Effects of Age and Nutrition on Expression of CD25, CD44, and L-Selectin (CD62L) on T-cells from Neonatal Calves*

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ABSTRACT

Effects of the plane of nutrition and age on the proliferation and activation of lymphocyte subsets from milk replacer-fed calves were investigated *in vitro*. Holstein calves were fed a standard (0.45 kg/d of a 20% crude protein, 20% fat milk replacer, $n = 4$) or intensified (1.14 kg/d of a 28% crude protein, 20% fat milk replacer, $n = 4$) diet from 1 to 8 wk of age. Average daily weight gain of intensified-diet (0.66 kg/d) calves was greater than that of standard-diet (0.27 kg/d) calves. Relative to the pokeweed mitogen-induced responses of CD4⁺ cells from steers (5 to 6 mo of age), CD4⁺ cells from 1-wk-old calves showed decreased proliferative activity, delayed increase in CD25 expression, and no demonstrable increase in CD44 expression or decrease in CD62L expression. Calf CD8⁺ and $\gamma\delta$ T-cell receptor⁺ cells, unlike T-cells from the older animals, did not demonstrate decreased expression of CD62L after stimulation with mitogen. The increased expression of CD44 by mitogen-stimulated $\gamma\delta$ T-cell receptor⁺ cells from older animals was not seen in $\gamma\delta$ T-cell receptor⁺ cells from 1-wk-old calves. At wk 8 of age, mitogen-induced proliferation and expression of activation antigens by T-cells from standard-fed calves were similar to responses of T-cells from steers indicating rapid maturation of T-cell function during the neonatal period. Feeding calves an intensified milk replacer was associated with decreased proliferation of mitogen-stimulated CD4⁺, CD8⁺, and $\gamma\delta$ T-cell receptor⁺ cells; decreased CD25 expression by mitogen-stimulated CD4⁺ and CD8⁺ cells; and decreased CD44 expression by mitogen-stimulated CD8⁺ cells. These results indicate that the functional capacity

of the calf's T-cell population becomes more adult-like during the first weeks of life and suggest that nutrition modulates T-cell function during this period of immune maturation.

(**Key words:** calf, immune function, nutrition)

Abbreviation key: CD62L = L-selectin, CMI = cell-mediated immune, FBS = fetal bovine serum, PBMC = peripheral blood mononuclear cells, PEM = protein energy malnutrition, PI = proliferation index, PWM = pokeweed mitogen, TCR = T-cell receptor.

INTRODUCTION

Activation, differentiation, trafficking, and migration of T-cells through sites of inflammation or infection are essential for an effective immune response. The α chain of the IL-2 receptor, CD25, is expressed on activated T-cells, B-cells, and monocytes. Formation of the high-affinity IL-2 receptor allows T-cell proliferation and differentiation to be driven by IL-2. In cattle, proliferating CD4⁺, CD8⁺, and $\gamma\delta$ T-cell receptor (TCR⁺) cells exhibit increased expression of CD25 (Waters et al., 2003b). Expression of CD25 on CD4⁺, CD8⁺, and $\gamma\delta$ TCR⁺ cells has been used to monitor responses of bovine T-cells to inactivated bovine herpesvirus-1 (Endsley et al., 2002), *Mycoplasma bovis* (Vanden Bush and Rosenbusch, 2003), bovine respiratory syncytial virus (Sandbulte and Roth, 2002), *Mycobacterium bovis* (Waters et al., 2003a,b; Nonnecke et al., 2005) and mitogenic stimulation (Nonnecke et al., 1993; Franklin et al., 1994).

Leukocyte trafficking is regulated by surface adhesive interactions between adjacent T-cells or between cells and the extracellular matrix. The leukocyte adhesion molecule, CD44, binds to components of the extracellular matrix and is considered essential for extravasation of T-cells at sites of inflammation (Dailey, 1998). Expression of CD44 is upregulated on antigen-activated murine T-cells and remains elevated on memory T-cells (Dailey, 1998). In *Mycobacterium bovis*-infected cattle, expression is greater in CD4⁺, CD8⁺, and $\gamma\delta$ TCR⁺

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T-cells proliferating in response to antigen than in T-cells unresponsive to antigen (Waters et al., 2003b).

The lymph node homing receptor, L-selectin (CD62L), is required for entry of cells into lymph nodes through high endothelial venules. Its expression on murine lymphocytes is downregulated after polyclonal and antigenic stimulation in vivo and in vitro (Dailey, 1998). Activated T-cells with reduced CD62L expression do not adhere to lymph node high endothelial venules in vitro or traffic to lymph nodes in vivo in the mouse (Dailey, 1998). In *Mycobacterium bovis*-infected cattle, CD62L expression is decreased in antigen-activated CD4⁺, CD8⁺, and $\gamma\delta$ TCR⁺ cells (Waters et al., 2003b). Decreased expression reduces clearance of activated cells from the blood by lymph nodes, allowing them to traffic to sites of activated endothelium.

Although the effects of nutrient supply on body composition and performance of neonatal calves are established (Diaz et al., 2001; NRC, 2001), few studies have considered effects of nutrient supply on immune function of the young calf (Nonnecke et al., 2003; Foote et al., accepted). Nutritional status influences broad aspects of immune function (Scrimshaw et al., 1968; Watson and McMurray, 1979; Sullivan et al., 1993). In prepubescent mice, protein-energy malnutrition (PEM) depresses thymus-dependent immunity (Woodward, 1998). In mice, PEM is associated with an overabundance of CD4⁺CD45RA⁺ T-cells (CD4⁺ naïve-phenotype) and CD8⁺CD45RA⁺CD62L⁺ T-cells (CD8⁺ naïve-phenotype) that are quiescent compared with CD45⁺ effector and memory phenotypes (Woodward et al., 1999; ten Bruggencate et al., 2001). Similarly, feed-restricted rats have a decreased capacity to respond to recall antigens when compared with ad libitum-fed rats (Fernandes et al., 1997). Depressed humoral and cell-mediated immune (CMI) responses in calves (Griebel et al., 1987) and decreased numbers of circulating CD8⁺ and $\gamma\delta$ TCR⁺ cells in cattle experimentally infected with virulent *Mycobacterium bovis* (Doherty et al., 1996) also have been associated with PEM.

