Immune response in nonspecific mastitis: What can it tell us?

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ABSTRACT

We analyzed a large number of immune response parameters from quarter milk samples with distinct bacteriological and quarter somatic cell count (qSCC) statuses. Furthermore, we sought to explore and identify displayed immune response patterns in milk samples from mammary glands with nonspecific mastitis. Thus, 92 quarter milk samples from 28 cows were stratified into 4 groups, as follows: (1) 49 culture-negative control quarters with a low qSCC ($<1 \times 10^5$ cells/mL) from 19 dairy cows (so-called healthy quarters); (2) 15 culture-negative quarters with high qSCC ($>2 \times 10^5$ cells/mL; so-called quarters with nonspecific mastitis) from 10 dairy cows; (3) 8 culture-positive quarters with low qSCC (noninflammatory quarters with low qSCC) from 5 dairy cows; and (4) 20 culture-positive quarters with high qSCC (so-called truly infected quarters) from 8 dairy cows. Using flow cytometry, we evaluated the percentage of milk neutrophils and their viability, intracellular reactive oxygen species production, phagocytosis, and the expression of CD62L, CD11b, and CD44 for each of the 4 quarter strata. Furthermore, the percentage of monocyte/macrophages, B cells, and T lymphocyte subsets were evaluated by flow cytometry. Milk samples from bacteriologically negative quarters (both with a low and elevated qSCC) had a lower qSCC than those with bacteriologically positive outcomes (both with a low and elevated qSCC). As expected, the healthy quarters showed the lowest percentage of neutrophils and also showed a higher percentage of

milk monocytes/macrophages and lower percentage of T lymphocytes than truly infected quarters. The most prominent result of the present study is that quarters with nonspecific mastitis showed the highest percentage of milk CD4⁺ T lymphocytes. The healthy quarters had a lower percentage of apoptotic neutrophils than noninflammatory and truly infected quarters, although it did not differ from those from the quarters with nonspecific mastitis. Our study supports the role of differential cell counting in the diagnosis of mastitis, as the milk leukocyte populations markedly fluctuate under healthy and inflammatory conditions. Furthermore, an increase in milk CD4⁺ T cells was associated with nonspecific mastitis, suggesting an increase in this leukocyte subpopulation is correlated with low bacterial shedding. Our study allows us to go further in our understanding of mammary gland immunity, providing further insights on potential protective mammary gland immunity, which we hypothesize can open new avenues for the development of novel targets that can promote bovine udder health.

Key words: mastitis, intramammary infection, differential leukocyte count, somatic cell count, dairy cow

INTRODUCTION

Mastitis is the costliest disease in dairy production with important public health and animal well-being concerns (Oliver et al., 2011; Stevens et al., 2016). Still, the definition of mastitis is not always consistent, and often used interchangeably with IMI. Intramammary infection requires the presence of a pathogen, although mastitis entails inflammation but not always infection of the mammary gland (Andersen et al., 2010). Thus, routine diagnosis of mastitis is based on the indicators of inflammation, and consequently the milk SCC has

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long been included as a component of the definition of mastitis (Pyörälä, 2003). Somatic cell count measures all somatic cells in milk; nevertheless, it does not distinguish the different cell populations present. Thus, it has been proposed that differential cell counting (DCC) could provide a more reliable inflammatory status of the mammary gland (Pyörälä, 2003; Koess and Hamann, 2008; Takano et al., 2018).

Culture of approximately 25 to 50% of all milk samples with high SCC does not yield bacterial growth (Makovec and Ruegg, 2003; Taponen et al., 2009; Souza et al., 2016). In the last few years, growing evidence is available that no-growth of mastitis pathogens from quarters with subclinical (Souza et al., 2016) and clinical mastitis (Pinzón-Sánchez and Ruegg, 2011) is associated with higher spontaneous bacteriological cure rates. In other words, in these cases, the milk somatic cells could be (more) effective in reducing the number of bacteria and could consequently reduce the likelihood of isolating the viable pathogen, suggesting the immune system is capable of overcoming the infection (Pinzón-Sánchez and Ruegg, 2011; Souza et al., 2016). We hypothesize that the likelihood of bacterial shedding correlates with the immune response of quarters within cows. Mapping the immune response by measuring specific immune parameters (i.e., by comparing parameters from samples with distinct bacteriological and quarter milk SCC statuses) will allow identification of potential immune parameters that are potentially correlated with a successful pathogen-host immune response. This could eventually reveal an immune response pattern that is able to improve existing mastitis prevention and control programs.

