Journal of Dairy Research (2018) **85** 167–173. © *Hannah Dairy Research Foundation 2018*
doi:10.1017/S0022029918000316

Effects of herd and physiological status on variation of 16 immunological and inflammatory parameters in dairy cows during drying off and the transition period

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Received 29 May 2017; accepted for publication 9 April 2018

During drying off and transition period, cows are subject to changes in endocrine status, metabolic stressors and altered immune functions, which could lead to an increased risk of disease. To expand our knowledge on the immune/inflammatory status and to identify markers to define cow status during this interval, the pattern of 9 different cellular parameters, 5 cytokines, 2 enzymes and 3 cellular ratios in blood samples were assessed in 15 primiparous cows belonging to three different dairy herds in Lombardy. Our data showed that the variation of almost all parameters was influenced by the physiological period in which the samples were collected, except for apoptosis, IL-1β, IL-6, lysozyme and granulocyte/monocyte ratio. Several markers were directly correlated either to the herd alone (IL-1β, IL-6, lysozyme, granulocyte/lymphocyte ratio and granulocyte/monocyte ratio) or in association with the sampling time (white blood cell count, necrosis, lymphocytes count, $CD4^+$ lymphocytes proportion). Hierarchical cluster analysis identified three herd-associated sample clusters showing different frequency along the follow-up period. The results of this field study highlight the importance of the herd factor in the immune/inflammatory response. Furthermore, these results suggest that cellular parameters are probably the most suitable markers to define cow status during drying-off and the peripartum period.

Keywords: Dairy cows, transition period, drying off, immunity, markers.

Drying off is a very peculiar and important period for dairy cows. In this period, milk production ceases, secreting epithelial tissue renovates and the mammary gland prepares for the next lactation. In the last part of the dry period, the so-called 'transition' period starts. This period is defined as the three weeks before and three weeks after parturition, and represents a time of physiological stress for the dairy cows (Goff & Horst, 1997). During drying off and the transition period, cows are subject to major changes in endocrine status, metabolic stressors and altered immune function, that could entail an increased risk of disease (Jonsson et al. 2013). Considerable research has been conducted into the changes in immune function that occur around the time of calving, mostly from the perspective that these changes might increase susceptibility to periparturient infectious diseases (Goff & Horst, 1997; Karcher et al. 2008) such as rumen acidosis, milk fever, lameness, metritis, and mastitis (Drackley, 1999). Moreover, multiple stressors can reroute resources like amino acids and energy, causing immune system depression, especially if they are severe and prolonged (Drackley et al. 2005). Together with the immune competence reduction, transition cows were also shown to display an overt inflammatory response related to pregnancy and lactation, even without signs of infection and/or other pathology (Bionaz et al. 2007; Bertoni et al. 2008; Trevisi et al. 2010, 2012). This can increase the metabolic stress through alteration of the host's immune defenses, thus causing a vicious circle. However, previous field studies of milk and blood immune and inflammatory response parameters during periparturient period are influenced by herd (Piccinini et al. 2004, 2005), suggesting that metabolic adaptation in the animal as well as

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https://doi.org/10.1017/S0022029918000316 Published online by Cambridge University Press

management of the herd could have an influence on cow immune and inflammatory response. For all these reasons, identifying accurate markers and their patterns to define the immune and inflammatory status of the healthy cow during drying-off and transition period would be very useful to increase our knowledge on cow response during these important parts of its productive life. Therefore, the purpose of this study was to determine several humoral and cellular parameters at specific time points during the drying off and transition period in 15 healthy primiparous cows from three dairy herds in Lombardy, and identifying the effects of physiological status and herd on the immune and inflammatory response of these cows.

Materials and methods

Herd and cow selection

Herds were selected based on their proximity to Milan to assure the delivery of samples in the shortest time. All herds kept cows indoors and applied total mixed rations based on corn silage all year-round. In these herds, all cows entering drying off period received local antibiotic treatment with a dry-cow product. The inclusion criteria for cows in their first lactation were to be pregnant, with parturition expected in 3 months-time, to be of the same breed (Italian Holstein-Friesian), and to be clinically healthy, without receiving any treatments or vaccines after breeding. In each herd, five cows were selected at random among the eligible ones.

