



Surface expression of CD11b, CD62L, CD44 receptors on blood and milk neutrophils during subclinical and clinical mastitis in Sahiwal cows

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ABSTRACT

Surface expression of cell adhering molecules and CD44 was studied in Sahiwal (SW) cows suffering naturally from subclinical (SCM) and clinical mastitis (CM). Cows were divided into groups, viz. healthy (12), SCM (12) and CM (12) groups on the basis of CMT scoring, bacteriological culture, gross morphological changes in the milk and by counting milk SCC after screening of 136 SW cows. Bacteriological culture indicated infection of *Staphylococcus aureus* as the causative agent of SCM and CM. Healthy cows showed significantly higher CD62L expression as compared to the SCM and CM group of animals in both blood and milk neutrophils but no significant difference was found between blood and milk neutrophils. The results revealed a significant upregulation of CD11b positive cells in the CM group of cows. Significantly higher expression of CD44 was found in the neutrophils of both blood and milk of SCM group as compared to CM group. From the study, it was evident that neutrophils exhibit dynamic interplay between the integrins (CD11b) and selectins (CD62L) along with CD44 during SCM and CM *Staphylococcal* mastitis to offer host protection against the foreign invaders.

Key words: CD11b, CD44, CD62L, Mastitis, Neutrophils, Sahiwal cows

Mastitis is considered the most economically important disease of high producing cows. The pathology of mastitis becomes very complex due to involvement of multiple pathogens and diverse cross talking agents. There is involvement of immune cells like neutrophils, macrophages and lymphocytes which make immune response of the disease very complex (Paape *et al.* 2003). Out of all the cells, neutrophils are the first cells to be recruited to the site of pathogen invasion. Massive influx of neutrophils to the udder is one of most significant process that occurs during the invasion of the pathogen as a mode to protect the udder from pathogen attack. The recruitment of neutrophils is mediated by a dynamic process known as tethering, which is mediated by differential and stage specific expression of cell adhering molecules known as selectins and integrins (Diez Fraile *et al.* 2002). Selectins are associated with loose contact of neutrophils with surface endothelium, whereas, integrins are involved in tight contact

with the endothelium mediating diapedesis across the endothelium (Diez Fraile *et al.* 2003, Borregard 2010).

The immune response of the host differs on the basis of pathogen and their associated molecules (Bannerman 2009). After the process of recruitment, timely removal of the neutrophils from the site of infection is mediated by the surface expression of CD44 and its recognition through the macrophages. Neutrophils after carrying phagocytosis undergo apoptosis and are removed by macrophages. Any deviation in the process of neutrophil removal from the lumen of the udder elicits onset of secondary inflammation and further tissue damage (Paape *et al.* 2003). In Indian dairy herds, 70 % of SCM and CM are caused by *Staphylococcus aureus* (Sentitula *et al.* 2012), which is also considered as one of the most virulent strains causing bovine mastitis (Smith *et al.* 2005). Till date, scanty information is available in the literature regarding the pattern of expression of cell surface adhesion molecules and CD44 in indigenous cows suffering from subclinical mastitis (SCM) and clinical mastitis (CM). Therefore, the present study was undertaken to study the differential expressions of CD62L, CD11b and CD44 in both blood and milk neutrophils isolated from SCM and CM Sahiwal (SW) cows.

MATERIALS AND METHODS

Sahiwal (SW) cows (36) reared under semi-intensive system of management at cattle yard of NDRI, Karnal, were taken after screening 136 SW cows and categorized into

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three groups of 12 cows each. All the cows were screened for mastitis by California Mastitis Test (CMT), gross/morphological changes in milk, bacteriological culture and its evaluation and microscopic counting of milk SCC (Swain *et al.* 2014, 2015a, b). The first group of 12 cows apparently free of infection was classified as control or healthy group and cultured milk samples were negative for the presence of bacteria. The second group of 12 cows having CMT score of single positive, milk SCC of 2.14 to 2.46 ($10^5/\text{ml}$), apparently no change in the milk morphology was classified as cows suffering from subclinical mastitis (SCM). The third group of cows having CMT score of double and triple positive based on the intensity of gel formation and SCC count of 4.5 to 5.85 ($10^5/\text{ml}$) was classified as clinical mastitis group (CM). This group of cow's milk was showing the presence of flakes and clots with very high milk SCC.