Furthermore, although milk leukocyte patterns in mammary glands with distinct bacteriological and quarter SCC (qSCC) statuses have been studied using microscopic DCC independent of the mastitis-causing pathogen (Pilla et al., 2012), we wanted to deepen our understanding of the immunological profile of quarters under healthy and inflammatory conditions. For instance, using flow cytometry, we provide detailed information on lymphocyte subsets and several PMN functions, including viability, reactive oxygen species (ROS) production, phagocytosis, and expression of adhesion molecules (i.e., β -chain of β_2 -integrin, one of the 3 endothelial-selectin ligands, and L-selectin). Thus, to the best of our knowledge, this study is the first to compare a large number of immune response parameters from quarter milk samples with distinct bacteriological and qSCC results independent of the mastitis-causing pathogen, when present.

Thus, in the present study we sought to explore and identify displayed immune response patterns specific for milk samples from mammary glands suffering from

nonspecific mastitis (i.e., milk samples with high qSCC and microbiological negative results). Furthermore, our study provided detailed immunological profile of quarters under healthy and inflammatory conditions.

MATERIALS AND METHODS

Samples and Sample Strata

Twenty-eight clinically healthy Holstein dairy cows at different lactation stages were enrolled from one commercial herd located at Tambaú city, Brazil. First, milk samples from 112 individual mammary quarters were aseptically collected for microbiological analysis as described by the National Mastitis Council (1999). Then, milk samples were collected in sterile plastic bottles by hand milking for the qSCC (40 mL) and flow cytometric analyses (1 L of milk). Samples were kept at 4°C until arriving at the laboratory.

Quarters with qSCC between $>1 \times 10^5$ and $<2 \times 10^5$ cells/mL were excluded ($n = 20$), as there are some variations in widely accepted qSCC thresholds (Schepers et al., 1997; Pyörälä, 2003; Bansal et al., 2005; Schwarz et al., 2010).

Based on bacteriological culture and qSCC results, the remaining 92 samples were stratified into 4 strata, as follows: (stratum 1) 49 culture-negative control quarters from 19 dairy cows with no abnormal secretions in the strip cup test and a qSCC $<1 \times 10^5$ cells/mL (so-called healthy quarters), using the threshold for qSCC described by Bansal et al. (2005) and Schwarz et al. (2010) in uninfected quarters; (stratum 2) 15 culture-negative quarters from 10 dairy cows with high qSCC ($>2 \times 10^5$ cells/mL; so-called nonspecific mastitis), as the threshold for qSCC proposed by Schepers et al. (1997) and Schukken et al. (2003); (stratum 3) 8 culture-positive quarters from 5 dairy cows with low qSCC ($\leq 1 \times 10^5$ cells/mL; so-called noninflammatory infected quarters); and finally (stratum 4) 20 culture-positive quarters from 8 dairy cows with high qSCC ($>2 \times 10^5$ cells/mL; so-called truly infected quarters with high qSCC).

Afterward, samples were randomized and codified, and the additional milk analyses were carried out without knowledge of the status of the quarter. This study complied with the Ethical Principles in Animal Research (Brazil, 2008) and was approved by the Bioethics Commission of the Faculty of Veterinary Medicine and Animal Science University of São Paulo.

Bacteriological Culture

The bacteriological culture was performed 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 observation of colony morphology, Gram staining, and biochemical testing (Oliver et al., 2004). A sample was considered culture-negative if no growth was observed (no colony from a 0.01-mL sample; <100 cfu/mL).

Determination of the SCC

For SCC measurement, milk samples were collected in tubes containing microtablets of bronopol (2-bromo-2-nitropane-1,3-diol) and were carried out using an automated somatic cell counter (Somacount 300, Bentley Instruments, Chaska, MN).

Separation of Milk Cells

For flow cytometric analysis, 1 L of milk from each mammary quarter was diluted with 1 L of PBS, and milk cells were separated as previously described by Koess and Hamann (2008) and Della Libera et al. (2015). The cells were placed in 1 mL of RPMI-1640 nutrition medium (R7638, Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Cultilab, Campinas, Brazil) and counted using a Neubauer chamber. The cell viability was first assessed using trypan blue exclusion. The number of cells per quadrant was similar during counting in the Neubauer chamber, which indicates the homogeneous distribution of milk cells. Afterward, we gently mixed the cell suspension before taking an aliquot for every assay to ensure the homogeneous distribution of cells. The milk cells were then diluted with the nutrition medium and 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 stained for CD21, CD3, CD4, and CD8 for 30 min at room temperature. The identification of lymphocytes subsets was based on the their cytoplasmatic granularity and mean fluorescence intensity following 2-step fluorescent immunolabeling with primary anti-bovine mAb and the secondary antibody coupled to the long-wavelength fluorescent probes (Table 1). After washing with PBS, the cells were incubated for 30 min at room temperature with the secondary antibodies. The cells were then washed with PBS and immediately analyzed by flow cytometry (FACSCalibur, BD Bioscience, San Jose, CA). Here, 20,000 milk cells, excluding most of the cell debris, were analyzed in each sample. An unstained control, secondary antibody control, and single stained milk samples were also prepared as compensation con-

trols. FlowJo software (TreeStar Inc., Ashland, OR) was used to examine the data.

