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Nutrient-sensing kinase signaling in bovine immune cells is altered during the postpartum nutrient deficit: A possible role in transition cow inflammatory response

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ABSTRACT

Transition dairy cows experience a nutrient deficit, particularly in the immediate postpartum period. At the same time, the inflammatory balance is altered and cows exhibit an immune response primed for inflammatory response rather than tolerance. The mechanistic link that might be underlying the immunological effects due to the lack in nutrients is not fully understood. Studies in other species demonstrate an orchestrating role of nutrient-sensing kinases in the determination of immune phenotypes and immune cell proliferation and differentiation. Our primary objective was to investigate changes in energy storage and signaling through the protein kinase B (AKT)/mechanistic target of rapamycin complex 1 (mTOR) pathway in bovine immune cells in the transition period, as well as the association with cytokine expression profiles. A secondary objective was to test if supplementation with branched-chain amino acids alone or in combination with oral propylene glycol had any effect on the measured parameters. To assess cellular energy storage, glycogen concentration was measured by an enzymatic-fluorometric method in peripheral blood mononuclear cells (PBMC) of multiparous Holstein cows (n = 72) at 3 time points in the transition period (21 d before, 7 and 28 d after calving). At the same time points, phosphorylation of proteins in the AKT/mTOR pathway was assessed by immunoblotting in PBMC from 60 animals. Whole-blood leukocyte cytokine gene expression of *IL12B*, *IL6*, *IL1B*, *TNF*, and *IL10* was measured in samples from 50 animals by reverse-transcription quantitative PCR with and without stimulation of samples with 10 ng/mL of lipopolysaccharide. Compared with glycogen concentration of prepartum PBMC, glycogen concentration decreased by 37% on d 7 postpartum. The activation of AKT/mTOR in bovine PBMC postpartum was reduced compared with prepartum values. Results of reverse-transcription quantitative PCR showed an increase in cytokine gene expression postpartum compared with prepartum values. Supplementation with branched-chain amino acids alone or in combination with oral propylene glycol did not alter glycogen storage, AKT/mTOR activity, or inflammatory balance as assessed by the measured parameters in this study. We conclude that the nutrient deficit of the immediate postpartum period is sensed by bovine immune cells, and that it affects their energy storage as well as cellular signaling pathways postpartum. Temporal associations with changes in cytokine gene expression are intriguing and warrant further investigation of the role of this pathway as a possible link between metabolism and immune phenotype postpartum.
Key words: transition, AKT kinase, mTOR kinase, inflammation

INTRODUCTION

The transition from late pregnancy to early lactation is a critical period for dairy cows that is associated with pronounced negative nutrient balance and an increase in the risk for metabolic and infectious diseases. At the same time, decreased efficiency in pathogen clearance and an increase in the magnitude and duration of inflammation is typically seen. The changes in nutrient availability early postpartum include a lack of energy substrates, as well as AA. This deficit is caused by a prioritization of nutrient use by the mammary gland, associated with a dramatic decrease in circulating glucose, insulin, and EAA (Bell et al., 2000; Kuhla et al., 2011; Mann et al., 2016a). At the same time, we observe an increase in plasma nonesterified fatty acids from lipolysis, as well as ketone bodies originating primarily from hepatic fatty acid metabolism (McCarthy et al., 2015).

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The degree and persistence of inflammation in the transition period show individual variation, which is associated with differences in disease risk and production (Bionaz et al., 2007; Bradford et al., 2015; Huzzey et al., 2015). Several factors are discussed as contributors to the inflammatory state. Adipose tissue mobilization is regarded as a contributor to immune dysfunction postpartum, as both fatty acids as well as ketone bodies can have direct effects on the functionality of immune cells in early lactation (Sordillo and Raphael, 2013). Reactive oxygen species are produced at an increased rate in early lactation and may have a direct effect on immune cell function when produced in excess (Sordillo and Raphael, 2013). Additionally, the availability of energy and AA changes rapidly during this time, although less is known about the effect of the lack of these macronutrients on bovine immune cells.

Immune cells require large amounts of glucose upon activation (Pearce and Pearce, 2013). This large demand for glucose upon activation of the innate immune system was recently demonstrated in lactating dairy cows by infusion of lipopolysaccharide and was estimated to require more than 1 kg of glucose in a 12-h period (Kvidera et al., 2017). Glucose is stored in the form of glycogen in white blood cells and increases with the maturity of the cell (Wachstein, 1949). This storage plays a crucial role in the rapid availability of glucose upon activation (Wei et al., 2017). It was previously demonstrated that neutrophils, which depend on glycolysis to perform phagocytosis and chemotaxis, have diminished glycogen stores early postpartum, and that this depletion is more pronounced in cows with metritis (Galvao et al., 2010). Glycogen storage in immune cell types other than neutrophils warrants investigation in peripartum dairy cows.

Decreased availability of glucose, insulin, and AA, particularly the branched-chain AA (**BCAA**) Leu, are known to decrease the activity of the protein kinase B (**AKT**)/mechanistic target of rapamycin complex 1 (**mTOR**) pathway (Beugnet et al., 2003; Atherton et al., 2010; Altomare and Khaled, 2012). The blood concentrations of free EAA experience a sudden drop around the time of calving and for the first weeks postpartum (Meijer et al., 1995) that occur parallel to the decreased concentration of glucose and insulin in the bloodstream. In addition, the cellular energy balance is sensed by the AMP-activated kinase as a ratio between AMP:ATP. Activation of AMPK leads to an increase in cellular catabolic pathways, and further inhibits the PI3K/AKT/mTOR-pathway upon activation (Blagih et al., 2012). The AKT/mTOR kinase signaling cascade is an important nutrient-sensing pathway in mammalian cells and this pathway is central in determining the type, magnitude, and duration of innate immune responses (Weichhart et al., 2015; Vergadi et al., 2017). We have shown previously that the activity of the AKT/mTOR pathway is decreased in tissues of postpartum dairy cows when the nutrient deficit is most severe (Mann et al., 2016b,c). The motivation of this work was to address the gap in knowledge by investigating temporal changes in nutrient-sensing pathways in the transition period that are known to play a critical role in regulating the magnitude and duration of the inflammatory response in other mammals. Our hypothesis was that changes in nutrient availability in the postpartum period are also associated with changes in energy storage and activity of the described nutrient-sensing cellular signaling pathways in bovine immune cells. In addition, and because of the well documented association of mTOR activity and BCAA availability, as well as the documented immunological benefits of BCAA in other species (Calder, 2006), we wanted to test if supplementation with these AA alone or in combination with a glucose precursor in the postpartum period has an effect on these signaling pathways.

