Differential Transcriptional Response To *Staphylococcus aureus* Infection In Two Divergent Lines Of Sheep Selected On Milk Somatic Cell Score

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Introduction

Amongst infectious diseases, mastitis is of major importance in dairy ruminants because of its high frequency and related costs. Accumulating literature data over the last decades give strong evidence that dairy ruminant's ability to control udder health is under genetic control (Axford *et al.* 2002). Many countries have implemented selection programmes based on milk somatic cell score (SCS), the log transformed values of somatic cell count, to improve resistance to mastitis. Although it has a moderate heritability (0.13) (Rupp and Boichard 2003), its genetic correlation with bacterial infection is assumed to be near the unit. To assess the effect of SCS-based selection, two divergent lines of dairy sheep were created in an INRA experimental facility (Rupp *et al.* 2009). The Low SCS line showed a significantly lower frequency of intra-mammary infections when compared to the High SCS line. More information about the divergent lines will be presented in the present WCGALP congress by Rupp *et al*.

Microarray technology enables comparison of several thousands of gene expression in the context of complex biological functions. Different cell types were chosen for transcriptomic analyses according to their complementary roles in the host's response to intramammary infections: 1) capacity of pathogen recognition and signalling to other cells; 2) abundance at the pathogen entry site and 3) capacity of bacterial phagocytosis and killing. As mammary epithelial cells (MEC) are numerous and present at the first line of defence, they may play a crucial role in the early host-pathogen interactions. Dendritic cells wear lots of antigens, and circulate in corporal fluid, so that they can detect pathogen intrusion and spread information, which represent the first step in host's response to a pathogen. In addition, milk cells, mainly composed of neutrophils, are recruited in the udder after an infection to contain bacteria spreading. Therefore transcriptomic analyses of these three cell types may provide crucial information to understand molecules and pathways involved in early host-pathogen interactions and control of mammary infections.

The present study aims at identifying the main differences of gene expression between two divergent SCS lines of ewes upon *Staphyloccocus* infections. Transcritomic profiling was applied to epithelial and dendritic cells, and milk cells (mainly neutrophils), in order to better understand genetically determined mechanisms and genes involved in mastitis resistance.

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Material and methods

Animals came from two lines of Lacaune dairy sheep divergently selected on the SCS breeding values of their parents at INRA experimental facility of La Fage (UE 321, Roquefort, France) (Rupp *et al.* 2009). Two groups of thirteen ewes of High and Low SCS lines were included in three experiments.

1. Milk cells were collected *in vivo* after intramammary inoculation of two different *Staphylococcus* species, respectively a mild pathogenic specie that is very frequent in small ruminant mastitis, *S epidermidis*, and a much more virulent strain, *S. aureus* (SA9A). They were inoculated at the beginning of two successive lactations separated by an antibiotic treatment to clear off the first infection. For both experimental infections, citernal lavage was performed to collect milk cells twelve hours after challenge.
2. For two groups of four of the above ewes, dendritic cells (DC) were differentiated from bone marrow (BM) progenitors and cultured *in* vitro as described by Foulon (Foulon and Foucras 2008). Then, BMDC were stimulated with heat-killed *S. aureus*. Cells were sampled before stimulation (T0), or after three and eight hours of culture.
3. Mammary epithelial cells (MEC) were collected and prepared *in vitro* from two groups of six additional lactating primiparous ewes after culling. MEC were stimulated either with 2 x 10^5 cfu/ml of the previously characterized ovine *S. aureus* strain, that was used for mammary inoculations or by a 20% supernatant obtained from its culture. Cells were sampled without stimulation (T0), or after one and five hours of culture.

After an amplification-labelling step, samples dyed with Cy3 or Cy5 were hybridised on an ovine oligonucleotide microarray (Agilent) harbouring 15208 probes, at the Genotoul genomic platform in Toulouse. A 4000B Axon scanner and Agilent Technologies' Feature Extraction software were used to extract data. Annotations of probes were completed from http://www.sigenae.org/ (sheep oligo annotation, version 5 of 2009/11/10).

Analyses were conducted separately on the three experiments. Differentially expressed genes were detected using ANOVA applied to gene by gene linear models. Benjamini Hochberg (BH) selection procedure with a threshold of 5% was subsequently applied for multiple test corrections. Pre-processing steps and statistical analysis were performed with Genespring version 11 and SAS. Meta analysis of the three experiments was then pursued by comparing lists of genes differentially expressed between High SCS and Low SCS sheep.

Results

Gene expression profiling in three cell types upon *Staphylococcus* stimulation

After experimental infections, inoculation of different *Staphylococcus* bacteria led to differences in gene expression of milk cells. Actually, 4355 probes were differentially expressed (BH1%) when comparing *S. epidermidis* to *S. aureus challenge*. Moreover, 198 probes had a $\log_2$-fold change superior to two. Among those probes, 150 and 48 were up-regulated and down-regulated, respectively, after *S.aureus* challenge when compared to *S. epidermidis* challenge.

