Characterization and comparison of cell-mediated immune responses following ex vivo stimulation with viral and bacterial respiratory pathogens in stressed and unstressed beef calves¹

Veronica M. Buhler, Kaycee R. Cash, David J. Hurley, and Brent C. Credille²

Food Animal Health and Management Program, Department of Population Health, College of Veterinary Medicine, University of Georgia, Athens, GA 30602

Abstract: The goal of this study was to compare the cell-mediated immune responses of highly commingled, sale-barn origin calves (STR; $n = 10$) to those of single source calves that had been weaned for 60 d (UNS; $n = 10$). Peripheral blood mononuclear cells and neutrophils (PMNs) were isolated from jugular venous blood of each calf. Peripheral blood mononuclear cells were stimulated with Concanavalin A (ConA), BVDV-1, BVDV-2, BHV-1, *Mannheimia haemolytica*, and *Pasteurella multocida* and evaluated for clonal proliferation and secretion of IL-8 into cell culture supernatants. The native functional capacities of PMNs were evaluated in response to stimulation with heat-killed *Escherichia coli* and *Staphylococcus aureus*. Complete blood counts and serum biochemical profiles were performed for each animal at the time of sample collection. Compared with STR calves, UNS calves had

greater lymphocyte proliferative responses following stimulation BVDV1 ($P = 0.041$), BVDV2 ($P = 0.002$), BHV-1 ($P = 0.001$), *M. haemolytica* ($P = 0.016$), and *P. multocida* ($P = 0.049$). In addition, PMNs isolated from UNS calves had a greater ability to phagocytose *E. coli* ($P = 0.001$) and *S. aureus* ($P = 0.003$) when compared with STR calves. Serum nonesterified fatty acids were higher in STR calves ($P < 0.001$). Serum β -hydroxybutyrate was lower in STR calves ($P < 0.003$). These data suggest that immunologic and physiologic differences exist between STR and UNS calves. Although the underlying mechanisms for these differences are not clear, it is possible that combinations of energy imbalances, stress-induced immunosuppression, and general immune naiveté may predispose STR calves to an increased risk of morbidity and mortality due to bovine respiratory disease.

Key words: beef cattle, bovine respiratory disease, immune function, stress

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INTRODUCTION

Bovine respiratory disease (BRD) is the most common cause of morbidity and mortality in North American beef cattle (Magstadt et al., 2018). It is estimated that BRD costs the cattle industry in excess of US\$1 billion every year (Griffin, 1997;

Watts and Sweeney, 2010). One part of the cattle industry that is particularly affected by BRD is the stocker cattle segment (Crosby et al., 2018). These cattle are typically light-weight, unweaned, commingled, and of unknown health status (Snyder et al., 2017). Although BRD in stocker cattle is a multifactorial disease syndrome, clinical signs are often associated with colonization of the upper and lower airway with both viral and bacterial pathogens (Snyder et al., 2017). As a result, it is common for stocker calves to be vaccinated against the most common disease-causing agents at arrival to stocker facilities (Richeson et al.,

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²Corresponding author: bc24@uga.edu

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2008). Unfortunately, stocker calves experience numerous stressors that could affect the efficacy of vaccination and it has been established that stress can suppress immune function in cattle (Carrasco and Van de Kar, 2003; Charmandari et al., 2005). In addition, stress can also contribute to increased shedding of viral respiratory pathogens (Fulton et al., 2016; Richeson et al., 2016). However, more recent work uses dexamethasone to simulate stress-induced immunosuppression in otherwise healthy cattle with a known health and transportation history (Richeson et al., 2016). Therefore, the objectives of this study were to characterize the cell-mediated immune responses of multiple source, highly commingled calves of unknown health history and compare the responses of these calves to a group of calves that were single source, of a known health history, and weaned for 60 d.

MATERIALS AND METHODS

This study and all procedures were approved by both the University of Georgia College of Veterinary Medicine Clinical Research Committee and University of Georgia Institutional Biosafety Committee using guidelines established in the Guide for the Care and Use of Agricultural Animals in Research (Societies, 2010).

Animals and Housing

This study was performed with private cattle producers in Covington and Crawford, GA. Sample collections were performed on each individual operation and not at a research facility. All animal health protocols used at each operation were in place prior to beginning this study and were not altered for the purposes of data collection.

