Effect of proinflammatory mediators and glucocorticoids on L-selectin expression in peripheral blood neutrophils from dairy cows in various stages of lactation

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Objective—To determine whether proinflammatory mediators and glucocorticoids affect CD62L(L-selectin) expression on peripheral blood neutrophils from cows in various stages of lactation.

Animals—100 healthy dairy cows during early (13.1 ± 0.79 days after parturition; n = 31), peak (58.7 ± 1.64 days after parturition; 31), and mid (137.2 ± 2.59 days after parturition; 38) lactation.

Procedure—In vitro effects of relevant proinflammatory mediators that are released in response to mastitis caused by gram-negative bacteria such as lipopolysaccharide (endotoxin), tumor necrosis factor-α, and platelet-activating factor (PAF) on CD62L expression on bovine neutrophils were assessed by flow cytometry. Influences of cortisol and dexamethasone on CD62L expression on bovine neutrophils were also investigated.

Results—Basal CD62L expression on neutrophils from cows during early, peak, and mid lactation were similar. Lipopolysaccharide and tumor necrosis factor-α had no effect on CD62L expression on neutrophils from cows at any stage of lactation. Conversely, PAF elicited a time- and dose-dependent, down regulatory effect on CD62L expression. However, no differential shedding of CD62L from neutrophils of cows at any stage of lactation were detected. In addition, no effects on CD62L expression on bovine neutrophils after whole blood incubation with cortisol or dexamethasone were observed. Incubation with glucocorticoids did not prevent the down regulatory effect of PAF on CD62L expression.

Conclusions and Clinical Relevance—Comparable basal CD62L expression on bovine neutrophils and equal amounts of CD62L shedding from bovine neutrophils during all stages of lactation suggest that variations in CD62L density are not a likely cause of susceptibility of cows to coliform-induced mastitis during early lactation. (*Am J Vet Res* 2004;225:1421–1426)

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Parturition and the onset of lactation are periods of considerable stress for dairy cows during which time they undergo physiologic alterations. It is during this time that cows have an increased susceptibility to intramammary infection and clinical mastitis, most likely as a result of a decreased immunocompetence. Among the phagocytic cells affected at peripartum, neutrophils are of particular interest as a result of their primary role in the innate immune defense against mastitis.[1] The inability of neutrophils to rapidly migrate to the site of infection has been proposed as a major determinant in increased severity mastitis caused by gram-negative bacteria.[2,3]

The recruitment of neutrophils to sites of inflammation is regulated by a series of cellular adhesion and activation events. The early stages of this recruitment process (ie, tethering and rolling) are mediated partly by the leukocyte adhesion molecule L-selectin (CD62L) through initiation of both neutrophil-neutrophil and neutrophil-endothelial cell interactions.[4] This molecule is considered to be constitutively expressed on mature neutrophils, and recently, the possible existence of a reservoir at the cell surface has been described in cattle.[5] Upon infection of surrounding tissues, invading neutrophils undergo rapid disengagement of ligated CD62L molecules, which is accomplished by a zinc-based metalloprotease that cleaves the CD62L ectodomain at a membrane-proximal site.[6,7]

It has been proposed that margination and migration of neutrophils during the periparturient period are negatively affected by the reduced expression of CD62L.[8,9] Indeed, peripheral blood neutrophils no longer expressing CD62L loose their ability to home to inflammatory sites in vivo and consequently remain in circulation.[10] This makes tissues considerably more vulnerable to infections. In addition, it has been suggested that CD62L becomes refractory to efficient shedding during the periparturient period.[11] If neutrophils are unable to shed CD62L, they may not be able to regulate the balance between the initial rolling and the next steps during extravasation.[7,12]

Periparturient dairy cattle are particularly susceptible to *Escherichia coli*-induced mastitis. The release of **lipopolysaccharide** (**LPS**, endotoxin) and the induction of **tumor necrosis factor-α** (**TNF-α**) and **platelet-activating factor** (**PAF**) are known to play a preeminent role in the pathogenesis of this inflammatory disease.[13,14] These stimuli are therefore particularly relevant for the current study. In addition, certain observa-

tions point to a relationship between increased blood cortisol concentrations and altered trafficking and phagocytic ability of peripheral blood neutrophils around the time of parturition.[15] More specifically, it has been shown that glucocorticoids induce CD62L shedding on bovine neutrophils.[16] The purpose of the study reported here was to determine whether proinflammatory mediators and glucocorticoids are able to affect expression of CD62L in peripheral blood neutrophils from dairy cows in early lactation.

