Short communication: Inflammation, migration, and cell-cell interaction-related gene network expression in leukocytes is enhanced in Simmental compared with Holstein dairy cows after calving

V. Lopreiato,¹ A. Minuti,^{1*} V. M. Morittu,² D. Britti,² F. Piccioli-Cappelli,¹ J. J. Loor,³ and E. Trevisi¹

¹Department of Animal Sciences, Food and Nutrition, Faculty of Agriculture, Food and Environmental Science, Università Cattolica del Sacro Cuore, 29122 Piacenza, Italy

²Interdepartmental Services Centre of Veterinary for Human and Animal Health, Department of Health Science, Magna Græcia University, Catanzaro 88100, Italy

³Department of Animal Sciences and Division of Nutritional Sciences, University of Illinois, Urbana 61801

ABSTRACT

The aim of this study was to investigate changes in the abundance of genes involved in leukocyte function between cows highly specialized for milk production (Holstein, $n = 12$) and cows selected for meat and milk (Simmental, $n = 13$). Blood was collected on d 3 after calving in PAXgene tubes (Preactix, Hombrechtikon, Switzerland) to measure mRNA abundance of 33 genes. Normalized gene abundance data were subjected to MIXED model ANOVA using SAS (SAS Institute Inc. Cary, NC). Simmental cows had greater transcript abundance of proinflammatory cytokines and receptor genes (*IL1B*, *TNF*, *IL1R*, *TNFRSF1A*), cell migration- and adhesion-related genes (*CX3CR1*, *ITGB2*, *CD44*, *LGALS8*), and the antimicrobial *IDO1* gene. In contrast, compared with Holstein cows, Simmental cows had lower abundance of the toll-like receptor (TLR) recognition-related gene *TLR2*, the antimicrobial-related gene *LTF*, and *S100A8*, which is involved in cell maturation, regulation of inflammatory processes, and immune response. These results revealed that breed plays an important role in the modulation of genes involved in immune adaptation and inflammatory response, and the immune system of Simmental cows could potentially have a more acute response in early lactation. In turn, this might be beneficial for mounting a more efficient response after calving and allow for a smoother homeorhetic adaptation to lactation.

Key words: PAXgene, Simmental, leukocytes, transition period

Short Communication

Simmental is a cattle breed selected for meat and milk production, which, during the last decades in intensive production systems, has undergone intense selection for higher milk yield while still maintaining the dual-purpose characteristic. The different features in Simmental (selected for both milk and beef production traits) and Holstein cows (selected for milk traits) are due to distinct genetic patterns and breeding histories, which has led to the hypothesis that innate and adaptive immune responses differ between these breeds when managed under an intensive dairy production system with similar environmental or experimental conditions. For instance, whole-blood transcriptome analysis revealed that Simmental and Holstein cows have similar patterns but varying degrees of activation of metabolic and physiological mechanisms associated with environmental adaptation. In fact, compared with Simmental cows, Holsteins had a greater number of affected genes and impacted pathways, suggesting differences in metabolic activity (Sandri et al., 2015; Pošćić et al., 2017). However, no previous studies have attempted to investigate differences in abundance of genes involved in immune adaptations and inflammatory responses between these breeds immediately after calving.

Polymorphonuclear leukocyte cells (PMNL) and lymphocyte functions decrease gradually starting about 2 wk before calving, with the lowest degree of responsiveness between time of calving and 2 d afterward (Kehrli et al., 1989; Batistel et al., 2018). This dysfunction encompasses reduced activity of the superoxide production, phagocytosis, and expression of cell surface adhesion molecules, as well as a reduction of capacity for migration (Kehrli et al., 1989; Burton et al., 1995, 2005). According to Kehrli et al. (1989), impaired

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*Corresponding author: andrea.minuti@unicatt.it

neutrophil function during the periparturient period is primarily attributable to the hormonal and metabolic changes that prepare the mammary gland for lactation. In fact, around this critical period, metabolism of dairy cows shifts from the demands of pregnancy to those of lactation. Notably, most studies on impairment of innate and adaptive immune functions during the transition period have been undertaken using high-yielding dairy cows. Hence, the objective of the present study was to explore gene network profiles of 33 genes involved in recognition, immune mediation, migration, cell adhesion, antimicrobial mechanisms, the inflammatory cascade, oxidative stress, and the leukotriene pathway in leukocytes from Simmental and Holstein cows immediately after calving. We hypothesized that leukocyte function and response would differ between cows highly specialized for milk production such as Holstein and dual-purpose cows selected for meat and milk production such as Simmental.