S. aureus stimulation of BMDC caused a strong modification of gene expression in both lines. Indeed, 4332 genes were differentially expressed along the experiment (BH5%) and 94

had a fold-change superior to three. Main gene expression differences occurred between T0 and 8 hours after stimulation (67 genes).
Regarding mammary epithelial cells, 2455 and 2463 probes were differentially expressed (BH5%) along experiment with respectively bacteria and supernatant stimulations. Two thirds of those genes were common. After one hour of stimulation, very few genes had an expression twice higher (n=0 and n=14, for bacteria and supernatant stimulations, respectively), indicating a latency period before gene activation upon stimulation.

Line effect in separate analyses of cell types

In the three cell types, the number of differentially-expressed genes was much smaller between the two divergent lines than between the different stimulations (*S. aureus* vs *S. epidermidis*; *S. aureus vs* supernatant) or the stimulated/unstimulated cultures.
Transcriptomic profiling of milk cells allowed the identification of a list of 162 genes that were significantly differentially-expressed (BH5%) between sheep lines. Indeed, 77 and 85 genes were respectively up and down regulated in the Low SCS line (resistant sheep) when compared to the High SCS line (susceptible sheep). Moreover, 15 genes were confirmed by RTqPCR. Some genes were over-expressed in the resistant line, such as SC5 an antimicrobial peptide, or ITGAL, ST3GAL and CAPN3 involved in neutrophil adhesion and diapedesis.
Microarray analysis of BM-DC showed 1080 differentially expressed genes between the sheep lines (BH5%), with no less than 46 genes having a fold-change higher than three. We can focus on MAPRE1, an important gene in regulating microtubule dynamics and GST, a gene for detoxification. Whereas MAPRE1 and GST were up regulated in the susceptible line, a MHC class I-related gene and UQCRQ, the subunit VI of the mitochondrial complex III were up-regulated in resistant ewes. Some differences in gene expression between the two lines existed before stimulation (25 genes with FC>3), and some others occurred only after stimulation (21 genes with FC>3). Therefore, the basal gene expression in dendritic cells is not exactly identical between the divergent lines and stimulation by bacteria increased some of these differences.
Transcriptomic profiling of MEC allowed the identification of 324 and 572 differentially-expressed probes (BH5%) between the susceptible and resistant lines after *S. aureus* and supernatant stimulation, respectively. But only 16 and 11 genes, respectively, had a fold-change superior to two. Out of the 324 and 572 probes, 242 were common to both experiments, including 10 genes with a fold-change superior to two. Among these 10 genes, SLC2A, a glucose transporter, PLAT, a plasminogen activator, and PRDX, a peroxiredoxin that play different roles in cellular redox regulation and antioxidant protection, were up-regulated in susceptible ewes. On the contrary, CAT, the catalase gene was down regulated.
Therefore, although only few genes were differentially expressed between the susceptible and resistant lines, and although fold changes were rather low, genetic differences between ewes divergently selected on somatic cell score are likely to exist. The genes communally regulated between the sheep line in at least two cell types were studied together

Meta-analysis of transcriptomic profiles in three different cell types

Though the three transcriptomic profiling experiments were conducted on different cell types, time scale and animals, a subset of 57 genes, that were consistently differentially expressed between the High and Low SCS lines, was identified. Two genes were up-

regulated in the susceptible line for the three cell types: MAPRE1, whose protein plays an important role in microtubule function, including mitotic spindle formation and chromosome segregation (Su 2001) and a non annotated sequence. This sequence might be an interesting functional gene candidate, so its investigation is in progress. Several genes were grouped in a pathway analysis (IPA) network in close connection with TLRs. This result indicated that TLR signalisation pathways may be partly responsible for the genetic differences between the SCS-selected divergent lines. A study comparing dairy cattle carrying different alleles on a quantitative trait locus affecting udder health highlighted an up-regulation of Toll-likereceptor 2 in less-susceptible animals (Griesbeck-Zilch 2009). Apoposis pathway may also be involved in mechanisms that discriminate the sheep lines with the differentially regulation of one caspase gene, which is the mayor components of the cell suicide machinery. Some genes are good positional and functional QTL candidate since they are localised near QTL regions for SCS reported in bovine or ovine experiments.

Conclusion

Transcritomic profiling analyses enabled to identify some candidate mechanisms involved in host's response to intramamamry infections in various cell types. Comparison of the High and Low SCS lines of sheep further allowed some insight into genetically determined difference in mastitis resistance. Some differentially expressed genes have already been confirmed by RTqPCR, other confirmations are in progress. SNP studies in hand, on the divergent SCS lines, are expected to be complementary to transcriptomic information and to allow the identification of some positional and functional candidates for mastitis resistance QTLs.

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