Veterinary Immunology and Immunopathology 188 (2017) 21–26

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Full Length Article

# Blood and milk polymorphonuclear leukocyte and monocyte/macrophage functions in naturally caprine arthritis encephalitis virus infection in dairy goats

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ARTICLE INFO

*Keywords:*
Lentivirus
Goat
Small ruminant
Leukocyte function
Milk cells

ABSTRACT

The exact influence of caprine arthritis encephalitis virus (CAEV) infection on blood and milk polymorphonuclear leukocytes (PMNLs) and monocyte/macrophages of goats remains unclear. Thus, the present study sought to explore the blood and milk PMNL and monocyte/macrophage functions in naturally CAEV-infected goats. The present study used 18 healthy Saanen goats that were segregated according to sera test outcomes into serologically CAEV negative (n = 8; 14 halves) and positive (n = 10; 14 halves) groups. All milk samples from mammary halves with milk bacteriologically positive outcomes, somatic cell count $\geq 2 \times 10^6$ cells $mL^{-1}$, and abnormal secretions in the strip cup test were excluded. We evaluated the percentage of blood and milk PMNLs and monocyte/macrophages, the viability of PMNLs and monocyte/macrophages, the levels of intracellular reactive oxygen species (ROS) and the nonopsonized phagocytosis of *Staphylococcus aureus* and *Escherichia coli* by flow cytometry. In the present study, a higher percentage of milk macrophages ($CD14^+$) and milk polymorphonuclear leukocytes undergoing late apoptosis or necrosis (Annexin-$V^+$/Propidium $iodide^+$) was observed in CAEV-infected goats; we did not find any further alterations in blood and milk PMNL and monocyte/macrophage functions. Thus, regarding our results, the goats naturally infected with CAEV did not reveal pronounced dysfunctions in blood and milk polymorphonuclear leukocytes and monocytes/macrophages.

## 1. Introduction

Caprine arthritis-encephalitis virus (CAEV) belongs to the genus *Lentivirus* of the family *Retroviridae* and is widespread in goat populations around the world. CAEV causes a persistent and slowly progressive degenerative inflammatory disease in multiple organs, comprising joints, lungs, nervous system, and mammary glands (Ponti et al., 2008).

The major tropism of CAEV is for monocytes, macrophages, and dendritic cells in which virus expression is influenced by maturation of mononuclear phagocytes (Ponti et al., 2008). In this context, it is known that macrophages and dendritic cells and their precursors, monocytes, are important sentinels and effector cells in combating microbial pathogens, and they have a crucial function bridging both innate and adaptive responses (Bieber and Autenrieth, 2015). Thus, because CAEV has a tropism to these cells, it can be hypothesized that CAEV can affect both adaptive and innate immunities.

In tissues, other cell types may also be infected and act as virus reservoirs. These additional targets include mammary epithelial cells that readily sustain CAEV infection and also may be implicated in the pathogenesis of mastitis (Mselli-Lakhal et al., 2001; Lechat et al., 2005). Viral infections of epithelial cells in target organs usually result in the secretion of biologically active factors that regulate the pathobiology of mastitis in infected goats because CAEV-infected mammary epithelial cells render high virus titers *in vitro* (Mselli-Lakhal et al., 2001).

*Abbreviations:* IMI, intramammary infection; SCC, somatic cell count; PMNL, polymorphonuclear leukocyte; ROS, reactive oxygen species; GMFI, geometric mean fluorescence intensity; AGID, agar gel immunodiffusion; PBS, phosphate-buffered saline; PE-Cy5, phycoerythrin-Cy5; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; Ab, antibody; PI, propidium iodide; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DCF, 2′,7′-dichlorofluorescein; MCP-1, monocyte chemotactic protein 1; LDH, lactate dehydrogenase (LDH) activity; NAGase, *N*-acetyl-β-D-glucosaminidase activity

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http://dx.doi.org/10.1016/j.vetimm.2017.04.009
Received 2 May 2016; Received in revised form 16 April 2017; Accepted 25 April 2017

Furthermore, the high virus titers *in vivo* may in turn induce an inflammatory response by the recruitment and activation of inflammatory and immune effector cells (Mselli-Lakhal et al., 2001). Therefore, the exact influence of CAEV infection on mammary gland immunity of goats remains unclear.

In addition, the CAEV serological status has been associated with bacterial intramammary infections (IMIs), especially in herds with a high prevalence of CAEV and bacterial IMIs (Bergonier et al., 2003; Jiménez-Granado et al., 2014). These facts would contribute, at least in part, to the worsening of production records (Martínez-Navalón et al., 2013; Jiménez-Granado et al., 2014) and to a reduced cheese yield (Nowicka et al., 2015), both of which have previously been reported for CAEV-seropositive goats. Altogether, these scenarios appear suitable for an investigation of the effects of CAEV on mammary gland immunity, which is largely dependent on phagocyte function (Souza et al., 2012a,b).

To the best of our knowledge, this is the first report that tries to explore specific functions of blood and milk polymorphonuclear leukocyte (PMNL) and monocyte/macrophage functions (i.e., viability, phagocytosis of *Staphylococcus aureus and Escherichia coli* and intracellular ROS production) in naturally CAEV-infected goats. Thus, the present study was performed to evaluate the blood and milk PMNL and monocyte/macrophage functions in naturally CAEV-infected goats.