For bacteriological studies, a 0.02 ml aliquot of each sample was spread on 5% sheep blood agar. The plates were incubated aerobically at 37°C and examined after 24 and 48 h. The plates having monoculture colonies were further confirmed by morphological assessment and by performing standard biochemical tests. Catalase test was positive as air bubbles appeared in the culture plate. Blue and purple colored cocci in bunches appeared in Gram's staining. Further, the bacteria were confirmed by inoculating the culture in mannitol salt agar. Yellow colonies appeared which confirmed the presence of *Staphylococcus aureus* (Dang *et al.* 2007, Swain *et al.* 2014).

Sampling of milk and blood: Milk and blood samples were collected from all the three groups of cows as per the protocols approved by the animal ethics committee of the institute. Teats were disinfected with 70% ethyl alcohol prior to collection of milk. Fifty ml of milk was collected in sterile plastic centrifuge tubes. The tubes were kept in ice box and immediately transferred to the laboratory for further processing and evaluation. Autologous blood samples of 9 ml were also taken after milk sampling by venipuncture from the external jugular vein using heparin as an anticoagulant.

Isolation of blood neutrophils: All materials and reagents used for the isolation of blood and milk polymorphonuclear neutrophils (PMN) were sterile and of cell culture grade. Isolation of PMN from peripheral blood samples was performed as per the protocol described earlier (Swain *et al.* 2014). The purity of the blood PMN was more than 90% as evaluated by Field's stain under oil immersion lens (100 \times). Different types of blood and milk PMNs were estimated by Field's stain and May Grunwald Giemsa stain and were observed under oil immersion lens (100 \times).

Isolation of milk neutrophils: Isolation of PMN from milk was performed as per the method described earlier (Swain *et al.* 2014, 2015a,b) for milk samples. The purity of milk PMNs was more than 85% after staining with Field's stain. The cell were suspended in RPMI medium and counted.

Expression of CD62L, CD11b, CD44 in blood and milk neutrophils: Expressions of CD62L, CD11b and CD44 were quantified by using flow cytometry. The isolated blood and

milk neutrophils were taken at a concentration of 10^5 cells/ml of the suspension. The experiment was carried out in triplicate. Briefly, 250 μL of cells suspended in PBS were taken in four tubes. The first tube was only having cell suspension without any antibodies and was considered as the control. The second, third and fourth tubes were taken as experimental/test tubes. In each of the test tube, 250 μL of cell suspension was taken and to this, the primary antibody was added which was tagged with FITC as a probe. For studying the expression of CD11b, the primary antibody (CD11b-FITC) was used at 1:50 dilutions. The cell suspension along with the primary conjugated antibody was incubated for 1 h at 37°C in dark. After 1 h of incubation, the cell suspension was washed twice with PBS (pH=7.4) at 750 RPM for 5 min. After second washing, the cell pellet was resuspended in 250 μL of PBS. To this, 250 μL of 1% paraformaldehyde was added and stored in dark at 4°C till use. After 24 h of cell labeling, the cells were analyzed for the quantification of expression of CD11b. Similar procedure was employed for evaluation of CD62L and CD44 expression. The antibodies used were at 1:100 dilutions (0.5 mg/ml for CD62L and 0.5 mg/ml for CD44).

Flow cytometry: Mean fluorescence intensity was quantified using a flow cytometer. The instrument used the Diva software for the analysis of the data. PMNs were gated on dot plots representing cell size based on forward light scattering and granularity based on side light scattering. 10,000 events were acquired for the evaluation of the relative expression of neutrophil surface receptors in control/healthy, SCM and CM group samples of blood and milk. During the study, forward light scatter (FSC), orthogonal light scatter (SSC), FITC fluorescence (FL1) parameters of blood and milk PMNs were quantified by using the flow cytometry. The neutrophils were identified and gated on the basis of their size (FSC) and granularity (SSC). The results obtained were expressed in percent expression on neutrophils.

Statistical analysis was carried out by using the Sigma Plot software package version 7.01. Means and standard errors of the mean (SEM) were calculated and the data were presented as means \pm SEM. To compare the groups with respect to different variables in blood and milk, two way analysis of variance (ANOVA) was used and the significance was tested at 0.05 (5%) for all the observations.

