# Shifts in Bovine CD4+ Subpopulations Increase T-helper-2 Compared with T-helper-1 Effector Cells During the Postpartum Period

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## ABSTRACT

This study determined the cytokine profile of $CD4^+$ T-helper cells to elucidate the specific $CD4^+$ T-helper phenotype during the postpartum period. Peripheral blood mononuclear cells were isolated from cows during periods of increased susceptibility (3 d postpartum, n = 7) and decreased susceptibility (mid- to late lactation, n = 6) to mastitis. Isolated mononuclear cells were magnetically separated into $CD4^+$-enriched or $CD4^+$-depleted populations using specific bovine monoclonal antibodies and were confirmed to be enriched or depleted by flow cytometric analysis. T-helper-1 and T-helper-2 subpopulations were distinguished by cytokine profiles, at both the molecular and protein level, by competitive quantitative reverse transcriptase-polymerase chain reaction and specific bioassays, respectively. The $CD4^+$-enriched cultures isolated postpartum had enhanced interleukin-4 and interleukin-10 mRNA transcript expression; cultures isolated during the mid- to late lactating period had enhanced interleukin-2 mRNA transcripts. Depletion of $CD4^+$ lymphocytes decreased, and enrichment of $CD4^+$ lymphocytes increased interferon-$\gamma$ transcripts in cultures isolated from mid- to late lactation cows. Interferon-$\gamma$ and interleukin-2 bioassays revealed that cytokine secretion paralleled mRNA transcript levels. These data suggest that $CD4^+$ lymphocytes act predominantly as T-helper-2 compared with T-helper-1 within 3 d after calving. Alterations in the T-helper-1 and T-helper-2 responses, and therefore the repertoire of cytokines produced, may be an underlying reason for diminished host immune response during the postpartum period.
(**Key words**: T-helper lymphocytes, interleukin-2, interleukin-4, mastitis)

**Abbreviation key**: **Con A** = concanavalin A, **HBSS** = Hanks balanced salt solution, **$^3$HTdr** = $^3$H-[methyl] thymidine, **IFN** = interferon-$\gamma$, **IL** = interleukin, **MTT** = (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide, **PBMC** = peripheral blood mononuclear cells, **rcRNA** = recombinant RNA, **RT-PCR** = reverse transcriptase-polymerase chain reaction, **$T_H$** = T-helper lymphocyte.

## INTRODUCTION

Dairy cows are more susceptible to mastitis during certain times of the lactation cycle including the postpartum period (2 wk after calving). During this period, increased incidence of mastitis has been correlated with diminished immune responses (20, 40). Neutrophil and lymphocyte functions such as phagocytosis, cellular proliferation, antibody development, and cytokine production are reduced at this time (8, 11, 14, 15, 19, 39). Reduction of lymphocyte function during the postpartum period may contribute to delayed host responsiveness during the early stages of pathogenesis. However, the underlying factors causing postpartum immunosuppression are not fully understood.

Several mechanisms for immunosuppression, observed around the time of calving, have been suggested. High circulating levels of hormones including estrogen, progesterone, and glucocorticoids near the onset of parturition can suppress important lymphocyte functions (3, 29, 46). Diminished leukocyte functions postpartum also have been correlated to shifts in leukocyte trafficking patterns at this time (33). Lower percentages and total numbers of T-lymphocyte subpopulations are observed in bovine peripheral blood, mammary secretions, and mammary parenchyma during the postpartum period (23, 33, 45, 47). In addition to changes in T-lymphocyte numbers, there are changes in function of certain subpopulations as well. The $CD8^+$ suppressor lymphocytes are more predominant than are $CD8^+$ cytotoxic lymphocytes postpartum compared with the mid- to late lactating period (35). Although $CD8^+$ suppressor lymphocytes diminish the responsiveness of $CD4^+$ lymphocytes postpartum (35), it is not known

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whether shifts also occur in $CD4^+$ subpopulations during this stage of lactation.

The participation of $CD4^+$ T-helper (**$T_H$**) lymphocytes is integral for an effective immune response. T-helper lymphocytes can be separated into two distinct subpopulations, $T_H$-1 or $T_H$-2, based on the repertoire of cytokines they produce (1, 32). The $T_H$-1 $CD4^+$ lymphocytes mainly secrete interleukin (**IL**)-2 and interferon-$\gamma$ (**IFN-$\gamma$**) and promote cellular responses against intracellular pathogens and viruses. The $T_H$-2 $CD4^+$ lymphocytes mainly secrete IL-4, IL-5, and IL-10 and promote humoral immunity. Shifts in the proportion of $T_H$-1 and $T_H$-2 subpopulations, and therefore the cytokines produced, have been implemented in host susceptibility to disease (4, 6, 27). Reduced secretion of IL-2 and IFN-$\gamma$ during the postpartum period has been correlated to increased incidence of mastitis and is partially due to lower numbers and functions of lymphocytes at this time. However, it is not known whether lowered production of IL-2 and IFN-$\gamma$ is also due to a shift in $CD4^+$ populations, similar to the shift observed with $CD8^+$ lymphocytes, during the postpartum period when dairy cows are more susceptible to mastitis. We investigated whether altered IL-2 and IFN-$\gamma$ production during the postpartum period was due to shifts in $T_H$-1 to $T_H$-2 bovine CD4+ lymphocytes.

## MATERIALS AND METHODS

### Isolation of Mononuclear Cells

Peripheral blood mononuclear cells (**PBMC**) were isolated from mid- to late lactating (n = 6) and postpartum (n = 7) Holstein dairy cows. All experimental cows were free of mastitis and in third or fourth lactation. Cells isolated from postpartum cows were obtained within 3 d of calving. Mononuclear cells were isolated and purified as previously described (33). Purified cells were then washed three times in Hanks balanced salt solution (HBSS; Sigma Chemical Co., St. Louis, MO) and resuspended in phosphate buffered saline containing 2% BSA (Sigma Chemical Co.). Mononuclear cell-enriched preparations contained more than 95% mononuclear cells as determined by Wright's Giemsa staining and were more than 95% viable as assessed by trypan-blue exclusion. Total white blood cell counts were made on an automatic cell counter (Coulter Electronics Ltd., Miami, FL).

