In vitro methionine supplementation during lipopolysaccharide stimulation modulates immunometabolic gene network expression in isolated polymorphonuclear cells from lactating Holstein cows

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ABSTRACT

Methionine (Met) is one of the 2 most limiting amino acids for milk production in dairy cow diets. The accepted “ideal” ratio of lysine (Lys) to Met (L:M) when formulating diets is 3:1. However, blood from cows fed corn silage-based diets without supplemental rumen-protected Met averages approximately 3.6:1 L:M. Recent in vivo research on cattle immunonutrition has revealed that the immune system could benefit from greater Met supply. To study more closely the effects of different L:M ratios, blood polymorphonuclear cells (PMN) were isolated from 5 Holstein cows in mid-lactation (238 ± 20 d postpartum, 33.8 ± 3.8 kg of milk/d; mean $\pm$ SD). The PMN were incubated at 3 different levels of L:M (3.6:1, 2.9:1, or 2.4:1) and stimulated with lipopolysaccharide (LPS) at either 0 or 50 μ g/mL for 2 h at 37°C. Target genes were associated with cytokines, pathogen recognition, nuclear receptors, killing mechanisms, and Met and glutathione metabolism. Data were subjected to ANOVA using PROC MIXED in SAS, with L:M, LPS, and their interaction as fixed effects. Stimulation with LPS upregulated genes related to cytokines (*IL1B*, *TNF*, *IL10* and *IL6*) and nuclear receptors, including nuclear factor kappa B (*NFKB1*) and glucocorticoid receptor (*NR3C1*), and downregulated the mRNA abundance of chemokine receptor 1 (*CXCR1*), lysozyme (*LYZ*) and glutathione reductase (*GSR*). A linear decrease was observed in the mRNA abundance of *TNF* when L:M was decreased. A similar response was observed for interleukin-1 receptor-associated kinase 1 (*IRAK1*) and *NFKB1* abundance in cells stimulated with LPS (linear effect). A linear increase

of *LYZ* mRNA expression as L:M decreased was detected in unstimulated cells. Furthermore, a decrease in L:M led to a linear decrease of superoxide dismutase 1 (*SOD1*) mRNA abundance in cells challenged with LPS. Overall, LPS challenge triggered the activation of isolated PMN from mid-lactation cows. However, data suggest the use of a shorter incubation time to capture the peak response and not the resolution of the inflammatory response as in the present study. Our results indicate a possible involvement of Met in modulating PMN inflammatory and oxidative stress status and in helping the resolution of inflammation after initial stimulation.

Key words: PMN, lactating cows, inflammatory response, gene expression

INTRODUCTION

Methionine (Met) and Lysine (Lys) have been identified most frequently as major limiting essential amino acids (AA) in dairy cattle nutrition (NRC, 2001). Besides being an essential constituent of milk casein and a driver of milk production, availability of Met is important for other biological functions (Loor et al., 2013; Osorio et al., 2013; Du et al., 2018). For example, as a lipotropic agent, Met is directly involved in very low density lipoprotein synthesis via the generation of S-adenosylmethionine, the most important methyl donor (Martinov et al., 2010). Being the only essential sulfur-containing AA, Met acts as the precursor for other sulfur-containing AA such as cysteine and homocysteine (Brosnan and Brosnan, 2006) and as precursor for the synthesis of the major antioxidants glutathione and taurine (Atmaca, 2004).

Not only does the immune system benefit greatly from proper nutrition but, indirectly, proper nutrition will also prepare the cow for periods of stress (Zhu et al., 2019), reducing adverse effects and enhancing re-

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covery from them. In humans, Met, along with several of its metabolites, displays an immunonutritional role; that is, they help support and boost certain activities of the immune system (Grimble and Grimble, 1998; Redmond et al., 1998; Grimble, 2006; Li et al., 2007). Increasing the supply of Met by feeding it in rumen-protected form has shown a positive effect on systemic inflammatory indices and immune function of lactating cows, underscoring the positive effects of this AA reported in humans (Osorio et al., 2013; Zhou et al., 2016b; Batistel et al., 2017, 2018; Vailati-Riboni et al., 2017; Zhou et al., 2018).

