Far-off and close-up feeding levels affect immunological performance in grazing dairy cows during the transition period¹

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ABSTRACT: During the peripartum period, dairy cows often have signs of inflammation. Various stresses, including infectious and metabolic diseases, have been discussed as causative for this inflammation. In this study, expression profiles for 17 immune markers were measured in whole blood preparations from 78 dairy cows over a time frame starting 1 wk before calving to 4 wk after calving. Additionally, the effects of far-off and close-up feeding on immune function of dairy cows during the peripartum period were investigated. Cows were assigned to 1 of 2 feeding levels in late lactation to achieve a low and high BCS at the time of dry-off (approximately 4.25 and 5.0 on a 10-point scale). Following dry-off, both herds were managed to achieve a BCS of 5.0 one month before calving; this involved controlled feeding (i.e., maintenance) and over-feeding of ME during the far-off dry period. Within each far-off feeding-level treatment, cows were offered 65, 90, or 120% of their precalving

ME requirements for 3 wk precalving in a 2 × 3 factorial arrangement. Analysis of gene expression profiles from blood cells revealed effects of time indicating that the transition cow's immune system counteracts the peripartum inflammation, whereas later postcalving it becomes activated to provide protection against postpartum infections. Far-off feeding affected ($P < 0.05$) the expression of 2 of the investigated genes at calving. Interleukin-6 (IL-6) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression in unstimulated, peripheral leukocytes were lower ($P < 0.05$) in animals from the Far-Off_Over-fed group compared with the Far-Off_Control-fed group. Close-up feeding had several effects on gene expression, indicating that immune function in Feed120 animals was distinct from the Feed90 and Feed65. In conclusion, feeding management precalving becomes an important intervention to ensure immunocompetence at and after calving.

Key words: immunity, immunocompetence, peripartum period, transition cow

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INTRODUCTION

An estimated 30 to 50% of dairy cows experience metabolic or infectious disease in the period immediately postcalving (LeBlanc, 2010). This increased incidence of diseases has a significant effect on production and reproduction. It is likely caused by the metabolic challenges dairy cows are exposed to while transitioning from late pregnancy to early lactation (Bell, 1995; Drackley et al., 2005), which also induce immunologic changes (Goff and Horst, 1997; Ingvarsen and Moyes, 2013; Heiser et al., 2015). The peripartum period of dairy cows is often, if not always, accompanied by inflammatory conditions, including the release of proinflammatory cytokines (Bertoni et al., 2008). Various causes for peripartum inflammation in dairy cows, such as infectious or metabolic diseases, trauma, endotoxins from the gut, or stresses, have been reviewed by Drackley et al. (2005).

Previous studies have investigated the phenotype and function of neutrophils (Gilbert et al., 1993; Madsen et al., 2002; Hammon et al., 2006; Graugnard et al., 2012; Crookenden et al., 2016; Crookenden et al. 2017) and lymphocytes (Kehrli et al., 1989; Lessard et al., 2004; Lacetera et al., 2005; Karcher et al., 2008; Schwarm et al., 2013; Heiser et al., 2015; Nightingale et al., 2015; Lange et al., 2016). These studies provide evidence that essentially all cows experience some degree of systemic subacute inflammation in the days after parturition (Bradford et al., 2015). Immune function is modulated during the peripartum period, and this likely affects susceptibility to infectious diseases after calving. In our earlier studies (Heiser et al., 2015; Lange et al., 2016), we reported that feeding management precalving can influence immune function at and after calving and suggested that there may be a benefit to a restricted precalving diet in optimally conditioned cows, whereas under-conditioned cows should be fed to requirements.

Studies of the past 10 yr have established gene expression analysis of immune cells as a powerful method to characterize immune cell function (Touzot et al., 2015). This study utilizes the analysis of gene expression in white blood cells. Genes have been selected for analysis that are known to be key regulators or effector molecules, particularly for T helper cells (Th cells) (further details on the selected genes are provided in the Appendix). Th cells release characteristic cytokines to help other cells of the immune system, mainly B cells and cytotoxic T cells (Janeway et al., 2001). Several subtypes of Th cells are known, e.g., Th1, Th2, and Th17. Th1

cells are important for the defense against intracellular bacteria and protozoa. Th2 cells are effective against extracellular parasites including helminths. Th17 cells are important for clearing pathogens at mucosal surfaces.

Several studies have indicated an association between dietary energy intake before calving and metabolic and molecular changes (Schmitt et al., 2011; Graugnard et al., 2012; Ji et al., 2014; Akbar et al., 2015; Lange et al., 2016). The BCS at calving has an effect on metabolic profiles (Roche et al., 2013) and it has been reported that the effect of precalving level of feeding is dependent on precalving BCS (Roche et al., 2015). In the current study, the hypothesis was investigated that different trajectories of BCS gain precalving affect immune function during the peripartum period. A better understanding of these effects can inform feeding recommendations for improved immune function.

MATERIALS AND METHODS

Animals and Treatment

In accordance with the New Zealand Animal Welfare Act (1999), all animal manipulations were approved by the Ruakura Animal Ethics Committee (Hamilton, New Zealand). The experiment was undertaken at the DairyNZ Lye Farm facility, Hamilton, New Zealand (37°46'S 175°18'E) between January and October 2014. One hundred and fifty mid-lactation dairy cows of mixed age and breed (Holstein-Friesian, Jersey, Holstein-Friesian × Jersey) that passed a veterinary clinical examination were enrolled in the experiment ~20 wk before the planned start of calving. Cows were allocated randomly to 1 of 2 treatment groups (75 cows per group). All cows were dried off 79 ± 9.5 d before calving.