Stressed calves (STR) were mixed-breed bull calves that had been purchased from auction markets in northeast Georgia and western North Carolina on a single day. All calves were of unknown age and health history, light weight (209–248 kg), and commingled. The calves were transported 68 to 290 km to the stocker facility on the day of purchase and allowed to rest in receiving pens bedded with loose Coastal bermudagrass (*Cynodon dactylon*) hay overnight. All calves were given access to free-choice Coastal bermudagrass hay and water following arrival and processed the following morning. Arrival processing involved the administration of an autogenous modified live viral (IBR, PI3, BVDV, BRSV, Nold Animal Supply, Gettysburg SD) and killed bacterial respiratory

vaccine (*Mannheimia haemolytica*, *Pasteurella multocida*, *Streptococcus durans*, *Histophilus somni*, *Mycoplasma bovis* (Nold Animal Supply, Gettysburg, SD), treated for external and internal parasites with topical moxidectin (Cydectin Pour-On, Bayer Animal Health, Shawnee Mission, KS) and oral fenbendazole (Safeguard, Merck Animal Health, Madison, NJ), implanted with Revalor G (Merck Animal Health, Madison, NJ), and had an ear notch taken for BVDV testing (IDEXX Laboratories, Westbrook, ME). Those calves that were BVDV positive were quarantined in a pen that was isolated from other calves. These BVDV positive calves were retested 14 d later to confirm the diagnosis of persistently infected (PI) status. During the course of this study, no animals were positive for PI with BVDV. All calves were castrated at arrival using the California Bander (InoSol, El Centro, CA) and administered tetanus antitoxin (Colorado Serum Co, Denver, CO). After processing, calves were held in a 0.45-ha pen with ad libitum access to water and coastal bermudagrass hay (8% CP, 31.1% CF, and 55.6%TDN on a DM basis). They were also provided a supplement consisting of 50% corn gluten feed, 50% soyhulls (18.7% CP, 15.1% CF, and 79.4% TDN), and added mineral (Free Choice Performance Mineral, Godfrey's Feed, Madison, GA, 15–19% Ca; 5% P; 18.2–21.8% salt; 1% Mg; 300,000 IU/lb Vitamin A; 1,250 ppm Cu; 100 IU/lb Vitamin E; 3,750 ppm Zn; 26 ppm Se; 2,000 ppm Mn) at rates that increased up to a maximum of 4.5 kg/calf/d.

Unstressed calves (UNS) were mixed-breed, single source steer calves between 6 and 8 mo of age that had resided on the same farm since birth and had been weaned from the dams for a period of 60 d. Steer calves had been castrated at birth and all calves received 2 doses of a pentavalent modified live respiratory vaccine containing a *M. haemolytica* leukotoxin (Bovishield Gold OneShot, Zoetis Inc, Florham Park, NJ), a multivalent clostridial toxoid (Vision 7, Merck Animal Health, Madison, NJ), and a mutant *Salmonella* Typhimurium bacterin (Endovac Beef, Immvac, Columbia, MO) prior to weaning. In addition, UNS calves were treated for external and internal parasites with a combination of injectable ivermectin/clorsulon (Ivomec Plus, Merial, Duluth, GA) and oral fenbendazole (Safeguard, Merck Animal Health, Madison, NJ) at the time of weaning. After weaning, UNS calves were housed on a 30-acre annual ryegrass pasture and fed a soybean hull based supplement (14.6% CP, 5.9% CF, 77.2% TDN) at rates that increased up to a maximum of 4.5 kg/calf/d.

Sample Collection and Processing

Ten calves were randomly selected for sampling from each group using a random number generator application (Random.org iOS app, version 1.1.2, Randomness and Integrity Services Ltd., Dublin, Ireland). In the STR group, the 10 calves were selected from a population of 64 bull calves. In the UNS group, the 10 calves were selected from a population of 60 steers. Samples from the STR calves were collected at the time of arrival processing, prior to the administration of any animal health products and at no other point. Calves that were selected for sampling were restrained with a rope halter securing the head to the side of the chute for safe and proper sample collection. Sixty milliliters of blood were aseptically obtained from the jugular vein of each calf via venipuncture with a sterile 16-gauge hypodermic needle and sterile 30-mL syringes containing 1 mL of 0.1 M EDTA. An additional 20-mL blood sample was collected and transferred to tubes containing EDTA (BD Vacutainer K2-EDTA Blood Tubes, Becton, Dickinson and Company, Franklin Lakes, NJ) and plain tubes without anticoagulant (BD Vacutainer Red Top Blood Tubes, Becton, Dickinson and Company, Franklin Lakes, NJ) for complete blood count (CBC) and serum biochemical analyses, respectively. Samples for CBC and serum biochemical analysis were submitted to the University of Georgia Athens Veterinary Diagnostic Laboratory, an American Association of Veterinary Laboratory Diagnosticians (AAVLD) accredited laboratory, for processing and test completion according to standard laboratory protocols.

