Effect of a DNA-based immunostimulant on growth, performance, and expression of inflammatory and immune mediators in beef calves abruptly weaned and introduced to a complete ration¹

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ABSTRACT: The effect of a DNA immunostimulant on inflammatory and immune responses, performance, and health in calves following abrupt weaning and introduction to a concentrate diet was tested. Sixty-four single source Angus crossbred steers were weaned on day 1 and assigned to receive a DNA immunostimulant (TRT) or saline (CON) on days 0, 2, 4, and 6. On day 0, steers received clostridial and respiratory vaccines and anthelmintic; they were then transported 2 h, allocated to pens ($n = 8$ per pen), and introduced to total mixed ration. Daily intake, ADG, and feed efficiency were measured. Serum haptoglobin, tumor necrosis factor- α (TNF- α), and interleukin-1 beta (IL-1 β) were assayed by ELISA or AlphaLISA on days 0, 2, 4, 6, 14, and 28; serum-neutralizing antibodies (SNA) to bovine herpesvirus-1 and bovine viral diarrhea virus-1 (BVDV-1) were quantified on days 0, 28, 68, and 135. In a subset of cattle ($n = 6$ to 8 per treatment group), the percent macrophages and activated gamma delta ($\gamma\delta$) T cells in blood was determined by flow cytometry on days 2 and 6, and expression of mRNA for TNF- α , interferon-gamma (IFN- γ), IL-4, and IL-10 by stimulated

blood mononuclear cells was assessed by real-time reverse transcriptase PCR on day 6. After 70 d, cattle were shipped 1,205 km to a feedlot and performance and health were followed. There was a significant effect of time on serum TNF- α , IL-1 β , haptoglobin, and SNA ($P < 0.001$); the range in concentration among cattle on each day was large. The ratio of IFN- γ to IL-4 expression was significantly higher ($P = 0.03$) for TRT cattle, suggesting that treatment activated T-helper type 1 cells. There was a trend toward an improved feed conversion ($P = 0.10$) for TRT steers over the 70-d backgrounding period. There was no effect of treatment on feedlot performance or carcass merit ($P > 0.10$). During backgrounding, 1 TRT steer died of enterocolitis. In spite of backgrounding, cattle experienced an outbreak of bovine respiratory disease (BRD) in the feedlot and 1 of 31 TRT cattle and 5 of 32 CON cattle died of BRD. The immunostimulant modified some immune responses during backgrounding. Large variability in inflammatory responses during backgrounding indicated that events around weaning induce systemic inflammation that varies substantially among cattle.

Key words: beef cattle, cytokines, haptoglobin, immunostimulant, inflammation

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INTRODUCTION

Events occurring around the time beef calves are weaned, such as vaccination, transportation, and introduction to a complete ration, can increase their risk for infection or systemic inflammation that may induce morbidity, mortality, and decreased production. Although knowledgeable producers strive to limit the impact of these factors through careful management, they may at times be unavoidable. Administration of a recently released DNA immunostimulant (Zelnate, Bayer HealthCare LLC, Shawnee Mission, KS) has been shown to decrease lung lesion scores in cattle experimentally challenged with the bacteria *Mannheimia haemolytica* (Nickell et al., 2016) and to decrease mortality in high-risk cattle after feedlot placement (Rogers et al., 2016). This DNA immunostimulant is made of a noncoding DNA plasmid containing multiple cytosine-guanine rich (CpG) sequences that is encased in cationic liposomes (Ilg, 2017). CpG DNA motifs are well-described pathogen-associated molecular patterns (PAMP) that activate inflammation and immune responses through interaction with various cytoplasmic and endosomal receptors in host cells (Warshakoon et al., 2009; Krieg, 2012). Cationic liposomes are included in this DNA immunostimulant to improve uptake of the plasmid, but they can also contribute to activation of host inflammation and immunity (Lonez et al., 2012). Given this background, the objective of this research was to test the effect of this DNA immunostimulant on inflammatory responses, health, and production of single source calves that were abruptly weaned, vaccinated, subjected to a short (2 h) episode of transport, and introduced to a complete ration. The hypothesis was that measures of inflammation would be decreased and health and performance would be improved during backgrounding in cattle treated with the DNA immunostimulant. Little has been reported regarding the specific effects of this immunostimulant on bovine immune cell responses; therefore, a secondary objective was to evaluate the immune response in a subset of the cattle.

