

Effect of Holstein genotype on *ex-vivo* cytokine response to lipopolysaccharide (LPS) and lipoteichoic acid (LTA) during the periparturient period

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ABSTRACT

Effects of Holstein genotype on innate immune response were assessed with *ex-vivo* lipopolysaccharide (LPS) and lipoteichoic acid (LTA) stimulation of whole blood from unselected (UH, $n = 10$) and contemporary (CH, $n = 11$) Holsteins that differ in production by more than 4,500 kg/lactation. Blood was collected at -14, 7, 28, and 49 days in milk (DIM), mixed with a pathogen-associated molecular pattern (PAMP) molecule (0.01 or 1.0 μg LPS or 10 or 100 μg LTA per mL blood) and incubated (4 h, 37 °C). Plasma cytokines were quantified by ELISA, \log_{10} -transformed and analyzed by repeated measures with DIM as the repeated effect. Cytokine responses increased with PAMP dose and decreased as DIM increased. There was a genotype by LPS dose interaction for IL-1 β as response to the low dose was greater in UH but did not differ between genotypes for the high dose. The IL-1 β response was greater while the IL-6 response to LTA tended to be greater in UH than in CH cows. The more negative energy balance of CH cows did not impact genotype difference in cytokine responses. Results indicate selection since the mid-1960s has decreased *ex-vivo*, whole blood cytokine response of CH cows to LPS and to LTA.

1. Introduction

The dairy industry has selected for high-producing cows to achieve greater profitability and efficiency. The contemporary Holstein (CH) produces more milk and components than her ancestors but concerns about greater susceptibility to diseases and reduced health and fitness traits continue (Curone et al., 2018; Cole et al., 2021). The University of Minnesota has maintained a herd of unselected Holsteins (UH) that have been continually bred to 1964 breed average Holstein sires since 1964 (Young, 1977; Ma et al., 2019). Our studies have demonstrated that CH cows have greater milk and component yields (Crooker et al., 2001; Weber et al., 2007; Ma et al., 2019) and that adaptations required to support these greater yields include altered metabolic, endocrine and

immune profiles (Weber et al., 2007; Cousillas Boam, 2018) relative to those of their UH herdmates. Consistent with the greater incidence of health problems in CH cows (Jones et al., 1994; Council on Dairy Cattle Breeding, 2022), we have demonstrated that UH heifers and cows have greater cytokine responses to systemic administration of LPS (Cousillas Boam, 2018; Cousillas-Boam et al., 2020) and that UH cows mount a more effective defense against intramammary administration of *E. coli* (Lippolis et al., 2022) than CH cows. We have also demonstrated substantial genomic differences between these Holstein genotypes including a greatly reduced genomic diversity among the CH cows (Kim et al., 2013; Ma et al., 2019) and multiple nucleotide sequence differences within regions known to contain genes associated with immune function (Ma et al., 2019). The continued presence of these UH cows

Abbreviations: CH, contemporary Holstein; DIM, day in milk; DMEM, Dulbecco's Modified Eagle Medium; LTA, lipoteichoic acid; UH, unselected Holstein; WOL, week of lactation.

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enable evaluation of the impact of changes in the bovine genome since 1964 on physiological and metabolic functions and on how these functions are regulated.

As part of the normal homeorhetic adjustments that occur during the transition from gestation to lactation, dairy cattle experience inflammation and immunosuppression due to a variety of hormonal, physiological, and metabolic alterations associated with changes in pregnancy status and the onset of lactation that are independent of their exposure to pathogens (Bradford and Swartz, 2020; LeBlanc, 2020; Horst et al., 2021). As a result, early postpartal dairy cattle can be more susceptible to bacterial infections. Lipoteichoic acid (LTA) and LPS are the primary pathogen-associated molecular pattern (PAMP) molecules in Gram-positive and Gram-negative bacteria, respectively. Recognition of these PAMP by their pattern recognition receptors on macrophages and other cells initiates an immune response directed toward elimination of the bacterial threat. The pattern recognition receptors for LPS and LTA differ and although the overall immune response can be similar, expression of the induced immune response components, including tumor necrosis factor- α , interleukin-1 beta (IL-1 β) and interleukin-6 (IL-6), differs between these PAMP and reflects differences between the signaling pathways these PAMP stimulate (Bannerman, 2009; Wellnitz et al., 2011; Wellnitz and Bruckmaier, 2021).