Identification of Neutrophils and Monocytes/Macrophages

Milk neutrophils and monocyte/macrophages were differentiated from other cells by indirect fluorescent labeling by incubating for 30 min at room temperature with unlabeled primary mAb (Table 1). A gating strategy was used to differentiate granulocytes and monocytes (Figure 1) because CD14, although abundantly expressed on the surface of monocytes, can also be expressed to a lesser extent on bovine PMN (Ibeagha-Awemu et al., 2008; Piepers et al., 2009), as previously described (Blagitz et al., 2017). Next, 1 mL of PBS was added to the cell suspension and centrifuged at $400 \times g$ for 8 min. Finally, a labeled secondary antibodies (Table 1) were added and incubated for 30 min at room temperature in the dark to visualize CH138A and CD14 binding. An unstained control, secondary antibody control, and single stained milk samples were also prepared as compensation controls. The neutrophils were identified based on the cells' cytoplasmic granularities and CH138A positivity (Figure 1), as previously described by Piepers et al. (2009), Blagitz et al. (2013), and Blagitz et al. (2015a). A subpopulation of CH138A⁺ cells with low SSC-H property were excluded, representing nonspecific binding of CH138A⁺ to milk cells and cellular debris (Figure 1), as proposed by Piepers et al. (2009). The monocytes/macrophages were identified based on CD14 positivity after excluding CH138A⁺ cells (Figure 1). Here, 20,000 milk cells, excluding most of the cell debris, were analyzed in each sample. FlowJo software (TreeStar Inc.) was used to examine the data.

Detection of PMN Apoptosis by Flow Cytometry

The death of the neutrophils (CH138⁺) was measured by dual labeling with annexin V and propidium iodide (PI; K2350, APOPTEST-FITC, Dako Cytomation, Mijdrecht, the Netherlands) and flow cytometry analysis as previously described (Della Libera et al., 2015; Santos et al., 2017). The milk PMN were identified using mAb, as described above.

To analyze the data, scatterplots were generated for the gated PMN. The living, nonapoptotic cells were negative for the fluorescein isothiocyanate (FITC)-labeled annexin V and PI staining. The cells that were reactive with the FITC-labeled annexin V but negative for PI staining were classified as apoptotic cells (Della Libera et al., 2015). The necrotic subpopulation was positive for both FITC-labeled annexin V and PI staining.

Table 1. Monoclonal antibodies used for labeling and differentiation of bovine milk leukocytes in flow cytometric analysis^{1,2}

Item	Primary antibody				Secondary antibody									
	Name	Type	Amount (μL)	Specificity	Host	Company	Isotype	Name	Type	Amount (μL)	Specificity	Host	Company	Isotype
PMN	CH138A	CH138A	1	Bovine	Mouse	VMRD	IgM	M31504	IgM – PE	1	Mouse	Goat	Invitrogen	IgM
CD14	MM61A	CD14	1	Bovine	Mouse	VMRD	IgG ₁	M31505	IgM – APC	2	Mouse	Goat	Invitrogen	IgM
		CD44	1	Bovine	Mouse	VMRD	IgG ₃	A10541	IgG ₁ – APC	1	Mouse	Goat	Invitrogen	IgG1
One of the endothelial selectin ligands	BAG40A	CD44	1	Bovine	Mouse	VMRD	IgG ₃	M32701	IgG ₃ – FITC	2	Mouse	Goat	Invitrogen	IgG3
β ₂ -Integrin	MM12A	CD11b	1	Bovine	Mouse	VMRD	IgG ₁	M32018	IgG ₁ – PE-Cy5	1	Mouse	Goat	Invitrogen	IgG1
	MM1A	CD3	1	Bovine	Mouse	VMRD	IgG ₁	M32018	IgG ₁ – PE-Cy5	1	Mouse	Goat	Invitrogen	IgG1
T Lymphocyte	ILA11	CD4	1	Bovine	Mouse	VMRD	IgG _{2a}	M32204	IgG _{2a} – PE	1	Mouse	Goat	Invitrogen	IgG2a
CD4 T Lymphocyte	BAQ111A	CD8	1	Bovine	Mouse	VMRD	IgM	M31501	IgM – FITC	1	Mouse	Goat	Invitrogen	IgM
CD8 T Lymphocyte	BAQ15A	CD21	1	Bovine	Mouse	VMRD	IgM	M31501	IgM – FITC	1	Mouse	Goat	Invitrogen	IgM
B Lymphocyte	MCA1649F	CD62L with FITC ³	2	Mouse	Goat	AbD Serotec								

¹VMRD Pullman Inc. Corp., Pullman, WA; Invitrogen, Carlsbad, CA; AbD Serotec, Oxford, UK.²PE = R-phycoerythrin; APC = allophycocyanin; FITC = fluorescein isothiocyanate; PE-Cy5 = phycoerythrin cyanine 5.

