Short communication

Effect of Holstein genotype on ex-vivo interleukin-1β response to lipopolysaccharide (LPS), lipoteichoic acid (LTA) and heat-killed Gram-negative and Gram-positive bacteria

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ABSTRACT

Effects of Holstein genotype on interleukin-1β response were assessed by *ex-vivo* stimulation of whole blood with lipopolysaccharide (LPS), lipoteichoic acid (LTA), or sonicated, heat-killed Gram-negative or Gram-positive bacteria. Holstein genotypes were unselected Holsteins (UH, n = 14) not subjected to selection pressures since the mid-1960s and contemporary Holsteins (CH, n = 13). Milk yield of UH and CH cows differ by more than 4500 kg/lactation. Whole blood was mixed with 0.01 μg LPS, 10 μg LTA or 2.5×10^6 CFU of sonicated, heat-killed *E. coli, K. pneumoniae, S. marcescens, S. aureus, S. dysgalactiae,* or *S. uberis* per mL of blood and incubated (4 h, 37 °C). Plasma IL-1β was quantified by ELISA and $\log_{10}$-transformed concentrations analyzed with a multivariate linear mixed effects model. Responses to bacteria were greater than responses to LPS or LTA. Responses to LPS, LTA and the Gram-negative stimulants were greater in UH than in CH cows while responses to the Gram-positive bacteria did not differ between Holstein genotypes. In both genotypes, strong correlations were detected among IL-1β responses to the Gram-negative stimulants and to LTA. There were strong correlations among IL-1β responses to the Gram-positive bacteria in CH cows but only between *S. aureus* and *S. dysgalactiae* in UH cows. The IL-1β response to *S. uberis* was highly correlated with responses to all of the Gram-negative stimulants in CH cows but only with *E. coli* in the UH cows. The reduced immune response could make contemporary cows more susceptible to infection by Gram-negative bacteria. Results confirm selection practices since the mid-1960s have altered immune response in the Holstein, at least to Gram-negative bacteria, and validate the need for additional studies to further evaluate the impacts of these selection practices on immune function in contemporary Holsteins.

1. Introduction

Inflammation of the mammary gland, more commonly known as mastitis, is detrimental to cow health and has a large negative economic impact on the dairy industry. Economic losses are associated with sub-clinical and clinical mastitis and include decreased income due to reduced milk yield and quality, increased labor for treatments and care, increased treatment costs, and increased culling and death (Bradley,

2002; Ashraf and Imran, 2020). Mastitis can be caused by Gram-negative and Gram-positive bacteria, but infection severity differs among the various pathogens (Schukken et al., 2011). Mastitis caused by Gram-negative bacteria tends to develop quickly and is often of short duration while mastitis caused by Gram-positive bacteria frequently progresses more slowly and tends to develop into chronic mastitis. Lipopolysaccharide (LPS) and lipoteichoic acid (LTA) are pathogen-associated molecular pattern (PAMP) molecules for

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Gram-negative and Gram-positive bacteria, respectively. Recognition of these and other PAMP from pathogens by host cells induces immune responses that target the invading bacteria. Release of pro-inflammatory cytokines, including interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-α (TNFα), is an initial immediate response following PAMP recognition. These host cell responses have been reported to be similar between Gram-negative bacteria and LPS but often less similar and more variable between Gram-positive bacteria and LTA (Bannerman, 2009; Khatun et al., 2021).