The study was designed to have 2 feeding levels during the far-off nonlactating period (i.e., 80 to 40 d before calving) but a similar BCS at approximately 3 wk prior to calving. Therefore, the treatment groups needed to be at different BCS at the end of lactation. One of the treatment groups was managed to gain very little BCS (<0.25 BCS units; all BCS units in 10-point scale, unless otherwise stated; Roche et al., 2004) during the last 8 wk of lactation and 0.75 to 1.0 BCS units in the 7 wk after the completion of lactation (i.e., over-feeding during the far-off nonlactating period; **Far-Off Over-fed**). In comparison, the other group was managed to gain approximately 0.5 to 0.75 BCS units during

the last 8 wk of lactation, so that they had <0.25 BCS units to gain in the 7 wk after the completion of lactation (i.e., controlled feeding during the far-off nonlactating period; **Far-Off_Control-fed**). During this BCS establishment period, 3 groups of cows were managed separately to achieve the treatment target BCS at dry-off by being assigned to gain, maintain, or lose BCS groups. Cows in all groups grazed an allowance of fresh pasture and were supplemented with pasture silage, corn silage, and palm kernel extract

From 19 ± 5.4 d before calving (i.e., close-up nonlactating period), cows within each BCS gain group were randomly assigned to 1 of 3 levels of ME intake in a 2×3 factorial arrangement of treatments. Feed allowances were managed to achieve 3 levels of ME intake up until calving (65, 90, and 120% of estimated ME requirements; 1.05 MJ of ME/kg of BW^{0.75}; (Roche et al., 2005); **Feed65**, **Feed90**, and **Feed120**, respectively). The 3 close-up treatment groups were grazed within the same paddock before calving and separated by an electric fence to control pasture allowance/treatment. Cows were allocated to treatment twice per week based on the expected calving date.

Although cow allocation to treatment was random, groups were assessed to ensure they were balanced for age, breed, BCS at the time of enrolment, and expected calving date. The complete details for the animal management have been reported elsewhere (Roche et al., 2017).

Blood Samples

Of the 25 animals in each treatment group, 13 were randomly selected for gene expression analysis. Blood was sampled on multiple days via coccygeal venipuncture at approximately 0800 h. To allow analysis of gene expression for a time period starting before calving and covering an extended time after calving (4 wk), while maintaining feasibility and considering animal welfare, blood was sampled at 4 time points; 5 to 11 d before calving (Pre) and 7 ± 2 d (week 1), 14 ± 2 d (week 2), and 28 ± 3 d (week 4) after calving. Blood was collected from 13 animals per treatment group into evacuated blood tubes containing lithium heparin as an anticoagulant (Vacutainer, Becton & Dickinson, NJ). Neutrophils are short-lived cells with a half-life of ≤ 7 h that should be analyzed within 2 to 4 h of collection; they are also extremely easily activated. Hence, analysis of gene expression in neutrophils was performed using different sampling and processing methods and will be reported separately.

Here, following a procedure that has been established in previous studies of gene expression in blood lymphocytes and monocytes (Heiser et al., 2015; Lange et al., 2016), samples were shipped overnight (16 to 25 h) at ambient temperature to the Hopkirk Research Institute (Palmerston North, New Zealand) for further processing.

In Vitro Stimulation of Whole Blood

For in vitro stimulation, 2 mL of whole blood was transferred to a 50 mL tube. Stimulation was performed by adding Staphylococcal enterotoxin B (SEB) and LPS (both from Sigma-Aldrich, St. Louis, MO) to a final concentration of 200 ng/mL. For each blood sample, another 2 mL of whole blood was used without stimulation. Tubes were incubated for 22 ± 0.5 h at 37 °C in 5% CO₂. Prewarmed (37 °C) Tris-buffered ammonium chloride lysis buffer was added for lysis of red blood cells and samples incubated for 10 min at 37 °C. After centrifugation, 2 washes with PBS (300 × g, 7 min, room temperature) were performed, cell pellets were resuspended in TRIzol (Invitrogen, Carlsbad, CA) and frozen at -80 °C.

To obtain baseline gene expression, unstimulated whole blood samples were analyzed and results expressed as RNA copy number. They were compared with samples that were stimulated in vitro. Expression of stimulated samples is presented as fold change in RNA copy number compared with the unstimulated samples.

RNA Preparation

Samples were retrieved from -80 °C storage and quickly thawed for subsequent preparation of RNA. RNA was prepared using Direct-zol RNA Kits (Zymo Research, Irvine, CA) according to the manufacturer's recommendations. Briefly, Trizol samples were mixed with an equal volume of ethanol (95 to 100%) and transferred to a separation column. Columns were washed twice with RNA PreWash buffer and once with RNA Wash Buffer. RNA was eluted using 15 µL of RNase-free water and archived at -80 °C.

Gene Expression Analysis

Gene expression analysis from RNA samples was performed using Nanostring technology and the nCounter Analysis System (Geiss et al., 2008). This system utilizes molecular barcodes on gene sequence-specific probes and single molecule

imaging to count RNA copies. This allows the analysis of multiple genes from the same sample (multiplexing) using a set of probes with distinct barcodes, called a CodeSet.

RNA samples were analyzed using a CodeSet consisting of probes specific for 17 genes from *Bos taurus* (Table 1). Genes were chosen based on their involvement in the induction of T lymphocyte responses (*CD40* and its ligand *CD40LG*, *IL12*, and *IL23R*), because they are expressed by activated leukocytes (*IFNG*, *TNF*, *IL2*, *IL2R*, *IL4*, *IL17*, *PRF*), are involved in lymphocyte inhibition (*IL-10*, *CTLA4*, *PDCD1*), or are otherwise of interest (*IL6*, *IL23*, *GAPDH*) (see Appendix for further information on target genes). Equipment, reagents, and supplies for the Nanostring analysis were purchased from Nanostring (Seattle, WA).

The Nanostring technology allows for the analysis of multiple reference genes in each sample and the use of multiple reference genes is advised. Therefore, for this study, 3 reference genes were chosen: 1) *RPL15* is the symbol for “ribosomal protein L15” (gene ID 507151); 2) *YWHAZ* is the symbol for “tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide” (gene ID 287022); 3) *GUSB* is the symbol for “glucuronidase, beta” (gene ID 515687). Anstaett et al. (2010) found *YWHAZ* among the 3 most stable genes in bovine lymphocytes under their experimental conditions. Focusing on expression in oocytes others found *RPL15*, *PPIA*, and *GUSB* to be most stable (Macabelli et al., 2014). For their studies of bovine liver Lisowski et al. (2008) reported, among others, *YWHAZ* to be most stable. It has also been identified as a suitable reference gene in whole blood neutrophils from beef calves (Vorachek et al., 2013). Additionally, we analyzed gene expression data from one of our collaborators (Bhuju et al., 2012; Aranday-Cortes et al., 2012, 2013), confirming the value of *RPL15*, *YWHAZ*, and *GUSB* as reference genes for bovine neutrophils.