Our primary objective was to investigate the glycogen storage in bovine peripheral blood mononuclear cells (**PBMC**) around the time of calving, as well as to describe the activity of nutrient-sensing cellular signaling pathways during the transition period and associate this with the inflammatory profile of bovine whole blood. Our secondary objective was to investigate if supplementation with BCAA alone, or BCAA in combination with the glucose precursor propylene glycol, in postpartum dairy cows is associated with changes in energy storage, cellular signaling, and inflammatory profile in the transition period.

MATERIALS AND METHODS

Animals and Treatments

Samples were collected from animals enrolled in a larger study investigating the role of BCAA supplementation alone or in combination with propylene glycol in postpartum dairy cows. All procedures for this experiment were approved by the Cornell University Institutional Animal Care and Use Committee (number 2014–0118). Cows were housed in the Cornell University Ruminant Center in Harford, New York, and samples were collected between February and June 2016. All cows were fed the same dry period and fresh period TMR ad libitum, and samples were analyzed throughout the study using near infrared analysis (Cumberland Valley, Maugansville, MD). Cows were fed once daily at 0700 h using individual feed buckets in a tiestall. Animals were enrolled in 1 of 3 treatment groups on the day of calving: cows that received 550 g

of a rumen-protected (**rp**; hydrogenated vegetable oil-soy based) BCAA supplement (Balchem Corporation, New Hampton, NY) as a top-dress mixed into 200 g of molasses for the first 35 DIM, cows that received the same treatment as BCAA as a top-dress mixed in molasses once a day for the first 35 DIM plus 300 mL of propylene glycol as a drench once a day for the first 7 DIM (**BCAAPG**), and an untreated control group that only received 200 g of molasses (**CTRL**). The BCAA treatment was composed of 375 g of 27% rp L-leucine, 85 g of 48% rp L-isoleucine, and 90 g of 67% rp L-valine. The dose was determined based on being able to achieve the minimum supplementation rate shown in a previous study to increase free BCAA in blood using a postruminal infusion (Mackle et al., 1999; Larsen et al., 2014). One sample of each AA was analyzed for chemical composition using wet chemistry analysis (Cumberland Valley).

Seventy-two animals were included in this study: 23 in group BCAA, 25 in group BCAAPG, and 24 in CTRL. Parity distribution was as follows: 38 (52.8%) entered second lactation, 20 (27.8%) entered third lactation, 10 animals (13.9%) entered fourth lactation, and 4 animals (5.6%) entered fifth lactation. Cows in the beginning of the study received a 50% lower dose of leucine. To account for this difference and other changes associated with these 2 distinct time periods of the study (i.e., diet composition and ambient temperature), a period effect was included in the analysis.

Peripheral Blood Mononuclear Cell Analysis

Separation of PBMC. Blood was taken from the coccygeal vessels after disinfection of the puncture site with 70% ethanol approximately 21 d before, as well as 7 and 28 d after calving into sterile evacuated glass tubes containing 143 USP units of freeze-dried sodium heparin (BD, Franklin Lakes, NJ) consistently at 1400 h. To prevent contamination of the blood sample and activation of immune cells by perforating the skin, at least one whole 10-mL vacutainer tube was filled to flush the needle and was used for a different purpose. Samples for PBMC separation were immediately placed on ice. For transport to the laboratory, 15 mL of anticoagulated blood was diluted 1:1 with PBS that contained phosphatase inhibitors 1 m*M* sodium orthovanadate, 10 m*M* sodium β-glycerophosphate, and 10 m*M* sodium fluoride. In the laboratory the sample was transferred into Leucosep tubes prefilled with 15 mL Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) and centrifuged for 15 min at 1,000 × *g* and 17°C. The layer containing PBMC was transferred into 50-mL conical tubes and washed 3 times with ice-cold PBS containing the same phosphatase inhibitors as described above. For each sample, PBMC were enumerated using a hemocytometer (Leica, Wetzlar, Germany) with Trypan Blue (Sigma-Aldrich) exclusion staining at a dilution of 1:1. For glycogen analysis, aliquots of 10^7 cells were snap frozen in liquid nitrogen and stored at −80°C until processing. For immunoblotting, one aliquot of 10^7 PBMC was lysed in 250 μL of ice-cold lysis buffer containing 150 m*M* sodium chloride, 1% Triton X, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 m*M* Tris, and 1 m*M* phenylmethane sulfonyl fluoride with the addition of a protease and phosphatase inhibitor cocktail (Halt, Thermo Fisher Scientific, Waltham, MA).

