. *BMC Genomics* 12: 208–223, 2011.
9. **Bossers K, Ylstra B, Brakenhoff RH, Smeets SJ, Verhaagen J, van de Wiel MA.** Intensity-based analysis of dual-color gene expression data as an alternative to ratio-based analysis to enhance reproducibility. *BMC Genomics* 11: 112–124, 2010.
10. **Bottero V, Imbert V, Frelin C, Formento JL, Peyron JF.** Monitoring NF-kappa B transactivation potential via real-time PCR quantification of I kappa B-alpha gene expression. *Mol Diagn* 7: 187–194, 2003.
11. **Boudjellab N, Chan-Tang HS, Zhao X.** Bovine interleukin-1 expression by cultured mammary epithelial cells (MAC-T) and its involvement in the release of MAC-T derived interleukin-8. *Comp Physiol Biochem* 127: 191–199, 2000.
12. **Bougarn S, Cunha P, Gilbert FB, Meurens F, Rainard P.** Technical note: Validation of candidate reference genes for normalization of quantitative PCR in bovine mammary epithelial cells responding to inflammatory stimuli. *J Dairy Sci* 94: 2425–2430, 2011.
13. **Bougarn S, Cunha P, Harmache A, Fromageau A, Gilbert FB, Rainard P.** Muramyl dipeptide synergizes with Staphylococcus aureus lipoteichoic acid to recruit neutrophils in the mammary gland and to stimulate mammary epithelial cells. *Clin Vaccine Immunol* 17: 1797–1809, 2010.
14. **Brand B, Hartmann A, Repsilber D, Griesbeck-Zilch B, Wellnitz O, Kuhn C, Ponsuksili S, Meyer HH, Schwerin M.** Comparative expression profiling of *E. coli* and *S. aureus* inoculated primary mammary gland cells sampled from cows with different genetic predispositions for somatic cell score. *Genet Sel Evol* 43: 24–40, 2011.
15. **Calvinho LF, Almeida RA, Oliver SP.** Influence of bacterial factors on proliferation of bovine mammary epithelial cells. *Rev Argent Microbiol* 33: 28–35, 2001.
16. **Casel P, Moreews F, Lagarrigue S, Klopp C.** sigReannot: an oligo-set re-annotation pipeline based on similarities with the Ensembl transcripts and Unigene clusters. *BMC Proc* 3: S3, 2009.
17. **Van Damme J, Wuyts A, Froyen G, Van Coillie E, Struyf S, Billiau A, Proost P, Wang J, Opdenakker G.** Granulocyte chemotactic protein-2 and related CXC chemokines: from gene regulation to receptor usage. *J Leukoc Biol* 62: 563–569, 1997.
18. **Davies G, Genini S, Bishop SC, Giuffra E.** An assessment of opportunities to dissect host genetic variation in resistance to infectious diseases in livestock. *Animal* 3: 415–436, 2009.
19. **Fournier B, Philpott DJ.** Recognition of Staphylococcus aureus by the innate immune system. *Clin Microbiol Rev* 18: 521–540, 2005.
20. **de la Fuente R, Díez RM, Domínguez-Bernal G, Orden JA, Martínez-Pulgarín S.** Restoring catalase activity in Staphylococcus aureus subsp. *anaerobius* leads to loss of pathogenicity for lambs. *Vet Res* 41: 41–52, 2010.
21. **Gilot P, Lina G, Cochard T, Poutrel B.** Analysis of the genetic variability of genes encoding the RNA III-activating components Agr and TRAP in a population of Staphylococcus aureus strains isolated from cows with mastitis. *J Clin Microbiol* 40: 4060–4067, 2002.
22. **Griesbeck-Zilch B, Meyer HHD, Kuhn CH, Schwerin M, Wellnitz O.** Staphylococcus aureus and Escherichia coli cause deviating expression profiles of cytokines and lactoferrin messenger ribonucleic acid in mammary epithelial cells. *J Dairy Sci* 91: 2215–2224, 2008.
23. **Griesbeck-Zilch B, Osman M, Kuhn C, Schwerin M, Bruckmaier RH, Pfaffl MW, Hammerle-Fickinger A, Meyer HHD, Wellnitz O.** Analysis of key molecules of the innate immune system in mammary epithelial cells isolated from marker-assisted and conventionally selected cattle. *J Dairy Sci* 92: 4621–4633, 2009.
24. **Günther J, Esch K, Poschadel N, Petzl W, Zerbe H, Mitterhuemer S, Blum H, Seyfert HM.** Comparative kinetics of Escherichia coli- and Staphylococcus aureus-specific activation of key immune pathways in mammary epithelial cells demonstrates that S. *aureus* elicits a delayed response dominated by interleukin-6 (IL-6) but not by IL-1A or tumor necrosis factor alpha. *Infection Immunity* 79: 695–707, 2011.