Hybridization. RNA samples were hybridized with the CodeSets according to manufacturer’s instructions (nCounter Gene Expression Assay User Manual (MAN-C0003-03)). Briefly, RNA samples were thawed on ice. MasterMix was prepared by adding 130 μ L of hybridization buffer and 13 μ L of RNAase-free water to Reporter CodeSet tubes. Twenty-one microliters of MasterMix, 4 μ L of RNA sample, and 5 μ L of Capture ProbeSet were added to each tube of a 12-tube-strip immediately before placing the strip at 65 °C for 18 $\pm$ 0.5 h.

Purification and immobilization. After hybridization, excess probe was removed and the probe/target complexes aligned and immobilized in the nCounter cartridge. These steps were performed automatically by the nCounter Prep Station.

Counting. Sample cartridges were placed in the nCounter Digital Analyzer for data collection. Color codes on the surface of the cartridge are counted and tabulated for each target molecule. Data were retrieved from the Analyzer as raw data (RCC files).

Quality control for gene expression analysis. For analysis, the raw data (RCC files) were imported into nSolver Analysis Software v2.5 (free to download at <http://www.nanostring.com/products/nSolver>).

nSolver’s Quality Control routine was performed using the corresponding RLF file (BoNtr01_C3862_plex2.rlf) which contains the CodeSet information. All samples passed the QC routine by meeting any of the following criteria: 1) Imaging: fields of view registration is less than 75%; 2) Binding density: binding density is outside the 0.05 to 2.25 range; 3) Positive control linearity: positive control R^2 value < 0.95; 4) Positive Control limit of detection: 0.5 fM (femtomolar) positive control is $\leq$ 2 standard deviations above the mean of the negative controls.

Background subtraction was performed by subtracting the geometric mean of 8 internal negative controls from each sample.

Positive control normalization was performed by using the geometric mean of 6 internal positive controls to compute the normalization factor. The normalization factor of all samples was inside the 0.3 to 5.0 range. Reference gene normalization was performed using the geometric mean of counts for the reference genes *RPL15*, *GUSB*, and *YWHAZ*. The normalization factor of all samples was inside the 0.1 to 99.0 range. Inter-CodeSet Calibration was performed by repeat testing 1 sample per run.

Statistical Analysis

Since repeated measurements were made on the same experimental unit (cows), a repeated measures ANOVA with 2 experimental factors, Far-off Feeding, with 2 levels (Far-Off_Control-fed and Far-Off_Over-fed and Close-up Feeding, with 3 levels (Feed65, Feed90, and Feed120) and 1 repeated measures factor (4 time points) has been used. Linear mixed effects models were utilized with appropriate