Materials and Methods

Cows and blood sample collection—Blood samples were obtained from clinically normal Holstein-Friesian cows from the Ghent University dairy herd that were in their first to fourth lactation. Three groups of cows were tested as follows: cows in early (13.1 ± 0.79 days after parturition; n = 31), peak (58.7 ± 1.64 days after parturition; 31), and mid (137.2 ± 2.59 days after parturition; 38) lactation. Blood was collected aseptically in pyrogen-free heparinized evacuated tubes by jugular venipuncture between 8:00 and 9:00 AM.

Total leukocyte count and degree of neutrophil maturity—Total leukocyte count was determined with an electronic particle counter.[a] Briefly, whole blood (25 µL) was diluted in 20 mL of isotonic counting solution[b] before addition of 200 µL of lysis solution.[c] The cell number was determined with optimized instrument settings by use of a dual threshold model. Counting was performed in triplicate for each sample. Smears were prepared from whole blood and stained with Giemsa-quick.[d] Differential microscopic counts were determined by counting 200 cells. Only cows with neutrophil counts greater than 90% of the total granulocyte population were included in the study. Proportions of mature, band, and immature (ie, myelocytes and metamyelocytes) neutrophils were evaluated.

Reagents for adhesion molecule mobilization—The LPS (*E coli* O111:B4)[e] was dissolved in pyrogen-free saline. Recombinant human TNF-α[f] was diluted in Dulbecco PBS solution containing 0.1% fetal calf serum. Platelet-activating factor(1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine)[f] and cortisol were dissolved in ethanol, and dexamethasone was dissolved in dimethyl sulfoxide. All products were of the highest purity available.

To test the effect of the different compounds, blood was divided into aliquots (90 µL) in pyrogen-free, round-bottom tubes. Samples were incubated with LPS, TNF-α, PAF, cortisol, or dexamethasone at 37°C to elaborate a time-course and dose-dependent curve. To evaluate the effect of glucocorticoids on the PAF-induced adhesion molecule mobilization, samples were pretreated with cortisol or dexamethasone for 30 minutes prior to stimulation with PAF for 1 hour.

Immunofluorescence staining—Samples were incubated for 30 minutes at 37°C with 50 µL of anti-bovine monoclonal antibody recognizing CD62L (clone 11G10,[g] mouse IgG1 isotype)[17] at saturating concentrations as determined by flow cytometric titration. Control samples were incubated with 50 µL of RPMI 1640, 1% bovine albumin fraction V,[d] and 0.2% NaN_3, a potent inhibitor of redistribution (eg, as caused by shedding or internalization) of cell membrane antigens. Red blood cells were lysed with 300 µL of a cold sterile buffered solution (pH 7.4) containing 21.47 mmol of Trizma base/L and 138.34 mmol of NH_4Cl/L and gently mixed for 6 minutes at room temperature (approx 21°C). After centrifugation (200 × *g* for 10 minutes at 4°C), cells were washed twice in control solution (ie, 300 µL RPMI 1640, 1% bovine albumin fraction V, and 0.2% NaN_3). A second incubation on ice was performed in the dark for 30 minutes with 50 µL of the **fluorescein isothiocyanate (FITC)**-labeled secondary antibody goat anti-mouse IgG[e] diluted in control solution. Cells were washed twice in 300 µL of PBS solution (200 × *g* for 10 minutes at 4°C) and subsequently pelleted and resuspended in 1% paraformaldehyde.

