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Neutrophils expressing major histocompatibility complex class II molecules circulate in blood and milk during mastitis and show high microbicidal activity

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ABSTRACT

Bovine mastitis is mainly caused by bacterial infection and is responsible for important economic losses as well as alterations of the health and welfare of animals. The increase in somatic cell count (SCC) in milk during mastitis is mainly due to the influx of neutrophils, which have a crucial role in the elimination of pathogens. For a long time, these first-line defenders have been viewed as microbe killers, with a limited role in the orchestration of the immune response. However, their role is more complex: we recently characterized a bovine neutrophil subset expressing major histocompatibility complex class II (MHC-II) molecules (MHC-II^{pos}), usually distributed on antigen-presenting cells, as having regulatory capacities in cattle. In this study, our objective was to evaluate the implication of different neutrophils subsets in the mammary gland immunity during clinical and subclinical mastitis. Using flow cytometry, we analyzed the presence of MHC-II^{pos} neutrophils in blood and in milk during clinical mastitis at different time points of inflammation ($n = 10$ infected quarters) and during subclinical mastitis, defined as the presence of bacteria and an SCC $>150,000$ cells/mL ($n = 27$ infected quarters). Our results show, for the first time, that in blood and milk, neutrophils are a heterogeneous population and encompass at least 2 subsets distinguishable by their expression of MHC-II. In milk without mastitis, we observed higher production of reactive oxygen species and higher phagocytosis capacity of MHC-II^{pos} neutrophils compared with their MHC-II^{neg} counterparts, indicating the high bactericidal capacities of MHC-II^{pos} neutrophils. MHC-II^{pos} neutrophils are enriched in milk compared with blood during subclinical mastitis but not during clinical mastitis. Moreover, we observed a positive and highly

significant correlation between MHC-II^{pos} neutrophils and T lymphocytes present in milk during subclinical mastitis. Our experiments involved a total of 47 cows (40 Holstein and 7 Normande cows). To conclude, our study opens the way to the discovery of new biomarkers of mastitis inflammation.

Key words: major histocompatibility complex class II, bactericidal response, mastitis, neutrophils

INTRODUCTION

Mastitis is an inflammation of the mammary gland resulting primarily from bacterial infection. It affects the health and welfare of animals and leads to economic losses due to lower milk production and quality, antibiotic treatments, and premature drying off and culling (Halasa et al., 2007). Mastitis, the most prevalent and costly disease in dairy herds worldwide, presents a spectrum of clinical conditions ranging from severe clinical mastitis to subclinical mastitis, where the animals do not show signs of discomfort. When clinical mastitis occurs, local signs of mammary inflammation, such as swollen and red mammary gland and a painful and warm udder, which are a concern for the welfare of the animal, is accompanied by lumps in milk, which renders it unproper for consumption. In contrast, subclinical mastitis displays no apparent clinical signs of inflammation, whereas milk SCC is increased to levels $>200,000$ cells/mL, which affects milk quality and lowers the price (Smith et al., 2001). Therefore, subclinical mastitis represents 70 to 80% of the economic losses linked to mastitis (Gonçalves et al., 2017), and measures to better understand and prevent these forms of the disease are needed.

The SCC, by providing an indication of the inflammatory status of the mammary gland, is now a well-established parameter in milk control. Values above 150,000 to 200,000 cells/mL of milk are often set as a marker to identify mastitis, even though this threshold varies depending on countries (Smith et al., 2001). The increase

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in SCC during mastitis is mainly due to an influx of PMN from blood to the mammary gland (Rainard and Riollet, 2003; Koess and Hamann, 2008; Schwarz et al., 2011). The PMN are rapidly recruited during the acute inflammatory response to defend the mammary gland against invading pathogens and represent the signature of inflammation by being the predominant cell type in milk (Paape et al., 1979; Rainard and Riollet, 2003). In addition to PMN, macrophages and lymphocytes are also present in milk and counted in the SCC.

For decades, genetic tools are available to help dairy farms to control mastitis. Somatic cell score (a logarithmic transformation of SCC) has been used to select mastitis resistant cows (Rupp et al., 2009; Brand et al., 2011; Weigel and Shook, 2018). A divergent selection experiment in goats based on SCS showed favorable results with reduction of clinical and subclinical mastitis in the low SCS group and higher incidence in the high SCS group (Rupp et al., 2009). Other authors conducted a divergent genetic selection on Holstein and Normande breeds based on sire breeding values. In the Holstein breed, a lower SCS and a lower prevalence of clinical mastitis in the mastitis resistant group were observed compared with the control group. However, results in the Normande breed were much less marked compared with the Holstein breed in first lactation (Lefebvre et al., 2020).

Although divergent selection based on genetic or SCS seems promising to select mastitis-resistant cows, improvements in the selection criteria are still required, especially to refine cell phenotyping, particularly of PMN, when measuring SCC in different breeds.

Polymorphonuclear neutrophils are efficient phagocytes and release a large amount of reactive oxygen species (ROS) into phagocytic vacuoles to rapidly kill pathogens (Nguyen et al., 2017). Although PMN are generally described as an innate cell type with proinflammatory roles, some studies in humans and mouse models highlighted new phenotypes and functions for unconventional PMNs, such as suppression of T-cell proliferation (Mantovani et al., 2011; Pillay et al., 2013). In cattle, we recently characterized a new subset of PMN expressing major histocompatibility complex class II (MHC-II) molecules. These unconventional MHC-II^{POS} PMN were identified in bone marrow, spleen, and blood of healthy cows. Classical MHC-II^{NEG} PMN and MHC-II^{POS} PMN displayed distinct transcriptomic profiles and functions. Importantly, we also showed that MHC-II^{POS} PMN were able to inhibit the proliferation of T cells in vitro, whereas classical PMN were not. Because of this function, we proposed to name these “bovine regulatory PMN” (Rambault et al., 2021b).

The presence of regulatory PMN during infection remains to be investigated. Therefore, in the present study, our objective was to address this question in mastitis. We analyzed MHC-II^{NEG} and MHC-II^{POS} PMN subsets in blood and milk from healthy Normande and Holstein cows, as well as during clinical or subclinical mastitis in Holstein cows. We demonstrated that both subsets were present in milk from healthy cows and during mastitis, and we observed a higher bactericidal capacity of milk MHC-II^{POS} PMN compared with their classical counterparts. Interestingly, we also observed a positive correlation between counts of MHC-II^{POS} PMN and T cells in milk during subclinical mastitis.