MATERIALS AND METHODS

Animals and Management

This research was approved by the Mississippi State University Institutional Animal Care and Use Committee (approved protocol #15-071). Sixty-four Angus crossbred steers born at the

Mississippi Agricultural and Forestry Experiment Station Prairie Research Unit were abruptly weaned on study day - 1. On the day of weaning, the median age (range) of cattle was 224 d (193 to 247 d). Body weight was measured on days - 1 and 0 (30 September 2015) and the average value was used for the baseline in comparison of ADG between experimental groups. On day 0, cattle were vaccinated with modified-live 5-way viral vaccine containing bovine herpesvirus-1 (BoHV-1), bovine viral diarrhea virus-1 (BVDV-1), BVDV-2, bovine respiratory syncytial virus (BRSV), parainfluenza type 3 virus (PI3V), and *M. haemolytica* (Pyramid-5 + Presponse, Boehringer Ingelheim Vetmedica Inc., Duluth, GA) and multivalent clostridial bacterin-toxoid (Ultrabac 8, Zoetis, Kalamazoo, MI), and dewormed with ivermectin and closulon (Ivomec Plus, Boehringer Ingelheim Vetmedica Inc., Duluth, GA) by injection at the label dose. The cattle were stratified by body weight and assigned to receive either a label dose of the DNA immunostimulant by intramuscular (IM) injection (TRT) or an equivalent volume of sterile isotonic saline IM (CON). After processing, the cattle were subjected to a 2-h episode of transport. After transport, they were unloaded and placed into pens by treatment group (8 cattle per pen, 4 pens per treatment). Each pen contained bunks which utilized the GrowSafe System (GrowSafe Systems Ltd., Airdrie, Alberta, Canada) to monitor feed intake during the course of the study. The cattle were considered to be medium risk, because all originated from the Prairie Research Unit, but were abruptly weaned and introduced to a complete ration and transported by truck. Between days 0 and 70, the cattle were observed daily by the investigators or Prairie Research Unit staff members to identify signs of illness. In addition to the day 0 treatment, cattle were treated with the DNA immunostimulant or saline per their treatment group assignment on days 2, 4, and 6. This regimen was used because the study objective was to test the hypothesis that immunostimulant treatment would mitigate inflammatory responses associated with abrupt weaning and introduction to complete ration, but it was not clear when, relative to these events, a single dose would be most effective. Multiple doses were therefore administered in an effort to prove the concept.

After cattle were backgrounded for 70 d at the Prairie Research Unit, they were transported on 9 December 2015 to Gregory Feedlot in Tabor, Iowa to be finished as part of the Tri County Steer Carcass Futurity, where assessment of health and

performance was continued through harvest. All cattle from both treatment groups were fed together in a single pen for the entire feeding period.

Diets

Table 1 provides the ingredients and nutrient composition of the diet used during the back-grounding phase of the experiment. Feed was mixed by a commercial feed mill (Southern Seed and Feed, Macon, MS). A sample was collected from each new batch of feed and composited. Composite samples were sent to a commercial laboratory (Cumberland Valley Analytical Services, Maugansville, MD) for nutritional analysis.

Assessment of ADG, Intake, and Performance Indicators

From days 0 to 70, individual feed intake was measured using the GrowSafe system. Cattle were weighed on days 1, 0, 14, 28, 56, 69, and 70. Cattle were weighed unshrunk and the days 1 and 0 weights were averaged to determine the initial weight for analysis, and the days 69 and 70 weights were averaged to determine the final weight. At the feedlot, cattle were weighed at arrival (day 76), at reimplanting (day 135), on day 196, and at closeout.