Isolation of Peripheral Blood Mononuclear Cells

Sixty milliliters of EDTA blood were pooled for each individual animal into 2 50-mL tubes and centrifuged for 15 min at $650 \times g$ at 23 °C. Each buffy coat plus the top most 3 to 4 mm of red blood cell (RBC) layer was collected. These samples were pooled for each animal in a new sterile 50-mL tube, suspended in 50 mL of phosphate buffered saline (PBS), and centrifuged for 5 min at $650 \times g$ at 23 °C. The PBS layer was removed and the cell pellet was suspended in 40 mL of PBS. The diluted cell suspension was then layered over 10 mL of single step-density gradient (1.077 g/mL, ThermoFisher, Pittsburgh, PA). For controlled layering, needles (18 gauge, 1.5 inch) were attached to sterile 60-mL syringe barrels that were set against the side of the 50-mL centrifuge tube preloaded with density

gradient warmed to room temperature. The diluted cells were loaded slowly into the syringes, allowing the cells to form layers over the density gradient by gravity flow, and centrifuged for 40 min at $800 \times g$ at 23 °C.

After centrifugation, the peripheral blood mononuclear cells (PBMCs) were aspirated from the interface of the PBS and density gradient. The PBMCs were then suspended in an equal volume of PBS and washed by centrifugation for 5 min at $650 \times g$ at 23 °C. The PBS was discarded and the PBMC pellet was suspended in 10 mL of PBS solution. A 50- μ L aliquot of each cell suspension was transferred to a vial containing 450 μ L of a 0.04% solution of trypan blue dye^b for cell counting by hemocytometer and viability assessment on the basis of dye exclusion by PBMCs. Viable PBMCs were quantified microscopically and suspended at a concentration of 3×10^6 viable cells/mL in RPMI-1640 containing L-glutamine and 10% heat inactivated gamma-irradiated fetal bovine serum (Atlanta Biological, Atlanta, GA) containing 50 μ g/mL of gentamicin and 2 mM sodium pyruvate (complete RPMI).

PBMC Proliferation

One hundred microliters of the 3×10^6 cells/mL PBMC suspension was placed in quadruplicate wells for each proliferation assessment of a 96 well round-bottom plate for each animal. The wells also had 100 μ L of one of the following added: complete RPMI containing live BVDV-1 (NADL strain; 0.25 Multiplicity of Infection, MOI), live BVDV-2 (A125 strain; 0.25 MOI), inactivated BHV-1 (0.5 MOI), heat-killed *M. haemolytica* (10^8 CFU/mL, diluted 1:300), heat-killed *P. multocida* (10^8 CFU/mL, diluted 1:200), Concanavalin A (1 μ g/mL), or complete RPMI medium. The plates were incubated in 5% CO₂ at 37 °C for 5 d. After this incubation, 10 μ L containing 0.2 μ Ci of ³H-thymidine in sterile PBS was added to each well. The plates were incubated in 5% CO₂ at 37 °C for an additional 6 h. Well contents were harvested onto glass fiber filters using a 96-well plate automated harvester, and the disks were removed from the paper and placed in 7-mL scintillation vials. All disks allowed to dry for 12 to 24 h in a radiation hood with continuous air flow. Three milliliters of high efficiency scintillation cocktail was added to each vial. The vials were read on a beta scintillation counter (using a low energy profile window—34% efficiency for tritium) for determination of cell proliferation in response to each antigen or mitogen. Results were expressed

as the stimulation index and was calculated as the mean counts per minute for PBMC cultured with ConA, BVDV1, BVDV2, BHV-1, *M. haemolytica*, or *P. multocida* divided by the mean counts per minute for PBMC cultured in cell culture media alone.