PBMC Immunoblotting. Cell aliquots were thawed on ice and sonicated for 6 s to achieve complete membrane rupture (550 Sonic Dismembranator, Thermo Fisher Scientific). Samples were left on ice for 20 min before centrifugation at 10,000 × *g* for 10 min at 4°C. The supernatant was transferred and the protein concentration determined as previously described (Mann et al., 2016b). Lysates were adjusted to a protein concentration of 1 μg/μL and Western blot analysis was performed with 8 and 12.5% Tris-glycine SDS-polyacrylamide gels with loading amounts of 20 μg per lane. Aliquots of a single PBMC lysate sample from 1 cow at 21 d postpartum were used as the control sample and loaded on every gel, and results of the densitometry of each gel were standardized to the control sample to control for variation between gels. Transfer, blocking, and incubation with primary and secondary antibodies were carried out as described previously (Mann et al., 2016b). Primary antibodies were to phosphorylated (**p**)-AMPKα (Thr172); AMPKα, p-AKT(Ser473), AKT, non-p-4EBP1 (Thr46), 4EBP1, p-S6RP (Ser235/236), and the β-subunit of the insulin receptor (**INSR**; all Cell Signaling Technology, Danvers, MA). Beta-actin served as the loading control and to express relative expression of INSR, whereas all other targets were quantified as the ratio of phosphorylated to total protein, respectively. For antibodies without documented cross-reactivity with bovine samples (INSRβ, non-p-4EBP1, 4EBP1, pS6RP, S6RP), epitope homology was 100%, except for 4EBP1 which was predicted at 93%.

Densitometry was performed using VisionWorks software (v. 8.20, UVP, Upland, CA).

Determination of Glycogen Concentration in PBMC. Glycogen content of PBMC was determined using a commercially available fluorometric assay (Glycogen assay kit, Cayman Chemical, Ann Arbor, MI). Aliquots of 10^7 PBMC were thawed by addition of 600 μL of ice-cold assay buffer. Samples were sonicated for 2 s on ice as described above, vortexed, and centrifuged at 800 × *g* for 10 min at 4°C. Samples were handled

on ice until addition of assay reagents according to the manufacturer's instructions (www.caymanchem.com). Samples were assayed in duplicate. The interassay coefficient of variation calculated from pooled control samples included on all 5 plates used in this study was 6.9%. Results are presented as micrograms of glycogen/10^6 PBMC.

Whole-Blood LPS Stimulation and Inflammatory Profile

Whole-Blood LPS Stimulation. Heparinized whole blood was collected as described above with the difference that samples were kept at room temperature for transport to the laboratory. Blood samples were mixed by inversion and 2 mL was transferred into sterile, endotoxin-free culture tubes (VWR, Radnor, PA). Purified LPS from *Escherichia coli* O111:B4 was purchased from Millipore Sigma (Burlington, MA) and a stock solution of 1 mg/mL frozen in aliquots. Aliquots of the stock solution were thawed and diluted with RPMI 1640 cell-culture medium (Thermo Fisher Scientific). One tube per sample was stimulated at concentration of 10 ng of LPS/mL of blood in a total volume of 10 μL of LPS in RPMI/mL of blood. A second tube served as the unstimulated control with the addition of the medium used to dilute the LPS stock solution. The dose of 10 ng/mL of LPS for whole-blood stimulation was chosen as it is close to the neutralizing capacity of plasma and similar to the concentration of LPS found in septic patients (Dedrick and Conlon, 1995).

Both tubes were incubated at 38°C for 2 h in a water bath. The reaction was stopped by placing tubes on ice, followed by centrifugation at 4°C for 10 min at 1,500 × *g*.

RNA Extraction and Reverse-Transcription Quantitative PCR from Whole-Blood Leukocytes. Plasma was separated from the cell pellet and the cell pellet mixed with ice-cold ammonium-chloride-potassium red cell lysis buffer (Lonza, Basel, Switzerland). Cells were pelleted at 300 × *g* for 5 min at 4°C, and washed twice with ice-cold PBS. The white cell pellet was then lysed in RNA lysis buffer according to manufacturer's instructions (RLT buffer, Qiagen, Hilden, Germany). Extraction of RNA was performed with the RNeasy Plus Mini Kit after rapid homogenization of the cell lysate using a spin column (QIAshredder, Qiagen, Hilden, Germany). The optical density 260:280 ratio was measured with a spectrophotometer (NanoVue Plus, GE Healthcare, Life Sciences, Little Chalfont, UK) for purity of RNA. In addition, a subset of samples from 6 cows used for a separate experiment were analyzed with a fragment analyzer for RNA quality control (Advanced Analytical, Ankeny, IA) showing RQN values ranging from 8.8 to 10.0. For cDNA synthesis on the same day, up to 500 ng of RNA was reverse-transcribed (SuperScript III First-Strand Synthesis SuperMix, Life Technologies, Thermo Fisher Scientific) and stored at −20°C until analysis. Bovine-specific primer probe sets (TaqMan Gene Expression Assays, Applied Biosystems, Thermo Fisher Scientific) with exon spanning probes were purchased for the genes of interest (*IL10*: Bt03212724, *IL12B* (p40): Bt03213923, *TNF*: Bt03259154, *IL1B*: Bt03212741, *IL6*: Bt03211905). Two housekeeping control genes (*RPLP0*, assay Bt03218086 and *TBP*, assay Bt03241948) were chosen according to Brym et al. (2013). Real-time reverse-transcription quantitative PCR was performed using a 1:2 dilution of cDNA at 10% of the final reaction volume and each sample was analyzed in triplicate using a StepOne Plus system and 2× master mix (TaqMan Gene Expression Master Mix, Applied Biosystems, Thermo Fisher Scientific). The PCR protocol consisted of denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s (denaturation) and 1 min at 60°C (annealing and extension). Results were analyzed using the comparative quantification algorithms-standard curve method ($\Delta\Delta C_t$ method, StepOne Software, v 2.3). Results were expressed as relative quantity ($RQ = 2^{-\Delta\Delta Ct}$).

Analytical Approach

Differences in baseline characteristics of the study population were analyzed using Fisher's exact test for categorical variables (parity) and ANOVA for continuous outcomes (sampling day relative to calving, days dry). Concentration of glycogen, densitometry results of nonp4EBP1:4EBP1, pS6RP:S6RP, pAKT:AKT, as well as all gene expression data had to be log-transformed to achieve normality of residuals to satisfy the assumptions of the ANOVA. Leukocyte cytokine expression after stimulation was analyzed as the net increase in gene expression after subtracting the expression level before stimulation.