Figure 1. Successive gating scheme for identifying milk PMN (CH138A^+ cells) and monocytes/macrophages ($\text{CH138A}^-/\text{CD14}^+$). Recordings of scatter angle light scatter (SSC-H) versus fluorescent properties (FL1-H; CH138A-fluorescein isothiocyanate) were performed on a gated standard population excluding most debris (A), which allowed us to identify PMN based on their cytoplasmic granularity and mean fluorescence intensity following a 2-step fluorescent immunolabeling protocol using primary anti-bovine monoclonal antibody specific for PMN identification (CH138A) and secondary antibodies coupled to long-wavelength fluorescent probe (FL1-H; B). At this moment, we also excluded PMN by gating the CH138A^- population (B). A subpopulation of CH138A^+ cells with low SSC were excluded representing nonspecific binding of CH138A^+ to cellular debris and milk cells (NB). Subsequently, the monocytes/macrophages were identified within the CH138A^- population based on mean fluorescence intensity following a 2-step fluorescent immunolabeling protocol using primary anti-bovine monoclonal antibody specific for monocyte/macrophage identification (CD14) and secondary antibodies coupled to long-wavelength fluorescent probe (FL4-H; allophycocyanin).

Intracellular ROS Production

Intracellular ROS production was evaluated by flow cytometry using 2',7'-dichlorofluorescein diacetate (D6883, Sigma-Aldrich) as a probe (Hasui et al., 1989). An unstained control and single stained samples were also prepared as compensation controls. For this assay, 10,000 gated PMN were analyzed in each milk sample. FlowJo software (TreeStar Inc.) was used to examine the data.

Preparation of PI-Labeled Bacteria

The preparation of PI-labeled *Staphylococcus aureus* (ATCC 25923) was performed as proposed by Hasui et al. (1989), with some modifications described by Blagitz et al. (2013, 2015a,b).

Phagocytosis Assay

The unopsonized phagocytosis assay was carried out by flow cytometry using PI-labeled *Staphylococcus aureus*, as previously described by Hasui et al. (1989). Briefly, 2×10^5 viable milk cells were incubated with 100 μL of heat-killed, PI-labeled *S. aureus* for 30 min at 37°C . Thereafter, 2 mL of 3 mM EDTA was added to substantially decrease the number of adherent bacteria to the leukocyte membrane that could be mistakenly be recognized as ingested (Batista et al., 2018), and after centrifugation at $400 \times g$ for 10 min at 4°C , the leukocytes were resuspended in 300 μL of PBS and examined by flow cytometry (FACSCalibur, BD Bioscience, San Jose, CA). An unstained control and single stained samples were also prepared as compensation controls.

At least 20,000 cells were analyzed in each sample of milk. The FlowJo TreeStar software (TreeStar Inc.) was used to examine the data.

Expression of L-Selectin, β_2 -Integrin, and CD44

The identification of PMN expressing β -chain of β_2 -integrin (CD11b), one of the 3 endothelial-selectin (E-selectin) ligands (CD44), and L-selectin (CD62L) was carried out by flow cytometry (FACSCalibur, BD Bioscience) as previously described (Della Libera et al., 2015). An unstained control, secondary antibody control, and single stained samples were also prepared as compensation controls. For this assay, 10,000 gated neutrophils cells were analyzed in each sample. The FlowJo TreeStar software (TreeStar Inc.) was used to examine the data.

Statistical Methods

First, the Kolmogorov-Smirnov test was performed to evaluate the normality and homoscedasticity of the different outcome parameters. Then, those data that did not present normality or homoscedasticity were submitted to base-10 logarithmic (SCC; percentage of T and B cells; percentage of apoptotic, viable and necrotic PMN; percentage and intensity of ROS production by PMN; intensity of phagocytosis by PMN, percentage and expression of CD44 by PMN, and expression of CD11b by PMN) or angular transformation (percentage of $\text{CD4}^-\text{CD8}^+$, $\text{CD4}^+\text{CD8}^-$, and $\text{CD4}^-\text{CD8}^-$ T cells) according to the type of response. Subsequently, the data were analyzed using a multivariate ANOVA.

The ANOVA test for comparison of the means used was the Duncan test. As transformation of the percentage of CH138A⁺ cells and the percentage of CD44⁺ by PMN were not possible to get normally distributed data, the Kruskal-Wallis test was applied. The model was carried out with cow as a random effect to take into account potential clustering of quarters within cows. The statistical analysis were performed using the InfoStat statistical software version 2018 (Di Rienzo et al., 2018). The mean and standard error of mean of the original variable were retained. The mean and the standard error of the original variable were retained to improve the interpretation of the results. The significance was set at $P \leq 0.05$.