IL-8 Secretion by PBMC

Six hundred microliters of the 3×10^6 cells/mL PBMC suspension were placed in triplicate wells of a 24-well plate for each stimulation. This was followed by 600 μ L of media containing one of the following: live BVDV-1 (NADL strain; 0.5 MOI), live BVDV-2 (A125 strain; 0.5 MOI), inactivated BHV-1 (0.5 MOI), heat-killed *M. haemolytica* (10^8 CFU/mL, 1:300), heat-killed *P. multocida* (10^8 CFU/mL, 1:200), Concanavalin A (1 μ g/mL), or complete RPMI (as control) added to each set of triplicate wells. These plates were incubated at 37 °C in 5% CO₂ for 72 h. At this point, the plates were centrifuged at $650 \times g$ at 23 °C for 5 min and the supernatant collected into clear 1.5-mL centrifuge tubes that were stored frozen at -80 °C until cytokine assessment. Secretion of IL-8 into cell culture supernatant by PBMCs was measured via a commercially available ELISA antibody pair and standards according to the manufacturer's instructions (Bovine IL-8 ELISA VetSet, Kingfisher Biotech Inc, St Paul, MN). Secretion of IL-8 into cell culture supernatants of stimulated cells was normalized to secretion of the cytokine into the cell culture supernatants of unstimulated cells by subtracting baseline cytokine concentrations from medium of unstimulated cells from cytokine concentrations in the medium from stimulated cells for each mitogen or antigen. As a consequence, results are expressed as Δ IL-8 to better reflect the change in IL-8 secretion by PBMCs associated with antigenic stimulation.

Polymorphonuclear Cell Isolation

After collecting PBMC separated over the density medium, the remaining cells in the density gradient medium were recovered. This combination was diluted in PBS to a volume of 50 mL per tube. The tube was centrifuged for 10 min at $650 \times g$ at 23 °C. After centrifugation, the supernatant was removed. Next, the contaminating red blood cells were lysed by adding 20 mL of sterile water to the pellet, vortexing for 30 s, and then immediately returned to an isotonic environment using 20 mL of $2\times$ PBS. Each 50-mL conical tube was mixed well and the volume of the tube brought to 50 mL

with PBS. Polymorphonuclear cells (PMN) were pelleted via centrifugation at $800 \times g$ for 5 min at 23 °C. Supernatants (900 μ L) were removed and cell pellets broken with a 10-mL pipette in 10 mL of PBS. The single cell suspension was brought to a volume of 50 mL with PBS in order to wash PMN free of the remaining red blood cell fragments, membrane, and debris. After two washes, the supernatant (900 μ L) was removed. The pellet was suspended to 10 mL with PBS. Cells were counted by placing 50 μ L of this PMN suspension in 450 μ L of 0.04% Trypan blue solution in a clean 1.5-mL tube. The PMN density and viability were assessed on the basis of dye exclusion by PMN in an Incyto DHC-N01-5 Neubauer hemocytometer (Bulldog Bio, Portsmouth, NH). The PMN suspension was again centrifuged and the supernatants were removed. The PMN were diluted to a concentration of 2×10^6 cells/mL in complete RPMI.

Polymorphonuclear Cell Phagocytosis

To measure PMN phagocytic activity, PMN were incubated with commercial stocks of bodipy-labeled killed *Escherichia coli* and *Staphylococcus aureus*. Incubations were performed for 30 min at 37 °C in the dark, and then assay tubes were fixed at 4 °C with 300 μ L of 2% paraformaldehyde. The tubes were held overnight at 4 °C before analysis. Phagocytosis of internalized bacteria, measured in the presence of 0.04% trypan blue to quench externally bound bacterial signal, was then quantified in the population of PMN as defined by the high forward angle and high-side scatter population gated in the sample using a flow cytometer. The percent of PMN positive for phagocytosis, and indication of the total number of labeled bacteria ingested by the PMNs (as determined by assessment of mean fluorescence intensity [MFI]) was measured using BD Accuri C6 Generation 2 cytometer with BD data capture and analysis software (BD Biosciences, San Jose, CA) according to techniques described previously (Ryman et al., 2013).

Statistical Analysis

Normality of the data was assessed based on histograms and normal Q-Q plots of the residuals. Constant variance of the data was assessed by plotting residuals against predicted values. For the percent of neutrophils positive for phagocytosis by flow cytometry, data were normally distributed and were therefore compared between groups using a two-sided *t*-test. For CBC, serum biochemistry,

NEFA, BHB, and neutrophil phagocytosis MFI, data were compared between groups using the Wilcoxon rank-sum test because data were not normally distributed. For PBMC proliferation and Δ IL-8 secretion by PBMCs, data were not normally distributed and are compared between groups using a Wilcoxon rank-sum test for each mitogen or stimulus evaluated. For all tests, a value of $P < 0.10$ was considered and tendency and value of $P < 0.05$ were considered significant. All analyses were performed with commercially available statistical software (Stata, Version 15.1, StataCorp, LP, College Station, TX).