Serum Haptoglobin, Tumor Necrosis Factor-Alpha, Interleukin-1 Beta, and Neutralizing Antibodies

To assess the impact of treatment on systemic inflammatory response, serum concentrations of

haptoglobin (acute phase reactant) and tumor necrosis factor-alpha (TNF- α) and interleukin-1 beta (IL-1 β ; proinflammatory cytokines) were measured in all cattle on days 0, 2, 4, 6, 14, and 28. Blood was collected by jugular venipuncture into tubes with no additive and allowed to clot. Serum was separated by centrifugation at $2000 \times g$ for 15 min at room temperature. Serum was removed and stored frozen at -80°C until analysis. Serum concentrations of haptoglobin, TNF- α , and IL-1 β were measured by a commercially available ELISA for haptoglobin (Bovine Haptoglobin ELISA Kit, ICL Inc., Portland, OR) or AlphaLISA Assay for Bovine TNF- α or IL-1 β (Perkin-Elmer, Waltham, MA), per kit instructions. Serum samples were diluted as needed to keep the optical density value in the linear region of the standard curve and then multiplied by the dilution factor to determine the final concentration in ng/mL for haptoglobin and pg/mL for TNF- α and IL-1 β .

To determine whether DNA immunostimulant treatment at the time of MLV 5-way viral vaccination affected the humoral immune response to vaccination, serum-neutralizing antibody titers to BoHV-1 and BVDV-1 were measured as described previously (Woolums et al., 2013) at the University of Georgia Athens Veterinary Diagnostic Laboratory from serum samples collected on days 0, 28, 68, and 135.

Circulating Macrophages and Activated Gamma Delta T Cells

In an effort to characterize the mediators of immunity that are activated in cattle treated with the DNA immunostimulant, detailed immune assessments were made in a subset of cattle on days 2 and 6. On days 2 and 6, blood was collected from 8 randomly selected cattle in each treatment group (2 from each pen); an adequate number of peripheral blood mononuclear cells (PBMC) were isolated from 6 TRT cattle and 8 CON cattle and stained with FITC-conjugated anti-CD11b (MCA1425F, Bio-Rad, Hercules CA) and RPE-conjugated anti-CD14 (MCA1568PE, Bio-Rad) to identify macrophages, and RPE-conjugated anti-CD25 (MCA2430PE, Bio-Rad), anti-WC1 (MCA1655, Bio-Rad) followed by FITC-conjugated anti-mouse IgG2 secondary as described (Seo et al., 2009). Cells were assayed on a FACScalibur flow cytometer with unstained cell preparations used for setting gates.

Table 1. Ingredient and nutrient composition¹ of the diets fed to cattle during the growing phase of the experiment

Items	Diet
Ingredient, % of DM	
Corn	47.02
Cottonseed hulls	18.06
Soybean hull pellets	7.04
Cottonseed meal	20.00
Molasses	5.03
Vitamin/Mineral Premix	1.06
Nutrient composition	
DM, %	87.03
CP, % of DM	14.07
NDF, % of DM	34.04
NEm, Mcal/kg	1.59
NEg, Mcal/kg	0.99
Soluble CP, % of DM	1.07

¹Nutrient composition was conducted by Cumberland Valley Analytical Services using a composite of diet samples.

Stimulated Cytokine mRNA Expression by Peripheral Blood Mononuclear Cells

To assess the effect of DNA immunostimulant treatment on expression of cytokines that contribute to modulation of host inflammatory responses and to development of host immunity, production of mRNA for the cytokines TNF- α , interferon-gamma (IFN- γ), IL-4, and IL-10 by mitogen-stimulated or superantigen-stimulated PBMC from the randomly selected subset of cattle described above was measured on day 6. Nonspecific stimulation with a mitogen (concanavalin A, con A) or superantigen (staphylococcal enterotoxin B, **SEB**) was used to activate relatively large proportions of T cells, increasing the possibility of identifying effects of the DNA immunostimulant on T cell activation. TNF- α and IL-10 were assessed due to their effects to increase and decrease inflammation, respectively; IFN- γ and IL-4 were assessed due to their status as prototypical cytokines representing T helper type 1 and T helper type 2 immune responses, respectively. The PBMC were isolated as described previously (Woolums et al., 2013); an adequate number of cells for culture were obtained from 8 TRT cattle and 7 CON cattle. The PBMC were suspended in Roswell Park Memorial Institute (**RPMI**) medium + 10% fetal calf serum (**FCS**) at 5×10^6 cells per mL. One milliliter of cells was added to each of 3 wells of a 24-well plate; one milliliter of media alone was added to the first well (negative control), 1 mL of media with 10- μ g/mL con A was added to the second well (final con A concentration was thus 5 μ g/mL), and 1 mL of media with 0.2- μ g/mL SEB was added to the third well (final SEB concentration was thus 0.1 μ g/mL). Cells were incubated for 72 h at 37 °C in 5% CO₂, then PBMC were transferred to RLT buffer (QIAGEN, Valencia CA) and stored at -80 °C until RNA was extracted. RNA was extracted using the RNeasy purification kit (Qiagen, Valencia, CA). First-strand cDNA was generated from 1- μ g RNA using Superscript III RT and oligo dT primers (Invitrogen, ThermoFisher Scientific, Waltham, MA). The qRT-PCR was performed using primers (sequences shown in [Supplementary Table 1](#)), Power SYBR Green Master Mix (ThermoFisher Scientific, Waltham, MA), and the ABI 7500 real-time PCR system (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions. Real-time PCR data were analyzed using sequence detector system software (Applied Biosystems, Carlsbad, CA); C_T were then normalized by calculating ΔC_T [C_T of target - C_T of the internal control (β -actin gene)].