Current recommendations for duodenal supply of Lys and Met to maximize milk protein content and yield in established lactation are 7.2 and 2.4% of MP, respectively, with an “ideal” ratio of 3:1 (L:M; NRC, 2001). This recommendation already decreases the ratio of between 3.6 and 3.8:1 observed in Met-unsupplemented dairy diets based on corn/corn silage and soybeans (Osorio et al., 2013; Zhou et al., 2016b). However, the aforementioned improvements to immune function indices in lactating dairy cows were obtained with ratios of approximately between 2.7 and 2.8:1, surpassing the recommended 3:1.

We hypothesized that a decrease in the L:M ratio by increasing Met supply and holding Lys constant would alter gene networks associated with bactericidal response and killing capacity of the polymorphonuclear cells (PMN; e.g., neutrophils), the first responders to pathogen invasion. The specific objective of the present study was to measure alterations in immune gene network expression in response to different ratios of L:M with or without exogenous lipopolysaccharide (LPS) in PMN from dairy cows. To achieve this, we applied an *in vitro* assay based on freshly isolated circulating PMN, which we previously validated in dairy calves (Abdelmegeid et al., 2017). To avoid the influence of stressors and endocrine changes peculiar to early lactation, we isolated the cells from mid-lactation Holstein cows.

MATERIALS AND METHODS

Animal Handling and Blood Sampling

Five Holstein cows were selected from the University of Illinois Dairy Unit herd. Cows were in mid-lactation (238 ± 20 DIM), had an average daily production in the week of sampling of 33.8 ± 3.8 kg, and were considered healthy (e.g., absence of clinical scenarios, not treated during the current lactation, and milk SCC < 100,000). Cows were housed in a freestall barn with sand-bedded cubicles and access to a concrete lot, where the feed

bunks were located. Cows were fed a corn silage-based TMR diet once a day at 0700 h. Ingredients and nutrient composition of the diet are reported in the supplemental materials (Supplemental Tables S1–S3; <https://doi.org/10.3168/jds.2018-15737>). Cows were milked 3 times a day at 0500, 1200, and 1900 h. Blood was collected from the jugular vein at 0600 h, before feeding, into Vacutainer tubes (BD Vacutainer, Becton Dickinson and Company, Franklin Lakes, NJ), containing 1.5 mL of ACD solution A (trisodium citrate, 22.0 g/L; citric acid, 8.0 g/L; and dextrose 24.5 g/L). From each animal 15 tubes were collected, for a total of 120 to 150 mL of blood. Blood was immediately placed on ice and transported to the laboratory for immediate isolation of PMN within 40 min of the time of sampling.

Isolation of PMN. The PMN were isolated based on procedures previously described (Abdelmegeid et al., 2017). The final PMN pellets from the 5 animals were then mixed together to obtain a homogeneous pool. Before incubation, a 50- μ L aliquot of the PMN pool was incubated for 15 min on ice with 100 μ L of the primary antibody CH138A solution (Washington State University, Pullman, WA; 15 μ g/mL in $1 \times$ PBS). The aliquot was then washed twice with 2 mL $1 \times$ PBS and incubated for another 15 min on ice, protected from light, with 50 μ L of secondary antibody solution PE (Southern Biotech, Birmingham, AL; 4 μ g/mL in $1 \times$ PBS) and 50 μ L of propidium iodide (PI; Thermo Fisher Scientific, Waltham, MA; 50 μ g/mL). Cells were analyzed via flow cytometry (BD LSR II, Becton Dickinson), using PE and PI as markers of PMN and viability, respectively. The PMN purity (cells PE⁺) and viability (cells PE⁺/PI⁺) were greater than 90%.

Incubation and Treatments. The isolated pool of PMN was diluted to a final concentration of 6×10^6 cells per mL with warm (37°C), Met-free incubation medium (RPMI 1640, Gibco, Thermo Fisher) and 8% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) previously heat-inactivated. From the diluted pool, aliquots of 1 mL were incubated in 14-mL Falcon tubes (Thermo Fisher) with a snap cap design to allow air flow, and allocated to each experimental group. The PMN were assigned to 1 of 3 treatments based on the ratio of Lys to Met, as follows: 3.6:1, 2.9:1, or 2.4:1, calculated based on medium concentration of Lys. The basal ratio of 3.6:1 was chosen based on available plasma AA data from lactating dairy cows (Verbeke et al., 1972; Rogers et al., 1987; Zhou et al., 2016a; Sadri et al., 2017; Marczuk et al., 2018). Actual levels of the 2 AA in the medium were 40 mg/L for Lys in all treatment groups, and 11.11, 13.79, and 16.67 mg/L of methionine for the 3.6:1, 2.9:1, and 2.4:1 groups, respectively. These amounts were, on average, 4 times larger than those