25. **Günther J, Koczan D, Yang W, Nurnberg G, Repsilber D, Schuberth HJ, Park Z, Maqbool N, Molenaar A, Seyfert HM.** Assessment of the immune capacity of mammary epithelial cells: comparison with mammary tissue after challenge with Escherichia coli. *Vet Res* 40: 31–44, 2009.
26. **Holland DB, Bojar RA, Farrar MD, Holland KT.** Differential innate immune responses of a living skin equivalent model colonized by Staphylococcus epidermidis or Staphylococcus aureus. *FEMS Microbiol Lett* 290: 149–155, 2009.
27. **Korhonen H, Marnila P, Gill HS.** Milk immunoglobulins and complement factors. *Br J Nutr* 84: 75–80, 2000.

28. **Kühn C, Reinhardt F, Schwerin M.** Marker assisted selection of heifers improved milk somatic cell count compared to selection on conventional pedigree breeding values. *Arch Tierz Dummerstorf* 51: 23–32, 2008.
29. **Lahouassa H, Moussay E, Rainard P, Riollet C.** Differential cytokine and chemokine responses of bovine mammary epithelial cells to Staphylococcus aureus and Escherichia coli. *Cytokine* 38: 12–21, 2007.
30. **Lutzow YC, Donaldson L, Gray CP, Vuocolo T, Pearson RD, Reverter A, Byrne KA, Sheehy PA, Windon R, Tellam RL.** Identification of immune genes and proteins involved in the response of bovine mammary tissue to Staphylococcus aureus infection. *BMC Vet Res* 4: 18–41, 2008.
31. **Lutzow SYC, Gray C, Vuocolo T, Donaldson L, Broadway M, Tellam R.** Lipopolysaccharide and lipoteichoic acid induce different innate immune responses in bovine mammary epithelial cells. *Cytokine* 31: 72–86, 2005.
32. **Martinez-Pulgarin S, Dominguez-Bernal G, Orden JA, de la Fuente R.** Simultaneous lack of catalase and beta-toxin in Staphylococcus aureus leads to increased intracellular survival in macrophages and epithelial cells and to attenuated virulence in murine and ovine models. *Microbiology* 155: 1505–1515, 2009.
33. **Matthews KR, Almeida RA, Oliver SP.** Bovine mammary epithelial cell invasion by Streptococcus uberis. *Infect Immun* 62: 5641–5646, 1994.
34. **Moreilhon C, Gras D, Hologne C, Bajolet O, Cottrez F, Magnone V, Merten M, Groux H, Puchelle E, Barbry P.** Live Staphylococcus aureus and bacterial soluble factors induce different transcriptional responses in human airway cells. *Physiol Genomics* 20: 244–255, 2005.
35. **Paape MJ, Bannerman DD, Zhao X, Lee JW.** The bovine neutrophil: structure and function in blood and milk. *Vet Res* 34: 597–627, 2003.
36. **Paape MJ, Mehrzad J, Zhao X, Detilleux J, Burvenich C.** Defense of the bovine mammary gland by polymorphonuclear neutrophil leukocytes. *J Mammary Gland Biol* 7: 109–121, 2002.
37. **Pareek R, Wellnitz O, Van Dorp R, Burton J, Kerr D.** Immunorelevant gene expression in LPS-challenged bovine mammary epithelial cells. *J Appl Genet* 46: 171–177, 2005.
38. **Pawitan Y, Michiels S, Koscielny S, Gusnanto A, Ploner A.** False discovery rate, sensitivity and sample size for microarray studies. *Bioinformatics* 21: 3017–3024, 2005.
39. **Pisoni G, Moroni P, Genini S, Stella A, Boettcher PJ, Cremonesi P, Scaccabarozzi L, Giuffra E, Castiglioni B.** Differentially expressed genes associated with Staphylococcus aureus mastitis in dairy goats. *Vet Immunol Immunopathol* 135: 208–217, 2010.
40. **Rainard P, Corrales JC, Barrio MB, Cochard T, Poutrel B.** Leucotoxic activities of Staphylococcus aureus strains isolated from cows, ewes, and goats with mastitis: importance of LukM/LukF′-PV leukotoxin. *Clin Diagn Lab Immunol* 10: 272–277, 2003.
41. **Rainard P, Fromageau A, Cunha P, Gilbert FB.** Staphylococcus aureus lipoteichoic acid triggers inflammation in the lactating bovine mammary gland. *Vet Res* 39: 52–64, 2008.
42. **Rainard P, Riollet C, Berthon P, Cunha P, Fromageau A, Rossignol C, Gilbert FB.** The chemokine CXCL3 is responsible for the constitutive chemotactic activity of bovine milk for neutrophils. *Mol Immunol* 45: 4020–4027, 2008.