Relative quantification of the target gene was determined by the comparative C_T method ($\Delta\Delta C_T$) of calculating $\Delta\Delta C_T = [\Delta C_T \text{ of ConA or SEB treated} - \Delta C_T \text{ of medium only}]$, according to the manufacturer's instructions.

Statistical Analysis

Data related to feed intake and weight gain in the backgrounding period, and weight gain and carcass merit in the feedlot period, were analyzed using the MIXED procedure of SAS 9.4 (SAS Institute, Cary, NC). The animal was the unit of analysis. All results are reported as least squares means and were separated using the PDIF option of SAS. Significance was set at $P \leq 0.05$, and tendencies were determined if $P > 0.05$ and ≤ 0.10 .

Bovine haptoglobin, IL-1, and TNF- α concentration measurements were transformed to the log10 scale for analysis and are presented as the geometric mean with 95% confidence intervals. A linear mixed model (PROC MIXED; SAS 9.4, SAS Institute, 2013) was used to compare the mean protein concentration considering groups, time, and their interactions as fixed effects and animal as a random effect. A heterogeneous compound symmetry, antedependence, and autoregressive covariance structures resulted in the best fit for the bovine haptoglobin, IL-1, and TNF- α concentration data, respectively, and thus were used to model the correlations between protein concentration measurements of the same animal along the different time points. Tukey's test was used to adjust the P values resulting from multiple comparisons. All of the analyses were conducted using a level of significance at P values of <0.10 .

Results from cytokine RT-PCR in each treatment group were tested for normality using the Shapiro-Wilk test; data that were not normally distributed were log or natural log transformed which resulted in a normal distribution. Treatment groups were compared using an unpaired t -test with Welch's correction (Prism 7, Version 7.0a, GraphPad Software Inc., La Jolla, CA). The ratio of IFN- γ to IL-4 was calculated by dividing the fold-change over unstimulated value for IFN- γ by the same value for IL-4 for each sample. Results for flow cytometry (percent cells positive) were tested for normality using the Shapiro-Wilk test; data that were not normally distributed were natural log transformed which resulted in a normal distribution. The treatment group average was used for missing values. Treatment groups were compared by 2-way ANOVA (Prism 7, Version 7.0d, GraphPad

Software Inc., La Jolla, CA) with assessment of significant effects of treatment, time, and treatment by time interaction. A Fisher's exact test was used to test the significance of the number of cattle that died during the feeding period in each treatment group. All of the analyses were conducted using a level of significance of P values of <0.10 .

RESULTS

Health and Performance of Cattle: Backgrounding Phase

In the first week after weaning, some cattle in both treatment groups displayed coughing and nasal discharge. However, the feed intake remained normal, and no cattle displayed signs of depression associated with need for treatment, so they were not treated for bovine respiratory disease (BRD). Over the 70-d backgrounding phase at the Prairie Research Unit, 1 steer in the TRT group required treatment for a rectal prolapse, and a second steer died on study day 60. Necropsy of that steer indicated that the cause of death was clostridial enterocolitis.