The dairy industry has selectively bred cows to produce more milk which has increased efficiency and profit and reduced the environmental footprint of the industry (Capper et al., 2009). Although the modern, contemporary Holstein (CH) produces more milk and milk components than her ancestors, this greater productivity has been linked to detrimental changes in reproduction and animal health (Ma et al., 2019; Cole et al., 2021; Council on Dairy Cattle Breeding, 2022; United States Department of Agriculture - National Agricultural Statistics Service (USDA-NASS), 2022). A herd of unselected Holsteins (UH) maintained by the University of Minnesota has not been subjected to selection pressures since the mid-1960s and provides opportunities for unique evaluations of the impacts of selective breeding (Young, 1977; Weber et al., 2007; Ma et al., 2019). Our previous efforts have demonstrated that systemic administration of LPS to growing UH heifers and lactating UH cows generated a greater IL-6 response relative to their CH herdmates (Cousillas Boam, 2018; Cousillas-Boam et al., 2020). Our initial work with a whole blood stimulation assay (WBSA) demonstrated that IL-6 and IL-1β responses to LPS and LTA were greater in UH than in CH cows (Brink et al., 2022). Consistent with the premise that differences in immune responses could help explain differences in susceptibility to mastitis, we have also demonstrated that UH cows mounted a more effective immune response to intramammary administration of *E. coli* than CH cows (Lippolis et al., 2022).

The *ex-vivo* WBSA is a minimally invasive and relatively inexpensive approach to assess or screen animals for their immune response to disease causing agents (Jahan et al., 2015; Khatun, 2021). We recently demonstrated that IL-1β and IL-6 responses in WBSA with LPS and LTA were less in CH than in UH cows during the periparturient period (Brink et al., 2022). Although energy deficit was greater in the CH cows, this did not impact the differences in cytokine response between the genotypes. The new work described in this paper extends our evaluation of selection-induced, immune response differences between these Holstein genotypes. Our main objective was to expand our assessment of effects of selection to include IL-1β response in WBSA with sonicated, heat-killed Gram-negative and Gram-positive bacteria and to determine if these IL-1β responses were similar to those obtained with purified PAMP. Based on our earlier work, we hypothesized the IL-1β response to these immune stimuli would be less in CH than in UH cows.

2. Materials and methods

2.1. Animals and Management

Experimental and animal care procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee. All cows were managed uniformly and housed at the University of Minnesota Dairy Cattle Teaching and Research Center in St. Paul, MN. All cows were fed the same dry-cow or lactating cow diet. Both diets were formulated to meet nutritional needs of Holsteins (National Research Council, 2001) and non-limiting amounts fed as total mixed rations. All cows were monitored from the week before through the week after sampling. Jugular blood samples were collected in 10 mL vacutainers (Vacutainer Beckton Dickinson and Co., Franklin Lakes, NJ) containing sodium heparin, immediately placed on ice, transported to the lab and used in WBSA within 90 min of sampling.

2.2. Preparation of killed bacteria

Growth and storage of isolates of 3 Gram-negative (*Escherichia coli* P4; *Klebsiella pneumoniae,* an unknown strain isolated from a Minnesota dairy farm; and *Serratia marcescens* of unknown origin) and 3 Gram-positive (*Staphylococcus aureus,* Newbould; *Streptococcus dysgalactiae,* an unknown strain, isolated from a mastitic cow in Minnesota; and *Streptococcus uberis,* 0140J) bacteria were prepared. Each bacterial isolate was grown in fresh brain heart infusion broth at 37 °C . Prior to reaching stationary growth, an aliquot was removed to determine the number of colony forming units (CFU) and the residual immediately incubated in a 70 °C water bath for 1 h to kill the bacteria. Serial dilutions of the live aliquots were plated in triplicate on blood agar plates, inverted and incubated at 37 °C for 48 h prior to counting (countable range 25–250 colonies/plate). Mean replicate counts from these dilutions indicated the corresponding heat-killed preparations had the equivalent of 6.3×10^9 CFU/mL for *E. coli,* 6.0×10^9 CFU/mL for *K. pneumoniae,* 4.6×10^9 CFU/mL for *S. marcescens,* 5.4×10^9 CFU/mL for *S. aureus,* 5.9×10^8 CFU/mL *S. dysgalactiae* and 9.8×10^8 CFU/mL for *S. uberis.* Upon removal from the 70 °C water bath, 0.2 mL aliquots of each heat-killed bacterial stock were plated in triplicate on blood agar plates which confirmed the lack of viable bacteria. Aliquots of the heat-killed bacteria were stored at − 80 °C until used. Less than 60 min before use, the heat-killed bacterial preparations were thawed and sonicated on ice with three 5 s bursts at 50% power (Ultrasonic homogenizer, Model FS-150 N; Chuanghe Laboratory Equipment, Zhengzhou City, Henan Province, China).

