A CHARACTERIZATION OF INFLAMMATORY AND STRUCTURAL MARKERS WITHIN THE RUMEN EPITHELIUM DURING GRAIN-INDUCED RUMINAL ACIDOSIS IN LACTATING DAIRY CATTLE

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ABSTRACT

The objective of this study was to characterize the mRNA and protein expression of inflammatory and structural genes in the rumen epithelium during grain-induced ruminal acidosis in lactating dairy cattle. A total of 16 rumen-fistulated, lactating Holstein dairy cattle (618±35 kg of body weight, 221±32 days in milk) were used in a randomized complete block design study. All cattle were initially fed a high-forage diet (HF; 88.9% of dry matter) and after a baseline (wk 0) measurement, half of the cattle were randomly assigned and transitioned to a high-concentrate diet (HC; 62.2% of dry matter) which was fed for 3 weeks (weeks 1, 2 and 3). Continuous ruminal pH, ruminal LPS and plasma LPS-binding protein were measured each week followed by a rumen papillae biopsy used for mRNA and protein quantification. After the baseline period, ruminal LPS was higher in HC compared to HF cattle (28851±6905 vs. 5771±3042 EU mL^{-1}). There was no difference in mRNA expression of inflammatory and structure genes in rumen papillae between HF and HC cattle during all weeks. With regard to protein expression, there was an up regulation (p = 0.02) of nuclear factor of activated T-cells cytoplasmic 2 expression during weeks 1, 2 and 3; however, all other inflammatory markers within the rumen epithelium were unchanged by treatment. These results suggest that although grain-induced ruminal acidosis leads to characteristic whole-animal inflammatory response, only marginal changes in inflammatory and structural gene and protein expression in the rumen epithelium were detected.

Keywords: Rumen Epithelium, Ruminal Acidosis, Inflammation, Gene Expression

1. INTRODUCTION

To meet the demands of milk production in early lactation, it has become common to feed dairy cattle diets rich in rapidly fermentable carbohydrates. When cattle are fed rapidly fermentable diets, typically achieved by high inclusion levels of grains, the rate of ruminal acid production may exceed the rate of ruminal absorption and buffering, causing a digestive disorder termed ruminal acidosis. The sub acute form of ruminal acidosis, termed SARA, has become common in North American dairy production systems and is typically diagnosed when ruminal pH drops below 5.6 for more than three hours per day (AlZahal *et al.*, 2007). Sub acute ruminal acidosis has been shown to depresssruminal fiber digestion, feed intake, milk production and milk fat and has become a significant economic issue in our dairy industry (Plaizier *et al.*, 2008).

Dairy cattle health and welfare can be compromised by grain-induced SARA as it has been associated with rumenitis, bloat, liver abscesses and laminitis (Plaizier *et al.*, 2008). A more recent finding is that grain-induced SARA causes an increase in the concentration of acute phase proteins such as Serum Amyloid A (SAA) and Haptoglobin (Hp) in peripheral blood, indicative of systemic inflammatory response (Gozho *et al.*, 2007;

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Plaizier *et al.*, 2008). The whole-animal inflammatory response during grain-induced SARA is thought to be initiated through altered permeability of the gut, such as the Rumen Epithelium (RE) (Penner *et al.*, 2011), which enables the transmigration of microbes and immunogenic compounds into portal circulation. The structural transformations and inflammation of the RE during grain-induced SARA have been characterized (Steele *et al.*, 2011b), but the precise molecular mechanisms triggering these events are unknown.