MATERIALS AND METHODS

Dairy Herds

Three French dairy herds were recruited for this study. The first herd (**H1**), located in Normandy, was chosen because it housed both Holstein and Normande cows. The second herd (**H2**) was located in the INRAE Centre Val de Loire campus (45 lactating Holstein cows) and was chosen because of its proximity to the laboratory, which facilitated functional experiments and repetitions. The third (**H3**) was located in Maine-et-Loire department and was chosen to investigate neutrophils during the course of either clinical or subclinical mastitis. Because of its size (>100 lactating Holstein cows), we had access to numerous naturally occurring clinical and subclinical cases of mastitis during the time of our study. Trained staff and absence of a milking robot were determinant for inclusion of this herd.

All procedures were approved by local Ethics Committee (agreements APAFIS#29498–2021020410061759, APAFIS#26730–202007241825481, and APAFIS#23901–20200203095344). Our protocol did not interfere with farms' breeding practices. All animals used in our study remained in the herd with their herdmates, without any space or food restrictions.

Experimental Design and Justification of Sample Size

Blood and Milk Sampling from Healthy Holstein and Normande Cows. Healthy Holstein and Normande cows from H1 were all in early second lactation (60–94 d after calving). Milk was aseptically collected, plated on blood agar plates (Oxoid) and analyzed 24 and 48 h after incubation at 37°C to confirm the absence of infection in the mammary gland. Blood samples were collected in 10-mL Vacutainer K₂EDTA tubes (Becton Dickinson) for flow cytometry

and 4-mL Vacutainer K₃EDTA tubes for complete blood count. Based on the heterogeneity observed in terms of percentage of immune cell populations or phagocytosis in our previous experiments (Rambault et al., 2021b), a sample size calculator estimated the required size of our experimental groups to be at least 5 cows per breed, with a classical α error rate of 0.05 and a power of 80% to observe a difference of at least 20% between breeds. Accordingly, 2 independent experiments with 5 Holstein and 7 Normande cows were performed.

Collection of Milk for Functional Analysis of Neutrophils. Thirty milliliters of milk was collected aseptically from 7 Holstein cows (H2) without any signs of clinical mastitis. The sample size was set at $n \geq 6$ cows to allow a Wilcoxon signed-rank test to be carried out on paired samples (2 subsets of neutrophils from the same milk compared).

Collection of Samples from Cows with Clinical Mastitis. As soon as milking technicians identified a cow with a clinical mastitis (H3), the animal was recruited for the experiment: milk from the single infected quarter and blood were collected on the day of diagnosis (d 0; S1) and at 2 time points (S2 and S3 as illustrated in Figure 3A). After the diagnosis of clinical mastitis and the first milk sampling, antibiotics (Mastijet, MSD) was administered intramammarily during 4 consecutive milkings. Because of the heterogeneity of the clinical mastitis cases (pathogen diversity and level of inflammation), we analyzed 10 cows. Every animal was investigated independently.

Collection of Samples from Cows with Subclinical Mastitis. Quarter milk samples and blood samples were collected from 20 lactating cows (H3) suspected of having subclinical mastitis based on a composite milk SCC >300,000 cells/mL on the last milk cell count and with normal-appearing milk the week before. Individual quarters with SCC >150,000 cells/mL and a positive bacterial culture were considered to have subclinical mastitis. Sample size was justified by the heterogeneity of SCC. We performed 2 independent experiments with 10 cows each. Among the 20 cows tested, 3 had SCC <150,000 cells/mL in their 4 quarters and were excluded from the study.

Cell Preparation from Blood Samples

Vacutainer 10-mL K₂EDTA tubes were centrifuged at $1,000 \times g$ for 10 min at 20°C. Plasma and buffy coat were discarded, then ACK lysing buffer (Gibco; 5 vol/1 vol of blood cells) was added for 5 min at room temperature to lyse the red blood cells. Cells were washed twice in Dulbecco's PBS (D-PBS; without calcium and magnesium) with 2 mM EDTA.

Milk Samples

After cleaning and disinfection of the teat, the first 2 streams of milk were discarded and 30 mL of milk of each quarter was collected in sterile tubes. For animals with mastitis, milk was collected before any antibiotic treatment.

Microbial Culture and Bacterial Identification. Thirty microliters of milk from each quarter was plated on blood agar plates (Oxoid). Plates were incubated at 37°C and examined at 24 and 48 h. Bacterial identification was performed using conventional bacteriology techniques as described in the *Laboratory Handbook on Bovine Mastitis* (Adkins et al., 2017). If conventional bacteriology tests were not sufficient for bacterial identification, MALDI-TOF MS was performed. If 3 or more types of colonies were visible after incubation, the milk sample was considered contaminated and no identification was performed.

Preparation of Cells from Milk Samples. Milk SCC was determined using an automated cell counter (Fossomatic model 90, Foss Food Technology). Milk was diluted 2 times in D-PBS with 2% (vol/vol) horse normal serum (Gibco) and centrifuged at $1,000 \times g$ for 10 min at 4°C. The cream layer and supernatant were discarded and the cell pellet was resuspended and washed twice in D-PBS with 2% horse normal serum ($400 \times g$, 10 min, 4°C).

Flow Cytometry

Cells prepared from milk and blood samples were resuspended in D-PBS (without Ca and Mg) containing 10% (vol/vol) horse normal serum (Gibco) and 2 mM EDTA, and labeled for 30 min with primary antibodies (30 min, 4°C). After washes in D-PBS with 10% horse normal serum ($400 \times g$, 10 min, 4°C), cells were labeled with the corresponding fluorescent-conjugated secondary antibodies. Two panels of antibodies were designed for the analysis of markers of myeloid and lymphoid cells. All antibodies used are listed in Table 1. Cells were washed and fixed with BD cell fix solution (Becton Dickinson) diluted 4 times in D-PBS (without Ca and Mg). Data were acquired with a LSR Fortessa X-20 Flow cytometer (Becton Dickinson) and results were analyzed with the Kaluza software (Beckman Coulter).

Phagocytosis

Following the labeling of cells with antibodies listed in Table 1, 2 methods were used to assess the phagocytosis capacity of PMN.

pHrodo Red Escherichia coli BioParticles. Phagocytosis was measured using pHrodo Red *Esch-*

Table 1. Antibodies used for flow cytometry analysis

Antibody	Target ¹	Reactivity	Clone	Supplier	Isotype	Dye ²
Primary antibodies	Granulocytes (G1)	Bovine	CH138A	KingFisher Biotech	Mouse IgM	—
	MHC-II	Feline	CAT82A	KingFisher Biotech	Mouse IgG ₁	—
	CD14	Human	CC-G33	Bio-Rad	Mouse IgG ₁	PE–Alexa Fluor 750
	CD45	Sheep	1.11.32	Bio-Rad	Mouse IgG ₁	PE
	CD3	Bovine	MM1A	KingFisher Biotech	Mouse IgG ₁	—
Secondary antibodies	IgM	Mouse	—	Invitrogen	Goat IgG	Alexa Fluor 647
	IgG ₁	Mouse	—	Invitrogen	Goat IgG	Alexa Fluor 488
	IgG ₁	Mouse	—	Invitrogen	Goat IgG	PE
	Fixable viability dye (FVD)	—	—	BioLegend	—	Zombie aqua
	FVD	—	—	eBiosciences	—	efluor 780

¹CD14 and CD45 antibodies are directly conjugated, whereas secondary antibodies were used to measure G1, MHC-II, and CD3 expression.