Sampling

Cows were sampled from 7 d before drying off up to 4 weeks post-calving. A weekly sampling frequency was adopted with the exception of the period between 7d before expected date of calving and 7d after calving, when samples were taken every 3 d. For each cow and sampling time, three blood samples were collected from the jugular vein and put in either in EDTA or in sterile tubes to analyse respectively granulocyte population, enzymes, and cytokines; in this latter case, after addition of 10 parts of RNAlater solution (Life Technologies, USA) for total RNA isolation.

Cellular markers

White blood cells count (WBC), polymorphonuclear neutrophils (PMN), lymphocytes (LYM) and monocytes (MON) counts and proportion were assessed by an emocytometer (Sysmex XT-2000Iv, J). Proportion of $CD4^+$, $CD8^+$, and $WC1^+\gamma\delta$ T cell (WC1) were assessed by flow cytometric analysis following protocols routinely applied in our laboratories (Comazzi et al. 2011). Cells were labelled with these antibodies: ILA11 (VMRD, USA), BAQ111A (VRMD, USA) and CC15 (Serotec, UK) for detection respectively of $CD4^+$, $CD8^+$ and WC1 subsets. Apoptosis (APO) and necrosis (NEC) were assessed by flow cytometric analysis, adding APC-conjugated Annexin V in conjunction with propidium iodide (PI) for identification of early and late apoptotic cells. All these assessments were performed using a FACScan flow cytometer and Cell Quest software (Becton Dickinson, USA).

Cytokine analysis

Total RNA was extracted from lysed blood cells using the RNeasy mini kit (RNeasy® mini kit; Qiagen, Germany). The cDNA was synthesised in 20 µl RT mix containing 50 U MuLV reverse transcriptase, 5 mm $MgCl_2$, 10 mM Tris-HCl (pH 8·3), 50 mM KCl, 1·25 µM random hexadeoxyribonucleotide (pd $(N)_6$) primers, 0·5 U/µl RNase inhibitor (GeneAmp® RNA PCR kit, ThermoFisher, USA), 1 mM dNTPs (Promega, USA). The mixture was subjected to 42 °C for 60 min and inactivated at 95 °C for 5 min. The final volume was adjusted to 100 µl with RNase free water. The cDNA was stored at −80 °C until used. Real-time RT-PCR was performed using QuantiTect SYBR Green RT-PCR (Qiagen, D) by a standard protocol recommended by the manufacturer in a iCycler iQ® (BioRad, USA). The primers used for interleukin 6 (IL6), interleukin 8 (IL8) and tumour necrosis factor-α (TNFα), as well as the reference gene (glyceraldehyde 3-phosphate dehydrogenase (GAPDH) derived from the paper of Leutenegger et al. (2000). Whereas interleukin 1 beta (IL1) primers derived from Prgomet et al. (2005) and interferon-γ (IFN) primers from Waldvogel et al. (2000). The cytokines transcriptional level was calculated according to the comparative threshold cycle (C_T) method (Leutenegger et al. 2000).

Cellular ratios

Three commonly used cellular ratios were also calculated: (a) $CD4^+/CD8^+$ defined as the ratio of the proportion of $CD4^+$ to the proportion of $CD8^+$ as a marker of immune homeostasis (Mehrzad & Zhao, 2008); (b) PMN/LIN, and (c) PMN/MON,defined as the ratio of proportion of PMN to, respectively, the proportion of LIN (P/L) or MON (P/M). These latter two ratios are considered as markers of inflammation (Rivas et al. 2001).