It has been known for some time that increases in grain intake lead to a large and sudden increase in ruminal gram negative bacterial load (Motoi *et al.*, 1993). It has recently been shown that there is an increase in lysis of gram-negative bacteria, causing an increase in ruminal LPS during grain-induced ruminal acidosis, which may be triggering a localized inflammation of the RE (Gozho *et al.*, 2007; Nagaraja and Titgemeyer, 2007). Consequently, increases in acidity, coupled with LPS, may work together to damage the RE, thereby initiating a local inflammatory response that will eventually lead to antigen clearance, wound healing and the reestablishment of homeostasis (Thibault *et al.*, 2010). Currently it is not known whether changes in RE integrity are the result of immune-mediated events corresponding to changes in gene and protein expression. A review of the literature reveals that one of the most ubiquitous and important transcription factors controlling the expression of pro-inflammatory cytokine and chemokine genes is Nuclear Factor κB (NF-κB) (Hoffmann and Baltimore, 2006; Calder, 2008). It has been extensively characterized that LPS interacts with Toll-Like Receptors (TLR), thereby activating mitogen-activated protein kinases and subsequently nuclear NF-κB. It is possible that the elevation in ruminal LPS could be triggering an immune response during grain-induced SARA; therefore, an examination of expression patterns of cytokines and chemokines involved in this immunomodulatorycascade is warranted.

Recent efforts investigating the molecular adaptation of the RE during SARA in dairy cattle have focused on characterizing the expression profiles of metabolic and transporter genes at the mRNA level (Penner *et al.*, 2009; 2011; Steele *et al.*, 2011b; 2012). There is a scarcity of information regarding the expression of key inflammatory pathways at both the mRNA and protein levels. By studying inflammatory pathways, we can better understand the biochemical and perhaps the genetic factors that lead to disease. Therefore, the aim of this study is to characterize the mRNA and protein expression of key inflammatory and structural gene targets during grain-induced SARA. We hypothesized that the grain-induced ruminal acidosis in lactating dairy cattle is associated with the differential mRNA and protein expression of inflammatory genes in the RE.

2. MATERIALS AND METHODS

2.1. Animals, Experimental Treatments and Feeding

The outline of the experimental design and treatments has been described previously by Steele *et al.* (2012). In brief, sixteen multiparous, rumen-cannulated, lactating Holstein dairy cattle (618±35 kg of BW, 221±32 DIM, mean ± SD) housed in a tie-stall facility at the Ponsonby Dairy Research Station were used in this study (University of Guelph, Guelph, Ontario, Canada). All experimental procedures were approved by the University of Guelph Animal Care Committee in accordance with the Canadian Council on Animal Care (Olfert *et al.*, 1993).

All cattle were gradually transitioned to a high-forage diet (HF; 88.9% hay, 6.7% grain pellet, 4.4% supplement % of DM; 16.2% CP, 46.9% NDF, 27.9% NFC, 7.4% starch) five wk preceding the experiment. The protein supplement and hay were fed at 0800 h and 1600 h in equal allotments and a grain mix (40% ground wheat, 40% ground barley, 20% ground corn) was fed at 0800, 1200 and 1600 h, also in equal allotments. To maintain consistency of dietary intake between cattle, the concentrate portion was fed in plastic trays and all leftovers were introduced into the rumen via the fistula if animals did not consume it within 60 min post feeding. At the end of week 0, the cattle were randomly assigned to be maintained on the control group (HF) diet or transitioned to an HC diet (HC; 37.8% hay, 57.8% grain pellet, 4.4% supplement % of DM; 14.5% CP, 32.3% NDF, 46.0% NFC, 27.9% starch). The transition to the HC diet was conducted in gradual increments over five days and HC cattle were fed the diet until the end of week 3.

2.2. Physiological Measurements

Physiological measurements were performed during the final two days of each week (weeks 0, 1, 2 and 3). For the last 48 h of each experimental week (weeks 0, 1, 2 and 3), ruminal pH was recorded in the ventral sac as described previously by our research group (AlZahal *et al.*, 2007). Ruminal fluid samples were collected at 1600h for the last two days of each experimental week and assessed in duplicate for ruminal SCFA concentration by gas chromatography as previously described (Steele *et al.*, 2012). Another portion of the rumen fluid sample was processed for determination of free LPS concentrations in HC cattle by a chromogenic Limulus Amoebocyte