²PE = phycoerythrin.

erichia coli BioParticles Conjugate (Molecular Probes) following the manufacturer's instructions. Briefly, 10⁵ cells/well were incubated in a 96-well microplate for 1 h at 37°C or 4°C (negative control) with 20 µg/well of pHrodo *E. coli* BioParticles in RPMI-1640 supplemented with 2 mM L-glutamine, 10 mM HEPES, and 1 mg/mL BSA with extremely low endotoxin level (≤ 1.0 endotoxin units/mg; hereafter referred to as RPMI complete medium). The fluorescence linked to ingestion of the *E. coli* BioParticles was directly measured with the LSR Fortessa X-20 Flow cytometer.

Green Fluorescent Protein-*E. coli* P4. *Escherichia coli* strain P4 was transformed with the *gfp*-carrying plasmid pFPV25.1 (Bramley, 1976; Valdivia and Falkow, 1996). Transformants were grown in brain-heart infusion (BHI) medium in the presence of 100 µg/mL ampicillin. Then, we used the *E. coli* P4-green fluorescent protein (GFP) strain to test the phagocytosis capacity of PMN. The *E. coli* P4-GFP strain was grown in 10 mL of BHI medium with 100 µg/mL ampicillin overnight at 37°C without agitation. Bacteria were then diluted in BHI medium (1 vol/100 vol) and incubated for 6 h at 37°C without agitation. Bacterial concentration was determined by optical density measurement at 600 nm and adjusted to 8 × 10⁶ cfu/mL in RPMI complete medium. After labeling of milk cells, 2 × 10⁵ cells were plated in a 96-well microplate and infected at a multiplicity of infection (MOI) of 2 in RPMI complete medium for 30 min at 37°C or 4°C.

ROS Production

Reactive oxygen species produced by PMN were quantified using the CellROX Orange Flow Cytometry Assay Kits (C10493; Molecular Probes) following the manufacturer's instructions. Briefly, 10⁵ milk cells were first incubated for 1 h at 37°C in RPMI complete medium with or without 400 µM *tert*-butyl hydroperoxide (TBHP) in a 96-well black microplate and then for 30

min with 100 nM CellROX and 1 µL/mL eFluor780 fixable viability dye. Fluorescence was directly measured with the LSR Fortessa X-20 flow cytometer.

Statistical Analysis

Individual data and medians are presented in the figures. Statistical analyses were performed with R Studio (<https://www.rstudio.com/>) and Prism 6.0 software (GraphPad) using nonparametric tests because of small sample sizes. Wilcoxon signed-rank test and Spearman correlation test were used. Represented *P*-values were indicated as follows: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

RESULTS

MHC-II^{pos} PMN with High Phagocytic Capacity and ROS Production Circulate in Blood of Healthy Holstein and Normande Cows

To better characterize PMN subsets in dairy cows, we analyzed their proportions in the blood of healthy Holstein and Normande cows, the 2 main dairy breeds in western France. To limit physiological variations due to different stages of lactation, we selected cows within the first trimester of the second lactation. We then analyzed MHC-II^{pos} and MHC-II^{neg} PMN according to the gating strategy, as described in our previous study (Rambault et al., 2021b). We did not observe any difference between the 2 breeds for the ratio of total PMN circulating in blood (Figure 1A). Similarly, the ratio of MHC-II^{pos} among total PMN was comparable for the 2 breeds (Figure 1B).

We next compared by flow cytometry the phagocytic activity of PMN from Holstein and Normande cows using conjugated pHrodo Red *E. coli* BioParticles (Rambault et al., 2021b), allowing us to quantify phagocytosis by mean fluorescence intensity as a direct