Ex-vivo stimulation of whole blood or blood cells (Damsgaard et al., 2009; Skovbjerg et al., 2010; Jahan et al., 2015) with killed bacteria (Hessle et al., 2005; Khatun et al., 2017, 2021) or their PAMP (Jahan et al., 2015; Khatun et al., 2021) is a minimally invasive assay used to assess immune responses. The main objective of this study was to determine effects of selection since the mid-1960s on Holstein whole blood IL-1 β and IL-6 responses to *ex-vivo* LPS and LTA stimulation. Based on our previous work (Cousillas Boam, 2018; Cousillas-Boam et al., 2020; Lippolis et al., 2022), we hypothesized the cytokine responses to PAMP would be less in CH than UH cows due to a less responsive innate immune system in CH cows.

2. Materials and methods

2.1. Animals and Management

Animal care and experimental procedures were conducted in compliance with the University of Minnesota Institutional Animal Care and Use Committee. Cows (11 UH; 11 CH) were paired (1/genotype) by parity and expected calving date. There were 3, 5, and 3 cows in their 1st, 2nd, or 3rd or greater lactation from each genotype. Cows were housed at the University of Minnesota Dairy Cattle Teaching and Research Center in St. Paul, MN, and managed uniformly. All cows calved between 09/13/20 and 12/05/20. One 5th lactation UH cow had a displaced abomasum, was removed, and data from her not included in the analysis. Cows were fed the same dry cow total mixed ration prepartum and the same lactating cow total mixed ration postpartum. Diets were formulated to meet the nutritional needs of Holsteins (NRC, 2001). Cows were milked twice daily. Daily feed intakes and milk yields were determined and reported as weekly means. Cows were weighed weekly after a p.m. milking. Cows were observed for health abnormalities and treated when necessary. Energy balance was determined according to NRC, (2001) as described by (Caixeta et al., 2020) and reported as weekly means.

Coccygeal blood samples were collected at $-14, 7, 28,$ and 49 ± 3 days in milk (DIM). Expected calving date was used to determine the day to collect the -14 DIM sample and actual calving date to determine postpartum sampling days. Blood samples were collected in 10 mL vacutainers (Vacutainer Beckton Dickinson and Co., Franklin Lakes, NJ) that contained sodium heparin, immediately placed on ice, transported to the lab and processed within 90 min.

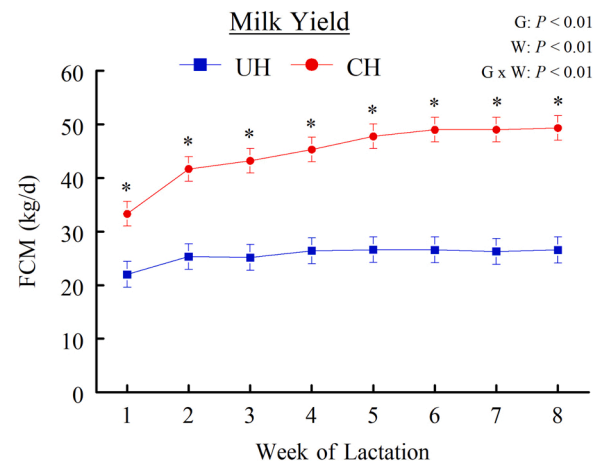


Fig. 1. Fat corrected milk yield (3.5% FCM) of unselected (UH, $n = 5$) and contemporary Holstein (CH, $n = 7$) cows. Results reported as weekly least squares means \pm SEM. Yields differed between genotypes (G, $P < 0.01$) and increased with week of lactation (W, $P < 0.01$). There was an interaction ($P < 0.01$) of the main effects as the difference between genotypes increased from week 1 through 8. * $P < 0.01$ between weekly genotype means.