Repeated measures analysis was performed in Proc MIXED with the fixed effect of treatment group and sample time point, as well as their interaction. An unstructured covariance structure was chosen in all models. A fixed effect of study period as explained above was forced into every model. A treatment group × period interaction allowing for the identification of effects in the treatment groups alone compared with the control group (attributable to supplementation), as well as a treatment group × period × sample time point interaction were tested in all models and retained if $P < 0.05$. Outlier diagnostics were performed as defined a priori with the INFLUENCE statement and removed if Cook's distance exceeded 0.5. Differences in least

squares means were controlled for multiple comparisons using Tukey's test. After each model fit, residuals were visually inspected for normality and homoscedasticity. All results are presented as geometric means and 95% CI unless otherwise stated.

RESULTS

Description of Study Population

The analysis of pre- and postpartum diets is summarized in Table 1. The chemical composition of the BCAA supplement was as follows: L-leucine, 26.7% CP, 58.1% crude fat, 15% NDF; L-isoleucine, 36.5% CP, 37.6% crude fat, 14.6% NDF; and L-valine, 55.3% CP, 23.9% crude fat, 3% NDF. Parity distribution was not different between groups ($P = 0.96$). The actual sampling days relative to calving were −17.0 (−17.7 to −16.3), 7.0 (6.3 to 7.6), and 27.6 (27.0 to 28.3) for the 21 d prepartum, d 4 and 28 postpartum time points, respectively. The sampling days relative to calving were evenly distributed between the groups for all 3 time points ($P \geq 0.37$). Cows were dry for an average of 57 d (55.6 to 58.7), and no difference existed between the groups ($P = 0.67$). Average weekly DMI (±SD) was 14.0 ± 1.9, 15.2 ± 1.5, and 14.5 ± 2.0 for d 21 prepartum, 17.8 ± 3.7, 18.3 ± 2.1, and 18.9 ± 2.4 for d 7 postpartum, and 23.2 ± 4.3, 23.7 ± 2.2, and 24.5 ± 2.5 for d 28 postpartum in groups CTRL, BCAA, and BCAAPG, respectively. Two cows (1 in group BCAA, 1 in group BCAAPG) were missing the d 7 postpartum sample, and 2 cows had the d 21 prepartum sample only (1 in group BCAA, 1 in CTRL). Health events were diagnosed, recorded, and treated according to standard farm protocols. All health events occurred postpartum. Hypocalcemia occurred in 3, 2, and 1 animals in groups CTRL, BCAA, and BCAAPG, respectively. Pneumonia was diagnosed in 2 and 1 animals in groups CTRL and BCAA, respectively. Retained placenta was diagnosed in 4, 3, and 7 animals in groups CTRL, BCAA, and BCAAPG, respectively. Metritis occurred in 1 animal in group BCAAPG. One left displacement of the abomasum was diagnosed in a cow in group BCAA. One case of clinical mastitis was diagnosed in a cow in group CTRL.

Glycogen concentration was analyzed for all available samples. For immunoblot analysis, 20 blocks of cows with complete samples from all time points were selected for Western blot analysis in each group to achieve equal representation (n = 20 for CTRL, BCAA, and BCAAPG, respectively). For gene expression data, samples from 17, 16, and 17 animals in groups CTRL, BCAA, and BCAAPG were included. Cows produced an average of 39.0 (37.1 to 39.2), 40.9 (38.8 to 43.0), and 41.4 (39.6 to 43.2) kg of milk during the first 28 DIM in groups CTRL, BCAA, and BCAAPG, respectively ($P = 0.15$).

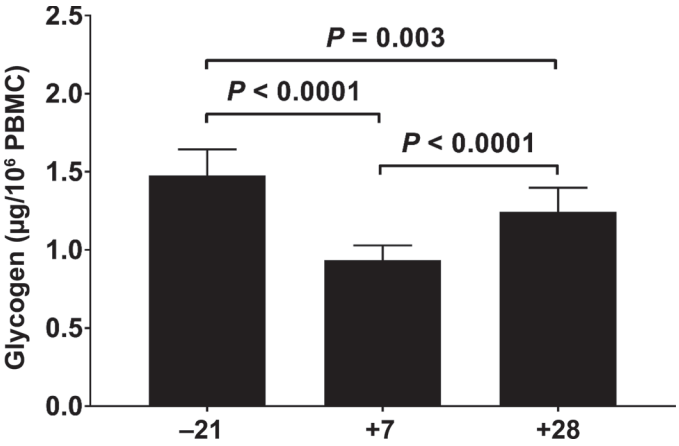

Figure 1. Glycogen concentration in peripheral blood mononuclear cells (PBMC; µg/10^6) at 3 different time points in the transition period (21 d prepartum, 7 and 28 d postpartum; n = 72). *P*-values for all pairwise comparisons between time points controlled for multiple comparisons using Tukey's procedure derived from repeated measures ANOVA. For results of other main effects (group and group × time interaction), see Supplemental Table S1 (https://doi.org/10.3168/jds.2018-14549). Error bars represent 95% CI.

