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The neutrophil function and lymphocyte profile of milk from bovine mammary glands infected with Streptococcus dysgalactiae

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Streptococcus dysgalactiae is a bacterium that accounts for a notable proportion of both clinical and subclinical intramammary infections (IMIs). Thus, the present study explores the function of milk neutrophils and the lymphocyte profile in mammary glands naturally infected with *Streptococcus dysgalactiae*. Here, we used 32 culture-negative control quarters from eight clinically healthy dairy cows with low somatic cell counts and 13 *S. dysgalactiae*-infected quarters from six dairy cows. Using flow cytometry, we evaluated the percentage of milk monocytes/macrophages and neutrophils, expression of CD62L, CD11b and CD44 by milk neutrophils, the levels of intracellular reactive oxygen species (ROS) production and phagocytosis of *Staphylococcus aureus* by milk neutrophils, and neutrophil viability. Furthermore, the percentages of B cell ($CD21^+$) and T lymphocyte subsets ($CD3^+/CD4^+/CD8^-$; $CD3^+/CD8^+/CD4^-$; and $CD3^+/CD8^-/CD4^-$), and the expression of CD25 by T milk lymphocytes ($CD3^+$) and T $CD4^+$ milk cells were also assessed by flow cytometry using monoclonal antibodies. The present study showed a higher SCC and percentage of milk neutrophils, and a decrease in the percentage of milk monocytes/macrophages from *S. dysgalactiae*-infected quarters when compared to uninfected ones. We also observed a higher expression of CD11b by milk neutrophils and a tendency toward a decrease in neutrophil apoptosis rate in *S. dysgalactiae*-infected quarters. In addition, the *S. dysgalactiae*-infected quarters had higher percentages of milk T cells ($CD3^+$) and their subset $CD3^+CD8^+CD4^-$ cells. Overall, the present study provided new insights into *S. dysgalactiae* IMIs, including distinct lymphocyte profiles, and a tendency toward an inhibition of apoptosis in milk neutrophils.

Keywords: Immune response, mastitis, somatic cell count, dairy cow.

Mastitis is a disease of major economic importance, causing reduced milk production and quality and increasing the use of veterinary drugs worldwide. Several bacterial genera and species are capable of causing mastitis, and one of the most common groups of bacteria isolated from intramammary infections (IMIs) is *Streptococcus* spp., which includes *Streptococcus dysgalactiae* (Osteras et al. 2006; Whist et al. 2007; Souza et al. 2009; Zadoks & Fitzpatrick, 2009; Botrel et al. 2010; Schwarz et al. 2010; Beecher et al. 2012; Abrahmsén et al. 2014; Leelahapongsathon et al. 2014). For instance, Botrel et al. (2010) described that *S. dysgalactiae* was isolated in 8·8 and 9·4% of the milk samples from clinical and subclinical cases of mastitis, respectively.

Mastitis organisms are categorised as contagious or environmental pathogens based on their distinct characteristics of distribution and interaction with the teat and teat duct.

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Environmental pathogens are those whose primary reservoir is the environment rather than infected mammary glands. The most frequently isolated environmental pathogens are coliform bacteria and streptococci other than *S. agalactiae*. Among the environmental streptococci, *S. uberis* and *S. dysgalactiae* are the most prevalent (Calvinho et al. 1998; Osteras et al. 2006; Whist et al. 2007; Zadoks & Fitzpatrick, 2009; Schwarz et al. 2010; Leelahapongsathon et al. 2014), although these bacteria have been found to be contagious in some dairy herds (Whist et al. 2007).

In the last few decades, with the improvement of mastitis control programs leading to herds with lower somatic cell counts (SCCs), environmental mastitis has become a major problem in many well-managed dairy herds that have successfully controlled contagious pathogens (Schukken et al. 1989; Calvinho et al. 1998; Green et al. 2004). In these herds, *S. dysgalactiae* accounts for a significant number of subclinical and clinical cases of IMIs (Calvinho et al. 1998; Zadoks & Fitzpatrick, 2009). Furthermore, mastitis control programs have had minimal effects on reducing the incidence of IMIs caused by *S. dysgalactiae* (Bolton et al. 2004; Lundberg et al. 2014). Despite improvements in management practices, *S. dysgalactiae* mastitis has remained a problem in many dairy herds. However, few studies have investigated the immune response against *S. dysgalactiae* (Calvinho et al. 1998; Song et al. 2001; Wyder et al. 2010; Beecher et al. 2012) and its effects on udder health.

Striving for a complete elimination of antimicrobial use in dairy cattle is unrealistic, although a reduction in antimicrobial use is recommended and feasible. From that point of view, one of the most practical means for dealing with mastitis may be to enhance the natural ability of animals to resist infection. The greatest obstacle in establishing this type of strategy is a lack of understanding of many aspects of the host immune response. Currently, the roles of various immune system components in the defence of the mammary gland against infection by several pathogens are not well understood. Altogether, it is clear that a more comprehensive understanding of the immune response is essential to the development of effective prevention strategies of mastitis (Soltys & Quinn, 1999).

The present study aimed to investigate the function of milk neutrophils and the milk lymphocyte profile in *S. dysgalactiae*-infected mammary glands.