RESULTS AND DISCUSSION

Counting of milk SCC was done microscopically in all the groups of SW cows. In the control group, milk SCC was in the range of 0.92 to 1.36 ($10^5/\text{ml}$) of milk, whereas, the SCM group exhibited a range of 2.14 to 2.46 ($10^5/\text{ml}$) of milk and the CM group revealed a highly significant ($P < 0.05$) SCC range between 4.5 to 5.85 ($10^5/\text{ml}$). Control/healthy animals showed negative CMT score, whereas, the SCM group showed single positive; and the CM group of cows showed double and triple positive CMT score along with thick gel formation as already discussed in the earlier section.

Bacteriological evaluation of milk samples revealed the presence of *Staphylococcus aureus* in the milk samples of infected cows. The milk samples of control animals were negative for the presence of pathogen as confirmed from the culture. The results of expression of CD62L in the blood and milk neutrophils are presented in Fig 1a,b. In the control healthy animals, the CD62L expression was significantly high ($P < 0.05$) as compared to the SCM and CM group of animals. No significant difference was found between blood

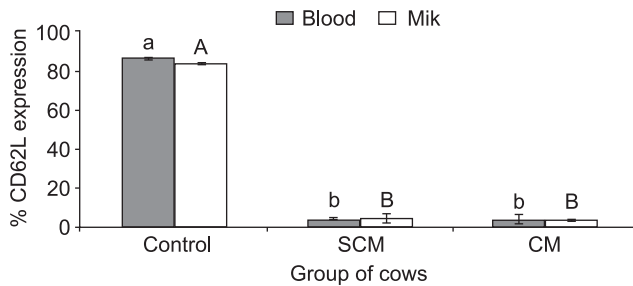


Fig. 1a. Per cent CD62L expression on neutrophils of different groups of SW cows. Significance of the difference among the three groups is indicated with asterisks ($*P < 0.05$; $n=12$). Bars represent the standard error of the mean.

and milk neutrophils with respect to the expression of CD62L.

The relative expression of CD11b in different groups of cows is presented in Figs 2,3. The results revealed a significant ($P < 0.05$) upregulation of CD11b positive cells in the CM group of cows as compared to SCM and control groups of cows. In the study, we noted a reverse trend of expression of CD62L (L-selectins) and $\beta 2$ integrins

(CD11b). There was no difference between the blood and milk neutrophils with respect to expression of CD11b. The degree of expression of CD11b increased significantly ($P < 0.05$) in SCM and CM group of cows as compared to control group of cows.

CD44 expression in both blood and milk neutrophils isolated from different groups of SW cows are presented in Fig. 4a,b. CD44 expression was lowest in blood neutrophils of control group and highest in SCM group. CD44 was significantly ($P < 0.05$) higher in SCM group as compared to control and CM group. In all the three groups, the expression of CD44 was significantly ($P < 0.05$) higher in milk neutrophils as compared to the blood neutrophils.

The present study was designed to evaluate the relative expressions of surface adhesion receptors on the surface of both blood and milk neutrophils during natural occurrence of *Staphylococcal* subclinical and clinical mastitis in

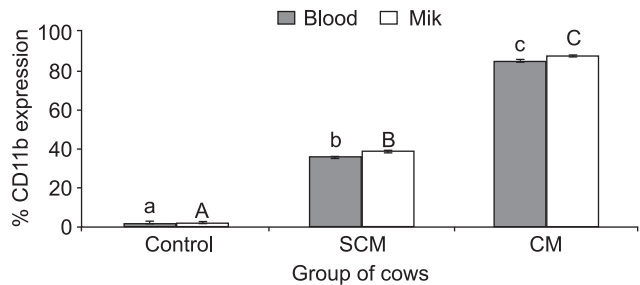


Fig.2. Percent CD11b expression on neutrophils of different groups of SW cows. Significance of the difference among the three groups is indicated with asterisks ($*P < 0.05$; $n=12$). Bars represent the standard error of the mean.