PBMC Glycogen Concentration

Glycogen content did not differ between groups at any time (Supplemental Table S1; https://doi.org/10.3168/jds.2018-14549), but the average concentrations decreased to 63.2% of the prepartum levels on d 7 postpartum ($P < 0.0001$). On d 28 postpartum, average concentrations were 83.8% of the prepartum values and

Table 1. Analyzed composition (mean ± SD) of diets[1]

Item	Dry	Fresh
DM (%)	49.2 ± 4.6	52.0 ± 2.9
NE_L (Mcal/kg of DM)	1.48 ± 0.05	1.63 ± 0.03
CP (% of DM)	13.7 ± 0.9	15.5 ± 0.8
ADF (% of DM)	28.2 ± 2.3	21.6 ± 1.6
NDF (% of DM)	42.2 ± 3.3	31.7 ± 2.1
Starch (% of DM)	19.5 ± 3.1	25.7 ± 2.7
Ether extract (% of DM)	3.1 ± 0.3	3.4 ± 0.2
Ash (% of DM)	8.2 ± 0.7	7.9 ± 0.5
Ca (% of DM)	1.54 ± 0.35	0.89 ± 0.09
P (% of DM)	0.31 ± 0.04	0.36 ± 0.03
Mg (% of DM)	0.52 ± 0.07	0.46 ± 0.05
K (% of DM)	1.20 ± 0.16	1.28 ± 0.41
S (% of DM)	0.42 ± 0.05	0.37 ± 0.03
Na (% of DM)	0.13 ± 0.02	0.77 ± 0.11
Cl (% of DM)	0.64 ± 0.07	0.51 ± 0.05
DCAD (mEq/100 g of DM)	−7.8 ± 5.5	31.7 ± 6.1

[1]Near-infrared spectroscopy of 22 dry-period and fresh-period TMR composite samples, respectively (Cumberland Valley, Maugansville, MD).

this was different from both the 21 d prepartum concentrations ($P = 0.01$), as well as the d 7 postpartum values ($P < 0.0001$; Figure 1).

PBMC Immunoblotting

None of the proteins of interest differed between groups at any time point and no interaction between group and time was found except for the ratio of p-AKT ($P = 0.08$). Detailed results are summarized in Supplemental Table S1 (https://doi.org/10.3168/jds.2018-14549).

Results showed differences over time (Figure 2). The ratio of phosphorylated AKT to total AKT decreased to the lowest point on d 7 postpartum compared with the prepartum value ($P = 0.001$), and increased again on d 28 postpartum. Values on d 28 postpartum did not differ from d 21 prepartum ($P = 0.59$), but were higher than those on d 7 postpartum ($P = 0.01$, Figure 2A). The ratio of phosphorylated S6RP to total S6RP did not differ between time points ($P > 0.27$ for all pairwise comparisons; Figure 2B). The ratio of nonphosphorylated 4EBP1 to total 4EBP1 increased from d 21 prepartum to d 7 postpartum ($P = 0.07$) and decreased again on d 28 postpartum ($P = 0.51$). Both postpartum time points did not differ from each other ($P = 0.54$, Figure 2C).

Phosphorylation of AMPK increased from d 21 prepartum and remained elevated through the postpartum period. Both postpartum time points differed from the prepartum values ($P < 0.0001$), but did not differ from each other ($P = 0.39$, Figure 2D). The protein expression of INSR increased from d 21 prepartum to d 7 postpartum and further increased on d 28 postpartum. All time points differed from each other ($P < 0.02$, Figure 2E).

Whole-Blood Leukocyte Cytokine Expression

The baseline expression of all cytokines, except *IL1B*, changed over time (Figure 3, left panel). The RQ of *IL10* increased from the lowest expression on d 21 prepartum to the highest expression on d 28 postpartum (Figure 3A, left). The expression of *IL12B* was highest on d 7 postpartum compared with d 21 prepartum and d 28 postpartum (Figure 3B, left). The ratio of *IL12B:IL10* expression was highest on d 7 postpartum and differed from both d 21 prepartum as well as from d 28 postpartum (Figure 3C, left). The gene expression of *TNF* and *IL6* increased linearly from the prepartum to the postpartum time points, showing the highest expression on d 28 postpartum (Figure 3D and 3E, left). In contrast, the expression of *IL1B* decreased from the