RESULTS

SCC and Bacteriological Analysis

The results of the logarithm milk qSCC are presented in Figure 2. Milk samples from bacteriologically negative quarters (both with a low and elevated qSCC) had a lower qSCC than those with bacteriologically positive outcomes (both with a low and elevated qSCC). *Corynebacterium bovis* were isolated from all noninflammatory infected quarters (stratum 3). *Streptococcus dysgalactiae* and *C. bovis* were isolated from 55% ($n = 11$) and 45% ($n = 9$), respectively, of the truly infected quarters (stratum 4).

Figure 2. Logarithm quarter milk somatic cell count (qSCC) from healthy quarters (bacteriologically negative with low qSCC; stratum 1), nonspecific mastitis (bacteriologically negative with high qSCC; stratum 2), and noninflammatory (bacteriologically positive with low qSCC; stratum 3) and truly (bacteriologically positive with high qSCC; stratum 4) infected quarters. Different letters indicate $P \leq 0.0001$. Results are reported as mean $\pm$ SEM.

Percentage of Milk PMN, Monocyte/Macrophage, and Lymphocyte Subsets

Overall, the results of the percentage of the distinct milk leukocyte subpopulations are summarized in Table 2, and their fluctuation in comparison with healthy mammary glands is shown in Figure 3. The healthy quarters (stratum 1) presented the lowest percentage of PMN and T lymphocytes (CD3⁺), in contrast to the highest percentage of milk monocytes/macrophages. The percentage of monocytes/macrophages was also higher in noninflammatory infected quarters (stratum 3) than truly infected quarters (stratum 4). The percentage of B cells was not statistically related to qSCC and bacteriological statuses. The percentage CD8⁺ T lymphocytes was highest in truly infected quarters, followed by the nonspecific mastitis (stratum 2) and noninflammatory infected quarters, in contrast to the healthy quarters, which showed the lowest level. The percentage of CD4⁺ T lymphocytes was the only observation with a difference in the percentage between strata 2 and the other strata. Furthermore, there is a tendency toward a higher CD4:CD8 ratio in quarters with nonspecific mastitis in comparison to healthy quarters ($P = 0.06$). The percentage of CD4⁺CD8⁺ T lymphocytes was highest in truly infected quarters, followed by quarters with nonspecific mastitis and healthy quarters, in contrast to the noninflammatory quarters, which presented the lowest level.

Milk PMN Functions

The results of function of milk PMN are summarized in Table 3. The healthy quarters also had a lower percentage of apoptotic PMN than bacteriologically positive quarters (strata 3 and 4), although it did not differ from those from nonspecific mastitis. There was no relation between bacteriological and qSCC statuses and phagocytosis, intracellular ROS production, and expression of adhesion molecules by milk PMN.

DISCUSSION

Differential cell counts can be obtained by light microscopy and flow cytometry. Nonetheless, the accuracy of flow cytometry is related to the difficulty in differentiating cell populations by light microscopy and the much higher number of cells per sample that are analyzed by flow cytometry (Koess and Hamann, 2008; Pilla et al., 2013; Takano et al., 2018). Still, even using flow cytometry, the mAb used can affect the DCC outcomes. We accurately identified PMN, monocyte/macrophage, and lymphocyte subsets, whereas others evaluating milk DCC by flow cytometry did not use

Table 2. Percentage of milk PMN, monocyte/macrophage and lymphocyte subsets from healthy quarters [bacteriologically negative with low quarter milk SCC (qSCC); stratum 1], nonspecific mastitis (bacteriologically negative with high qSCC; stratum 2), and noninflammatory (bacteriologically positive with low qSCC; stratum 3) and truly (bacteriologically positive with high qSCC; stratum 4) infected quarters¹

Item	Negative		Positive	
	Stratum 1	Stratum 2	Stratum 3	Stratum 4
qSCC	Low ($\leq 1 \times 10^5$ cells/mL)	High ($> 2 \times 10^5$ cells/mL)	Low ($\leq 1 \times 10^5$ cells/mL)	High ($> 2 \times 10^5$ cells/mL)
% CH138A ⁺ (PMN)	9.41 $\pm$ 1.79 ^b	24.06 $\pm$ 4.88 ^a	25.78 $\pm$ 5.30 ^a	30.84 $\pm$ 3.30 ^a
% CD14 ⁺ /CH138A ⁻ (monocytes/macrophages)	51.96 $\pm$ 3.00 ^a	26.55 $\pm$ 5.16 ^{bc}	39.38 $\pm$ 4.30 ^{ab}	11.04 $\pm$ 1.66 ^c
% CD21 ⁺ (B lymphocytes)	11.64 $\pm$ 1.72 ^a	18.97 $\pm$ 2.31 ^a	10.09 $\pm$ 2.57 ^a	11.98 $\pm$ 1.24 ^a
% CD3 ⁺ (T lymphocytes)	9.00 $\pm$ 0.83 ^b	13.65 $\pm$ 2.80 ^{ab}	10.04 $\pm$ 1.41 ^{ab}	19.54 $\pm$ 2.00 ^a
% CD3 ⁺ CD4 ⁻ CD8 ⁺ (CD8 ⁺ T lymphocytes)	3.79 $\pm$ 0.42 ^c	5.66 $\pm$ 1.36 ^b	5.57 $\pm$ 1.08 ^b	7.55 $\pm$ 1.05 ^a
% CD3 ⁺ CD8 ⁻ CD4 ⁺ (CD4 ⁺ T lymphocytes)	1.38 $\pm$ 0.20 ^b	3.85 $\pm$ 0.97 ^a	1.36 $\pm$ 0.31 ^b	2.55 $\pm$ 0.51 ^b
% CD3 ⁺ CD8 ⁻ CD4 ⁻ (CD4 ⁻ CD8 ⁻ T lymphocytes)	2.94 $\pm$ 0.40 ^b	3.20 $\pm$ 0.78 ^b	2.21 $\pm$ 0.79 ^c	5.97 $\pm$ 0.75 ^a
CD4:CD8 ratio	0.42 $\pm$ 0.04 ^{a*}	0.71 $\pm$ 0.11 ^{a*}	0.37 $\pm$ 0.12 ^a	0.66 $\pm$ 0.12 ^a