The trial was carried out in accordance with Italian laws on animal experimentation (DL n. 26, 04/03/2014) and ethics (Authorization of Italian Health Ministry N 403/2017-PR). The experimental design of this study involved 13 Simmental and 12 Holstein cows representing the control group from a larger cohort. Details of management, diet, health status, and metabolic status of cows have been published previously (Lopreiato et al., 2019). Briefly, management procedures for Simmental and Holstein cows were similar: cows were fed once daily (0700 and 0800 h, lactating and dry cows, respectively) with a TMR, and after parturition were milked twice daily (at 0500 and 1700 h). On d 3 after calving, blood was sampled (2.5 mL) from the jugular vein into PAXgene Blood RNA System tubes (Preanalytix, Hombrechtikon, Switzerland) for RNA extraction. The use of PAXgene tubes in cattle has been reported previously (Sandri et al., 2015; Pošćić et al., 2017).

The complete procedure for RNA extraction from PAXgene RNA tubes was performed according to the manufacturer's protocol (Blood RNA Kit Handbook, PreAnalytix GmbH, Qiagen, Hilden, Germany). Complete details of the procedure are presented in Supplemental File S1 (<https://doi.org/10.3168/jds.2019-17298>). After extraction, RNA was quantified using the Qubit RNA BR Assay Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA), and RNA quality was assessed with the Experion Automated Electrophoresis System (Bio-Rad, Hercules, CA). The average ($\pm$ SD) RNA quality index was 9 ± 0.52 . All samples used in the analysis were diluted with nuclease-free water to a concentration of 100 ng of RNA/ μ L, and synthesis of cDNA was performed using a reverse transcription kit (RevertAid RT Reverse Transcription Kit; Thermo

Fisher Scientific). The qPCR was performed using 4 μ L of diluted cDNA combined with 6 μ L of mixture composed of 5 μ L 1 $\times$ SYBR Green Master Mix (Applied Biosystems, Woolston Warrington, UK), 0.4 μ L each of 10 μ M forward and reverse primers, and 0.2 μ L of nuclease-free water. Three replicates were run for each sample. The qPCR efficiency and quantification cycle values were obtained for each reaction using LinReg-PCR (Version 2017.1; Amsterdam UMC, Amsterdam, the Netherlands). The final data were normalized using the geometric mean of 3 internal control genes: *ACTB*, *SDHA*, and *YWHAZ*. Additional details are reported in Supplemental File S1 (<https://doi.org/10.3168/jds.2019-17298>). Normalized gene abundance data were analyzed using the MIXED procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC), in which the fixed effect was breed, and cows were included as random effect. All means were compared using the PDIF statement of SAS. Significant differences were declared at $P \leq 0.05$.

The abundance of genes investigated for leukocyte functions in Simmental and Holstein dairy cows is presented in Table 1. Compared with Holstein, Simmental dairy cows had greater ($P \leq 0.05$) transcript abundance of the proinflammatory cytokines *IL1B* and *TNF*, the cytokine receptors *IL1R* and *TNFRSF1A*, the cell migration-related gene *CX3CR1*, the cell adhesion-related genes *ITGB2*, *CD44*, and *LGALS8*, and the cell antimicrobial-related gene *IDO1*. In contrast, Holstein cows had greater ($P < 0.05$) abundance of the toll-like receptor (**TLR**) recognition-related gene *TLR2*, the antimicrobial-related gene *LTF*, and *S100A8*, which is involved in cell maturation, regulation of inflammatory processes, and immune response.

Directed migration to inflammatory sites is an important function of PMNL but also of monocytes and lymphocytes. Chemokine receptors are pivotal for chemotaxis, and chemokine signaling is essential for immune cell migration during bacterial infection (Salusto and Baggiolini, 2008). The CX3CR1 protein is the receptor for CX3CL1, also known as fractalkine. According to several studies, CX3CR1 is expressed by monocytes, macrophages, dendritic cells, T cells, and natural killer cells; depending on environmental condition, its interaction with CX3CL1 mediates chemotaxis of immune cells in either a pro- or an anti-inflammatory response (Lee et al., 2018).

Previous studies with Holstein cows reported down-regulation of chemokine and chemokine-receptor genes after calving (Seo et al., 2013; Crookenden et al., 2016), confirming that homeorhetic adaptations after parturition are associated with a reduction of leukocyte responsiveness and migration to inflammatory sites.

The fact that abundance of *CX3CR1* was greater in Simmental leukocytes after parturition may be indicative of a greater migration capacity of leukocytes (chemotaxis).