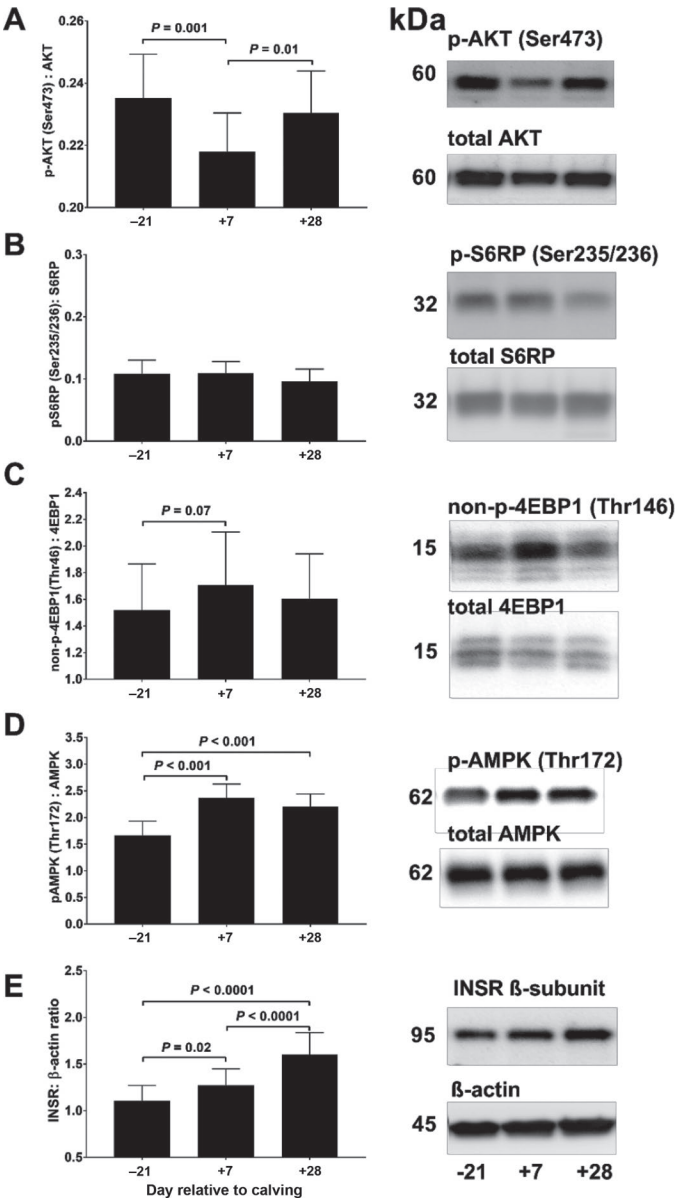

Figure 2. Immunoblotting results for phosphorylated proteins and insulin receptor protein expression in peripheral blood mononuclear cells (PBMC) from cows (n = 60) at 3 time points in the peripartum period (left panel), as well as representative Western blots from 1 animal (right panel). Samples were taken approximately 21 d before calving (−21), 7 d postpartum (+7), and 28 d postpartum (+28). Least squares means and 95% CI of densitometry results for the main effect of time on the ratio of phosphorylated (p) protein kinase B (pAKT; Ser473): AKT (A), the mechanistic target of rapamycin complex (mTOR) substrate p-S6 ribosomal protein (pS6RP; Ser235/236): S6RP (B), the mTOR substrate nonphosphorylated eukaryotic translation initiation factor 4E-binding protein 1 (non-p4EBP1; Thr46): 4EBP1 (C); p-AMP-activated protein kinase (pAMPK; Thr172): AMPK (D), and insulin receptor β-subunit (INSR): β-actin ratio (E) are presented. *P*-values for pairwise comparisons of the main effect of time were controlled for multiple comparisons using Tukey's procedure. For results of other main effects (group and group × time interaction), see Supplemental Table S1 (https://doi.org/10.3168/jds.2018-14549). Error bars represent 95% CI.