### Cell Separation Using Magnetic Beads

Enriched and depleted cultures of $CD4^+$ T-lymphocytes were obtained with magnetic beads using a two-step procedure. Aliquots of isolated PBMC were first incubated for 30 min at 4°C with 50 $\mu$l of mouse anti-bovine CD4 (CACT83B) per $10^7$ cells. The cells then were washed once with PBS + 2% BSA and incubated an additional 30 min at 4°C with 10 $\mu$g of goat-anti-mouse IgG coated magnetic beads (Miltenyi Biotec., Auburn, CA) per $10^7$ cells. The bead-cell complex then was extracted from noncomplexed cells with a magnetic field (VarioMACS Magnetic Separation Systems; Miltenyi Biotec., Auburn, CA), and the positive ($CD4^+$-enriched) and negative ($CD4^+$-depleted) fractions were collected. The purity of each fraction was determined by flow cytometric analysis. Isolation of these cultures did not activate the cells or alter their viability (data not shown) and, therefore, should not have altered cytokine expression at either the molecular or protein level.

### Flow Cytometric Analysis

To phenotypically characterize the isolated PBMC, $CD4^+$-enriched and $CD4^+$-depleted cultures, flow cytometry was performed by incubations with monoclonal antibodies specific for bovine leukocyte antigens (12). Cells were incubated with each of the following lineage specific bovine monoclonal antibodies (VMRD, Pullman, WA): CD2 ($\alpha\beta$-T lymphocytes BAQ95A at 1:100 vol/vol), CD3 (T-cell receptor MM1A at 1:100 vol/vol), CD4 (T-helper CACT83B at 1:160 vol/vol), CD8 (T-cytotoxic and suppressor at CACT80C at 1:400 vol/vol), TcR-N24 ($\gamma\delta$-T lymphocytes $\delta$-chain 1:100 vol/vol), B-B2 (B-lymphocytes BAQ44A at 1:150 vol/vol), MG (monocyte/granulocyte DH59B at 1:100 vol/vol), A2 (activated CD8 lymphocytes CACT77A at 1:100 vol/vol), A3 (activated CD4 lymphocytes CACT114A at 1:100 vol/vol), and IL-2R (IL-2 receptor, $\alpha$-chain CACT116A at 1:100 vol/vol). Cell surface markers were visualized using goat anti-mouse F(ab)2 IgG-fluorescein isothiocyanate (Becton Dickinson Immunocytometry Systems, San Jose, CA) with a previously described staining procedure (7). Mononuclear cells were gated by forward and side light scatter, and data were collected for 10,000 events. Immunofluorescence was expressed as the percentage of positive cells less controls, which accounted for nonspecific labeling because of Fc receptor binding.

### RNA Isolation

Total PBMC, enriched (CD4+), and depleted (CD4−) cultures were resuspended ($1 \times 10^7$ cells/ml) in RPMI-1640 containing 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 1% antibiotic-antimycotic solution (Sigma Chemical Co.), and 1%

TABLE 1. Primer sequences used in reverse transcriptase-polymerase chain reaction (RT-PCR).[1]

| Target gene | Primer | Annealing temperature | PCR product size |
|---|---|---|---|
| Interleukin-2 | Forward 5′-TTAACGCTACAGAATTGAAACAT-3′<br>Reverse 5′-TTAAATAAATAGAAAGCCTGATAG-3′ | 56°C | 311 bp |
| Interleukin-4 | Forward 5′-CATTGTTAGCGTCTCCTGGTA-3′<br>Reverse 5′-GCTCGTCTTGGCTTCATTC-3′ | 66°C | 403 bp |
| Interleukin-10 | Forward 5′-GCCGAGATGCGAGCACCCTGTC-3′<br>Reverse 5′-CCTTCTCCACCGCCTTGCTCTTGT-3′ | 66°C | 369 bp |
| Interferon-$\gamma$ | Forward 5′-AGCCAAATTGTCTCCTTCTACTTC-3′<br>Reverse 5′-CTGACTTCTCTTCCGCTTTCTG-3′ | 56°C | 261 bp |
| $\beta$-globulin | Forward 5′-CAACTTCATCCACGTTCACC-3′<br>Reverse 5′-GAAGAGCCAAGGACAGGTAC-3′ | NA[2] | NA |

[1]Cytokine primers were generated from the specific bovine cytokine gene. Primer sequences were chosen using a primer selection program (DNAstar). The $\beta$-globulin primers were generated from the human $\beta$-globulin gene and also used to generate recombinant RNA for internal standards. A search of Genebank sequence database was performed on each primer. Only primer sets that produced PCR products that matched the specific gene of interest were utilized in RT-PCR.

[2]Not applicable.

L-glutamine (Sigma Chemical Co.). Cultures were stimulated with concanavalin A (**Con A**; Sigma Chemical Co.) at 2.5 $\mu$g/ml for 8 and 12 h at 37°C. After incubation, each cell culture was resuspended at $5 \times 10^6$ cells/ml in TRIZOL™ Reagent (Life Technologies, Grand Island, NY), and total RNA was isolated according to manufacturer instructions.

## Quantitative Competitive Reverse Transcriptase-Polymerase Chain Reaction

Quantitative competitive reverse transcriptase-polymerase chain reaction (**RT-PCR**) was performed to compare the relative expression of IL-2, IL-4, IL-10, and IFN mRNA in PBMC, enriched (CD4+), and depleted (CD4−) cultures. Forward and reverse specific bovine primers were designed from the known cytokine sequences with DNAStar primer select software (Table 1). Internal standards, recombinant RNA (**rcRNA**), were constructed as previously described (9). Briefly, each forward rcRNA primer contained the T7 promoter, target mRNA forward primer, and spacer-gene forward primer ($\beta$-globulin) (Table 1). Each reverse primer contained the spacer-gene reverse primer, target mRNA reverse primer, and poly$(dT)_{18}$. The PCR reactions for internal standards were carried out in a final volume of 50 $\mu$l with 200 ng of human genomic DNA (Promega, Madison, WI) and containing 3 m*M* $MgCl_2$, 0.2 m*M* of each dNTP, 30 pmol each of rcRNA forward and reverse primers, and 2.5 units *Taq* polymerase (Promega). The reactions were heated to 94°C and then subjected to a 30-s annealing step at 59°C and a 45-s extension step at 72°C. Following the final cycle, an additional extension step was included at 72°C for 5 min. The products were purified (Wizard PCR Prep; Promega) and transcribed into RNA using an in vitro transcription system (Riboprobe System-T7; Promega).