Table 1. CodeSet specifications

Gene	Protein name	Accession	Position	Target sequence
<i>CD40</i>	CD40 or TNF receptor superfamily member 5	NM_001105611.1	1043–1142	CCGCTCAGTCTCCCAACCTGCTTTTAAACGATGGTAAACAAAACCTTGG GGGAGGACATACATAATATCCACGACCTTCCACCCAGCGGGTTCAT
<i>CD40LG</i>	CD154 or “tumor necrosis factor (ligand) superfamily, member 5”	NM_174624.2	609–708	AGTGGATCAGAGAGAACTCTTACTGAGAGCTGCAACACCCACAGTCTTCCAAA CCATGGGGCAGCAATCCATTCACTTAGGAGGAGTCTTTGAATTGC
<i>CTLA4</i>	CD152 or cytotoxic T-lymphocyte-associated protein 4	NM_174297.1	36–135	GTGGACATCTAGGACCTGGCCCTGCACCTGCCCTATTTTCTTGCTTCATTCCTGT TTTCTCTAAAGGATGATGTGACCCAGCTCCAGTGGTGCTG
<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase	NM_001034034.1	213–312	TGATTCCACCCACGGCAAGTTCAACGGCACAGTCAAGGCAGAGAACGGGAAGCT CGTCATCAATGGAAGGCCATCACCATCTTCCAGGAGCGAGATCCT
<i>GUSB</i>	Glucuronidase, beta	NM_001083436.1	1815–1914	GATTTTCATGACTAACACAGTCCAGTTCGGATGATTGGGAACAGAAAAGGATCTT CACTCGCAGAGACAAACAAAGAGTGCAGCTTTCTCTGTTCGAG
<i>IFNG</i>	Interferon-gamma type 2 or IFN- γ	NM_174086.1	503–602	TCAAAGTGATGAATGACCTGTACCAAAATCTAACTCAGAAAGCGAAGAGAA GTCAGAATCTCTTCGAGGCGGAGAGCATCAAGTAATGGTCCTC
<i>IL-10</i>	Interleukin-10	NM_174088.1	145–244	GGCGAGGCGAAGACTTTCTTTTCAATGAAGGACCAACTGCACAGCTTACTGTTGAC CCAGTCTCTGCTGGATGACTTTAAGGGTTACCTGGGTGCGCAAG
<i>IL12A</i>	Interleukin-12, p35	NM_174355.1	410–509	ACGTGGAGTTCCAGGCCATGAATGCAAGCTTCTGATGATCCTAAGAGGCAAAAT CTTTCTAGACCAGAAACATGCTGGCAGCTATTGCTGAGCTGATGCA
<i>IL17A</i>	Interleukin-17A	NM_001008412.1	148–247	TCCAGGCTGCCCCACTACTGAGGACAAGAACTTCCACAGCATGTGAGGGTCAA CCTAAACATCGT TAAACGGAGCACAACTCCAGAAAGGCCACCGAT
<i>IL2</i>	Interleukin-2	NM_180997.2	218–317	CAGGATGCATACATTTGACTTTTACGTGCCCAAGGTTAACGCTACAGAAATTGAAAC ATCTTAAGTGTCTACTAGAAGAACTCAAACTTCTAGAGGAAGTG
<i>IL22</i>	Interleukin-22	NM_001098379.1	389–488	CCAATCTGACAAATCCACCCCTATATGAAAGGTGGTGCCTTCTCTCCAGG CTCAGCAAAAAGCTAAGCCAAATGTCATGTTGAGTCTGACAACCCAG
<i>IL23R</i>	Interleukin-23 receptor	NM_001127172.1	588–687	CCAAGCTCAAAATGTTCTGGGCATGGAGAAGTCGAAACAACACTACAAATTCATC TGACGATATAGTGATACCTTCTGCAATCCATTATTTCCAGGGCTGAG
<i>IL2RA</i>	Interleukin-2 receptor, alpha	NM_174358.2	1642–1741	CTGTTGAGCTTCAGAGTTTGGAAAGGATTTTCACTGGAGTTAATGCCAGGAGAA GGGGAGAAAGCTGGGTCACGTAGTGGGTTATCCTTAACATTTAGCTTC
<i>IL4</i>	Interleukin-4	NM_173921.2	237–336	TGCTGCCCCAAGAACAACAACACTGAGAAGGAACCTTCTGCAGGGTTGGAATTGA GCTTAGGCGTATCTACAGGAGCCACAGTGTCTGAACAAATTCCTG
<i>IL6</i>	Interleukin-6	NM_173923.2	293–392	CACGGCAGAAAATAAGCTGAATCTTCCAAAATGGAGGAAAAGGACGGATGC TTCCAATCTGGGTTCATATCAGGCGATTGCTTGATCAGAAACCATGTC
<i>PDCD1</i>	Programmed cell death 1 or PD-1 or CD279	NM_001083506.1	321–420	TGCCGATGGGACAGAGTTTAACATAGCATCTGGCGGCCACGCGCAATGACAG CGCGCTCTACTTCTGGGGCCATCTACCTGCCACCCCGGACGCA
<i>PRF1</i>	Perforin 1 (pore forming protein)	NM_001143735.1	1271–1370	CAGGTCGTGGGAGACTGGATCACTGCCACGGACGCCTATCTGAAGGTTTTTT CGCCGGCCAGAGCAGAGGACCGCCACAGTATGGAACGATAACAA
<i>RPL15</i>	Ribosomal protein L15	NM_001077866.1	984–1083	ACCTGTAGGTTTGTGATGCATGTGTTGAGACCTGCAGCTTATGGGGTGATG CAACACTCTGCAGTTTACGGAGTTTGGCAGGGACAAACATTTTCAG
<i>TNF</i>	Tumor necrosis factor ¹ (TNF superfamily, member 2) or TNF- α	NM_173966.2	950–1049	CTTCAGGCCCTCTCATCCCTCTGTTTAAAGGGAATATGGGCTCAGGGCTG GGCTCCAAAGCATCCAACITTAACAACAGCTACATTTAGAAAT
<i>YWHAZ</i>	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide ²	NM_174814.2	147–246	CAAGGAGCTGAATTATCCAATGAGGAGGGAATCTTCTCTCAGTGTCTTATAAAAATG TTGTAGGAGCCCCGTAGTCACTCTTGGAGGGTCTGCTCCAGTA

variance function for modeling heteroscedasticity and with Cow as the random effect. None of the response variables met (at least approximately) the assumptions for ANOVA. All nonstimulated genes/response variables met (at least approximately) the ANOVA assumptions with “natural LOG” transformation. For those genes meeting the ANOVA assumptions only marginally, we evaluated permutation tests (with 5,000 randomizations) and the resulting significance of the various effects was very similar to those of the usual ANOVA; thus, the results of the ANOVA were retained. For fold-change data, Sample ID 753 (i.e., Animal ID 9162, treatments Far-Off_Over-fed & Feed65 for week 4) resulted in unusually large model residual (outside of [-6,6] interval in terms of standardized residuals; thus, it was regarded as a probable “outlier”) for genes *CD40LG*, *CTLA4*, *IL12A*, *IL4*, *IL22*, *PDCD1*, and *PRF*. These were removed from analyses. A square-root (for genes *CD40*, *CD40LG*, *CTLA4*, *IL2*, *IL23R*, *IL2RA*, *PRF1*, *TNF*) or log (for genes *GAPDH*, *IFNG*, *IL-10*, *IL12A*, *IL17A*, *IL4*, *IL6*, *IL22*, *PDCD1*) transformation was required for satisfactory ANOVA assumptions for the fold-changes. *P*-values < 0.05 were considered statistically significant (with smaller values providing stronger evidence for significance); *P*-values ≥ 0.05 and < 0.1 were considered a trend.

In order to analyze gene expression profiles together for both the unstimulated as well as the stimulated cells, a Canonical Discriminant Analysis (CDA) was performed. CDA determines linear functions of variables called Canonical Discriminant Functions (CDFs) that maximally separate the groups of individuals while keeping the variation within groups as small as possible. The groups of interest here are the combinations of Far-off Feeding with 2 levels and Close-up Feeding with 3 levels.

All analyses were undertaken using the R software version 3.3.1 (R Core Team, 2015).

RESULTS AND DISCUSSION

A previous report of the current study showed that over-feeding of ME to low BCS cows during the far-off nonlactating period had multiple effects (Roche et al., 2017). It increased blood albumin to globulin ratio compared with the cows that were dried off close to recommended calving BCS and control-fed during the far-off dry period. It also reduced blood fatty acid and β -hydroxybutyrate concentrations in early lactation. Cows in the Feed65 group 1) had lower fatty acids and

β -hydroxybutyrate in early lactation, 2) produced less milk, particularly during the first 21 d of lactation, 3) had more than 3-fold greater concentration of haptoglobin immediately postcalving, and 4) had a lower blood cholesterol concentration and albumin to globulin ratio, when compared with the Feed90 and Feed120 groups.