Materials and methods

Animals and experimental design

The present study utilised milk samples from 45 quarter of14 Holstein dairy cows, which were collected at various stages of lactation from a commercial herd. From these samples, 32 culture-negative quarters from eight dairy cows exhibiting no abnormal secretions in the strip cup test and a milk somatic cell count (MSCC) lower than 2×10^5 cells/ml, which is the MSCC threshold proposed by Schepers et al. (1997) and Schukken et al. (2003) for uninfected quarters, were defined as the control group. Additionally, 13 *S. dysgalactiae*-infected quarters from six dairy cows were included. The parity and days in milk of all animals were also recorded.

Regarding the criteria used here, Dohoo et al. (2011) assessed the sensitivity (SE) and specificity (SP) of a single milk quarter sample and described that milk samples with SCC ⩾ 200 000 cells/ml and with at least two pure colonies of *S. dysgalactiae* or *S. uberis* have a SE and SP of 66·4 and 100%, respectively. In addition, these authors reported that 89% of all *Streptococcus* spp. isolates in the weekly data had more than 1000 colony forming per unit/ml in culture, suggesting that these infections shed a large number of microorganisms.

Sample collection

First, the strip cup test was performed to identify the presence of clots, flakes or otherwise obviously abnormal secretions. Then, the pre-dipping was performed, and one towel was used for each teat. After discarding the first three milk streams, teat ends were scrubbed with cotton containing 70% ethanol, and single milk samples from individual mammary quarters were aseptically collected into sterile vials for bacteriological analysis (approximately 3 ml). Finally, milk samples for the SCC (approximately 40 ml) and evaluation of neutrophilic function and milk lymphocyte profile (approximately 1 litres) were collected. Samples were kept at 4 °C for about 3 h until arriving at the laboratory. Milk samples for bacteriological analysis were cooled at –20 °C until the analysis.

Bacteriological analysis

The bacteriological analysis was performed by culturing 0·01 ml of each single milk-quarter sample on 5% bovine blood agar plates (Bio Express, São Paulo, Brazil). The plates were incubated for 72 h at 37 °C, and *S. dysgalactiae* subsp. *dysgalactiae* was identified as Gram-positive cocci, alpha-hemolytic, catalase negative, esculin negative and negative reaction in Christie-Atkins-Munch-Petersen (CAMP) (Raemy et al. 2013). A milk sample was considered culture-positive when the growth of ⩾10 (10^3 colony forming per unit) pure *S. dysgalactiae* subsp. *dysgalactiae* colonies was detected. Samples yielding no bacterial growth were regarded as culture-negative.

Determination of the SCC

Milk samples for SCC were collected in 40 ml vials containing microtablets of bronopol (2-bromo-2-nitropane-1,3-diol) as a preserving agent. Subsequently, SCC measurements were performed using an automated, fluorescent, microscopic somatic cell counter (Somacount 300 – Bentley Instruments®, Chaska, USA).

Separation of milk cells

The separation of the milk cells was performed as described by Koess & Hamann (2008). Briefly, 1 L of milk was diluted

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with 1 litre of phosphate-buffered saline (PBS; pH 7·4; 1·06 mM Na_2HPO_4, 155·17 mM NaCl and 2·97 mM $Na_2HPO_4{\cdot}7H_2O$). After centrifugation at 1000 ***g*** for 15 min, the cream layer and supernatant were discarded. The cell pellet was then washed once using 30 ml of PBS and centrifuged at 400 ***g*** for 10 min. The cells were resuspended in 1 ml of RPMI-1640 nutritional medium (cat. n. R7638, Sigma Aldrich, USA) supplemented with 10% fetal bovine serum (Cultilab, Campinas, Brazil) and counted using a Neubauer chamber. Cell viability was evaluated using trypan blue exclusion (Jain & Jasper, 1967). The milk cells were then diluted with nutritional medium containing 10% fetal bovine serum to a concentration of 2×10^6 viable cells/ml.

Enumeration of lymphocyte subpopulations

The cells were washed with PBS and incubated with the primary monoclonal antibodies (mAbs) for 30 min on ice to detect CD21, and the combination of CD3, CD4 and CD8. The identification of lymphocyte subsets was based on their cytoplasmic granularities and mean fluorescence intensities following a two-step fluorescence immunolabeling protocol using primary anti-bovine mAbs and secondary mAbs coupled to long-wavelength fluorescent probes (Table 1). Next, 1 ml of PBS was added to the cell suspension and centrifuged at 400 ***g*** for 8 min. Finally, the labelled secondary mAb was added to the cell suspension, and the cells were incubated for 30 min on ice with the secondary mAbs. The cells were then washed with PBS and immediately analysed using flow cytometry. A total of 20 000 milk cells, excluding the cellular debris, were examined per sample. FlowJo software (TreeStar Inc., Ashland, OR, USA) was used to analyse the data. The results were corrected for autofluorescence, which was defined as the fluorescence that was associated with non-labelled freshly isolated milk cells from the same cow (Della Libera et al. 2015).

Expression of CD25

A crucial event in the initiation of an immune response is the activation of T cells, which requires IL-2 binding to its high-affinity IL-2 receptor for optimal signalling. The IL-2 receptor α-chain (CD25) is needed for the high affinity binding of IL-2 to effector cells and is potently induced after T cell activation (Waters et al. 2003; Maslanka et al. 2014). Thus, here we evaluated the expression of CD25 in bovine T cells ($CD3^+$) and T $CD4^+$ lymphocytes by flow cytometry using the mAbs listed in Table 1. Firstly, unlabelled primary mAbs that were directed against CD3, CD4 and CD25 were added to the cell suspension and incubated for 30 min on ice. The isolated milk cell suspension was centrifuged at 400 ***g*** for 8 min, and the labelled secondary mAbs were added. Finally, the isolated milk cells were incubated for 30 min on ice in the dark to allow the visualisation of cells expressing CD3, CD4 and CD25. Expression of CD25 was determined for gated T cells ($CD3^+$) and T $CD4^+$ lymphocytes ($CD3^+$ $CD4^+$) populations. Data are presented as median fluorescence intensity (MFI). For this assay, 10 000 gated T cells were examined per sample. FlowJo Tree Star Software (TreeStar Inc., Ashland, OR, USA) was used to analyse the data.