^{a-c}Different superscripts within a row indicate $P \leq 0.05$.

¹The results are shown as the mean $\pm$ SEM.

* $P = 0.06$.

CD14 and CH138A mAb in combination (Pillai et al., 2001; Rivas et al., 2001; Dosogne et al., 2003; Koess and Hamann, 2008; Schwarz et al., 2011; Pilla et al., 2013). Not doing so can lead erroneous identification of some PMN as monocyte/macrophages, considering that PMN can also express CD14 on their surface (Paape et al., 1996; Sladek et al., 2002; Ibeagha-Awemu et al., 2008). Even worse, some studies did not use a specific mAb to accurately differentiate and identify many milk cell types (Pillai et al., 2001; Dosogne et al., 2003). Other studies used CD11b mAb to identify PMN or to differentiate PMN (CD11b⁺/CD14⁻) and macrophages (CD11b⁻/CD14⁺; Koess and Hamann, 2008; Schwarz et al., 2011; Pilla et al., 2013) though lymphocytes and macrophages can also express CD11b (Christensen et al., 2001; Duan et al., 2016).

It is well known that the percentage of neutrophils can differentiate between bacteriological statuses of quarter milk samples, even in those with low qSCC (qSCC $\leq 1 \times 10^5$ cells/mL), and therefore should be regarded as of utmost importance for the identification of inflamed or infected quarters. Here, we observed that the percentage of milk PMN was lower in healthy quarters compared with the other strata. In contrast to our findings, Pilla et al. (2012) showed that the percentage of PMN from healthy quarters did not statistically differ from bacteriology positive quarters with a low qSCC.

Although there is no consensus on the main leukocyte population in healthy mammary quarters (Dosogne et

al., 2003; Schwarz et al., 2011; Pilla et al., 2012; Pilla et al., 2013; Blagitz et al., 2015a; Takano et al., 2018), our study strengthens the idea that milk monocytes/macrophages represent the major leukocyte population in healthy mammary gland, as previously proposed (Sarikaya et al., 2004, 2005; Merle et al., 2007; Damm et al., 2017; Gonçalves et al., 2017). In contrast to our results, Dosogne et al. (2003), Schwarz et al. (2011), and Pilla et al. (2012) described that lymphocytes represent the predominant cell population, which counterposes the higher percentage of monocyte/macrophages in healthy mammary glands and higher percentage of T cells (CD3⁺) and CD8⁺ T lymphocytes in the truly infected quarters from our study. In this regard, although previous studies have determined the percentage of lymphocytes in quarter milk samples with distinct SCC, our study allowed us to provide accurate information of distinct lymphocyte subpopulations.

In agreement with our results, T cells, especially CD8⁺ T cells, recruitment has been previously reported during IMI (Soltys and Quinn, 1999; Tassi et al., 2013). An increased number of $\gamma\delta$ T cells, mainly represented here by CD4⁻CD8⁻ T cells (Nieto Farias et al., 2018) in milk from cows with staphylococcal and streptococcal mastitis, has also been reported (Soltys and Quinn, 1999), which may explain the highest level of this lymphocyte subpopulation in truly infected quarters, mostly caused by streptococci. Therefore, it is difficult to compare the DCC of milk obtained by distinct methods because the type of material of the sample bottle

Group/ Leukocyte Population	Stratum 2	Stratum 3	Stratum 4
% CH138A ⁺ (PMNLs)	↑	↑	↑
% CD14 ⁺ /CH138A ⁻ (Monocytes/Macrophages)	↓	-	↓
% CD21 ⁺ (B lymphocytes)	-	-	-
% CD3 ⁺ (T Lymphocytes)	-	-	↑
% CD3 ⁺ CD4 ⁺ CD8 ⁺ (CD8 ⁺ T Lymphocytes)	↑	↑	↑↑
% CD3 ⁺ CD8 ⁻ CD4 ⁺ (CD4 ⁺ T Lymphocytes)	↑	-	-
% CD3 ⁺ CD8 ⁻ CD4 ⁻ (CD4 ⁺ CD8 ⁻ T Lymphocytes)	-	↓	↑