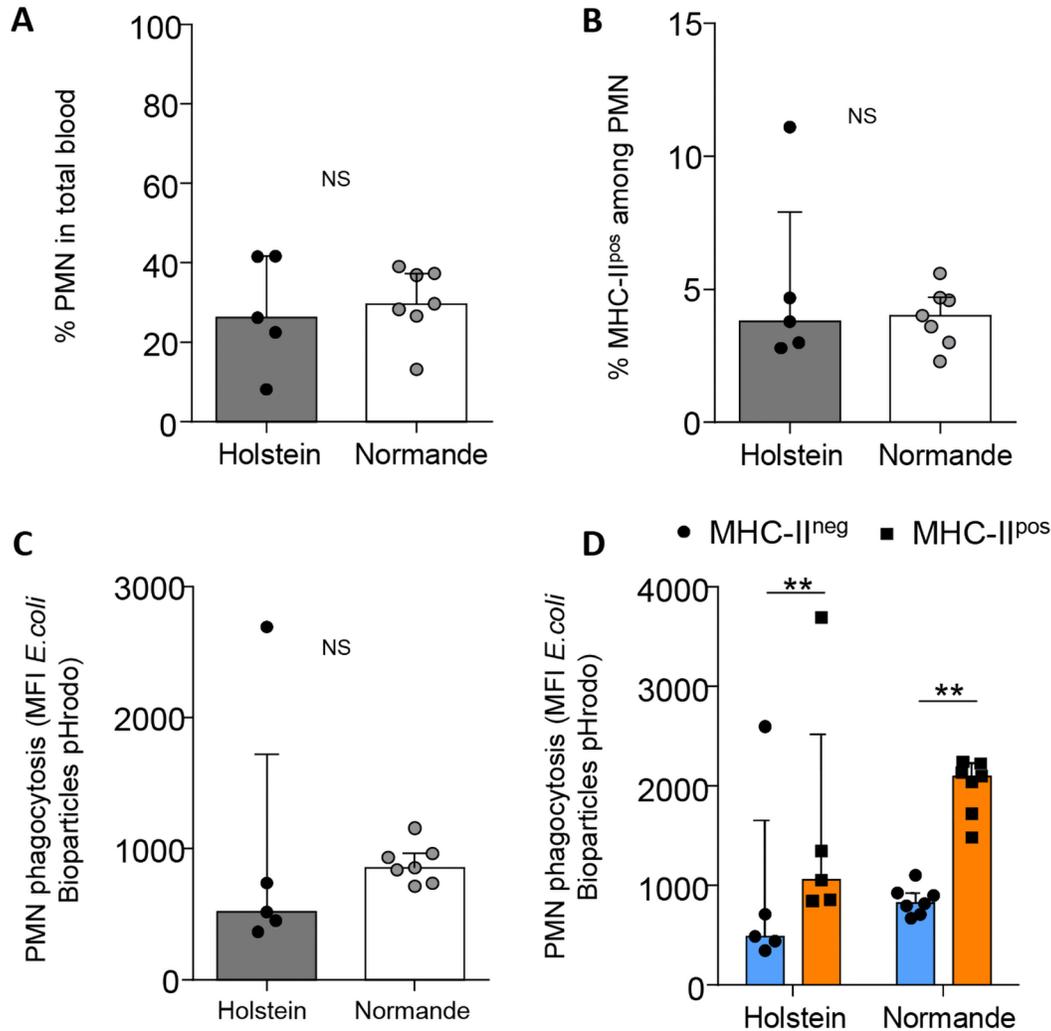


Figure 1. Blood neutrophils expressing major histocompatibility complex class II (MHC-II^{pos} PMN) display better phagocytosis activity than MHC-II^{neg} (i.e., not expressing MHC-II) PMN in Holstein and Normande cows. Cells were prepared from blood from the 2 breeds. (A) Proportion of total PMN in blood was determined by complete blood count, and (B) percentage of MHC-II^{pos} PMN was determined by flow cytometry among CD45+ G1+ live cells. (C, D) Phagocytosis of pHrodo *Escherichia coli* BioParticles by total PMN (C) or MHC-II^{pos} and MHC-II^{neg} PMN was assessed by measuring fluorescence intensity directly correlated with ingested particles. (A–D) Data are medians with interquartile range (n = 5 Holstein, n = 7 Normande); data are representative of 1 experiment over 2 independent experiments. **P < 0.01 (Wilcoxon signed-rank test). MFI = mean fluorescence intensity.

correlation of ingested particles. Phagocytosis by total PMN was comparable for both breeds (Figure 1C). Interestingly, for both breeds, MHC-II^{pos} PMN were significantly more efficient phagocytes than MHC-II^{neg} PMN (Figure 1D).

MHC-II^{pos} PMN with High Microbicidal Activities Are Recruited to the Mammary Gland

It is established that PMN represent the largest proportion of cells in SCC during mastitis. However, the presence of different subsets of PMN in milk has not been investigated. Because we did not observe any difference between breeds, we focused our analysis on milk

Holstein cows, the most represented breed in French dairy herds. We analyzed the presence of MHC-II^{neg} and MHC-II^{pos} PMN in milk according to the gating strategy described in Figure 2A. As in blood, the MHC-II^{pos} PMN subset was observed in milk. Next, we compared phagocytosis by the 2 subsets of milk PMN using an *E. coli* P4 strain expressing GFP. After incubation of milk cells with *E. coli* P4-GFP at 37°C, we analyzed by flow cytometry the percentage of the 2 subsets of PMN carrying green fluorescence. We normalized this ratio on cells stimulated with *E. coli* P4-GFP at 4°C where phagocytosis is blocked (Peterson et al., 1977). As in blood, milk MHC-II^{pos} PMN phagocytosed significantly more bacteria than MHC-II^{neg} PMN ($P =$

0.0156; Figure 2B). As ROS production is an important mechanism used by PMN to kill bacteria, we also measured the potential of milk cells to produce these bactericidal molecules after incubation with the nonspecific chemical inducer TBHP. Milk MHC-II^{pos} PMN produced significantly more ROS than MHC-II^{neg} PMN ($P = 0.0078$; Figure 2C). Therefore, as observed in blood, MHC-II^{pos} PMN recruited to the mammary gland are better equipped with bactericidal arsenal compared with their classical MHC-II^{neg} counterparts.

Ratios of MHC-II^{pos} and MHC-II^{neg} PMN Circulating in Blood and Recruited in Milk Do Not Vary During the Course of Clinical Mastitis

Because total PMN in SCC are a marker of mastitis, we next addressed how the proportion of MHC-II^{neg} and MHC-II^{pos} subsets varied during clinical mastitis. To do that, we followed 10 cows as soon as clinical mastitis was detected. We sampled blood and milk from the infected quarter at 3 different time points: S1 (d 0), S2 (between d 3 and 6), and S3 (d 21) (Figure 3A). According to animal care protocols, antibiotic treatment was administered into the mammary gland after milk sampling at d 0. Most clinical mastitis cases were associated with mild symptoms, and only gram-positive bacteria were detected after microbial culture (Table 2). Two bacterial identifications were unsuccessful due to milk contamination, and no bacteria were detected in one milk sample. As generally observed for clinical mastitis, inflammation signs at S1 were high and SCC ranged from $1,000 \times 10^3$ to $35,000 \times 10^3$ cells/mL. At S2, from 3 to 6 d after antibiotic treatment, milk SCC was generally lower: 4 quarters had $<300,000$ cells/mL, of which 3 had negative microbial culture. At S3 (d 21), milk SCC from 6 quarters were $<300,000$ cells/mL and had negative microbial cultures, indicating recovery. When bacteria were detected in S2 or S3 milk samples, they belonged to the same species as that isolated from S1 (4/10 cows).

Next, we thoroughly analyzed blood and milk SCC composition by flow cytometry and designed 3 antibody labeling panels and gating strategies to distinguish key cells involved in mastitis physiopathology, including the 2 PMN subsets (Figure 3B). The myeloid panel allowed us to distinguish the 2 PMN subsets from macrophages, and the lymphoid panel informed us on CD3 lymphocytes. We could not perform flow cytometry analysis on 2 S3 milk samples because the SCC was too low. As expected, in S1 milk samples, PMN were the main cell type and low proportions of macrophages and T lymphocytes were detected (Figure 3C). In S2 and S3, ratios of the different cell types were relatively more heterogeneous. In our analysis, we distinguished

samples with SCC above the threshold of 300,000 cells/mL, indicating that the episode of clinical mastitis was still ongoing (Figure 3C, red dots), from samples with a quarter SCC below the threshold (Figure 3C, blue dots). In S2 and S3, PMN were still the main cell type in most samples. However, in 3 S2 and 4 S3 milk samples, PMN ratios started to decrease, which correlated with SCC below the threshold of 300,000 cells/mL. The samples with lowest SCC had also the highest proportions of macrophages, suggesting that the mammary gland was returning to homeostasis. Interestingly, no difference in proportion of T lymphocytes was observed between the 3 time points. MHC-II^{pos} PMN were present in blood and milk at each time point (Figure 3D). There was a trend toward higher MHC-II^{pos} to MHC-II^{neg} PMN ratios in milk than in blood, although this did not reach significance due to high heterogeneity. We could not correlate the MHC-II^{pos} to MHC-II^{neg} PMN ratios with the threshold of 300,000 cells/mL in SCC. Moreover, the proportion of MHC-II^{pos} PMN did not seem to be related to bacterial clearance (see Table 2). In conclusion, even though we detected the new subset of MHC-II^{pos} regulatory neutrophils in blood and milk during each clinical mastitis case, we could not correlate their presence to the level of inflammation, the sampling time (onset vs. treated mastitis), or bacterial clearance.

