Methane emission, metabolism, and performance of Holstein dairy cows with low, medium, and high lymphocyte proliferation during transition

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ABSTRACT

This study aimed to identify interactions between state of lactation (dry or early lactating) and immune responder group (low, medium, or high) for energy metabolism traits as well as metabolic and immunological traits in dairy cows. In early lactation, when the energy priority of cows shifts toward the mammary gland, the energy available to be partitioned toward the immune system may differ among individuals. The equilibrium between energy supply from feed, digestion, and body reserve mobilization and energy expenditure with milk, immune system, methane, and heat production is delicate in this stage. Seventeen Holstein cows entering their second to fifth lactation were kept under comparable feeding, housing, and management conditions and were studied from 14 ± 6 d before calving to 11 ± 3 d after calving. Feed intake, milk yield, body condition, blood metabolites, and cortisol as well as gaseous exchange in respiration chambers were measured. The latter was used to quantify methane emission and to calculate resting metabolic rate and heat production. Subsets of blood leukocytes and peripheral blood mononuclear cells (PBMC) were monitored. Activation and proliferation of the PBMC in response to the mitogen phytohemagglutinin ante- and postpartum were assessed using the oxygen consumption rate (24-h cell culture assay) and the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay (72-h cell culture assay). Cows were classified based on the in vitro proliferative response of the PBMC measured postpartum in low ($n = 6$), medium ($n = 5$), and high

($n = 6$) responders. We found no interaction of state of lactation with responder group for feed intake, milk yield, efficiency, metabolic traits, and immune cell activation ante- and postpartum. However, after calving, low-responder cows produced less methane per unit of body weight and per unit of energy-corrected milk compared with the other cows. This might be indicative of a low rumen fermentation intensity. Low responders might therefore suffer from a lower availability of digestible energy in early lactation and not be able to sustain the shift from immune cell activation to proliferation. If so, the selection of environmentally friendly low-methane emitters could promote phenotypes with a compromised immune response in the critical early lactation.

Key words: immune system, lymphocyte activation, mitogen, ruminant

INTRODUCTION

In early lactation, the proliferative response of leukocytes can largely differ among dairy cows. This is mostly related to differences in metabolic and endocrine adaptations accomplished during transition, when the partitioning of energy and nutrients prioritizes the mammary gland. In addition, a mobilization of body reserves compensates for the faster increase of energy output through milk production than energy input by feed uptake (Gross et al., 2011). Thus, nutritional status is directly linked to the capability of the immune system (Mallard et al., 1998; Sordillo, 2016). The main factor contributing to energy supply is ruminal digestion. Cows with a higher energy loss from ruminal methane emissions might lack energy to support the immune system. Alternatively, higher methane emissions could be indicative of a greater ruminal fermentation activity and thus nutrient digestibility of such cows.

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The energy consumed by activated leukocytes was found to account for 25% of the basal metabolic rate (Straub et al., 2010). Therefore, cows with an energy deficiency, which can support immunological maintenance but not activation, might also display smaller resting metabolic rates (**RMR**) and energy expenditures (heat production; **HP**), both indicators of lower metabolic energy turnover.

The effect of state of lactation on immunological traits has been reported repeatedly. For example, in vitro activation and proliferation of peripheral blood mononuclear cells (**PBMC**) was observed to be lower (Kashiwazaki et al., 1985; Schwarm et al., 2013), higher (Burton et al., 1993), or the same (Lacetera et al., 2005; Dang et al., 2013) in early-lactating cows compared with dry cows. The PBMC consist predominantly of T and B lymphocytes as well as some monocytes. The immune response to a mitogen in vitro is assumed to reflect the response to an antigen in vivo. Consequently, the risk for infectious diseases is considered to be higher when PBMC activation or proliferation are low. The immune response is modulated by metabolites in the extracellular fluid, such as nonesterified fatty acids (**NEFA**) and BHB. These metabolites, which frequently occur at higher concentrations in plasma in early lactation due to body reserve mobilization, can modulate PBMC proliferation (Lacetera et al., 2004; Wang et al., 2018) and acute inflammation (Zarrin et al., 2014).

Upon stimulation, immune cells require glucose for their metabolism, and extrinsic signals are needed to utilize such nutrients to maintain metabolic activity (Rathmell et al., 2000). This explains why the level of PBMC activation is related to plasma glucose concentration (Jones et al., 2005; Schwarm et al., 2013) and thus de novo synthesis of glucose from nutrients absorbed from the gut rather than to lipids mobilized from body reserves. Upon stimulation, PBMC enter the cell cycle by shifting from the quiescent G_0 phase to the activation phase, the G_1 phase. In an unfavorable extracellular environment, cells do not proceed from the G_1 phase to the DNA synthesis phase and the cell cycle is aborted. This phenomenon is most likely the cause of a reduced PBMC proliferation.

