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Original article

High milk neutrophil chemiluminescence limits the severity of bovine coliform mastitis

Jalil MEHRZAD[a,b,c], Luc DUCHATEAU[a], Christian BURVENICH[a]*

[a] Ghent University, Faculty of Veterinary Medicine, Department of physiology, Biochemistry and Biometrics, Salisburylaan 133, 9820 Merelbeke, Belgium
[b] McGill University, Department of Animal Science, Macdonald-Stewart Building 21, 111 Lakeshore Road, Ste. Anne de Bellevue, H9X 3V9, Quebec, Canada
[c] Dairy and Swine Research and Development Centre, Agriculture and Agri-Food Canada, PO Box 90, 2000 Route 108 East, Lennoxville, J1M 1Z3, Quebec, Canada

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Abstract – Polymorphonuclear neutrophil (PMN) function changes during mastitis. To investigate the contribution of milk PMN to the severity of *Escherichia coli* (*E. coli*) mastitis, chemiluminescence (CL) of blood and milk PMN and their efficiency to destroy coliform bacteria in the mammary gland were examined following the induction of *E. coli* mastitis in early lactating cows. To better assess and define the degree of mastitis severity, cows were classified as moderate and severe responders according to milk production loss in the non-infected quarters at post-infection hour (PIH) 48. There was an inverse relationship between pre-infection milk PMN CL and colony-forming units at PIH 6. In moderate cows, the pre-infection blood and milk PMN CL was ~ 2-fold higher than that of severe cows. The probability of severe response increased with decreasing pre-infection PMN CL. At the beginning of the infection blood and milk PMN CL was consistently higher, and milk PMN CL increased faster after infection in moderate cows. At PIH > 48 milk PMN CL in severe cows exceeded that of moderate cows. The somatic cell count (SCC) in moderate cows increased faster than colony-forming units, whereas in severe cows the results were reversed. The kinetics of CL activity for blood and milk PMN before and during the early phase of infection confirmed an impairment in PMN CL activity for severe responding cows. High pre-infection blood and milk PMN CL and the immediate increase of milk PMN CL and SCC after infection limited bacterial growth thereby facilitating the recovery of *E. coli* mastitis in moderate cows. Our study strengthens the idea that pre-existing milk PMN (a static part of the udder's immune defense) functions as a "cellular antibiotic" before and during infection, and low milk PMN CL is a risk factor for bovine coliform mastitis.

bovine / chemiluminescence / mastitis / neutrophil / severity

1. INTRODUCTION

The most immediate and effective response to *E. coli* mastitis is the massive recruitment of polymorphonuclear neutrophils (PMN) through the blood-milk barrier and increased myelopoiesis [7]. This represents the mobilized or inflammatory (dynamic) immune defense of the mammary gland. It remains unknown if resident PMN, that arrived in milk by non inflammatory stimuli, may act as a static part of the innate immune defense

* Corresponding author: christian.burvenich@ugent.be

as described for several soluble factors, such as lactoferrin in dry secretions. Although several antimicrobial systems exist in the bovine mammary gland [21, 29], it is likely that the presence of PMN in milk might provide a central natural defense for the gland [6, 30]. Ingestion of bacteria by PMN triggers many bactericidal mechanisms [7, 35] including a marked increase in cyanide-insensitive oxygen consumption and the generation of reactive oxygen species (ROS) such as O_2^-, H_2O_2, OH°, $^1O_2$ and HOCl [2, 4]. These ROS are pivotal for the killing of endocytosed bacteria [13, 20, 25, 41, 45].

The PMN ROS load can be simply quantified by phagocytosis-induced and/or non-induced chemiluminescence (CL) techniques [1, 25, 31, 45]. The different CL responsiveness of blood and milk PMN to soluble and/or particle stimuli during infection could be due to different factors such as differences in protein kinase C, NADPH-oxidase, myeloperoxidase (MPO) activity [2, 44] and PMN survival [25]. Since these enzyme activities reflect intracellular and extracellular redox reactions, observed differences might offer some explanation about the disparities in the cow's response against pathogens in the mammary gland. Mastitic cows show a large variability in illness and a wide range of pathological responses [7, 17, 42, 43]. Previous studies conducted during physiological [25, 27] and mastitic [26] conditions of dairy cows have also highlighted variations in blood and milk PMN CL. While *E. coli* is eliminated from the mammary gland, the cow still faces another tough challenge: resolution of inflammation. All of these host factors determine the outcome of mastitis [7]. So, a study on how fast and how much ROS is produced extra-and-intracellularely by PMN during *E. coli* mastitis could add some new insight into the cow-*E. coli* interactions in the mammary gland.

A key challenge for bovine mammary gland immunologists now is where to focus their studies, on blood or milk PMN function. During the lactation cycle, milk PMN CL activity closely parallels blood PMN CL activity [25]. Despite the importance of PMN ROS in *E. coli* mastitis, only a few studies exist concerning the relationship between "blood" PMN ROS before and during *E. coli* mastitis and the severity of mastitis. Little information is available about the "kinetics" of blood PMN ROS, e.g., ROS_{max} and T_{max} in bovine PMN. The ROS_{max} and T_{max} could be "potentially" strong parameters for the assessment of the efficiency of PMN-*E. coli* interactions and the outcome of the infection. Most distinctively, resident milk PMN (as direct effector cells against pathogens in the mammary gland) would have an enormous impact on the elimination of bacteria, and their ROS_{max} and T_{max} would be crucial for the host. However, the concept of milk PMN ROS load versus the severity of coliform mastitis is largely theoretical.

The permanent gap between the knowledge on bovine blood PMN ROS versus milk PMN's led us to simultaneously assess blood and "milk" PMN ROS load to single out some novel contributing host factors to the severity of coliform mastitis. The impact of pre-infection milk PMN CL on bactericidal capacity in the gland, and on milk yield (as an indicator for mastitis severity) was therefore investigated. Blood PMN CL activity was used as a reference to explain the fluctuations in milk PMN CL activity. The shift of blood and milk PMN to immature forms during *E. coli* mastitis was also determined to get insight into the observed changes in PMN CL. The detailed assessment of the kinetics of blood and milk PMN CL activity before and during infection was aimed to shed fresh light on the prediction of the severity of bovine mastitis during early lactation.