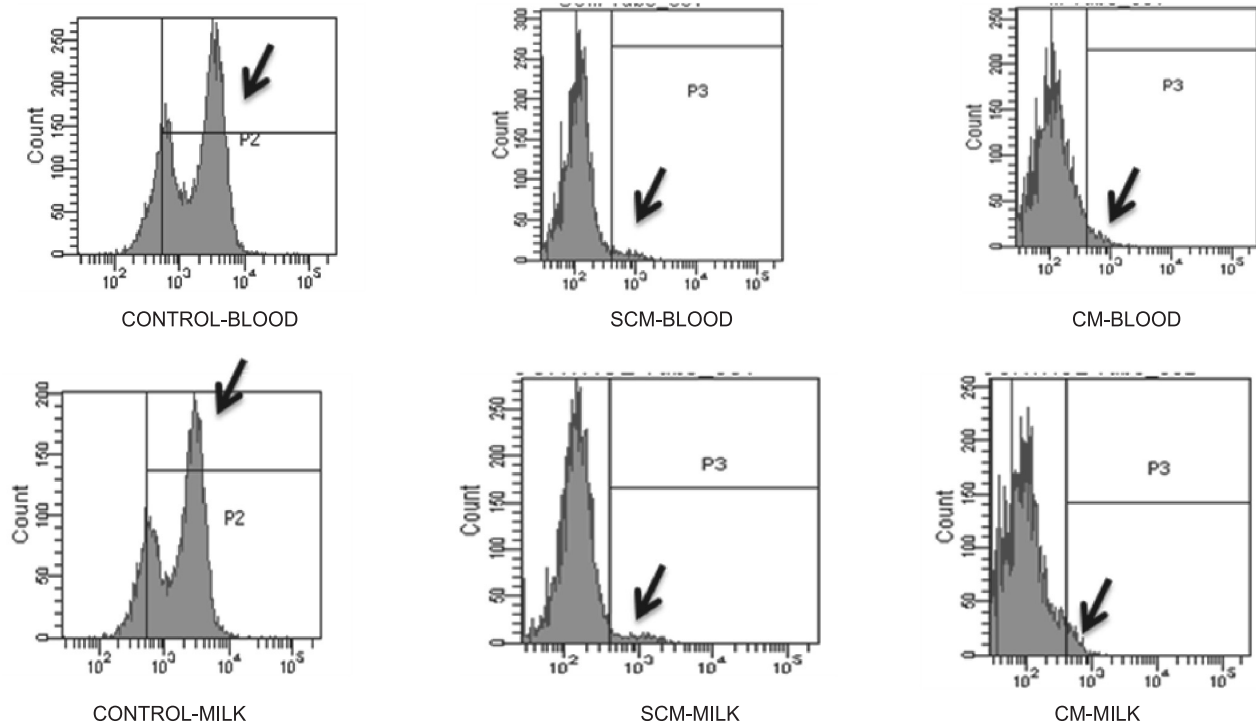


Fig. 1b. Per cent CD62L expression on neutrophils of different groups of SW cows.