observed in vivo (e.g., 2.62 and 9.56 mg/L Met and Lys, respectively; Verbeke et al., 1972; Rogers et al., 1987; Zhou et al., 2016a; Sadri et al., 2017; Marczuk et al., 2018). Each treatment was stimulated with LPS at either 0 or 50 µg/mL. Final combination of L:M and LPS were incubated in triplicate. Both Met and LPS (both from Sigma-Aldrich) were added to the test tubes from stock solution previously prepared, using the incubation medium as a diluent. Incubations lasted 2 h at 37°C in a sterile incubator with a controlled CO₂ (5%) atmosphere.

RNA Extraction. After incubation, PMN were pelleted by centrifugation and the supernatant decanted. Total RNA was extracted using Qiazol reagent (Qiagen, Hilden, Germany). The cell pellet was placed in 1 mL of Qiazol, and tissue was homogenized with a Bead Beater 16 (Biospec Products, Bartlesville, OK) using 2 30-s cycles of the homogenizer at full speed, and placed on ice after homogenization for 1 minute. Homogenized samples were centrifuged to remove any remaining cell debris. Chloroform was then added to the homogenized sample, which was centrifuged at 16,000 × *g* for 15 min, at 4°C, and the aqueous phase removed carefully. Precipitation of RNA was achieved with the addition of ethanol (Deacon Laboratories Inc., King of Prussia, PA), and the subsequent RNA pellet was washed and cleaned using miRNeasy mini spin columns (Qiagen). Genomic DNA was removed during purification with on-column DNase digestion (Qiagen). RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), and RNA quality was assessed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). All samples had an RNA integrity value greater than 8.0.

cDNA Synthesis and qPCR. Genes selected for transcript profiling in PMN were associated with cytokine production (*IL1B*, *IL10*, *IL6*, *TNF*), pattern recognition (*CXCR1*, *JSP.1*, *SELL*, *TLR2*, *TLR4*), inflammatory-related nuclear receptors (*IRAK1*, *NFKB1*, *NR3C1*), pathogen-killing mechanisms (*LYZ*, *MPO*, *SOD1*), and the glutathione pathway (*GPX1*, *GSR*, *DNMT3A*, *MAT1A*, *SAHH*). Information regarding gene function and primers sequences can be found elsewhere (Abdelmegeid et al., 2017). A portion of the RNA was diluted to 100 mg/L using nuclease-free water before reverse transcription. We synthesized cDNA using 100 ng of RNA, 1 µg of dT18 (Operon Biotechnologies, Huntsville, AL), 1 µL of 10 mmol/L dNTP mix (Invitrogen, Carlsbad, CA), 1 µL of random primers (Invitrogen), and 10 µL of nuclease-free water. The mixture was incubated at 65°C for 5 min and kept on ice for 3 min. A total of 6 µL of master

mix composed of 4.5 µL of 5× First-Strand Buffer, 1 µL of 0.1 M DTT, 0.25 µL (50 U) of SuperScript III RT (Invitrogen), and 0.25 µL of RNase Inhibitor (10 U, Promega, Madison, WI) was added. The reaction was performed in an Eppendorf (Hamburg, Germany) Mastercycler Gradient using the following temperature program: 25°C for 5 min, 50°C for 60 min, and 70°C for 15 min. The cDNA was then diluted 1:4 (vol/vol) with nuclease-free water.

Quantitative PCR (qPCR) was performed using 4 µL diluted cDNA combined with 6 µL of a mixture composed of 5 µL SYBR Green master mix (Applied Biosystems, Waltham, MA), 0.4 µL each of 10 µM forward and reverse primers, and 0.2 µL of nuclease-free water in a MicroAmp Optical 384-Well Reaction Plate (Applied Biosystems). Each sample was run in triplicate, and a 6-point relative standard curve plus the non-template control were used. The reactions were performed in an ABI Prism 7900 HT SDS instrument (Applied Biosystems), using the following conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C (denaturation), and 1 min at 60°C (annealing + extension). The presence of a single PCR product was verified by the dissociation protocol, using incremental temperatures to 95°C for 15 s plus 65°C for 15 s. Data were calculated with the 7900 HT Sequence Detection Systems Software (version 2.2.1, Applied Biosystems). The final data were normalized using the geometric mean of 3 internal control genes: *GAPDH*, *RPS9*, and *UXT*. Details of the qPCR performance can be found in Supplemental Table S4 (<https://doi.org/10.3168/jds.2018-15737>).