Competitive RT-PCR was performed as previously described (44). The reactions were heated to 94°C for 3 min and cycled 30 times through a 20-s denaturing step at 94°C, a 30-s annealing step at either 56 or 66°C, and a 30-s extension step at 72°C. After the final cycle, an extension step was included at 72°C for 5 min. The RT-PCR products were visualized using a 2% NuSeive agarose gel (FMC BioProducts, Philadelphia, PA) stained with ethidium bromide. Relative cytokine mRNA transcript expression was measured by a densitometry system (Eagle Eye II; Stratagene, La Jolla, CA) and quantified as previously described (9, 44).

## Cytokine Production

The ability of PBMC and $CD4^+$-enriched and $CD4^+$-depleted mononuclear cell cultures to produce cytokines in vitro was examined following stimulation with Con A. Cell cultures ($2.5 \times 10^6$ cells/ml) were incubated either in the presence or absence of Con A (2.5 $\mu$g/ml). Supernatants were collected after incubation at 37°C for 8, 12, and 24 h and stored at −70°C until analyzed.

## Analysis of IL-2 Activity

The level of IL-2 activity present in cell culture supernatants was determined by measuring $^3H$-

[methyl]thymidine (**$^3$HTdr**) incorporation by IL-2-dependent bovine T-lymphocytes (26). The IL-2-dependent lymphocytes were resuspended ($1 \times 10^6$ cells/ml) in RPMI-1640 containing 10% fetal bovine serum and 0.01 m*M* 2-mercaptoethanol (Sigma Chemical Co.). The cells (100 $\mu$l) were incubated with 100 $\mu$l of supernatant collected from Con A-stimulated cultures. After a 24-h incubation, 0.4 $\mu$Ci of $^3$HTdr was added to all wells and cultured for an additional 18 h. The cells were harvested with a semiautomatic harvester (Skantron, Sterling, VA), and the amount of $^3$HTdr incorporated into cellular DNA was quantified with a liquid scintillation counter (Beckman, Columbia, MD). The amount of IL-2 activity present in the supernatants was extrapolated from a standard curve generated with serial dilutions (1:2) of recombinant human IL-2 (Boehringer Mannheim, Indianapolis, IN). In parallel, samples were cocultured with a neutralizing bovine IL-2 antibody (6H14.H8A; 1:10) produced at the Monoclonal Antibody Facility, The Pennsylvania State University.

### Analysis of IFN Activity

The IFN levels in collected supernatants were analyzed through a modification of a colorimetric antiviral assay as previously described (2, 33). Briefly, 100 $\mu$l of Maden Darby bovine kidney cells ($1 \times 10^5$ cells/ml) (CCL22; ATCC, Rockville, MD) was added to a 96-well flat-bottom plate and incubated for 24 h at 37°C, 5% $CO_2$. Serial dilutions of standard and samples were added to triplicate wells and incubated for an additional 24 h. The supernatants were removed, and 150 $\mu$l of vesicular stomatitis virus (1:1000) was added to the plate and incubated for an additional 24 h. After the incubation period, 25 $\mu$l of (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (**MTT**, 5 $\mu$g/ml) was added to the plate and incubated for 2 h; the MTT was solubilized by the addition of extraction buffer (100 $\mu$l). The concentration of live cells was determined through colorimetric analysis with a microplate reader (Bio-Rad, Melville, NY) at 595 nm. Antiviral activity was used to determine IFN concentration in culture supernatants. The amount of IFN was extrapolated from a standard curve generated with serial dilutions (1:2) of recombinant human IFN (Boehringer Mannheim).

### Statistical Analysis

Data were analyzed by least squares analyses of variance using the general linear models procedure of SAS (31). Statistical analysis included the effects of cow, stage of lactation, and cell culture type. Preplanned comparisons of least square means from the overall model were made by pairwise *t* test. Means were contrasted between the two stages of lactation. Additionally, paired comparisons with respect to stages of lactation were made between cell culture types.

## RESULTS

Phenotypic analyses of isolated PBMC, CD4$^+$-enriched, and CD4$^+$-depleted cultures are summarized in Table 2. A lower percentage of mononuclear cells that stained positive for $\alpha$-$\beta$-T-lymphocytes was observed postpartum compared with the mid- to late lactating period. Reduced percentages of CD2$^+$ ($P \leq 0.01$) and both CD4$^+$ and CD8$^+$ subpopulations ($P \leq 0.05$) with a concomitant increase in monocytes were observed at this time. Depleted cultures contained <3% CD4$^+$ lymphocytes. Similar shifts in lymphocyte trafficking were observed in CD4$^+$-depleted cultures, except no differences were seen in the percentage of CD4$^+$ lymphocytes with respect to lactation stage. Regardless of lactation stage, cultures enriched for CD4$^+$ lymphocytes contained >60% CD4$^+$ lymphocytes, and the percentage of the other leukocyte phenotypes appeared to be fairly consistent. Cell cultures also were stained with various activation markers (Table 3). The percentage of cells staining positive for the IL-2 receptor and the A2 (activated CD8$^+$ lymphocytes) marker remained constant regardless of lactation stage or cell culture. Cultures enriched for CD4$^+$ lymphocytes had a higher percentage ($P \leq 0.05$) of cells staining positive for the A3 (activated CD4$^+$ lymphocytes) marker.