2.2. Whole blood stimulation assay with LPS and LTA

A preliminary dose-incubation study was conducted with 6 non-study cows to determine concentrations of LPS (0.01–10 μg) from *Escherichia coli* 0111:B4 (Sigma-Aldrich L4931) and LTA (10–500 μg) from *Staphylococcus aureus* (Sigma-Aldrich L2515) and incubation time (2, 4, and 6 h) in whole blood (data not reported). Stock solutions of LPS and LTA were prepared in Dulbecco's Modified Eagle Medium (DMEM) and stored at -80°C until utilized. On sample collection days, heparinized blood from vacutainers were combined by cow, gently mixed, and aliquoted for isolation of plasma (1,300 \times g for 10 min at 4°C) or for incubation with and without a PAMP. Aliquots were mixed with DMEM (10 $\mu\text{L}/\text{mL}$ blood) that contained no PAMP, 0.01 or 1.0 μg LPS or 10 or 100 μg LTA per mL blood. Mixed blood was incubated (4 h, 37°C), centrifuged (1,300 \times g for 10 min 4°C), and the isolated plasma stored at -20°C until analyzed.

2.3. Plasma analysis

Plasma concentrations of IL-1 β and IL-6 were measured by ELISA (Invitrogen, Thermo Fisher Scientific, Vienna, Austria; kit ESS0027 and ESS0029, respectively) according to the manufacturer's instructions. Samples from each pair of cows were analyzed on the same plate in duplicate with standards and pool samples. The intra- and inter-assay coefficients of variation were 6.8 and 9.9 for IL-1 β and 6.0 and 7.1 for IL-6, respectively.

2.4. Calculations and statistical analysis

Non-incubated, basal blood samples were used to determine circulating cytokine profiles during the periparturient period. Cytokine concentration in incubations with DMEM only were subtracted from corresponding concentrations in incubations with PAMP to determine cytokine responses. Cytokine concentrations were \log_{10} transformed prior to analysis. Production data were summarized by week of lactation (WOL). Data were analyzed by repeated measures with PROC MIXED (SAS 9.4; SAS Institute Inc., Cary, NC). Week of lactation or DIM was used as the repeated effect with covariance structure AR(1) for production parameters and compound symmetry for cytokine measurements. The model included main effects (genotype, DIM or WOL, dose) and their interactions. There were an insufficient number of animals to assess effects of parity. Production data are reported as least squares

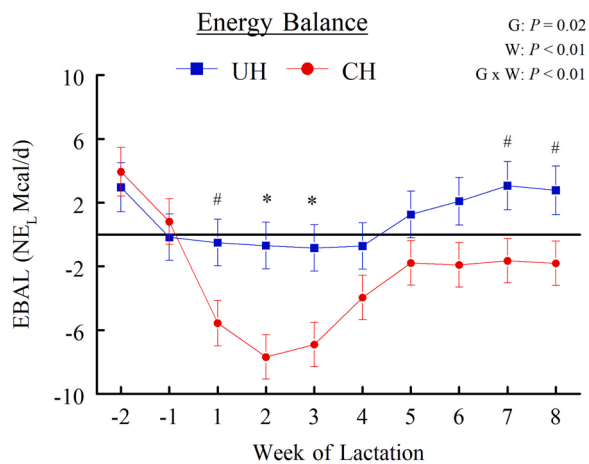


Fig. 2. Energy balance (EBAL) reported as net energy of lactation for unselected (UH, $n = 5$) and contemporary Holstein (CH, $n = 7$) cows. Results reported as weekly least squares means \pm SEM. Energy balance differed between genotypes (G, $P < 0.02$), by week of lactation (W, $P < 0.01$) and by the interaction ($P < 0.01$) of these main effects. # $P < 0.05$ or * $P < 0.01$ between weekly genotype means.

means \pm SEM and cytokine data reported as least squares means \pm SEM of the \log_{10} transformed pg/mL values. Means were considered to differ when $P \leq 0.05$ and trends identified when $0.05 < P \leq 0.10$.