2. MATERIALS AND METHODS

This experiment has been approved by the ethical committee of the Faculty of Veterinary Medicine from the Ghent University.

2.1. Animals

All Holstein-Friesian cows were in their 215 ± 6 days of first pregnancy (2.2 ± 0.3 yr) on arrival at the experimental dairy farm. The animals were on a system of zero-grazing from arrival till the end of the experiment; they were put in individual stalls and were fed with a special ration for pregnancy and lactation and always had free access to water and hay. After gestation, clinically healthy cows (free from typical periparturient diseases before and after calving) were selected (n = 20) on the basis of 2 consecutive bacteriologically negative milk samples and a milk somatic cell count (SCC) of $< 2 \times 10^5$ mL^{-1} milk per individual quarter. One week before infection, the animals were fed a daily ration of approximately 8 kg of concentrate and had free access to water and hay. They were milked twice daily at 8 a.m. and 6 p.m. with a quarters milking machine (Packo & Fullwood, Zeddelgem, Belgium). The cows were experimentally infected in the mammary gland with *E. coli* at 19 ± 5 days after parturition.

2.2. Bacterial challenge

Escherichia coli strain P4:O32 (H37, ß-glucuronidase$^+$, haemolysin$^-$) was obtained from a clinical case of mastitis. This strain has been frequently used to induce bovine mastitis by several researchers. The stock of *E. coli* was maintained in lyophilized medium at –20 °C until use. The cultures were frequently observed for viability and purity. Before infection, the bacteria were subcultured in brain-heart infusion broth (CM225; Oxoid, Nepean, ON, USA) at 37 °C. The bacterial suspension was washed three times with pyrogen free saline solution (9 g·L^{-1}) and resuspended in the solution. Bacterial counting was performed using the plate count method. The teat ends were disinfected with ethanol (70%) mixed with 0.5% chlorohexidine. *E. coli* mastitis was induced in the left front and hindquarters by a single intramammary injection of 10 mL of 10^4 *E. coli* per quarter using a sterile teat cannula (7 cm; Me. Ve. Mat., Deinze, Belgium). After injection, each quarter was massaged for 30 s to distribute the bacterial solution in the gland.

2.3. Milk and blood sampling and clinical signs

Individual quarter milk samples were aseptically collected for determination of cfu (10 mL), SCC (50 mL) and isolation of PMN (200 mL) at 24 h before, immediately before and at 6, 12, 18, 24, 48, 72, 144, 216 and 312 h following *E. coli* injection. For diagnostic bacteriology and determination of bacterial cfu, 0.5 mL of quarter milk was serially diluted in a pyrogen free saline solution (9 g·L^{-1}) and 0.01 mL of the samples of different dilutions were streaked in duplicates on Columbia Sheep Blood Agar (Biokar Diagnostics, Beauvais, France) plates using an inoculation loop. The plates were incubated for 24 h at 37 °C. Peripheral blood (80 mL) was collected aseptically from each cow by venipuncture from the external jugular vein into evacuated tubes (Laboratory EGA, 28210 Nogent-le-Roi, France) containing 125 i.u. heparin as the anticoagulant. The blood sampling was carried out after milk sampling at 24 h before, immediately before and at 6, 12, 18, 24, 48, 72, 144, 216 and 312 h following *E. coli* injection. Measurements of rectal temperature, heart rate, rumen motility and clinical examination of the mammary gland were performed at the time of blood and milk sampling. Evening and morning milk were pooled to obtain quarter daily milk production (MP). The cows were divided into two different severity groups (moderate (M; n = 15) with MP loss < 50% and severe (S; n = 5) with MP loss ≥ 50%) based on the MP loss of non-infected udder halves at post-infection hours (PIH) 48 of mastitis induction compared to the pre-infection MP [38].

2.4. Blood and milk parameters before and after *E. coli* challenge

Blood and milk was collected from healthy cows for PMN isolation before *E. coli* challenge, and blood and milk PMN ROS production capacity was determined. The MP loss of non-infected quarters and the cfu of infected quarters were measured at PIH 48 and 6, respectively. The relationship between pre-infection milk PMN CL and cfu at PIH 6 was also examined. The PMA (phorbol 12-myristate, 13-acetate) and latex stimulated respiratory burst activity of blood and milk PMN, cfu, SCC and differential circulating leukocyte counts were followed for several days after *E. coli* infection.

2.5. Blood and milk PMN preparation, enumeration and differentiation

All materials and reagents used for the isolation of blood and milk PMN were sterile. The isolation of PMN from peripheral blood was performed using two periods after [8]. The isolation procedure of PMN from blood yielded > 98% granulocytes (PMN + eosinophils) that were predominantly PMN (> 87%) and a viability of > 98%. After counting the cells using an electronic programmable particle counter (Coulter counter Z2, Coulter Electronics Ltd., Luton, UK) and determining the viability and percentage of PMN, the cell suspension was adjusted to a concentration of 5×10^6 cells·mL^{-1} in Dulbecco phosphate buffered saline (DPBS; Gibco BRL, Life Technologies Inc., Gaithersburg, MD, USA) supplemented with gelatin (0.5 mg·mL^{-1}; Merck, Darmstadt, Germany). Individual quarter milk samples were used for subsequent PMN isolation, as described previously [26]. Briefly, the pooled milk of the two *E. coli*-infected quarters of each cow was filtered separately through a nylon filter (40 µm pore size) and diluted to 60% v/v with cold DPBS. The isolation of PMN from milk was performed using three centrifugation steps as previously described [25, 26]. The isolation procedure yielded 65–98% PMN with viability (determined in duplicate by means of flow cytometry (FACSScan, Becton Dickinson Immunocytometry Systems, San José, CA, USA) using propidium iodide exclusion) of 70–98% throughout the experiment. The total number of circulating leukocytes and isolated blood and milk cells were determined using an electronic particle counter [25]. Differential circulating leukocyte counts were determined by differentiating 200 eosin-Giemsa-stained cells from smears using light microscopy, with identification based on morphological characteristics as described previously [25]. To quantify the percentages of each cell type in the samples, PMN (mature and immature), monocytes/macrophages, lymphocytes, eosinophils and epithelial cells (only in milk) were identified on 200 cells per slide and expressed as a percentage of particular cells in respective samples.