RESULTS

CBC and Serum Biochemical Parameters

Compared with UNS calves, STR calves had greater neutrophil to lymphocyte (N/L) ratio ($P = 0.041$) and a tendency towards a greater segmented neutrophil count ($P = 0.060$, Table 1). STR calves had greater serum sodium ($P = 0.009$), chloride ($P = 0.001$), anion gap ($P = 0.004$), creatinine ($P = 0.002$), albumin ($P = 0.008$), aspartate aminotransferase (AST, $P = 0.007$), creatine kinase (CK, $P = 0.013$), γ -glutamyl transferase (GGT, $P = 0.032$), and nonesterified fatty acid (NEFA, $P < 0.001$) concentrations than UNS calves (Table 2, Figure 1). In addition, STR calves had reduced serum bicarbonate ($P = 0.004$), globulin ($P = 0.028$), total calcium ($P < 0.001$), and beta-hydroxy butyrate (BHB, $P = 0.003$) concentrations than UNS calves (Table 2, Figure 1).

PBMC Proliferative Responses and IL-8 Secretion by PBMC

Induced IL-8 production is a widely used marker for the induction of proinflammatory activation related to stress (DeForge et al., 1993). Its production is modulated by stress conditions, most acutely by oxidant stress. Peripheral blood mononuclear cells isolated from UNS calves had greater ex vivo proliferative responses than STR calves in response to exposure to BVDV1 ($P = 0.041$), BVDV2 ($P = 0.002$), BHV-1 ($P = 0.001$), *M. haemolytica* ($P = 0.016$), and *P. multocida* ($P = 0.049$). In contrast, there was no difference in ex vivo PBMC proliferative response to ConA between groups ($P = 0.199$, Figure 2).

Proliferation of circulating memory lymphocytes in response to recall antigens is widely used as an indicator of the efficacy of the immune response to prior exposure (Donovan et al., 2003; Reber et al., 2006). To normalize the daily variation in culture conditions, the stimulation index is utilized as the most common expression of differential response. To allow the reader insight into the basal variation in animal responses, the mean, SEM, and range of the endogenous proliferation of PBMC for the UNS animals used in this study (in counts per minute [CPM] of ^3H incorporation) were 167.6 ± 8.8 (95% CI 149.1–185.9) cpm and for the STR animals were 276.3 ± 56.1 (95% CI 158.9–393.6) cpm ($P = 0.290$). Peripheral blood mononuclear cells isolated from UNS calves had greater Δ IL-8 than STR calves in response to stimulation with ConA ($P = 0.008$), BVDV-2 ($P = 0.003$), BHV-1 ($P = 0.004$), *M. haemolytica* ($P = 0.007$), and *P. multocida* ($P = 0.013$, Figure 3).

Table 1. Comparison of complete blood count parameters (median, 10th–90th percentiles) in STR and UNS calves

Parameter	Reference range	Group		P-value
		Unstressed	Stressed	
WBC, $\times 10^3/\mu\text{L}$	4.9–12	9.6 (8.0–12.1)	10.4 (5.6–15.9)	0.391
HCT, %	22–33	28.4 (26.7–31.6)	29.5 (25.2–35.6)	0.142
Platelets, $\times 10^3/\mu\text{L}$	N/A	392 (344–513)	410 (266–538)	0.713
Seg neutrophils, $\times 10^3/\mu\text{L}$	0.6–4.0	2.2 (1.5–3.9)	3.6 (1.1–5.7)	0.060
Lymphocytes, $\times 10^3/\mu\text{L}$	2.5–7.5	6.6 (4.3–8.7)	5.2 (2.8–10.2)	0.221
N/L ratio	0.4–2.3	.35 (.2–.9)	.58 (.2–1.3)	0.041
Monocytes, $\times 10^3/\mu\text{L}$	0.0–0.9	.43 (.2–.9)	.31 (0–.6)	0.253
Eosinophils, $\times 10^3/\mu\text{L}$	0.0–2.4	.09 (0–.6)	.16 (0.1–1.2)	0.158
Fibrinogen, mg/dL	100–600	400 (110–870)	500 (300–900)	0.339

Reference ranges reported here were established by the University of Georgia Athens Veterinary Diagnostic Laboratory.

WBC = total white blood cell count; HCT = hematocrit; Seg neutrophils = segmented neutrophil; N/L ratio = neutrophil/lymphocyte ratio.