Flow cytometry—Specimens were analyzed on a flow cytometer.[h] For each sample, 20,000 events were recorded in list mode and displayed on a logarithmic scale. The neutrophil population was characterized by forward and side light scattering characteristics and by use of an antibody stain for bovine granulocytes.[i] For evaluation of the results, the **mean fluorescence intensity (MFI)** of the gated population was used as an indicator of the relative receptor number per cell. In addition, we determined the percentage of the neutrophil population with positive fluorescence for CD62L. Preliminary experiments with isotype-matched control antibodies (mouse IgG1 isotype)[j] and FITC-labeled secondary antibody had no substantial difference in the amount of background staining, compared with samples incubated with the secondary antibody alone. Therefore, nonspecific background fluorescence was further assessed with FITC-labeled secondary antibody.

Statistical analysis—The MFI of CD62L and percentage of $CD62L^+$ neutrophils in resting cells at time 0 and their time evolution were compared among cows in early, peak, and mid-lactation by use of a mixed model. The parameter cow was introduced in the analysis as a random effect, and time, lactation stage, and their interaction were added as categoric fixed effects. The total leukocyte count and number of mature, band, and immature neutrophils of cows in various stages of lactation were compared by use of a fixed effect model. The effect of LPS, TNF-α, PAF, cortisol, and dexamethasone concentration on CD62L expression (MFI and percentage of $CD62L^+$ cells) was analyzed by use of a mixed model. The parameter cow was introduced as a random effect, whereas time, concentration, and their interaction were added as categoric fixed effects. Finally, the effect of pretreatment with cortisol and dexamethasone prior to the addition with PAF on CD62L expression was assessed by use of a mixed model, where the parameter cow was introduced as a random effect and the treatment as a fixed effect. Pairwise comparisons were adjusted by use of the Tukey method. Values of $P < 0.05$ were considered significant. Data are presented as mean (± SEM) values.

Results

Expression of CD62L in unstimulated neutrophils—A time-course experiment was performed to compare the expression of CD62L among peripheral blood neutrophils from early-, peak-, and mid-lactating cows. With increasing incubation time, the MFI and percentage of $CD62L^+$ neutrophils moderately but significantly ($P < 0.001$) decreased. After 2 hours, a decrease in CD62L expression from an MFI of 35.84 ± 1.59 to 25.13 ± 1.00 and a decrease in the percentage of $CD62L^+$ neutrophils from 98.1 ± 0.26% to 95.1 ± 0.41% were measured. All 3 curves, representing unstimulated neutrophils of cows in various stages of lactation, had similar amounts of expression at time 0, and values paralleled at each subsequent time point evaluated (**Figure 1**).

Total leukocyte count and degree of neutrophil maturity—Upon analysis of blood samples, significant differences relative to the stage of lactation were

observed in the total leukocyte count and degree of neutrophil maturity. Both parameters were lowest during early lactation (**Table 1**). In addition, the number of band and immature peripheral blood neutrophils was higher during early lactation and decreased as cows entered the later stages of lactation.

Effect of LPS and TNF-α on CD62L expression—A wide range of doses for each compound tested was combined with several incubation periods, and a time course was constructed. First, blood samples obtained from 7 cows in mid lactation were treated with LPS at concentrations of 1, 10, and 100 ng/mL. Differences among stages of lactation (8 cows/lactational stage) were subsequently evaluated with LPS at a concentration of 1 ng/mL. Blood samples obtained from 4 cows in each lactational stage were also incubated with TNF-α at 1, 10, and 50 ng/mL. Subsequently, CD62L expression was measured on peripheral blood neutrophils. Different to findings in humans,[18-20] LPS and TNF-α had no detectable effect at any of the concentrations, incubation times, or stages of lactation tested (data not shown).

Effect of PAF on CD62L expression—At concentrations of 0.1, 1, and 10 µmol/L, PAF was added to blood samples obtained from 9 cows and CD62L expression on neutrophils was evaluated by flow cytometry. Different from the effects of LPS and TNF-α additions, a clear effect was seen when neutrophils were incubated with PAF at various concentrations (**Figure 2**). A significant ($P < 0.001$) decrease in the MFI of CD62L was detectable at a PAF concentration of 0.1 µmol/L, and significant ($P < 0.001$) decreases in surface-bound CD62L were detectable at PAF concentrations of 1 and 10 µmol/L. For each concentration of PAF, a time-dependent significant ($P < 0.001$) effect

Table 1—Mean (± SEM) total leukocyte and neutrophil counts in blood samples from cows in various stages of lactation.

