

Journal of the Hellenic Veterinary Medical Society

Vol 73, No 2 (2022)



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doi: [10.12681/jhvms.26718](https://doi.org/10.12681/jhvms.26718)

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To cite this article:

Safak, T., & Risvanli, A. (2022). Udder defense system: Effect of milk SCC level on Th1/Th2 cytokine balance. *Journal of the Hellenic Veterinary Medical Society*, 73(2), 4135–4140. <https://doi.org/10.12681/jhvms.26718>

Udder defense system: Effect of milk somatic cell count level on Th1/Th2 cytokine balance

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ABSTRACT: The immune system of the cow is kept strong to protect the cows from mastitis, which causes economic losses for dairy cattle herds. Cellular immunity, especially, plays an important role as a first-line defense system. In the case of inflammation, cytokines play a decisive role in monitoring this process. In this study, the relationship between somatic cell count (SCC) and cytokine (tumor necrosis factor alpha, interferon gamma, and interleukins TNF- α , IFN- γ , IL-2, -4, -5, and -10, respectively) concentrations in milk were determined. It was decided to support the humoral and cellular aspects of the udder defense system by determining the T-helper cell (Th1/Th2) cytokine polarization in high SCC milk from cows with signs of mastitis. Milk samples from 180 cows were divided into five groups according to the somatic cell count level ($< 150 \times 10^3$, 151×10^3 - 400×10^3 , 401×10^3 - 600×10^3 , 601×10^3 - $1,000 \times 10^3$, and $> 1,000 \times 10^3$ cells/mL). It was understood that the Th1 direction was determined before mastitis was formed, that is when SCC was low ($< 150 \times 10^3$). As a result, it was concluded that the Th1 polarization should be supported to protect cows from mastitis.

Keywords: Bovine mastitis, Somatic cell count, Th1/Th2 cytokine

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Date of initial submission: 07-04-2021
Date of acceptance: 29-09-2021

INTRODUCTION

Mastitis is defined as a common and costly disease affecting dairy cattle worldwide. The mammary gland has various defense mechanisms, such as anatomical, cellular, and humoral, that act to protect it from mastitis. These structures help prevent the invasion of pathogens into the udder. Microorganisms that exceed the anatomical defense, which is the first line of defense, begin to invade and colonize in the udder. In the presence of pathogens, cellular defense, which forms the second line of defense, becomes active. Under these conditions, an increase in the somatic cell count (SCC) is observed in milk along with pathological changes in the udder (Rainard and Riollet, 2006; Alnakip, 2014; Sordillo, 2018).

The SCC in milk consists of epithelial cells, polymorphonuclear leukocytes, monocytes, and macrophages. In general, the SCC should be below 200,000 cells/mL in milk obtained from a healthy udder, but SCC increases after mastitis begins. Macrophages constitute the majority of SCC in a healthy cow's milk. After macrophages recognize bacteria, they secrete several chemicals that pull polymorphonuclear leukocytes from the vessels to the infection site. Also, polymorphonuclear leukocytes are attracted to the area by specific chemical substances produced by microorganisms. Since polymorphonuclear leukocytes will migrate to the milk in cases of mastitis, the majority (90%) of SCC in milk is composed of neutrophils, whose main task is to destroy and phagocytose microorganisms (Kelly et al., 2000; Fox, 2009; Akers and Nickerson, 2011).

The third line of defense, acquired immunity, is established by the lymphocytes. Lymphocytes are divided into two groups of T-lymphocytes (T- and B-cells). T- and B-cells originate from hematopoietic stem cells in the bone marrow (Abbas et al., 2015). When naive T cells encounter antigens (protein, polysaccharide, or a part of the pathogen), they differentiate into effector T-helper (CD4+) cells, a process that allows B-cells to transform into plasma cells and T-cytotoxic (CD8+) cells that kill pathogens (Dembic, 2015). T-cells in milk are divided into two as $\alpha\beta$ T- and $\gamma\delta$ T-cells containing CD4+ (T-helper) and CD8+ (T-cytotoxic) cells. The healthy mammary gland contains $\alpha\beta$ T lymphocyte cells, mostly CD8+ cells. In mastitis, the CD4+ phenotype is intense (Park et al., 2004).