Gene expression profiles for immune markers were obtained from whole blood preparations from cattle exposed to various feeding regimes from 1 wk before calving to 4 wk after calving. No significant effect was observed for Breed nor for any of the interactions of groups (Far-Off group $\times$ Feed group, Far-Off group $\times$ Time, Feed group $\times$ Time, Far-Off group $\times$ Feed group $\times$ Time; all *P* > 0.2).

Effects of Transition on Gene Expression Profiles of Circulating Leukocytes

Genes encoding inducers of immune responses revealed higher expression at week 2 and week 4 (Fig. 1) compared with precalving: expression of *CD40* and *IL23R* was greater (*P* < 0.05) and *CD40LG* tended (*P* < 0.10) to be more highly expressed at week 2. *CD40* is constitutively expressed on antigen-presenting cells, explaining the relatively high RNA copy number (Murphy et al., 2007). *CD40LG* is expressed on naive T lymphocytes upon recognition of antigen (Murphy et al., 2007). Both molecules drive further activation of their respective cell types. *IL12A* in leukocytes was unaffected by calving, but the expression in bovine neutrophils has been reported to be lower on the day of calving compared with postcalving time points (Crookenden et al., 2016). Higher expression of *IL23R* was evident in week 2 and week 4 compared with week 1. *IL23* and *IL12* have different receptors and different effects. *IL12* induces development of Th1 cells, which produce *IFNG* (Croxford et al., 2014); in comparison, *IL23* is known to drive Th17-type responses (Chognard et al., 2014; Floss et al., 2015). IL-23 is involved in the differentiation of Th17 cells in a proinflammatory context, particularly in the presence of TGF- β and IL-6 (Gaffen et al., 2014). Activated Th17 cells produce IL-17, IL-6, IL-22, TNF, and GM-CSF. *IL23R* mRNA expression is highest in CD8 and $\gamma\delta$ T cells (Chognard et al., 2014). Inflammatory macrophages also express IL-23R and are activated by IL-23 to produce IL-1, TNF, and IL-23 (Duvallet et al., 2011). No effect on expression of any of these markers at or immediately after calving was evident. Taken together, the higher expression of genes encoding inducers

Figure 1. Gene expression profiles (RNA copy number) during the peripartum period. *CD40*, *CD40LG*, *IL12A*, and *IL23R* are markers for the activation of T cells. *IFNG*, *TNF*, *IL2*, *IL2RA*, *IL4*, *IL17A*, and *PRF1* are expressed in activated T cells. *IL10*, *CTLA4*, and *PDCD1* are inhibitory molecules. Each data point represents the mean from a group of animals ($n = 78$; $\pm$ SEM). The arrows indicate a trend for a difference (broken arrow; $0.1 > P > 0.05$) or a statistically significant difference (solid arrow; $P < 0.05$).

of immune responses at week 2 and week 4 indicate the onset of postpartum infections and the immune response against them.

Expression of activation markers was slightly lower for all genes at week 1 (Fig. 1). Although not statistically significant for individual genes, the accumulated effect of this lower expression most likely reflects dampened activity of the immune system after calving. This is likely an effort to confine peripartum inflammation. Dairy cows go through systemic inflammation of some degree around calving (Drackley et al., 2005; Bertoni et al., 2015) and this is likely caused by metabolic processes in liver and adipose tissue (Gerner et al., 2013; Masoodi et al., 2015; Vailati-Riboni et al., 2016). Shortly after calving and due to tissue damage in the uterus and the mammary gland, an increased immune response is needed to fight off the onset of infections that otherwise lead to clinical metritis or mastitis. All the leukocyte activation markers demonstrated greater expression at week 2 and week 4 compared with precalving and week 1 although significant only for *TNF*, *IL2RA*, and *PRF1*. *IL2RA* is expressed on a large proportion of activated and memory T cells

(Janeway et al., 2001; Triplett et al., 2012). The proportion of IL-2R-positive lymphocytes increases in early lactation relative to the mid-dry period (Meglia et al., 2005). Perforin (*PRF1*) is an effector molecule required for the cytotoxic function of T lymphocytes (Trapani, 1995). With the accumulation of antigen-experienced T cells with age, the proportion of cells expressing perforin increases (Hogg et al., 2011). The same can be expected—on a smaller scale—during an infection. T lymphocytes of particular importance for the defense of mucosal surfaces are Th17 cells (Dubin and Kolls, 2008). Increased expression of IL-17 was demonstrated in mammary tissue and somatic cells in milk but not in circulating leukocytes, indicating that IL-17 plays a role locally but not systemically (Tao and Mallard, 2007; Bruno et al., 2010). In this study too, *IL17A* expression in blood was widely unaffected during the peripartum period.

Further evidence for an anti-inflammatory activity peripartum is the increased expression of the anti-inflammatory cytokine IL-10 (Karcher et al., 2008; Heiser et al., 2015), which was confirmed here, although only as a trend ($P < 0.10$).

Others have also reported that IL-10 expression in leukocytes is minimal in mid and late lactation compared with postpartum (day 0 to 3) (Shafer-Weaver et al., 1999). Furthermore, IL-10 expression in bovine neutrophils has been reported to be higher on the day of calving compared with pre- and postcalving time points (Crookenden et al., 2016). Whereas IL-10 is a rather general anti-inflammatory cytokine, CTLA4 and PDCD1 are inhibitory regulators specific to T lymphocytes. At week 2 and week 4, *PDCD1* expression was lower ($P < 0.05$) compared with precalving and week 1. Another inhibitory molecule in T lymphocytes, *CTLA4*, had a greater expression at week 4 ($P < 0.05$). *CTLA4* inhibits T cells during activation early (Schildberg et al., 2016), whereas *PDCD1* is expressed later. *PDCD1* has a role in T cell exhaustion (Okagawa et al., 2016; Schildberg et al., 2016). Following acute and chronic infection, *PDCD1* is upregulated on T lymphocytes (Attanasio and Wherry, 2016). Expression of *PDCD1* returns to low levels after resolution of acute infections (Barber et al., 2006). Together, these observations support the interpretation that shortly before and after calving, circulating lymphocytes express *PDCD1* as a consequence of the systemic inflammation. Later (week 2 and week 4) they were replaced by lymphocytes that have low *PDCD1* and only little *CTLA4* expression, the latter a result of recent activation from the onset of postpartum infections.