Indicator traits of adaptive immunity can help identify cows with a low or high ability to mount an immune response (Catalani et al., 2013). Stoop et al. (2016) and Amadori et al. (2018) classified cows as low, medium, and high immune responders at one state of lactation. No difference in production traits among low, medium, and high responders was observed by Stoop et al. (2016). No energy metabolism traits were reported by these authors.

The present study aimed to identify interactions of the degree of PBMC proliferation with state of lac-

tation (dry or early lactating) for energy metabolism traits as well as metabolic and immunological traits in dairy cows. We hypothesized an interaction of state of lactation (before or after calving) with animal group (low, medium, or high responders) for the aforementioned traits.

MATERIALS AND METHODS

Experimental Design

The experimental protocol complied with the Swiss legislation for animal welfare and was approved by the Committee on Animal Experimentation of the Cantonal Veterinary Office Zurich (license no. 06/2014). Seventeen Holstein cows in transition to their second to fifth lactation were selected from the AgroVet-Strickhof herd (Eschikon, Lindau, Switzerland). The dry period was initiated 8 to 10 wk before expected calving. Cows were studied from (mean $\pm$ SD) 14 ± 6 d before calving to 11 ± 3 d after actual calving. Based on the in vitro proliferative response of their PBMC after calving, cows were grouped into low [$n = 6$; proliferation index (**PI**) of 1.3–1.8], medium ($n = 5$; PI of 2.0–2.4), and high ($n = 6$; PI of 2.6–4.3) responders (see Figure 1A, postpartum). The proportion of low and high responders was defined as 1 SE below and above the mean, respectively. The average lactation numbers of the low, medium, and high groups were 2.8 ± 0.40 , 3.0 ± 0.45 , and 3.0 ± 0.52 , respectively. The experiment was conducted in subsequent runs of 1 to 2 cows each because only 2 respiration chambers were available. The procedures carried out lasted for 6 d antepartum and 6 d postpartum for each cow. All cows were clinically healthy at these times. They calved normally in proximity to the anticipated calving date. One cow in the fourth lactation from the medium-responder group had a twin birth.

Management of the Cows

The cows remained in their usual tiestalls for most of the experiment except for 2 stays of 2 d in respiration chambers, where they were tied as well. During the dry period, the cows received a mixed forage ration consisting of chaffed wheat straw, grass silage, corn silage, mineral–vitamin pellets, and extra hay (Table 1). During lactation, the mixed ration consisted of corn silage, grass silage, concentrate, hay, and NaCl, with part of the corn silage being replaced by sugar beet pulp in winter. In cases where milk yield exceeded 32 kg/d, an energy concentrate was offered on top at 1 kg/2 kg of milk in 4 portions/d. Otherwise, feed was provided at 0400 and 1415 h. The cows had

Figure 1. (A) Proliferation index (PI) of peripheral blood mononuclear cells (PBMC); methane emissions (B) per day, (C) per unit of BW, and (D) per unit of ECM; and (E) activation index of PBMC on d 14 ± 6 before calving (antepartum) and 11 ± 4 DIM (postpartum) in cows with low (white circles), medium (gray circles), and high (black circles) PI after calving. The activation index is the ratio of oxygen consumed by stimulated and nonstimulated PBMC after 24 h of incubation. The PI is the ratio of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide-reducing activity of stimulated and nonstimulated PBMC after 72 h of incubation. Data are presented as LSM $\pm$ SE. Effects were for $PI_{(time, group \times time)}$ ($P < 0.05$) and for $PI_{(group)}$ ($P < 0.10$). Observed effects were for methane emissions per day and per unit of BW $_{(group, group \times time)}$ ($P < 0.10$), for methane emissions per day and per unit of BW $_{(time)}$ ($P < 0.05$), and for methane emissions per unit of ECM $_{(group)}$ ($P < 0.05$). No effects ($P > 0.10$) were found for activation index. Means without common letters (A, B at $P < 0.05$ and a–d at $P < 0.10$) differ.

Table 1. Nutrient content (mean $\pm$ SD) of the feed components and the respective amounts offered during the dry and lactation periods