2.6. Chemiluminescence assay

Luminol-enhanced PMA and-latex beads (polystyrene 0.76 µm diameter, 4×10^{11} particles·mL^{-1}; Sigma Chemical Co., St. Louis, MO, USA)-stimulated cellular CL was used to measure the CL activity of PMN isolated from blood and milk of *E. coli*-infected quarters. CL was measured in duplicate for 30 min at 37 °C with a microtiterplate luminometer (type LB96P; EG&G Berthold, 75312 Bad Wildbad, Germany). PMA-stimulated CL was measured immediately after the addition of 100 ng·mL^{-1} PMA and 0.3 mM luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione, Sigma Chemical Co.) to 2×10^6 cells·mL^{-1} in a total volume of 200 µL per well. Similar concentrations of luminol and cells per well were used for latex beads (final concentration of 500 particles·PMN^{-1}) stimulated CL. Stock solutions of PMA and luminol were prepared in dimethyl sulphoxide (Sigma Chemical Co.) and were always stored at –20 °C. The area under the curve (AUC) was calculated for the registered impulse rates (relative light unit (RLU)·s^{-1}) over the whole measurement period of 30 min. The CL response was expressed per 10^3 viable PMN in each

isolated cell sample. Since the contribution of milk macrophages to luminol-dependent CL is negligible [25], the CL response was expressed per 10^3 viable PMN. For milk PMN, the CL assay formula, $CL_{PMN} = 10^3 \times Cl_{isolated\ cells} \cdot (4 \times 10^5 \times \%\ PMN \times \%\ V)^{-1}$, was used to perform the corrections, where Cl = mean $RLU \cdot s^{-1}$, 4×10^5 = total number of cells per well, %PMN = total percentage of PMN in isolated cells, %V = percentage of viable PMN. The CL of blood PMN was calculated with the same formula as for milk PMN applying the corrections described by Heyneman et al. [17] for interference by eosinophils. The CL kinetics of blood and milk PMN stimulated by PMA and latex was performed prior to and during the course of the *E. coli* infection in all individual cows throughout the study.

2.7. Statistical analyses

The relationship between CL (AUC) immediately before challenge and the reduction of milk production 48 h after challenge was first studied by a linear regression model, and the null hypothesis of the slope being equal to 0 was tested. Alternatively, logistic regression analysis was performed to investigate whether CL (AUC) immediately before challenge can predict whether a cow will be a severe responder (reduction of milk production 48 h after challenge > 50%) or not. Again the null hypothesis of the slope in the logistic regression model being equal to 0 was tested. Furthermore, cfu at 6 h was linearly regressed on the CL (AUC) immediately before challenge and the null hypothesis of the slope being equal to 0 was tested.

The differences in CL after challenge between moderate and severe cows were analysed by a mixed model with the cow as the random effect and time as the categorical variable with five levels (0, 6, 12, 18–24 and ≥ 48 h) for each of the two locations (blood or milk) and for each of the two methods of stimulation (latex or PMA). Such analyses were done for AUC, RLU_{max} and T_{max} as dependent variables in order to study the different aspects of the CL process. As analyses are done at five different time points, each comparison between moderate and severe cows is performed at the 0.01 significance level in order to ensure an overall size equal to 0.05 (Bonferroni multiple comparisons technique).

3. RESULTS

3.1. Pre-infection PMN CL in blood and milk in relation to the severity of mastitis

The level of pre-infection CL (AUC) in milk PMN had a significant influence on the severity of mastitis. Both MP loss and the probability of a severe response decreased significantly with increasing values of pre-infection CL (AUC) in milk PMN with both PMA and latex stimulation (Figs. 1b and 1d, Tab. I). Although the same trend was observed in blood PMN, a significant result was only obtained for the effect of PMA stimulated pre-infection CL on the probability of a severe response ($P = 0.038$), and the linear effect was generally smaller for blood PMN.

Pre-infection CL for both milk and blood PMN significantly influenced another parameter of mastitis severity, cfu at PIH 6, but again the relationship was far stronger for milk PMN (Tab. II); this inverse relationship was significant both for latex and for PMA stimulated CL.

Figure 2 shows the kinetics of blood and milk PMN CL activity immediately "before" infection after stimulation with PMA and latex beads of the individual M and S cows prior to infection. For both milk and blood PMN stimulated with either latex or PMA, the lowest CL values were observed in S cows. In the presence of PMA, pre-infection blood PMN RLU_{max} in M cows was never below 2000 $RLU \cdot s^{-1}$, whereas in S cows the RLU_{max} never reached 2000 $RLU \cdot s^{-1}$. In milk, although lower than in blood, the average RLU_{max} in the M cows was above 200 $RLU \cdot s^{-1}$,

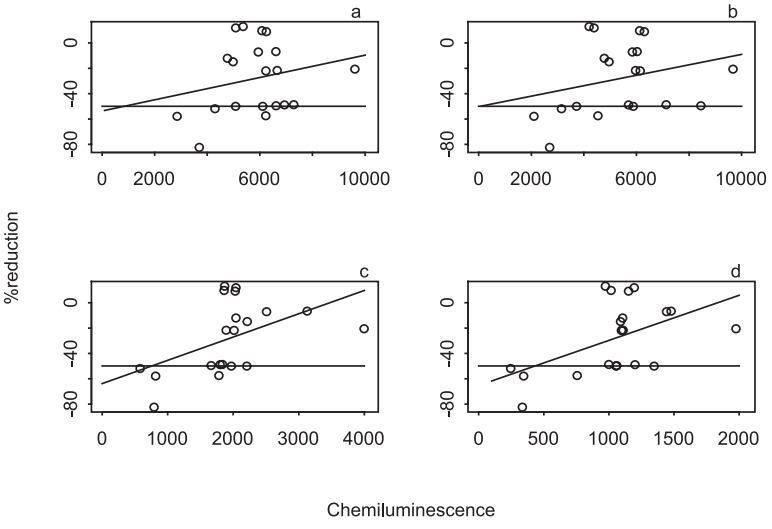

Figure 1. Relationship between PMA (a, c)-and-latex (b, d) stimulated blood (a, b) and milk (c, d) PMN CL (AUC of 1000 viable PMN) prior to the inoculation of *E. coli* and milk production loss at PIH 48 ($n = 20$). Circles represent individual cows through which the regression line has been fitted. The horizontal line corresponds to the 50% milk production loss (severity threshold).