Statistical Analysis

After normalization with the geometric mean of the internal control genes, the triplicate averages of the qPCR data were log₂ transformed before statistical analysis, to obtain a normal distribution. The incubation tubes were treated as individual observations. Statistical analysis was performed with SAS. Normalized, log₂ transformed data were subjected to ANOVA with PROC MIXED. Normality of the residuals was tested via PROC UNIVARIATE in SAS. All observations were normally distributed. The statistical model included L:M ratio (3.6:1, 2.9:1, 2.4:1), LPS stimulation (yes, no), and their interactions (L:M × LPS) as fixed effects. The Kenward-Roger statement was used for computing the denominator degrees of freedom. Orthogonal contrasts were used to determine the linear or quadratic effect of L:M overall, or within LPS levels. Data were considered significant at $P \leq 0.05$, using the PDIFF statement in SAS. For ease of interpretation,

Table 1. Main effects of lysine-to-methionine ratio (L:M) and LPS challenge in cultures of polymorphonuclear cells isolated from Holstein cows (238 ± 20 DIM, 33.8 ± 3.9 kg of milk/d, mean $\pm$ SD)

Gene category	L:M					LPS					P-value		
	3.6:1	2.9:1	2.4:1	SEM		NO	YES	SEM	L:M	LPS	Linear	Quadratic	
Cytokines													
<i>IL1B</i>	0.95	0.96	0.90	0.04		0.74 ^a	1.18 ^b	0.04	0.42	<0.01	0.31	0.41	
<i>TNF</i>	0.98 ^b	0.91 ^{ab}	0.87 ^a	0.04		0.75 ^a	1.12 ^b	0.04	0.13	<0.01	0.05	0.99	
<i>IL10</i>	0.91	0.95	1.00	0.07		0.78 ^a	1.17 ^b	0.06	0.59	<0.01	0.33	0.87	
<i>IL6</i>	0.85	0.86	1.01	0.05		0.64 ^a	1.28 ^b	0.06	0.09	<0.01	0.06	0.25	
Recognition													
<i>CXCR1</i>	0.96	0.93	0.89	0.04		1.01 ^b	0.85 ^a	0.03	0.52	0.01	0.27	0.83	
<i>JSP1</i>	0.98	0.93	0.93	0.02		0.95	0.95	0.01	0.14	0.89	0.07	0.40	
<i>SELL</i>	0.94	0.97	0.92	0.03		0.93	0.95	0.03	0.60	0.50	0.73	0.35	
<i>TLR2</i>	0.97	0.95	0.92	0.02		0.93	0.96	0.02	0.30	0.19	0.13	0.79	
<i>TLR4</i>	1.05	1.01	1.00	0.05		1.06	0.98	0.04	0.71	0.15	0.41	0.97	
Nuclear receptors													
<i>IRAK1</i>	1.02 ^b	0.98 ^b	0.88 ^a	0.02		0.96	0.96	0.02	<0.01	0.99	<0.01	0.15	
<i>NFKB1</i>	0.95 ^b	0.85 ^{ab}	0.84 ^a	0.04		0.74 ^a	1.05 ^b	0.04	0.10	<0.01	0.05	0.44	
<i>NR3C1</i>	0.96	0.93	0.91	0.02		0.90 ^a	0.97 ^b	0.02	0.29	0.02	0.13	0.91	
Killing mechanisms													
<i>LYZ</i>	0.90	0.95	0.95	0.06		1.10 ^b	0.79 ^a	0.05	0.70	<0.01	0.43	0.80	
<i>MPO</i>	0.96	0.94	0.89	0.04		0.93	0.92	0.03	0.44	0.81	0.23	0.74	
<i>SOD1</i>	1.03	0.95	0.95	0.03		0.96	0.99	0.03	0.14	0.46	0.07	0.39	
Methionine and glutathione pathway													
<i>GPX1</i>	0.95	0.93	0.92	0.02		0.95	0.91	0.02	0.52	0.07	0.27	0.83	
<i>GSR</i>	0.82 ^c	0.70 ^b	0.62 ^a	0.02		0.76 ^b	0.66 ^a	0.01	<0.01	<0.01	<0.01	0.78	
<i>DNMT3A</i>	0.96 ^b	0.96 ^b	0.87 ^a	0.03		0.91	0.94	0.02	0.05	0.30	0.03	0.17	
<i>MAT1A</i>	0.70	1.22	0.71	0.34		0.65	1.10	0.24	0.29	0.11	0.83	0.13	
<i>SAHH</i>	0.92	0.90	0.89	0.02		0.91	0.90	0.02	0.62	0.69	0.34	0.88	