Enzyme analysis

Lysozyme (LYS) in blood serum was assessed in duplicate by the procedure described by Piccinini et al. (2004). The concentration of unknown samples, in µg/ml, was calculated by a standard curve obtained by adding a standard amount of lysozyme in each plate on a microplate spectrophotometer (Spectramax 340, Molecular Devices, USA). N-Acetyl-β-glucosaminidase (NAG) was assessed in duplicate by the procedure described by (Kitchen et al. 1978), and expressed as units (pmol of 4-methylubelliferon released per min at 25 °C catalysed by 1 µl of blood serum) on a Fluoroskan

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Table 1. Cellular and humoral markers. Results of variance analysis of factors considered and their interaction by general linear model for repeated measurements

			Factors		
Marker	Acronym	Units	Herd	Time	Time × Herd
White cells count	WBC	Cells/µl	n.s.†	0·0005	0·0065
Neutrophil granulocytes	PMN	Cells/µl	0·0012	0·0082	n.s.
Monocytes	MON	Cells/µl	0·0008	0·0445	n.s.
Lymphocyte	LYM	Cells/µl	n.s.	0·0043	0·0246
CD4 + lymphocytes	CD4+	%	n.s.	<0·0001	0·0204
CD8 + lymphocytes	CD8+	%	n.s.	<0·0001	n.s.
$WC1^{+}\gamma\delta$ T cell	WC1	%	n.s.	0·0347	n.s.
Necrosis	NEC	%	n.s.	0·0103	0·0440
Apoptosis	APO	%	n.s.	n.s.	n.s.
Interferon γ	IFN	RE‡	n.s.	0·0004	n.s.
Interleukin 1β	IL1	RE	0·0053	n.s.	n.s.
Interleukin 6	IL6	RE	0·0286	n.s.	n.s.
Interleukin 8	IL8	RE	n.s.	<0·0001	n.s.
Tumour Necrosis Factor α	TNF	RE	n.s.	0·0072	n.s.
Lysozyme	LYS	µg/ml	0·0308	n.s.	n.s.
N-Acetyl-β-glucosaminidase	NAG	units	0·0127	<0·0001	0·0003
CD4 + /CD8 + ratio	$CD4^{+}/CD8^{+}$	ratio	n.s.	<0·0001	n.s.
PMN/LIN ratio	P/L	Ratio	0·0035	0·0008	n.s.
PMN/MON ratio	P/M	ratio	0·0002	n.s.	n.s.

†n.s.: not significant at $\alpha = 0{\cdot}05$.
‡RE: relative expression.

Ascent microplate fluorimeter (Thermo Labsystems, Finland) at 355 exc and 460 em.

Statistical analysis

All data were collected in a database and statistically analysed by mixed model procedure for repeated measurements (MIXED) on SAS software (SAS 9·4, Sas Institute, USA) (Hatcher & Stepanski, 1994). The between-subjects factor was represented by herds (3 levels), the within-subjects factor was represented by sampling time (12 levels) and the model applied was a full factorial, with polynomial contrasts for within-subjects factor. Cluster analysis was performed on SPSS 2·4 (IBM Corp, USA), while difference in cluster frequencies and markers' mean values were analysed respectively with FREQ and GLM procedures of on SAS software (SAS 9·4, Sas Institute, USA).

Results

The pattern of 9 different cellular markers (APT, $CD4^{+}$, $CD8^{+}$, PMN, LYM, MON, NEC, WBC, WC1), five cytokines (IFN-γ, IL-1β, IL-6, IL-8, and TNF-α), two enzymes (LYS and NAG), and three cell ratios ($CD4^{+}/CD8^{+}$, PMN/LIN, PMN/MON) were assessed during drying off and transition periods in 15 primiparous cows (online Supplementary File Tables S1–S4). Our data showed that the variation of almost all parameters was influenced by the physiological period in which the samples were collected, except for APO, IL1, IL6, LYS and P/M (Table 1). Several markers were directly correlated to the herd, either alone like IL1, IL6, LYS, P/L and P/M (Table 1), or in association with the sampling time like WBC, NEC, CD4+, LYM, while NAG variability was affected by both the factors and their interaction (Table 1). Finally, three markers, PMN, MON and P/L varied depending on both time and herd, but not on the interaction between the two factors. Herd, sampling time or their interaction (Table 1), did not influence APO variation.

Among the parameters in which variation was significantly influenced by the interaction between herd and time, WBC showed large differences among herds (Fig. 1). Values observed in herd A were higher than the ones observed in the other two herds until calving was approaching. After calving, values dropped, and increased slightly 14 d after calving. On the other hand, values in herd B slightly increased close to calving to drop 7 d after calving.