3. Results and discussion

Results from this study extend our evaluation of the impact of selection on the Holstein immune system and demonstrate for the first time that blood from UH cows generates greater IL-1 β and IL-6 responses to a prototypical Gram-positive PAMP (LTA) than blood from CH cows. These results also support our previous findings that UH cows generate greater cytokine responses to a prototypical Gram-negative PAMP (LPS) than CH cows (Cousillas Boam, 2018; Cousillas-Boam et al., 2020).

The CH cows have consistently produced more milk than their UH herdmates (Jones et al., 1994; Weber et al., 2007; Ma et al., 2019) and did in this study (44.8 ± 2.1 vs 25.6 ± 2.3 kg FCM/d; $P < 0.01$; Fig. 1). Consistent with our previous reports, although the CH cows ate more (18.8 ± 1.0 vs 14.7 ± 1.0 kg DM/d; $P < 0.01$) it was not enough to compensate for their greater milk yield so they experienced a more negative energy balance (-2.65 ± 0.98 vs 0.92 ± 1.02 Mcal NE $_L$; $P <$

0.02) than the UH cows through 8 WOL. There were interactions of genotype by WOL for DMI ($P < 0.01$) and energy balance ($P < 0.01$) as neither differed between the genotypes prepartum but intake was greater and energy balance (Fig. 2) was lower in the CH cows postpartum. Adverse health events did not impact the study as the only cow with a substantial problem (displaced abomasum) was removed and her data not included in the analysis. Two CH cows were treated for metritis between 8 and 15 DIM and one UH cow treated for mastitis between 6 and 10 DIM. Cytokine concentrations from these 2 cows were within 1 SEM for the mean of the other cows and their data were included in the analysis because inclusion did not alter interpretation of the results. One UH cow was dried-off early due to only having 2 functional quarters. A total of 21, 21, 21 and 20 blood samples were analyzed at -14 , 7, 28 and 49 ± 3 DIM, respectively. The actual DIM for the prepartum sample ranged from -26 to -6 d and averaged -15 d with a SD of 5.8 d.

Cytokine concentration profiles and responses to stimuli can provide useful assessments of the physiological status of an animal but interpretation can be complicated as cytokines are released in response to multiple stimuli and impact multiple cell types (Tarrant, 2010). Interleukin-1 β and IL-6 recruit immune cells to sites of infection and activate pathogen-killing mechanisms that stimulate inflammatory responses. As primary proinflammatory cytokines, increases in IL-1 β and IL-6 concentrations in body fluids can serve as early indicators of inflammatory stimuli.

Back-transformed plasma concentrations of IL-6 in the non-incubated, basal samples ranged from 160 to 795 pg/mL and were similar to previous reports (Jahan et al., 2015; Trevisi et al., 2015; Bochniarz et al., 2017). Concentrations of IL-6 in non-incubated, basal samples were greater ($P < 0.05$) in CH than UH cows (2.64 ± 0.15 vs. $2.19 \pm 0.15 \log_{10}$ pg/mL) and decreased ($P < 0.01$) as DIM increased with concentrations at -14 and 7 DIM greater than those at 28 and 49 DIM. These postpartum decreases occurred in both genotypes, are consistent with previous reports of concentrations of IL-6 and other cytokines decreasing during the periparturient period (Røntved et al., 2005; Trevisi et al., 2015) and have been interpreted as indicating immunosuppression (LeBlanc, 2020; Horst et al., 2021). There was no interaction of genotype by day ($P = 0.54$) on IL-6 concentrations in non-incubated, basal samples. Incubating the basal blood samples at 37 °C for 4 h increased mean concentrations of IL-6 ($P < 0.01$) in CH and UH cows to 2.91 ± 0.15 and $2.45 \pm 0.15 \log_{10}$ pg/mL, respectively. These increases were relatively consistent among the samples and impacts of main effects and interactions were consistent with those for the non-incubated, basal samples. Plasma concentrations of IL-1 β are generally lower than IL-6 concentrations in healthy animals (Jahan