Of the remaining markers, *IL6* had a greater expression at week 2 and week 4 (Fig. 1). Compared with healthy animals, stimulated monocytes from cows with metritis had lower *IL6* gene expression at 7 and 14 d after calving (Galvão et al., 2012). *IL6* expression in bovine neutrophils has been reported to be highest on the day of calving compared with pre- and postcalving (Crookenden et al., 2016). These circulating neutrophils are probably a result of the systemic inflammation peripartum which lessens later, at which time activated T lymphocytes, also expressing IL-6, enter the circulation.

Taken together, these observations indicate that during the peripartum period the transition cow's immune system is expressing less activating and more inhibitory pathways to counteract a systemic subacute inflammation (Bradford et al., 2015), whereas, later it becomes activated, providing antigen-presenting cells, naive T lymphocytes and Th17 cells specialized in defending mucosal surfaces when and where they are needed to fight postpartum infections of the uterus and mammary gland.

Effects of Transition on Gene Expression Profiles of In Vitro Stimulated Peripheral Leukocytes

Antigen-presenting cells (*CD40*) and T lymphocytes (*CD40LG*) were less able to respond to a stimulus, further supporting the hypothesis of a dampened immune response at calving. Both early inducers of immune responses, *CD40*, and its ligand *CD40LG* had a lower ($P < 0.05$) fold change at week 1 compared with precalving (Fig. 2). Furthermore, *CD40* also tended ($P < 0.10$) toward an even lower response at week 4 compared with week 2. This indicates that antigen-presenting cells (*CD40*) and T lymphocytes (*CD40LG*) were less able to respond to a stimulus further supporting the hypothesis of a dampened immune response at calving. Fold change of *IL12A* tended to be increased at week 1 and be lower again at week 2; compared with precalving, this does not fit the hypothesis of a dampened immune response at calving. However, the IL-12 response to stimulation in vitro increased significantly at week 4 indicating the onset of adaptive immune responses to infections. Surprisingly, *IL23R* on the stimulated leukocytes had an opposite response, with expression decreasing ($P < 0.05$) at week 2 and week 4. IL-23 contributes to differentiation of Th17 cells, especially in the presence of TGF- β and IL-6 (Duvall et al., 2011), with gene expression of the latter being decreased as well (Fig. 2). In this context, the decrease in *IL23R* expression after stimulation could be interpreted as a shift away from the stimulation of Th17 and towards Th1 cells, which provide protection within tissues rather than at the mucosal surfaces.

All of the investigated activation markers, except for *IL17*, had a lower expression at week 1 (Fig. 2), but the difference was only significant for *IL2* and *PRF1*. Through its effects on T cells, IL-2 plays an important role in regulating immunity and immunological tolerance (Liao et al., 2011). Perforin is expressed by cytotoxic T lymphocytes and NK cells and crucial for their cytotoxic function (Trapani, 1995). Decreased expression by in vitro stimulated bovine leukocytes during the peripartum period has been reported previously for IFN- γ (Ishikawa et al., 1994; Heiser et al., 2015) and for TNF and IL-17 (Heiser et al., 2015). Decreased expression of these lymphocyte activation markers provides further support for the interpretation that the immune system is counterbalancing the inflammation in the liver and adipose tissue.

The gene expression data indicate a shift from innate immunity (TNF) to adaptive immunity (e-2)

Figure 2. Changes in gene expression profiles in in vitro stimulated vs unstimulated cells during the peripartum period. *CD40*, *CD40LG*, *IL12A*, and *IL23R* are markers for the activation of T cells. *IFNG*, *TNF*, *IL2*, *IL2RA*, *IL4*, *IL17A*, and *PRF1* are expressed in activated T cells. *IL10*, *CTLA4*, and *PDCD1* are inhibitory molecules. Each data point represents the mean from a group of animals ($n = 78$; $\pm$ SEM). The arrows indicate a trend for a difference (broken arrow; $0.1 > P > 0.05$) or a statistically significant difference (solid arrow; $P < 0.05$).

occurred during the weeks postpartum. After calving, *TNF* had a lower expression at week 4 compared with week 2 and *IL2* expression had a trend to increase from week 1 to week 2 and again to week 4. The lower expression of *IL2RA* at week 4 compared with week 2 is harder to interpret. Complex interactions of IL-2 and IL-2RA expression exist that likely influence both the Th1/Th2 differentiation as well as the development of memory T cells (Liao et al., 2011).

The T lymphocyte-inhibitory genes, *CTLA4* and *PDCD1*, demonstrated opposite changes in gene expression at week 1 compared with precalving. Expression of *CTLA4* was lower and *PDCD1* greater (Fig. 2). The increase in IL-10 expression that we reported in previous work (Heiser et al., 2015) was not evident in this study. *PDCD1* is a late inhibitor of T cells (Francisco et al., 2010). Its increased expression by in vitro stimulated T lymphocytes indicates that these samples from shortly after calving contain previously stimulated T cells that are now being switched off. This is further support for the hypothesis that the immune system tries to counterbalance the inflammation. In contrast, *CTLA4* is an early inhibitor of T cells (Buchbinder and Desai, 2016) and lower expression by in vitro stimulated T lymphocytes indicates that

the ability to activate naive T cells is maintained, if not improved, after calving.