Leukocyte adhesion and transmigration across the endothelial barrier represent a process that is a consequence of a combined action involving cytokines and chemoattractants. The adhesion of leukocytes is guaranteed by the expression of selectins, which bind to adhesion molecules located on the surface of endothelial cells near the inflamed tissues (Kansas, 1993). In addition, CD44 was identified as one of the 3 endothelial-selectin (E-selectin) ligands on PMNL, which causes the leukocytes to slow down, activating rolling of PMNL, and then their extravasation during inflam-

mation (Hidalgo et al., 2007). After selectins allow for binding between leukocytes and endothelial cells, β 2-integrins interact with intercellular adhesion molecules, thereby attaching more firmly with endothelial cells (Engelhardt and Wolburg, 2004). Thus, these latter molecules allow leukocytes to leave blood circulation to act as phagocytes at the site of inflammation (Taooka et al., 1999).

Compared with those of Holsteins, the upregulation of *LGLAS8* in Simmental leukocytes could reflect a difference in neutrophil migration and phagocytic capacity between these breeds. In fact, *LGALS8* (Galectin-8) is involved in cell-extracellular matrix adhesion (reversible), which plays an important role in the migration of neutrophils into target tissues (Zick et al., 2002; Nishi

Table 1. Normalized arbitrary mRNA abundance of genes involved in leukocyte function from Simmental or Holstein dairy cows at 3 d after calving, grouped according to primary function

Target	Breed		SEM ¹	P-value
	Holstein	Simmental		
Recognition and immune mediation				
<i>CD14</i>	72.94	63.09	3.76	0.38
<i>CD16</i>	88.38	115.40	15.90	0.10
<i>MYD88</i>	145.46	171.05	13.83	0.07
<i>TLR2</i>	44.70	39.09	2.65	0.04
Migration and adhesion				
<i>CCR2</i>	34.85	39.23	3.87	0.26
<i>CX3CR1</i>	18.19	26.72	3.09	0.01
<i>ITGB2</i>	182.94	223.05	16.25	0.02
<i>ITGAL</i>	45.72	53.99	4.98	0.11
<i>TLN1</i>	256.30	276.70	20.35	0.32
<i>SELL</i>	500.96	544.13	33.01	0.20
<i>SELPLG</i>	236.77	298.35	31.86	0.06
<i>CD44</i>	163.76	214.77	9.75	<0.01
<i>LGALS8</i>	36.20	45.28	2.65	<0.01
<i>IL8</i>	26.65	17.46	2.47	0.14
Antimicrobial strategies				
<i>MMP9</i>	73.69	41.13	3.97	0.10
<i>LTF</i>	3.72	0.68	0.44	0.04
<i>MPO</i>	2.58	4.19	0.87	0.07
<i>LCN2</i>	23.59	16.13	3.48	0.43
<i>IDO1</i>	33.28	58.17	5.70	0.03
Inflammatory cascade				
<i>CASP1</i>	27.37	28.04	2.43	0.78
<i>TNFRSF1A</i>	209.39	248.15	20.22	0.05
<i>TNF</i>	9.96	12.06	0.73	<0.01
<i>IL1B</i>	64.60	117.64	18.73	<0.01
<i>IL1R</i>	26.69	31.86	2.10	0.02
<i>IRAK1</i>	43.40	42.65	2.56	0.77
<i>IL18</i>	9.22	10.83	1.04	0.13
<i>NLRP3</i>	29.47	31.85	0.71	0.31
<i>S100A8</i>	936.47	586.68	181.27	0.06
<i>RPL13A</i>	986.24	1175.07	111.98	0.10
Leukotrienes and oxidative status				
<i>ALOX5</i>	15.87	23.13	1.57	0.10
<i>ALOX15</i>	27.64	38.24	6.75	0.12
<i>SOD1</i>	59.40	62.57	5.00	0.52
<i>SOD2</i>	164.32	217.22	13.98	0.18

¹Greatest SEM.

et al., 2003). Additionally, *LGALS8* has been described as a receptor with the ability to activate intracellular bacterial killing (Thurston et al., 2012), a feature indicating that downregulation of *LGALS8* may reduce not only the capacity of neutrophils to migrate into target tissues but also the ability of neutrophils to kill pathogens.

Together, the upregulation of *ITGB2*, *CD44*, *CXCR1*, and *LGALS8* after calving suggests that leukocytes in Simmental cows possess a greater degree of functionality, thereby contributing to a lower incidence of infection postpartum (greater influx of leukocytes into tissues). Furthermore, the upregulation of adhesion and migration molecules in Simmental cows was likely associated with the greater abundance of cytokine- and cytokine receptor-related genes (*IL1B*, *IL1R*, *TNF*, *TNFRSF1A*), functioning as checkpoint regulators of leukocyte recruitment, trafficking, and maturation during the inflammatory response (Schmidt et al., 2013; Heiser et al., 2018).