	Cows		
Cell count	**Early lactation (n = 24)**	**Peak lactation (24)**	**Mid lactation (24)**
Total leukocyte (cells/µL)	6,094 ± 315*	6,940 ± 317*†	7,578 ± 38†
Mature neutrophil (cells/µL)	1,287 ± 215*	2,279 ± 255†	2,337 ± 164†
Band neutrophil (cells/µL)	364 ± 65.7*	54.4 ± 10.6†	21.9 ± 3.82†
Immature neutrophil (cells/µL)	136 ± 34.0*	45.1 ± 10.3†	9.82 ± 5.15‡

*,†, ‡ Stages with distinct superscript annotations are significantly ($P < 0.05$) different from each other.

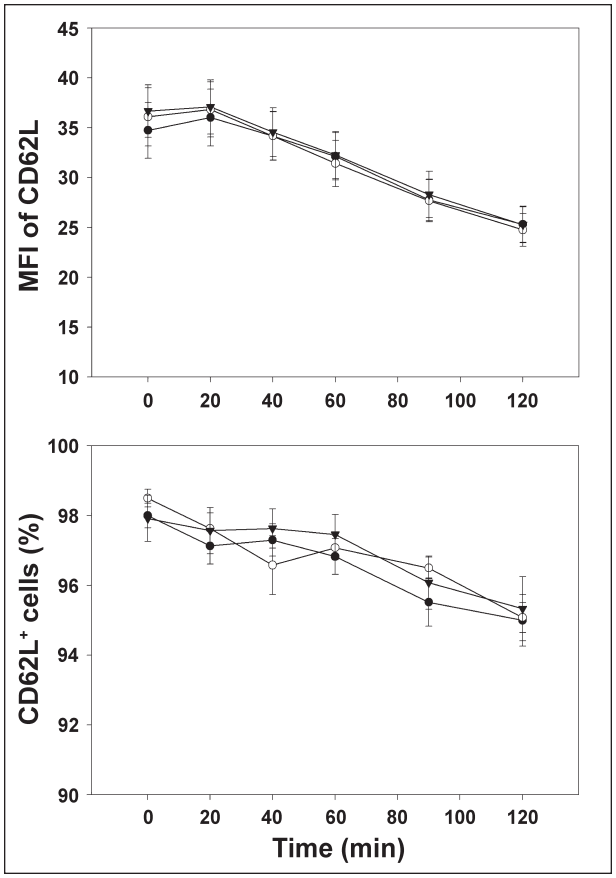

Figure 1—Mean (± SEM) values of CD62L expression versus time in neutrophils from cows in early (closed circle; n = 24), peak (open circle; 24), and mid (closed triangle; 24) lactation. Expression of CD62L was measured as mean fluorescence intensity (MFI; top panel) and percentage of CD62L+ cells (bottom panel).