In addition, the pattern of LYM showed large differences among herds (Fig. 1). Indeed, herd A showed the lowest values until calving when a peak was observed. Values for herd B showed an opposite trend with higher values during drying-off, and a large drop around calving. Finally, herd C values, as for WBC pattern, had a lesser degree of variation, when compare to the other two herds.

Within the lymphocyte population, the $CD4^{+}$ proportion showed a general increase during the observed period (Fig. 1). Values for herd B were lower than the other two herds until calving. Then, after calving, an increase was observed in all the three herds, but at different sampling times.

When NAG pattern was considered (Figure 1), large differences among herds were already observed during

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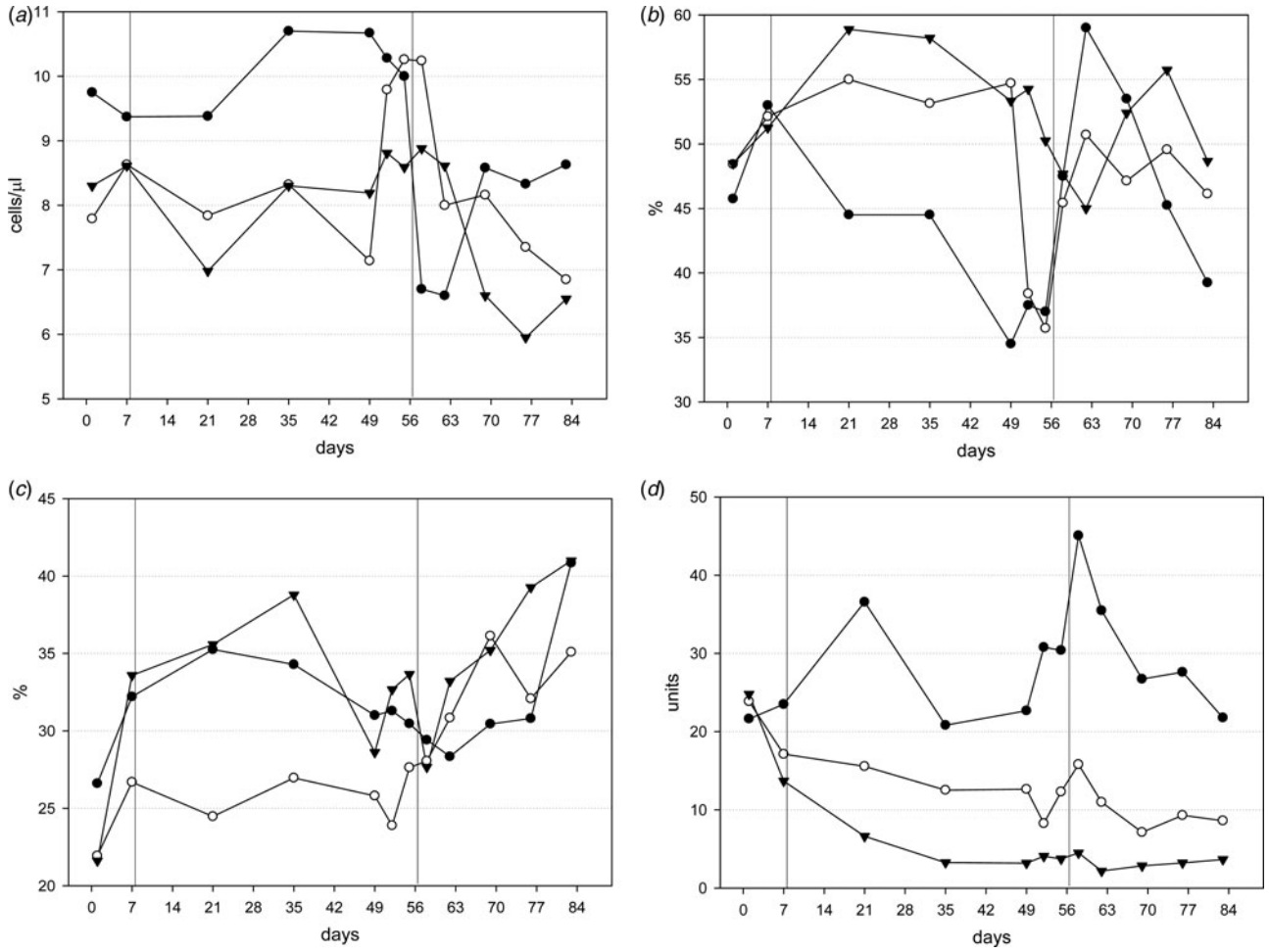