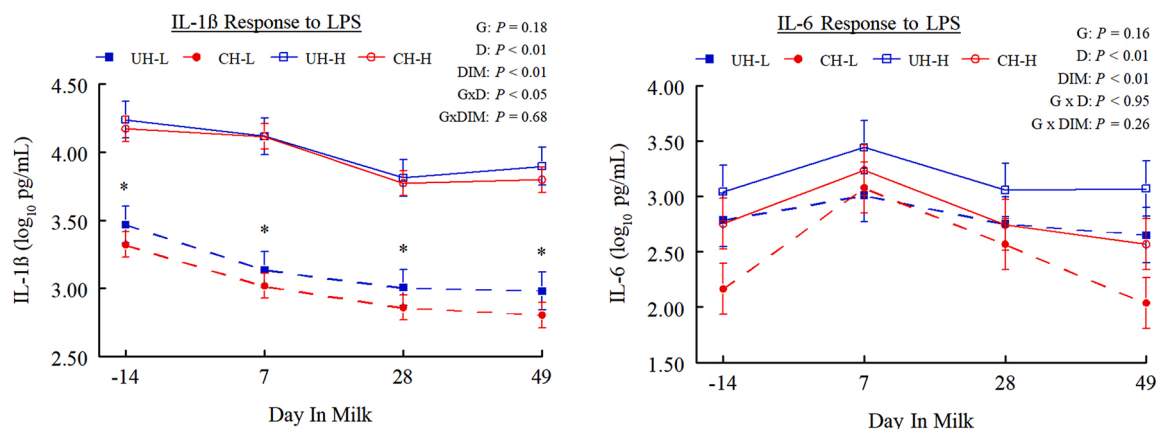


Fig. 3. Plasma \log_{10} -transformed concentrations (pg/mL) of IL-1 β and IL-6 following *ex-vivo* stimulation of whole blood from unselected (UH, $n = 5$) and contemporary (CH, $n = 7$) Holstein cows with a low (0.10 μ g; UH-L, CH-L) or high (1.0 μ g; UH-H, CH-H) dose of lipopolysaccharide (LPS) per mL of blood. Results reported as weekly least squares means \pm SEM. Concentrations of IL-1 β and IL-6 increased with dose (D, $P < 0.01$). Concentrations of IL-1 β decreased ($P < 0.01$) as day in milk (DIM) increased while concentrations of IL-6 were greatest at 7 DIM. There was a genotype (G) by dose interaction ($P < 0.05$) for IL-1 β as the response to the low dose of LPS was greater in UH than CH cows (* $P < 0.01$) but did not differ between genotypes for the high dose.