2.3. WBSA with PAMP and heat-killed bacteria

Preliminary WBSA pathogen dose studies were conducted with multiparous, mid-lactation (126–183 day in milk (DIM); 7 UH and 5 CH) cows for *E. coli* (Study A) and with multiparous, mid- to late lactation (177–258 DIM; 5 UH and 5 CH) cows for *S. uberis* (Study B). An equivalent of 2.5×10^5, 2.5×10^6, or 2.5×10^7 CFU/mL blood for these sonicated, heat-killed bacteria were used to assess IL-1β response. Impact of bacterial dose on IL-1β response to sonicated, heat-killed *K. pneumoniae, S. marcescens, S. aureus,* and *S. dysgalactiae* was similarly assessed but with only 2 multiparous, late lactation (260 DIM) CH cows.

Study C assessed effects of Holstein genotype on IL-1β response to LPS, LTA and to each heat-killed pathogen with 14 UH and 13 CH cows blocked (1/genotype) by parity and either their expected calving date or current DIM. There were 5 (3 UH, 2 CH) pregnant, non-lactating nulliparous cows, 4 (2 UH, 2 CH) primiparous first-lactation cows, and 18 (9 UH, 9 CH) multiparous cows in their second or greater lactation. Blood samples were collected from blocks of cows between February 11, 2022 and March 4, 2022 when non-lactating cows were at − 14 ± 10 DIM (−30 to −3 DIM) and lactating cows were 219 ± 91 DIM (109 to 330 DIM).

In brief, blood for the WBSA was collected and mixed with 10 μL PBS/mL blood (no stimulant) or 10 μL PBS with 0.01 μg LPS, 10 μg LTA, or dose of sonicated, heat-killed bacterial preparation per mL of blood and incubated at 37 °C for 4 h (Brink et al., 2022). The heat-killed bacterial preparations contain multiple PAMP so the pure (LPS and LTA) and mixed (each heat-killed bacterial preparation) PAMP preparations are collectively referred to as stimulants throughout this manuscript.

2.4. Plasma analysis

Plasma concentrations of IL-1β were measured by ELISA (Invitrogen, Thermo Fisher Scientific, Vienna, Austria; kit ESS0027) according to the manufacturer's instructions. Samples from each genotype were analyzed on the same plate in duplicate. The intra- and inter-assay coefficients of variation were 4.7% and 8.8%, respectively.

2.5. Calculations and statistical analysis

Cytokine concentration in incubations with only PBS (controls) were subtracted from corresponding concentrations in incubations with stimulant to determine cytokine response. Cytokine response concentrations were $\log_{10}$-transformed prior to statistical analysis.

Effects of bacterial dose in study A and B were assessed using PROC MIXED (SAS 9.4; SAS Institute Inc., Cary, NC). The model included main effects (dose and genotype) and their interaction. In study C, effects of Holstein genotype were assessed using the lmer package (version number 1.1.27.1) of R (version 4.1.1; R Core Team, 2021). A split-plot design was used with cow within block as the whole-plot unit, genotype as the whole-plot treatment, sample as the split-plot unit and stimulant as the split-plot treatment. A linear mixed model was fit with genotype, stimulant, and their interaction as fixed effects and block and cow as random effects. Additionally, Gram-type was compared using contrasts by comparing the average of Gram-negative stimulants with the average of the Gram-positive stimulants.

Cytokine data are reported as $\log_{10}$-transformed pg/mL least squares means ± SEM unless stated otherwise. Means were considered to differ when $P \leq 0.05$ and trends identified when $0.05 < P \leq 0.10$. Pearson correlation coefficients for responses between stimulants in study C are reported for each genotype.