Figure 3. Fluctuations of each leukocyte population in quarters with nonspecific mastitis [bacteriologically negative with high quarter milk SCC (qSCC); stratum 2], and noninflammatory (bacteriologically positive with low qSCC; stratum 3) and truly (bacteriologically positive with high qSCC; stratum 4) infected quarters in comparison to healthy quarters (bacteriologically negative with low qSCC). (–) not affected; single up arrow = higher than healthy quarters; double up arrow = higher than all groups; down arrow = lower than healthy quarters.

and method of preparation, including centrifugation steps, could affect the leukocyte populations (Schröder and Hamann, 2005), beyond the effect of distinct milk fractions on somatic milk cell populations (Sarıkaya et al., 2005). Moreover, one should keep in mind that the immune response between quarters in cows is not an independent process (Blagitz et al., 2015b), and that

is the reason why interdependence of udder quarters within cows was considered in the statistical analysis.

The most prominent result of the present study is that the nonspecific mastitis was associated with a higher percentage of milk CD4⁺ T lymphocytes. While the study size is relatively limited, the higher percentage of CD4⁺ T lymphocytes associated with nonspecific masti-

Table 3. Milk PMN functions from healthy quarters [bacteriologically negative with low quarter milk SCC (qSCC); stratum 1], nonspecific mastitis (bacteriologically negative with high qSCC; stratum 2), and noninflammatory (bacteriologically positive with low qSCC; stratum 3) and truly (bacteriologically positive with high qSCC; stratum 4) infected quarters¹

Item	Negative		Positive	
	Stratum 1	Stratum 2	Stratum 3	Stratum 4
qSCC	Low ($\leq 1 \times 10^5$ cells/mL)	High ($> 2 \times 10^5$ cells/mL)	Low ($\leq 1 \times 10^5$ cells/mL)	High ($> 2 \times 10^5$ cells/mL)
Annexin ⁻ V ⁺ /PI ⁻ (%)	43.05 $\pm$ 2.54 ^a	31.83 $\pm$ 5.58 ^{ab}	21.05 $\pm$ 2.96 ^b	26.29 $\pm$ 2.97 ^b
Annexin ⁻ V ⁺ /PI ⁺ (%)	22.49 $\pm$ 1.92 ^a	24.36 $\pm$ 5.44 ^a	14.21 $\pm$ 2.44 ^a	21.97 $\pm$ 5.89 ^a
Annexin ⁻ V ⁻ /PI ⁻ (%)	29.39 $\pm$ 2.27 ^a	33.83 $\pm$ 5.73 ^a	55.00 $\pm$ 4.54 ^a	42.23 $\pm$ 6.11 ^a
ROS production (%)	59.48 $\pm$ 3.08 ^a	73.68 $\pm$ 6.13 ^a	84.29 $\pm$ 2.15 ^a	71.29 $\pm$ 5.96 ^a
Intensity of ROS production (GMFI)	1,689 $\pm$ 132.6 ^a	1,275 $\pm$ 217.9 ^a	1,637 $\pm$ 164.6 ^a	1,856 $\pm$ 270.4 ^a
Phagocytosis (%)	58.03 $\pm$ 2.69 ^a	60.27 $\pm$ 4.39 ^a	51.69 $\pm$ 4.52 ^a	60.58 $\pm$ 3.28 ^a
Intensity of phagocytosis (GMFI)	193.7 $\pm$ 13.70 ^a	125.1 $\pm$ 19.69 ^a	150.3 $\pm$ 21.17 ^a	118.3 $\pm$ 16.32 ^a
CD44 ⁺ (%)	36.33 $\pm$ 3.10 ^a	37.86 $\pm$ 5.02 ^a	61.31 $\pm$ 13.13 ^a	28.19 $\pm$ 3.28 ^a
CD44 expression (GMFI)	178.6 $\pm$ 29.27 ^a	167.4 $\pm$ 67.67 ^a	105.4 $\pm$ 36.19 ^a	115.8 $\pm$ 22.36 ^a
CD11b ⁺ (%)	87.97 $\pm$ 1.84 ^a	94.09 $\pm$ 2.82 ^a	95.71 $\pm$ 1.03 ^a	85.02 $\pm$ 5.19 ^a
CD11b expression (GMFI)	793.9 $\pm$ 59.28 ^a	1,132 $\pm$ 166.3 ^a	832.1 $\pm$ 141.6 ^a	1,015 $\pm$ 134.7 ^a
CD62L (%)	23.95 $\pm$ 3.38 ^a	15.15 $\pm$ 5.87 ^a	13.90 $\pm$ 4.14 ^a	12.10 $\pm$ 2.96 ^a
CD62L expression	91.24 $\pm$ 14.17 ^a	73.57 $\pm$ 16.70 ^a	41.21 $\pm$ 9.05 ^a	90.61 $\pm$ 22.98 ^a

^{a,b}Different superscripts within a row indicate $P \leq 0.05$.