Feed intake, weight gain, and feed efficiency for steers during the backgrounding phase are shown in Table 2. There was a trend toward improved ADG for TRT steers between days 15 and 70 ($P = 0.06$), and a trend toward an improved feed conversion ratio (decreased feed to gain, $P = 0.10$) for TRT steers over the 70-d backgrounding period.

Health and Performance of Cattle: Feedlot Phase

In spite of backgrounding for 70 d, during the feedlot phase, several cattle in both groups were treated for BRD in the first 3 wk after arrival. Feedlot management reported that unusually cold

and wet weather may have contributed to the BRD outbreak. The entire group was mass-treated with tulathromycin on 30 December 2015 (study day 91). Six cattle died with gross lesions compatible with BRD between 18 December 2015 (study day 79) and 12 February 2016 (study day 134): 1 steer from the TRT group and 5 steers from the CON group ($P = 0.19$). Measures of feedlot performance are shown in Table 3 and results of carcass merit assessment are shown in Table 4. The steers were harvested on 3 May 2016 (study day 216) and 7 June 2016 (study day 251). There was no significant difference between groups for any measurement of performance or carcass merit ($P > 0.10$).

Serum Haptoglobin, TNF- α , IL-1 β , and Neutralizing Antibodies

Results of serum haptoglobin assay are shown in Figure 1. Serum haptoglobin concentrations increased substantially between days 0 and 2. There was a wide range in measured concentration of serum haptoglobin among cattle in each group (note log scale on the y-axis of Figure 1). There was a significant effect of time ($P < 0.001$) and a significant time by treatment interaction ($P = 0.03$), but no significant effect of treatment ($P > 0.10$). Although serum haptoglobin concentrations decreased over time, they were still relatively elevated over baseline on day 28. Results of serum TNF- α and IL-1 β assay are shown in Figures 2 and 3. Similar to the results for serum haptoglobin, there was a wide range in concentrations among cattle in each group. There was a significant time effect on serum TNF- α and IL-1 β concentrations ($P < 0.001$ for both), but no significant interaction or treatment effect ($P > 0.10$). Means and 95% confidence intervals for haptoglobin, TNF- α , and IL-1 β are presented

Table 2. Feed intake, growth performance, and efficiency during the 70-d backgrounding phase in Control (CON) and DNA immunostimulant-treated (TRT) cattle item

	CON ¹	TRT	P value
Intake			
Period 1 (1–14 d) DMI, kg/d	8.64 $\pm$ 0.32	8.21 $\pm$ 0.33	0.36
Period 2 (15–70 d) DMI, kg/d	8.52 $\pm$ 0.27	8.96 $\pm$ 0.28	0.27
Overall DMI, kg/d	10.7 $\pm$ 0.33	11.2 $\pm$ 0.33	0.30
Period 1 (0–14 d) ADG, kg/d	2.36 $\pm$ 0.22	2.24 $\pm$ 0.23	0.70
Period 2 (15–70 d) ADG, kg/d	0.92 $\pm$ 0.07	1.11 $\pm$ 0.07	0.06
Overall ADG, kg/d	1.21 $\pm$ 0.06	1.33 $\pm$ 0.06	0.12
Gain:feed ratio	0.112 $\pm$ 0.003	0.119 $\pm$ 0.003	0.16
Feed conversion ratio (feed:gain)	9.34 $\pm$ 0.31	8.60 $\pm$ 0.32	0.10

¹LS Means $\pm$ standard error.

Table 3. Performance of Control (CON) and DNA immunostimulant-treated (TRT) cattle during the feed-lot phase

Item	CON	TRT	P value
Delivery weight, kg	338.2 ± 5.2	345.2 ± 5.2	0.34
On Test weight, kg	359.7 ± 5.5	361.0 ± 5.4	0.86
Reimplant weight, kg	401.8 ± 5.7	397.3 ± 5.4	0.66
Final live weight, kg	555.4 ± 8.4	557.7 ± 8.0	0.84
Days on feed	156.7 ± 3.3	156.6 ± 3.1	0.98
Overall ADG, kg/d	1.38 ± 0.04	1.36 ± 0.04	0.65
On Test ADG, kg/d	1.57 ± 0.05	1.59 ± 0.04	0.73

Table 4. Carcass characteristics of control (CON) and DNA immunostimulant treated (TRT) cattle