3. Results and discussion

Studies A and B indicated detectable IL-1β responses were generated with each dose of heat-killed *E. coli* and *S. uberis* (Fig. 1). The IL-1β responses differed among doses for *E. coli* ($P < 0.01$) and for *S. uberis* ($P < 0.01$) and differed between genotypes for *E. coli* ($P < 0.01$) but not for *S. uberis* ($P = 0.90$). There was no interaction of dose and genotype ($P \geq 0.63$) for either bacterium. Similar dose response profiles were obtained with heat-killed *K. pneumoniae, S. marcescens, S. aureus,* and *S. dysgalactiae* (not reported). The mid-dose of 2.5×10^6 CFU/mL blood was chosen for subsequent efforts as it generated a substantial response that did not require excessive dilution of the sample for analysis. This dose is the same as that used for similar WBSA studies (Khatun et al., 2021).

Despite the considerable among-animal variation in IL-1β response within both genotypes (Fig. 2), there was an overall genotype by Gram-type interaction ($P < 0.01$) as the response to Gram-negative stimulants was greater in blood from UH ($3.58 \pm 0.10 \log_{10}$ pg/mL; 95% CI: 3.38, 3.78) than from CH ($3.27 \pm 0.10 \log_{10}$ pg/mL; 95% CI: 3.06, 3.47) cows
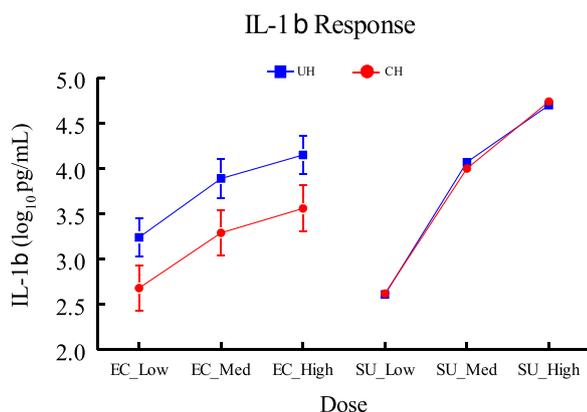

Fig. 1. Plasma IL-1β concentrations after *ex-vivo* stimulation of whole blood with sonicated, heat-killed *E. coli* (EC; Study A) or *S. uberis* (SU; Study B). Blood from 7 unselected (UH) and 5 contemporary (CH) cows in Study A and from 5 UH and 5 CH cows in Study B was incubated with 2.5×10^5, 2.5×10^6, or 2.5×10^7 bacteria/mL of blood (Low, Med or High dose, respectively). Results are reported as $\log_{10}$-transformed pg/mL least squares means ± SEM. Response to each dose of *E. coli* differed ($P < 0.01$) between genotypes.

while the overall response to Gram-positive stimulants did not differ between UH ($3.54 \pm 0.10 \log_{10}$ pg/mL; 95% CI: 3.34, 3.74) and CH ($3.50 \pm 0.10 \log_{10}$ pg/mL; 95% CI: 3.29, 3.71). Responses to LPS, *S. marcescens* and LTA were greater ($P < 0.01$) and responses to *E. coli* and *K. pneumoniae* tended to be greater ($P < 0.09$) in UH blood while response to the Gram-positive bacteria did not differ ($P > 0.44$) between genotypes. The greater IL-1β response to *E. coli* in UH blood and the lack of a genotype difference in IL-1β response to *S. uberis* are consistent with results from our dose studies (Study A and B, respectively). The greater IL-1β responses to LPS and LTA in blood from UH cows are consistent with our previous findings (Brink et al., 2022). We had insufficient observations to assess parity but there were no obvious differences in responses among parities (Fig. 2) and interpretation of the results was not altered by inclusion or exclusion of data from nulliparous or primiparous cows. Parity did not affect TNFα response in a similar WBSA study (Khatun et al., 2021).