Peripheral blood mononuclear cell, CD4$^+$-enriched, and CD4$^+$-depleted cultures were assessed for IL-2, IL-4, IL-10, and IFN-$\gamma$ mRNA transcript expression. Greater ($P \leq 0.05$) IL-2 mRNA transcripts were expressed in PBMC and CD4$^+$-enriched cultures isolated from mid- to late lactation cows compared with postpartum cows (Figures 1 and 2). Depletion of CD4$^+$ lymphocytes decreased ($P \leq 0.05$) the level of IL-2 transcripts in cultures isolated from mid- to late lactation cows. The CD4$^+$ enrichment of these cultures increased IL-2 transcript levels, although not significantly, over those of PBMC cultures. No differences with respect to cell culture were observed in IL-2 transcript levels during the postpartum period. Figure 2 is representative of the standard curve and sample data from the competitive RT-PCR. Similar results were observed for IFN-$\gamma$ mRNA transcript levels (Figures 3 and 4).

In contrast to IL-2 and IFN-$\gamma$, expression of IL-4 and IL-10 transcripts were higher in cell cultures

TABLE 2. Flow cytometric analysis of isolated peripheral blood mononuclear cells (PBMC), CD4+-enriched, and CD4+-depleted cultures isolated from mid- to late lactation (Mid-late) and postpartum dairy cows.

| Monoclonal antibody | Antigen distribution | Stage of lactation | PBMC[1] | | CD4+ -depleted[2] | | CD4+ -enriched[3] | |
|---|---|---|---|---|---|---|---|---|
| | | | (% staining positive)[4] | | | | | |
| | | | $\overline{X}$ | SE | $\overline{X}$ | SE | $\overline{X}$ | SE |
| CD2 | $\alpha\beta$ T lymphocytes | Mid-late | 41.4** | 1.4 | 43.4* | 2.5 | 76.9 | 8.0 |
| | | Postpartum | 22.7 | 1.5 | 12.1 | 1.9 | 71.3 | 8.3 |
| CD3 | T cell receptor | Mid-late | 43.4** | 2.5 | 31.3* | 3.8 | 78.1 | 7.3 |
| | | Postpartum | 25.7 | 1.5 | 17.6 | 3.0 | 77.9 | 9.6 |
| CD4 | T-helper cells | Mid-late | 26.7* | 0.5 | 2.5 | 0.9 | 63.1 | 6.2 |
| | | Postpartum | 14.3 | 0.6 | 1.6 | 0.2 | 65.1 | 1.1 |
| CD8 | T-cytotoxic-suppressor | Mid-late | 13.1* | 0.9 | 18.4** | 4.6 | 9.0 | 4.0 |
| | | Postpartum | 6.6 | 0.9 | 8.7 | 1.5 | 4.2 | 1.1 |
| $\gamma\delta$ | $\gamma\delta$ T lymphocytes | Mid-late | 7.9 | 1.7 | 13.0 | 4.2 | 6.5 | 2.1 |
| | | Postpartum | 5.7 | 1.3 | 7.3 | 1.7 | 3.7 | 1.1 |
| B | B lymphocytes | Mid-late | 31.8 | 4.0 | 25.4 | 4.0 | 9.1 | 4.0 |
| | | Postpartum | 24.2 | 3.6 | 22.2 | 3.6 | 16.2 | 4.7 |
| MG | Monocytes | Mid-late | 20.2 | 2.4 | 32.4 | 2.6 | 6.5 | 3.0 |
| | | Postpartum | 36.7** | 1.7 | 56.5* | 4.7 | 4.5 | 0.7 |

[1]Cultures contained all mononuclear cell phenotypes.

[2]Cultures contained <3% CD4+ lymphocytes.

[3]Cultures contained >60% CD4+ lymphocytes.

[4]Percentage of cultures staining positive. Values are least square mean (± SEM); n = 6 for mid- to late lactation cows, and n = 7 for postpartum cows.

*Stage of lactation differed ($P < 0.05$).

**Stage of lactation differed ($P < 0.01$).

isolated from postpartum cows. In PBMC cultures, greater ($P \leq 0.05$) IL-4 transcript expression was observed in cell cultures isolated from postpartum compared with mid- to late lactation cows (Figure 5). Depletion of CD4+ lymphocytes did not alter IL-4 transcript level, and CD4+ enrichment enhanced IL-4 transcript levels in cultures isolated during both stages of lactation. However, the level of IL-4 transcript expression was much greater ($P \leq 0.01$) in CD4+-enriched cultures isolated from postpartum

TABLE 3. Flow cytometric analysis utilizing activation marker for isolated peripheral blood mononuclear cells (PBMC), CD4+-enriched, and CD4+-depleted cultures isolated from mid- to late lactation (Mid-late) and postpartum dairy cows.

| Monoclonal antibody | Antigen distribution | Stage of lactation | PBMC[1] | | CD4+ -depleted[2] | | CD4+ -enriched[3] | |
|---|---|---|---|---|---|---|---|---|
| | | | (% staining positive)[4] | | | | | |
| | | | $\overline{X}$ | SE | $\overline{X}$ | SE | $\overline{X}$ | SE |
| ACT2 | Activated CD8 | Mid-late | 11.6 | 1.4 | 11.6 | 0.8 | 14.6 | 1.3 |
| | | Postpartum | 9.6 | 0.7 | 8.9 | 0.7 | 9.5 | 1.7 |
| ACT3 | Activated CD4 | Mid-late | 3.8 | 2.4 | 2.7 | 1.6 | 7.5 | 1.9 |
| | | Postpartum | 7.6 | 0.9 | 2.7 | 1.1 | 23.2* | 4.7 |
| IL-2R | $\alpha$-chain | Mid-late | 4.0 | 1.4 | 2.1 | 1.1 | 7.6 | 2.4 |
| | | Postpartum | 8.0 | 3.3 | 3.4 | 1.4 | 5.9 | 0.3 |

[1]Cultures contained all mononuclear cell phenotypes.

[2]Cultures contained <3% CD4+ lymphocytes.

[3]Cultures contained >60% CD4+ lymphocytes.

[4]Percentage of cultures staining positive. Values are least square mean (± SEM); n = 6 for mid- to late lactation cows, and n = 7 for postpartum cows.

*Stage of lactation differed ($P < 0.05$).