Fig. 1. Pattern of variation during the periparturient period for white blood cells *(a)*, lymphocytes *(b)*, CD4+ lymphocytes *(c)* and N-Acetyl-β-glucosaminidase *(d)* in the three herds considered (herd A ●, herd B ○, herd C ▼). Vertical line at day 7 represents the start of drying off period, while vertical line at day 56 represents calving.

dry-off period. Furthermore, herd A showed two peaks in NAG levels, one seven days after drying off and one other, higher, 3 d after calving. After calving, the levels of NAG remained high in herd A, low in herd C, while values from herd B were always between the ones observed for the other two herds.

Data were evaluated by hierarchical cluster analysis to identify relative homogeneous groups based on the values of the sixteen parameters observed in each sampling time. This analysis allowed identifying 3 clusters, including, respectively, 19·1, 33·5 and 47·4% of the samples. These three clusters had different profiles along the follow-up period (Figure 2). Moreover, there is a significant association ($P < 0{\cdot}05$ at χ^2 test) between clusters and herds. Indeed, cluster 1 was significantly associated with herd C (62·2% of the samples); cluster 2 with herd A (66·7% of the samples), while cluster 3 was numerically, but not statistically, associated with herd A (50·6% of the samples).

Table 2 reports the least-square mean values (±S.E.) observed in the three clusters, calculated by GLM

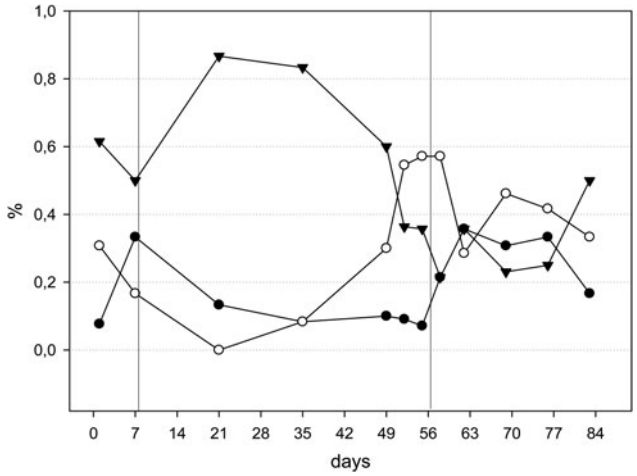

Fig. 2. Frequency of sample clusters during the periparturient period in the three herds considered (herd A ●, herd B ○, herd C ▼). Vertical line at day 7 represents the start of drying off period, while vertical line at day 56 represents calving.

https://doi.org/10.1017/S0022029918000316 Published online by Cambridge University Press

Table 2. Cellular markers. Least square means and standard errors observed in the three cluster defined by hierchical cluster analysis

Marker	Acronym	Units	Cluster 1	Cluster 2	Cluster 3	Model (P)
White cells count	WBC	Cells/μl	6·88 ± 0·33[a,†]	9·10 ± 0·25[b]	8·48 ± 0·21[b]	<0·0001
Neutrophil granulocytes	PMN	Cells/μl	27·54 ± 1·14[a]	51·25 ± 0·85[b]	36·47 ± 0·72[c]	<0·0001
Monocytes	MONO	Cells/μl	8·97 ± 0·85[a]	6·29 ± 0·64[b]	5·01 ± 0·54[b]	<0·0007
Lymphocyte	LYM	Cells/μl	60·51 ± 1·32[a]	38·92 ± 0·99[b]	51·71 ± 0·84[c]	<0·0001
$CD4^+$ lymphocytes	$CD4^+$	%	36·3 ± 1·28[a]	28·71 ± 0·97[b]	29·30 ± 0·81[b]	<0·0001
$CD8^+$ lymphocytes	$CD8^+$	%	18·82 ± 0·93[a]	14·96 ± 0·70[b]	18·31 ± 0·59[a]	0·0003
$WC1^+\gamma\delta$ T cell	WC1	%	7·44 ± 0·59[a]	7·03 ± 0·48[a]	7·24 ± 0·42[a]	n.s.[‡]
Necrosis	NEC	%	10·38 ± 1·045[a]	9·23 ± 0·79[a]	10·93 ± 0·66[a]	n.s.
Apoptosis	APO	%	5·39 ± 0·47[a]	5·15 ± 0·36[a]	5·63 ± 0·30[a]	n.s.
$CD4^+/CD8^+$ ratio	$CD4^+/CD8^+$	Ratio	2·09 ± 0·12[a]	2·04 ± 0·09[a]	1·71 ± 0·08[b]	0·0047
PMN/LIN ratio	P/L	Ratio	0·47 ± 0·08[a]	1·43 ± 0·06[b]	0·72 ± 0·05[c]	<0·0001
PMN/MON ratio	P/M	Ratio	6·87 ± 1·96[a]	11·82 ± 1·49[b]	11·86 ± 1·26[b]	n.s.