MHC-II^{pos} Regulatory PMN Positively Correlate with T Cells in Milk During Subclinical Mastitis

Subclinical mastitis is characterized by the absence of visible signs of inflammation of the mammary gland. However, PMN still account for most cells in the milk SCC. Thus, we investigated whether MHC-II^{pos} PMN were present in milk and blood during subclinical forms of mastitis. To do so, we selected 20 Holstein cows presenting a composite milk SCC $>300,000$ cells/mL the week before our experiments, based on information provided by monthly milk quality control. On the day of the experiment, milk samples were collected from individual quarters. All milk samples had normal appearance and, after microbial culture and SCC, subclinical mastitis in analyzed quarters was ascertained based on positive microbial culture and SCC $>150,000$ cells/mL. Three cows showed no sign of subclinical mastitis and were excluded. Among the 17 remaining animals sampled (i.e., 68 quarters), 27 quarters were classified with subclinical mastitis with SCC ranging from 168×10^3 to $28,858 \times 10^3$ (Table 3). Proportions of different leucocytes present in blood and milk were analyzed by flow cytometry (see Figure 3B for gating strategy). As expected, total PMN were still the main cell type among total milk leukocytes during subclinical masti-

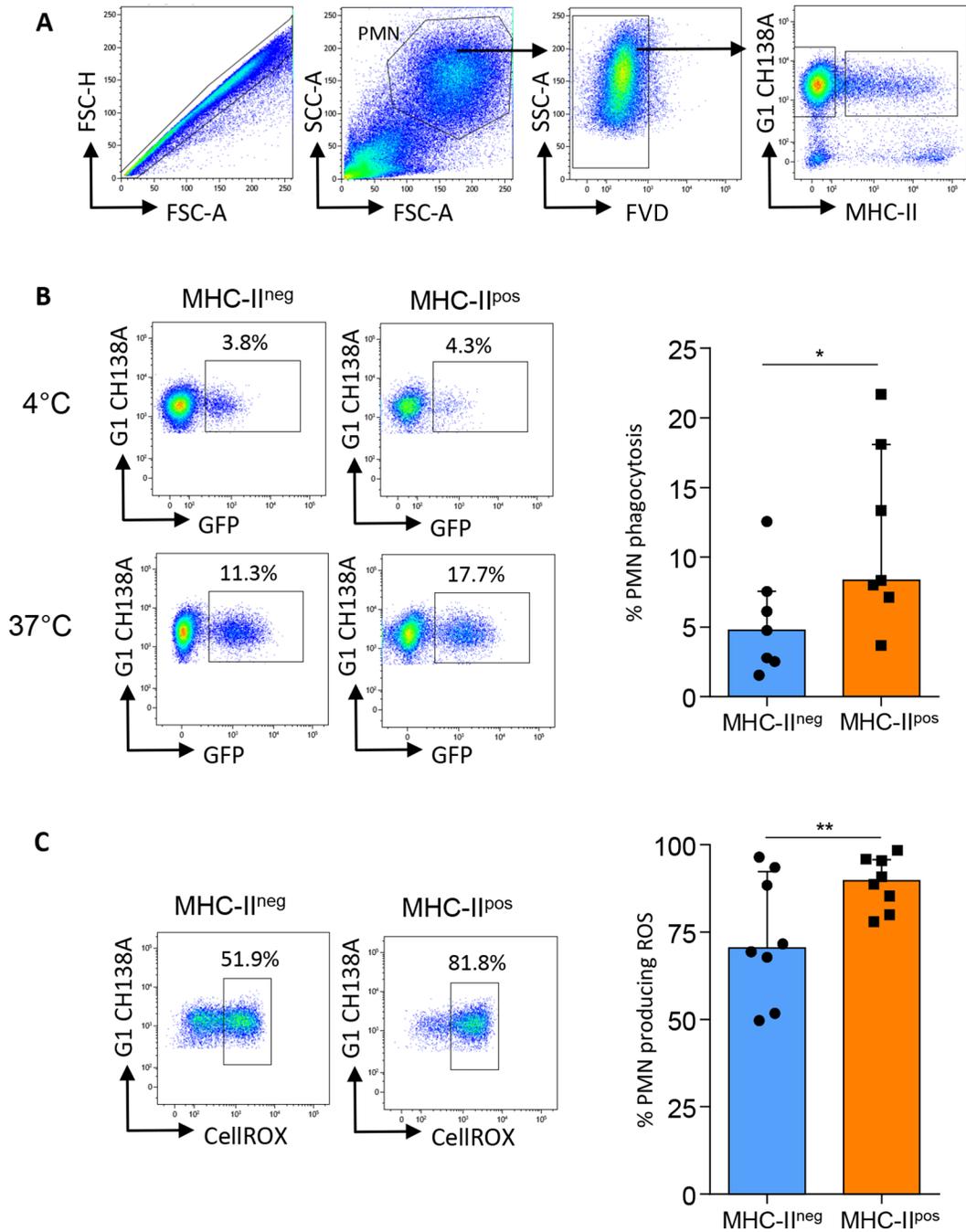


Figure 2. Neutrophils expressing major histocompatibility complex class II (MHC-II^{pos} PMN) have higher bactericidal capacity than MHC-II^{neg} (i.e., not expressing MHC-II) PMN. (A) Flow cytometry gating strategy used to identify MHC-II^{neg} and MHC-II^{pos} PMN in milk. (B) After labeling milk cells, phagocytosis by MHC-II^{pos} or MHC-II^{neg} PMN was assessed using the *Escherichia coli* P4-GFP (green fluorescent protein) strain at 37°C. Phagocytosis by PMN was normalized on PMN stimulated with *E. coli* P4-GFP at 4°C. Data represent n = 2 experiments with n = 7 cows. (C) Oxidative stress was measured in MHC-II^{pos} and MHC-II^{neg} neutrophils using the CellROX Orange probe, which reacts with all reactive oxygen species (ROS). Cells were activated with *tert*-butyl hydroperoxide (TBHP), and levels of ROS were measured by flow cytometry. Data represent n = 2 experiments with n = 8 cows. (B, C) Fluorescence for each sample is depicted, and paired MHC-II^{pos} and MHC-II^{neg} samples were analyzed for each animal. Data are medians with interquartile range. FSC-A = forward scatter, area; FSC-H = forward scatter, height; SSC-A = side scatter, area; FVD = fixable viability dye. Dots = MHC-II^{neg} PMN; squares = MHC-II^{pos} PMN. **P* < 0.05; ***P* < 0.01 (Wilcoxon signed-rank test).