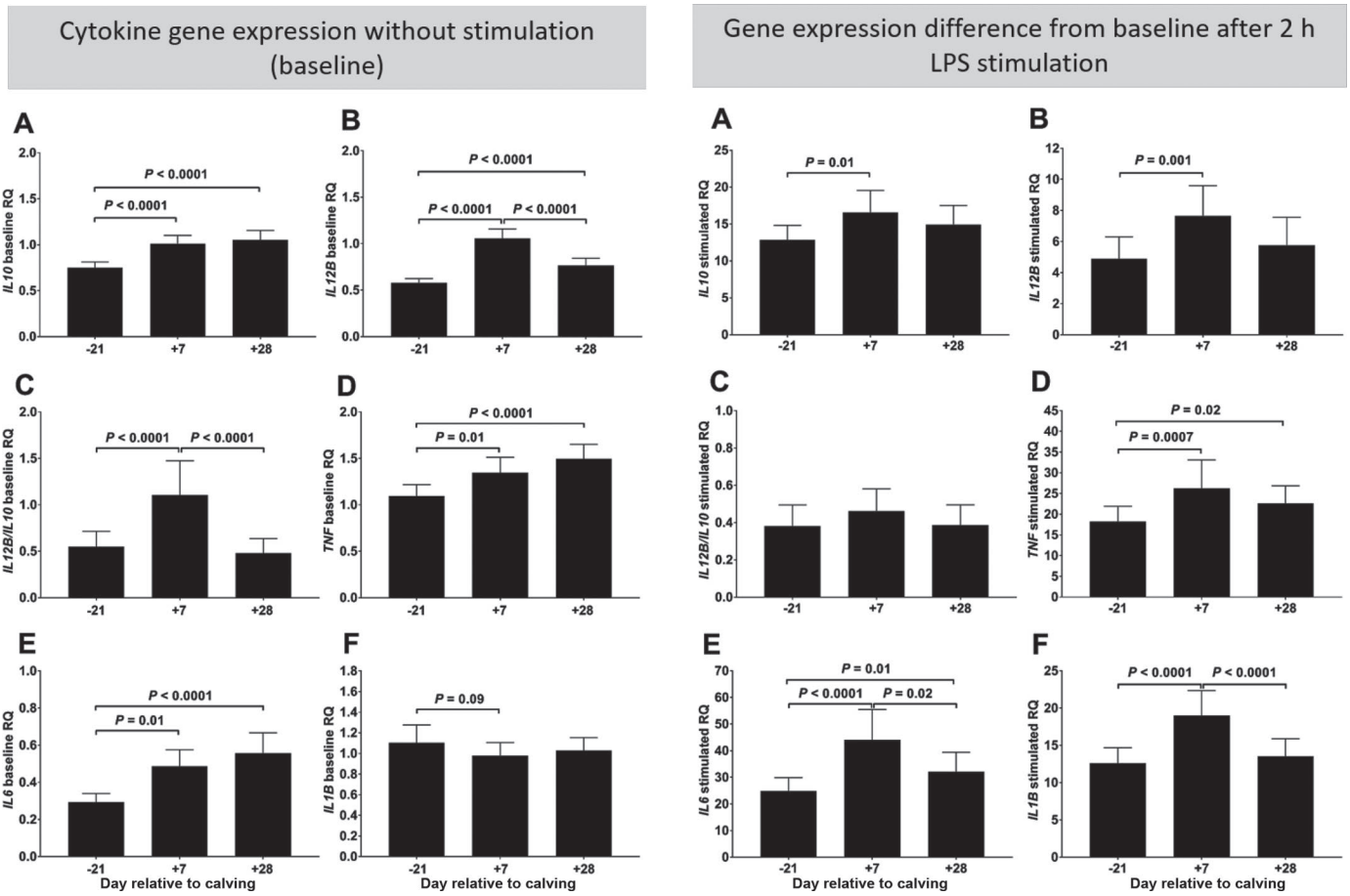

Figure 3. Cytokine gene expression (relative quantity, RQ) measured by reverse-transcription quantitative PCR in whole-blood leukocytes at 3 time points in the peripartum period from 50 cows before LPS stimulation (left panel A–F) and the net increase in RQ after 2 h of stimulation with 10 ng/mL of LPS at 38°C (right panel A–F). Results are presented as geometric LSM and 95% CI. Samples were taken approximately 21 d before calving (−21), 7 d postpartum (+7), and 28 d postpartum (+28). Gene expression of *IL10* (A), *IL12*B (B), the ratio of *IL12B:IL10* (C), *TNF* (D), *IL6* (E), and *IL1B* (F) are shown at baseline (left panel) and as the net increase (total expression – baseline; right panel). *P*-values for pairwise comparisons of the main effect of time were controlled for multiple comparisons using Tukey's procedure. For results of other main effects (group and group × time interaction), see Supplemental Table S2 (https://doi.org/10.3168/jds.2018-14549). Error bars represent 95% CI.

d 21 prepartum value to d 7 postpartum, but was not different on d 28 postpartum (Figure 3F, left).

Cytokine expression in whole-blood leukocytes before stimulation for each group over time is summarized in Supplemental Table S2A (https://doi.org/10.3168/jds.2018-14549). An interaction between group and time was found for the baseline expression of *IL6* ($P = 0.001$).

The expression of all cytokines, except the ratio between *IL12B:IL10*, changed over time when whole blood was stimulated with LPS (Figure 3, right panel). The expression of *IL10* was highest on d 7 postpartum compared with the prepartum values (Figure 3A, right), and the same was true for *IL12B* (Figure 3B, right). Although the ratio of *IL12B:IL10* followed the same pattern, statistical differences were not found between the time points. The *TNF*, *IL6*, as well as *IL1B* expression were highest on d 7 postpartum (Figure 3D, 3E, and 3F, right).

The difference in cytokine expression in whole-blood leukocytes after stimulation for each group and each time point is shown in Supplemental Table S2B (https://doi.org/10.3168/jds.2018-14549). No interaction between time and treatment group was found except for the ratio of *IL12B:IL10* expression after stimulation ($P = 0.09$).

DISCUSSION

The primary objective of this study was to describe changes in the activity of nutrient-sensing kinases in immune cells in the peripartum period. Our findings show that the activation of nutrient-sensing pathways experiences a measurable degree of change in immune

cells of early lactating dairy cows. This is evidenced by the lower phosphorylation ratio of AKT, higher phosphorylation ratio of AMPK, and a tendency for an increase in the nonphosphorylated form of 4EBP1. We have previously demonstrated the lower degree of activity of AKT in adipose tissue (Mann et al., 2016b), as well as of AKT and the mTOR substrate 4EBP1 in muscle tissue (Mann et al., 2016c) in the immediate postpartum period. The work presented here expands this finding to circulating immune cells. We hypothesize that the alteration in this pathway could serve as a possible link between energy status and inflammation, as well as other immune cell functions, given the well-documented role of these nutrient-sensing kinases in immunomodulation of other mammals.

Cytokine gene expression showed a clear increase in response in the postpartum period, with generally maximal response after stimulation on d 7 postpartum. At baseline, only *IL12B* reached a maximum on d 7 postpartum, whereas *TNF*, *IL6*, and *IL10* reached their maximum expression on d 28 postpartum. Interestingly *IL1B* was expressed lower at baseline early postpartum compared with prepartum, but increased similarly to other cytokines after stimulation.

The role of nutrient-sensing kinases in orchestrating the inflammatory response has been investigated in several recent studies. Stimulation of immune cells with LPS activates the AKT/mTOR-pathway as part of a survival response (Bauerfeld et al., 2012). Inhibition of AKT was shown to increase TNF-α and IL-6 production in murine macrophages (Bauerfeld et al., 2012), as well as IL-12 production in murine splenic dendritic cells (Fukao et al., 2002). Inhibition of mTOR signaling in human dendritic cells and monocytes increased IL-12, IL-6, and TNF-α and decreased IL-10 production (Haidinger et al., 2010; Macedo et al., 2013). Bovine monocyte-derived dendritic cells from postpartum cows showed a significantly lower upregulation of IL-10 after stimulation with *E. coli* compared with monocyte-derived dendritic cells from cows in the dry period (Pomeroy et al., 2015). Although we investigated the response of whole-blood leukocytes and thus did not single out individual cell subsets, the upregulation of the pro-inflammatory cytokines IL-12, IL-6, and TNF-α is consistent with the above-mentioned studies. The fact that IL-10 expression was also increased postpartum, albeit the maximum being delayed, could be interpreted as a physiological feedback response following expression of pro-inflammatory cytokines and may be enabled by the fact that all cell populations were represented in our sample. However, alternative explanations of the increase in IL-10 should be considered. Under reduced cellular energy status, AMPK is activated. The robust phosphorylation of AMPK in postpartum PBMC may contribute to an inhibition of the AKT/mTOR pathway due to its known suppressive effect on mTORC1 (Shaw, 2009; Blagih et al., 2012; O'Neill and Hardie, 2013) as described above. However, AMPK activation was shown to decrease TNF-α and IL-6 production while increasing IL-10 production in LPS-stimulated macrophages in vitro, pointing to a possible independent role of AMPK as a counter-regulator of inflammation (Sag et al., 2008).

Although we are showing a temporal association between the changes in nutrient-sensing kinases and the inflammatory profile, we cannot demonstrate a causal relationship. Clearly, exposure to LPS from other sources or other immunological stimuli may be the reason for the changes in cytokine expression at baseline in the postpartum period, particularly during the high-risk period postpartum. However, this would not explain the net increase in response after stimulation. Further characterization of the role of the AKT/mTOR-pathway in bovine immune cell subsets, particularly in the transition period, and experimental alteration of the pathway is necessary to better understand the effect of our findings on immune dysfunction and inflammation when cows experience the greatest degree of nutrient deficit.