Item	Dry period		Early-lactation period		
	Forage mix ^{1,2}	Extra hay	Forage protein concentrate–mineral mix ³	Energy concentrate ⁴	Extra hay
No. of samples	30	30	34	1	22
DM (%)	35.0 $\pm$ 4.1	89.6 $\pm$ 4.1	42.3 $\pm$ 3.4	93.0	88.9 $\pm$ 2.9
Nutrient (% of DM)					
No. of samples	28	7	34	1	10
OM	93.2 $\pm$ 0.8	93.6 $\pm$ 0.3	92.6 $\pm$ 0.68	93.5	91.2 $\pm$ 0.7
CP	8.52 $\pm$ 0.96	6.43 $\pm$ 0.96	13.78 $\pm$ 1.71	24.23	13.80 $\pm$ 3.40
NDF	55.9 $\pm$ 4.0	66.3 $\pm$ 2.9	42.8 $\pm$ 1.9	21.4	57.4 $\pm$ 1.5
ADF	36.5 $\pm$ 2.8	41.5 $\pm$ 1.7	27.7 $\pm$ 1.6	8.5	33.3 $\pm$ 1.4
ADL	5.52 $\pm$ 1.08	7.49 $\pm$ 0.94	4.40 $\pm$ 0.67	3.50	5.46 $\pm$ 0.56
Ether extract	2.39 $\pm$ 0.33	1.65 $\pm$ 0.96	2.94 $\pm$ 0.26	6.88	1.77 $\pm$ 0.33
Gross energy (MJ/kg of DM)	18.3 $\pm$ 0.3	18.1 $\pm$ 0.1	18.4 $\pm$ 0.2	17.9	17.8 $\pm$ 0.9
Average amount offered (kg of DM/d)	10.56 $\pm$ 1.47	2.84 $\pm$ 1.53	19.44 $\pm$ 3.33	3.23 $\pm$ 0.82	1.05 $\pm$ 0.64

¹Comprised (DM basis) chaffed wheat straw, 40%; grass silage, 35%; and corn silage, 25%.

²An extra mineral–vitamin supplement (100 g/d per cow; Kroni Locher, Altstätten, Switzerland) that comprised (per kg) Ca, 120 g; Mg, 60 g; Na, 30 g; Zn, 440 mg; Mn, 130 mg; Se, 4 mg; Fe, 100 mg; I, 2 mg; Co, 0.7 mg; vitamin A, 20,000 IU; vitamin D₃, 2,000 IU; and vitamin E, 50 mg.

³Comprised (DM basis), on average, summer and winter diet (DM basis) of corn silage, 49.5%; grass silage, 28.2%; concentrate, 12.5%; hay, 9.4%; and NaCl, 0.4%. During the winter period, 11% of corn silage was replaced with sugar beet pulp. The concentrate (no. 50109, Getreidesammelstelle, Thalheim, Switzerland) comprised (DM basis) rapeseed cake, 50%; soybean meal, 17%; corn gluten, 15%; wheat, 13%; dicalcium phosphate dehydrate, 3%; and limestone, 2%. Mineral content (per kg): Ca, 17.8 g; P, 13.2 g; and Mg, 3.35 g.

⁴The energy concentrate (163 Extra MegaDigest, UFA, St. Margrethen, Switzerland) comprised corn, soybean meal, wheat, distillers grains, rapeseed cake, corn gluten, wheat starch, mill by-product mix, feed-grade rice, feed-grade fatty acids, molasses, minerals, dextrose, and wheat bran. Mineral and vitamin content (per kg): P, 50 g; Ca, 7.5 g; Mg, 3.0 g; Na, 1.5 g; Zn, 100 mg; Mn, 50 mg; Fe, 30 mg; Cu, 10 mg; I, 1 mg; Co, 0.4 mg; Se, 0.15 mg; vitamin A, 15,000 IU; vitamin D₃, 2,250 IU; vitamin E, 15 IU; and niacin, 1 g.

permanent access to water. Milking started at 0415 and 1600 h. Mastitis occurred in 1 low-responder cow in fourth lactation at 3 to 5 DIM, and metritis occurred in 1 high-responder cow in second lactation at 10 to 13 DIM. Sampling dates were shifted to after the diseases were cured. Data from these 2 cows were in the range of the other data; therefore, they were not excluded from the data set.

Collection of Data and Sampling

Individual feed intake was recorded by weighing the total amounts offered and leftovers for 6 d (4 d in the tiestall followed by 2 d in the respiration chamber). The mixed dry-off and lactation diets were sampled twice per week over the experimental period. Samples were stored at -20°C and composited periodically before drying. The extra hay was sampled repeatedly over the experimental period ($n = 7$ antepartum, $n = 10$ postpartum) and composited per time point. The leftovers were weighed individually. Leftovers amounting to 10 to 20% of the amount offered were analyzed separately. Feed samples were dried at 65°C to constant weight and ground through a 1-mm screen with a centrifugal mill. Milk yield was recorded in total for 3 d (1 d in the tiestall followed by 2 d in the respiration chamber) at each milking using a mechanical milk meter (DeLaval, Sursee, Switzerland). Milk from each

milking was sampled and conserved with bronopol. Once per experimental week, BW was measured with a floor scale and backfat thickness was determined by ultrasonic measurement (model Tringa Linear, Esaote/Pie Medical, Maastricht, the Netherlands) in the sacral region according to Schröder and Staufenbiel (2006). Concomitantly, BCS was assessed on 1- to 5-point scale according to Edmonson et al. (1989). Blood was collected from a jugular vein into Vacutainer tubes (4–6 mL; Greiner Bio One, St. Gallen, Switzerland) at 0600 h (2 h after feeding) on d 14 ± 6 before parturition and on d 11 ± 4 after parturition. For reasons not related to the experiment, no blood could be collected from 3 cows antepartum (2 medium-responder cows and 1 high-responder cow). For the cell assays, the blood was collected into tubes containing sodium heparin for later analyses. For the other analyses, the plasma was prepared from tubes containing potassium EDTA (BHB, NEFA, and cortisol) or sodium fluoride/potassium oxalate (glucose) by centrifugation at $1,200 \times g$ for 10 min at 4°C and stored at -80°C .