Table 2. Comparison of serum biochemical parameters (median, 10th–90th percentiles) in STR and UNS calves

Parameter	Reference Range ¹	Group		P-value
		Unstressed	Stressed	
Sodium, mmol/L	132–152	140 (137–144)	144 (140–149)	0.009
Potassium, mmol/L	3.9–5.8	4.6 (4.3–4.9)	4.7 (4.3–6.2)	0.302
Chloride, mmol/L	97–111	97 (94.1–99.9)	101 (98–105)	0.001
Bicarbonate, mmol/L	17–29	23.5 (17.4–27)	18 (14.1–22.9)	0.004
Anion gap, mmol/L	14–20	23.5 (19.2–33.3)	29 (29.1–36.9)	0.004
Creatinine, mg/dL	1.0–2.0	.8 (.6–1.2)	1.3 (1–1.7)	0.002
Total protein, g/dL	6.7–7.5	7.6 (7.0–8.0)	7.3 (6.6–7.9)	0.147
Albumin, g/dL	3.0–3.5	3.6 (3.3–3.9)	3.9 (3.7–4.1)	0.008
Globulin, g/dL	3.0–3.5	3.9 (3.1–4.7)	3.4 (2.8–4.1)	0.028
Glucose, mg/dL	45–75	79 (70.6–99.9)	74 (63.1–152)	0.241
Calcium, mg/dL	9.7–12	10.4 (10.1–11.3)	9.6 (8.8–9.9)	0.000
AST ² , U/L	78–132	66.5 (53.7–90.2)	94 (65.3–227)	0.007
Creatine kinase, U/L	44–211	247 (82.4–342)	468 (57.2–2280)	0.013
GGT ² , U/L	15–39	13 (8.2–15.8)	15 (12–20.8)	0.032

¹Reference ranges reported here are derived from Smith's Large Animal Internal Medicine (Smith B.P., 2015).

²AST = aspartate aminotransferase; GGT = gamma-glutamyl transferase.

Figure 1. Box and whisker plot depicting serum BHBA (mg/dL) and NEFA (mEq/L) concentrations in UNS and STR calves. On the left y-axis is serum BHBA concentration in mg/dL. On the right y-axis is serum NEFA concentration in mEq/L. Experimental group for each treatment is found on the x-axis. The ends of the box represent the upper and lower quartiles, the horizontal line inside the box represents the median, and the vertical lines outside the box represent the range. *Treatments differ $P < 0.05$. BHBA = beta-hydroxybutyrate; NEFA = nonesterified fatty acids.

PMN Phagocytosis

Two phagocytosis parameters were assessed: 1) the percent of PMN that phagocytosed the labeled bacteria, and 2) the mean fluorescent intensity (MFI) of the PMN that phagocytosed bacteria as an indicator of avidity of phagocytosis. The percentage of PMN exhibiting phagocytic uptake of *E. coli* was not different ($P = 0.191$) between UNS and STR calves. However, the percentage of PMN exhibiting phagocytic uptake of *S. aureus* was greater ($P = 0.008$) in STR than UNS calves (Figure 4). In contrast, PMN isolated from UNS calves phagocytosed a greater number of labeled *E. coli* ($P = 0.001$) and *S. aureus* ($P = 0.003$) than PMN from STR calves (Figure 5).

DISCUSSION

Stocker production represents a significant portion of the beef cattle industry in the Southeastern United States. Most calves that originate from this region are considered to be at high-risk of developing BRD as they are often lightweight, unweaned, intact males that have an unknown health history, and are in transit for long periods of time (Snyder et al., 2017). It has been shown that BRD in stocker populations can reduce average daily gain and, as a result, decrease per animal returns by 10% to 20% (Pinchak et al., 2004).

Because BRD is so common and economically important in stocker facilities, many producers use multiple tools in an attempt to reduce the impact

Figure 2. Box and whisker plot depicting PBMC proliferation (as stimulation index) in STR and UNS calves in response to ex vivo stimulation with viral and bacterial respiratory pathogens. The ends of the box represent the upper and lower quartiles, the horizontal line inside the box represents the median, and the vertical lines outside the box represent the range. ConA = Concanavalin A; BVDV1 = Bovine Viral Diarrhea Virus -1; BVDV2 = Bovine Viral Diarrhea Virus -2; BHV1 = Bovine Herpes Virus -1; *M. haemolytica* = *Mannheimia haemolytica*; *P. multocida* = *Pastuerella multocida*. *Treatments differ $P < 0.05$.