Excess nutrition also modulates immune function. Expression of IL-2 receptor (CD25) by mitogen-stimulated lymphocytes from rats fed an ad libitum diet is reduced when compared with cells from rats fed a 40% food-restricted diet (Iwai and Fernandes, 1989). Similarly, high-fat diets suppress CD25 expression on splenic lymphocytes in mice (Peck et al., 2000). Although CD62L expression by circulating leukocytes from adult cattle is not affected by energy balance (Perkins et al., 2001), effects of energy on CD25, CD44, and CD62L expression on activated bovine lymphocytes have not been reported.

The objective of this study was to evaluate effects of age and increased nutrient supply provided by intensi-

fied milk replacer on the functional capacity of peripheral blood T-cell subsets from young dairy calves. We hypothesized that increasing dietary protein and energy would enhance proliferative responses and activation antigen (CD25, CD44, and CD62L) expression on mitogen-stimulated T-cell subsets from neonatal calves.

MATERIALS AND METHODS

Animals and Treatments

All procedures involving animals were approved by the National Animal Disease Center, Animal Care and Use Committee. Holstein bull calves (n = 8) were housed at the Land O'Lakes, Inc., Research Farm in Webster City, IA, and 4 Holstein steers (5 to 6 mo of age) were housed at the National Animal Disease Center in Ames, IA. Calves were housed on raised stalls in an environmentally controlled building. Steers were housed on straw in a building with southern exposure. Calves were allotted randomly to a standard (n = 4) or intensified (n = 4) diet. The standard diet consisted of a 20% CP, 20% fat milk replacer fed at 0.45 kg/d with calf starter (18% CP, Land O'Lakes, Inc., St. Paul, MN) offered ad libitum. The intensified diet consisted of a 28% CP, 20% fat milk replacer fed at 1.14 kg/d, with calf starter (22% CP, Land O'Lakes, Inc.) offered ad libitum. Calves were fed these diets from 1 to 8 wk of age.

Blood Mononuclear Cell Recovery and Enrichment

Blood was collected from calves at 1 wk (prior to initiation of dietary treatments) and at 8 wk of age (7 wk after initiation of dietary treatments) by jugular venipuncture. Blood was collected from the steers at the same times. Sixty milliliters of blood was collected into 10% (vol/vol) 2× acid-citrate-dextrose (a sterilized solution containing sodium citrate [77 μ mol/L], citric acid [38 μ mol/L], and dextrose [122 μ mol/L]).

Peripheral blood mononuclear cells (PBMC) were enriched by density gradient centrifugation as described previously (Nonnecke et al., 1991). The PBMC-enriched populations were resuspended in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 25 mM HEPES buffer, 2 mM L-glutamine (Sigma, St. Louis, MO), antibiotics (100 U/mL of penicillin and 0.1 mg/mL of streptomycin, Sigma), 50 μ M 2-mercaptoethanol (Sigma), 1% nonessential AA (Sigma), 2% essential AA (Sigma), 1% sodium pyruvate (Sigma), and 10% (vol/vol) heat-inactivated fetal bovine serum (FBS, Hyclone Laboratories, Inc., Logan, UT).

Table 1. Primary and secondary antibodies used in the flow cytometric analysis of PBMC populations from neonatal calves and steers.

Primary antibody specificity	Clone ¹	Isotype	Secondary antibody ^{2,3}
CD4 ⁺	GC50A1	IgM	IgM-APC
CD8 ⁺	BAQ111A	IgM	IgM-APC
$\gamma\delta$ TCR ⁺	CACT61A	IgM	IgM-APC
CD25	CACT116A	IgG ₁	IgG ₁ -PE
CD44	BAT31A	IgG ₁	IgG ₁ -PE
CD62L	BAQ92A	IgG ₁	IgG ₁ -PE

¹Primary mouse-generated monoclonal antibodies from VMRD (Pullman, WA).

²Rat anti-mouse IgM-allophycocyanin (APC) [$\sim 0.2 \mu\text{g}/\text{well}$] from Pharmingen, San Diego, CA.

³Goat anti-mouse IgG₁-phycoerythrin (PE) ($1 \mu\text{g}/\text{well}$) from Southern Biotechnology Associates (Birmingham, AL).

Blood Mononuclear Cell Cultures

Staining of isolated PBMC with PKH67 green fluorescent dye (Sigma) for flow cytometric analysis of lymphocyte subset proliferation was performed following manufacturer directions as described previously (Waters et al., 2003a). Briefly, 4×10^7 PBMC were centrifuged (10 min, $400 \times g$) the supernatants were decanted, and the cells were resuspended in 2 mL of diluent C that was provided in the PKH67 kit. Diluted cells were added to 1 mL of diluted ($4 \mu\text{M}$) PKH67 green fluorescent dye, incubated for 5 min, and then incubated 1 min with 4 mL of inactivated FBS to adsorb excess dye and stop further uptake of dye. Cells were then washed twice with RPMI 1640 medium. Individual wells of 96-well tissue culture plates were seeded with 2×10^5 PKH67-stained PBMC in a total volume of 200 μL (10 replicates for each treatment) in 96-well, round-bottom microtiter plates. Cultures were nonstimulated (media only) or stimulated with pokeweed mitogen (PWM, $1 \mu\text{g}/\text{mL}$) and incubated for 3 and 6 d at 39°C in a humidified atmosphere with 5% CO_2 .