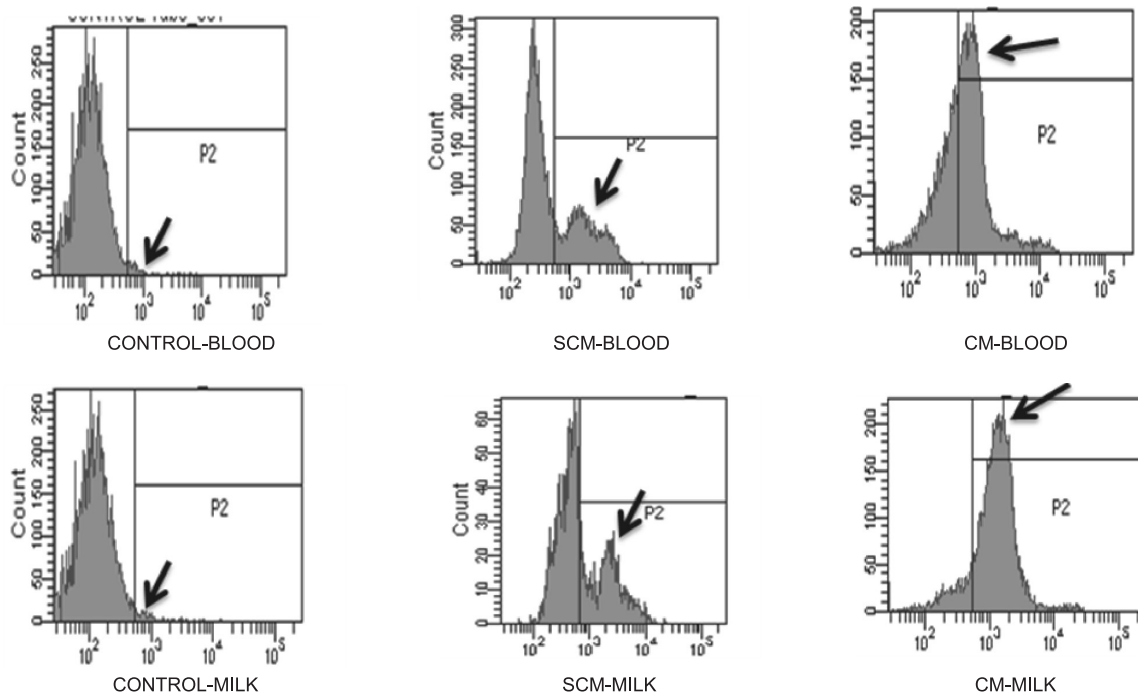


Fig. 3. Percent CD11b expression on neutrophils of different groups of SW cows.

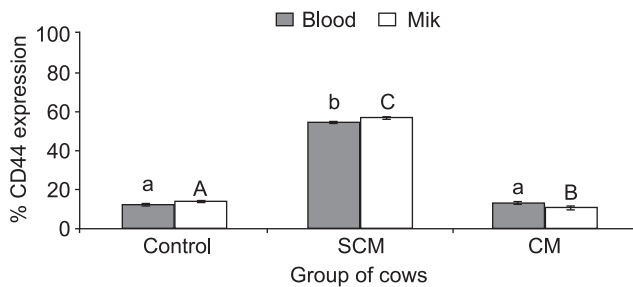


Fig. 4a. Percent CD44 expression on neutrophils of different groups of SW cows. Significance of the difference among the three groups is indicated with asterisks (*P < 0.05; n=12). Bars represent the standard error of the mean.

indigenous Sahiwal cows. The level and degree of adhesion molecule expression is dependent on the stage as well as severity of infection by the pathogens (Diez Fraile *et al.* 2003).

The expression of CD62L increased in early phases of infection, whereas, with the progression of time, the expression of CD62L got decreased and the expression of CD11b increased. With the peak of infection, the PMNs shed CD62L and express more CD11b for tight binding with the endothelium and rapid diapedesis to tissue spaces. This biphasic expression of adhesion molecules on the surface of the PMNs regulates the degree of PMN function (Diez Fraile *et al.* 2004). We noted this biphasic pattern of

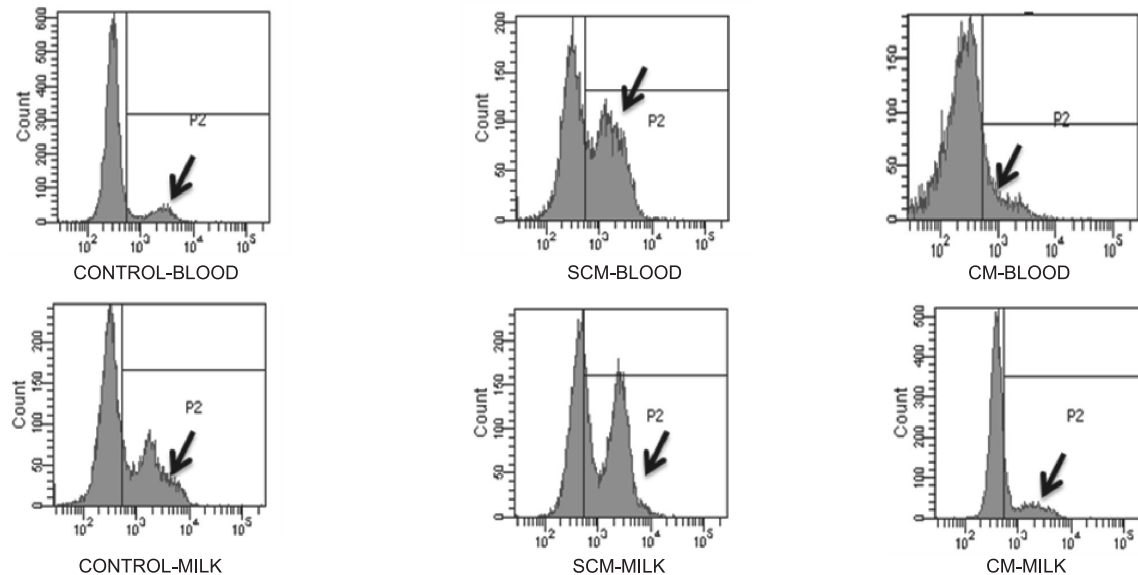


Fig. 4b. Percent CD44 expression on neutrophils of different groups of SW cows.