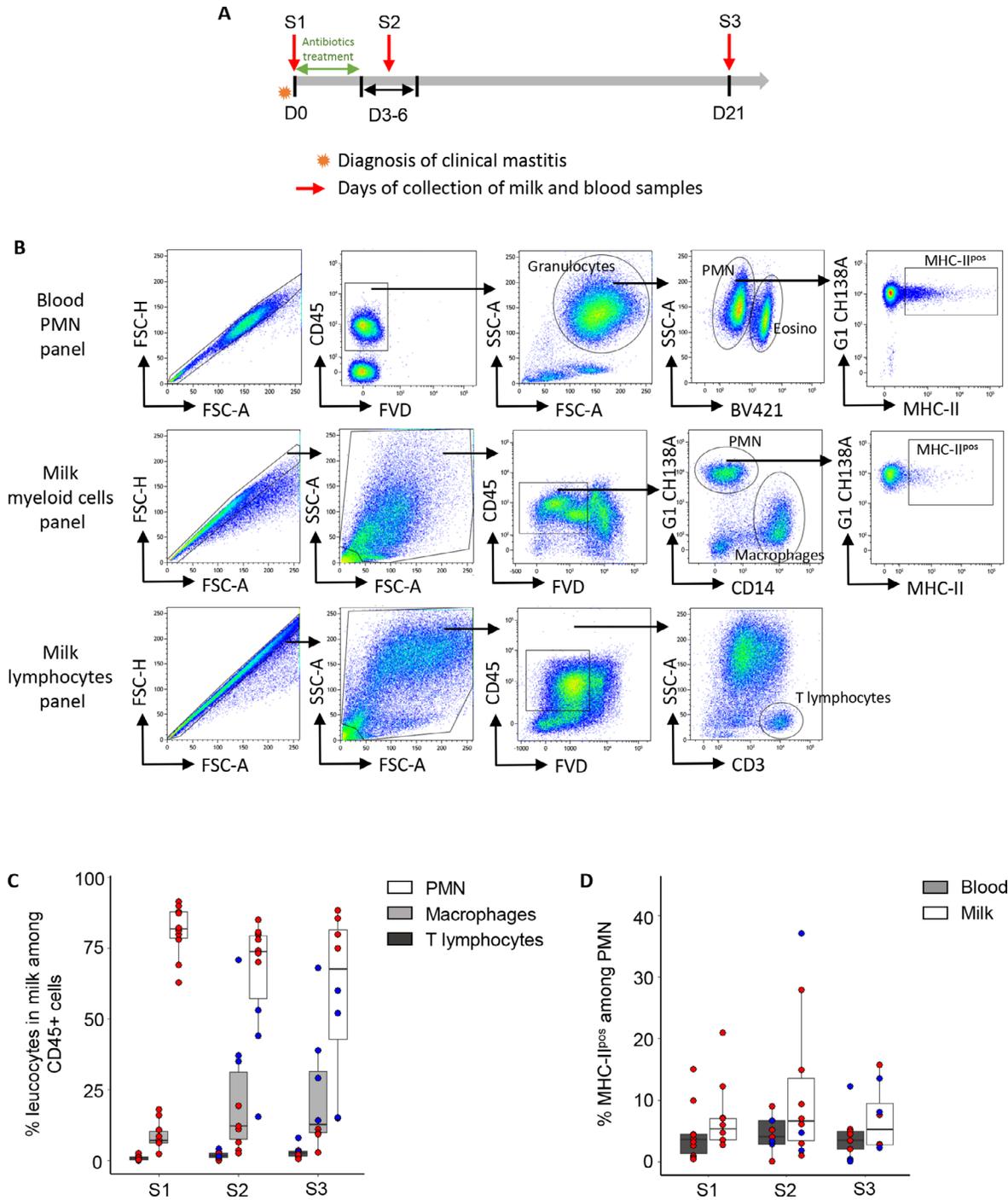


Figure 3. Neutrophils expressing major histocompatibility complex class II (MHC-II^{pos} PMN) are present in blood and milk during clinical mastitis and their proportion does not vary over time. (A) Description of sampling protocol. Blood and milk samples from the infected quarter (S1) were immediately collected after the diagnosis of the clinical mastitis. After antibiotic treatment for 5 milkings of the infected quarter, second blood and milk samples (S2) were collected between d 3 and 6 (the day was chosen to avoid weekends), and third blood and milk samples (S3) were collected at d 21. (B) Flow cytometry gating strategy used to identify different cells populations in blood and in milk. Blood MHC-II^{pos} PMN are defined as CD45⁺ SSC^{high} FSC^{high} G1⁺ MHC-II⁺. Eosinophils were excluded by excitation with violet laser in the “granulocytes” gate. Milk PMN, macrophages, and T lymphocytes are defined as CD45⁺ G1⁺ CD14⁻, CD45⁺ G1⁻ CD14⁺, and CD45⁺ CD3⁺, respectively. Milk MHC-II^{pos} PMN are defined as MHC-II⁺ in the “PMN” gate. (C) Percentage of PMN, macrophages, and T lymphocytes in milk at each sample collection time. Red dots = milks with SCC >300,000 cells/mL; blue dots = milks <300,000 cells/mL. (D) Percentage of MHC-II^{pos} PMN in blood and milk at each time point. (C, D) Data are boxes, and whiskers show minimum to maximum range of values (n = 10 cows and 27 independent experiments). FSC-A = forward scatter, area; FSC-H = forward scatter, height; SSC-A = side scatter, area; FVD = fixable viability dye.