Analysis of Proliferation and Expression of Activation Markers

Culture replicates were pooled after 3- and 6-d incubation periods. Approximately 2×10^5 pooled PKH67 stained cells in 150 μL of culture medium were added to individual wells of 96-well, round-bottomed microtiter plates. The PKH67-stained cells were double-labeled with 1 of 3 phenotype markers (CD4, CD8, or $\gamma\delta$ TCR) and 1 of 3 activation markers (CD25, CD44, or CD62L). Primary and secondary antibodies used in the analyses are listed in Table 1. Monoclonal primary antibody ($1 \mu\text{g}/\text{well}$) diluted in PBS containing 0.02% NaN_3 and 1% inactivated FBS was added to individual wells in 25- μL aliquots. Cells were incubated for 15 min at room

temperature and centrifuged ($400 \times g$ at room temperature for 2 min). Supernatant was decanted and cells were double-labeled with 100 μL of a cocktail containing 2 secondary isotype-specific antibodies conjugated to fluorochromes (IgM-allophycocyanin and IgG₁-phycoerythrin; listed in Table 1). Secondary antibodies were diluted in PBS containing 0.02% NaN_3 and 1% inactivated FBS. Cells were incubated for 15 min at room temperature in the dark and then centrifuged as described above. Cells were resuspended in 200 μL of PBS and examined by flow cytometry the same day.

Ten thousand cells exhibiting light-scattering properties consistent with bovine PBMC were analyzed. Data were acquired using a BDLSR flow cytometer (Becton Dickinson, San Jose, CA). Lymphocyte proliferation (CD4⁺, CD8⁺, and $\gamma\delta$ TCR⁺ cells) data were acquired using CellQuest (Becton Dickinson) software and analyzed using Modfit LT (Verity Software House, Inc., Topsham, ME) software. Proliferation profiles were determined for the gated CD4⁺, CD8⁺, and $\gamma\delta$ TCR⁺ T-cell subsets within the PBMC population. Lymphocyte proliferation was estimated by measuring the fluorescence intensity of PKH67-stained cells. Because fluorescence intensity decreases as cells proliferate, cell generation can be mathematically determined by fluorescence intensity using Modfit LT software. Data are presented as the mean ($\pm$ SEM) percentage of T-cells proliferating beyond the parent population, as determined by Modfit LT software, and as a proliferation index (PI), also determined by Modfit LT software. The PI is the sum of cells in all generations divided by the theoretical number of parent T-cells present at the beginning of the incubation period. Thus, PI is a measure of the increase in cell number in culture over the course of the incubation period.

Activation marker (i.e., CD25, CD44, CD62L) data were acquired using CellQuest (Becton Dickinson) software and were analyzed using FlowJo (Tree Star Inc., San Carlos, CA) software. Data are presented as geometric mean fluorescence intensities (mean $\pm$ SEM) of gated cells (i.e., CD4⁺, CD8⁺, and $\gamma\delta$ TCR⁺ cells) within the PBMC population.

Statistical Analyses

Data were analyzed as a completely randomized design using Statview software (version 5.0, SAS Inst., Inc., Cary, NC) using the GLM for all analysis. Animal served as the experimental unit in the analysis of all data. Growth was analyzed as a split-plot with repeated measures ANOVA. The model included the fixed effects of dietary treatments (standard vs. intensified diet), time (weeks on experiment), and the treatment $\times$ time interaction, and calf was included in the model as the

random effect. Fisher's protected-LSD test was applied when significant effects ($P < 0.05$) were detected. Proliferative responses (i.e., percentage proliferating and PI) and activation marker expression (i.e., mean fluorescence intensity of activation antigens on specific T-cell subsets) were analyzed as a split-plot with factorial ANOVA. Model included fixed effects of dietary treatments (standard vs. intensified diet), age (calf vs. steer), and stimulation (nonstimulated vs. stimulated cultures). Significance was declared at $P < 0.05$ and trends from $P > 0.05$ to < 0.10 . Fisher's protected-LSD test was applied when trends or significant ($P < 0.05$) effects were detected.

RESULTS

Growth Performance of Standard-Diet and Intensified-Diet Calves

Although mean BW of standard-diet calves and intensified-diet calves were not different ($P > 0.05$) at the beginning of the trial, weights of calves on the intensified diet were greater ($P < 0.01$) than weights of calves on the standard diet from wk 2 through 7 of the experimental period. The average daily weight gain of intensified-diet calves (0.66 kg/d) exceeded ($P < 0.0001$) the daily gain of standard-diet calves (0.27 kg/d) during the experimental period.

Proliferative Responses of T-cells Subsets from Steers and 1-Wk-Old Calves

Proliferative responses of PWM stimulated lymphocytes from steers and calves at 1-wk of age, are shown in Figure 1. Percentages of $CD4^+$ cells proliferating in response to PWM did not differ between the two age groups (Figure 1A), although the PI of $CD4^+$ cells from steers was greater than the PI of calf $CD4^+$ cells (Figure 1B). Proliferative responses of $CD8^+$ cells from steers and 1-wk-old calves were not different; however, responses (i.e., percentage proliferating and PI) of calf $\gamma\delta TCR^+$ T-cells were greater than responses of steer $\gamma\delta TCR^+$ cells (Figure 1A). Responses of mitogen stimulated PBMC from calves and steers were similar to the corresponding responses of their $CD4^+$ T-cell populations (data not shown).

Proliferation of T-Cell Subsets from Steers and 8-Wk-Old Calves

Proliferative responses of mitogen-stimulated lymphocytes from 8-wk-old calves (i.e., 7 wk after initiation of dietary treatments) are shown in Figure 2. The hypo-responsiveness of T-cell subsets from 1-wk-old calves to mitogenic stimulation (Figure 1) was not observed

Figure 1. Pokeweed mitogen-induced proliferative responses of peripheral blood $CD4^+$, $CD8^+$, and $\gamma\delta TCR^+$ T-lymphocytes from 1-wk-old calves ($n = 4$) and steers (5 to 6 months of age, $n = 4$). The T-cell responses are presented as percentages proliferating (A) and as proliferation indices (B). Mononuclear cell cultures were incubated for 6 d. Values represent proliferative responses (mean $\pm$ SEM) of PWM stimulated cultures minus responses of nonstimulated cultures. Asterisk (*) indicates that proliferative responses in calves differed from steers ($P < 0.05$).