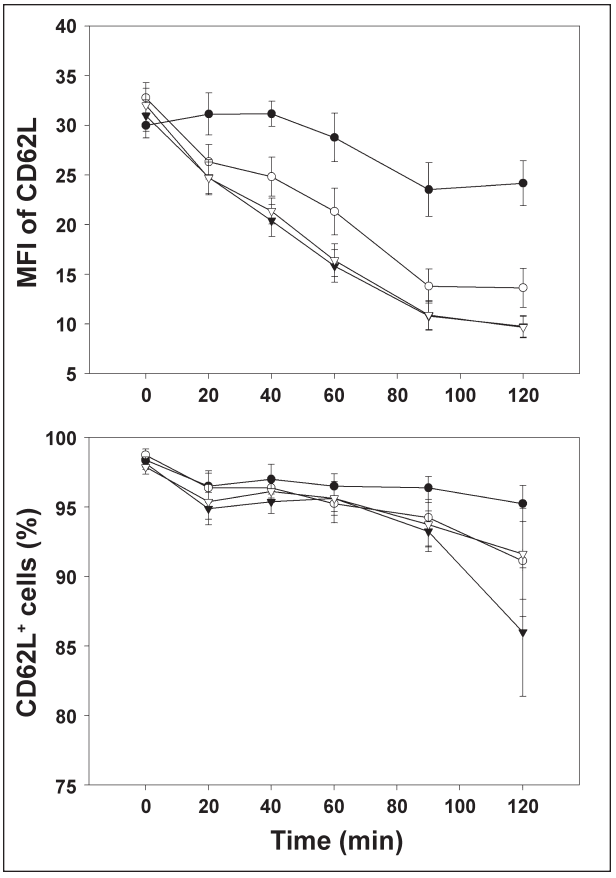

Figure 2—Mean (± SEM) values of CD62L expression versus time in bovine neutrophils in response to platelet-activating factor (PAF) at concentrations of 0 (closed circle; n = 9), 10^{-1} (open circle; 9), 1 (closed triangle; 9), and 10 (open triangle; 9) µmol/L. Expression of CD62L was measured as MFI (top panel) and percentage of CD62L+ cells (bottom panel).

was observed. Similar changes were observed with respect to the percentage of $CD62L^+$ neutrophils, albeit a significant ($P = 0.032$) change was only reached with a PAF concentration of 1 µmol/L. In preliminary experiments, no significant differences in CD62L expression on neutrophils from blood samples of 4 cows were detected at lower PAF concentrations of 10^{-4} and 10^{-2} µmol/L (data not shown).

Testing the effects of PAF at a concentration of 1 µmol/L on CD62L expression on neutrophils from blood samples from cows in various stages of lactation (6 cows/lactational stage) resulted in essentially the same decrease in CD62L expression during the 3 periods under study (**Figure 3**). Taken together, these data indicate that PAF induces a concentration-dependent and progressive decrease in cell surface expression of CD62L on neutrophils of cows in all stages of lactation.

Effect of cortisol or dexamethasone on CD62L expression—Peripheral blood was treated with cortisol (50, 100, and 200 nmol/L) or dexamethasone (5, 10, and 40 nmol/L) at 37°C, and CD62L expression on neutrophils was subsequently measured by flow cytometry. Blood samples from a group of 9 cows (3 cows/stage of lactation) were evaluated for either the effect of cortisol or for dexamethasone on CD62L expression on neutrophils. Cortisol concentrations corresponding to pathophysiologic concentrations in plasma were selected.[1] On the basis of the results of a pharmacokinetic study[21] of dexamethasone in cows, we tested a dose range between minimal and maximal values detected in plasma. Similar to findings in humans,[22-26] none of the concentrations of cortisol or dexamethasone had a measurable effect on CD62L expression in bovine neutrophils in vitro. This applied for any stage of lactation or incubation time tested (data not shown).