Table 2. Description of clinical mastitis cases in samples collected at diagnosis (d 0; S1), after antibiotic treatment (d 3 to 6; S2), and at d 21 (S3)

Case	Lactation stage ¹	Lactation number	Pathogen	Clinical grade ²	S1		S2		S3	
					SCC × 10 ³	Microbial culture ³	SCC × 10 ³	Microbial culture	SCC × 10 ³	Microbial culture
CM1	Near calving	7	<i>Staphylococcus aureus</i>	1	8,400	+	10,500	-	1900	-
CM2	Early	7	<i>Enterococcus</i> spp.	1	8,000	+	632	-	83	-
CM3	End	2	<i>Streptococcus uberis</i>	1	4,800	+	1,500	+	14,600	+
CM4	End	1	<i>Staphylococcus arlettae</i> ⁴	1	3,400	+	6,000	-	13,200	+
CM5	Early	5	Contaminated sample	1	6,400	+	63	-	7	+
CM6	Mid	3	<i>Staph. aureus</i>	2	35,000	+	31,000	+	24,000	+
CM7	End	7	No bacteria	2	9,000	-	257	-	81	-
CM8	Near calving	1	<i>Strep. uberis</i>	1	26,500	+	6,300	+	78	-
CM9	End	4	Contaminated sample	1	1000	+	316	+	41	-
CM10	Early	1	<i>Strep. uberis</i>	1	5,200	+	78	-	45	-

¹Lactation stage: Near calving = <7 d after calving, Early = 7 < days after calving <100, Mid = 100 < days after calving <200, Late = >200 d after calving.

²Clinical grade: 1 = slight damage (lumps in milk only); 2 = moderate damage (swollen and painful udder).

³Microbial culture: + indicates presence of colonies on blood agar, - indicates no colonies on blood agar.

⁴Bacterial identification by MALDI-TOF MS.

Table 3. Bacterial identification and SCC of subclinical mastitis cases

Case	Quarter ¹	SCC × 10 ³	Pathogen(s)
SM1	LR	4,705	CNS
	LF	423	CNS
SM2	RR	699	Contaminated sample ²
SM3	LR	842	CNS
	LF	627	CNS
SM4	RR	2,703	CNS
	LR	759	CNS
	RF	1,066	CNS
SM5	LF	677	<i>Corynebacterium</i> spp.
	LF	981	Yeast
SM6	RR	440	CNS + <i>Enterococcus</i> spp.
	LF	508	CNS + <i>Corynebacterium</i> spp.
SM7	LF	2,769	CNS
SM8	LR	9,389	<i>Streptococcus uberis</i>
	LF	311	No bacteria
SM9	RR	2,966	CNS
SM10	LR	28,858	<i>Staphylococcus arlettae</i>
SM11	LR	169	CNS
	RR	5,576	<i>Staphylococcus aureus</i>
SM12	RF	203	<i>Staph. aureus</i>
SM13	LR	3,457	<i>Staph. aureus</i>
SM14	RF	1,738	Contaminated sample
SM15	LR	2,618	No bacteria
SM16	RF	915	<i>Staph. aureus</i>
	LR	361	No bacteria
SM17	LR	606	<i>Staph. aureus</i>
	RR	491	<i>Staph. aureus</i>

¹LF = left front; RF = right front; LR = left rear; RR = right rear.

²Contaminated sample = 3 or more types of colonies.

tis, whereas lower proportions of macrophages and T lymphocytes were observed (Figure 4A). Interestingly, among total PMN, the MHC-II^{POS} subset was present in a significantly higher proportion in milk than in blood (Figure 4B, $P = 0.0042$). Thus, contrary to what was observed during clinical mastitis, the regulatory PMN subset seemed to accumulate in milk during subclinical mastitis. Moreover, we observed a positive and highly significant correlation between counts of MHC-II^{POS} PMN and T lymphocytes present in milk (Figure 4C, $P < 0.0001$).

DISCUSSION

In agreement with a long record of data on mastitis and follow-up of high milk SCC as a marker of mammary gland inflammation, we observed in our study that total PMN represented the most abundant leucocytes in milk on the day of clinical mastitis diagnosis (Leitner et al., 2000; Schwarz et al., 2011). However, we described here that, in blood as in milk, PMN during mastitis are a heterogeneous population and encompass at least 2 subsets. In humans and mice, PMN heterogeneity is now well established (Silvestre-Roig et al., 2016; Li et al., 2019). In cattle, despite PMN being the main cell type recruited to the mammary gland during mastitis, they are still simply described as G1-positive

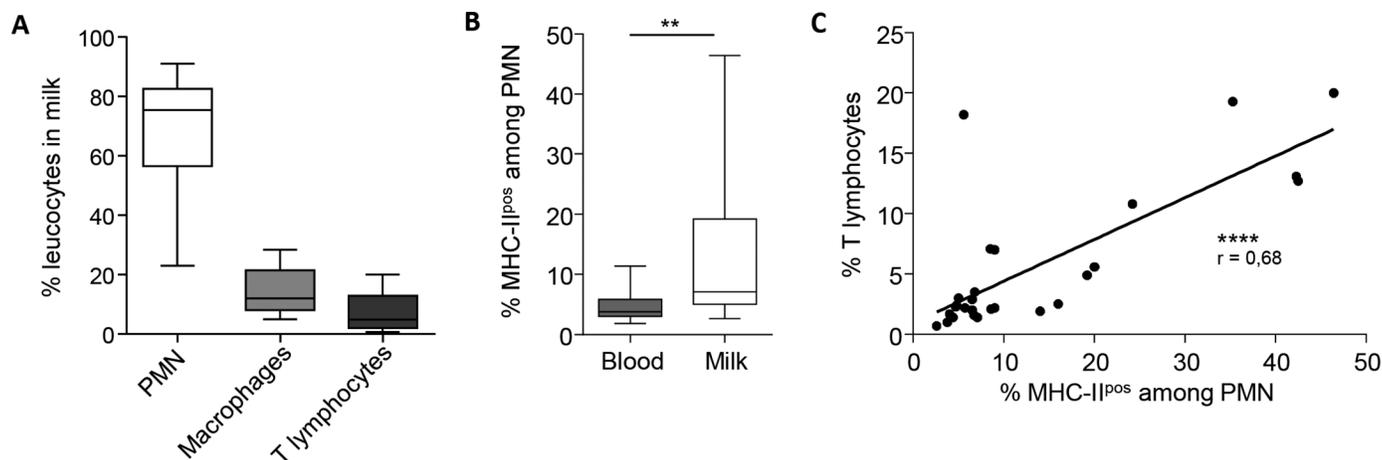


Figure 4. Neutrophils expressing major histocompatibility complex class II (MHC-II^{pos} PMN) accumulate in milk during subclinical mastitis and correlate with T cells. (A) Percentage of PMN, macrophages, and T lymphocytes among CD45+ cells in milk during subclinical mastitis. (B) Percentage of MHC-II^{pos} PMN in blood and in milk during subclinical mastitis. (A, B) Data are boxes and whiskers show minimum to maximum range of values. (C) Correlation between the percentage of T lymphocytes and MHC-II^{pos} PMN in milk during subclinical mastitis. Data represent n = 2 independent experiments with n = 27 quarters from 17 cows. ** $P < 0.01$; **** $P < 0.0001$ (Wilcoxon signed-rank test, B; Spearman correlation test with 95% CI, C).