Lysate (LAL) end-point assay (QCL-1000, Lonza Group Ltd., Basel, Switzerland) as described by Gozho *et al.* (2005). To monitor blood LPS-Binding Protein (LBP) concentration, a 14-gauge catheter (Becton Dickinson Angiocath, Franklin, NJ) was inserted into one jugular vein of each cow under local anaesthesia in the morning before blood collection. Blood samples were collected at 800h and 1600h during the final day of each experimental week from HF and HC cattle. Immediately after collection, blood samples were put on ice, centrifuged at 3000 g for 15 min and frozen at -20°C until analysis. The concentration of LBP in plasma was determined in duplicate using previously described methods (Gozho *et al.*, 2005).

2.3. Rumen Papillae Biopsies for mRNA and Protein Quantification

Rumen papillae were biopsied from the ruminal ventral sac at the end of weeks 0, 1, 2 and 3 (0700h) based upon previously developed methodology (Steele *et al.*, 2011a; 2011b). In brief, the reticulo-rumen contents were partially evacuated to facilitate the retraction of the ventral sac. Rumen papillae were excised (150 mg) using surgical scissors each week from previously unbiopsied sites and washed 20 times in ice-cold PBS (pH 7.4) (Steele *et al.*, 2011a; 2011b).

Rumen papillae were snap-frozen in liquid nitrogen, then stored at -80°C until total RNA or protein could be isolated. The target genes to assess immune changes in the RE Included Interleukin-1 (IL1), Interleukin-1 Receptor-Associated Kinase 1 (IRAK1), Nuclear Factor of Activated T-Cells Cytoplasmic 2 (NFATC2), Tumor Necrosis Factor-Alpha (TNFA), Interleukin 6 (IL6) and Nuclear Factor Kappa B (NFKB), B-Cell Lymphoma/leukemia 10 (BCL10),and Inhibitor of nuclear factor Kappa-B Kinase subunit Alpha (IKKA). In addition, Collagen Alpha-1-IV chain (COL4A1), Fibronectin (FN1), Laminin subunit Beta-1 (LAMB1) were assessed as markers for changes in rumen epithelial structure.

Total RNA was isolated as previously described by Steele *et al.* (2012) using an RNeasy midi kit (Qiagen, Missisauga, Ontario, Canada) and the concentration was determined using a NanoDrop (ND-1000, NanoDrop Technologies, Wilmington DE). RNA was then treated with DNase (Invitrogen, Burlington, Ontario, Canada) prior to assessing the quality using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA) and the RNA 6000 Nano kit (Caliper Life Sciences, Mountain View, CA). After RNA isolation and quality assessment, samples (5 µg each) were reverse-transcribed before iTaq SYBR Green (Bio-Rad Laboratories) qPCR analysis in duplicate using an ABI Prism 7000 (Applied Biosystems). The sequences, R^2, standard curve slope and primer efficiencies of primers are presented in **Table 1**. Exon-spanning primers

for target genes were designed using NCBI/Primer BLAST Primer Express (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and bovine sequences listed in Gen Bank (National Center for Biotechnology Information, Bethesda, MD). The amp icons of all primers designed for this study were verified using BLASTN in NCBI and dissociation curves were generated at the end of amplification to verify the presence of a single product. On the basis of previous experimental protocols (Steele *et al.*, 2011a), GAPD was determined to be the most stable housekeeping gene in the rumen papillae and was therefore used in this study. For each week, the relative mRNA expression of genes was calculated using the inverse of qPCR efficiency raised to Delta Ct (Pfaffl *et al.*, 2004). The HF cattle were pooled for each week to create a control value to which each HC sample was normalized for determination of relative mRNA expression, as previously described (Xue *et al.*, 2010).