Item	Control	Treated	P value
Hot carcass weight, kg	343.4 ± 5.6	341.3 ± 5.3	0.79
Dressing percentage, %	61.8 ± 0.3	61.2 ± 0.3	0.19
Fat thickness, cm	1.27 ± 0.06	1.23 ± 0.06	0.65
Ribeye area, sq cm	81.3 ± 1.44	83.8 ± 1.37	0.22
KPH, %	2.44 ± 0.09	2.37 ± 0.08	0.51
USDA yield grade	2.54 ± 0.13	2.31 ± 0.12	0.20
Percent retail product, %	63.0 ± 0.5	63.9 ± 0.4	0.13
Marbling score ¹	484.6 ± 12.5	497.6 ± 11.8	0.46
USDA quality grade ²	17.9 ± 0.3	18.1 ± 0.3	0.55

LS Means ± standard error.

¹Marbling score: 400 = Slight⁰; 500 = Small⁰.

²USDA quality grade: 17 = Select⁰; 18 = Select⁺; 19 = Choice⁻.

in [Supplementary Tables 2 to 4](#), and the data for individual cattle are depicted in [Supplementary Figure 1](#).

The distribution of serum-neutralizing antibodies (SNA) to BoHV-1 and BVDV-1 in TRT and CON cattle is shown in [Figure 4](#). Titers to both viruses went up between days 0 and 28 following vaccination on day 0. There was no significant difference in titers between the groups at any time point ($P > 0.10$) ([Figure 4](#)).

Percent Circulating Blood Macrophages and Activated Gamma Delta T Cells

The percent macrophages (CD11b+, CD14+) and activated gamma delta T cells (WC1+, CD25+) in isolated PBMC are shown in [Figure 5](#). In both the TRT and CON groups, there was a wider range in the percent of circulating macrophages among steers on day 2 than on day 6. There was no significant effect of treatment ($P > 0.10$), and no significant effect of time ($P > 0.10$) on percent activated gamma delta T cells; however, there was a significant effect of time on the percent circulating macrophages ($P = 0.004$).

Expression of Cytokine mRNA by Stimulated PBMC

The fold-change in expression of mRNA for IFN- γ , IL-4, and IL-10 by PBMC stimulated with either Con A or SEB, relative to unstimulated cells, is shown in [Figure 6](#). The ratio of fold-change for IFN- γ to IL-4 is also shown. The fold-change for expression of IL-4 was significantly lower ($P = 0.04$) for TRT than CON. The ratio of IFN- γ to IL-4 was significantly higher for TRT than CON cattle, both for Con A ($P = 0.03$) and also SEB ($P = 0.03$, [Figure 6](#)).

Although the differences between groups were not statistically significant ($P > 0.10$), the pattern of expression of mRNA for TNF- α by stimulated PBMC was different in the 2 groups ([Figure 7](#)). Expression of mRNA for TNF- α was elevated (≥ 2 -fold increase over response by unstimulated cells) in 3 of 7 CON cattle, versus 0 of 8 TRT cattle.

DISCUSSION

Previous reports have described the effect of the DNA immunostimulant evaluated here to decrease pulmonary lesion scores following experimental *M. haemolytica* challenge ([Nickell et al., 2016](#)), and

Figure 1. Serum haptoglobin concentration in DNA immunostimulant-treated and control cattle ($n = 32$ per group). Dots represent outliers. Within treatment, time points with different letters (lowercase letters for Control, uppercase letters for Treated) are significantly different ($P < 0.001$). Treatment by time interaction was also significant ($P = 0.03$), and comparisons within groups across time are shown in [Supplementary Table 2](#). The effect of treatment was not significant ($P > 0.10$).

Figure 2. Serum TNF- α concentration in DNA immunostimulant-treated and control cattle ($n = 32$ per group). Dots represent outliers. Within treatment, time points with different letters (lowercase letters for Control, uppercase letters for Treated) are significantly different ($P < 0.006$). Neither the effect of treatment nor the treatment by time interaction were significant ($P > 0.10$). Comparisons within groups across time are shown in [Supplementary Table 3](#).

to decrease mortality in high-risk cattle after feedlot entry (Rogers et al., 2016). However, to the best of our knowledge, this is the first study to assess the impact of this immunostimulant on inflammatory and immune responses, feed intake, and production measures in cattle when given at the time of abrupt weaning and introduction to complete ration. The trend toward improved feed-to-gain ratio seen in TRT cattle was of interest; this contrasted with the results of a large study evaluating the effect of the DNA immunostimulant in high-risk feedlot heifers (Rogers et al., 2016), in which treatment had no effect on feed efficiency (feed to gain).