Measurement of Gaseous Exchange

The individual gaseous exchange was measured for 2 d starting at 11 ± 5 d before parturition ($n = 13$) and 13 ± 3 d after parturition ($n = 14$) equivalent to d 5 of the 6-d experimental period. Unforeseen problems

did not allow measurements in all 17 animals at both time points. The 2 open-circuit respiration chambers, as described in detail by Buehler and Wanner (2014), had volumes of 19.3 m³ each and were air-conditioned to maintain 18°C, 55% relative humidity, and an air pressure of -60 Pa with respect to ambient pressure. Airflows were set to 700 L/min (Promethion FG-1000 flow generators, Sable Systems Europe GmbH, Berlin, Germany). Concentrations of CH₄, CO₂, and O₂ were analyzed using a gas analyzer (Promethion GA-4, Sable Systems Europe GmbH). The analyzer was calibrated automatically before each measurement using pure N₂ (99.9%) and a mixed gas (19.8% O₂, 0.5% CO₂, and 0.1% CH₄ in N₂ as carrier). During the experiment, a total of 12 recovery tests of O₂ and CO₂ in each chamber were performed by burning propane gas. The average recovery of both chambers was 93% for O₂ and 101% for CO₂. The O₂ and CO₂ data from the experimental cows were corrected with the corresponding recovery rates obtained closest in time to the respective measurement. The 10 lowest values of O₂ consumption during the night (2000 to 0400 h) of both measurement days were used to determine the RMR according to Dittmann et al. (2015):

$$\text{RMR (kJ/kg of BW}^{0.75} \text{ per day)} = \text{O}_2 \text{ (L)} \times 20.08 \text{ (kJ)/BW}^{0.75} \text{ (kg)}.$$

The HP, corrected for assumed CO₂ production from microbial fermentation, was calculated according to Chwalibog et al. (1996):

$$\begin{aligned} \text{HP (kJ)} = & 16.18 \times \text{O}_2 \text{ (L)} + 5.02 \times [\text{CO}_2 \text{ (L)} \\ & - 3 \times \text{CH}_4 \text{ (L)}] - 2.17 \times \text{CH}_4 \text{ (L)} - 5.99 \\ & \times \text{urine N (50 g)}. \end{aligned}$$

Laboratory Analyses

Feed. For analyses of DM and total ash (method 942.05; AOAC International, 1995), a thermo-gravimetric device (TGA 701, Leco Corporation, St. Joseph, MI) was used. Using a C-N analyzer (Leco-CN 2000, Leco Corporation), nitrogen and carbon contents were determined (method 968.06; AOAC International, 1995). Crude protein was calculated as 6.25 × N. Fiber contents were analyzed with the Fibertec System M (1020 Hot Extraction; Foss, Hillerød, Denmark); NDF was analyzed using the method of Van Soest et al. (1991), and ADF and ADL were analyzed using method 973.18 of AOAC International (1995). For NDF analysis, heat-stable α-amylase was used, but no sodium sulfite. The ADL was analyzed sequentially after the ADF step by

incubation in sulfuric acid (72%) for 3 h. A calorimeter (C7000, IKA-Werke GmbH and Co. KG, Staufen, Germany) was applied for quantifying gross energy. Ether extract was assessed with a Soxhlet extraction system (Extraktionsapparatur B-811, Büchi Labortechnik AG, Flawil, Switzerland; method 963.15, AOAC International, 1995).

Milk. The bronopol-conserved milk was analyzed for contents of fat, protein, and lactose as well as SCC using a Fourier-transform infrared spectrophotometer (MilkoScan FT6000, Foss) at the Swiss routine milk analysis laboratory (SuisseLab AG, Zollikofen, Switzerland). The fat:protein ratio of milk was calculated as an indicator of ketosis (Buttcher et al., 2010). The ECM was calculated based on Agroscope (2019):

$$\begin{aligned} \text{ECM (kg)} = & \text{milk (kg)} \times [0.39 \times \text{fat (\%)} + 0.24 \\ & \times \text{protein (\%)} + 0.17 \times \text{lactose (\%)}] / 3.14. \end{aligned}$$

Blood. Plasma was analyzed for glucose, NEFA, and BHB with an automated analyzer (Cobas Mira 2, Hoffmann-La Roche, Basel, Switzerland) using commercial kits (Randox Laboratories Ltd., Schwyz, Switzerland) as described by van Dorland et al. (2009) and Kreipe et al. (2011). Cortisol in plasma was analyzed with a RIA according to Thun et al. (1981).