Total protein from rumen papillae samples was extracted by the addition of a lysis buffer (Ready Prep Protein Extraction Kit; Bio-Rad Laboratories) before homogenization and centrifugation at 13,000 g for 25 min at 4°C. Protein concentrations were determined using the Bio-Rad Protein Assay Kit. Proteins were resolved using glycerol-based SDS-polyacrylamide gels (12%) and transferred to polyvinylidene difluoride membranes (Millipore) and immunoblotting was carried out using the SNAP I.D. System vacuum (www.Millipore.com). All primary antibodies were obtained from Santa Cruz Biotechnology (www.scbt.com) (IL1, cat. #sc-7884; IRAK1, cat. #sc-7883; NFATC2, cat. #sc-13034; TNFA, cat #sc-1351; BCL10, cat. #sc-5611; IKKA, cat. #sc-7120; COL4A1, cat. #sc-9301; FN1, cat. #sc-6952; LAMB1, cat. #sc-23410; PCNA, cat. #sc-7907, IL6, cat. #sc-1265, NFKB, cat. #sc-1190). After incubation with the appropriate secondary antibodies, the complexes were detected using the enhanced chemiluminescence method (ECL Advance Western Blotting Detection Kit; GE/Amersham,), visualized using the ChemiGenius2 Bioimaging system (Syngene; Cambridge, United Kingdom) and quantified (Gene Tools software; PerkinElmer). Equal loading was confirmed using alpha tubulin as a control (cat. #sc-31782).

2.4. Statistical Analysis

Data was generated weekly using methods previously described (Steele *et al.*, 2012). The data analysis for this study was generated using [SAS/STAT] software, Version 9.1 of the SAS System for Windows. Copyright © 2002-2003, SAS Institute Inc:

$$Y_{ijk} = \mu + D_i + T_j + (D \times T)_{ij} + B_k + \varepsilon_{ijk}$$

Where:
Y_{ijk} = The dependent variable

μ = The overall mean
D_i = The fixed effect of diet (i = 1, 2)
T_j = The fixed effect of time or week (j = 1,.., 4)
$(D \times T)_{ij}$ = The effect of the interaction of diet by time (ij = 1,.., 8)
B_k = The fixed effect of block or phase (k = 1, 2)
ε_{ijk} = The random residual error

Diet, time and block were considered fixed effects and week of experiment was used as a repeated measurement with cow as the subject. Cow was subjected to covariance structures and the covariance structure that gave the smallest Bayesian information criterion was used. As described previously, the Helmert contrasts (week 0 vs. 123, week 1 vs. 23 and week 2 vs. 3) were used to evaluate significant changes due to treatment.

Table 1. Primers for quantitative real time PCR (qPCR)

Gene symbol	Name	Genbank accession	Primer (5'-3')	Amplicon size (bp)	Efficiency (%)
BCL10	B-cell CL/lymphoma 10	NM_001078028.1	F-CAAAGGACTGGATACCCTGGTCGAA R-GGCTCACAGCTGCTACATTTCAGTC	92	89
COLAA1	Bost Taurus collagen type IV, alpha 1	NM_ NM_001166511.1	F-GAGTCCAGGGTTTCCAGGCGAC R-CCCAACGGTCCCGTGCCAAT	104	87
FN1	Fibronectin 1	NM_001163778.1	F-CCAGCACAGCCACTTCCGTG R-GGGCGTCAGGTGCTGTGGTC	78	93
IKKA/CHUK	Conserved helix-loop-helix ubiquitous kinase	NM_174021.2	F-GAATCTCTGGAACAGCGCGCAA R-ACCATCTCCGTGCTGTCGCT	120	94
IL1	Interleukin 1, beta	NM_174093.1	F-TGAACCGAGAAGTGGTGTTCTGCAT R-CAGCTGCAGGGTGGGCGTAT	145	87
IRAK1	Interleukin-1 receptor-associated kinase 1	NM_001040555.1	F-GTGGATCAACCGCAACGCCC R-GGGAGGGTGCCAAGCAGTGA	98	85
LAMB1	Laminin, beta 1	NM_001206519.1	F-ACGACATTTGCTCCAAACCGCC R-AGCTGGACGGAATGTCTTGAAGGT	89	91
TNFA	Tumor necrosis factor	NM_173966.2	F-CCCTCCATCAACAGCCCTCTGG R-TGATGTCGGCTACAACGTGGGC	135	91
NFATC2	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2, transcript variant 3	XM_608872.3	F-ACGGCGGCCAGCAGATGATT R-AGCATGTTAGGCTGGCTCTTGTCT	110	98
GAPDH	Glyceraldehyde-3-Phosphate dehydrogenase	NM_001034034.1	F-TGGAAAGGCCATCACCATCT R-CCCACTTGATGTTGGCAG	129	84