^{a-c}Means within a row differ ($P \leq 0.05$).

Table 2. Interactions between lysine-to-methionine ratio (L:M) and LPS challenge in cultures of polymorphonuclear cells isolated from Holstein cows (238 ± 20 DIM, 33.8 ± 3.9 kg of milk/d, mean $\pm$ SD)

Gene category	LPS	L:M			SEM	P-value		Linear P-value		Quadratic P-value	
		3.6:1	2.9:1	2.4:1		L:M × LPS	No	Yes	No	Yes	
Cytokines											
<i>IL1B</i>	No	0.76	0.75	0.72	0.04		0.84	0.40	0.55	0.83	0.36
	Yes	1.18	1.23	1.12	0.08						
<i>TNF</i>	No	0.83	0.72	0.72	0.04		0.44	0.07	0.27	0.37	0.45
	Yes	1.16	1.16	1.05	0.07						
<i>IL10</i>	No	0.67 ^a	0.76 ^{ab}	0.91 ^b	0.08		0.08	0.03	0.33	0.63	0.84
	Yes	1.25	1.19	1.10	0.13						
<i>IL6</i>	No	0.60	0.63	0.70	0.05		0.85	0.17	0.15	0.62	0.26
	Yes	1.21	1.19	1.44	0.11						
Recognition											
<i>CXCR1</i>	No	1.04	0.96	1.02	0.06		0.27	0.77	0.21	0.33	0.27
	Yes	0.88	0.90	0.78	0.06						
<i>JSP.1</i>	No	0.94 ^{ab,A}	0.99 ^{a,A}	0.92 ^b	0.02		0.01	0.74	0.03	0.05	0.01
	Yes	1.02 ^{a,B}	0.88 ^{b,B}	0.94 ^b	0.02						
<i>SELL</i>	No	0.90	0.94	0.95	0.04		0.30	0.42	0.21	0.76	0.34
	Yes	0.98	0.99	0.90	0.05						
<i>TLR2</i>	No	0.94	0.92	0.92	0.03		0.60	0.65	0.10	0.93	0.68
	Yes	1.00	0.97	0.92	0.04						
<i>TLR4</i>	No	1.09	1.02	1.06	0.06		0.64	0.72	0.42	0.49	0.58
	Yes	1.00	1.01	0.93	0.07						
Nuclear receptors											
<i>IRAK1</i>	No	1.06 ^a	0.92 ^{b,A}	0.91 ^b	0.04		0.02	0.005	0.006	0.18	0.007
	Yes	0.99 ^a	1.04 ^{a,B}	0.86 ^b	0.04						
<i>NFKB1</i>	No	0.79	0.67	0.76	0.04		0.13	0.48	0.03	0.07	0.51
	Yes	1.15 ^a	1.08 ^{ab}	0.94 ^b	0.07						
<i>NR3C1</i>	No	0.92	0.87	0.91	0.03		0.18	0.81	0.06	0.24	0.37
	Yes	1.01	0.99	0.91	0.04						
Killing mechanisms											
<i>LYZ</i>	No	0.96 ^a	1.12 ^{ab}	1.25 ^b	0.09		0.06	0.03	0.22	0.98	0.72
	Yes	0.84	0.80	0.73	0.07						
<i>MPO</i>	No	1.00	0.88	0.92	0.05		0.17	0.21	0.63	0.24	0.15
	Yes	0.91	0.99	0.87	0.06						
<i>SOD1</i>	No	1.00	0.91	0.98	0.04		0.25	0.56	0.05	0.12	0.78
	Yes	1.06 ^a	0.99 ^{ab}	0.90 ^b	0.05						
Methionine and glutathione pathway											
<i>GPX1</i>	No	0.95	0.94	0.97	0.03		0.27	0.73	0.07	0.64	0.90
	Yes	0.95 ^a	0.91 ^a	0.88 ^a	0.03						
<i>GSR</i>	No	0.82 ^a	0.74 ^{b,A}	0.73 ^{b,A}	0.02		<0.01	0.01	<0.01	0.29	0.21
	Yes	0.82 ^a	0.67 ^{b,B}	0.53 ^{c,B}	0.02						
<i>DNMT3A</i>	No	0.95	0.91	0.88	0.04		0.36	0.16	0.07	1.00	0.08
	Yes	0.97	1.01	0.86	0.05						
<i>MAT1A</i>	No	0.79	0.90	0.39	0.31		0.16	0.21	0.13	0.24	0.29
	Yes	0.62	1.65	1.30	0.71						
<i>SAHH</i>	No	0.91	0.92	0.91	0.03		0.54	0.94	0.17	0.87	0.74
	Yes	0.94	0.89	0.88	0.04						