Identification of neutrophils

Milk neutrophils were differentiated from other cells by indirect fluorescence labelling. The cells were incubated with an unlabelled primary anti-bovine granulocyte mAbs (Table 1) for 30 min on ice. Next, 1 ml of PBS was added to the cell suspension, which was centrifuged at 400 ***g*** for 8 min. Finally, a labelled secondary mAb (Table 1) was added, and the sample was incubated for 30 min on ice in the dark to visualise the bound of CH138A. The neutrophils were identified using flow cytometry based on the cells' cytoplasmic granularities and CH138A positivity as previously described by Piepers et al. (2009) and Blagitz et al. (2013) (Fig. 1). A total of 20 000 milk cells, excluding cellular debris, were examined per sample. FlowJo software (TreeStar Inc., Ashland, OR, USA) was used to analyse the data. The results were corrected for autofluorescence, which was defined as the fluorescence that was associated with non-labelled freshly isolated milk cells from the same cow.

Identification of macrophages/monocytes

CD14 has long be used to identify macrophages/monocytes, however, milk neutrophils can also express CD14 (Paape et al. 1996; Sladek et al. 2002). Thus, the macrophages/monocytes were identified using flow cytometry based on CD14 positivity and CH138 negativity ($CD14^+/CH138^-$). The cells were washed with PBS and incubated with an unlabelled primary anti-bovine CH138A and CD14 mAbs (Table 1) for 30 min on ice. Next, 1 ml of PBS was added to the cell suspension, which was centrifuged at 400 ***g*** for 8 min. Finally, a labelled secondary mAbs (Table 1) was added, and the sample was incubated for 30 min on ice in the dark to visualise the bound of CD14 and CH138A. A total of 20 000 milk cells, excluding cellular debris, were examined per sample. FlowJo software (TreeStar Inc., Ashland, OR, USA) was used to analyse the data. The results were corrected for autofluorescence, which was defined as the fluorescence that was associated with non-labelled freshly isolated milk cells from the same cow.

Detection of apoptosis using flow cytometry

The death of neutrophils ($CH138^+$) was assessed using dual-labelling with an annexin V antibody and propidium iodide (PI) using a commercial kit (cat. n. K2350, APOPTEST-FITC™, Dako Cytomation, Mijdrecht, The Netherlands). Flow cytometric analysis was performed as previously described (Piepers et al. 2009; Blagitz et al. 2013). Briefly, 2×10^5 viable milk cells were suspended in 100 µl of

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Table 1. Monoclonal antibodies used for immunophenotyping bovine milk leucocytes by flow cytometry

Description	Primary antibody							Secondary antibody						
	Name	Type	Amount (μl)	Specificity	Host	Company	Isotype	Name	Type	Amount (μl)	Specificity	Host	Company	Isotype
Polymorphonuclear	CH138A	CH138A	1	Bovine	Mouse	VMRD†	IgM	M31504	IgM – PE	1	Mouse	Goat	Invitrogen‡	IgM
								M31505	IgM – APC	2	Mouse	Goat	Invitrogen‡	IgM
Macrophages / Monocytes	MM61A	CD14	1	Bovine	Mouse	VMRD†	IgG1	A10541	IgG1 – APC	1	Mouse	Goat	Invitrogen‡	IgG1
One of the endothelial-selectin ligands	BAG40A	CD44	1	Bovine	Mouse	VMRD†	IgG3	M32701	IgG3 – FITC	2	Mouse	Goat	Invitrogen‡	IgG3
β_2-integrin	MM12A	CD11b	1	Bovine	Mouse	VMRD†	IgG1	M32018	IgG1 – PE-Cy5	1	Mouse	Goat	Invitrogen‡	IgG1
T Lymphocyte	MM1A	CD3	1	Bovine	Mouse	VMRD†	IgG1	M32018	IgG1 – PE-Cy5	1	Mouse	Goat	Invitrogen‡	IgG1
CD4 T Lymphocyte	ILA11	CD4	1	Bovine	Mouse	VMRD†	IgG2a	M32204	IgG2a – PE	1	Mouse	Goat	Invitrogen‡	IgG2a
CD8 T Lymphocyte	BAQ111A	CD8	1	Bovine	Mouse	VMRD†	IgM	M31501	IgM – FITC	1	Mouse	Goat	Invitrogen‡	IgM
α-chain of IL-2 receptor	LCTB2A	CD25	1	Bovine	Mouse	VMRD†	IgG3	M32701	IgG3 – FITC	2	Mouse	Goat	Invitrogen‡	IgG3
B Lymphocyte	BAQ15A	CD21	1	Bovine	Mouse	VMRD†	IgM	M31501	IgM – FITC	1	Mouse	Goat	Invitrogen‡	IgM
L-selectin	MCA1649F	CD62L With FITC marker§	2	Mouse	Goat	AbD Serotec§								