It should be noted that previous work tested the effect of a single dose of the DNA immunostimulant,

Figure 3. Serum IL-1 β concentration in DNA immunostimulant-treated and control cattle ($n = 32$ per group). Dots represent outliers. Within treatment, time points with different letters (lowercase letters for Control, uppercase letters for Treated) are significantly different ($P < 0.001$, except for control d 14 vs. 28, where $P = 0.03$). Neither the effect of treatment nor the treatment by time interaction were significant ($P > 0.10$). Comparisons within groups across time are shown in [Supplementary Table 4](#).

whereas in this study, the TRT cattle were given the DNA immunostimulant every 48 h for 4 treatments beginning on day 0. This regimen was used in an effort to prove the concept that treatment could mitigate inflammatory responses associated with abrupt weaning and introduction to a complete ration, when it was not clear when a single dose would be most effective, relative to the time of these events. Additional research will be needed to assess the effect of a single dose on host immune or inflammatory responses.

The DNA immunostimulant is labeled for use to aid in the treatment of BRD due to *M. haemolytica* when administered at the time of, or within 24 h after, a perceived stressful event. Although the cattle in this study were considered to be at medium risk for BRD, they were subjected to stressful events including abrupt weaning and shipment. The cattle in this study were considered medium risk, because all originated from the Prairie Research Unit, but were abruptly weaned and introduced to a complete ration, and transported by truck for 2 h. Thus, they were not expected to experience a high rate of BRD during the study. This held true in the backgrounding phase. However, given the 70-d backgrounding phase and the healthy status of the cattle when they were shipped to the feedlot, there was an unexpectedly high rate of BRD morbidity and mortality after the cattle entered the feedlot. Six of the 63 cattle died of BRD in the feedlot; five of these cattle were in the CON group and one was in the TRT group. These numbers are too small to make any conclusions, but they suggest that administration of the DNA immunomodulator at the beginning of the backgrounding phase may have induced some effect to prevent

Figure 4. Distribution of serum neutralizing antibody titers to BVDV (left) and BoHV-1 (right) and on days 0, 28, 68, and 135 ($n = 32$ per group except on day 68 when $n = 31$ in the DNA immunostimulant-treated group, and on day 135 when $n = 30$ in the treated group and 27 in the control group). Geometric mean titer in each group is indicated by the horizontal bar. Cattle were given vaccine containing modified-live BoHV-1 and BVDV-1 on day 0. There was no significant difference ($P > 0.10$) between the titers in the 2 groups on any experimental day.

Figure 5. The percent of peripheral blood mononuclear cells (PBMC) that were (a) activated gamma delta T cells (WC1+CD25+) or (b) activated macrophages (CD14+ CD11b+) in a subset of steers in the DNA immunostimulant-treated group. An adequate number of cells were available for analysis for 8 of 8 sampled calves in the control group and 6 of 8 sampled calves in the treated group. Within treatments, time points with different letters are significantly different ($P = 0.004$; lowercase letters for control and uppercase letters for treated). The effect of treatment was not significant ($P > 0.10$).

mortality in the feedlot phase, and further investigation of this relationship may be worthwhile.

The exact mechanisms by which the DNA immunostimulant decreases lung pathology following *M. haemolytica* challenge, and decreases mortality in cattle after feedlot entry, are currently not well defined. In light of the fact that the inflammatory response resulting from neutrophils activated and killed by leukotoxin is believed to contribute to the pathogenesis of the lung lesions due to *M. haemolytica* infection (Slocombe et al., 1985; Weiss et al., 1991), we hypothesized that the DNA immunostimulant might moderate the host inflammatory response. Although it may be counterintuitive to attribute anti-inflammatory responses to an immunostimulant, decreased inflammation can be the net effect of some immune processes. Although serum haptoglobin, TNF- α , and IL-1 β concentrations were not different between TRT and CON

cattle, expression of TNF- α mRNA by stimulated PBMC was elevated in some CON cattle, but was low in all TRT cattle. This finding suggests that the immunostimulant may in fact modulate inflammatory responses, but at sites or at times not reflected by serum concentrations of haptoglobin, TNF- α , or IL-1 β .