Table I. The relationship between milk production loss (linear regression)/severity (logistic regression) and CL in blood and milk PMN stimulated with PMA and latex beads during experimentally induced *E. coli* mastitis. The slope and its standard error are based on the data of 15 moderate cows and 5 severe cows.

Source of PMN	Stimulator	Statistical analyses			
		Slope (SE) Linear	*P*-value Linear	Slope (SE) Logistic	*P*-value Logistic
Blood	PMA	0.0044 (0.0045)	0.34	0.0017 (0.0008)	0.038
Blood	Latex	0.0041 (0.0035)	0.26	0.0038 (0.0025)	0.13
Milk	PMA	0.0185 (0.0077)	0.029	0.0037 (0.002)	0.057
Milk	Latex	0.036 (0.014)	0.022	0.0062 (0.0031)	0.043

approximately 2-fold higher than that of S cows. For both milk and blood PMN CL induced with PMA, T_{max} was always higher in the M cows (Figs. 2c and 2d). In the presence of latex, although slightly lower than PMA, similar patterns of blood and particularly milk PMN CL for RLU_{max} and T_{max} were observed (Figs. 2a and 2b). Pre-infection PMN AUC of M cows was approximately 2-fold higher than those of S cows in each particular combination of blood and milk PMN with latex and PMA. Furthermore, the PMA and latex stimulated blood PMN CL in M cows increased faster, remained substantially higher for a longer time and decreased more slowly than in S cows (Figs. 2a and 2c). This pattern was similar to that of milk PMN (Figs. 2b and 2d).

3.2. Blood and milk differentiation between M and S cows and clinical symptoms

Although no significant differences on pre-infection WBC and PMN were observed between the M and S groups, leukopenia and neutropenia were far more pronounced in S cows, with neutropenia existing even at PIH > 48 in S cows (Tab. III). Light microscopic comparison of blood and milk differential leukocyte counts of M and S cows during initiation and resolution of coliform mastitis revealed different results (Fig. 3). The appearance of immature PMN in blood and milk of M cows was much quicker when compared to S cows. The untimely appearance of massive PMN in the infected quarters of S cows showed that the resolution of inflammation in the S cows was significantly delayed. The adhesion (not aggregation) of milk PMN to each other and the almost total absence of mononuclear cells (3 ± 2% without macrophage for S cows and 43 ± 11% with 34 ± 7% macrophages for M cows) in the milk of S cows at PIH 48 was also remarkable. A representative example of this result is shown in Figure 3.

Table II. Relationship between pre-infection PMA- and latex-stimulated blood and milk PMN CL and cfu at PIH 6 (linear regression) during experimentally induced *E. coli* mastitis. The slope and its standard error are based on the data of 20 cows.

Source of PMN	Stimulator	Statistical analyses	
		Slope (SE)	*P*-value
Blood	PMA	–3.29 (1.49)	0.014
Blood	Latex	–3.39 (1.056)	0.0007
Milk	PMA	–7.91 (2.63)	0.0013
Milk	Latex	–16.78 (4.5)	0.0001

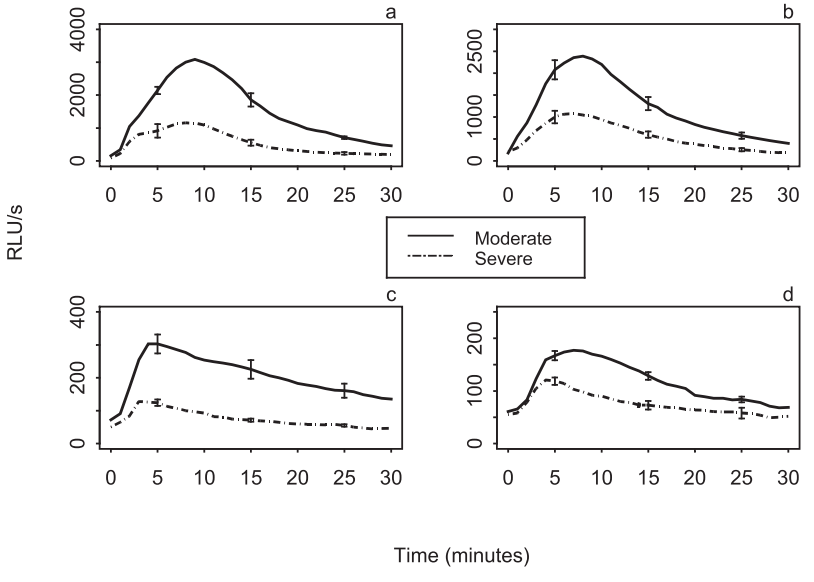

Figure 2. Comparison of pre-infection chemiluminescence (CL) kinetics of PMA (a, c) and latex (b, d) stimulated blood (a, b) and milk (c, d) PMN in moderate (n = 15, solid lines) and severe cows (dashed lines). The generation of CL was monitored continuously for 30 min after the addition of 100 ng·mL^{-1} PMA and/or 500 latex bead particles·PMN^{-1} and 0.3 mM luminol to the 4 × 10^5 isolated cells suspension in 200 µL. Bars represent the standard error of the mean.

Table III. Comparison of some circulating leukocytes and neutrophils as well as SCC and cfu in milk between moderate and severe cows to experimentally induced *E. coli* mastitis in dairy cows. Values are means ± SEM of 15 cows (moderate; M) and means ± SEM of 5 cows (severe; S).