^{a-c}Means within a row, among L:M levels within an LPS stimulation level, with different superscripts differ ($P \leq 0.05$).^{A,B}Means within a gene and L:M level differ between unstimulated (No) and stimulated (Yes) cells ($P \leq 0.05$).

estimates and standard errors reported in Tables 1 and 2 were properly back-transformed.

RESULTS

LPS Effect

The main effect of LPS challenge on blood PMN gene expression is reported in Table 1. LPS stimulation induced an increase ($P < 0.05$) in all cytokine mRNA expression (*IL1B*, *TNF*, *IL10*, and *IL6*). However, the expression of genes related to pattern recognition (*TLR2* and *TLR4*), PMN adhesion (*SELL*), or antigen presentation (*JSP.1*) were not changed by LPS challenge. The expression of the interleukin-8 receptor *CXCR1* was downregulated ($P < 0.05$) in PMN stimulated with LPS. Among the nuclear receptors, both *NFKB1* and *NR3C1*, but not *IRAK1*, were upregulated ($P < 0.05$) in the LPS-challenged group. In addition, the expression of *MPO* and *SOD1* were unchanged ($P > 0.05$) by stimulating PMN with LPS, whereas *LYZ* expression was downregulated in the LPS groups ($P < 0.05$). Among the targeted genes related to Met and glutathione pathways, only *GSR* mRNA expression was markedly decreased in LPS-stimulated PMN ($P < 0.05$).

Methionine Effect

A linear decrease ($P < 0.05$) was observed in the mRNA expression of *TNF* when the ratio of L:M decreased (i.e., when Met supply increased). Similarly, *IRAK1* and *NFKB1* had a linear decrease ($P < 0.05$) as L:M decreased. Among the genes related to Met and glutathione pathway, *GSR* and *DNMT3A* were affected by the L:M ratio, with a linear decrease ($P < 0.05$) of their mRNA expression as L:M decreased.

Interactions Between Methionine and LPS

Results for the interaction of L:M ratio with LPS challenge are reported in Table 2. When the L:M ratio decreased without exogenous LPS there was a linear increase ($P < 0.05$) for *IL10* mRNA expression. However, when stimulated with LPS, this response was not observed ($P > 0.05$). Expression of *JSP.1* responded quadratically to the decrease of L:M ratio both with and without LPS challenge ($P < 0.05$). In contrast, in unstimulated samples the response was opposite, with L:M ratio of 2.9:1 causing the highest expression, whereas it caused the lowest response when LPS was added. This led to a significant ($P < 0.05$) interaction between L:M and LPS, with a higher *JSP.1* mRNA ex-

pression in stimulated samples at an L:M ratio of 3.6:1, and a lower expression in stimulated versus unstimulated samples when L:M ratio was 2.9:1. No differences ($P > 0.05$) were observed at the highest L:M ratio of 2.4:1. In addition, no interactions ($P > 0.05$) between L:M and LPS were detected for other genes related to pathogen recognition, including *CXCR1*, *SELL*, *TLR2*, and *TLR4*.

