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An explant of heifer mammary gland to study the immune response of the organ

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ABSTRACT

Continuous or primary epithelial cell lines have been extensively used to study the mammary gland immune response, but they are constituted by a single cell population. Our aim was to test whether an explant of heifer gland, where the tissue structure is maintained, might be a valid model to investigate the innate immune response to infection. The study was carried out on 2 mm^3-sections of heifer udders, in 2 consecutive trials, using LPS or LTA in the first trial and two different concentrations of *Staphylococcus aureus* (*Staph. aureus*) in the second. Treated and untreated sections were collected after 1 h, 3 h and 6 h incubation; in the first trial, a final time-point at 18 h was considered. The mRNA expression of TNFα, IL-1β, IL-6, IL-8 and LAP was analyzed by quantitative real-time PCR. Histological examination showed well-preserved morphology of the tissue, and apoptosis only showed a slight, not significant increase throughout the experiment. IL-1β and IL-6 were significantly up-regulated, in response to LPS or *Staph. aureus*, while TNF-α and IL-8 significantly increased only under LPS treatment. LAP expression showed a significant late increase when stimulated by LPS. The immunochemical staining of the sections demonstrated a higher number of T lymphocytes within the alveolar epithelium, in comparison with interstitial localization. Since the explants belonged to pubertal non-pregnant heifers, T cells may be regarded as resident cells, suggesting their participation in the regulation of mammary homeostasis. Therefore, applying our model would give new insights in the investigation of udder pathophysiology.

1. Introduction

Mastitis is considered the most important disease of dairy cattle, due to its economic impact. It is hard to estimate the losses associated to clinical or subclinical mastitis because of the costs of treatment, lower milk production, milk discard, premature slaughter, and antibiotic usage (McDougall et al., 2009). Most studies focus on the development of vaccines or on herd management, to reduce/eliminate bacterial reservoirs. Other strategies address the modulation of the cow immune response, in order to increase the mammary gland resistance to invading pathogens. The udder immunity is principally based on the innate response, and the clinical outcome of mastitis is not only related to the infectious agent, but also to the immune response of the mammary gland. The cellular effectors are both leukocytes and mammary epithelial cells (MEC). Cytokines play a crucial role in the balance between humoral and cell-based immune responses. The pro-inflammatory cytokines Tumor Necrosis Factor alpha (TNF-α) and Interleukin-1 beta (IL-1 β) induce an acute reaction, Interleukin-8 (IL-8) attracts neutrophils into the infected udder, while Interleukin-6 (IL-6) facilitates the transition of the inflammatory process, decreasing neutrophils and favouring monocyte recruitment. Innate immunity includes also antimicrobial peptides, such as the Lingual Antimicrobial Peptide (LAP), a member of the β-defensin family, exhibiting antibacterial activity against a broad spectrum of microorganisms (Tomasinsig et al., 2010). Investigating the role of innate immunity in the mammary gland is essential to better understand the pathogenesis of mastitis.

To that end, it is essential to select an appropriate *in vitro* model. A widespread approach is the use of established mammary epithelial cell lines, since the cells are theoretically identical in each passage, deriving from a single monoclonal line. However, the comparison of the results from different papers is still problematic, since changes in cell morphology and metabolism may occur as well as genetic instability in cells maintained in *in vitro* systems for prolonged time periods (Buhering et al., 2004). Primary MEC cultures are also utilized in many studies regarding mammary gland pathophysiology. The cells are obtained from mammary parenchymal tissue collected from lactating animals (Zhao et al., 2010). Both models are considered as reliable tools to investigate the functions of mammary epithelial cells, but the mammary tissue consists of different cell types, including fibrocytes, resident macrophages, endothelial cells,

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and adipocytes. Notably, all these cells produce cytokines and other immune-modulatory molecules, orchestrating a complex play. Therefore, the aim of the present study was to set up an explant of the mammary gland, using a healthy heifer gland, in order to investigate the tissue immune response to infection.

2. Materials and methods

The study was carried out in 2 consecutive trials, using the udder of two different heifers, both sent to the abattoir for infertility reasons. Different treatments were applied, but sampling and culture conditions were identical.

2.1. Sampling and culture conditions

In both trials, the udder of a heifer was taken at the slaughterhouse and immediately transported to the laboratory. The skin and fat were removed and a section of mammary parenchyma was aseptically taken and washed with Hanks' Balanced Salts Solution (HBSS) added with 1:100 antibiotic-antimycotic solution containing 100 units penicillin, 0.1 mg streptomycin and 0.25 μg amphotericin B per mL. The tissue was then trimmed into smaller sections of 2 mm^3 weighing 0.02 g. The udder sections were cultured at 37 °C with 5% CO_2 in 96-well plates in the following medium, supplemented with 10% fetal calf serum (FCS): 50% Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DMEM/F12), 30% Roswell Park Memorial Institute (RPMI) 1640 medium, 20% NCTC-135 medium, containing 0.1% lactose, 0.1% lactalbumin hydrolysate, 1.2 mM glutathione, 10 μg/mL L-ascorbic acid, 1 μg/mL hydrocortisone, 1 μg/mL insulin, 5 μg/mL transferrin, and 0.5 μg/mL progesterone. The media were purchased from Thermo Fisher Scientific (Waltham, USA), HBSS and supplements from Sigma-Aldrich (St. Louis, USA).