†Within a row, values with different superscript statistically differ (α = 0·05).
‡n.s., not significant.

procedure. A significant difference among clusters was observed for all the cellular markers considered out of WC1, NEC, APO and P/M ratio. When humoral markers were considered, only two of them showed a significant difference among clusters (IL1 and IL8) (Table 3).

Discussion

Despite clear evidence of changes in immune and inflammatory markers during drying-off and periparturient period in absence of any apparent disease status, the attempts to identify markers or indexes to define cows at risk during this period in a consistent way are still unsatisfactory (Trevisi et al. 2012; Hailemariam et al. 2014). Moreover, a range of normality for several of the parameters considered was not set or, when such ranges are available, they are not relevant to this peculiar period of cow life. Therefore, with the aim of contributing to filling this gap, we screened several parameters related to innate immunity and inflammation to identify their pattern during drying off and periparturient period, and to assess the effects of physiological status and herd on parameters' values. Despite the relatively small number of individuals, and the expected biological variations, the statistical analysis showed the presence of a significant influence of these periods on several markers.

During drying-off and transition period significant variations among immune and inflammatory markers are expected (Goff & Horst, 1997; Trevisi et al. 2010) even in healthy animal, as in this study. Six markers (WC1, $CD8^+$, INF, IL8 and TNF $CD4^+/CD8^+$), were influenced only by time. The absence of significant effects due to herd and its interaction with time suggests that these parameters could be useful to assess changes in inflammation or immune competence only in relation to physiological status, in the absence of an overt disease status. More interestingly, there are other four markers (IL1, IL6, LYS and P/L), that varied only depending by the herd. Therefore, they could be good candidate markers of the general status of the herd, as previously suggested (Amadori et al. 2015).

The observed changes of PMN and MON were depending on time and herd, but not on their interaction, thus, for each specific point of time, it is not possible to know whether to attribute any differences to the physiological status or to the herd. The variability of remaining markers (WBC, LYM, $CD4^+$, NAG, NEC) was influenced by the interaction between time and herd. This latter evidence, despite the small sample size, further supports the role of these factors as potential marker candidates to assess immunological and inflammatory status in cows in apparent health status during drying-off and periparturient period.

Table 3. Humoral markers. Least square means and standard errors observed in the three cluster defined by hierchical cluster analysis

Marker	Acronym	Units	Cluster 1	Cluster 2	Cluster 3	Model (P)
Interferon-γ	IFN	RE[†]	1·17 ± 0·04[a,‡]	1·27 ± 0·03[a]	1·22 ± 0·02[a]	n.s.[§]
Tumour necrosis factor-α	TNF	RE	1·13 ± 0·02[a]	1·16 ± 0·02[a]	1·13 ± 0·02[a]	n.s.
Interleukin 1β	IL1	RE	1·13 ± 0·29[a]	1·19 ± 0·02[a]	1·10 ± 0·02[b]	0·0105
Interleukin 6	IL6	RE	1·13 ± 0·03[a]	1·06 ± 0·02[a]	1·08 ± 0·02[a]	n.s.
Interleukin 8	IL8	RE	1·22 ± 0·26[a]	1·19 ± 0·02[a,b]	1·14 ± 0·02[b]	0·0471
Lysozyme	LYS	μg/ml	28·70 ± 6·55[a]	30·05 ± 4·81[a]	35·7 ± 3·85[a]	n.s.
N-Acetyl-β-glucosaminidase	NAG	units	13·63 ± 2·97[a]	13·10 ± 2·21[a]	17·00 ± 1·96[a]	n.s.