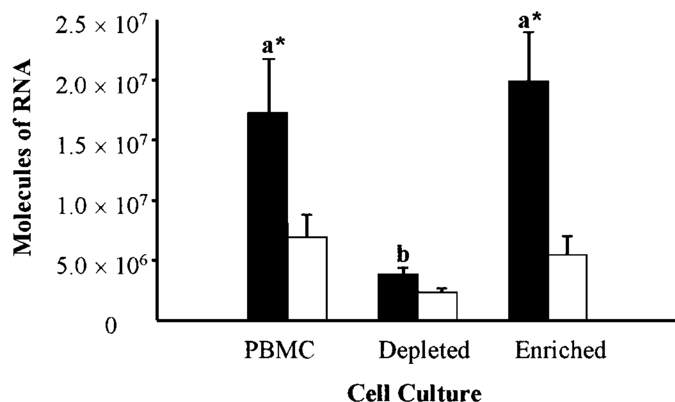


Figure 1. Level of interleukin-2 mRNA transcripts in concanavalin A-stimulated cultures isolated from mid- to late lactation (■) and postpartum (□) cows. Data are expressed as least square mean of mRNA molecules (± SEM); n = 6 for mid- to late lactation, and n = 7 for postpartum. Peripheral blood mononuclear cells (PBMC) contained all mononuclear cell phenotypes. Depleted cultures contained <3% $CD4^+$ lymphocytes, and enriched cultures contained >60% $CD4^+$ lymphocytes. [a,b,c]Means within a stage of lactation with different superscripts differ ($P < 0.05$). *Means within a cell culture category differ with respect to stage of lactation ($P < 0.05$).

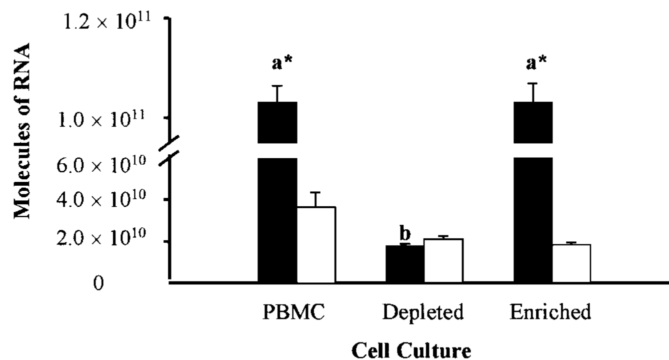


Figure 3. Level of interferon-$\gamma$ mRNA transcripts in concanavalin A-stimulated cultures isolated from mid- to late lactation (■) and postpartum (□) cows. Data are expressed as least square mean of mRNA molecules (± SEM); n = 6 for mid- to late lactation, and n = 7 for postpartum. Peripheral blood mononuclear cells (PBMC) contained all mononuclear cell phenotypes. Depleted cultures contained <3% $CD4^+$ lymphocytes and enriched cultures contained >60% $CD4^+$ lymphocytes. [a,b,c]Means within a stage of lactation with different superscripts differ ($P < 0.05$). *Means within a cell culture category differ with respect to stage of lactation ($P < 0.05$).

compared with mid- to late lactation cows. Figure 6 is representative of the standard curve and sample data from the competitive RT-PCR.

The expression of IL-10 mRNA transcripts in all cultures isolated from mid- to late lactation cows was minimal (Figure 7) compared with those isolated from postpartum cows. Both PBMC ($P \leq 0.05$) and $CD4^+$-enriched ($P \leq 0.01$) cultures from postpartum cows had greater IL-10 transcript levels compared with their counterparts from mid- to late lactation cows. Depletion of $CD4^+$ lymphocytes decreased ($P \leq 0.05$) and enrichment increased ($P \leq 0.05$) the level of IL-10 transcripts in cultures from postpartum cows. Figure 8 is representative of the standard curve and sample data from the competitive RT-PCR.

At the protein level, the expression of IL-2 and IFN paralleled the expression of cytokine transcripts. Depletion of $CD4^+$ lymphocytes decreased ($P \leq 0.05$) and enrichment enhanced the amount of IL-2 produced by cultures isolated from mid- to late lactation cows (Table 4). Greater IL-2 production was observed in PBMC ($P \leq 0.01$) and depleted ($P \leq 0.05$) cell cultures isolated from mid- to late lactation cows compared with postpartum cows. Enrichment of cultures with $CD4^+$ lymphocytes increased ($P \leq 0.05$) IL-2 production in cultures regardless of lactation stage. However, addition of an IL-2 neutralizing antibody decreased ($P \leq 0.05$) the amount of IL-2 measured in cultures from mid- to late lactation cows but not from postpartum cows. The production of IFN by the different cell cultures is summarized in Table 5.

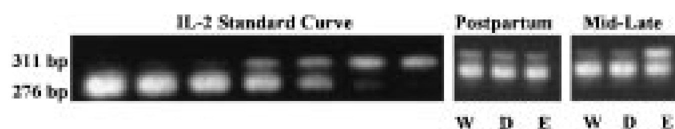


Figure 2. Quantitation of bovine interleukin-2 mRNA using competitive reverse transcriptase-polymerase chain reaction. The molecules of interleukin-2 (IL-2) mRNA in the cell cultures were estimated by determining when the volume of the rcRNA (276 bp) equalled that of the target (311 bp). Peripheral blood mononuclear cells (W) contained all mononuclear cell phenotypes. Depleted cultures (D) contained <3% $CD4^+$ lymphocytes, and enriched cultures (E) contained >60% $CD4^+$ lymphocytes. Shown is one representative sample from each lactation stage and culture type. Samples were obtained from either postpartum or mid- to late lactation (Mid-Late) cows.

Figure 4. Quantitation of bovine interferon-$\gamma$ (IFN) mRNA using competitive reverse transcriptase-polymerase chain reaction. The molecules of interferon-$\gamma$ mRNA in the cell cultures were estimated by determining when the volume of the rcRNA (324 bp) equalled that of the target (261bp). Peripheral blood mononuclear cells (W) contained all mononuclear cell phenotypes. Depleted cultures (D) contained <3% $CD4^+$ lymphocytes, and enriched cultures (E) contained >60% $CD4^+$ lymphocytes. Shown is one representative sample from each lactation stage and culture type. Samples were obtained from either postpartum or mid- to late lactation (Mid-Late) cows.