PIH	Parameter	Blood		Parameter	Milk	
		M	S		M	S
	WBC/μL	8209 ± 250	8433 ± 1165	SCC/μL	72 ± 15	161 ± 130
0	PMN/μL	2412 ± 263	2980 ± 856	cfu/μL	0	0
	Immature N/μL	652 ± 68	928 ± 203			
	WBC/μL	8504 ± 637	7536 ± 2403	SCC/μL	5431 ± 1179	79 ± 28
6	PMN/μL	1811 ± 210	2252 ± 722	cfu/μL	3535 ± 548	24562 ± 3752
	Immature N/μL	1518 ± 208	2233 ± 410			
	WBC/μL	4271 ± 682	2661 ± 349	SCC/μL	5265 ± 1124	4709 ± 1740
12	PMN/μL	601 ± 141	273 ± 83	cfu/μL	113 ± 51	17146 ± 3912
	Immature N/μL	1018 ± 193	745 ± 167			
	WBC/μL	6752 ± 766	5360 ± 578	SCC/μL	3953 ± 867	11550 ± 1321
18–24	PMN/μL	1341 ± 231	776 ± 144	cfu/μL	24 ± 4	9665 ± 2541
	Immature N/μL	2325 ± 347	2263 ± 376			
	WBC/μL	8899 ± 324	11329 ± 931	SCC/μL	1829 ± 453	5701 ± 836
> 48	PMN/μL	2042 ± 139	2825 ± 417	cfu/μL	7 ± 2	1790 ± 318
	Immature N/μL	1815 ± 117	3205 ± 331			

The overall pre-infection SCC was the same for M and S cows. The SCC in M cows, however, increased faster (maximal at PIH 6). In S cows the maximal SCC appeared at PIH 18–24 (Fig. 4 and Tab. III). The intramammary *E. coli* infection induced an increase in rectal temperature and heart rate that peaked at PIH 6 to 12, as well as swelling and pain of the infected quarters; the appearance of flecks and milk leakage in infected quarters was observed at PIH 6 to 12 (data not shown) for both groups. All clinical symptoms disappeared in the M cows within PIH 24, but lasted longer in S cows. Clinical signs were more pronounced in S cows and even at PIH > 72 the infected quarters had pain and abnormal secretions.

3.3. Effect of *E. coli* mastitis on blood and milk PMN CL kinetics in M and S cows

Figure 5 shows blood and milk PMN CL kinetics after stimulation with PMA and latex beads during mastitis in M and S cows. In latex and PMA activated blood PMN, consistently higher ROS capacity in M cows was observed throughout the infection. A more rapid shift in activity to higher AUC and RLU_{max} by latex ingestion in M cows was remarked (Tab. IV and Fig. 5: 1.a, 2.a, 3.a and 4.a; 1.c and 2.c). The T_{max} at PIH 18–24 for latex stimulated blood PMN was substantially lower in S cows (Tab. IV).

Figure 5 and Table IV also show the PMA and latex activated CL kinetics of milk PMN harvested from *E. coli* infected quarters during infection. Compared to pre-infection PMN CL activity, large disparities between M and S groups during *E. coli* infection were observed. For latex stimulated milk PMN CL kinetics, in addition to an overall higher AUC and T_{max}, the average RLU_{max} in the M group at PIH 6 and 12 was approximately 2000, whereas it was below 650 in the S cows (Tab. IV and Fig. 5: 2.b and 3.b). There was also a prolonged shift of the curve from the left to the