CD4+ helper T-cells differentiate into different sub-cell types, such as Th1, Th2, Th17, and induced regulatory T (iTreg) that produce different types of

cytokines and therefore have different functions. Among the cytokines produced by Th1 cells are interferon (IFN)- γ , interleukin (IL)-2, tumor necrosis factor (TNF- α), and IL-1 β , while cytokines, such as IL-4, -5, -10, and -13 are produced by Th2 cells. While Th1 cells strengthen the cellular immune response as a result of the cytokines they secrete, Th2 cells activate the humoral immunity as a result of their cytokine secretion. In general, cytokines secreted from Th1 cells participate in cellular responses, while cytokines secreted from Th2 cells participate in humoral responses. Depending on the Th1 or Th2 cytokine group that is active, it should support and contribute to the inflammation process (Romagnani, 2000; Zhu and Paul, 2008; Zhang et al., 2011).

When previous publications were reviewed, studies in which the cytokines produced by Th1 and Th2 are investigated one-by-one or concurrently in cases in which mastitis develops could be found. In this study, cytokines were evaluated separately as cytokine groups produced from Th1 and Th2 lymphocytes. Thus, this study aimed to determine in which direction the Th1/Th2 cytokine balance progressed according to the SCC level and whether this balance shifted to humoral or cellular responses.

MATERIALS AND METHODS

Animal and Somatic Cell Count

For the study, the ethics committee approval was obtained from the Firat University Animal Experiments Local Ethics Committee (FU-2018/98). Milk samples were obtained from cows aged 3-5 years old that were milked twice daily from 180 multiparous cattle breeds (between parities two and three) on seven farms. SCC was performed with fresh milk samples using DeLaval Cell Counter® (Cellcounter DCC; DeLaval, Sweden) device by following the manufacturer's procedure. The raw milk was grouped according to their somatic cell count levels ($< 150 \times 10^3$, 151×10^3 - 400×10^3 , 401×10^3 - 600×10^3 , 601×10^3 - $1,000 \times 10^3$, and $> 1,000 \times 10^3$ cells/mL).

Cytokine Analysis

Milk samples were stored at -70°C until analysis of cytokines from Th1 (TNF- α , IFN- γ , and IL-2) and Th2 (IL-4, IL-5, and IL-10) cells. Species-specific commercial enzyme-linked immunosorbent assay (ELISA) kits (USCN Life Science Inc., Wuhan, China) were used to determine the levels of Th1 (TNF- α , IFN- γ , and IL-2) and Th2 (IL-4, IL-5, and

IL-10) cytokines in the milk samples. The application steps of the ELISA test were carried out as specified by the manufacturer in the booklet and methods in the literature (Can-Sahna and Risvanli, 2015). After all steps were completed, milk cytokine levels were determined by reading the plates at 450 nm in an ELISA reader (Bio Tek Instruments, USA)

Statistical Analysis

The normality distribution of the cytokines (TNF- α , IFN- γ , IL-2, -4, -5, and -10) and SCC were tested using the visual (histogram and probability graphs) and Kolmogorov-Smirnov test. It was determined whether the data showed a normal distribution or not. The data of Th1 (TNF- α , IFN- γ , and IL-2) and Th2 (IL-4, -5, and -10) to normality distribution was determined by visual (histogram and probability graphs) and analytical methods (Kolmogorov-Smirnov/Shapiro-Wilk). It was then determined whether or not the data showed a normal distribution. As a result of the evaluation of the cytokine data (TNF- α , IFN- γ , IL-2, -4, -5, and -10), it was determined that they did not meet the parametric test assumptions and showed a normal distribution. Therefore, the Kruskal-Wallis test, one of the nonparametric tests, was used for

inter-group comparisons. The Bonferroni corrected Mann-Whitney-U test was used for post-hoc group comparisons. A value of $P < 0.01$ was accepted as significant in the pairwise group comparisons.