The IL-1β responses to heat-killed bacteria were greater ($P < 0.05$) than the responses to purified LPS and LTA (Fig. 3). This was expected as these sonicated bacterial preparations should expose blood cells to more than a single PAMP. A much larger dose of LPS (10 μg/mL blood) than what we used generated a TNFα response equivalent to that from sonicated heat-killed *E. coli* in a similar bovine WBSA study (Khatun et al., 2021). In a similar assay with human monocytes, IL-1β response to UV-killed *E. coli* was about 2-fold greater than that to an equivalent amount of LPS (Hessle et al., 2005). Our relative bacteria:LPS ratio was 50% of the 1:1 ratio of Hessle et al. (2005) so our greater relative response (80 vs. 50%) might reflect greater PAMP exposure due to greater disruption (sonication) of our *E. coli* preparation. Hessle et al. (2005) also detected a greater IL-1β response to killed *S. aureus* than to peptidoglycan and peptidoglycan is generally more stimulatory than LTA. The IL-1β response to *K. pneumoniae* was less than that to *S. marcescens* ($P > 0.05$) and response to *S. aureus* was less ($P < 0.05$) than responses to any of the other heat-killed bacteria. Differences among the IL-1β responses generated by the bacteria in this study likely reflect differences in the type, number and quantity of PAMP within the bacteria and the receptors they activate.

Although there was considerable among-animal variation in IL-1β response to individual stimulants, strong correlations ($r \geq 0.84$, $P < 0.01$) were detected among IL-1β responses to the 4 Gram-negative stimulants and LTA for each genotype (Table 1). Strong correlations were also detected among the 3 Gram-positive bacteria in CH cows ($r \geq 0.74$, $P < 0.01$) but only between *S. aureus* and *S. dysgalactiae* in UH cows. The IL-1β responses between LTA and the 3 Gram-positive bacteria were weak ($r \leq 0.43$, $P > 0.10$) except for *S. uberis* in CH cows ($r = 0.58$, $P < 0.05$). In both genotypes, IL-1β responses to LTA were more strongly correlated with those for LPS and *E. coli* than with any of the Gram-positive bacteria ($r \leq 0.58$). Correlations between IL-1β responses to *S. uberis* and all Gram-negative stimulants were strong ($r \geq 0.63$, $P < 0.01$) in CH cows but only for *E. coli* in UH cows. When data from both genotypes were combined, overall interpretations were consistent with those for the CH cows although all correlation coefficients and *P*-values associated with *S. uberis* were reduced (data not reported). Strong correlations among TNFα responses to LPS, *E. coli*, *S. aureus*, *S. dysgalactiae*, and *S. uberis* have been reported (Khatun et al., 2021). These strong correlations among responses to the various stimulants indicates one or more of these stimulants could be used to rank individual cows as low or high responders. Identification of associations between responses to WBSA and intramammary challenges could reduce the need to use bacterial infections to distinguish less and more susceptible cows.