PE:R-Phycoerythrin; APC: Allophycocyanin; FITC: fluorescein isothiocyanate; PE-Cy5: Phycoerythrin cyanine 5
†VMRD Pullman Inc. Corp®, Pullman, WA, USA
‡Invitrogen, Carlsbad, CA, USA
§AbD Serotec, Oxford, England

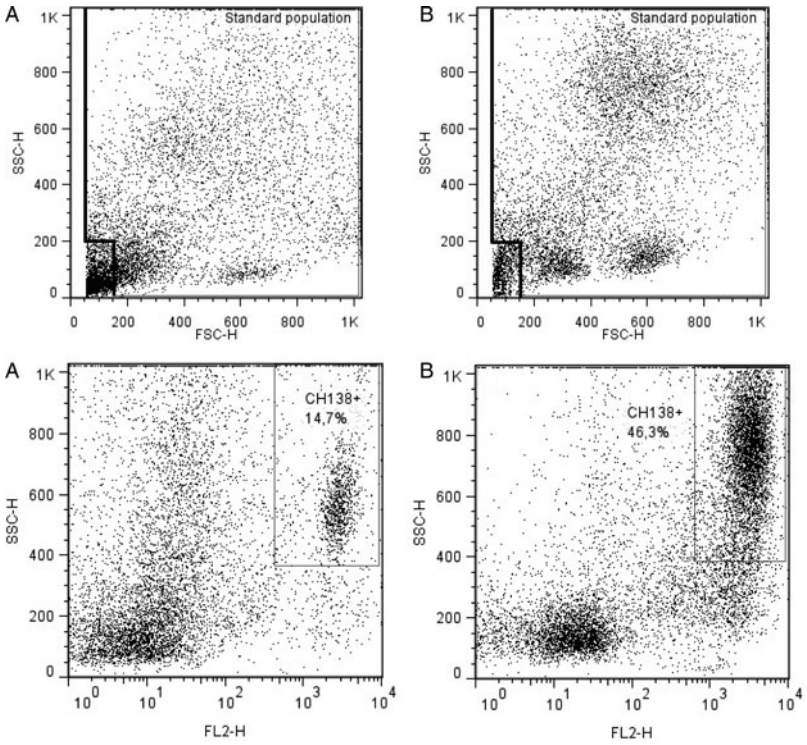

Fig. 1. Flow cytometric identification of the milk polymorphonuclear leucocytes (neutrophils) isolated from a representative uninfected (A) and *S. dysgalactiae*-infected (B) quarters. Recordings of scatter (SSC-H) and fluorescent properties (FL2-H; CH138A-Phycoerythrin) were performed on 20 000 events gated in a standard population excluding most of cell debris.

binding buffer (10 mM HEPES, 150 mM NaCl, 1 mM $MgCl_2$ and 1·8 mM $CaCl_2$) containing an anti-annexin V-FITC antibody and incubated on ice for 20 min in the dark. Immediately before flow cytometric analysis, 5 µl of a 250 µg/ml PI solution was added (final concentration 2·5 µg/ml). Next, the neutrophils were labelled using mAbs as described above.

To analyse the data, scatterplots were generated for the gated neutrophils. The living, non-apoptotic cells were negative for both FITC-labelled anti-annexin V and PI. The cells that were positive for FITC-labelled anti-annexin V but negative for PI were classified as apoptotic cells. The necrotic and late apoptotic cells were positive for both FITC-labelled anti-annexin V and PI (Piepers et al. 2009; Blagitz et al. 2013). A total of 20 000 milk cells, excluding cellular debris, was examined per sample. FlowJo software (TreeStar Inc., Ashland, OR, USA) was used to analyse the data.

Intracellular reactive oxygen species production

Intracellular reactive oxygen species (ROS) production was assessed with flow cytometry using 2′,7′-dichlorofluorescein diacetate (DCFH-DA) as a probe (Hasui et al. 1989). Briefly, 2×10^5 viable milk cells from each quarter, which were previously assessed using trypan blue exclusion, were incubated at 37 °C for 30 min with 0·3 µM DCFH-DA (D6883, Sigma Aldrich, St. Louis, MO, USA).

The intracellular 2′,7′-dichlorofluorescein (DCF) fluorescence of the neutrophils ($CH138^+$) was determined with flow cytometry using an excitation wavelength of 488 nm. DCFH-DA, which is a cell-permeable, nonfluorescent probe, is converted to DCF by ROS in a dose-dependent manner, resulting in fluorescence emission. The green fluorescence of DCF was detected at 500–530 nm.

The percentage of neutrophils producing ROS was calculated as the number of fluorescent neutrophils divided by the total neutrophil count and multiplied by 100. The MFI of ROS production was estimated from the median of DCF fluorescence (Blagitz et al. 2015; Della Libera et al. 2015). For this assay, 10 000 gated neutrophils were examined per sample. FlowJo software (TreeStar Inc., Ashland, USA) was used to analyse the data. The results were corrected for autofluorescence, which was defined as the fluorescence that was associated with non-labelled freshly isolated milk cells from the same cow.