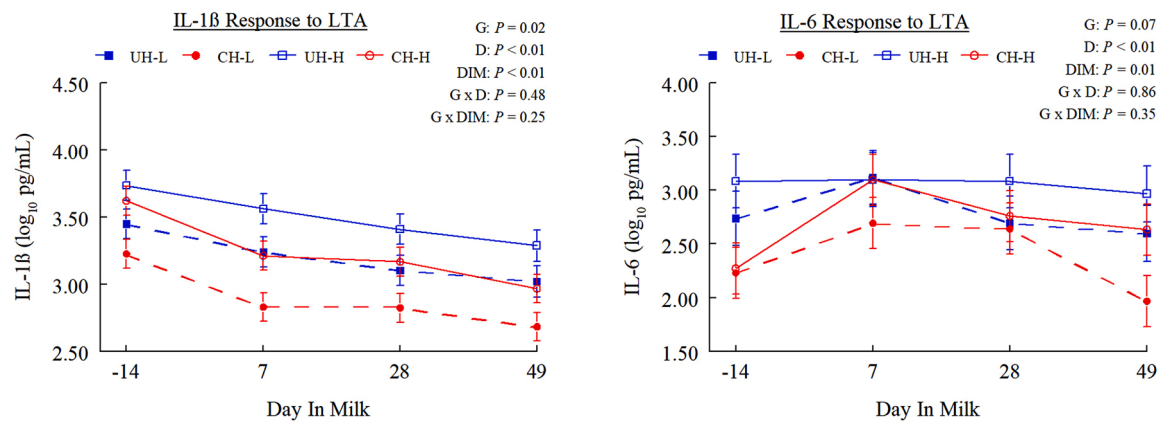


Fig. 4. Plasma \log_{10} -transformed concentration (pg/mL) of IL-1 β and IL-6 following *ex-vivo* stimulation of whole blood from unselected (UH, $n = 5$) and contemporary (CH, $n = 7$) Holstein cows with a low (10 μg ; UH-L, CH-L) or high (100 μg ; UH-H, CH-H) dose of lipoteichoic acid (LTA) per mL of blood. Results reported as weekly least squares means \pm SEM. Concentrations of IL-1 β and IL-6 increased with dose (D, $P < 0.01$). Concentrations of IL-1 β decreased ($P < 0.01$) as day in milk (DIM) increased while concentrations of IL-6 were greatest at 7 DIM. Genotype (G) affected the response to LTA as the IL-1 β response was greater ($P < 0.03$) and the IL-6 response tended ($P < 0.07$) to be greater in UH than in CH cows.

et al., 2015; Trevisi et al., 2015) and these authors frequently detected IL-1 β concentrations lower than 50 pg/mL with the same ELISA kits used in this study. Concentrations of IL-1 β in most (72 of 84) of our incubated basal samples were below the detection limit so the non-incubated basal samples were not analyzed for IL-1 β .

Responses of IL-1 β and IL-6 were increased ($P < 0.01$) with increased LPS dose (Fig. 3). There was a genotype by dose interaction ($P < 0.05$) for IL-1 β as the response was greater in UH than CH cows (3.15 ± 0.11 vs $2.86 \pm 0.10 \log_{10}$ pg/mL) with the 0.01 μg dose but did not differ between genotypes with the 1.0 μg dose (4.02 ± 0.11 vs $3.91 \pm 0.10 \log_{10}$ pg/mL). Although these results indicate the potential for a greater IL-1 β sensitivity to LPS in UH blood and for similar maximal IL-1 β responses for the genotypes, an expanded dose response analysis is needed before this can be ascertained. There was no genotype by DIM interaction ($P > 0.26$) and no dose by DIM interaction ($P > 0.26$) for either cytokine in the LPS incubations. The IL-1 β response decreased ($P < 0.01$) as DIM increased while the IL-6 response was greatest ($P < 0.01$) at 7 DIM. The reduced responses of both cytokines at 28 and 49 DIM in both genotypes are consistent with reductions in the baseline cytokine concentrations at these DIM. These results are similar to the *ex-vivo* IL-1 β and IL-6 responses when blood from multiparous, periparturient cows was challenged with a similar source of LPS at 0.01 and 5 $\mu\text{g}/\text{mL}$ blood (Jahan et al., 2015). These decreases have been interpreted as indicating a diminished immune response during this postpartum interval (LeBlanc, 2020; Horst et al., 2021).

The *ex-vivo* IL-6 response to LPS did not differ ($P = 0.16$) between the periparturient UH and CH cows in this study but the numerical differences were consistent with our previous determinations of greater *in-vivo* IL-6 response to systemic LPS by growing UH heifers (Cousillas-Boam et al., 2020) and mid to late lactation UH cows (Cousillas-Boam, 2018). Differences in physiological status and the overall immunosuppression experienced by cows during the periparturient period could contribute to differences in the magnitude of the responses between this and our previous studies. The *ex-vivo* response and assay sensitivity is likely less than the *in-vivo* response given the *ex-vivo* approach only reflects the response of blood cells and does not include those caused by PAMP interactions with other cell types. A reduced response could require a greater number of observations to detect biologically important differences. Although additional refinement of this *ex-vivo* screening approach could be beneficial, the results did identify immune response differences between the UH and CH cows which support results from our *in-vivo* evaluations.