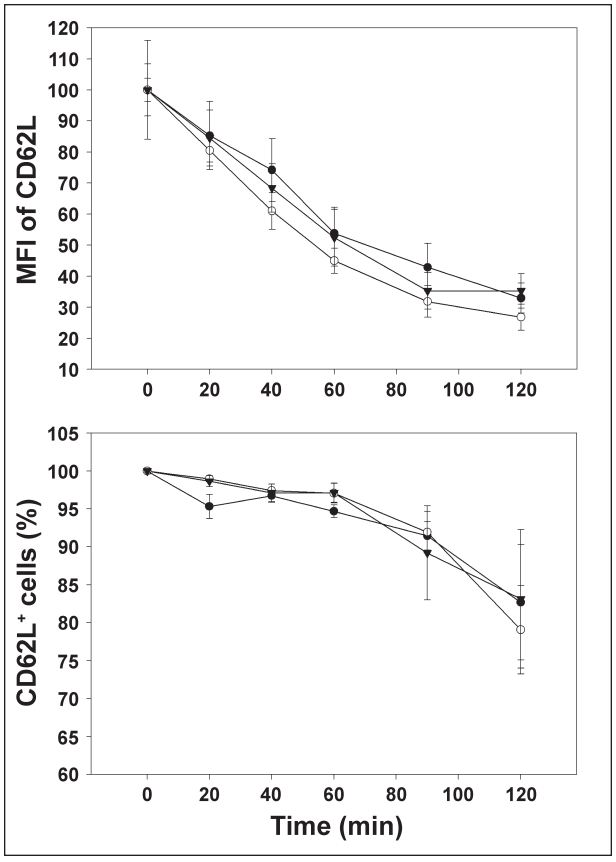

Figure 3—Effect of PAF (1 µmol/L) on mean (± SEM) values of CD62L expression versus time in neutrophils from cows in early (closed circle; n = 6), peak (open circle; 6), and mid- (closed triangle; 6) lactation. Expression of CD62L was measured as MFI (top panel) and percentage of $CD62L^+$ cells (bottom panel) and expressed as a percentage of control (100%) values.

Effect of cortisol or dexamethasone combined with PAF on CD62L expression—To determine whether glucocorticoids affect PAF-induced down regulation of CD62L, whole blood obtained from 7 cows in each lactational stage was pretreated with various concentrations of cortisol or dexamethasone for 30 minutes at 37°C prior to addition of PAF (1 µmol/L) for 1 hour. The PAF-induced decrease in CD62L expression on neutrophils was not abrogated by preincubation with cortisol nor with dexamethasone at any of the concentrations tested or stages of lactation (data not shown).

Discussion

Cows in early lactation have peripheral blood neutrophils with a decreased ability to rapidly migrate to the site of inflammation. The mechanisms for this decreased capacity are, however, poorly understood. It has been proposed that low amounts of CD62L adhesion molecule on neutrophils, decreased ability to shed the molecule from the cell surface, or both contribute to the high susceptibility of cows in early lactation to coliform-induced mastitis.

Results of our study indicate that CD62L expression on bovine neutrophils decreases as cells age in vitro. This decrease probably reflects the loss of CD62L from the cell surface, which is possibly caused by nonspecific activation occurring during incubation. Indeed, spontaneous shedding of CD62L has been observed for neutrophils from humans.[20] The in vitro results of our study extend these observations to bovine neutrophils. Our data also agree with those of other studies[27,28] in which most peripheral blood neutrophils stain positively for CD62L.

In our study, unstimulated neutrophils from cows in early lactation expressed slightly lower amounts of CD62L, although not significantly lower amounts, than neutrophils from cows in later stages of lactation. A previous study[3] comparing neutrophils from cows in early and mid lactation yielded similar results. In contrast, several investigations on neutrophils obtained from cows around the time of parturition have reported significantly decreased amounts of CD62L expression.[8,9] In the latter studies, a gradual decrease during the second week before parturition with minimal values at calving was observed before returning to baseline amounts of expression at approximately 4 days after parturition. For our experiments, cows in the first 3 weeks after parturition were selected. This difference in experimental setup may well account for the apparent discrepancy between study results. In addition, results of our study indicate that the kinetics of CD62L

in unstimulated neutrophils was similar for the 3 stages of lactation under evaluation.

It has been reported that LPS and TNF-α induce CD62L shedding from the neutrophil surface in humans.[18-20] However, these 2 proinflammatory mediators did not affect CD62L expression on bovine neutrophils in our study. We speculate that species-related differences in signal transduction patterns are responsible for this outcome. The unresponsiveness of CD62L at the neutrophil surface upon LPS treatment is in agreement with findings of Monfardini et al,[9] who attributed the result to the immunosuppression occurring in cows in early lactation. However, results of our study indicate that the absence of CD62L shedding by LPS cannot be directly linked to the stage of lactation.