†RE, Relative expression.
‡Within a row, values with different superscript statistically differ (α = 0·05).
§n.s., not significant.

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These observations are supported by the results of hierarchical cluster analysis, which allowed us to identify three clusters of cellular parameters with significant differences in their mean values, their frequencies during the follow-up period (Fig. 2), and with a significant association with the three herds considered.

Cluster 1 showed significantly low values for PMN, PMN/LIN, PMN/MON and WBC, while slightly higher mean values for $CD4^+/CD8^+$ and IL8 compared to the other clusters. This pattern suggests the absence of an inflammatory reaction (Rivas et al. 2001). Moreover, the low variation in frequencies observed during the follow-up period, suggests an association to cows with a sufficient immunologic homeostasis (Mehrzad & Zhao, 2008).

Cluster 2 is characterised by high level of PMN, P/L, IL1 and low level of $CD8^+$ and LYM, suggesting that samples derived from cows with some impairments of immune response and/or a subclinical inflammatory reaction (Rivas et al. 2001; Mehrzad & Zhao, 2008).

Cluster 3 showed the highest frequency during drying-off and its characterised by significant high levels of LYM, WBC, and significant low levels for $CD4^+/CD8^+$, IL-1 and IL8. This pattern suggests that the cluster includes samples from cows with a good homeostasis (Danicke et al. 2016).

Conclusions

Drying off and periparturient period was confirmed to have a large and significant influence on the variability of several, but not all, immune and inflammatory parameters. The results of this study under field conditions, despite the relatively small sample size, demonstrated several markers of innate immunity and inflammation that varied during drying-off and transition period due to the effects of herd, as observed previously (Piccinini et al. 2005; 2004).

Among the sixteen markers and the three ratios considered, most of the parameters related to cellular immunity were significantly influenced by herd and its interaction with physiological status of the cow. These results support previous evidence on the role of herd factor on immune/inflammatory response, despite the apparent similarity among herds.

The presence of a significant effect of the interaction between herd and physiological status for most of the cellular parameters suggests these as suitable markers to define cow status during drying-off and peripartum period, when the aim is to assess differences among herds, or when data from a 'reference' herd are available.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0022029918000316.

References

Amadori M, Fusi F, Bilato D, Archetti IL, Lorenzi V & Bertocchi L 2015 Disease risk assessment by clinical immunology analyses in periparturient dairy cows. *Research in Veterinary Science* **102** 25–26

Bertoni G, Trevisi E, Han X & Bionaz M 2008 Effects of inflammatory conditions on liver activity in puerperium period and consequences for performance in dairy cows. *Journal of Dairy Science* **91** 3300–3310

Bionaz M, Trevisi E, Calamari L, Librandi F, Ferrari A & Bertoni G 2007 Plasma paraoxonase, health, inflammatory conditions, and liver function in transition dairy cows. *Journal of Dairy Science* **90** 1740–1750

Comazzi S, Gelain ME, Martini V, Riondato F, Miniscalco B, Marconato L, Stefanello D & Mortarino M 2011 Immunophenotype predicts survival time in dogs with chronic lymphocytic leukemia. *Journal of Veterinary Internal Medicine* **25** 100–106

Danicke S, Meyer U, Winkler J, Ulrich S, Frahm J, Kersten S, Valenta H, Rehage J, Haussler S, Sauerwein H & Locher L 2016 Haematological and immunological adaptations of non-pregnant, non-lactating dairy cows to a high-energetic diet containing mycotoxins. *Archives of Animal Nutrition* **70** 1–16

Drackley JK 1999 Biology of dairy cows during the transition period: the final frontier? *Journal of Dairy Science* **82** 2259–2273