## 2. Materials and methods

### 2.1. Animals and experimental design

The present study used 18 healthy Saanen goats from a commercial herd that were segregated according to sera test outcomes into serologically CAEV negative (n = 8; 14 halves) and positive (n = 10; 14 halves) groups. Because of the effects of bacterial mastitis pathogens on leukocyte profile and function (Souza et al., 2012b), the following exclusion criteria were applied for milk samples: 1) halves with at least one bacteriological positive outcome in two consecutive single milk samplings within an interval of a week; 2) halves with somatic cell count (SCC) higher than $2 \times 10^6$ cells $mL^{-1}$ as the milk SCCs proposed for goats with IMI (Souza et al., 2012a); 3) halves with abnormal secretions in the strip cup test; and 4) halves with alteration(s) in the clinical examination of the mammary glands and teats (Dirksen et al., 1993). To exclude the effect of mastitis pathogens on milk leukocyte profile and function, milk samples for bacteriological examination were collected twice (seven days before and on the day of flow cytometry analysis) to determine PMNL and macrophage functions. In addition, no animal had any hematological alterations (Pugh, 2005). The lactational status and parity of all animals were also recorded. The present study was approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine and Animal Science, University of São Paulo (Certificate n. 1684/2009).

### 2.2. Blood sampling

Blood samples were aseptically collected in the jugular vein from each goat after milking, and the samples were stored as follows: 1) in tubes containing potassium EDTA for hematological evaluation; 2) in heparin tubes for assessment of PMNL and monocyte function by flow cytometry; or 3) in tubes without an anticoagulant to obtain serum for serodiagnosis of CAEV.

### 2.3. Milk sampling

First, the presence of abnormal secretions was identified by the strip cup test. Then, pre-dipping was done, and one towel was utilized for each teat. Afterwards, the first three milk streams were discarded, teat ends were scrubbed with cotton soaked in 70% ethanol solution, and a single milk sample was aseptically collected from individual mammary halves into a sterile vial for bacteriological examination. Lastly, milk samples for the milk SCC and flow cytometric analysis were collected. Samples were kept at 4 °C until arriving at the laboratory. Milk samples for bacteriological examination were thawed at −20 °C until the analysis.

### 2.4. Serodiagnosis of CAEV

The dairy goats were sera tested for CAEV by agar gel immunodiffusion (AGID) (Biovetech®, Recife, Brazil) and ELISA (VMRD Pullman Inc., USA, cat. number 289-5) utilizing the antigen p28.

### 2.5. Hematological procedures

The total leukocyte counts were performed on an automatic cell count (BC-2800 VET, MINDRAY®, Shenzhen, China). The differential leukocyte count was determined by routine smears.

### 2.6. Bacteriological analysis

Bacteriological analysis was done by culturing 0.01 mL of each milk sample on 5% sheep blood agar plates. The plates were incubated for 72 h at 37 °C, followed by Gram staining, colony morphology visualization and biochemical testing (Oliver et al., 2004).

#### 2.6.1. Determination of the SCC

Milk samples for SCC were collected in a 40-mL containers in which there were microtables of bronopol (2-bromo-2-nitropane-1,3-diol). Then, the SCC measurements were determined by an automated fluorescent microscopic somatic cell counter (Somacount 300–Bentley Instruments®, Chaska, EUA) utilizing ethidium bromide that binds to DNA in the cell nuclei, allowing us to exclude cytomasmic particles (Hall and Rycroft, 2007; Souza et al., 2012a).

### 2.7. Separation of milk cells

Here, 300 mL of milk from each half were collected in a flask containing 300 mL of phosphate-buffered saline (PBS; pH 7.4; 1.06 mM $Na_2HPO_4$, 155.17 mM NaCl and 2.97 mM $Na_2HPO_4{\cdot}7H_2O$). Then, the separation of milk cells was done as previously described by Blagitz et al. (2013). Afterwards, the cell viability was performed by trypan blue exclusion to obtain a concentration of $2 \times 10^6$ viable cells $mL^{-1}$ in nutrition medium (RPMI-1640, cat. n. R7638, Sigma Aldrich, USA) with 10% fetal bovine serum.

### 2.8. Identification and quantification of blood and milk leukocyte populations

First, aliquots of 100 μL of blood in tubes were lysed by hypotonic lysis to eliminate contaminating erythrocytes. After centrifuging at 250 × *g* for 8 min, cells were washed in PBS and stained for 30 min at room temperature to detect CD14 and PG68A following incubation with the primary antibodies (Abs). The identification of the PMNL population was based on their side scatter measure and PG68A positivity (Fig. 1), and the identification of monocytes/macrophages was performed using phycoerythrin-Cy5 (PE-Cy5)-conjugated mouse anti-human CD14 (Fig. 1). The PMNLs were identified with swine IgG1 anti-mouse PG68A (VMRD Pullman Inc. Corp®, Pullman, WA, USA), which was specie-reactive with goat cells (Leitner et al., 2011). The $CD14^+$ leukocytes, mainly monocytes and macrophages (Winnicka et al., 1999; Boulaaba et al., 2011), were identified with a PE-Cy5-conjugated mouse anti-human CD14 (MCA2804C, AbDSerotec, Oxford, England) that was specie-reactive with goat cells. After washing with PBS, the cells for identification of PMNLs were incubated for 30 min at room temperature with the secondary Abs goat anti-mouse IgG1 conjugated to PE-Cy5 (M32018; Invitrogen, Carlsbad, CA, USA).