PBMC Activation and Proliferation Assay. The PBMC were isolated by density-gradient centrifugation and resuspended with RPMI 1640 medium (Merck AG, Zug, Switzerland) with additives (2 mM L-glutamine, 10 mM HEPES, 10% fetal bovine serum). Cell number and viability were determined using an automatic cell counter (Eve, NanoenTek, Seoul, Korea). The PBMC activation was assessed by measuring O₂ consumption rate (**OCR**) for 24 h as a measure of ATP production (Schwarm et al., 2013). This activation assay was conducted in the presence or absence of the T cell mitogen phytohemagglutinin L (4 µg/mL; Merck AG). Isolated cell suspensions were seeded in quadruplicate per treatment onto fluorophore-coated 96-well Oxoplates (PreSens, Regensburg, Germany) after adjusting cell concentration to 2 × 10⁶ cells/mL. The PBMC were incubated in humidified air with 5% CO₂ at 39°C for 24 h. Fluorescence was detected with a microplate reader (Cytation 3 M, Biotek, Lucerne, Switzerland) from the bottom side in a dual kinetic mode (540/650 nm and 540/590 nm). Fluorescence intensity was converted into oxygen concentration following the instruction manual of PreSens. The oxygen partial pressure in percent air saturation was used to adjust oxygen concentration (cO₂; µM) by multiplication with 1.93 (PreSens template; www.presens.de/support/download-center/tools-utilities.html) for 39°C

and 965 hPa. The OCR (fmol/min) in the well was calculated according to Wang et al. (2005) using 0.71 cm as diffusion path length and 0.35 cm² as surface area exposed to the atmosphere. The OCR was scaled to cell number, which was assessed from plates incubated for 24 h in parallel. The relative activation index (**AIx**) was calculated using OCR [nmol/(min × 10⁷ cells)] as

$$\text{AIx} = \text{OCR}_{\text{presence of mitogen}} / \text{OCR}_{\text{absence of mitogen}}.$$

The PBMC proliferation in the presence or absence of the mitogen phytohemagglutinin L was assessed using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (**MTT**; Sigma-Aldrich, Buchs, Switzerland) as described by Meese et al. (2018). The PBMC incubation procedure was as described for activation except that the cell culture plate was conventional. After 72 h, cells were treated with MTT and incubated for 4 h and then overnight after application of SDS (Sigma-Aldrich). The optical density (**OD**) was measured with the same microplate reader with test and reference wavelengths of 550 and 690 nm, respectively. The PI was calculated as

$$\text{PI} = \text{OD}_{\text{presence of mitogen}} / \text{OD}_{\text{absence of mitogen}}.$$

Flow Cytometry. The proportion of leukocyte subsets (granulocytes, lymphocytes, and monocytes) in aliquots of blood and isolated PBMC as well as the cell cycle status (G₀, G₁, synthesis, mitosis) of PBMC were assessed with a Cytomics flow cytometer (FC500 MPL; Beckman Coulter, Nyon, Switzerland) equipped with 488- and 635-nm argon lasers. Erythrocytes in the blood aliquots were lysed using VersaLyse (Beckman Coulter). Blood cells and PBMC were then preserved in 4% paraformaldehyde at 4°C; the PBMC were also preserved in 70% alcohol and stored at −20°C. Cells (7,000–20,000) were gated based on side (proportional to cellular granularity) and forward (proportional to cell size) scatter characteristics of each population and differentiated based on Jones et al. (2007) using the dyes fluorescein isothiocyanate-peanut agglutinin and propidium iodide (Sigma-Aldrich).

Statistical Evaluation

Repeated-measurement ANOVA was performed by applying the Mixed procedure of SAS (version 9.3; SAS Institute Inc., Cary, NC). Time (before or after calving), animal group (low, medium, or high responders), and the animal group × time interaction were considered as fixed effects. Individual cow was considered as experimental unit. Inclusion of lactation number as random factor in the model did not change effects and

associated interactions of immunological traits (data not shown). Therefore, lactation number was not included in the final model. Multiple comparisons among means were performed using the Tukey-Kramer test. Differences were considered significant at $P \leq 0.05$ and a trend at $P \leq 0.10$. Data are presented as least squares means ± standard error of the mean unless otherwise stated. Spearman correlation coefficients between immunological traits and traits of energy metabolism and their significance were calculated using the Corr procedure of SAS.

RESULTS

Feed Intake, Body Condition, and Performance

Dry matter intake increased ($P < 0.001$) and BCS ($P < 0.05$) and BW ($P < 0.001$) decreased after calving (Table 2). Backfat thickness did not change ($P > 0.10$) with time. There were no significant ($P > 0.10$) interactions between animal group (low, medium, or high responders) and time (before or after calving) and no effect ($P > 0.10$) of group in any of these variables.