cells (anti-granulocyte CH138A antibody; Leitner et al., 2000, 2003; Piepers et al., 2009). We recently described in healthy cattle a new population of regulatory PMN characterized by MHC-II expression and T-cell suppression capacity (Rambault et al., 2021b). Here, we show that this same population is present in milk during clinical and subclinical mastitis. Of note, the proportion of milk MHC-II^{pos} PMN was highly variable in both forms of the disease and a possible explanation is the diversity of pathogens involved inducing different levels of inflammation between animals. Similarly, it is worth pointing out that not all clinical mastitis cases were cured at d 21 as pathogens were sometimes still detected in milk with remaining high SCC. These heterogeneous clinical situations might also explain our variable results on the PMN MHC-II^{pos} versus MHC-II^{neg} ratio. For future investigations, it would be interesting to include additional time points to see whether the MHC-II^{pos} subset increases in proportion when the acute phase of the mastitis episode has passed. Indeed, during our study of clinical mastitis limited to 21 d, MHC-II^{pos} PMN were observed at each sampling time point—at onset of the disease or after antibiotic treatment and beginning of recovery—in blood and milk, and we could not correlate their presence with the course of the disease.

Among differences in functions between the 2 subsets, we demonstrated here that MHC-II^{pos} PMN had higher phagocytic and ROS production capacities compared with classical MHC-II^{neg} PMN. In our previous report, we observed that blood MHC-II^{pos} PMN were higher producers of ROS than their MHC-II^{neg}

counterparts, but we did not observe any difference in phagocytosis (Rambault et al., 2021b). This apparent discrepancy can be explained by the different methods used in the 2 studies. In our previous work, we performed the phagocytosis assay on PMN purified by fluorescence-activated cell sorting, a process that triggers cell activation. In contrast, in the present study, we worked with total milk or blood cells to assess phagocytosis while avoiding PMN activation by cell sorting. This simpler and less harsh method for PMN purification allowed us to confirm that blood MHC-II^{pos} PMN had higher phagocytosis power than MHC-II^{neg} PMN. Moreover, we could also demonstrate by using an *E. coli* strain expressing GFP that superior phagocytosis by MHC-II^{pos} PMN compared with MHC-II^{neg} was retained after recruitment to the mammary gland. Similar results were obtained using pHrodo *E. coli* BioParticles (data not shown). These data support that PMN are the most important killer of bacteria invading the mammary gland during mastitis, and early control of infection mainly relies on this population, especially during infection with *E. coli* or *Staphylococcus aureus*, which are widespread mastitis pathogens (Rainard and Riollet, 2003). Therefore, a better understanding of the proportion, kinetics, and level of recruitment, as well as regulation of different PMN subsets recruited to the mammary gland could help to implement better control measures, for example, by introducing such markers in genetic selection programs. More work is needed and based on the low number of animals that we analyzed in this study, we could not correlate the levels of MHC-II^{neg} versus MHC-II^{pos} PMN with differential control of

bacteria multiplication in the mammary gland during the longitudinal study of clinical mastitis.

We observed that MHC-II^{POS} PMN reached milk during both clinical and subclinical forms of mastitis. MHC-II^{POS} PMN accumulated in high numbers in milk compared with those circulating in blood, particularly during subclinical mastitis. We cannot explain the reason for such a finding. Interestingly, we observed during this work that animals with the highest proportion of MHC-II^{POS} PMN in milk were those with repetitive high milk SCC recorded over several weeks or months (data not shown), which could suggest that this subset was associated with chronicity of mastitis. However, this interpretation warrants caution. Thus, because we did not have the information about SCC per quarter for these animals, we cannot accurately conclude that MHC-II^{POS} PMN numbers in each quarter were correlated with mastitis caused by the same pathogen in the same quarter. The highly significant correlation of MHC-II^{POS} PMN with T lymphocytes in milk, along with the capacity of blood MHC-II^{POS} PMN to suppress T-cell proliferation (Rambault et al., 2021b), supports the hypothesis that MHC-II^{POS} PMN may be involved in control of the adaptive immune response in the long term and are associated with chronic infection. Unfortunately, for technical reasons, we could not demonstrate that milk MHC-II^{POS} PMN suppressed T-cell proliferation. We attempted to sort MHC-II^{POS} PMN from milk both by flow cytometry and incubation with magnetic beads (Rambault et al., 2021a) but the high activation state and poor viability status of the cells after such procedures did not lead to conclusive data.

Several studies have reported that human PMN expressing MHC-II behave as antigen-presenting cells (Culshaw et al., 2008; Abi Abdallah et al., 2011). Blood PMN expressing MHC-II have also been reported in human patients with chronic inflammatory disease but not during acute bacterial infections (Iking-Konert et al., 2002). Altogether, this suggests that MHC-II^{POS} are involved in chronic mastitis or modeling of the adaptive immune phase of the immune response by interacting with T cells.

Milk control is routinely applied in animal facilities or farms, and abnormal SCC is one parameter to inform about animals' inflammatory status. Currently, differential SCC, allowing a more precise characterization of milk leukocytes, is being investigated as a new tool for mastitis diagnosis (Schwarz et al., 2011; Damm et al., 2017; Gonçalves et al., 2017). The proportion of leukocyte populations provides more precise information about the inflammatory status of the mammary gland, but the description of cell types is limited to total PMN, macrophages, and lymphocytes, with no information on specific subsets (Pilla et al., 2012; God-

den et al., 2017; Kirkeby et al., 2020). A better understanding of the involvement of MHC-II^{POS} neutrophils in mastitis would improve differential counts and pave the way for identification of potential biomarkers of chronic mastitis.

To conclude, we believe that the diversity of phenotypes and functions of PMN needs to be further investigated and we advocate that the classical description of PMN as a homogeneous cell type in cattle must be revisited. Mastitis remains one of the most important issues for dairy farming; thus, investigating how MHC-II^{POS} PMN behave during chronic mastitis would bring new views on the pathophysiology of this costly disease. Our study also suggests that new tools such as differential counting used for mastitis diagnosis can be improved with a more accurate characterization of PMN subsets. This could also open the way to the discovery of new biomarkers of chronic inflammation. Future studies should focus on deciphering the function of MHC-II^{POS} neutrophils, on whether their presence, because of their high microbicidal capacities, correlates with a good prognosis for mastitis or whether they contribute to mastitis chronicity by interfering with T-cell responses. Answering these questions will provide a new basis for the improvement of current diagnostic tools for breeders and could help producers make antibiotic treatment decisions, especially at the dry-off period.

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