Responses of IL-1 β and IL-6 increased ($P < 0.01$) with increased dose of LTA (Fig. 4) and there was no dose by DIM interaction ($P > 0.68$) for

either cytokine. The IL-1 β response to LTA was greater (3.35 ± 0.08 vs. $3.07 \pm 0.08 \log_{10}$ pg/mL; $P = 0.02$) in UH cows while the IL-6 response tended to be greater (2.92 ± 0.14 vs. $2.54 \pm 0.14 \log_{10}$ pg/mL; $P = 0.07$) in UH than in CH cows. There were no interactions ($P > 0.25$) of genotype with LTA dose or DIM for either cytokine. Consistent with the responses to LPS, response of both cytokines to LTA was affected ($P < 0.01$) by DIM as the IL-1 β response was greatest at -14 DIM and the IL-6 response was greatest at 7 DIM with both responses decreasing thereafter. These decreases are consistent with a diminished immune response during this postpartum interval (LeBlanc, 2020; Horst et al., 2021).

Prepartum energy balance did not differ between genotypes but postpartum energy balance was more negative in CH cows and remained negative through 8 WOL while UH cows achieved positive energy balance by 5 WOL. Responses of IL-1 β and IL-6 to LTA or LPS were affected by DIM but the lack of interactions of DIM with genotype or any other main effects indicates the effects of genotype were not impacted by the difference in energy balance between the genotypes. Interestingly, the UH cows were in positive energy balance when their response was lowest which indicates reductions in immune response of cows during the periparturient period are not driven entirely by negative energy balance.

These results demonstrate for the first time that whole blood IL-1 β and IL-6 responses to LTA are less in CH cows than in UH cows. This is also the first report of a greater IL-1 β response to LPS by UH than by CH cows. The lack of a difference in IL-6 response to LPS between the genotypes is not consistent with our previous findings of greater IL-6 responses in UH heifers and cows (Cousillas-Boam, 2018; Cousillas-Boam et al., 2020) but this might be influenced by differences in physiological status as cows experience immunosuppression during the periparturient period or by differences in sensitivity between the *ex-vivo* and *in-vivo* approaches. Actual magnitude of cytokine response differences between the genotypes might be greater than we report here because pre-incubation storage of blood samples at a 4 $^{\circ}$ C can reduce cell viability (Jerram et al., 2021). However, TNF α responses were similar when blood samples were incubated at 4 or 37 $^{\circ}$ C for 2 h prior to LPS stimulation (Segre and Fullerton, 2016). Pre-incubation conditions can alter cell-type profiles (Jerram et al., 2021) but previous reports indicate normalizing for cell count or type did not alter ranking of low and high responders and that there was a strong correlation between unadjusted and normalized cytokine response values (Segre and Fullerton, 2016; Smit et al., 2009; Spierenburg et al., 2018). Importantly, our *ex-vivo* results are consistent with our previous *in-vivo* results that demonstrated UH cows have greater cytokine responses to systemic

administration of LPS (Cousillas Boam, 2018; Cousillas-Boam et al., 2020) and that they can mount a more effective defense against intramammary administration of *E. coli* than CH cows (Lippolis et al., 2022). In addition, our results are consistent with overall trends for reduced health traits of CH cows (Council on Dairy Cattle Breeding, 2022).

In conclusion, the *ex-vivo* whole blood assays identified greater cytokine responses in the UH than in the CH cows. The UH cows represent US Holsteins from the mid-1960s and provide opportunities for direct comparisons of CH cows with representatives of their ancestors to assess impacts of selection in US Holsteins. Direct comparison of UH and CH cows in the same facility can identify impacts of selection without the influence of intervening improvements in cow management and care that confound historical records (Capper et al., 2009). The cytokine response differences warrant additional studies with the UH vs. CH model to identify immune function differences between the genotypes. Identification of genomic factors responsible for these immune function differences could be used to strengthen selection efforts to improve health traits of Holsteins.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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