We observed no differences ($P > 0.10$) between groups in ECM yield (42.3 ± 1.01 kg), milk production efficiency (6.56 ± 0.196 kg of ECM/100 kg of BW), and feed conversion efficiency (1.94 ± 0.063 kg of ECM/kg of DMI). The milk fat:protein ratio and the SCC were not different ($P > 0.10$) between the groups, with on average 1.18 ± 0.038 and $157,000 \pm 97.5$ cells/mL, respectively. No significant ($P > 0.10$) correlations occurred between immunological traits and DMI, BW, BCS, backfat thickness, or ECM variables except for a positive correlation between AIx and DMI ($r = 0.47$, $P < 0.05$).

Metabolic Rate, HP, and Methane Production

The RMR and HP increased ($P < 0.001$) to 1.30-fold values from antepartum to postpartum (Table 2), but there were no significant ($P > 0.10$) interactions between group (low, medium, or high responder) and time (before or after calving) and no effects ($P > 0.10$) of group. Daily methane production tended to differ ($P < 0.10$) between groups and increased after calving ($P < 0.05$) in all groups (Figure 1B). This increase was particularly great in medium responders (1.8-fold increase), resulting in a trend ($P < 0.10$) for a group × time interaction. After calving, methane yield (per unit of DMI and per unit of gross energy intake) decreased ($P < 0.01$). However, neither group × time interactions ($P > 0.10$) nor group effects ($P > 0.10$) were observed (Table 1). Methane emission intensity (per 100 kg of BW) tended to differ ($P < 0.10$) between groups and

Table 2. Metabolic and immunological traits (LSM) in dry and lactating cows¹

Item	Period		SEM	P-value
	Dry	Early lactation		
DMI (kg)	11.4	22.0	0.591	<0.001
BW (kg)	711	650	18.3	<0.001
BCS	2.95	2.75	0.118	0.025
Backfat thickness (cm)	0.91	0.79	0.102	0.187
Resting metabolic rate (kJ/kg of BW ^{0.75} per day)	645	865	31.9	<0.001
Heat production (kJ/kg of BW ^{0.75} per day)	694	909	25.2	<0.001
Methane yield				
g/kg of DMI	21.1	17.6	0.885	0.008
% of gross energy	6.43	4.74	0.273	<0.001
Plasma concentration				
Glucose (mM)	3.37	2.93	0.076	<0.001
Nonesterified fatty acids (μM)	216	477	101	0.109
BHB (μM)	380	834	193	0.097
Cortisol (ng/mL)	4.25	2.95	0.613	0.046
Blood leukocyte proportion (% of total leukocytes)				
Granulocytes	66.4	54.6	3.04	0.038
Lymphocytes	19.4	27.9	2.20	0.037
Monocytes	14.5	17.6	1.34	0.095
PBMC ² subsets (% of isolated PBMC)				
Lymphocytes	88.3	86.7	1.30	0.304
Monocytes	11.7	13.3	1.30	0.304
Cell cycle ³ status (% of isolated PBMC)				
G ₀ phase	80.5	81.5	2.93	0.650
G ₁ phase	3.69	1.67	0.85	0.100
Synthesis phase	9.13	10.7	1.73	0.364
Mitosis phase	6.75	5.76	1.58	0.628
PBMC ⁴ reaction after 24 h of incubation				
OCR ⁴ unstimulated	2.61	2.81	0.157	0.332
OD ⁵ unstimulated	0.640	0.558	0.056	0.176

¹Values of dry and lactating cows are given as the pooled mean of low-, medium-, and high-responder cows because the interaction of group (low, medium, or high) and state of lactation (dry or early lactating) was not significant.

²Peripheral blood mononuclear cells.

³G₀ phase = quiescent cells; G₁ phase = cell growth (RNA and protein syntheses); synthesis phase = DNA replication; mitosis phase = cell division.

⁴Oxygen consumption rate (nmol/min × 10⁷ cells) of PBMC incubated for 24 h without the mitogen phytohemagglutinin.

⁵Optical density of PBMC incubated for 72 h without the mitogen phytohemagglutinin.

increased ($P < 0.01$) postpartum in all groups (Figure 1C). The increase was greatest in the medium responders (2.0-fold increase) and smallest in low responders (group × time interaction, $P < 0.10$). Methane emission intensity (per kg of ECM) was smaller ($P < 0.05$) in low responders than in medium and high responders (Figure 1D). Among the immunological traits, AIX was correlated ($P < 0.05$) with RMR ($r = 0.42$), HP ($r = 0.44$), daily methane production ($r = 0.62$), and methane emission intensity (per 100 kg of BW, $r = 0.63$), and PI was correlated ($P < 0.05$) with methane emission intensity (per kg of ECM, $r = 0.72$; per 100 kg of BW, $r = 0.63$).