Figure 2. Pokeweed mitogen-induced proliferative responses of peripheral blood CD4⁺, CD8⁺, and γδTCR⁺ lymphocytes from standard- (n = 4) and intensified-diet (n = 4) calves, and steers (n = 4) at wk 7 of the trial. Calves were 8 wk old at the time of blood collection. The T-cell responses are presented as percentages proliferating (A) and as proliferation indices (B). Mononuclear cell cultures were incubated for 6 d. Values represent proliferative responses (mean ± SEM) of PWM stimulated cultures minus responses of nonstimulated cultures. ^{a,b}For a specific T-cell subset, values with different superscripts differ ($P < 0.05$).

in cultures consisting of PBMC from 8-wk-old calves (Figure 2). Percentages of CD4⁺, CD8⁺, and γδTCR⁺ cells proliferating in response to PWM from standard-diet calves and steers were not different (Figure 2A). Additionally, PI of CD4⁺, CD8⁺, and γδTCR⁺ cells from standard-diet calves and steers were not different (Figure 2B). In PWM-stimulated cultures, percentages of proliferating CD8⁺ and γδTCR⁺ cells were greater in PBMC populations from steers than in PBMC populations from intensified-diet calves. The PI of PWM-stimulated steer CD4⁺ and CD8⁺ cells were higher than the PI of CD4⁺ and CD8⁺ cells from intensified-diet calves. The PI of stimulated γδTCR⁺ cells was not affected ($P > 0.05$) by age or by diet (Figure 2B). The PWM-induced proliferative responses of complete PBMC populations were similar to responses of individual T-cell subsets (data not shown).

Dietary treatments also affected mitogen-induced proliferative responses of calf T-cell subsets. In PWM-stimulated cultures, percentages of proliferating CD4⁺, CD8⁺, and γδTCR⁺ cells were greater in cultures of PBMC from standard-diet calves than in cultures of PBMC from intensified-diet calves (Figure 2A). Proliferation indices of stimulated CD4⁺, CD8⁺, and γδTCR⁺ cells from standard-diet and intensified-diet calves; however, were not different (Figure 2B).

Expression of Activation Antigens by T-Cell Subsets from Steers and 1-Wk-Old Calves

Expression of CD25, CD44, and CD62L by nonstimulated and PWM-stimulated T-cell subsets from calves less than 1 wk of age and from steers is shown in Tables 2, 3, and 4. Expression of CD25 by CD4⁺, CD8⁺, and γδTCR⁺ T-cells from steers was higher in PWM-stimulated cultures than in nonstimulated cultures (Table 2). The only exception was CD25 expression on steer γδTCR⁺ T-cells, which was of similar magnitude in stimulated and nonstimulated, 6-d cultures. Expression of CD25 on CD4⁺ cells from 1-wk-old calves differed from expression by CD4⁺ cells from steers. Unlike CD4⁺ cells from steers, stimulated cells from calves tended to demonstrate increased CD25 expression in 6-d but not 3-d cultures. In mitogen-stimulated 6-d cultures, CD25 expression on calf CD4⁺ cells tended to be lower than steer CD4⁺ cells. Expression of CD25 on calf and steer CD8⁺ and γδTCR⁺ T-cells was similar in nonstimulated and stimulated cultures. The only exception was the higher CD25 expression by calf CD8⁺ cells in nonstimulated, 6-d cultures.

Expression of CD44 on CD4⁺, CD8⁺, and γδTCR⁺ T-cells from steers was higher in PWM-stimulated than in nonstimulated cultures (Table 3). The lower CD44

Table 2. Expression (mean fluorescence intensity) of CD25 on T-cell subsets from steers and 1-wk-old calves.^{1,2}

Cell Phenotype	3-d cultures			6-d cultures		
	NS	PWM	Mitogen effect ³ $P > F_i$	NS	PWM	Mitogen effect ³ $P > F_i$
CD4 ⁺						
Steers	34 ± 5	120 ± 4	<0.0001	74 ± 10	507 ± 27	<0.0001
Calves	51 ± 28	74 ± 24	0.54	104 ± 54	331 ± 80†	0.06
CD8 ⁺						
Steers	15 ± 1	169 ± 20	<0.001	24 ± 4	208 ± 30	<0.001
Calves	16 ± 3	211 ± 56	<0.05	70 ± 15*	221 ± 53	<0.05
γδTCR ⁺						
Steers	34 ± 3	119 ± 17	<0.01	33 ± 6	37 ± 3	0.55
Calves	33 ± 5	89 ± 5	<0.001	28 ± 4	36 ± 7	0.36

¹Cells from calves (n = 4) and steers (n = 4) were nonstimulated (NS) and stimulated with pokeweed mitogen (PWM) for 3 and 6 d.

²Values represent mean fluorescence intensity (mean ± SE).

³P-values.

*Values for a specific T-cell subset within a column differ, $P < 0.05$.

†Values for a specific T-cell subset within a column tend to differ, $P < 0.10$.

expression by γδTCR⁺ T-cells in stimulated relative to nonstimulated 6-d cultures was the only exception. Expression of CD44 by T-cell subsets from calves differed markedly from CD44 expression by T-cells from steers. Although CD44 expression by T-cell subsets from steers increased after PWM stimulation, CD44 expression by calf CD4⁺, CD8⁺, and γδTCR⁺ T-cells did not increase after stimulation. The only exception was that CD44 expression on calf CD8⁺ cells tended to be higher in PWM-stimulated than in nonstimulated 3-d cultures. Surprisingly, CD44 expression by calf γδTCR⁺ T-cells was lower in PWM-stimulated than in nonstimulated,

6-d cultures. Age-related differences of CD44 mean values also were observed. In nonstimulated 6-d cultures, CD44 expression by calf CD8⁺ cells was higher than expression by steer CD8⁺ cells. Expression of CD44 by calf γδTCR⁺ cells was lower than by steer γδTCR⁺ cells in PWM-stimulated 3-d cultures.