Preparation of PI-labelled bacteria

PI-labelled *Staphylococcus aureus* (ATCC 25923) was prepared as described by Hasui et al. (1989) with some modifications for the detection of phagocytosis of *S. aureus* by milk neutrophils. Briefly, *S. aureus* was cultured for 18 h at 37 °C on brain-heart infusion agar. Subsequently, the bacteria were heat-killed using incubation at 60 °C for 30 min,

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after which they were washed three times using a sterile saline solution (0·9% NaCl). The bacterial density was adjusted to an absorbance of 2·50 at 620 nm, yielding approximately $2{\cdot}4 \times 10^9$ bacteria/ml, as previously described (Hasui et al. 1989). The bacteria were then labelled using a 5% PI (P4170, Sigma Aldrich, St. Louis, MO, USA) solution for 30 min on ice. The fluorescent bacteria were washed three times and suspended in PBS containing 5 mM glucose and 0·1% gelatin, and aliquots were stored at −80 °C. Thereafter, the PI-labelling of the bacteria was confirmed using flow cytometry.

Phagocytosis assay

The phagocytosis assay was performed using flow cytometry of PI-labelled *S. aureus*. Briefly, 2×10^5 viable milk cells were incubated with 100 µl of heat-killed, PI-labelled *S. aureus* and 900 µl of PBS for 30 min at 37 °C. Subsequently, 2 ml of 3 mM EDTA was added, and after centrifugation at 400 ***g*** for 10 min, the leucocytes were resuspended in 300 µl of PBS and analysed using flow cytometry.

The percentage of neutrophils ($CH138^+$) that phagocytised the PI-labelled bacteria was equal to the number of fluorescent neutrophils divided by the total neutrophil count and multiplied by 100. The MFI of *S. aureus* phagocytosis was estimated from the median value of PI fluorescence (Blagitz et al. 2013, 2015; Della Libera et al. 2015). At least 20 000 cells were examined per sample. FlowJo Tree Star Software (TreeStar Inc., Ashland, OR, USA) was used to analyse the data.

Expression of L-selectin, β_2-integrin and CD44

The identification of neutrophils expressing L-selectin (CD62L), the β-chain of β_2-integrin (CD11b) and one of the three endothelial-selectin ligands (CD44) was performed with flow cytometry using the mAbs listed in Table 1. Unlabelled primary mAbs that were directed against CH138A, CD11b and CD44 were added to the cell suspension and incubated for 30 min on ice. The isolated milk cell suspension was centrifuged at 400 ***g*** for 8 min, and a CD62L mAb and secondary mAbs for the detection of the anti-CH138A, anti-CD11b and -CD44 mAbs were added. Finally, the isolated milk cells were incubated for 30 min on ice in the dark to allow the visualisation of cells expressing CD62L, CD11b and CD44. Expression of CD11b, CD44 and CD62L was determined for gated neutrophils ($CH138A^+$). We chose the relative MFI for analysis of adhesion molecules because this parameter was much more discriminating compared with the percentage of positive cells. The MFI provides an accurate measurement of the brightness of the stained cells and is thus an indicator of the number of receptors per cell (Diez-Fraile et al. 2003). For this assay, 10 000 gated neutrophils were examined per sample. FlowJo Tree Star Software (TreeStar Inc., Ashland, OR, USA) was used to analyse the data.

Statistical analyses

Descriptive statistics (mean ± SE) were initially calculated for all variables. To account for the nested nature of the data (quarters and cows nested within the cows), multilevel analyses were conducted using the Generalised Linear Latent and Mixed Models (GLLAMM) procedure. Multilevel models are the most appropriate technique for analysing nested data that are not independent of each other (Rabe-Hesketh & Skrondal, 2008). The potential moderating effect of number of days in milk and parity on observed associations was estimated in the model by including appropriate interactions terms. A two-tailed probability level of 0·05 was adopted. The statistical analyses were performed using STATA statistical software version 12 (Stata Corp., College Station, Texas, USA).

Results

The logarithmic SCC and the proportion of neutrophils ($CH138^+$, Fig. 1) were higher in the quarters that were infected with *S. dysgalactiae* compared with the mammary glands that were free from infection (Table 2). On the other hand, the macrophages/monocytes were the major cell type in milk from healthy quarters (Table 3). From the 13 *S. dysgalactiae*-infected quarters that were included in the present study, only two quarters were from clinical cases of mastitis (presence of abnormal secretion in the strip cup test). Furthermore, *S. dysgalactiae*-infected cows have a higher number of days in milk (222·77 ± 25·32) and parity (3·77 ± 0·48) than uninfected ones (133·38 ± 22·84, $P = 0{\cdot}007$; 2·50 ± 0·31, $P = 0{\cdot}001$).

The results of this study revealed a tendency toward a lower percentage of annexin-V^+/PI^- neutrophils (apoptotic neutrophils) from *S. dysgalactiae*-infected mammary glands compared with the uninfected glands (Table 2). No significant difference was observed in the viable (annexin-V^-/PI^-) milk neutrophils, necrotic or late apoptotic (annexin-V^+/PI^+) milk neutrophils, the percentage of milk neutrophils that produced intracellular ROS or phagocytised *S. aureus*, the MFI of intracellular ROS production, the MFI phagocytosis of *S. aureus*, and the expression of CD44 and CD62L by the milk neutrophils (Table 2). By contrast, the expression of CD11b by milk neutrophils in the *S. dysgalactiae*-infected quarters was higher than uninfected quarters (Fig. 2; Table 2).

In addition, the *S. dysgalactiae*-infected quarters had higher percentages of milk T cells ($CD3^+$) (Fig. 3; Table 3) and their subset $CD3^+CD8^+CD4^-$. The expression of CD25 by T cells ($CD3^+$) and $CD4^+$ T cells did not differ between groups. No significant difference was observed in the percentage of milk B cells (Table 3).