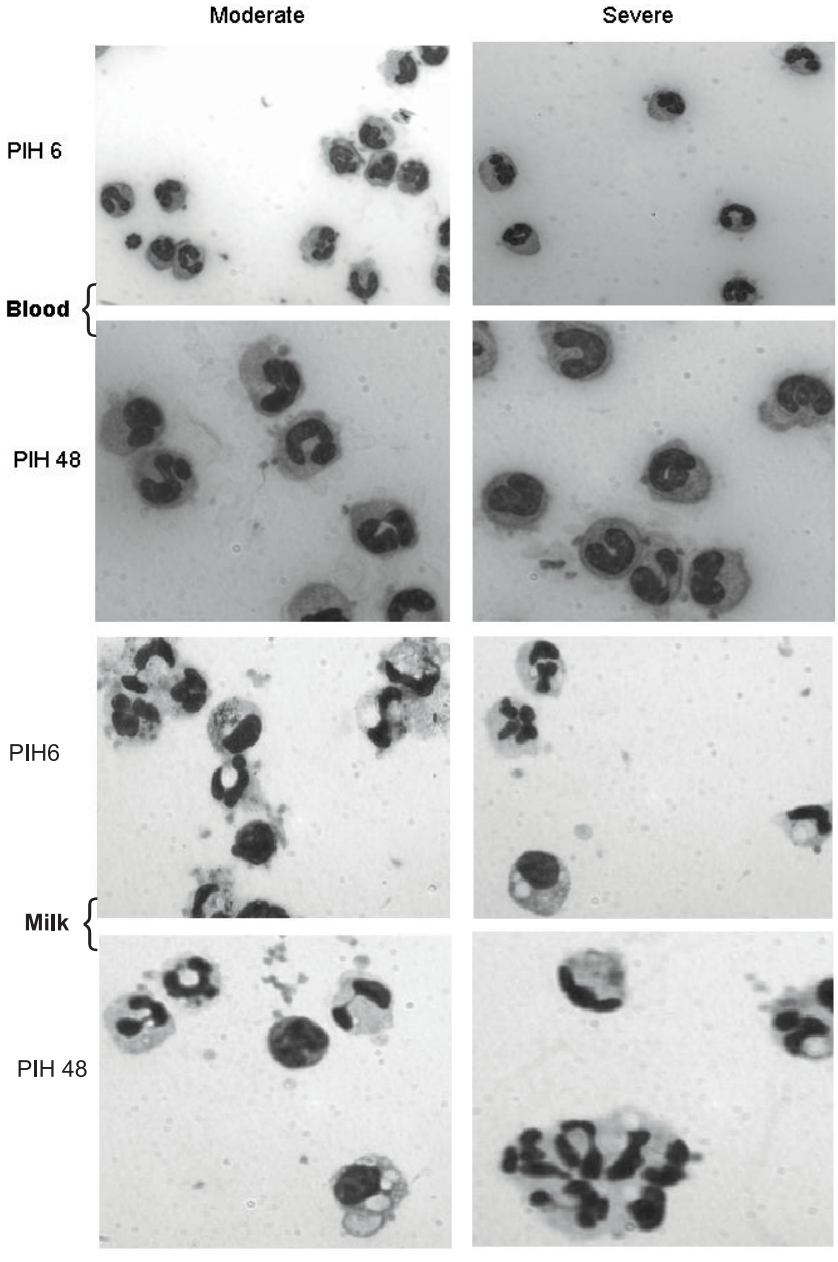

Figure 3. Representative light micrographs of isolated PMN from blood and *E. coli* infected quarters during the initiation and resolution of mastitis in severe (S) and moderate (M) cows. Fast appearance of immature neutrophils in blood and milk in M cows at PIH 6 reveals that the bone marrow in M cows is more alert. (Hematoxilin-Eosin stained × 1000, but × 400 for upper right and left blood samples.)

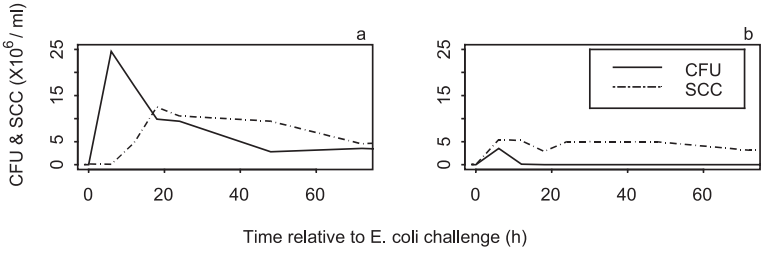

Figure 4. Concentration of *E. coli* (solid lines) in infected mammary glands and pattern of leukocyte influx (dashed lines) into milk in (a) severe cows ($n = 5$) compared with (b) those in moderate cows ($n = 15$) during experimentally induced *E. coli* mastitis.

Table IV. Comparison of AUC, RLU_{max} and T_{max} of blood and milk PMN between moderate and severe cows during experimentally induced *E. coli* mastitis. The data shown are means of 15 moderate (M) cows and 5 severe (S) cows. An asterisk corresponds to a significant difference ($P < 0.01$) between M and S cows.

PIH	Parameter	Latex-stimulated PMN				PMA-stimulated PMN			
		Blood		Milk		Blood		Milk	
		M	S	M	S	M	S	M	S
	AUC	6611	3310	1235	682	5949	4667	2332	1242
0	RLU_{max}	2565*	1097	197	127	3223*	1212	309	130
	T_{max}	7.1	7	7	4.2	8.8	8.6	4.7	3.2
	AUC	5739	3544	11587*	2768	6296	3367	14714*	5396
6	RLU_{max}	2599*	1112	1932*	623	3236*	1238	3970*	1795
	T_{max}	7.2	7.2	14.6	11.6	8.6	9.6	4.1	4.4
	AUC	4756	2982	18232*	3152	4783	3937	19326*	7564
12	RLU_{max}	2608*	727	2054*	507	3311*	1215	4588*	2219
	T_{max}	7.1	6.8	14.2	13.4	8.5	9.4	4.2	3
	AUC	8656*	2370	7977	4278	6897*	2332	10283	8785
18–24	RLU_{max}	2315*	527	1121	972	3401*	883	2305	2211
	T_{max}	7.5*	4.8	11.3*	5.8	8.6	8.6	6.2*	2.8
	AUC	12294*	6296	1483	2031	9731	8742	2942	4200
> 48	RLU_{max}	2947*	1674	228*	753	4079*	1450	329	988
	T_{max}	6	7.5	8.1	5.8	8.3	8	5.5	6

right for latex ingestion in M cows at PIH 6, 12, 18 and 24 (Tab. IV and Fig. 5: 3.b, 4.b and 5.b). At PIH 48 and 72 RLU_{max} and AUC of latex stimulated milk PMN of S cows revealed a reversed response compared to M cows (Tab. IV and Fig. 5: 6.b and 7.b). For PMA stimulated milk PMN CL kinetics, a significantly higher T_{max}, was only observed at PIH 18–24. Overall, higher AUC appeared in the M cows and the average RLU_{max} in