Blood Metabolites and Hormones

Compared with before calving, concentrations of glucose and cortisol decreased after calving ($P < 0.05$), and we observed a trend ($P < 0.10$) for an increase of

BHB concentration and no difference ($P > 0.10$) in NEFA concentrations (Table 2). There were no interactions ($P > 0.10$) between group (low, medium, or high responder) and time (before or after calving) and no group effects ($P > 0.10$) in any of these variables. No significant ($P > 0.10$) correlations occurred between immunological traits and blood metabolites and hormones.

Proportions of Leukocyte Subsets, Cell Cycle Status, and In Vitro Reactivity of PBMC

Compared with the dry period, we observed a decrease ($P < 0.05$) in granulocyte proportions and an increase in lymphocyte ($P < 0.05$) and monocyte ($P < 0.10$) proportions in early lactation (Table 2), but no interactions ($P > 0.10$) between group (low, medium, or high responder) and time (before and after calving) and no group effects ($P > 0.10$) were found. In PBMC,

there were no effects ($P > 0.10$) of group or time and no ($P > 0.10$) group $\times$ time interactions in the proportions of lymphocytes and monocytes or in the proportions of cells in G_0 , G_1 , synthesis, and mitosis phases of the cell cycle. There were no time differences ($P > 0.10$) in the basal oxygen consumption of PBMC cultured for 24 h without mitogen or in the basal MTT-reducing activity of PBMC cultured for 72 h without mitogen. Groups slightly differed ($P = 0.094$) in the basal OCR of PBMC, but no interaction ($P > 0.10$) between group and time was found. We observed a group $\times$ time interaction ($P = 0.097$) in the basal MTT-reducing activity (OD of unstimulated PBMC) but no effect ($P > 0.10$) of group. There were a group $\times$ time interaction ($P < 0.05$) and effects of group ($P < 0.10$) and time ($P < 0.05$) in PI (Figure 1A). The interaction resulted from different responses of responder groups in PI ante- and postpartum. However, we revealed no ($P > 0.10$) group $\times$ time interaction, group, or time effects for AIx (Figure 1E). No significant ($P > 0.10$) correlations occurred between the PBMC characteristics and traits of energy metabolism.

DISCUSSION

In Vitro PBMC Activation and Proliferation and *In Vivo* Leukocyte Status

In the present study, animal groups (low, medium, or high responders) did not differ in the proportions of leukocyte subgroups in the blood (in vivo) and in isolated PBMC before incubation (in vitro). The differences in proliferative responsiveness found in vitro are expected to reflect the capability to respond to an antigen in vivo. However, it should be noted that the immune response under in vivo conditions is modulated by metabolites and endocrine factors, which is discussed in the following section. The observed lack of responder group differences in the proportion of the cell cycle phases G_0 , G_1 , synthesis, and mitosis of the isolated PBMC (before incubation) implies that the groups did not differ in their in vivo stages of preactivation and preproliferation before the in vitro incubation started.

The level of AIx mostly reflected that of the basal OCR (OCR unstimulated). Different from that, the level of PI was inversely changing over time compared with the basal MTT-reducing activity (OD unstimulated). From these observations, we concluded that the PI of high responders was particularly high postpartum because of a comparatively low basal MTT-reducing activity.

The observation of a positive correlation between PBMC activation and DMI supports the linkage of the nutritional status of an animal and the capability to

start an immune response (Sordillo, 2016). However, the cows grouped by low, medium, and high PBMC proliferation postpartum did not differ in PBMC activation, which indicates that the cell cycle was completed to a smaller extent in the PBMC of the low-responder cows compared with the high-responder cows. Despite the optimized conditions in the in vitro incubation in complete medium, the PBMC of low responders seem to have been imprinted in vivo in an energetically less favorable condition, limiting their utilization of the surrounding nutrients. Different from that, though not completely comparable with the present study, Catalani et al. (2013) categorized dairy cows into low, medium, and high responders based on the average lymphoproliferative response to LPS over 7 peripartur weeks. They found that high-responder cows were the least susceptible to infectious diseases.

Relationship Between Performance and Efficiency with Immune Response During Transition

The onset of the lactation in high-yielding dairy cows is a critical time concerning nutrient supply. It may result in a massive increase in plasma concentrations of BHB and NEFA (Weber et al., 2013) and loss of BW and BCS, especially in previously overconditioned cows. However, this was not the case in the cows used in the present study. The increase in DMI from 11 kg/d in the dry period to 22 kg/d in early lactation was steep and obviously was widely sufficient to support a yield of 42 kg of ECM/d from the beginning. As a consequence, the loss of BCS was only from 3.0 to 2.75. Most of the 61 kg of BW loss could be attributed to calf weight, and we observed no significant change in backfat thickness. All of this coincides with the comparably small peripartur increase in plasma BHB and the lack of difference in plasma NEFA. In accordance with this, the fat:protein ratio in milk was only 1.17, which is clearly below the threshold of 1.5, which would indicate high lipolysis and the risk of developing ketosis (Buttchereit et al., 2010).