¹The results are shown as the mean $\pm$ SEM. PI = propidium iodide; ROS = reactive oxygen species; GMFI = geometric mean fluorescence intensity.

tis, strengthening the role of milk CD4⁺ T lymphocytes for mammary gland immunity. We hypothesize that the increase in milk CD4⁺ T lymphocytes can present a key role in reducing the number of viable bacteria in the milk and can consequently decrease the likelihood of isolating the pathogen through milk culture, suggesting the capability of the immune system to overcome the infection. In fact, CD4⁺ T lymphocytes have been associated with clearance activity against several bacterial pathogens (Dunkley et al., 1994; Rivas et al., 2000). These cells can also capture bacteria efficiently from dendritic cells through a process termed transphagocytosis, allowing to kill internalized bacteria in lysosomes (Cruz-Adalia et al., 2017). Rainard et al. (2015) also showed that bovine CD4⁺ T lymphocytes can produce either IL-17A and IFN- γ . The IL-17A is associated with T_H17 immune response, which is implicated in neutrophil mobilization and immune response modulation and antigen-specific inflammation (Rainard et al., 2013). The T_H17 immune response also mediates the communication between the immune system and mammary epithelial cells, culminating in the upregulation of genes that encode antimicrobial peptides in bovine mammary epithelial cells, providing an alternative process for killing mastitis pathogens (Bougarn et al., 2011; Tassi et al., 2013). Beyond IL-17A, it is regarded that IFN- γ , which is associated with an T_H1 immune response, is essential for the control and elimination of infections by intracellular pathogens, and also plays an important role in protection against extracellular pathogens through its capacity to stimulate IgG₂ production. The recognition of antigens by specific antibodies, especially IgG₂, is important for the phagocytic capacity of milk neutrophils (Paape et al., 2003). In agreement with our results, Tassi et al. (2013) described that the resolution of IMI by *Streptococcus uberis* was associated with an increase in milk IL-17A levels that was preceded by a temporal increase in the levels of CD4⁺ T lymphocytes. Tassi et al. (2013) also showed that the IL-17A levels concurred with the inversion of the CD4⁺ to CD8⁺ ratio. In our study, there is also a tendency toward a higher CD4⁺ to CD8⁺ ratio in quarters with nonspecific mastitis compared with healthy quarters. Thus, our results suggest that an increase in milk CD4⁺ T cells may help in overcoming IMI in dairy cows, although further (longitudinal) studies are needed. Therefore, we believe that if we map the responses of animals that have mounted successful pathogen-host immune response, we may uncover potential novel targets that provide a basis for the development of alternative therapeutics (Souza et al., 2019).

Neutrophils have a shorter lifespan than other immune cells (Bassel and Caswell, 2018). Resolution of an inflammation entails the removal of PMN and their toxic

products from the site of infection by a process called apoptosis. Thus, apoptosis is a most sophisticated way of removing PMN at the site of infection, supporting the resolution of inflammation (Rainard and Riollet, 2006; Piepers et al., 2009). If this process fails, it results in a chronic, persistent inflammation; severe tissue damage; or both (Van Oostveldt et al., 2002; Rainard and Riollet, 2006; Piepers et al., 2009). In fact, the process of removing apoptotic neutrophils by macrophages termed efferocytosis produces anti-inflammatory cytokines and resolves inflammation (Bassel and Caswell, 2018). Regarding that, here we report that the qSCC of infected quarters with either low and high qSCC were statistically different from healthy mammary glands, which can be related to the inhibition of neutrophil apoptosis to survive and cause disease (Boutet et al., 2004; Sladek et al., 2005). In contrast, the behavior of apoptotic PMN from nonspecific mastitis was not statistically different from healthy mammary quarters, suggesting resolution of inflammation.

CONCLUSIONS

Our study strongly supports the crucial role of DCC in the diagnosis of mastitis, as monocytes/macrophages represent the major leukocyte type in healthy mammary glands, in contrast to an increase of PMN and T cells, especially CD8⁺ T cells, in truly infected mammary quarters. Furthermore, an increase in milk CD4⁺ T cells was associated with nonspecific mastitis, suggesting an increase in this leukocyte subpopulation is correlated with low bacterial shedding and an increased likelihood of resolution of the infection. Future longitudinal studies are required to assess the role of milk CD4⁺ lymphocytes as a predictive marker for bacteriological cure of IMI. Thus, studying quarters or cows that have mounted a successful pathogen-host immune response will improve our understanding of mammary gland immunity, potentially creating opportunities for the development of novel targets that could confer protective immunity against IMI.

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