43. **Rainard P, Riollet C.** Mobilization of neutrophils and defense of the bovine mammary gland. *Reprod Nutr Dev* 43: 439–457, 2003.
44. **Rescigno M, Lopatin U, Chieppa M.** Interactions among dendritic cells, macrophages, and epithelial cells in the gut: implications for immune tolerance. *Curr Opin Immunol* 20: 669–675, 2008.
45. **Riollet C, Rainard P, Poutrel B.** Cell subpopulations and cytokine expression in cow milk in response to chronic Staphylococcus aureus infection. *J Dairy Sci* 84: 1077–1084, 2001.
46. **Rozen S, Skaletsky H.** Primer3 on the www for general users and for biologist programmers. *Meth Mol Biol* 132: 365–386, 2000.
47. **Rupp R, Bergonier D, Dion S, Hygonenq MC, Aurel MR, Robert-Granié C, Foucras G.** Response to somatic cell count-based selection for mastitis resistance in a divergent selection experiment sheep. *J Dairy Sci* 92: 1203–1219, 2009.
48. **da Silva MCA, Zahm JM, Gras D, Bajolet O, Abely M, Hinnrasky J, Milliot M, de Assis MC, Hologne C, Bonnet N, Merten M, Plotkowski MC, Puchelle E.** Dynamic interaction between airway epithelial cells and Staphylococcus aureus. *Am J Physiol Lung Cell Mol Physiol* 287: L543–L551, 2004.
49. **Smyth GK.** Limma: linear models for microarray data. In: *Bioinformatics and Computational Biology Solutions using R and Bioconductor*, edited by Gentleman R, Carey VJ, Huber W, Irizarry RA, Dudoit S. New York: Springer, 2005, p. 397–444.
50. **Sordillo LM.** Factors affecting mammary gland immunity and mastitis susceptibility. *Livest Prod Sci* 98: 89–99, 2005.
51. **Sutra L, Poutrel B.** Virulence factors involved in the pathogenesis of bovine intramammary infections due to Staphylococcus aureus. *J Med Microbiol* 40: 79–89, 1994.
52. **Swanson KM, Stelwagen K, Dobson J, Henderson HV, Davis SR, Farr VC, Singh K.** Transcriptome profiling of Streptococcus uberis-induced mastitis reveals fundamental differences between immune gene expression in the mammary gland and in a primary cell culture model. *J Dairy Sci* 92: 117–129, 2009.
53. **Tao WJ, Mallard B.** Differentially expressed genes associated with Staphylococcus aureus mastitis of Canadian Holstein cows. *Vet Immunol Immunopathol* 120: 201–211, 2007.
54. **Toufeer M, Bonnefont CMD, Foulon E, Caubet C, Tasca C, Aurel MR, Robert-Granié C, Rupp R, Foucras G.** Gene expression profiling of dendritic cells reveals important mechanisms associated with predisposition to staphylococcus infections. *PLoS One* 6: e22147–e22160, 2011.
55. **Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F.** Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3: RESEARCH0034.1–0034.11, 2002.
56. **Veldkamp KE, VanKessel KPM, Verhoef J, VanStrijp JAG.** Staphylococcal culture supernates stimulate human phagocytes. *Inflammation* 21: 541–551, 1997.
57. **Vetillard J, Drouet M, Neildez-Nguyen TM, Mestries JC, Mathieu J, Thierry D, Herodin F.** Interleukine-8 acts as a strong peripheral blood granulocyte-recruiting agent rather than as a hematopoietic progenitor cell-mobilizing factor. *J Hematother Stem Cell Res* 8: 365–379, 1999.
58. **Weber A, Weber AT, McDonald TL, Larson MA.** Staphylococcus aureus lipotechoic acid induces differential expression of bovine serum amyloid A3 (SAA3) by mammary epithelial cells: Implications for early diagnosis of mastitis. *Vet Immunol Immunopathol* 109: 79–83, 2006.
59. **Wells SJ, Ott SL, Seitzinger AH.** Key health issues for dairy cattle–new and old. *J Dairy Sci* 81: 3029–3035, 1998.
60. **Yang W, Zerbe H, Petzl W, Brunner RM, Gunther J, Draing C, von Aulock S, Schuberth HJ, Seyfert HM.** Bovine TLR2 and TLR4 properly transduce signals from *Staphylococcus aureus* (*S. aureus*) and *E coli*, but *S. aureus* fails to both activate NF-kappaB in mammary epithelial cells and to quickly induce TNFalpha and interleukin-8 (CXCL8) expression in the udder. *Mol Immunol* 45: 1385–1397, 2008.
61. **Zoumpopoulou G, Tsakalidou E, Dewulf J, Pot B, Grangette C.** Differential crosstalk between epithelial cells, dendritic cells and bacteria in a co-culture model. *Intl J Food Microbiol* 131: 40–51, 2009.