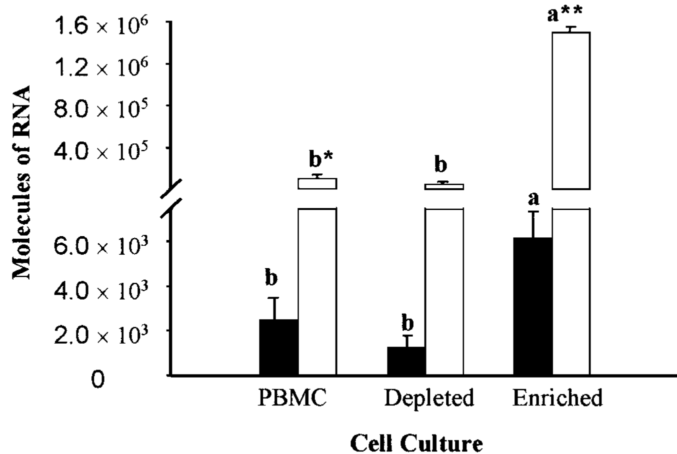


Figure 5. Level of interleukin-4 mRNA transcripts in concanavalin A-stimulated cultures isolated from mid- to late lactation (■) and postpartum (□) cows. Data are expressed as least square mean of mRNA molecules (± SEM); n = 6 for mid- to late lactation, and n = 7 for postpartum. Peripheral blood mononuclear cells (PBMC) contained all mononuclear cell phenotypes. Depleted cultures contained <3% $CD4^+$ lymphocytes, and enriched cultures contained >60% $CD4^+$ lymphocytes. [a,b,c]Means within a stage of lactation with different superscripts differ ($P < 0.05$). *Means within a cell culture category differ with respect to stage of lactation ($P < 0.05$). **Means within a cell culture category differ with respect to stage of lactation ($P < 0.01$).

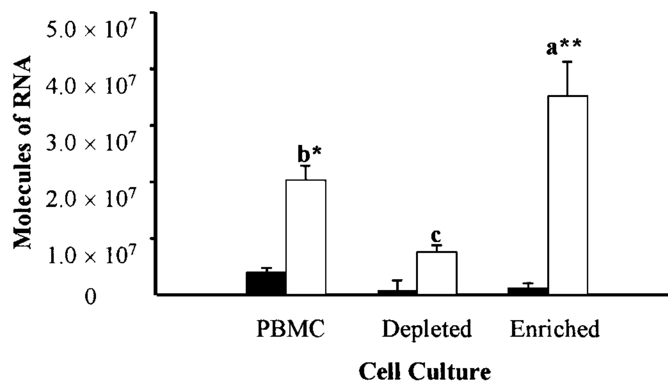


Figure 7. Level of interleukin-10 mRNA transcripts in concanavalin A-stimulated cultures isolated from mid- to late lactation (■) and postpartum (□) cows. Data are expressed as least square mean of mRNA molecules (± SEM); n = 6 for mid- to late lactation, and n = 7 for postpartum cows. Peripheral blood mononuclear cells (PBMC) contained all mononuclear cell phenotypes. Depleted cultures contained <3% $CD4^+$ lymphocytes and enriched cultures contained >60% $CD4^+$ lymphocytes. [a,b,c]Means within a stage of lactation with different superscripts differ ($P < 0.05$). *Means within a cell culture category differ with respect to stage of lactation ($P < 0.05$). **Means within a cell culture category differ with respect to stage of lactation ($P < 0.01$).

Regardless of cell culture, IFN production was higher ($P \leq 0.05$) in cultures isolated from mid- to late lactation cows compared with postpartum cows. Depletion of $CD4^+$ lymphocytes decreased ($P \leq 0.05$) and enrichment increased ($P \leq 0.05$) IFN productions by cultures from mid- to late lactation cows.

## DISCUSSION

During the mid- to late lactating period, $CD4^+$ lymphocytes act primarily as $T_H$-1 cells by expressing higher transcript and producing higher protein levels of both IL-2 and IFN compared with cultures isolated postpartum. This information is consistent with numerous studies (14, 33, 39) that demonstrated secretion of higher levels of IL-2 and IFN by both peripheral blood and mammary lymphocytes isolated during mid- to late lactation cows compared with the postpartum period. Increased IL-2 and IFN secretion by cultures isolated from mid- to late lactation cows cannot be completely explained by the two-fold increase of T-lymphocytes in these cultures. Because flow cytometric analysis revealed that $CD4^+$ and $CD8^+$ lymphocytes were not activated regardless of culture type, enhanced production of these cytokines also may be due to the predominance of $T_H$-1 sub-

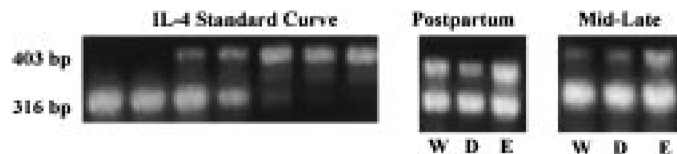


Figure 6. Quantitation of bovine interleukin-4 (IL-4) mRNA using competitive reverse transcriptase-polymerase chain reaction. The molecules of interleukin-4 mRNA in the cell cultures were estimated by determining when the volume of the rcRNA (316 bp) equalled that of the target (403 bp). Peripheral blood mononuclear cells (W) contained all mononuclear cell phenotypes. Depleted cultures (D) contained <3% $CD4^+$ lymphocytes, and enriched cultures (E) contained >60% $CD4^+$ lymphocytes. Shown is one representative sample from each lactation stage and culture type. Samples were obtained from either postpartum or mid- to late lactation (Mid-Late) cows.

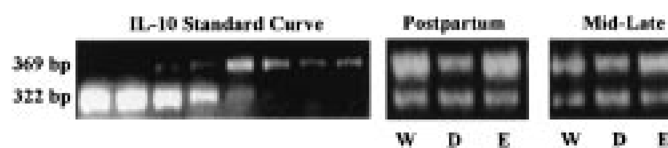


Figure 8. Quantitation of bovine interleukin-10 (IL-10) mRNA using competitive reverse transcriptase-polymerase chain reaction. The molecules of interleukin-10 mRNA in the cell cultures were estimated by determining when the volume of the rcRNA (322bp) equalled that of the target (369 bp). Peripheral blood mononuclear cells (W) contained all mononuclear cell phenotypes. Depleted cultures (D) contained <3% $CD4^+$ lymphocytes, and enriched cultures (E) contained >60% $CD4^+$ lymphocytes. Shown is one representative sample from each lactation stage and culture type. Samples were obtained from either postpartum or mid- to late lactation (Mid-Late) cows.