Regarding the statistical model used here, it was possible to determine an effect of the number of days in the following variables: percentage of annexin V^+/PI^+ milk neutrophils ($P = 0{\cdot}038$), percentage of milk neutrophils that produced ROS ($P = 0{\cdot}048$), MFI of ROS production by milk neutrophils

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Table 2. SCC and function of milk neutrophils from uninfected and Streptococcus dysgalactiae infected quarters

Group/variable	Uninfected quarters ($n = 32$)	*S. dysgalactiae* ($n = 13$)	*P* (value)
SCC (Log)	$4{\cdot}15 \pm 0{\cdot}12^{b}$	$6{\cdot}22 \pm 0{\cdot}12^{a}$	0·0001
$CH138^{+}$ (%)	$11{\cdot}52 \pm 2{\cdot}70^{b}$	$31{\cdot}35 \pm 4{\cdot}50^{a}$	0·004
Annexin V^{-}/PI^{-} (%)	$30{\cdot}41 \pm 2{\cdot}57^{a}$	$35{\cdot}80 \pm 7{\cdot}45^{a}$	0·18
Annexin V^{+}/PI^{-} (%)	$43{\cdot}43 \pm 3{\cdot}34^{a}$	$32{\cdot}83 \pm 2{\cdot}70^{a}$	0·07
Annexin V^{+}/PI^{+} (%)	$21{\cdot}00 \pm 2{\cdot}42^{a}$	$22{\cdot}89 \pm 7{\cdot}12^{a}$	0·70
ROS production (%)	$59{\cdot}39 \pm 3{\cdot}52^{a}$	$64{\cdot}83 \pm 8{\cdot}06^{a}$	0·93
Intensity of ROS production (MFI)	$1840 \pm 179{\cdot}4^{a}$	$1484 \pm 308{\cdot}2^{a}$	0·69
S. aureus phagocytosis (%)	$60{\cdot}67 \pm 3{\cdot}33^{a}$	$62{\cdot}47 \pm 4{\cdot}34^{a}$	0·37
Intensity of *S. aureus* phagocytosis (MFI)	$201{\cdot}4 \pm 19{\cdot}97^{a}$	$113{\cdot}7 \pm 21{\cdot}06^{a}$	0·21
CD44 expression (MFI)	$11{\cdot}21 \pm 3{\cdot}23^{a}$	$7{\cdot}37 \pm 6{\cdot}28^{a}$	0·94
CD62L expression (MFI)	$9{\cdot}32 \pm 2{\cdot}54^{a}$	$1{\cdot}01 \pm 0{\cdot}00^{a}$	0·61
CD11b expression (MFI)	$597{\cdot}2 \pm 63{\cdot}94^{b}$	$846{\cdot}7 \pm 194{\cdot}79^{a}$	0·001

Different superscripted letters within a row indicate significant differences ($P \leqslant 0{\cdot}05$) between the values The results are shown as the mean ± SE
SCC: somatic cell count; PI: propidium iodide; *S. aureus: Staphylococcus aureus*; ROS: reactive oxygen species; MFI: median fluorescence intensity

Table 3. Percentage of milk macrophages/monocytes and lymphocyte subsets from uninfected and Streptococcus dysgalactiae infected quarters

Group/variables	Uninfected quarters ($n = 32$)	*S. dysgalactiae* ($n = 13$)	*P* (value)
$CD14^{+}/CH138^{-}$ (%)	$65{\cdot}72 \pm 2{\cdot}73$	$27{\cdot}59 \pm 4{\cdot}49$	0·0001
$CD3^{+}$ (T cells) (%)	$9{\cdot}05 \pm 1{\cdot}10^{b}$	$17{\cdot}34 \pm 3{\cdot}03^{a}$	0·006
$CD4^{+}/CD8^{-}$ T cells (%)	$1{\cdot}57 \pm 0{\cdot}26^{a}$	$3{\cdot}77 \pm 1{\cdot}25^{a}$	0·30
$CD4^{-}/CD8^{+}$ T cells (%)	$3{\cdot}70 \pm 0{\cdot}42^{b}$	$6{\cdot}98 \pm 1{\cdot}51^{a}$	0·0001
$CD4^{-}/CD8^{-}$ T cells (%)	$3{\cdot}44 \pm 0{\cdot}61^{a}$	$6{\cdot}16 \pm 1{\cdot}08^{a}$	0·31
$CD3^{+}$ (T cells) (%)	$6{\cdot}72 \pm 0{\cdot}75^{b}$	$14{\cdot}20 \pm 2{\cdot}75^{a}$	0·023
Expression CD25 by $CD3^{+}$ (T cells)	$27{\cdot}14 \pm 5{\cdot}57^{a}$	$33{\cdot}53 \pm 6{\cdot}68^{a}$	0·73
$CD4^{+}$ T cells (%)	$1{\cdot}97 \pm 0{\cdot}31^{a}$	$4{\cdot}14 \pm 1{\cdot}35^{a}$	0·43
Expression CD25 by $CD4^{+}$ T cells	$70{\cdot}27 \pm 19{\cdot}45^{a}$	$52{\cdot}71 \pm 14{\cdot}89^{a}$	0·12
$CD21^{+}$ (B cells) (%)	$10{\cdot}47 \pm 1{\cdot}45^{a}$	$16{\cdot}18 \pm 2{\cdot}97^{a}$	0·71