Drackley JK, Dann HM, Douglas GN, Guretzky NAJ, Litherland NB, Underwood JP & Loor JJ 2005 Physiological and pathological adaptations in dairy cows that may increase susceptibility to periparturient diseases and disorders. *Italian Journal of Animal Science* **4** 323–344

Goff JP & Horst RL 1997 Physiological changes at parturition and their relationship to metabolic disorders. *Journal of Dairy Science* **80** 1260–1268

Hailemariam D, Mandal R, Saleem F, Dunn SM, Wishart DS & Ametaj BN 2014 Identification of predictive biomarkers of disease state in transition dairy cows. *Journal of Dairy Science* **97** 2680–2693

Hatcher L & Stepanski EJ 1994 *A Step-by-Step Approach to Using SAS System for Univariate e Multivariate Statistics*. Cary, NC: SAS Institute Inc

Jonsson NN, Fortes MRS, Piper EK, Vankan DM, de Cisneros JPJ & Wittek T 2013 Comparison of metabolic, hematological, and peripheral blood leukocyte cytokine profiles of dairy cows and heifers during the periparturient period. *Journal of Dairy Science* **96** 2283–2292

Karcher EL, Beitz DC & Stabel JR 2008 Modulation of cytokine gene expression and secretion during the periparturient period in dairy cows naturally infected with Mycobacterium avium subsp paratuberculosis. *Veterinary Immunology and Immunopathology* **123** 277–288

Kitchen BJ, Middleton G & Salmon M 1978 Bovine milk N-acetyl- beta -D-glucosaminidase and its significance in the detection of abnormal udder secretions. *Journal of Dairy Research* **45** 15–20

Leutenegger CM, Alluwaimi AM, Smith WL, Perani L & Cullor JS 2000 Quantitation of bovine cytokine mRNA in milk cells of healthy cattle by real-time TaqMan (R) polymerase chain reaction. *Veterinary Immunology and Immunopathology* **77** 275–287

Mehrzad J & Zhao X 2008 Tlymphocyte proliferative capacity and CD4 (+)/CD8(+) ratio in primiparous and pluriparous lactating cows. *Journal of Dairy Research* **75** 457–465

Piccinini R, Binda E, Belotti M, Casirani G & Zecconi A 2004 Evaluation of non-specific immune status of heifers in field conditions during the periparturient period. *Veterinary. Research* **35** 539–550

Piccinini R, Binda E, Belotti M, Casirani G & Zecconi A 2005 Comparison of blood and milk non-specific immune parameters in heifers after calving in relation to udder health. *Veterinary Research* **36** 747–757

Prgomet C, Sarikaya H, Bruckmaier RM & Pfaffl MW 2005 Short-term effects on pro-inflammatory cytokine, lactoferrin and CD14 mRNA expression levels in bovine immunoseparated milk and blood cells treated by LPS. *Journal of Veterinary Medicine Series a-Physiology Pathology Clinical Medicine* **52** 317–324

Rivas AL, Quimby FW, Blue J & Coksaygan O 2001 Longitudinal evaluation of bovine mammary gland health status by somatic cell counting, flow cytometry, and cytology. *Journal of Veterinary Diagnostic Investigation* **13** 399–407

https://doi.org/10.1017/S0022029918000316 Published online by Cambridge University Press

Trevisi E, Zecconi A, Bertoni G & Piccinini R 2010 Blood and milk immune and inflammatory profiles in periparturient dairy cows showing a different liver activity index. *Journal of Dairy Research* **77** 310–317

Trevisi E, Amadori M, Cogrossi S, Razzuoli E & Bertoni G 2012 Metabolic stress and inflammatory response in high-yielding, periparturient dairy cows. *Research in Veterinary Science* **93** 695–704

Waldvogel AS, Hediger-Weithaler BM, Eicher R, Zakher A, Zarlenga DS, Gasbarre LC & Heussler VT 2000 Interferon-gamma and interleukin-4 mRNA expression by peripheral blood mononuclear cells from pregnant and non-pregnant cattle seropositive for bovine viral diarrhea virus. *Veterinary Immunology and Immunopathology* **77** 201–212

https://doi.org/10.1017/S0022029918000316 Published online by Cambridge University Press