TABLE 4. Interleukin-2[1] production by peripheral blood mononuclear cells (PBMC), CD4+-enriched, and CD4+-depleted cultures isolated from mid- to late lactation (Mid-late) and postpartum cows.

| Stage of lactation | Antibody[2] | PBMC[3] | | CD4+ -depleted[4] | | CD4+ -enriched[5] | |
|---|---|---|---|---|---|---|---|
| | | $\overline{X}$ | SE | $\overline{X}$ | SE | $\overline{X}$ | SE |
| Mid-late | – | 147.6[b]** | 27.5 | 60.0[c]* | 6.1 | 271.6[a] | 47.0 |
| Postpartum | – | 38.0[b] | 15.5 | 27.8[b] | 8.0 | 152.6[a] | 47.2 |
| Mid-late | + | 20.6 | 1.3 | 8.3 | 1.2 | 39.5 | 2.9 |
| Postpartum | + | 25.5 | 2.9 | 12.7 | 3.1 | 98.6* | 9.7 |

[a,b,c]Means within a row with different superscripts differ ($P < 0.05$).

[1]Cell cultures were stimulated with 2.5 $\mu$g of concanavalin A for 24 h at 37°C under 5% $CO_2$. Activity is expressed as units per milliliter. Values are least square mean (± SEM); n = 6 for mid- to late lactation cows, and n = 7 for postpartum cows.

[2]Cultures were cocultured with neutralizing interleukin-2 antibody (6H14.H8A; 1:10).

[3]Cultures contained all mononuclear cell phenotypes.

[4]Cultures contained <3% CD4+ lymphocytes.

[5]Cultures contained >60% CD4+ lymphocytes.

*Means within a cell culture category differ with respect to stage of lactation ($P < 0.05$).

**Means within a cell culture category differ with respect to stage of lactation ($P < 0.01$).

populations during this time. Previous research utilizing neutralizing monoclonal antibodies against bovine CD4+ lymphocytes demonstrated that these cells were the main producers of IFN-$\gamma$ (14). Data from this study are supportive of those findings because enhanced IFN-$\gamma$ transcript levels and IFN production was observed in CD4+ enriched cultures from mid- to late lactation cows compared with postpartum cows.

Several researchers (10, 21, 22) have shown that promotion of $T_H$-1 lymphocyte differentiation can significantly enhance responses to certain bacterial infections. The $T_H$-1 cytokines, IL-2 and IFN-$\gamma$, promote cellular responses against intracellular pathogens and viruses by activating cell-mediated defense mechanisms and inducing specific immunity. The addition of an IL-2 neutralizing antibody confirmed that IL-2 and not IL-4 was measured in cultures from mid- to late lactating cows. These data along with the IFN-$\gamma$ results demonstrate that CD4+ cells, during the mid- to late lactating period, are capable of promoting cell-mediated antibacterial host defenses. Both IL-2 and IFN-$\gamma$ have been shown to activate and enhance microbicidal activity in macrophages (25). Recently, bovine lymphoid cells have been shown to mediate direct antibacterial activity, and this activity is dependent on IL-2 stimulation (34, 36). Therefore, the presence of $T_H$-1 subpopulations during the mid- to late lactating period most likely activates important antibacterial host defenses, which in turn can reduce the incidence of mastitis in dairy cattle at this time.

TABLE 5. Interferon[1] production by peripheral blood mononuclear cells (PBMC), CD4+-enriched, and CD4+-depleted cultures isolated from mid- to late lactation (Mid-late) and postpartum cows.

| Stage of lactation | PBMC[2] | | CD4+ -depleted[3] | | CD4+ -enriched[4] | |
|---|---|---|---|---|---|---|
| | $\overline{X}$ | SE | $\overline{X}$ | SE | $\overline{X}$ | SE |
| Mid-late | 188.8[b]** | 25.0 | 46.8[c] | 18.0 | 360.3[a]** | 57.4 |
| Postpartum | 65.8[a] | 11.2 | 26.5[b] | 7.3 | 9.0[b] | 4.5 |

[a,b,c]Means within a stage of lactation with different superscripts differ ($P < 0.05$).

[1]Cell cultures were stimulated with 2.5 $\mu$g of concanavalin A for 24 h at 37°C and under 5% $CO_2$. Activity expressed as units per milliliter. Values are least square mean (± SEM); n = 6 for mid- to late lactation cows, and n = 7 for postpartum cows.

[2]Cultures contained all mononuclear cell phenotypes.

[3]Cultures contained <3% CD4+ lymphocytes.

[4]Cultures contained >60% CD4+ lymphocytes.

**Means within a cell culture category differ with respect to stage of lactation ($P < 0.01$).

Although these cells mainly secreted IL-2 and IFN-$\gamma$, CD4$^+$ lymphocytes also have the propensity to produce IL-4 during the mid- to late lactating period. This phenomenon suggests that during this lactation stage $T_H$-2 lymphocytes also are present. The $T_H$-1 and $T_H$-2 pathways directly regulate each other and influence subsequent cytokine production (18). A balance between $T_H$-1 and $T_H$-2 subpopulations coordinates immune response to ensure pathogen elimination without causing disease. Perhaps the preponderance of IL-2 and IFN-$\gamma$ secreted during the mid- to late lactating period down regulates the production of IL-4 and IL-10 by $T_H$-2 CD4$^+$ lymphocytes. Such cross regulation would promote host defense against pathogens without initiating tissue-damaging effects and thus prevent self-inflicted injury as observed during delayed-type hypersensitivity or autoimmune reactions. Alternatively, a small percentage of CD4$^+$ lymphocytes may be uncommitted $T_H$-0 cells that are capable of producing both IL-2 and IL-4.