Different superscripted letters within a row indicate significant differences ($P \leqslant 0{\cdot}05$) between the values. The results are shown as the mean ± SE

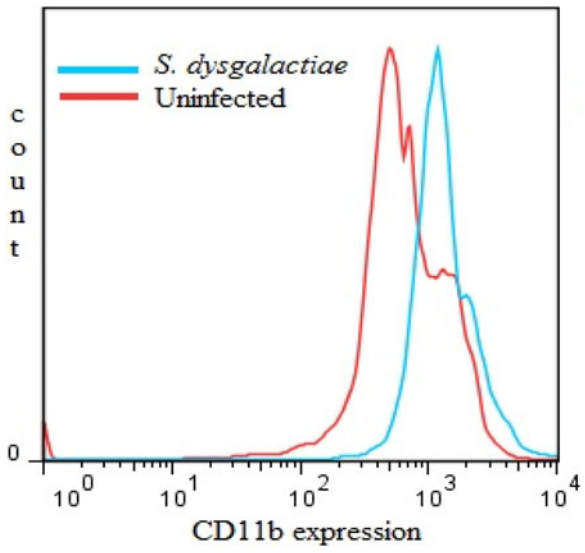

Fig. 2. Histogram overlays demonstrating CD11b expression on milk neutrophils. Histogram represents the log fluorescence (CD11b-Phycoerythrin-Cy5) of milk neutrophils ($CH138^{+}$).

($P = 0{\cdot}028$), percentage of milk neutrophils that phagocytised *S. aureus* ($P = 0{\cdot}063$), MFI of phagocytosis of *S. aureus* by milk neutrophils ($P = 0{\cdot}046$), expression of CD11b by milk neutrophils ($P = 0{\cdot}015$), percentage of milk monocytes/macrophages ($P = 0{\cdot}059$), and expression of CD25 by T ($P = 0{\cdot}007$) and T $CD4^{+}$ milk cells ($P = 0{\cdot}014$). Furthermore, an effect of parity on the following variables was observed: somatic cell count ($P = 0{\cdot}066$), percentage of milk neutrophils ($P = 0{\cdot}038$), percentage of annexin V^{+}/PI^{+} milk neutrophils ($P = 0{\cdot}0001$), percentage of milk monocytes/macrophages ($P = 0{\cdot}013$), and percentage of T $CD4^{+}$ milk cells ($P = 0{\cdot}007$).

Discussion

The mammary glands naturally infected with *S. dysgalactiae* had higher SCCs and neutrophil proportions in the present study, as previously described (Leitner et al. 2000; Djabri et al. 2002; Whist et al. 2007). This augmentation of milk SCC, mainly due to the increase of neutrophils might be at least partially due to the evocation of an eicosanoid response (prostaglandin $(PG)E_2$, $PGF_{2\alpha}$, and thromboxane B_2)

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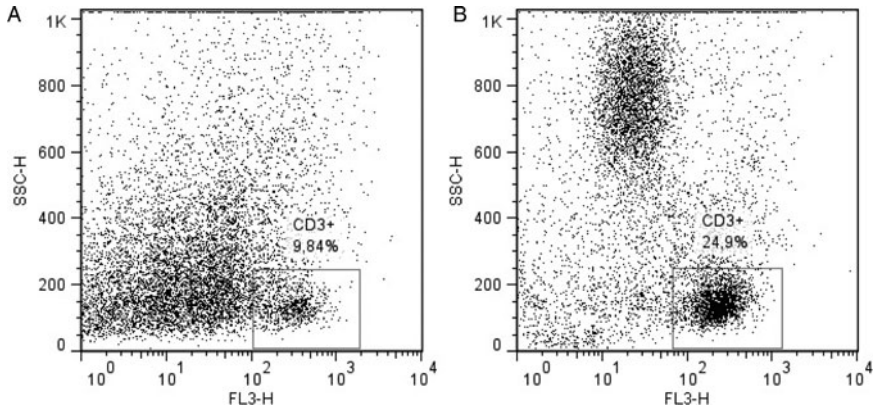

Fig. 3. Flow cytometric identification of the T lymphocytes ($CD3^+$) isolated from a representative uninfected (A) and *S. dysgalactiae*-infected (B) quarters. The recordings of scatter (SSC-H) and fluorescent properties (FL3-H; CD3-Phycoerythrin-Cy5) were performed on 20 000 events gated in a standard population excluding most of debris.

that leads to the recruitment of neutrophils in naturally infected mammary glands (Bannerman, 2009).

During mastitis, inflammatory chemoattractants guide neutrophils toward the foci of infection. The influx of neutrophils from the blood to the milk in the mammary gland is important for the defence of the mammary gland against bacterial infection (Paape et al. 2003; Rainard & Riollet, 2006). Several receptors on neutrophils participate in the migration into milk, including L-selectin, E-selectin and β_2-integrins. L-selectin plays a role in the first step of egress from the blood flow, allowing the initial, migratory rolling on the endothelium, which is a prerequisite for endothelial-dependent neutrophil arrest. Therefore, the expression of L-selectin on bovine neutrophils that migrate into tissues diminishes after activation and diapedesis (Diez-Fraille et al. 2004). Since these cells already down-regulated L-selectin during migration into the udder, little or no further down-regulation or shedding of L-selectin was observed in milk leucocytes isolated from cows with mastitis (Soltys & Quinn, 1999), as found here.