Our results are consistent with reports that immune responses to Gram-negative and Gram-positive bacteria differ (Bannerman et al., 2004; Bannerman, 2009; Hessle et al., 2005; Khatun et al., 2021) and that LPS provides a good representation of host response to Gram-negative bacteria while LTA by itself does not adequately represent host response to Gram-positive bacteria (Hessle et al., 2005; Seo
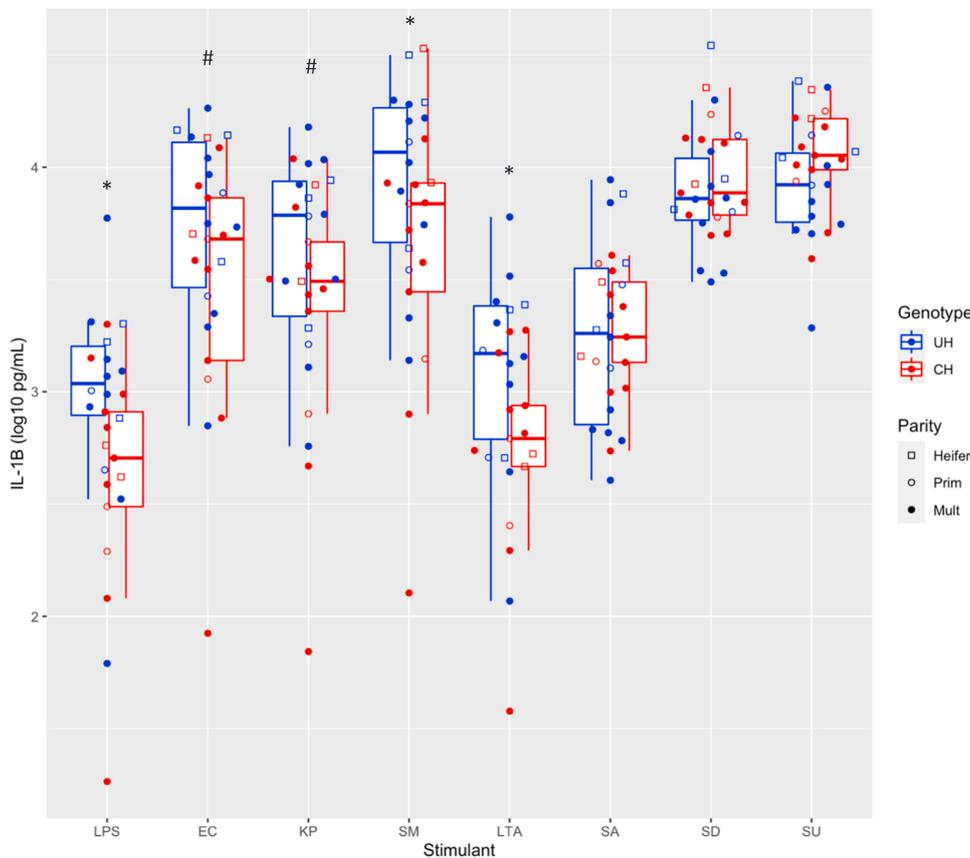
Fig. 2. Plasma IL-1β concentrations after *ex-vivo* stimulation of whole blood from unselected (UH; n = 14) and contemporary (CH; n = 13) Holsteins. Blood was incubated with 0.01 μg lipopolysaccharide (LPS), 10 μg lipoteichoic acid (LTA) or 2.5×10^6 CFU/mL blood of sonicated, heat-killed *E. coli* (EC), *K. pneumoniae* (KP), *S. marcescens* (SM), *S. aureus* (SA), *S. dysgalactiae* (SD) or *S. uberis* (SU). Results reported as $\log_{10}$-transformed pg/mL concentrations. There was a genotype by stimulant interaction ($P < 0.01$) as IL-1β response to Gram-negative stimulants were greater in UH than in CH cows (* $P < 0.05$; # $0.10 < P < 0.05$).
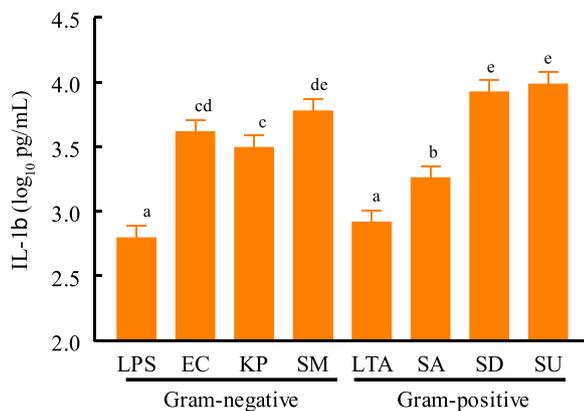

Fig. 3. Plasma IL-1β concentrations after *ex-vivo* stimulation of whole blood from unselected (UH; n = 14) and contemporary (CH; n = 13) Holsteins. Blood was incubated with 0.01 μg lipopolysaccharide (LPS), 10 μg lipoteichoic acid (LTA) or 2.5×10^6 CFU/mL blood of sonicated, heat-killed *E. coli* (EC), *K. pneumoniae* (KP), *S. marcescens* (SM), *S. aureus* (SA), *S. dysgalactiae* (SD) or *S. uberis* (SU). Results reported as overall $\log_{10}$-transformed pg/mL least squares means ± SEM. Lack of a common letter superscript indicates means differ among stimulants ($P < 0.05$).