The expression of *IRAK1* decreased linearly ($P < 0.05$) as L:M ratio decreased in PMN treated both with and without LPS, with lowest abundance for *IRAK1* mRNA observed at L:M ratio of 2.4:1. Furthermore, an interaction of L:M ratio and LPS challenge was also observed as a result of higher ($P < 0.05$) mRNA expression of *IRAK1* at L:M of 2.9:1 in PMN treated with LPS. The transcription factor *NFKB1* was linearly decreased ($P < 0.05$) as the L:M ratio decreased in LPS-challenged PMN cells but not in the unchallenged PMN.

Among the genes related to killing mechanisms, a linear increase of *LYZ* mRNA expression was found in unstimulated PMN as L:M ratio decreased ($P < 0.05$), causing higher expression in unstimulated PMN at both L:M of 2.9:1 and 2.4:1 compared with LPS-challenged PMN ($P < 0.05$). Furthermore, the decrease in L:M ratio led to a linear decrease of *SOD1* mRNA expression in challenged but not in the unchallenged PMN.

Concerning Met and glutathione metabolism, no interactions ($P > 0.05$) were detected for the expression of *GPX1*, *DNMT3A*, *MAT1A*, or *SAHH* mRNA. However, a linear effect of L:M ratio was detected for *GSR* expression in both stimulated and unstimulated PMN. This also led to an interaction between L:M ratio and LPS, with lower expression of *GSR* in stimulated PMN supplemented with Met to reach L:M ratios of 2.9:1 and 2.4:1.

DISCUSSION

Lactating dairy cows are typically supplemented with additional Met to reach an L:M ratio of 3:1 in the MP, as this relationship has been determined to be optimal for milk protein synthesis. Our choices of L:M ratios of 3.6:1, 2.9:1, and 2.4:1 for incubations represent levels obtained in typical corn silage-based diets not supplemented with rumen-protected Met, diets supplemented with rumen-protected Met to reach an approximately 3:1 ratio, and diets supplemented with additional Met to push the current industry standard, respectively. We surmise that additional Met would provide the cow with spare Met for other physiological functions, such as the immune response.

Effects of Methionine Supplementation

We recently reported the effect of Met supplementation in vivo on circulating PMN gene expression. Those results indicated an overall lower degree of activation, better redox status, and improved response upon challenge (Zhou et al., 2018). The present in vitro experiment confirmed those previous responses. The overall linear downregulation in *TNF*, *IRAK1*, *NFKB1*, *GSR*, and *DNMT3A*, together with the linear upregulation of the anti-inflammatory cytokine *IL10*, suggested a lower activation stage of PMN (Moyes et al., 2010) and a better redox status as Met supply increased. At the same time the upregulation of *JSP.1*, a component of the antigen presenting mechanism, and *LYZ*, a key antimicrobial enzyme in the PMN arsenal, seems to highlight a priming effect of Met on the capacity of these cells to respond readily upon stimulation.

The downregulation of antigen-presenting mechanisms (*JSP.1*) and regulatory receptors (*NFKB1* and *IRAK1*) when PMN were stimulated with LPS at the lower L:M ratio (i.e., higher levels of Met) suggested a diminished response to pathogens (Li et al., 2015). These data are somewhat opposite to our previous results highlighting how both PMN and monocytes from cows supplemented with Met had greater phagocytosis capacity and oxidative burst response when challenged ex vivo with *E. coli* (Osorio et al., 2013; Zhou et al., 2016b; Vailati-Riboni et al., 2017). In the current experiment, isolated PMN were stimulated with LPS for 2 h to determine how increasing Met supply could elicit effects during the activation stage of the cells. However, a previous report indicated a very rapid PMN response, on the order of minutes, to LPS stimulation, with maximum release of superoxide anions (O_2^-) within 15 to 60 min of stimulation and return to pre-challenge levels within 2 h (Aida and Pabst, 1991). Thus, we believe that our results more closely resemble the control stages of the inflammatory process, during the resolution stage of the PMN response.

The observed interactions between Met supply and LPS stimulation suggest better control and resolution of the inflammatory response when Met increases. A similar optimization and control of the inflammatory response was observed previously ex vivo (Vailati-Riboni et al., 2017). To corroborate this hypothesis, despite the lack of actual data, the downregulation of *GSR* and *SOD1* suggests a lower concentration of free radicals present after 2 h, which would indicate a faster resolution of the inflammatory response due to Met supply. Despite being considered a detrimental effect, as it leads to extended or chronic consequences in the inflammatory response, a downregulation of *SOD1*

expression might increase PMN life span, reducing apoptotic signals (Yasui et al., 2005).