The PROC MIXED of SAS was used to analyze the relative gene expression for HC cattle (generated relatively to HF cattle) with time (week) and block (phase) as fixed effects. The HC relative gene expression values different from 1 were determined using T-statistics.

3. RESULTS

3.1. Physiological Measurements

As reported in Steele *et al.* (2012), dramatic differences in ruminal pH were evident between HC and HF cattle during experimental weeks 1, 2 and 3. In summary, the mean and minimum daily ruminal pH was lower (p<0.01) in HC cattle compared to the HF cattle during experimental weeks 1, 2 and 3. The daily minimal ruminal pH reached levels below pH 5.0 for each experimental week in HC cattle, indicative of a severe state of SARA. Week 1 was marked by the largest change in ruminal pH (p<0.01) as HC cattle displayed 760 ± 71 min d^{-1} and 1139 ± 57 min d^{-1} below 5.6 and 6.0, respectively. As expected, total SCFA concentration was greater (p<0.05) in HC compared with HF cattle during weeks 1, 2 and 3 (Steele *et al.*, 2012).

With respect to ruminal and blood inflammatory markers, ruminal LPS significantly increased (p<0.05) in concentration from week 0 (4121 ± 478 EU mL^{-1}) through weeks 1 (20649 ± 522 EU mL^{-1}), 2 (21854 ± 694 EU mL^{-1}) and 3 (32310 ± 728 EU mL^{-1}) in HC cattle. LBP was higher (p<0.05) in HC cattle compared to HF cattle in the morning sampling (15.4 vs. 11.1 ± 1.1 µg mL^{-1}); however, there were no differences due to treatment detected during the 1600h sampling.

3.2. Gene Expression

The mRNA and protein expression of the all genes is presented in **Table 2 and 3**, respectively. There was no differential expression of mRNA of target genes between HF and HC cattle at any time point of this experiment. In accordance, there were minimal changes in the expression of all but one target protein. The relative protein expression of NFATc2 was upregulated (p = 0.02) by 2-fold in HC cattle compared to HF cattle.

4. DISCUSSION

The aim of this study was to investigate how the RE responds to the onset of grain-inducedruminal acidosis by characterizing the expression of immune-regulatory and structural genes. A comprehensive description of the nutritional model used to induce SARA by HC feeding in this study has previously been described by Steele *et al.*, (2012). The form of ruminal acidosis detected in this study (760±71 min d^{-1} below ruminal pH 5.6 in cattle fed HC diet) was severe compared to previous studies which have evaluated the effect of SARA on the RE and whole animal inflammation in the dairy cow (Gozho *et al.*, 2005; 2006; 2007; Khafipour *et al.*, 2009a; 2009b; Steele *et al.*, 2011a; 2011b).

Table 2. Least square means of fold change of the expression of genes in rumen tissue from cattle fed the high-concentrate diet (HC) relative to cattle fed high-forage diet (HF). All cows (n = 16) received HF diet during wk 0 and then assigned either HC (n = 8) or HF (n = 8) diet. Gene expression values for HF cattle were normalized to 1 within each week individually