A noteworthy finding was that the ratio of IFN- γ to IL-4 produced by stimulated PBMC of TRT cattle on day 6 (the only day when this was assessed) was significantly higher than the ratio for CON cattle. As the IFN- γ to IL-4 ratio can be viewed as a measure of relative activation of T helper type 1 (TH1) cells when compared with T helper type 2 (TH2) cells, these results suggest that the DNA immunostimulant induced a relatively strong TH1 response to immune stimulation, a relatively diminished response of TH2 cells, or both (Mosmann and Coffman, 1989). This is consistent with

Figure 6. The fold-change in expression of mRNA for (a) IFN- γ , (b) IL-4, and (d) IL-10 by PBMC stimulated with either Con A or SEB, relative to unstimulated cells. The PBMC were collected from a subset of steers ($n = 8$ in the DNA immunostimulant treated group and 7 in the control group) on study day 6. The ratio of expression of IFN- γ to IL-4 for each animal is also shown in (c). *Expression of IL-4 was significantly lower ($P = 0.04$) for Treated cattle compared with Control cattle. The ratio of IFN- γ to IL-4 was significantly higher ($P = 0.03$) for treated cattle, both when PBMC were stimulated with Con A ($P = 0.03$) and also SEB.

previous reports regarding the response of immune cells to CpG DNA in other systems (Ioannou et al., 2002; Ioannou et al., 2003), but this is to the best of our knowledge the first data indicating that this DNA immunostimulant preferentially stimulates a TH1 response in cattle. As classically understood, TH1 cells activate cell-mediated immune responses against viral and other intracellular pathogens,

whereas TH2 cells activate humoral responses, particularly production of IgA, IgE, and certain isotypes of IgG. It was also notable that, in spite of apparent preferential activation of TH1 cells, the humoral response to vaccination against BoHV-1 and BVDV-1 was not inhibited, as evidenced by comparable SNA in both groups. Collectively this suggests that the DNA immunostimulant can

Figure 7. Distribution of expression of mRNA for TNF- α by PBMC stimulated with concanavalin A (con A) or staphylococcal enterotoxin B (SEB) for cattle in the DNA immunostimulant-treated and control groups. Values are expressed as fold-change over mRNA expression by unstimulated cells from the same animal. The difference between groups was not significant ($P > 0.10$).

activate cell-mediated immunity while maintaining humoral immunity, both of which are important for protecting the host from pathogens that contribute to BRD.

Since this study was completed, it has been recognized that the DNA immunostimulant interacts with the cytoplasmic receptor cyclic guanylate adenylate synthase (cGAS) and the “stimulator of interferon genes” (STING) (Ilg, 2017); this interaction suggests that the immunostimulant activates interferon response factor 3 (IRF3), leading to production of type I interferons. Although type I interferons are well-known mediators of antiviral immunity, the role of these cytokines in antibacterial responses, and in modulation of inflammation in certain settings, is increasingly recognized (Gonzalez-Navajas et al., 2012). It remains to be determined whether type I interferons such as IFN- α and IFN- β are responsible for effects attributed to this DNA immunostimulant.

Notably, serum concentrations of TNF- α and IL-1 β were high in both groups on day 0, relative to day 28. At the time blood samples were collected on day 0, the cattle had not been vaccinated, transported, or introduced to complete ration, but they had been weaned the day before. Similarly, investigators found that concentrations of serum TNF- α and other inflammatory mediators were increased in dairy calves after weaning from milk feeding and movement to group pens (Kim et al., 2011). Overall, in the first month of this study, there was a wide range in concentrations of expression of haptoglobin and proinflammatory cytokines among the cattle tested, both in the serum (Figures 1–3), and in response to stimulation of PBMC. These data indicate that, in a group of calves experiencing management procedures often associated with weaning, there can be a wide range in the degree of inflammatory response among individuals, even in the absence of clinically important disease.

SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Animal Science* online.

Conflict of interest statement. None declared.

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