expression of adhesion receptors on milk PMNs isolated from milk infected with *Staphylococcus aureus*. The level of expression of adhesion receptors was less in milk as compared to that of blood neutrophils (92% to the blood PMNs) as evident from our study.

A significantly ($P < 0.05$) lower expression of surface receptors in the SCM group of cows was observed as compared to CM group of cows. The entry of the pathogen stimulates the expression of selectins and gradually the selectins get dislodged from the neutrophils and there is increased density of integrins over the surface of neutrophils (Hoeben *et al.* 2000). This dynamic kinetics of selectin and integrin on the surface of PMNs is the potential regulator of PMN function in terms of recruitment. However, in the present study, we were not able to understand why this kinetics was not seen in SCM group of cows but probably a weak chemokine and cytokine mediated signalling is associated with SCM caused by *Staphylococcus aureus*. Cleavage of L-selectin would be intended either as a signal to switch to a $\beta 2$ -integrin dependent adhesion, or to serve as a regulatory balance in PMN adherence by this receptor to the endothelium (Futosi *et al.* 2013). This may be another explanation to our results where we noted a higher expression of selectins in control neutrophils as compared to the SCM and CM group of cows.

In control animals, higher expression of selectins is an indication of slower and lower rolling of neutrophils along with their recruitment but with the progression of infection, expression of integrins increased which is an indication of strong binding and higher recruitment of neutrophils towards the site of infection. A reduction in the expression of L-Selectin causes a reduced recruitment of neutrophils to the site of infection and leads to a lower chemotaxis of PMNs (Kolaczowska and Cubs 2013). This may be a probable reason behind the higher expression of CD11b in both blood and milk neutrophils during SCM and CM in our study. During SCM, the bacteria and bacteria associated factors challenge is of lower degree as compared to CM group and hence the expression of CD11b was lower in SCM group as compared to the CM group (Hoeben *et al.* 2000). On the other hand, the expression of CD62L was more in the control healthy group as compared to the SCM and CM group.

CD44 serves as a surface signalling molecule during apoptotic signals to macrophages (Degrendele *et al.* 1996). The relatively higher expression of CD44 mediates a faster rolling of neutrophils along with faster recognition by macrophages for removal from the site of infection (Riollet *et al.* 2000a, 2000b). During SCM, more expression of CD44 mediates their faster removal by macrophages from the site of infection and this favours the local protection of mammary tissue, whereas, in the CM, significantly ($P < 0.05$) lower expression facilitates slower removal of the apoptotic neutrophils from the site of infection and this may also cause secondary tissue damage at the site due to the release of their constituents. We were not able to find out the exact logic behind this and it requires further

investigation. From the study, we speculate that higher degree of tissue damage in the CM may be due to the longer retention of the neutrophils and release of their secondary constituents as compared to SCM group.

Present study focuses on the significant roles played by both blood and milk neutrophils during mastitis in Sahiwal cows in terms of expression of surface adhesion molecules and CD44. This was the first study in elucidating the host response against the *Staphylococcus aureus* infection in terms of recruitment of neutrophils. This study also clearly indicated the differential pattern of expression of various receptors which is dependent on the type as well as severity of mastitis. CD44 potentially regulates the removal of the neutrophils from the site of infection and a lower expression of CD44 on the surface of neutrophils can be a major cause behind the secondary inflammation as well as tissue damage during clinical mastitis due to their delayed removal. However, further studies demonstrating the probable mechanism behind the expression of CD44 on the surface of neutrophils and modulation of the macrophage function in the removal of the neutrophils from the site of infection are required to understand the dynamics of neutrophils. Further studies on the basic and molecular mechanisms involved in the differential expression of CD11b and CD62L are required to validate the specific and early signalling pathways associated with the onset and progression of *Staphylococcal* mastitis.

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