Expression of CD62L by γδTCR⁺ T-cells from steers increased in 3-d, mitogen-stimulated cultures (Table 4). In 6-d cultures, CD62L expression by CD4⁺, CD8⁺, and γδTCR⁺ T-cells was lower in PWM-stimulated than in nonstimulated cultures. In 3- and 6-d cultures of calf PBMC, CD62L expression by CD4⁺, CD8⁺, and γδTCR⁺

Table 3. Expression (mean fluorescence intensity) of CD44 on T-cell subsets from steers and 1-wk-old calves.^{1,2}

Cell Phenotype	3-d cultures			6-d cultures		
	NS	PWM	Mitogen effect ³ $P > F_i$	NS	PWM	Mitogen effect ³ $P > F_i$
CD4 ⁺						
Steers	125 ± 12	264 ± 19	<0.001	352 ± 32	846 ± 87	<0.01
Calves	121 ± 43	208 ± 91	0.42	514 ± 134	840 ± 286	0.34
CD8 ⁺						
Steers	68 ± 3	388 ± 18	<0.0001	238 ± 22	460 ± 45	<0.01
Calves	83 ± 10	340 ± 113	0.06	457 ± 45*	568 ± 181	0.57
γδTCR ⁺						
Steers	163 ± 2	252 ± 15	<0.001	287 ± 13	102 ± 11	<0.001
Calves	144 ± 15	158 ± 28*	0.69	304 ± 40	103 ± 15	<0.01

¹Cells from calves (n = 4) and steers (n = 4) were nonstimulated (NS) and stimulated with pokeweed mitogen (PWM) for 3 and 6 d.

²Values represent mean fluorescence intensity (mean ± SE).

³P-values.

*Values for a specific T-cell subset within a column differ, $P < 0.05$.

Table 4. Expression (mean fluorescence intensity) of CD62L on T-cells subsets from steers and 1-wk-old calves.^{1,2}

Cell Phenotype	3-d cultures			6-d cultures		
	NS	PWM	Mitogen effect ³ $P > F_i$	NS	PWM	Mitogen effect ³ $P > F_i$
CD4 ⁺						
Steers	41 ± 3	34 ± 1	0.099	92 ± 19	39 ± 2	<0.05
Calves	47 ± 16	49 ± 14	0.92	124 ± 76	72 ± 22	0.53
CD8 ⁺						
Steers	83 ± 12	96 ± 16	0.13	165 ± 24	91 ± 17	<0.05
Calves	81 ± 18	161 ± 42	0.54	172 ± 68	101 ± 29	0.38
γδTCR ⁺						
Steers	153 ± 34	294 ± 40	<0.05	193 ± 17	99 ± 14	<0.01
Calves	233 ± 37	268 ± 57	0.63	308 ± 86	121 ± 44	0.10

¹Cells from calves (n = 4) and steers (n = 4) were nonstimulated (NS) or stimulated with pokeweed mitogen (PWM) for 3 and 6 d.

²Values represent mean fluorescence intensity (mean ± SE).

³P-values.

cells was similar in nonstimulated and PWM-stimulated cultures.

Activation Antigen Expression by T-Cell Subsets from Steers and 8-Wk-Old Calves

Expression of CD25, CD44, and CD62L by nonstimulated and PWM-stimulated T-cell subsets from steers and 8-wk-old calves is shown in Tables 5, 6, and 7. As observed at the beginning of the trial (Table 2), CD25 expression by steer CD4⁺, CD8⁺, and γδTCR⁺ cells was higher in PWM-stimulated than nonstimulated 3- and 6-d cultures (Table 5). For 8-wk-old calves, CD25 expression by CD4⁺, CD8⁺, and γδTCR⁺ cells was higher in PWM-stimulated than nonstimulated 3- and 6-d cultures (Table 5). The delayed expression of CD25 by stimulated CD4⁺ cells from 1-wk-old calves (Table 2) was not observed in PBMC cultures from standard- and intensified-diet, 8-wk-old calves (Table 5).

Expression of CD25 was affected by the plane of nutrition. In PWM-stimulated 3-d cultures, CD25 expression by CD4⁺ and CD8⁺ cells from standard-diet calves was higher than the CD25 expression by the same T-cell subsets from steers and intensified-diet calves. In PWM-stimulated 6-d cultures, CD25 expression by γδTCR⁺ T-cells from intensified-diet calves tended to be lower ($P < 0.1$) than the CD25 expression by γδTCR⁺ cells from steers.

As observed at the beginning of the trial (Table 3), expression of CD44 by CD4⁺, CD8⁺, and γδTCR⁺ T-cells from steers was higher in PWM stimulated than in nonstimulated cultures (Table 6). The exception was the lack of an effect of PWM on CD44 expression by CD4⁺ and γδTCR⁺ cells in 3-d cultures. Mitogen-induced

expression of CD44 by CD4⁺, CD8⁺, and γδTCR⁺ cells from 8-wk-old standard-diet calves was similar to expression by stimulated, steer CD4⁺, CD8⁺, and γδTCR⁺ subsets.

Expression of CD44 was affected by dietary treatment with responses of stimulated T-cell subsets from intensified-diet calves frequently being different or tending to be different from responses of cells from standard-fed calves or steers (Table 6). Unlike CD4⁺ cells from standard-diet calves, stimulated cells from intensified-fed calves did not demonstrate increased CD44 expression in 3-d cultures. In PWM-stimulated 6-d cultures, CD44 expression by CD4⁺ cells from intensified-fed calves tended to be lower than expression by CD4⁺ cells from standard-fed calves. Expression of CD44 on CD8⁺ cells from intensified-fed calves in mitogen-stimulated 3-d cultures was lower than expression by CD8⁺ cells from standard-fed calves. When compared with CD44 expression on γδTCR⁺ and CD4⁺ cells from steers, expression on the same T-cell subsets from intensified-fed calves was lower or tended to be lower in mitogen-stimulated 6-d cultures.

Effects of animal maturity and dietary treatment on CD62L expression by T-cell subsets are presented in Table 7. As observed at the beginning of the trial (Table 4), CD62L expression on CD4⁺, CD8⁺, and γδTCR⁺ T-cells from steers was lower in PWM-stimulated than in nonstimulated cultures (Table 7). The only exception was the lack of an effect of stimulation on CD62L expression by steer γδTCR⁺ cells in 6-d cultures.