The proinflammatory cytokines IL-1 β and TNF- α are characterized by similar biological effects on the host innate immune response to infection, including activation of endothelial cells and leukocytes (Bannerman, 2009). The protein encoded by the *IDO1* gene (indolamine 2,3-dioxygenase) is involved in the first step of tryptophan degradation through the kynurenine pathway. The antimicrobial mechanism generated by IDO1 induction is characterized by the reduction of tryptophan availability for pathogen growth at the infected site, and its activity is stimulated by proinflammatory cytokines (Mellor, 2005). Our findings on *IDO1* are consistent with the abundance of genes involved in the inflammatory cascade, which imply an enhanced inflammatory mediation in Simmental cows to support the innate and adaptive immune response.

A relevant relationship between immune response and metabolic adaptation after calving should be emphasized. In particular, during the first month after calving, metabolic data of cows involved in this study indicated that Simmental had significantly lower levels of BHB and free fatty acids compared with Holstein cows (Lopreiato et al., 2019). In vivo and in vitro experiments have revealed that leukocyte functions such as chemotaxis, phagocytosis, and superoxide anion release are reduced by high levels of BHB in circulation (Sartorelli et al., 1999, 2000; Suriyasathaporn et al., 1999). A recent study by Crookenden et al. (2019) highlighted the negative effects of high circulating free fatty acids, BHB, and liver triacylglycerol after calving on mRNA abundance of neutrophil function genes. Results indicated that cows with elevated levels of these biomarkers (defined as “high-risk” cows) were

characterized by downregulation of genes involved in recruitment, migration, and interferon signaling, suggesting that neutrophil inflammatory mediation may not be as efficient in high-risk cows as in low-risk cows. This underscores the pivotal role of the negative energy balance (**NEB**) in the innate and adaptive immune response after calving (Hammon et al., 2006).

In particular, among the genes investigated, Holstein leukocytes had greater mRNA abundance of *TLR2*. Toll-like receptors are expressed in immune cells such as neutrophils and macrophages, recognizing pathogen-associated molecular patterns on invading pathogens (Kumar et al., 2011). On the basis of our results, that Simmental leukocytes had upregulation of pro-inflammatory genes, this outcome was unexpected because TLR play a major role in inducing innate immunity and activating the pro-inflammatory response (Kumar et al., 2011). Moyes et al. (2010) highlighted the upregulation of mRNA abundance of *TLR2* and *TLR4* in circulating PMNL during an intramammary infection with *Streptococcus uberis* in Holstein dairy cows experiencing NEB compared with cows in positive energy balance. Thus, the increased amount of saturated fatty acids that occurs during postpartal NEB via adipocyte lipolysis (Douglas et al., 2007) may act as ligands for TLR, as reported previously in human macrophage cell lines (Lee et al., 2001, 2003). This could explain the upregulation of *TLR2* in Holstein cows, which had greater free fatty acid levels after calving than Simmental cows. Clearly, the relationship between lipids and immune response is not well understood, and further studies are needed to elucidate this aspect. Thus, NEB could be a key to understanding differences in leukocyte function between the 2 breeds investigated herein, because Simmental cows were also characterized by a lower milk production compared with Holsteins (Lopreiato et al., 2019).

Further efforts should be addressed to ascertain that, when different breeds are compared for innate and adaptive immune response during the transition period, other breed-specific mechanisms are involved. Data reported in the present study provide evidence of unique expression markers immediately after parturition between immune cells of Simmental cows (a dual-purpose breed for milk and meat production) and Holstein cows (a breed specialized for milk production). Hence, breed could play an important role in the modulation of those genes involved in immune adaptation and inflammatory response. However, further investigation should be focused on temporal changes to determine whether differences pointed out herein are still observed before parturition and over the first or second week after parturition.

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ORCIDS

- V. Lopreiato <https://orcid.org/0000-0001-6965-7340>
A. Minuti <https://orcid.org/0000-0002-0617-6571>
V. M. Morittu <https://orcid.org/0000-0001-6790-5734>
D. Britti <https://orcid.org/0000-0001-7355-8791>
F. Piccioli-Cappelli <https://orcid.org/0000-0003-1277-7821>
J. J. Loor <https://orcid.org/0000-0003-1586-4365>
E. Trevisi <https://orcid.org/0000-0003-1644-1911>