Phagocyte immune thresholds in bovine mastitis

PHAGOCYTE NON-PROTECTIVE AND PROTECTIVE RESPONSES IN BOVINE MAMMARY INFECTIONS

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SUMMARY

The presence of phagocyte-related cut-off points that distinguish protective from non-protective anti-
bacterial responses was investigated in bovine mastitis. Five lactating cows were intra-mammarily
infused with *Staphylococcus aureus* and CD11b surface density (expressed as median fluorescence
intensity or MFI) was assessed (before and three times after challenge) in blood and milk phagocytes
by flow cytometry. CD11b MFI was compared to bacterial counts of milk cultures, the somatic cell
count (SCC), as well as the surface density of several lymphocyte differentiation antigens. In addition,
the ratio of phagocytes (monocytes or macrophages [MØ] plus polymorphonuclear cells [PMN]) to
lymphocytes, and the ratio of PMN to MØ, were determined. These measures differentiated non-
mastitic from mastitic animals, identified very early (1 day post-infusion or pi), early (1 week pi) and
late (2 weeks pi) inflammations, and revealed CD11b MFI cut-off points above which no bacterial
growth in milk cultures and SCC <500,000 cells/ml were observed ("immune thresholds"). For any
given cell type, identical thresholds were shown in relation to both outcome indicators. Lower critical
cut-off points (resulting in protective effects) were observed at 2 wpi than at 1 dpi. Immune thresholds
distinguished individuals prone to mount efficient immune responses from those unable to do so, and
also provided predictions on future outcomes based on pre-challenge measures. It is suggested that
measurement of immune thresholds may improve prognosis of, and animal selection against, bovine
mastitis.

Control of bovine mastitis has historically been based on outcome measures, such as the Somatic Cell
Count (SCC) and bacterial counts (Sargeant et al., 2001). However, some measures used in the current
paradigm seem to be inconsistent. For instance, chronic mastitis may present lower SCC than legal
cut-off points (false negative results) and –although PMN are regarded to be the predominant cell type
measured by the SCC—, the percentage of milk mononuclear cells may exceed that of PMN (Leitner
et al., 2000; Shoshani et al., 2000; Boulanger et al., 2003; Burton & Erskine, 2003; Leitner et al.,
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2003). It has also been reported that SCC and bacterial counts may not correlate. Milk cultures may
show no bacterial growth, even when the SCC is high and vice versa (Albenzio et al., 2002; Winter et
al, 2003). In order to overcome these limitations, a new model for comprehensive assessment of the
host-pathogen interactions involved in bovine mastitis may be required.

Assessment of host-pathogen interactions relates to the rapidity and efficacy to respond to
bacterial invasions. Since most bacteria associated with bovine mastitis can multiply, approximately,
every 30 minutes (Burton & Erskine, 2003), the time elapsed between the moment bacteria are first
engaged by antigen-presenting cells and the time the immune response actually begins (after
phagocytes arrive to the inflammation site and bacterial clearance is initiated), is critical. In other
species, responses involving memory/effector T cells may require between 30 minutes and 2 hours of
T cell receptor (TCR) stimulation, whereas those involving naïve T cells require much longer
stimulations (6 to 30 hours) before engaged TCR molecules can trigger immune responses
(Lanzavecchia & Sallusto, 2001). This results in differences of biological significance. For instance, if
10 bacteria invade the mammary gland, memory/effector T cells are engaged and 4 bacterial
replication cycles occur within 2 hours, it may be expected that the resulting immune response will
have to clear only 40 bacteria. In contrast, if naïve T cells have been involved or if sub-optimal (or
non-sustained) T-cell stimulation has occurred, it may take > 6 hours to generate an immune response
which then would have to face a number of bacteria in the order of millions.

The bacterial agent can also determine the outcome of the immune response. For instance, S.
aureus invasions of the bovine mammary gland appear to result in poor (if not absent) IL-2 de novo
mRNA transcription (Riollet et al., 2000; Alluwaimi et al., 2003). Therefore, it may be assumed that
S. aureus invasions induce sub-optimal immune responses. That is, a “slow” TCR triggering process
may occur due to lack of IL-2 mediated signaling, which translates as an excessive number of
bacterial multiplications. In such case, the number of bacteria to be cleared may exceed the ability of
the cow’s immune system and, consequently, microbes may succeed in colonizing mammary tissues.
However, a different outcome is also possible. Since the threshold required to achieve earlier and sustained immune responses depend on signals facilitated by co-stimulatory molecules (such as CD11b), higher expression of CD11b per phagocyte may result in fewer TCRs actually required to elicit protective responses (Mestas & Hughes, 2001). For instance, Macey & al (2001) demonstrated (in humans) that increased CD11b MFI on PMN results in increased phagocytosis of \textit{S. aureus}. Similar findings have been observed in dairy cows (Rivas et al., 2001a). In spite of poor IL-2 synthesis induced by \textit{S. aureus} invasions, enhanced pro-adhesion and co-stimulatory functions (i.e., increased CD11b expression) may lead to efficient immune responses. Hence, measurement of this molecule may identify individual animals able to clear bacteria before infection develops.