Lowered production of IL-2 and IFN-$\gamma$ by peripheral blood and mammary mononuclear cells during the postpartum period can be partially explained by lower numbers of CD4$^+$ and the presence of suppressor CD8$^+$ cells (24, 33, 35, 39, 45). Data from the current study suggests that lowered production of these cytokines also might have been due to the predominance of $T_H$-2 CD4$^+$ lymphocytes at this time. The CD4$^+$ lymphocytes isolated from postpartum cows primarily expressed mRNA transcripts for $T_H$-2 cytokines, IL-4 and IL-10, and regardless of culture, secreted less IL-2 and IFN than their mid- to late lactating counterparts. Addition of an IL-2 neutralizing antibody did not alter cytokine level in these cultures, suggesting that these cultures mainly secreted IL-4 and not IL-2. The presence of other mononuclear cell phenotypes that can secrete IL-4, such as CD8$^+$ suppressor cells, may explain why CD4$^+$ depletion did not significantly decrease IL-4 transcript levels in these cultures. These data suggest that CD4$^+$ lymphocytes act mainly as $T_H$-2 compared with $T_H$-1 effector cells postpartum. Both IL-4 and IL-10 are considered to be immunosuppressive cytokines with respect to cell-mediated immunity. A preferential shift towards $T_H$-2 over $T_H$-1 has been correlated to disease progression during HIV infection (6). Therefore, the predominance of $T_H$-2 cells and $T_H$-2 derived cytokines during the postpartum period may diminish important cell-mediated defenses and render the cow more susceptible to disease.

It is unclear whether CD4$^+$ cells are already committed to the $T_H$-2 pathway and are activated to act as $T_H$-2 or whether they are driven to be $T_H$-2 during the postpartum period. Flow cytometric analysis of PBMC reveal no differences in the expression of activation markers between the lactation stages even though lower percentages of T-lymphocytes were observed during the postpartum period. This result indicates that T-lymphocytes are more activated during the postpartum compared with the mid- to late lactating period. Previous research has shown that CD8$^+$ cells are activated suppressor cells (24, 35), therefore CD4$^+$ cells may be activated to be $T_H$-2 subpopulations. This theory is supported by the fact that CD4$^+$-enriched cultures from postpartum cows expressed higher levels of the CD4$^+$ activation marker, and $T_H$ that are resting do not transcribe IL-4 until activated by pharmacological agents or binding of the T-cell receptor (42). Naïve CD4$^+$ lymphocytes can differentiate into either phenotype. Interleukin-4 critically influences the development of $T_H$-2 and can transform $T_H$-1 to $T_H$-2 cells (13, 17, 41). Suppressor CD8$^+$ cells present during the postpartum period can provide IL-4 needed for the development of $T_H$-2 at this time. There is also evidence that demonstrates that CD8$^+$ lymphocytes secrete chemokines that can modulate a shift to a response by $T_H$-2 compared with $T_H$-1 because of differential expression of chemokine receptors on these cells (16, 30). Additionally, hormones such as glucocorticoids, progesterone, or cortisol can influence differentiated $T_H$ lymphocytes to alter their cytokine repertoire from the $T_H$-1 to $T_H$-2 pattern of cytokine expression (28). Increased circulating levels of these hormones around the time of parturition may influence CD4$^+$ cells to favor $T_H$-2 over $T_H$-1 development. Therefore, it is logical that during the postpartum period, CD4$^+$ cells shift to be $T_H$-2 versus $T_H$-1 lymphocytes.

In addition to suppressing cell-mediated immune responses, IL-4 and IL-10 play a critical role in protecting the host against inflammation. Studies have shown a correlation between TNF-$\alpha$ production and the pathogenicity and severity of coliform mastitis during the postpartum period (37). Further research (38) revealed that bovine monocytes or macrophages are activated to produce higher levels of TNF-$\alpha$ postpartum. Interleukin-4 and IL-10 are potent anti-inflammatory cytokines that can suppress proinflammatory cytokines production by both monocyte or macrophages and neutrophils (5, 48). A switch to $T_H$-2 versus $T_H$-1 lymphocytes present during the postpartum period may serve a protective role aimed at down regulating the over production of TNF-$\alpha$ by monocyte or macrophages at this time. In support of this theory, it was recently shown (43) that a marked increase occurs in CD4$^+$ over CD8$^+$ phenotypes in the

milk of infected cows. This phenomenon was more pronounced during *Escherichia coli* infections. Additionally, IL-4 was shown (48) to modulate a balance between inflammatory and immune responses to bacterial infections in pigs. Along with protecting the host, increased production of $T_H$-2 cytokines may be essential for protecting the neonate. In bovine, it is well known that antibodies do not cross the placenta. Newborn calves obtain their humoral protection by the transfer of maternal antibodies from colostrum. The $T_H$-2 cytokines, in particular IL-4, help mediate the humoral immune response. Perhaps the greater percentage of $T_H$-2 cells during the postpartum period is, in part, due to the increased demand for antibody production to ensure survival of the neonate.

Although lymphocytes play a central role in immune responses, the function of specific subpopulations that reside in or traffic to the mammary gland during periods of heightened susceptibility to disease is not well established. Elucidation of the cytokines that participate in an immune response is useful to understanding immunity and immunopathology and provides information for long-term applications such as vaccine development and delivery and cytokine therapy. Data obtained from this study suggest that $CD4^+$ lymphocytes act predominantly as $T_H$-2 compared with $T_H$-1 within 3 d after calving. Alterations in the $T_H$-1 and $T_H$-2 responses, and therefore the repertoire of cytokines produced, may alter certain immune responses and thereby render the host more susceptible to bacterial infections.

## CONCLUSIONS

This study delineated the specific subpopulation of bovine $CD4^+$ lymphocytes during the postpartum period, a time of heightened susceptibility to mastitis. The $CD4^+$ lymphocytes act predominantly as $T_H$-2 compared with $T_H$-1 effector cells at this time. However, it is not clear whether these cells are activated to secrete IL-4 or are driven to be $T_H$-2. Current studies utilizing exogenous cytokines are investigating whether $CD4^+$ subpopulations are committed or driven toward $T_H$-2 versus $T_H$-1 differentiation during periods of immunosuppression.

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