et al., 2008). We have demonstrated that whole blood IL-1β responses to sonicated, heat-killed Gram-negative bacteria are greater in UH than in CH cows and confirm our previous findings that cytokine responses to LPS and LTA were greater in UH than in CH cows (Brink, et al., 2022). Differences in immune signaling can impact efficiency of immune response and can help explain animal variation in susceptibility to disease. The *in vitro* cytokine response differences we have identified between UH and CH cows might contribute to the more effective ability of

UH cows to combat bacterial growth and mastitis caused by intra-mammary administration of *E. coli* (Lippolis et al., 2022). In contrast, even though LTA generated a greater IL-1β response in the UH cows, IL-1β response to Gram-positive bacteria in our WBSA did not differ between these genotypes. Intramammary challenge studies are needed to determine if *in vivo* responses to Gram-positive bacteria or their PAMP differ between these Holstein genotypes.

Results from our intramammary *E. coli* challenge study (Lippolis et al., 2022) support our genomic analysis (Ma et al., 2019) that indicated continued selection for greater milk yield unintentionally carried along alterations that negatively impacted cow health. The complex, overlapping and multi-faceted nature of the immune system offers multiple opportunities for genomic alterations to impact immune response so the large variation in cytokine response among individual cows and the overlap between responses of UH and CH cows is not unexpected. However, the decreased heterozygosity of the CH genome (Ma et al., 2019) indicates beneficial polymorphisms should be more prevalent in UH cows.

The genetically stable herd of UH cows represents Holsteins in the US in the mid-1960s and provides a unique resource for direct comparison with CH cows to assess impacts of genetic selection. Results from the present study confirm selection practices since the mid-1960s have altered immune response in the Holstein, at least to Gram-negative bacteria, and validate the need for additional studies to further evaluate the impacts of these selection practices on immune function. Future selection efforts could be strengthened by identifying genomic sequences responsible for the differences in immune response to Gram-negative bacteria between these Holstein genotypes.

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Table 1

Pearson correlation coefficients between IL-1β responses to Gram-negative and Gram-positive stimulants[a] in whole blood from unselected (UH) and contemporary Holsteins (CH)[b].

Genotype	Stimulant	LPS	EC	KP	SM	LTA	SA	SD
UH	EC	0.90 * *						
	KP	0.92 * *	0.94 * *					
	SM	0.88 * *	0.95 * *	0.96 * *				
	LTA	0.96 * *	0.93 * *	0.97 * *	0.93 * *			
	SA	0.29	0.11	0.15	0.16	0.17		
	SD	0.46#	0.43	0.37	0.45	0.41	0.80 *	
	SU	0.43	0.57 *	0.39	0.44	0.35	0.36	0.55 *
CH	EC	0.92 * *						
	KP	0.92 * *	0.98 * *					
	SM	0.86 * *	0.98 * *	0.97 * *				
	LTA	0.97 * *	0.90 * *	0.91 * *	0.84 * *			
	SA	0.49#	0.42	0.54#	0.43	0.43		
	SD	0.27	0.30	0.38	0.33	0.20	0.74 * *	
	SU	0.63 *	0.71 * *	0.77 * *	0.78 * *	0.58 *	0.77 * *	0.76 * *

* *P < 0.01; * P < 0.05; # P < 0.10

[a] Lipopolysaccharide (LPS), lipoteichoic acid (LTA) or sonicated, heat-killed Gram-negative (*E. coli* (EC), *K. pneumoniae* (KP), *S. marcescens* (SM)) or Gram-positive (*S. aureus* (SA), *S. dysgalactiae* (SD), or *S. uberis* (SU)) bacteria.

[b] UH (n = 14), CH (n = 13).

Declaration of Competing Interest

The authors have not stated any conflicts of interest.

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