2.2. Treatments

Seven replicates of each treatment and control were used in both experiments. In the first trial, explants were treated with 1 μg/mL of lipopolysaccharide (LPS) or 0.5 μg/mL of lipoteichoic acid (LTA, both from Sigma-Aldrich, USA); in the second trial, two different concentrations of *Staphylococcus aureus* (*Staph. aureus*) strain MI 390 from our collection were used. After overnight incubation on blood agar plate at 37 °C, the bacteria were suspended in the culture medium and inoculated into the wells to a final concentration of 10^2 CFU/mL or 10^3 CFU/mL. Optical density was measured spectrophotometrically at 600 nm and was confirmed the following day by plate count method. Lipopolysaccharide was used as positive control in the second trial. Negative control wells were treated similarly, but no synthetic molecules or bacteria were added. In both trials, treated and untreated sections were collected after 1 h, 3 h and 6 h incubation; in the first trial, a final time-point at 18 h was considered. The sections of 6 out of the 7 repetitions, taken throughout the experiment, were immediately stored in RNAlater (Sigma-Aldrich, USA) at −20 °C until RNA extraction. The remaining sections were fixed in 10% neutral buffered formalin for at least 48 h at room temperature, routinely processed for paraffin embedding, and sectioned at 4-μm thickness.

2.3. Morphological analysis and inflammatory cells detection

Histological sections were stained with Hematoxylin-eosin (HE) to study the morphological aspects of the tissue; in the second trial, they were also evaluated by immunohistochemistry (IHC). Sections of formalin-fixed paraffin-embedded tissues were simultaneously dewaxed and unmasked by heat induced epitope retrieval and Buffer H (Bio-optica, Italy). Endogenous peroxidase activity was blocked by incubating sections in 3% H_2O_2 and slides were rinsed and treated with phosphate buffer saline (PBS, Sigma-Aldrich, USA) containing serum to reduce nonspecific background staining. As primary antibodies, rabbit polyclonal antibody anti-Iba1 (Ionized Calcium-binding Adapter molecule-1, Wako chemicals, Germany) or rabbit polyclonal antibody anti-CD3 (Dako, Denmark) were used, to detect histiocytes/macrophages or T cells, respectively. Sections were then incubated with biotinylated secondary antibody (goat anti-rabbit) and labelled by the avidin-biotin-peroxidase (ABC) procedure with a commercial immunoperoxidase kit (VECTASTAIN® Elite ABC-Peroxidase Kit Standard). The immunoreaction was visualized with 3,3′-diaminobenzidine (Peroxidase DAB Substrate Kit). All reagents were purchased from Vector Laboratories (USA). Substrate and sections were counterstained with Mayer's hematoxylin (Merck, USA). Known positive control sections were included in each immunolabelling assay. Sections were blind evaluated with a light microscopy by a veterinary pathologist and 3 different anatomical structures (alveolar, ductular and interstitial) semi-quantitatively scored as follows: − = absent, + = rare cells, ++ = some cells; +++ = numerous cells.

2.4. Proliferation and apoptosis

Both cell proliferation and apoptosis of the *ex vivo* sections were evaluated using an immunofluorescence-based assay. Sections were boiled for 5 min in antigen unmasking solution (Vector Laboratories, USA). Non-specific sites were blocked with PBS containing 5% Bovine Serum Albumin (BSA, Sigma-Aldrich, USA) and 10% non-immune serum (Thermo Fisher Scientific, USA). Samples were incubated overnight at 4 °C with a primary rabbit polyclonal antibody specific for the Ki67 marker of cell proliferation (1:50 dilution, Abcam, UK). Sections were washed with PBS and incubated with the secondary antibody (Alex Fluor® 488 goat anti-rabbit, Thermo Fisher Scientific, USA) for 1 h in darkness. Nuclei were stained with 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, USA). The percentage of apoptotic cells was evaluated using a commercial kit (*In situ* Cell Death Detection Kit, TMR red, Roche, Basel, Switzerland) following the manufacturer's instructions. The test is based on the Terminal deoxynucleotidyltransferase-mediated dUTP Nick-end Labeling (TUNEL) assay. For each slide, a minimum of 4 pictures were taken and blind observed under an Eclipse E600 microscope (Nikon, Japan). Pictures were acquired with Nis Elements Software (Version 4.0) with constant exposure parameters, then analyzed with the image analysis software ImageJ (http://rsbweb.nih.gov/ij/index.html). Threshold adjustments were applied to generate a black and white image and marker expression was normalized by DAPI fluorescence. Cell proliferation and apoptosis were evaluated by cell count, considering every cell type, and expressed as a percentage.

2.5. RNA extraction and quantitative real-time PCR

Tissue samples were lysed using 50 mg of acid-washed glass beads on a mixer mill Retsch MM301 (Retsch GmbH & co., Germany) and total RNA was extracted using TRIzol reagent (Sigma-Aldrich, USA), according to the manufacturer's recommendations. Total RNA yield and purity were determined by the NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Germany). Genomic DNA removal and reverse transcription was performed using the QuantiTect Reverse Transcription kit (Qiagen, Netherlands) and 1 μg RNA as a template.

The quantitative real-time PCR (qPCR) was performed following MIQE guidelines (Bustin et al., 2009) and qbase + software (Biogazelle, Belgium) was used for the gene expression analysis. All the samples were normalized to the negative control, in order to compare the results in the different time-points and experiments. For the qPCR reaction, 2 μL of cDNA was amplified in a 15-mL reaction volume with the Eco Real Time PCR Detection System (Illumina Inc., USA), using Eva Green Supermix (BioRad, USA) and different concentrations of primers. Three reference genes (Ubiquitously Expressed Transcript, UXT; Eukaryotic Translation Initiation Factor 3 K, EIF3K; Ribosomal Protein Large P0, RPLP0) validated with geNORM analysis, were selected as the best combination of internal standard for mammary gland tissue (Bonnet et al., 2013). Primers sequences of the target genes TNF-α