Figure 3. Box and whisker plot depicting Δ IL-8 secretion by PBMCs in response to ex vivo stimulation by viral and bacterial respiratory pathogens. The ends of the box represent the upper and lower quartiles, the horizontal line inside the box represents the median, and the vertical lines outside the box represent the range. ConA = Concanavalin A; BVDV1 = Bovine Viral Diarrhea Virus -1; BVDV2 = Bovine Viral Diarrhea Virus -2; BHV1 = Bovine Herpes Virus -1; *M. haemolytica* = *Mannheimia haemolytica*; *P. multocida* = *Pastuerella multocida*. *Treatments differ $P < 0.05$.

of BRD on their net return. One of the most common interventions used is vaccination. Surveys of feedlot veterinarians have revealed similar findings, with 100% recommending the use of a vaccine against BHV-1 and over 90% recommending the use of a vaccination against BVD-1 and 2 (Terrell et al., 2011; Lee et al., 2015). Unfortunately, studies have found that the use of vaccines at the time of arrival processing does little to reduce the risk of BRD in relevant cattle populations (Richeson et al., 2008; Richeson et al., 2009). In fact, more recent work has shown that delaying vaccination by 14 to 28 d might reduce retreatment risk, decrease mortality risk, and increase average daily gain relative to cattle vaccinated on arrival or not vaccinated at all (Richeson et al., 2015; Rogers et al., 2016; Hagenmaier et al., 2018). In addition, other work has found that high-risk calves vaccinated with a pentavalent modified-live viral vaccine on arrival to a stocker facility were more likely to be diagnosed

with and die from BRD than cattle not vaccinated (Griffin et al., 2018).

It is well recognized that cattle arriving at stocker facilities experience multiple stressors that could impair immune function (Richeson et al., 2008). It is believed that these stressors result in the release of endogenous glucocorticoids that can impair immune function (Carrasco and Van de Kar, 2003; Charmandari et al., 2005; Earley et al., 2012; Hodgson et al., 2012). Recent work on stress models has found that calves with a known background subjected to dexamethasone-induced immunosuppression had reduced interferon- γ secretion and impaired neutrophil function relative to control calves (Richeson et al., 2016; Hughes et al., 2017). While telling, this model might not accurately reflect the physiological and immunological status of high-risk calves under field conditions.

In our study, the goals were to evaluate the physiological and immunological status of

Figure 4. Box and whisker plot depicting phagocytosis of bodipy labeled *E. coli* and *S. aureus* in PMNs isolated from STR (gray) and UNS (black) calves. The ends of the box represent the upper and lower quartiles, the horizontal line inside the box represents the median, and the vertical lines outside the box represent the range. *Treatments differ $P < 0.05$.

Figure 5. Box and whisker plot depicting intensity of phagocytosis of bodipy labeled *E. coli* and *S. aureus* in PMNs isolated from STR (gray) and UNS (black) calves. The ends of the box represent the upper and lower quartiles, the horizontal line inside the box represents the median, and the vertical lines outside the box represent the range. MFI = mean fluorescence intensity. *Treatments differ $P < 0.05$.

high- and low-risk calves under field conditions. Here, we found that STR cattle had higher serum NEFA levels than UNS cattle, a factor that suggests fat mobilization is occurring to a greater extent in STR calves. In addition, STR calves had evidence of dehydration as reflected in increased serum concentrations of sodium, chloride, bicarbonate, creatinine, and albumin (Zabolotzky, 2011). These findings are not unexpected as many STR calves had been through sale barn channels prior to arrival at the stocker facility and have likely been without access to food or water for some period of time prior to processing. Our study also found that UNS calves had greater serum concentrations of BHBA, a ketone body often associated with negative energy balance, and fat mobilization in post-partum dairy cattle (McArt et al., 2013). Nevertheless, it has been shown that serum BHBA levels increase with maturation of rumen function in young dairy calves and it is possible that the increased serum BHBA concentrations in the UNS group in our study reflects enhanced maturation of rumen function associated with adaptation to feed for 60 d following weaning (Quigley et al., 1991).