Gene expression fold change from HC relative to HF						P (Interaction)		
Gene Immune	wk 0	wk 1	wk 2	wk 3	SE	0 vs 123	1 vs 23	2 vs 3
IKKA	0.96	0.74	0.82	0.90	0.10	0.27	0.34	0.61
ILI	0.73	0.66	0.73	0.72	0.15	0.89	0.76	0.96
NFATc2	0.86	1.17	1.23	0.94	0.15	0.43	0.54	0.35
IRAK1	0.89	0.90	1.02	1.07	0.14	0.35	0.24	0.71
TNFA	0.85	1.27	1.38	0.82	0.22	0.55	0.73	0.43
BCL10	0.76	0.78	0.79	1.08	0.12	0.40	0.34	0.12
Structural								
LAMB1	0.86	1.17	1.23	0.94	0.15	0.19	0.68	0.21
COL4	1.06	0.92	0.91	1.05	0.14	0.50	0.67	0.42
FN1	0.85	0.74	0.82	0.90	0.11	0.83	0.39	0.65

Table 3. Expression of proteins in rumen tissue from cattle fed the High-Concentrate diet (HC) relative to cattle fed High-Forage diet (HF). All cows (n = 16) received HF diet during wk 0 and then assigned either HC (n = 8) or HF (n = 8) diet

	Control				SARE					P (Interaction)		
Protein immune	wk 0	wk 1	wk 2	wk 3	wk 0	wk 1	wk 2	wk 3	SE	0 vs 123	1 vs 23	2 vs 3
IKKA	0.58	0.63	0.64	0.69	0.49	0.45	0.61	0.66	0.12	0.95	0.33	096
IL6	0.74	0.71	0.87	0.95	0.78	0.64	0.88	0.96	0.12	0.70	0.54	0.99
NFATc2	1.43	1.01	1.12	1.17	0.77	1.44	2.33	2.49	0.50	0.02	0.25	0.90
NFKB	1.08	1.08	1.30	1.51	1.27	1.80	1.85	2.38	0.47	0.33	0.99	0.63
TNFA	0.62	0.72	0.77	0.68	0.59	0.58	0.71	0.70	0.10	0.85	0.37	0.61
Bcl10	0.92	0.69	0.80	0.79	0.79	0.45	0.64	0.70	0.14	0.86	0.57	0.76
Structural												
LAMB1	1.04	1.17	1.84	1.65	1.10	1.02	1.36	1.33	1.30	0.30	0.51	0.70
COL4	1.80	1.69	1.99	1.95	1.62	1.20	1.88	1.17	0.32	0.44	0.93	0.16
FN1	1.08	0.88	1.13	1.10	0.69	0.65	0.75	1.09	0.20	0.38	0.88	0.15
Tub	2.47	2.55	2.29	1.97	2.39	2.92	2.57	2.66	0.41	0.27	0.82	0.48

It has been well established that gram-negative bacteria thrive in a carbohydrate rich environment. Therefore, a concerted overgrowth of ruminal gram-negative bacteria which shed LPS from their cell walls causes a rise in ruminal LPS. It can be speculated that a buildup of ruminal LPS can react locally on the RE or transmigrate through the RE, thus causing systemic inflammation (Khafipour *et al.*, 2009b). The ruminal LPS in HC cattle was six times greater during weeks 1, 2 and 3 compared to the baseline week (25310±478 EU mL^{-1} vs. 4121±478 EU mL^{-1}). However, the concentration of ruminal LPS in acidotic cattle reported in our study was lower than several studies. For example, Khafipour *et al.*, (2009a; 2009b) and Li *et al.* (2012) reported ruminal LPS concentrations higher than 100,000 EU mL^{-1} in spite of it being a more moderate form of

SARA. Several factors may play a role in this discrepancy, such as time of sampling, total feed intake, rumen volume and physiological state of the dairy cow. Nevertheless, the ruminal acidosis reported in this study was associated with an accumulation of ruminal LPS that may modulate an immune response locally in the RE or enter portal circulation and exert its effects systemically.