The calculations and tests of the data were carried out with the SPSS 22.0 version (Statistical Package for the Social Sciences for Windows SPSS 22.0 Edition for Windows, Chicago, Illinois, USA) program.

RESULTS

TNF- α and IFN- γ concentrations were found to be statistically lower in milk samples with high SCC ($P < 0.01$). Milk IL-5 concentration was found to be statistically high in milk with high SCC ($P < 0.01$). No statistically significant differences between changes in milk SCC levels and milk IL-2, -4, and -10 concentrations were found ($P > 0.05$) as shown in Table 1.

Table 2 shows the change in Th1/Th2 polarization according to the SCC levels in the milk samples. As seen from Table 2, TNF- α and IFN-concentrations in milk samples with $SCC < 150 \times 10^3$ according to the milk SCC levels were found to be higher than the other groups. Milk Th1/Th2 polarization balance was found to lean toward the Th1 direction.

Table 1. Milk cytokine concentrations at different levels of somatic cell count (Mean \pm Standard error of the mean)

SCC ($\times 10^3$) (cells/mL)		Cytokines (pg/mL)					
		TNF- α	IFN- γ	IL-2	IL-4	IL-5	IL-10
<150 (n = 56)	$\bar{x} \pm s_{\bar{x}}$	302.75 \pm 27.22 ^b	64.34 \pm 8.77 ^b	8.56 \pm 0.40	385.21 \pm 42.44	7.76 \pm 0.26 ^a	8.96 \pm 0.31
	Median	240.00	62.50	7.66	267.50	6.90	8.45
151-400 (n = 51)	$\bar{x} \pm s_{\bar{x}}$	224.19 \pm 34.60 ^a	40.12 \pm 7.10 ^a	9.04 \pm 0.55	459.82 \pm 55.79	8.78 \pm 0.30 ^b	8.38 \pm 0.22
	Median	67.50	11.5	8.02	300.00	8.30	8.40
401-600 (n = 20)	$\bar{x} \pm s_{\bar{x}}$	167.90 \pm 51.78 ^a	49.83 \pm 28.27 ^a	9.12 \pm 0.64	570.30 \pm 91.57	9.30 \pm 0.65 ^b	8.31 \pm 0.25
	Median	28.95	10.10	80.08	477.50	8.25	8.75
601-1000 (n = 17)	$\bar{x} \pm s_{\bar{x}}$	282.17 \pm 67.52 ^{ab}	32.57 \pm 6.36 ^a	9.38 \pm 0.96	548.05 \pm 90.18	8.36 \pm 0.47 ^{ab}	8.47 \pm 0.45
	Median	165.00	31.20	7.72	350.00	8.40	8.00
>1000 (n = 36)	$\bar{x} \pm s_{\bar{x}}$	125.07 \pm 32.39 ^a	20.34 \pm 3.46 ^a	11.62 \pm 1.94	514.22 \pm 65.03	9.12 \pm 0.42 ^b	8.21 \pm 0.25
	Median	28.95	10.10	8.23	425.00	8.70	8.40
P		***	***	-	-	**	-

∴ $P > 0.05$, **: $P < 0.01$, ***: $P < 0.001$

^{a, b}: The difference between groups with different superscripts in the same column is statistically significant, ($P < 0.01$)

Table 2. Th1/Th2 polarization according to milk SCC levels

SCC ($\times 10^3$) (cells/mL)	Th1		Th2			
	TNF- α	IFN- γ	IL-2	IL-4	IL-5	IL-10
<150 (n = 56)	↑	↑	↔	↔	↓	↔
151- 400 (n = 51)	↓	↓	↔	↔	↑	↔
401- 600 (n = 20)	↓	↓	↔	↔	↑	↔
601-1000 (n = 17)	↔	↓	↔	↔	↔	↔
>1000 (n = 36)	↓	↓	↔	↔	↑	↔

↑ : High concentration, ↓ : Low concentration, ↔ : That the concentration does not change.