Several age-related differences in CD62L expression by cells from 8-wk-old calves and steers were evident (Table 7). Unlike steers, CD62L expression by resting and PWM-stimulated γδTCR⁺ cells from standard-diet

Table 5. Expression (mean fluorescence intensity) of CD25 on T-cell subsets from steers and calves 7-wk after initiation of dietary treatments.^{1,2}

Cell Phenotype	3-d cultures			6-d cultures		
	NS	PWM	Mitogen effect ³ $P > F_i$	NS	PWM	Mitogen effect ³ $P > F_i$
CD4 ⁺						
Steers	131 ± 14	371 ± 35 ^b	<0.001	391 ± 97 ^b	1567 ± 208	<0.01
Standard-diet calves	122 ± 16	749 ± 77 ^a	<0.001	776 ± 135 ^a	1659 ± 121	<0.01
Intensified-diet calves	102 ± 19	477 ± 119 ^b	<0.05	662 ± 125 ^{ab}	1331 ± 174	<0.05
CD8 ⁺						
Steers	22 ± 2	464 ± 50 ^b	<0.0001	46 ± 7 ^{b,*}	1065 ± 130	<0.001
Standard-diet calves	33 ± 9	708 ± 26 ^a	<0.0001	86 ± 20 ^b	1194 ± 270	<0.01
Intensified-diet calves	45 ± 19	364 ± 111 ^b	<0.05	129 ± 9 ^a	829 ± 128	<0.01
γδTCR ⁺						
Steers	51 ± 10	297 ± 24	<0.0001	40 ± 4 ^b	928 ± 224 [†]	<0.01
Standard-diet calves	86 ± 44	347 ± 34	<0.01	69 ± 18 ^{ab}	627 ± 87	<0.001
Intensified-diet calves	127 ± 33	282 ± 45	<0.05	95 ± 8 ^a	496 ± 81	<0.01

^{a,b}For a specific T-cell subset within a column, values with differing superscript differ, $P < 0.05$.

¹Cells were nonstimulated (NS) or stimulated with pokeweed mitogen (PWM) for 3 and 6 d. Standard-diet calves ($n = 4$) were fed a 20% CP, 20% fat milk replacer fed at 0.45 kg/d and intensified-diet calves ($n = 4$) were fed a 28% CP, 20% fat milk replacer fed at 1.14 kg/d. Steers ($n = 4$) were fed a typical diet to allow for moderate growth.

²Values represent mean fluorescence intensity (mean ± SE).

³ P -values.

*CD25 mean fluorescence intensity on nonstimulated CD8⁺ cells from steers tends to differ from standard-diet calves, $P < 0.1$.

†CD25 mean fluorescence intensity on PWM-stimulated γδTCR⁺ from steers tends to differ from intensified-diet calves, $P < 0.1$.

calves was not different in 3-d cultures. In PWM-stimulated 3-d cultures, expression of CD62L was higher on CD4⁺ cells from standard-fed calves than from steers and tended to be higher on CD8⁺ cells from standard-diet calves compared with steers. The CD8⁺ cells from standard-diet calves had higher CD62L expression in nonstimulated 6-d cultures compared with CD8⁺ cells from steers. Expression of CD62L by stimulated γδTCR⁺ cells from standard-fed calves and steers tended to be different in 3- and 6-d cultures. In 6-d cultures, CD62L expression by nonstimulated γδTCR⁺ cells from standard-diet calves tended to be higher when compared with the same T-cell subset from steers.

Dietary treatments also affected CD62L expression. In PWM-stimulated 3-d cultures, CD62L expression by CD4⁺ cells from intensified-diet calves was lower than the expression by cells from standard-diet calves. In nonstimulated 3- and 6-d cultures, CD62L expression by CD8⁺ cells from intensified-diet calves was lower than the same T-cell subset from standard-fed calves. Similarly, γδTCR⁺ cells from intensified-diet calves had lower CD62L expression compared with the same T-cell subset standard-diet calves in nonstimulated 6-d cultures. Unlike T-cells from standard-fed calves, PWM stimulated CD8⁺ and γδTCR⁺ cells from intensified-fed

calves did not demonstrate decreased CD62L expression in 3- or 6-d cultures, respectively.

DISCUSSION

This study describes, for the first time, the expression of activation antigens (i.e., CD25, CD44, and CD62L) on nonstimulated and mitogen-stimulated CD4⁺, CD8⁺, and γδTCR⁺ cells from neonatal calves. This study also compared the expression of these antigens on T-cell subsets from calves and steers, and examined the influence of neonatal nutrition on their expression.

Although age-related changes in circulating mononuclear leukocyte population composition have been described in young and adult dairy cattle (Hein and Mackay, 1991; Ayoub and Yang, 1996; Nonnecke et al., 2003), functional changes in these populations are poorly described. Proliferation and expression of activation markers in response to polyclonal stimulation (i.e., PWM) were analyzed before initiation of dietary treatments in order to compare responses of adult and neonatal T-cell subsets to activation. The PWM-elicited responses of lymphocytes from steers in the current experiment were similar to antigen-specific responses previously reported in adult cattle (Endsley et al., 2002; Sandbulte and Roth, 2002; Waters et al., 2003b). Neo-

Table 6. Expression (mean fluorescence intensity) of CD44 on T-cell subsets from steers and calves 7 wk after initiation of dietary treatments.^{1,2}