Free LPS is delivered from its place of origin to antigen presenting cells such as macrophages by the soluble acute phase protein LBP (Muta and Takeshige, 2001). As such, LBP is partially responsible for the initiation of the immune response following the gram-negative bacterial overgrowth in the rumen. Since LBP levels are elevated by large circulating amounts of LPS, it is conceivable that following a SARA challenge, LBP levels

may indeed increase(Chen *et al.*, 2003; Gozho *et al.*, 2007). In our model, this is what accompanied changes in rumen LPS, which is in agreement with similar experimental models in dairy cattle that report elevated LBP (Khafipour *et al.*, 2009b; Zebeli and Ametaj, 2009) and other acute phase proteins such as SAA and HP (Kleen *et al.*, 2003; Plaizier *et al.*, 2008).

With regard to the RE, it has been widely established that increasing dietary rapidly fermentable carbohydrate alters RE proliferation (Goodlad, 1978), gene expression (Steele *et al.*, 2011b) and structure (Steele *et al.*, 2011a). It is becoming increasingly clearer that each response has different causes, time courses to ultimately impact RE function. To examine the responsiveness of the RE during a grain-induced SARA in this study, the relative expression value of mRNA and protein of key targets involved in immune-regulatory and structural repair pathways were determined. This study marks the first time that these immune-modulatory genes have been shown to be expressed in the RE. However, based on our results, there were minimal changes in the expression of genes involved in innate immunity and epithelial re-modeling.

We can propose that adaptation to a high grain challenge proceeds through minimal activation of a local immune response in the RE. The only marker which showed an upregulation of protein expression due to the HC diet was NFATC2. This growth factor is widely expressed in digestive tissues (Wang *et al.*, 2011) and belongs to a family of proteins responsible for the activation of T-cells (Maitra *et al.*, 2009). Ultimately responding to modulation of the calcium/calcineurin signaling network, NFATC2 is normally found in a phosphorylated and inactive form in the cytosol and once dephosphorylated, only translocates to the nucleus upon T cell receptor (Horsley and Pavlath, 2002) and TLR4 stimulation (Zanoni *et al.*, 2009). Within the nucleus, NFATc2 becomes a member of the nuclear factor of activated T cells transcription complex (Aliprantis and Glimcher, 2010; Sitara and Aliprantis, 2010). This complex not only plays a central role in inducing gene transcription during the immune response, but has been implicated as a necessary factor in the growth and differentiation of a number of tissues following a physiological insult (Santini *et al.*, 2001). For example, macrophage presentation of NFATc2 may facilitate the migration and subsequent activation of cells such as fibroblasts to stimulate wound healing (Lemaitre *et al.*, 2003; Sitara and Aliprantis, 2010). Not surprisingly, mRNA levels of NFATc2 did not indicate treatment differences as protein expression levels do not always coincide with gene expression (Gry *et al.*, 2009).

Although NFATc2 protein expression was increased due to treatment, there were marginal changes and variation in gene expression due to treatment. It is important to note that measurement of the relative expression patterns of LPS/TLR-mediated immune-regulatory pathways may not be indicative of general immune system stimulation. Indeed, the enhancement of an immune response through this pathway relies on a complex interplay of intermediates whose ability to transduce the signal into a downstream effect may only rely on their various states of phosphorylation and not on relative expression differences (Hoffmann and Baltimore, 2006). Furthermore, the time-course of the immune-molecular events may occur in the first hours of SARA (Cario and Podolsky, 2000). In addition, there was a large variation in gene expression values between animals, which coincides with many studies that note large variation in RE structure (Ellis *et al.*, 2012) and function (Penner *et al.*, 2009) between individual animals. In the future, assessing the expression of genes in immune cell types will be important to facilitate more cell specific outcomes.

5. CONCLUSION

A number of studies have been published indicating increases in LPS and the upregulation of acute phase proteins. However, this study was the first to our knowledge to directly characterize the expression of localized immune targets in the RE during the adaptation to an HC diet. In this experiment, it has been shown that adaptation was mediated by the immune system to a lesser extent than was previously thought based on gene expression profiling of targets involved in the innate immune system. Based on this research study, future research investigating the localized innate immune response of the RE during grain-induced SARA should consider evaluation of the time course of the response of the grain challenge and the specific immune cell types of the RE.

6. ACKNOWLEDGEMENT

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