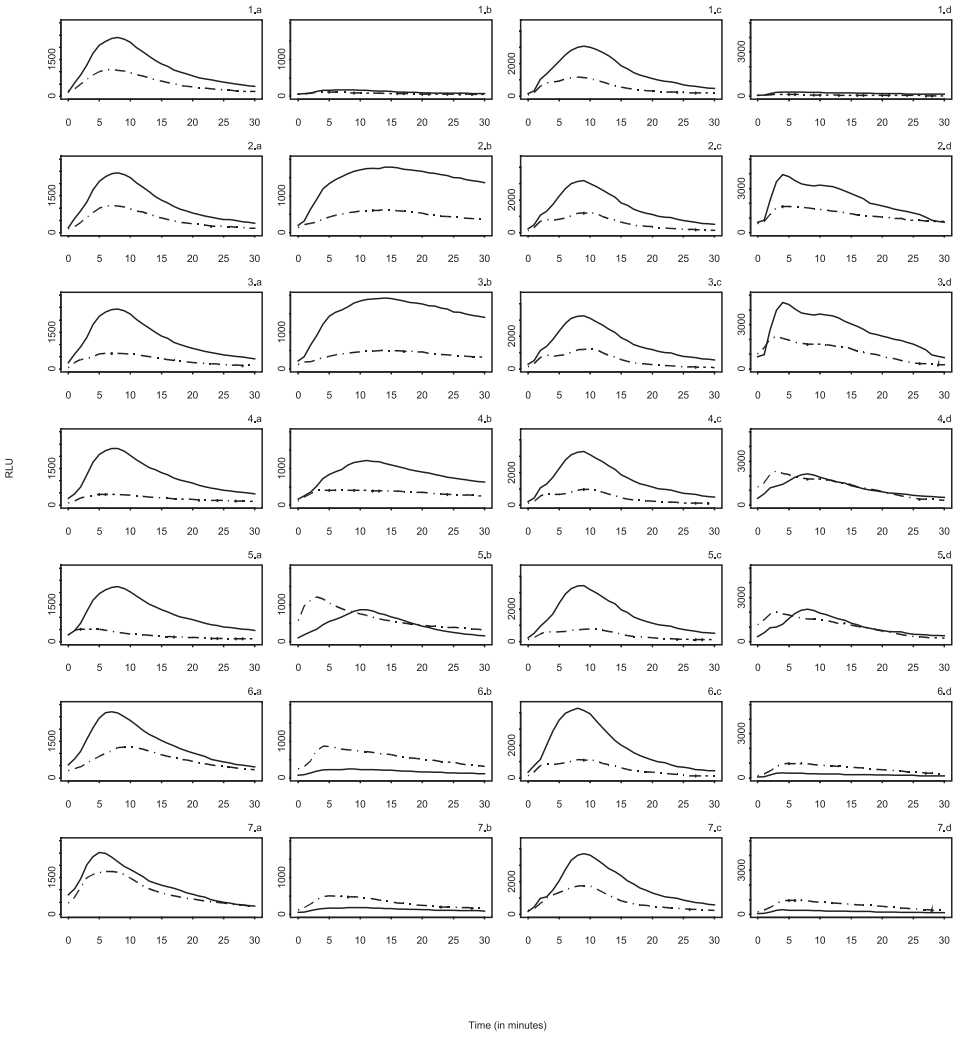

Figure 5. Blood and milk PMN CL kinetics prior to and during experimentally induced *E. coli* mastitis in moderate (solid lines) and severe (dashed lines) responding cows. The number of the figures corresponds to the time post-infection, with (1) for pre-infection, (2) for PIH 6, (3) for PIH 12, (4) for PIH 18, (5) for PIH 24, (6) for PIH 48 and (7) for PIH 72, whereas (a) corresponds to blood PMN with latex stimulation, (b) milk PMN with latex stimulation, (c) blood PMN with PMA stimulation and (d) milk PMN with PMA stimulation. The curves are averages of 15 moderate and 5 severe cows.

M cows at PIH 6 and 12 was 2-fold higher than in S cows (Tab. IV and Fig. 5: 2.d and 3.d). There was also a biphasic pattern in PMA-stimulated PMN CL at PIH 6, 12, 18 and 24 of M cows (Fig. 5: 2.d, 3.d and 4.d). While at PIH 6 and 12 the first peak was larger, at PIH 18 and 24 the second peak was higher (Fig. 5: 2.d, 3.d and 4.d). Surprisingly, at PIH 48 and 72 RLU_{max} and AUC of PMA stimulated milk PMN of

S cows revealed a reversed response compared to M cows (Tab. IV and Fig. 5: 6.d and 7.d).

4. DISCUSSION

The speed of PMN mobilization was different between the M and S cows. The relatively weaker and more sustained PMN recruitment in S cows might result in releasing their ROS and granules in the mammary tissue before reaching the pathogens. Furthermore, the delay of just 20 min of PMN influx into lacteal secretions is a significant effect, considering that *E. coli* bacteria double their number every 20 min [21]. A 3-h delay of PMN recruitment into the mammary gland could result in a 512-fold larger number of *E. coli* to be killed and that much more endotoxin be hydrolysed. So, fast recruitment of PMN with high bactericidal capacity into the udder at the right time is essential to prevent severe coliform mastitis [19, 32]; this phenomenon occurred only in the M cows. In contrast, the increased milk PMN recruitment, that constitutes the mobilized and/or inflammatory (dynamic) immune defense of the udder, in S cows was neither fast nor short-lasting, insufficiently reducing milk cfu (Fig. 4). Uncontrolled PMN recruitment and function at the wrong time is also harmful for many cell systems e.g., T cell hyporesponsiveness and lymphocyte proliferation inhibition caused by ROS [9, 28]. ROS also enhance natural killer cell and T cell activity [9, 18, 34, 40]. This evidence indicates that timely PMN functionality (at PIH < 6) may facilitate the recovery of *E. coli* mastitis. This rapid increased SCC and/or PMN was the reflection of a higher chemotactic response of blood PMN in M cows [22, 24], which quantitatively and qualitatively strengthened the dynamic innate defense of the udder against *E. coli*. Indeed, many sophisticated approaches have been adopted worldwide to alleviate the severity of coliform mastitis in high yielding dairy cows; none of these has yet made the early lactating cows more secure against this environmental mastitis, and peripartum high producing dairy cows are still extremely sensitive to *E. coli* mastitis. There is a tendency to believe that SCC, especially PMN, may be too low to protect the cows against *E. coli* mastitis [7]. However, this conception is far from straightforward, depending on the physiological conditions [25, 27] and genetic variabilities of the cows [37]. This immunological parameter, SCC and/or PMN, may be promising for the study of mastitis resistance. For example, cows with low SCC and mastitis incidence tend to exhibit a moderate response to *E. coli* mastitis [7, 37], and mastitis incidence and SCC levels are both lower in younger cows. This might be due to higher PMN quality in the milk of young cows [27] (see particular PMN CL in Figs. 2 and 5). It is therefore reasonable that the next phase in milk PMN research versus mastitis remedy would be to boost milk PMN quality, which matters more.

Accordingly Heyneman et al. [17] and Shuster et al. [38], the MP loss in non-infected contralateral quarters at PIH 48 was used as a criterion to classify cows as M or S responders to *E. coli* mastitis. We found that the extent of MP loss (an index for severity and mammary tissue damage) depends, in part, on the pre-infection blood and milk PMN CL activity: inflammation was less severe with less mammary tissue damage at higher PMN CL activity. Similar correlations were observed by Lohuis et al. [24], although they studied only the blood PMN CL. In our study this relationship was most pronounced in milk PMN CL, emphasizing the pivotal role of the pre-existing milk PMN in the udder's innate defense; this boosted bacteriostatic properties of the gland, enhancing rapid bacterial clearance at PIH approximately 12 in M cows (Tab. III and Fig. 4). Therefore, even more important than blood PMN, the impact of milk PMN CL on mastitis severity was crucial; e.g., every unit increase in pre-infection milk PMN CL (AUC) resulted in roughly 20 mL gain in MP loss at PIH 48, which coincided with a decrease of 0.5% in the probability of developing severe *E. coli* mastitis (Tabs. I,

II and Fig. 1). This was consistent with the finding of Zecconi et al. [47]. The milk PMN CL in M cows peaked at PIH 6 and 12, whereas in S cows it occurred much later, PIH 24, resulting in higher cfu in the infected quarters of S cows. This finding indicates that the initial bacterial growth is critical to producing further inflammation. The second milk PMN CL peak (as nonexistent in M cows) at PIH 72, at which all pathological consequences and mammary tissue damage had already happened, in the S cows was somewhat counter productive.