DISCUSSION

It is known that cytokines affect mastitis pathophysiology. Especially Th1 (IFN- γ , IL-1 β , -2, -8 and -12, TNF- α) and Th2 (IL-3, -6, -9, -10, -13, -25 and -31 and granulocyte-macrophage colony-stimulating factor [GM-CSF]) cytokine groups play an important role in inflammation. These cytokine groups increased or decreased according to the course of the infection. However, with respect to these changes, it is not expected that all cytokines belonging to the same group will increase or decrease at the same time. The results are interpreted according to the group with the majority of increasing or decreasing cytokines. Depending on which Th1 or Th2 cytokine groups are active, it should support and contribute to the inflammation process (Dembic, 2015; Sordillo, 2018).

It has been reported that levels of cytokine change due to the increase of SCC in milk in experimentally-induced and naturally occurring mastitis cases (Riollet et al., 2000; Riollet et al., 2001; Rambeaud et al., 2003; Bannerman, 2009). Lee et al. (2006) stated that the change in SCC is parallel to IL-8 and -12, but in the same study, no correlation could be established between SCC and TNF- α and IL-6 concentrations. In our study, a negative correlation between the increase of SCC and the concentration of TNF- α was found, while it was determined that IL-2, -4, and -10 concentrations were not affected by changes in SCC. It has been reported that there is an increase in TNF concentration in cases where mastitis occurs and SCC increases (Safak and Risvanli, 2021). It has also been reported that after 18 h of experimental infection, TNF- α and IL-8 concentrations began to decrease although SCC continued to increase (Riollet et al., 2000). Bannerman et al. (2004) could not establish a correlation between SCC change and IFN- γ and IL-12 and -10 concentrations. Unlike the study by Hisae-

da et al. (2001), in the present study, it was observed that as the SCC levels increased, the concentration of milk TNF- α and IFN decreased, while the concentration of IL-5 increased. The TNF- α concentration was higher in the *Escherichia coli* (*E. coli*) group than in the *Staphylococcus aureus* (*S. aureus*) and the *Streptococcus agalactiae* (*S. agalactiae*) groups (Safak et al., 2022).

Besides, according to the present study, the cytokine group (s) that was most active according to the SCC level was determined. Th1/Th2 polarization was found to lean toward the Th1 direction in the group with low milk SCC levels. In other words, it was determined that the Th1 direction was active in healthy milk. Applications, such as selenium, copper, zinc, vitamin E, vitamin A, D2 (Ergocalciferol), and D3 (Cholecalciferol), can be used to support the Th1 direction. Also, adjustments in nutrition to keep blood ketone levels low are among the practices that are used to support the Th1 direction (Hogan et al., 1990; Waller, 2000; Heinrichs et al., 2009; Lippolis et al., 2011). To support the Th2 direction, that is, humoral immunity, *Corynebacterium cutis* lysate applications (Saat et al., 2016), homeopathic drugs, including Healwell VT-6 and Dolisovet (Varshney and Naresh, 2005; Aubry et al., 2013) various peptides (Jeong et al., 2017), and vaccines that keep immunoglobulin levels high are recommended (Middleton et al., 2009).

As a result, in the present study, it was determined that the Th1/Th2 direction in healthy milk was active in the Th1 direction. Thus, it was understood that the cellular immunity was strong in the milk obtained from a healthy udder. It was concluded that supportive supplements in the direction of Th1 should be made to prevent mastitis, which causes huge economic losses in dairy cattle herds.

ACKNOWLEDGMENTS

This study was supported by the Scientific and Technological Research Council of Turkey [TUBITAK-119O787] and the Firat University Scientific Research Projects Unit [VF.19.07]. This manuscript

is part of the Ph.D. thesis of Tarık Şafak.

CONFLICT OF INTEREST

None declared by the authors.

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