In addition to the physiological changes seen, multiple immunological derangements were identified in the STR calves in this study. Compared with UNS calves, STR calves had reduced PBMC

proliferative responses to most of the stimuli assessed in these studies. Stimulation indices, as a normalized measure of PBMC proliferation, correlate with expansion of antigen-specific memory PBMC populations in circulation. An enhanced pool of antigen responsive cells provides a reasonable indicator of future antigen recall responses by immune individuals in vivo (Sandbulte and Roth, 2004). As a result, it is possible that this finding is reflective of lack of previous antigen exposure through vaccination or natural exposure in the STR cattle. It is interesting to note that STR cattle had reduced serum calcium concentrations that UNS calves. It is also possible that hypocalcemia might blunt the response of PBMCs to stimulation with exogenous antigen (Kimura et al., 2006). Indeed, calcium is an essential second messenger for immune cell activation and is responsible for regulation of cell proliferation, cytokine production, and cytokine receptor expression (Trebak and Kinet, 2019). Another potential reason for the decreased function of PBMCs seen in STR calves in this study is the increase in serum NEFA concentrations (Hammon et al., 2006). Since glucose is a major major fuel source for immune cells, it has been shown that elevated NEFA concentrations can contribute to impairment of in vitro PBMC function and might increase the risk of certain

diseases in post-partum dairy cattle (Ster et al., 2012). Although serum glucose concentrations were the same between the two groups of cattle evaluated in this study, it can be an insensitive indicator of systemic energy balance. As a result, measurement of intracellular glycogen concentrations would provide a better assessment as to what extent negative energy balance might contribute to the findings of this study (Galvao et al., 2010). Future work evaluating the association between increased serum NEFAs and risk of BRD in this type of calf is warranted and would further clarify this issue. Stressed calves had reduced ability to secrete IL-8 into cell culture supernatants relative to UNS calves. Interleukin-8 is produced by a wide variety of cell types, particularly PBMCs, in response to exposure to various inflammatory mediators (Kimura et al., 2002). It is likely that similar factors that are responsible for the decrease in PMBC proliferation (hypocalcemia, naïve immune function, negative energy balance) are responsible for the decreased secretion of IL-8 in STR calves.

Stressed calves had a reduced ability to phagocytose-labeled antigen compared with UNS calves. Polymorphonuclear cells play a critical role in microbial defense of the lung and are recruited to the alveoli in response to the release of cytokines from respiratory epithelial and immune cells, particularly IL-8 (Ackermann et al., 2010). Impairment of PMN activity, therefore, likely represents one reason for the increased incidence of BRD in high-risk populations of cattle. The decrease in PMN function likely arises as a result of multiple factors. First, the decreased secretion of IL-8 by PBMCs might impede the pathway for PMNs to migrate to the site of infection and might result in reduce PMN activation (Ackermann et al., 2010). Although IL-8 is not the only chemoattractant produced at the site of bacterial infection in cattle, it is produced by leukocytes in rather high quantities and exported from the cells making it. Furthermore, there are excellent commercial reagents available that allow for reproducible and quantitative measurements of IL-8 in culture supernatant that are sensitive enough to document the differences in activation and release of IL-8 by cells from stressed and nonstressed calves. In addition, it is possible that natural stressors such as transportation and commingling alter or exhaust PMN functional capacity. Transportation has been shown to alter neutrophil gene expression (Buckham Sporer et al., 2007; Buckham Sporer et al., 2008). Furthermore, abrupt weaning and commingling impairs neutrophil phagocytosis and reduced L-selectin expression (Lynch et al., 2010).

Although the data generated under this study assessed the impact of stress on the performance of several immune indicators, the differences observed were often quite small. It is not clear what the threshold point in the field is for decline in immune functional capacity to have an impact on the development of clinical disease in calves. Therefore, these studies provided a set of “marker points” that documented statistically significant changes that should be further explored for their capacity to affect the development of disease in a more reductive model where additional factors could be controlled.

One of the weaknesses of this study is the fact that the calves in this study were from different sources with different genetics, and the background of the STR calves was unknown. This could contribute to an increase in variability in the immune responses measured here and potentially bias the data. Another weakness of this study is that the calves in each group were housed at different locations for varying periods of time prior to sample collection. Different farms have different management styles that can influence many of the variables measured in this study. Nevertheless, this model is likely more representative of the variation encountered in field settings and, therefore, is more externally valid and a more realistic picture of cattle in the production setting. Additional samples collected from these calves serially at other time points would provide invaluable information as to the progression of immune function and, potentially, how immune dysfunction might lead to the development of BRD. Unfortunately, time and funding for this study were limited and these factors precluded more in depth analysis.

In conclusion, these results suggest that STR calves have a different immunological function from UNS calves. The need for future research investigating how these differences contribute to BRD risk, and how the modulation or alteration of immune activation and management of physiological balance is clear from the nearly universal difference between high- and low-stress cattle observed in this study.

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