In the present study, the immune response rank was not related to differences in energy availability as assessed by feed intake, body reserve mobilization (estimated via changes in BCS and backfat thickness), and milk yield. This is in accordance with Stoop et al. (2016), who found no difference in milk yield among low-, medium-, and high-responder cows. At unchanged DMI and ECM, feed conversion efficiency (ECM per unit of DMI) was similar among groups and thus did not coincide with the differences found in lymphoproliferative response postpartum. The decrease in plasma glucose concentrations postpartum was also similar among groups. The plasma BHB serves as fuel in acute

inflammations (Zarrin et al., 2014). However, high concentrations of NEFA (Lacetera et al., 2004) and ketone bodies (Targowski and Klucinski, 1983; Meese et al., 2018) would likely suppress the proliferation of bovine PBMC. However, at moderate concentrations, as was the case in the present study, these metabolites can be used as an energy source by bovine PBMC (Fox et al., 2005; Schulz et al., 2015; Wang et al., 2018). Around parturition, plasma glucocorticoid concentrations (e.g., that of cortisol) are typically elevated due to metabolic stress, and this can reduce T cell proliferation (Roth, 1985). However, in the present study, cortisol concentrations in plasma decreased periparturient, similarly to that reported by Weber et al. (2013) for cows with low liver fat contents. Bloom et al. (1979) reported that in vivo glucocorticoid treatment depleted the number of circulating lymphocytes, which corresponds well with our observation of smaller proportions of lymphocytes antepartum where cortisol concentrations were comparatively higher. Still, it is likely that a more pronounced energy deficiency at the beginning of lactation would have led to greater differences in performance and plasma metabolite concentrations between responder groups.

Relationship Between Metabolic Rate and Energy Losses Through Heat and Methane with Immune Response During Transition

In the present study, the generally higher metabolic energy turnover in response to milk formation was reflected in higher RMR and HP postpartum compared with antepartum. We anticipated that low-responder cows with reduced energy availability (to support immunologic activity) might display smaller RMR and HP compared with high-responder cows. However, groups did not differ in RMR and HP. In experiments inducing fever in sheep, RMR was increased by 28% (Fewell et al., 1991). In line with that, a positive correlation of PBMC activation with RMR and HP was observed. However, in the clinically healthy cows of the present study with their rather good supply with dietary energy, differences in PBMC proliferation might have been too small to lead to clear group variations in RMR and HP. In contrast to RMR, which is based on oxygen consumption of the cows, state of lactation did not affect the in vitro oxygen consumption (reflecting ATP production) of the unstimulated PBMC, suggesting that the energy partitioned by the cows toward immunological maintenance was the same ante- and postpartum.

We did not expect the observed difference between responder groups in the production of enteric methane. Methane formation is linearly related to DMI (Niu

et al., 2018) and thus largely increased when DMI doubled postpartum. However, the increase in methane was lower than the increase in DMI. Therefore, the methane yield (per unit of DMI or gross energy intake) concomitantly decreased. The low-responder cows were found to exhibit a smaller methane emission intensity per unit of BW and ECM. Consistent with that, there was a positive correlation of PBMC activation and proliferation with methane emission intensity. Retrospectively, the experimental set-up did not allow us to obtain information about ruminal and total-tract nutrient degradation and digestion apart from methane formation. The lower methane emission intensity of the low-responder cows may indicate a generally lower fermentation intensity, possibly because the rumen and the microorganisms in the rumen of low-responder cows do not achieve a similar degree of adaptation to the DMI increase as that of medium- and high-responder cows. In early lactation, low-responder cows might then gain comparably less digestive energy to sustain immune response, particularly the shift from activation to proliferation. However, it cannot be excluded that fermentation was simply shifted from acetate and methane formation to propionate (a glucogenic short-chain fatty acid) synthesis in the rumen, thus even improving energy availability to the mammary gland, but apparently not to the immune system.

CONCLUSIONS

Cows clearly differed in the postpartum immune response based on mitogen-induced lymphocyte proliferation in vitro, although no differences were observed in performance and metabolic modifications between cows at the onset of the lactation. The concomitant differences in enteric methane emissions may suggest that low-immune responders could still be at a disadvantage compared with medium- or high-responder cows in metabolic energy supply. They thus would be forced to favor milk production over the maintenance of a fully operative immune system. If so, the selection of cows that emit low levels of methane for environmental reasons could favor animals with a greater disease susceptibility. Further studies comprising early-lactating cows with a more critical energy status and an in-depth investigation of ruminal digestion will have to confirm this finding.

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