In our experiments, TNF-α was similarly unable to elicit CD62L shedding. No commercial source for recombinant bovine TNF-α is currently available. Therefore, we chose to use recombinant human TNF-α to perform our experiments on the basis of the high homology and similarities in activity between human and bovine TNF-α.[29] An up regulatory effect on CD11b expression on bovine neutrophils and an increase in CD18 mRNA concentrations in bovine neutrophils were found in vitro in previous studies[30,31] that used human TNF-α. In addition, pretreatment of bovine neutrophils with human TNF-α has been shown to enhance killing activity against *Staphylococcus aureus* and promote neutrophil apoptosis in vitro.[32,33]

As one of the earliest events in microvascular injury,[34] PAF is released and serves as a signal for neutrophils to bind tightly to the endothelium.[35,36] Although this potent proinflammatory mediator has been shown to decrease CD62L expression in human neutrophils, inconsistent results have been obtained with bovine neutrophils.[16,37] In our study, PAF caused CD62L shedding in a time- and concentration-dependent manner. Maximal down regulation was achieved with a PAF concentration of 1 µmol/L, as previously described for human neutrophils. However, to reach maximal differences, the incubation time (1 hour) was longer than required with human neutrophils (30 minutes).[23] Our results do not support the view that a decreased ability of neutrophils to shed CD62L molecule from their surface contributes to the increased susceptibility of cows to periparturient diseases. Indeed, no differences in the down regulatory effect of PAF on CD62L expression were detected for the 3 stages of lactation under evaluation.

Despite the well-described immunosuppressive consequences of glucocorticoids, the mechanisms through which they exert this effect have not been fully elucidated. An important aspect of glucocorticoid action is to inhibit leukocyte accumulation at the site of inflammation. It has been reported that cortisol and dexamethasone are able to reduce adhesion between leukocytes and endothelium.[38] Results of several studies[16,24,39] indicate that treatment of cows and humans with glucocorticoids leads to decreased CD62L expression on neutrophils, thus pointing to a possible mechanism through which neutrophil function may be negatively influenced. In vitro, however, we were unable to detect a down regulatory effect on CD62L after whole blood incubation with exogenous glucocorticoids at any stage of lactation. These results closely match those found with human neutrophils in vitro and seem to support the presence of an indirect glucocorticoid action on adhesion molecule expression.[22-26] It has been suggested by others that glucocorticoids can pretranslationally regulate CD62L gene expression in bovine neutrophils.[8] Furthermore, results of a study[26] in humans indicate that annexin I can serve as a mediator for the glucocorticoid-induced CD62L shedding in vivo.

The observation that cortisol and dexamethasone can attenuate LPS-induced upregulation of CD11b and CD18 in bovine neutrophils has resulted in the hypothesis that glucocorticoids may affect leukocyte traffic into inflamed tissues by a generalized inhibition of adhesion molecule mobilization.[30,40] Indeed, results of a study[23] in humans indicate that preincubation with glucocorticoids could avoid CD62L down regulation in response to neutrophil activation with PAF. Strikingly, we observed a lack of effect of both compounds (cortisol and dexamethasone) on the expression of CD62L in bovine neutrophils stimulated with PAF. We do not know whether this result reflects different mechanisms of shedding or whether it is caused by distinct in vitro conditions.

In conclusion, results of our study indicate that CD62L on bovine neutrophils undergo slow shedding in unstimulated cells and this process increases dramatically after activation by PAF. The in vitro kinetics of CD62L on bovine neutrophils had comparable basal amounts of expression during the 3 stages of lactation in quiescent cells and after induction with PAF. Collectively, results of our study suggest that the diminished migratory capacity of neutrophils in periparturient cows cannot be readily ascribed to a decreased CD62L expression or a reduced ability to shed CD62L.

[a]Coulter counter ZF, Coulter Electronics Ltd, Luton, UK.
[b]Isoton II, Counter Electronics, Kresfeld, Germany.
[c]Zap-Oglobin, Coulter Electronics Ltd, Luton, UK.
[d]Merck, Damstadt, Germany.
[e]Sigma-Aldrich, Bornem, Belgium.
[f]Calbiochem, San Diego, Calif.
[g]Gift of Dr. Max J. Paape, USDA, Agricultural Research Service, Immunology and Disease Resistance Laboratory, Beltsville, Md.
[h]FACScan, Becton-Dickinson, San José, Calif.
[i]CH138A, VMRD Inc, Pullman, Wash.
[j]DAKO A/S, Glostrup, Denmark.

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