The altered expression of *IL6* may indicate a shift in the T lymphocyte populations. *IL6* expression by in vitro stimulated leukocytes was lower ($P < 0.05$) at week 2 compared with week 1. In vitro stimulated *IL6* expression has been reported to be increased day 21 and day 27 after calving in cows that develop metritis in comparison to healthy cows (Galvão et al., 2012). IL-6 expression by LPS stimulated leukocytes was also reported to be greater around calving (± 3 d) compared with 6.5 wk post-calving (Jahan et al., 2015). Besides having several roles in the innate immune system (Schaper and Rose-John, 2015), *IL6* is also necessary for developing a Th17 cell response (Bettelli et al., 2006). The lower expression later after calving, in concert with the increased *IL2* expression, indicates a shift from a Th17 to a Th2 response.

After in vitro stimulation, leukocytes dropped in *GAPDH* expression during week 2 compared with week 1 (Fig. 2, row 5). Given its diverse functions (Nicholls et al., 2012), it cannot be concluded if these effects are in relation to the metabolic function of *GAPDH* or its role in the regulation of cell survival (Lee et al., 2011).

Effects of Far-off Feeding on Gene Expression Profile of Peripheral Leukocytes

The far-off feeding generated 2 groups of animals, Far-Off_Over-fed and Far-Off_Control-fed, by 5 wk after the completion of lactation. Currently, there is agreement that nutrition, microbiome, and immunity are interlinked, but very little is known about specific dependencies and regulation (Tilg and Moschen, 2015; Veldhoen and Ferreira, 2015), particularly with regards distant effects (Røntved et al., 2005; Ohtsuka et al., 2006). In this study, far-off feeding affected the expression of 2 of the investigated genes at calving.

IL6 expression in unstimulated, peripheral leukocytes was lower in animals from the Far-Off_Over-fed group compared with the Far-Off_Control-fed group (Fig. 3). During inflammation, IL-6 is expressed by a wide variety of cells, most importantly neutrophils and monocytes/macrophages (Schaper and Rose-John, 2015). Our results indicate that monocytes (and long-lived neutrophils in the blood samples) from animals from the Far-Off_Control-fed group had a higher baseline expression of *IL6*.

Far-off feeding level affected ($P < 0.05$) *GAPDH* expression in leukocytes. Animals from the Far-Off_Over-fed group expressed less *GAPDH* than those from the Far-Off_Control-fed group (Fig. 3). Interpretation of the biological relevance and function of this difference is difficult. Given its role in the regulation of cell survival (Lee et al., 2011) and the differences in *IL6* expression, it might provide signaling to increase the survival of cells of the innate immune system.

Effects of Close-up Feeding on Gene Expression Profile of Peripheral Leukocytes

Close-up feeding had several effects (Fig. 3) on gene expression. Baseline expression of *IL2RA* was lower in Feed90 and Feed120 compared with Feed65, whereas *IL4* was higher in Feed120 compared with Feed65 and Feed90. Given the role of IL-2 and IL-4 in the induction of Th1 and Th2 responses (Raphael et al., 2015), it can be speculated that the underfed animals favor developing a Th1 response, which targets mainly bacterial infections, whereas overfed animals rather develop a Th2 response, which is effective against extracellular parasites. *PDCDA1* had lower expression in Feed65 compared with Feed 90 and Feed120. *CTLA4* was affected, both at baseline, with Feed65, with higher expression than Feed90, and with stimulated cells

revealing lower expression in the Feed65 group compared with the Feed90 and Feed120. Stronger late inhibition of T cells via *PDCD1* in Feed90 and Feed120 animals indicates that these animals have an augmented need to counterbalance inflammation. *IL6* was unaffected at baseline level but had lower expression in Feed65 compared with Feed90 and Feed120 after in vitro stimulation. Cells from the latter 2 groups contributed to an inflammatory state by producing more IL-6, which is one of the most important proinflammatory cytokines (Schaper and Rose-John, 2015).

Multivariate Analysis of Gene Expression Profiles of Peripheral Leukocytes

The gene expression profiles, when considered together, for both the unstimulated as well as the stimulated cells demonstrated several statistical outcomes. Analysis of the baseline expression profiles revealed that, of the possible 5 canonical discriminant functions, only the first (CDF1), accounting for about 45% of the group separation, reasonably well distinguished Feed120 group from Feed90 and Feed65 groups for both Far-Off_Control-fed and Far-Off_Over-fed treatments (see Fig. 4A showing boxplots of canonical discriminant function scores). However, for each of the close-up feeding levels, no notable difference is sighted between the 2 Far-Off feeding groups. This led to looking at the Close-up feeding groups ignoring Far-off feeding, and the corresponding CDA results are shown in Fig. 4B. About 81% of the group differences are explained by the first CDF, and the remaining by the second CDF. As noted before, Fig. 4B (showing boxplots of CDF scores) and Fig. 4C (showing CDF scores of animals in the data) indicate that CDF1 separates Feed120 reasonably well from Feed90 and Feed65 groups. The linear functions (standardized coefficients, Fig. 4D) indicate that, CDF1 is a contrast of the genes, with *CD40*, *CTLA4*, *IL12A*, *IL23R*, *IL2RA*, *PDCD1*, and *TNF* on one side (positive coefficient) and the others on the other side, but dominated by *TNF* and *IL12A* versus *CD40LG*, *IL17A*, and *PRF1*. CDA of the expression profiles in in vitro stimulated cells did not reveal any notable separation of any of the feeding treatments (data not shown).

This implies that about 81% of the Close-up Feeding group differences are mainly due to the differences stated above. Relating back to the CDF1 linear function, it appears that Feed120 group generally has relatively higher expression levels for genes *CD40LG*, *IL17A* and *PRF1*, which represents

Figure 3. Gene expression profiles from animals receiving various feeding regimen. Gene expression profiles (RNA copy number) in unstimulated cells and change after in vitro stimulation (fold change) at week 1 from animals receiving various feeding regimen (Far-off feeding: Far-Off_Control-fed and Far-Off_Over-fed; Close-up feeding: Feed65, Feed90, and Feed120). Each data point represents the mean from a group of animals ($n = 26$; $\pm$ SEM). Different letters indicate statistically significant difference ($P < 0.05$).

a “Th17-like response” compared to *TNF* and *IL12A*, which represents a “Th1-like response”; and the other 2 groups behave opposite to this. This can be interpreted as the Feed120 animals being at an earlier phase of the immune response, mainly providing Th17 responses for protection of the mucosal surfaces, whereas the Feed90 and Feed65 groups are providing Th1 responses for protection within tissues.