Cell Phenotype	3-d cultures			6-d cultures		
	NS	PWM	Mitogen effect ³ $P > F_i$	NS	PWM	Mitogen effect ³ $P > F_i$
CD4⁺						
Steers	1244 ± 126 ^a	1241 ± 143	0.99	1033 ± 165 ^b	3060 ± 88	<0.0001
Standard-diet calves	959 ± 99 ^{ab}	1661 ± 160	<0.01	2010 ± 207 ^a	3071 ± 196	<0.01
Intensified-diet calves	634 ± 182 ^b	1278 ± 356	0.16	1824 ± 193 ^a	2665 ± 108 [‡]	<0.01
CD8⁺						
Steers	500 ± 62	1365 ± 181 ^{ab}	<0.01	627 ± 81 [†]	2717 ± 444	<0.01
Standard-diet calves	535 ± 105	1643 ± 78 ^a	<0.0001	925 ± 240	2757 ± 246	<0.01
Intensified-diet calves	511 ± 130	1088 ± 166 ^b	<0.05	1040 ± 89	1983 ± 140	<0.01
γδTCR⁺						
Steers	677 ± 79 [*]	636 ± 31	0.65	402 ± 28 [*]	2310 ± 434 ^a	<0.01
Standard-diet calves	446 ± 33	628 ± 9	<0.01	672 ± 118	1617 ± 71 ^{ab}	<0.001
Intensified-diet calves	614 ± 123	646 ± 28	0.81	589 ± 90	1315 ± 178 ^b	<0.05

^{a,b}For a specific T-cell subset within a column, values with differing superscripts differ, $P < 0.05$.

¹Cells were nonstimulated (NS) or stimulated with pokeweed mitogen (PWM) for 3 and 6 d. Standard-diet calves ($n = 4$) were fed a 20% CP, 20% fat milk replacer fed at 0.45 kg/d and intensified-diet calves ($n = 4$) were fed a 28% CP, 20% fat milk replacer fed at 1.14 kg/d. Steers ($n = 4$) were fed a typical diet to allow for moderate growth.

²Values represent mean fluorescence intensity (mean ± SE).

³Values represent P -values.

*CD44 mean fluorescence intensity on nonstimulated γδTCR⁺ cells from steers tends to differ from standard-diet calves, $P < 0.1$.

†CD44 mean fluorescence intensity on nonstimulated CD8⁺ cells from steers tends to differ from intensified-diet calves, $P < 0.1$.

‡CD44 mean fluorescence intensity on PWM-stimulated CD4⁺ cells from intensified-diet calves tends to differ from steers and standard-diet calves, $P < 0.1$.

nates were generally hyporesponsive to PWM stimulation when compared with older cattle. Studies in humans indicate CD25 is expressed on more circulating T-cells in older (7–14 d of age) than in younger (1–7 d of age) infants and that CD62L expression is lower on cord blood B-cells than on adult B-cells (Tasker and Marshall-Clarke, 2003; Hodge et al., 2004). Together, these results suggest that within the first week of life, neonatal lymphocytes may exhibit defects in activation and homing mechanisms. Interestingly, nonstimulated CD8⁺ cells from calves had a higher CD25 and CD44 expression compared with adults after 6 d of culture, suggesting that this cell phenotype may be activated nonspecifically in the young calf. Decreased or delayed expression of CD25 on CD4⁺ and γδTCR⁺ from neonates could result in reduced differentiation and proliferation of these subsets. Decreased CD44 expression on activated CD4⁺ cells could decrease extravasation of this subset to sites of inflammation. A failure to decrease CD62L expression on activated CD4⁺, CD8⁺, and γδTCR⁺ cells may promote clearance of these subsets from the circulation by lymph nodes decreasing their trafficking to sites of activated endothelium.

The age-related differences observed when calves were 1 wk old were not observed in 8-wk-old calves. In

particular, PWM-induced CD25 and CD44 expression by CD4⁺, CD8⁺, and γδTCR⁺ cells from standard-diet calves was similar to or higher when compared with the level of expression by T-cells from older cattle. The PWM-induced decrease in CD62L expression by CD4⁺, CD8⁺, and γδTCR⁺ cells from 8-wk-old standard-fed calves was more characteristic of responses of adult cattle than the 1-wk-old calf.

The neonatal calf has a heightened susceptibility to a variety of infectious diseases. The developmental immaturity of the neonate's immune system is thought to contribute to its increased susceptibility. New strategies are needed to enhance the functional capacity of the calf's immune system. One possible strategy is to optimize the calf's protein and energy status through dietary manipulation. In the current experiment, we analyzed the effects of increased dietary protein and energy on mitogen-induced CD25, CD44, and CD62L expression by T-cell subsets from neonatal calves. Increased dietary protein and energy decreased PWM induced proliferative responses of CD4⁺, CD8⁺, and γδTCR⁺ cells when compared with responses of the same cell populations from standard-fed calves and steers. In addition, the magnitude of PWM induced CD25 expression by CD4⁺ and CD8⁺ cells from intensi-

Table 7. Expression (mean fluorescence intensity) of CD62L on T-cell subsets from steers and calves 7 wk after initiation of dietary treatments.^{1,2}

Cell Phenotype	3-d cultures			6-d cultures		
	NS	PWM	Mitogen effect ³ $P > F_i$	NS	PWM	Mitogen effect ³ $P > F_i$
CD4 ⁺						
Steers	269 ± 44 ^a	55 ± 7 ^b	<0.01	869 ± 151	134 ± 23	<0.01
Standard-diet calves	210 ± 47 ^{ab}	108 ± 10 ^a	0.08	1376 ± 277	123 ± 17	<0.01
Intensified-diet calves	114 ± 16 ^b	48 ± 8 ^b	<0.05	836 ± 186	130 ± 55	<0.05
CD8 ⁺						
Steers	529 ± 71 ^a	130 ± 12	<0.01	920 ± 173 ^b	305 ± 26	<0.05
Standard-diet calves	449 ± 71 ^a	234 ± 36 [*]	<0.05	2047 ± 544 ^a	258 ± 74	<0.05
Intensified-diet calves	222 ± 50 ^b	136 ± 45	0.25	447 ± 130 ^b	159 ± 16 [‡]	0.07
γδTCR ⁺						
Steers	623 ± 59	324 ± 42	<0.01	566 ± 160 ^{ab}	339 ± 18 ^a	0.21
Standard-diet calves	561 ± 84	616 ± 108 [†]	0.70	1376 ± 404 ^{a,†}	272 ± 22 ^{ab,†}	<0.05
Intensified-diet calves	450 ± 82	483 ± 110	0.82	277 ± 80 ^b	222 ± 33 ^b	0.55

^{a,b}For a specific T-cell subset within a column, values with differing superscript differ $P < 0.05$.