The lower concentration of glycogen found in PBMC during the postpartum period reinforces the fact that these cells are undergoing a period of negative energy balance. Whether the degree of reduction in glycogen storage is biologically important was not tested in this study, but appears likely. We base this assumption on previous work by Galvao et al. (2010) who found that cows affected by metritis postpartum already had lower glycogen concentration in neutrophils on the day of calving and hypothesized that the lack of energy storage may affect cellular function. In addition, a lack of readily available glycogen to serve as energy substrates for the generation of ATP may further increase the activation of AMPK, which in turn inhibits the mTOR kinase. The results for glycogen concentration in PBMC are very similar to results by Naidu and Newbould (1973) on bovine white blood cells (approximately 1.3 $\mu g/10^6$). It should be noted that glycogen storage in PBMC is lower than that of neutrophils (Naidu and Newbould, 1973; Galvao et al., 2010) although most mononuclear cell subsets rely at least partially on glycolytic metabolism for survival, maturation, and function (Balmer and Hess, 2017).

The upregulation of the INSR protein expression could be due to a more activated immune cell state as certain immune cell subsets increase the expression of the receptor in response to stimulation and to direct glucose toward the cells (Maratou et al., 2007; Fischer et al., 2017). Alternatively, it is possible that the linear

increase in protein expression is part of the physiological adaptation to the nutrient deficit postpartum and upregulation may serve as a glucose scavenging mechanism during the time of the nadir in circulating insulin concentrations typically observed in the postpartum period (Mann et al., 2016a).

Our secondary objective was to investigate if changes in the AKT/mTOR signaling pathway, the amount of glycogen storage, as well as the inflammatory profile of bovine immune cells could be altered by supplementation with BCAA alone or BCAA with the addition of the glucose precursor propylene glycol. Our results do not support a measurable effect of BCAA supplementation on the parameters investigated here (as expressed by an interaction of time point and group) with the exception of the *IL6* expression at baseline in whole-blood leukocytes. Cows in group BCAAPG showed the smallest degree of change from the prepartum values immediately postpartum. In the absence of replication of these findings and demonstration of an effect on the inflammatory balance in a larger group of cows, the biological significance should be interpreted carefully. The fact that BCAA supplementation alone was not effective in dampening the decrease in the AKT/mTOR-pathway could point to the fact that although certain AA such as leucine are known to independently stimulate and be critical for the activation of mTOR kinase (Manjarin et al., 2016), all other AA, energy, and insulin have to be available in sufficiently increased quantities to produce a measurable effect (Calder et al., 2007; Bar-Peled and Sabatini, 2014). Alternatively, supplemented AA could have been used as energy precursors, and it may be necessary to also increase available energy in carbohydrate form to spare AA as energy substrates.

To our knowledge, this is the first study investigating these nutrient-sensing kinases in immune cells of transition dairy cows in vivo. The well-documented intricate link between nutrient-sensing kinase signaling and innate inflammatory response (Fruman et al., 2017) is intriguing given the fundamental changes in nutrient availability of transition dairy cows. Although these first results are encouraging to further investigate the role of these pathways in the bovine species, the study had several limitations that the reader should keep in mind when interpreting the findings. First, as we did not want to manipulate immune cell subsets due to the risk of loss of phosphorylation or activation, we studied PBMC, a mixed cell population that may change in composition in the transition period, as well as between individual cows. Therefore, observed changes in nutrient-sensing pathways might be at least partially associated with changes in the composition of PBMC. Future studies could employ a flow-cytometry based technology that would at least help to separate out cell subsets by size and granularity, and potentially by cell surface markers for populations of interest. The same is true for the inflammatory gene expression profile of whole-blood leukocytes. Our reasoning for stimulating whole-blood samples was that we did not want to change the environment and composition of the immune cells compared with the situation in the animal, as we believed that separating out immune cells may lead to alteration of nutrient status and alter our results. We believe that this is a valid approach to address our hypothesis that the systemic inflammatory profile changes over time. Using whole-blood leukocytes limits our ability to point to specific immune cell populations or changes in their representation that may be responsible for the changes in the observed inflammatory profiles.

We intentionally did not exclude animals with postpartum disease in this analysis, as this would have just affected animals with overt clinical signs but would not have allowed us to correctly classify animals with subclinical disease. Thus, the study group reflects the typical representation of animals in the transition period. Future studies could characterize nutrient-sensing kinase activity and inflammatory gene expression profiles specifically targeting animals with certain postpartum diseases compared with those that are healthy. However, the sample size in this current study would not have allowed for such comparisons, as this was not part of our study design.

Last, the role of the nutrient-sensing kinase pathways in inflammatory response and immune function in general is complex and comparison of literature findings in other species are complicated by the fact that most data are generated in vitro in immune cell subsets that are taken out of their metabolic and signaling context. As we begin to understand the role of these pathways in the bovine species, and given the crucial changes in nutrient availability and other stressors in transition dairy cows, efforts should be directed at preserving these relationships whenever possible.

CONCLUSIONS

We conclude that the signaling of the nutrient-sensing kinases AKT/mTOR and AMPK is altered in cows in the immediate postpartum period. The decrease in AKT/mTOR activation and the increase in AMPK phosphorylation are consistent with the state of nutrient deficit in the transition period. This was also demonstrated by a decrease in glycogen storage in bovine immune cells postpartum. Whole-blood leukocyte gene expression profiles changed concurrently, with greater cytokine gene expression at baseline and

after stimulation of cells with LPS postpartum. The temporal association is intriguing as nutrient-sensing kinases have been implicated in moderating the inflammatory cytokine profile in other species, but the presence or absence of a causal relationship needs to be demonstrated in future studies that extend this work and experimentally alter the identified pathways.

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