The β_2-integrins consist of three non-covalently linked heterodimers (CD11a,b,c/CD18), which are important cell surface adhesion molecules that are involved in neutrophil migration; the dominant β_2-integrin heterodimer that is expressed on bovine neutrophils is CD11b/CD18 (Smits et al. 2000; Paape et al. 2003; Rainard & Riollet, 2006). CD44 was identified as one of the three endothelial selectin (E-selectin) ligands on neutrophils responsible for slowing down and activating the rolling process. CD44 and two other E-selectin ligands (ESL-1 and PSGL-1) are required, but not essential, for neutrophil extravasation during inflammation (Gonen et al. 2008).

Here, the expression of CD62L and CD44 by milk neutrophils was not statistically significant. In that regard, Beecher et al. (2012) have demonstrated that the levels of IL-8 mRNA in *S. dysgalactiae* IMI, whose gene product enables leucocyte migration to the sites of infection, are not significantly different in milk somatic cells from uninfected control quarters *v.* infected quarters. Otherwise, a decreased transcription level of the β_2-integrin gene *cd18* was observed in milk somatic cells from experimental *S. dysgalactiae*-infected quarters at 7 h after challenge, and no difference was observed in the levels of *cd18* gene expression over the course of the infection. On the other hand, Soltys & Quinn (1999) reported that lymphocytes and neutrophils obtained from the milk of cows with mastitis exhibited significant up-regulation of β_2-integrins (CD18), consistent with an activated state due to the presence of bacterial pathogens, in agreement with our findings regarding the β_2-integrins (CD11b). Nagahata et al. (2011) found that the expression of L-selectin and CD18 molecules on neutrophils is higher in the milk from cows with *S. aureus* mastitis, although the expression of L-selectin in the milk was decreased compared to that in the blood. The reasons for these differences in phenotypic features between studies is unclear, though it may be associated with the difference in the virulence factors of these bacteria or the severity, extent and stage of the IMIs (Schukken et al. 2011).

The persistent accumulation of inflammatory cells at the inflammatory site requires both a continuous neutrophil influx and the increased survival of extravasated neutrophils (Boutet et al. 2004). The present study revealed a tendency toward a lower percentage of apoptotic neutrophils in the *S. dysgalactiae*-infected quarters; this finding confirms the results of Boutet et al. (2004), who described delayed neutrophil apoptosis in subclinical bovine mastitis.

It is noteworthy that neutrophils are the first line of defence against infection and that the active stage of immune defence requires viable, immune-competent cells (Baumert et al. 2009). In the present study, no significant difference in the viability rates was observed between the *S. dysgalactiae*-infected and uninfected quarters; moreover, there was no change in the percentage of neutrophils that produced intracellular ROS and phagocytised *S. aureus*.

Tassi et al. (2013), in an experimental infection by *S. uberis*, found that the proportion of $CD3^+$ lymphocytes

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was significantly elevated from 96 h post-challenge onwards, reaching its highest level at 312 h post-challenge. Regarding T cells subsets, these authors found that the proportion of $CD4^+$ and $CD8^+$ lymphocyte subsets increased 96 h post-challenge, with higher proportions of $CD4^+$ lymphocytes compared with $CD8^+$ lymphocytes present in the initial stages of infection. The proportion of $CD4^+$ cells stabilised after the initial increase, whereas the proportion of $CD8^+$ lymphocytes increased from 144 to 312 h post-challenge (the last sample collection). These data are in agreement with our findings, considering the higher proportion of milk T cells ($CD3^+$) and $CD8^+$ T lymphocytes in *S. dysgalactiae*-infected quarters found here. Furthermore, it appears that there is a selective recruitment of T-cell subsets to the udder during mastitis that depends on the pathogen (Soltys & Quinn, 1999).

The higher proportion of $CD8^+$ T lymphocytes found here may have implications in the mammary gland. For instance, Park et al. (1993) demonstrated that experimentally infected cows with *S. aureus* have higher number of activated $CD8^+$ T cells which are responsible for suppressing the proliferative response of milk $CD4^+$ T cells. Besides this, the preferential trafficking of $CD8^+$ suppressor lymphocytes into mammary gland tissues and secretion may be responsible for the lower responsiveness of local leucocytes compared with those from peripheral blood (Sordillo et al. 1997). Altogether, these data indicated that activation of $CD8^+$ T-cells during certain bacterial IMIs can suppress important host immune responses and predispose to the chronic pattern of the IMIs (Alnakip et al. 2014).

In milk from healthy mammary glands, the results of the percentage of each cell population are widely variable using flow cytometry, and consequently no consensus exists. Dosogne et al. (2003) and Schwarz et al. (2011) regarded lymphocytes as the predominant cell population in healthy mammary glands. In contrast, Leitner et al. (2000) and Leitner et al. (2003) showed that epithelial cells are the main cell type in milk from uninfected mammary glands. On the other hand, macrophages were also pointed out as the major cell type in milk from healthy mammary glands using flow cytometry (Koess & Hamann, 2008) or microscopy (Lee et al. 1980; Miller et al. 1991; Sarikaya et al. 2004, 2005; Merle et al. 2007), as found here.

Furthermore, the *S. dysgalactiae*-infected quarters used here came from cows with higher parity and days in milk than the uninfected cows, as previously described by Osteras et al. (2006).

Overall, the present study provided new insights into *S. dysgalactiae* IMIs, including distinct lymphocyte profiles, and a tendency toward an inhibition of apoptosis in milk neutrophils.

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