The current study provides further evidence that immune functions undergo changes during

the peripartum period. At calving or shortly thereafter, the transition cow’s immune system is counterbalancing inflammation caused by metabolic stress in liver and adipose tissue, before it shifts to an activated state providing cells and cytokine environments to fight postpartum infections of the uterus and mammary gland. Also, further proof is provided that changes in far-off and close-up nutrition can be driving forces for immunomodulation in transition dairy cows and support anti-inflammatory functions at calving

Figure 4. Canonical discriminant analysis (CDA) for nonstimulated samples. (A) Boxplots of canonical discriminant functions (CDF) 1 scores for the 6 Far-off/Close-up groupings. (B) Boxplots of CDF1 scores for the 3 Close-up groupings. (C) Scatter plots of CDF1 and CDF2 scores of all animals for the Close-up groupings. (D) Linear functions (standardized coefficients) for CDF1 for the Close-up groupings.

and innate and adaptive immune responses in the weeks thereafter. Consequently, feeding management precalving becomes an important intervention to ensure immunocompetence at and after calving.

Conflict of interest statement. None declared.

APPENDIX: FURTHER INFORMATION ON GENES ANALYZED

CD40 is expressed on antigen-presenting cells and is required for their activation after binding to the *CD40* ligand on T helper cells (Bedoui et al., 2016). *CD40LG* is the symbol for the “*CD40* ligand.” *CD40LG* is primarily expressed on activated T cells. It binds to *CD40* on antigen-presenting cells leading to their activation (Bedoui et al., 2016). *IL12A* is the symbol for “interleukin-12, p35.” Interleukin-12 (IL-12) is secreted by antigen-presenting cells in response to antigenic stimulation and serves as a costimulator differentiating naive T cells into T helper 1 cells (Croxford et al., 2014). IL-12 and IL-23 cytokines, respectively drive Th1 and Th17-type responses (Chognard et al., 2014). *IL23R* is the symbol for “interleukin-23 receptor” (IL-23R). IL-23 controls T helper 17 cell development and can contribute to autoimmune diseases; polymorphisms of the receptor have been discussed in the context of Crohn’s disease and similar diseases (Floss et al., 2015). Expression

of *IL-23R* is restricted to specific T cell subsets, a small number of B cells and innate lymphoid cells (Chognard et al., 2014).

Interferon-gamma type 2 (IFN- γ) is critical for innate and adaptive immune responses against viral and intracellular bacterial infections and is also a key activator of macrophages (Schroder et al., 2004). *TNF* is the symbol for “tumor necrosis factor” (*TNF* superfamily, member 2), also known as *TNF- α* . *TNF* regulates immune cells, with variable effects. Dysregulation of *IFNG* and *TNF* is associated with a number of diseases (Locksley et al., 2001; Kalliolias and Ivashkiv, 2016). *IL2* is the symbol for “interleukin-2” (IL-2). IL-2 has key roles in the control of immunity and immunological tolerance, primarily via its direct effects on T cells (Liao et al., 2011). *IL2RA* is the symbol for “interleukin-2 receptor, alpha.” *IL2RA* is expressed on various immune cells. With its ligand IL-2, it has key roles in the control of immunity and immunological tolerance, primarily via direct effects on T cells (Liao et al., 2011). *IL4* is the symbol for “interleukin-4” (IL-4). IL-4’s main function is to induce the differentiation of naive T helper cells into T helper 2 cells (Sokol et al., 2008). *IL17A* is the symbol for “interleukin-17A.” Interleukin-17 (IL-17) acts similarly to IFN- γ and synergistically with *TNF*. It is produced by T lymphocytes and supports migration of monocytes and neutrophils to the site of inflammation (Jin and Dong, 2013). *PRF1* is the symbol for “perforin 1 (pore forming

protein).” Perforin is expressed by cytotoxic T lymphocytes and NK cells. Upon degranulation, perforin binds to the target cell’s plasma membrane in which it forms pores that allow for the passive diffusion of proteases, known as the granzymes, into the target cell (Trapani, 1995).

In contrast to the proinflammatory cytokines, interleukin-10 (*IL10*) is considered to be an anti-inflammatory cytokine (Couper et al., 2008). *CTLA4* is the symbol for “cytotoxic T-lymphocyte-associated protein 4.” *CTLA4* is a protein receptor that downregulates immune responses. On conventional T cells, *CTLA4* is upregulated after activation and functions as an “off” switch when bound to CD80 or CD86 on the surface of antigen-presenting cells (Buchbinder and Desai, 2016). *PDCD1* is the symbol for the protein “programmed cell death 1,” also called PD-1. It is expressed by T and pro-B cells and prevents activation of T cells through a dual mechanism of promoting apoptosis (programmed cell death) in antigen-specific T cells in lymph nodes while simultaneously reducing apoptosis in regulatory T cells (Francisco et al., 2010).

Interleukin-6 (*IL-6*) acts as both a proinflammatory cytokine and an anti-inflammatory myokine. *IL-6* is secreted by neutrophils, monocytes, macrophages, fibroblasts, endothelial cells, smooth muscle cells, and T cells to stimulate immune responses (Schaper and Rose-John, 2015). *IL22* is the symbol for “interleukin-22.” *IL-22* is produced by activated DC and T cells and promotes innate immune responses against bacterial pathogens especially in epithelial cells (Wolk et al., 2004). *GAPDH* is the symbol for “glyceraldehyde-3-phosphate dehydrogenase”. *GAPDH*’s main function is the catalysis of the sixth step of glycolysis (Seidler, 2013) but has recently been implicated in several non-metabolic functions (Nicholls et al., 2012). *GAPDH* has successfully been used as reference gene in a large number of studies but on the other hand, significant differences in the expression levels of *GAPDH* mRNA were evident between tissue types and between donors of the same tissue (Barber, 2005).

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