The blood PMN response to PMA and/or latex beads did not decline substantially during the first day of infection in M cows, whereas in S cows the decline was substantial. Furthermore a decline in myelopoiesis was also observed as evident by a decline in the number of immature neutrophils. This was in accordance with previous findings [17, 42]; but in our study the decline was faster. The most probable reason for this discrepancy is the use of "primiparous cows" in the present study, whose PMN function in bone marrow, blood and milk is more pronounced and could react faster against *E. coli*, compared to pluriparous cows [27]. Latex-and PMA stimulated blood PMN CL in M cows revealed no substantial changes in the first 24 h after infection which was in agreement with Heyneman et al. [17], who used zymozan and PMA for PMN stimulation. Because the latex in our study was unopsonized, phagocytosis was nonspecific.

What made the M cows more resistant against *E. coli* mastitis was not merely higher PMN CL but also different PMN CL kinetics before infection and while *E. coli* infection played a role. This novel finding of CL kinetics induced by either latex or PMA stimulated blood and milk PMN during *E. coli* infection revealed important differences in M and S cows. The RLU_{max} in blood and milk PMN was always higher in the M cows compared to the S cows (except for PIH 48 and 72 for milk PMN). Thus, blood PMN and the newly migrated PMN in milk are functionally more efficient in M cows. The higher probability of developing clinical mastitis in S cows could result, at least in part, from lower milk PMN CL intensity before infection and at PIH 6 and 12.

Higher intensity and longer duration of CL for blood PMN before and during *E. coli* infection in M cows indicates a higher oxygen-dependent intracellular bactericidal capacity. The substantial decrease of blood PMN CL intensity in S cows at PIH 18–24 might be due to severe inflammation [26]. The observation of an early intense local inflammatory response in M cows and of late local but early systemic response in S cows was, in part, responsible for most of the disparities in CL activity between the M and S groups. At PIH 6, the number of mature neutrophils in M cows was lower, but not significant, with a higher CL activity. This revealed that the overall redox reactions in blood neutrophils (mature and immature) of M cows are higher. The appearance of immature neutrophils in the blood and milk of the M cows confirmed that the bone marrow in the M cows was more functional, compared to the S ones. This faster increase of young PMN in the milk of M cows during the initiation of mastitis and the shift from a predominant PMN population to mononuclear cells partially attributed to the severity of coliform mastitis. This is a physiologically critical compensatory mechanism during infection because of the bone marrow's timely reaction. Conversely, in S cows it is far less efficient to clear *E. coli* from the teat cistern with newly attracted young PMN at PIH > 48, which is too late. Furthermore, more pronounced adherence of milk PMN at PIH 48 (Fig. 3) and onwards can be the result of "extracellular" ROS production (e.g., Fig. 5: 6.b and 6.d), since these ROS are responsible for cell adhesion [11]. No such phenomenon was observed in the milk PMN of the M cows at PIH 48 and onwards.

The kinetics of milk PMN CL also indicated smaller intracellular bactericidal efficiency in S cows during pre-infection and

at PIH 6, 12 and 24. Although several intracellular bactericidal mechanisms of PMN have been described elsewhere [3, 33, 46], the central role of ROS on bactericidal mechanisms is nevertheless indisputable [1, 16, 35], especially for Gram-negative bacteria [6, 7]. Moreover, the CL kinetics of PMA and/or latex-induced luminol-dependent CL revealed some details on the location of the ROS that are produced intra- and extra-cellularly [12, 36]. In the study conducted "only on blood PMN" [17, 24, 43], the location of PMN ROS production remained unnoticed. In luminol dependent CL kinetics, ROS production after 3–4 min results mainly from intracellular events [12, 14, 15]. Since the luminol-dependent CL requires H_2O_2 [14, 15, 23], it is highly likely that the intracellular H_2O_2 production is higher in PMN from M cows than those from S cows. Subsequently, the impairment of the intracellular reactions of the MPO-H_2O_2 halide system is more pronounced in S cows, yielding less HOCl. HOCl is the major contributor to intracellular PMN ROS during phagocytosis and respiratory burst activity [10]. One of the underlying causes of a much higher milk cfu at PIH 6, 12 and 18 in S cows would be the inadequate intracellular ROS production during *E. coli* phagocytosis.

Latex- and PMA-stimulated milk PMN CL kinetics in S cows gave neither high AUC nor high RLU as compared to M cows. During the late stage of infection, at PIH 48 and later, however, the AUC and RLU values were higher in the S cows. This reaction could be unsuitable for the host because ROS could be produced extracellularly. The low T_{max}, as seen in S cows, is counter productive and would result in tissue damage. These findings lead us to consider the milk PMN ROS as a "double-edged sword". To minimize this extra-cellular ROS and mammary tissue damage, the application of antioxidants such as vitamin E and selenium [39] or the application of e.g. melatonin [5] in S cows would be effective.

In brief, high blood and milk PMN CL at the start and in the early phase of the infection is crucial for removing pathogens from the infected quarters. In M cows, increased milk PMN CL and SCC and decreased cfu are concurrent. It is conceivable that the static part of the innate resistance, pre-existing milk PMN, is a strong parameter for alleviating the severity of *E. coli* mastitis. Our study demonstrates for the first time that the pre-existing milk PMN CL is involved in the underlying mechanism of the static part of the udder's innate defense against bacteria. To shorten inflammatory reactions in the udder, boosting resident milk PMN ROS would be beneficial for dairy cows.

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