Effects of LPS Stimulation

Lipopolysaccharide, the major endotoxin of gram-negative bacteria, is recognized to be a major mediator of immune cell activation, PMN included (Guthrie et al., 1984; Doerfler et al., 1989; Aida and Pabst, 1991). Hence, upregulation of the transcription factor *NFKB1*, along with the pro-inflammatory cytokines *IL1B*, *IL6*, and *TNF* in the present study upon stimulation with LPS, agreed with previous data (Hayden and Ghosh, 2011) and underscored that the dose and timing of the challenge were adequate to obtain a response in the freshly isolated PMN from mid-lactation dairy cows.

Despite the clear activation of PMN upon LPS challenge, data also indicate the existence of a feedback mechanism to contain the activation of PMN. After activation, in fact, PMN showed downregulation of *CXCR1*, the receptor for chemokine IL-8, which causes them to migrate to sites of infection (Fujishima et al., 1993; Sabroe et al., 2005), and upregulation of *IL10* and *NRC31*, both of which have potent anti-inflammatory effects counteracting the expression of pro-inflammatory cytokines (Couper et al., 2008; Chinenov et al., 2013).

The downregulation of *LYZ*, encoding the antibacterial enzyme lysozyme, upon stimulation with LPS, agreed with previous data in both porcine and human peripheral blood mononuclear cells (Gao et al., 2010; Van Belleghem et al., 2017). The mechanistic reason for such effect is unknown. Previous study has shown that LPS binds to lysozyme and inhibits its activity and that, at the same time, binding reduces the endotoxicity of LPS (Ohno and Morrison, 1989). We know of no clear explanation for what seems to be a feedback regulatory mechanism of the PMN immune response to LPS.

The enzyme glutathione reductase (*GSR*) restores the reduced form of glutathione, an important cellular antioxidant that helps to eliminate H_2O_2 within cytosol, preventing oxidative damage to PMN (Cohen et al., 1987). Thus, the downregulation of *GSR* expression upon stimulation with LPS seems counterintuitive, as LPS causes PMN activation and engages the oxidative burst response. Because in *GSR*-deficient individuals (Roos et al., 1979) and mouse models (Yan et al., 2012) PMN immune response seems to be compromised, its downregulation would indicate a reduced functionality of the isolated PMN when challenged with LPS in our in vitro model. Despite the lack of PMN function data in the present study, previous in and ex vivo data

demonstrated increased functionality of bovine immune cells when additional Met was added to the diet to achieve L:M ratios of approximately 3 or lower (Zhou et al., 2016b; Vailati-Riboni et al., 2017; Zhou et al., 2018). Thus, we speculate that the reduction is due to a feedback mechanism to control and resolve the inflammatory response started 2 h prior. These mechanisms have already been observed at the enzyme activity level and could possibly exist at the transcriptional level as well. In a matter of seconds after stimulation, GSR activity is increased to withstand the marked production of reactive oxygen species, but 30 min later activity is already reduced due to consumption of H_2O_2 by myeloperoxidase as part of the burst response (Strauss et al., 1969). The production of nitric oxide (NO) concurrently with reactive oxygen species during activation has been found to reduce GSR activity, an effect that is thought to help counterbalance the effect of H_2O_2 on the enzyme. Because cellular reduced glutathione levels decrease in parallel with NO, it has been assumed that NO regulates its own synthesis through various negative feedback mechanisms on nitric oxide synthase (NOS) activity, including the reduction of cellular reduced glutathione (Butzer et al., 1999).

CONCLUSIONS

The current data suggest that the isolation of PMN from lactating cows is a suitable model to study the effect of nutrient supplementation or inflammatory stimulation—or both—in vitro. However, we suggest a pre-incubation period and a shorter time frame of stimulation to capture the response at peak activation. From a practical standpoint, targeting the supply of Met to an L:M ratio of at least 3, but with greater benefits at 2.4:1, might improve PMN inflammatory and oxidative status. As such, in the event of stimulation, this level of Met could help the resolution of inflammation. This effect would help cows avoid a prolonged or chronic detrimental result of the inflammatory response. Future work will have to assess concurrently immune function and supernatant markers to obtain a more comprehensive understanding.

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