¹Cells were nonstimulated (NS) or stimulated with pokeweed mitogen (PWM) for 3 and 6 d. Standard-diet calves ($n = 4$) were fed a 20% CP, 20% fat milk replacer fed at 0.45 kg/d and intensified-diet calves ($n = 4$) were fed a 28% CP, 20% fat milk replacer fed at 1.14 kg/d. Steers ($n = 4$) were fed a typical diet to allow for moderate growth.

²Values represent mean fluorescence intensity (mean ± SE).

³Values represent P -values.

^{*}CD62L mean fluorescence intensity on PWM-stimulated CD8⁺ cells from standard-diet calves had a tendency to be higher than steers and intensified-diet calves, $P < 0.1$.

[†]CD62L mean fluorescence intensity on γδTCR⁺ T-cells from standard-diet calves tends to differ from steers, $P < 0.1$.

[‡]CD62L mean fluorescence intensity on CD8⁺ cells from intensified-diet calves tends to differ from steers, $P < 0.1$.

fied calves was lower than in CD4⁺ and CD8⁺ populations from standard-fed calves. These data suggest that alterations in dietary protein and energy influence markedly CD25 expression by T-cell subsets. Although this does not prove a causal relationship, these data suggest that a possible explanation for the differences in proliferative responses may be due to dietary effects on CD25 expression. Reduced expression of the IL-2 receptor on T-cells resulting from increased dietary protein and energy has been described previously in rodents (Iwai and Fernandes, 1989; Peck et al., 2000).

Increased dietary fat has been linked to immunosuppression in rodents characterized by reduced lymphocyte responses to mitogenic stimulation and adhesiveness to endothelial cells (Erickson et al., 1980; Morrow et al., 1985; Sanderson and Calder, 1998). Immunosuppression induced by a high-fat diet may be an effect of certain adipokines, such as leptin and transforming growth factor- β , produced by adipocytes. Leptin links nutritional status with immune functions, acting as a signal to the body of energy stores (reviewed by Otero et al., 2005). Although exogenous leptin has been shown to reverse starvation-induced immunosuppression (Lord et al., 1998), leptin inhibits proliferative responses of and suppresses IL-2 production by PBMC in vitro (Lord

et al., 2002). Interestingly, leptin enhances naïve (CD4⁺ CD45RA⁺ T-cells) T-cell proliferation but inhibits memory (CD4⁺ CD45RO⁺ T-cells) T-cell proliferation (Lord et al., 2002), which may have consequences on vaccine recall responses. In addition, leptin enhances interferon- γ -induced expression of nitric oxide synthase in macrophages (Raso et al., 2002). We have reported previously that high plane of nutrition results in increased in vitro nitric oxide production by PBMC (Nonnecke et al., 2003; Foote et al., 2005). Obese mice also have elevated expression of transforming growth factor- β (Samad et al., 1997). Although transforming growth factor- β is known to play an important role in B cell isotype switching and immune regulation, it has been shown to decrease IL-2 production and proliferative responses of CD4⁺ and CD8⁺ T-cells (Wolfrum et al., 2004; McKarns and Schwartz, 2005). Plasma leptin concentrations or body fat composition were not measured in the current experiment, however, an enhanced plane of nutrition increases plasma leptin concentrations and percent body fat composition in preruminant calves and lambs (Block et al., 2003; Ehrhardt et al., 2003).

In the current experiment, feeding an intensified diet to calves decreased CD44 expression on PWM-stimulated CD8⁺ cells compared with standard-diet calves,

and delayed the increase of CD44 expression on PWM-stimulated CD4⁺ and $\gamma\delta$ TCR⁺ cells PWM compared with standard-diet calves. Diet also affected CD62L expression. Intensified-diet calves had lower CD62L expression on CD8⁺ and $\gamma\delta$ TCR⁺ cells in nonstimulated cultures compared with standard-diet calves, suggesting that these two populations may not be cleared efficiently from the circulation when compared with the same populations from standard-diet calves. Decreased CD62L expression on certain lymphocyte subsets from intensified-diet calves may explain the previous observation that intensified-diet calves have a higher percentage of circulating CD4⁺ and $\gamma\delta$ TCR⁺ T-cells compared with standard-diet calves (Foote et al., 2005). However, CD62L expression on mitogen-stimulated CD8⁺ cells from intensified-diet calves did not decrease until 6 d of culture, and CD62L on $\gamma\delta$ TCR⁺ did not decrease at either time-point. These data suggest that resting (i.e., nonstimulated) lymphocytes may not be cleared properly from circulation, and supports previous data (Iwai and Fernandes, 1989; Fernandes et al., 1997; Sanderson and Calder, 1998) indicating increased dietary energy affects negatively responses of PBMC to mitogenic stimulation. In addition, our observations that proliferative responses and expression of CD25, CD44, and CD62L by T-cells from 8-wk-old, standard-diet calves were similar to those of the older cattle suggests that feeding a standard milk replacer (i.e., 20% CP, 20% fat, 0.45 kg/d) provides sufficient nutrition to support normal immune cell function. The relationship between neonatal nutrition and the adaptive immune response requires further investigation.

Lymphocytes from 1-wk-old calves exhibited reduced proliferative responses, reduced expression of CD25 and CD44, and increased expression of CD62L following in vitro stimulation with PWM. In addition, neonatal nutrition influenced mitogen-induced proliferative responses and expression of the activation antigens, CD25, CD44, and CD62L. In conclusion, these results suggest that animal maturity and neonatal nutrition influence functional activities of T lymphocyte subsets essential in the development of CMI responses with possible consequences to the calf's susceptibility to infectious disease. Additional research is necessary to determine whether these changes in immune cell function are associated with an increased susceptibility of the calf to infection.

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