In other species, critical values of immune marker surface density appear to determine the outcome of host-pathogen interactions (Bikoue et al., 1996; Viola & Lanzavecchia, 1996). While the percentage and surface density of CD11b has been determined in bovines (Rivas et al., 2001a; Van Merris et al., 2002), interactions among markers, cell types, and/or time have not been evaluated. Accordingly, this study pursued four questions: i) whether immune marker density cut-off points can distinguish healthy from mastitic animals, ii) if so, whether they vary over time; iii) whether the time of the mastitis episode (i.e., early vs. late) can be estimated, and iv) whether individuals able to mount efficient immune responses may be differentiated from those unable to do so.

MATERIALS and METHODS

Animals

Five first-lactation, non-periparturient Holstein heifers were tested. They had no history of mastitis. At least three consecutive tests yielded no bacterial growth of specific pathogens from milk samples, and no individual mammary gland quarter showed somatic cell counts (SCC) > 200,000 cells/ml. Mastitic and non-mastitic animals were further identified by the relative ratio among leukocytes. When phagocytes predominated (the ratio of phagocytes [percentage of polymorphonuclear and
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...macrophages] was greater than the percentage of lymphocytes), mastitis was diagnosed. Absence of mastitis was concluded when lymphocytes predominated over phagocytes (Rivas et al., 2001b).

**Intra-mammary infusions**

*Staphylococcus aureus* ribotype 116-232-S3 (Rivas et al., 1997) was cultured in sterile Todd-Hewitt broth at 37°C until the exponential growth phase was reached. The number of colony-forming units (CFU) was determined, and cultures were diluted to 200 CFU/ml in sterile Todd-Hewitt broth and kept at 4°C until infused. After the morning milking on day 0, 1 ml of inoculum (200 CFU in Todd-Hewitt broth) was infused into each of the right front and left hind mammary gland quarters.

**Bacteriological analysis and SCC**

Decimal dilutions (100 µl each) of milk were cultured in triplicate onto blood agar plates and incubated at 37°C for 24 hours. The mean CFU in each replicate was determined as described (Rivas et al., 2001b). All *S. aureus* isolates recovered from milk of inoculated cows were the same ribotype as the inoculating strain (ribotype 116-232-S3). Duplicate somatic cell counts were determined with a cell counter at the North East Dairy Herd Improvement Association (Ithaca, New York, USA).

**Sample collection**

At least one liter of milk (collected at mid milking) was obtained before bacterial infusion (day 0), and after infusion. Three post-infusion times were selected in order to obtain samples representative of very early (1 day post-infusion or dpi), early (1 week pi or wpi) and late (2 wpi) cellular responses. Milk samples were transferred to sterile 1-liter bottles containing 10 ml (100X) of an antibiotic-antimycotic (penicillin, streptomycin, and fungizone) solution (# 15240-039, Gibco, Grand Island, NY, USA) and 12.5 µg/ml of gentamicin (Gibco). Blood samples were collected by tail vein venipuncture. Milk and blood were transported and stored at 4°C until analysis.
Isolation of blood and milk leukocytes

Blood cells were isolated by use of gradient centrifugation (Ficoll-Hypaque, Amersham-Pharmacia Biotech, Inc., Piscataway, NJ, USA), and washed 3 times. Milk leukocytes were isolated as described elsewhere (Rivas et al., 2001b). Briefly, milk was diluted (50:50 ratio) in PAE buffer (phosphate buffer solution or PBS with 10% acid citrate dextrose, 20mM EDTA and 0.1 sodium azide, pH 7.2) and centrifuged (350 X g) for 40 minutes at 15 C. The supernatant and fat layer were decanted, cells were washed 3 times in PAE buffer and then resuspended in 30 ml of Hank’s balanced salt solution (HBSS, Gibco). After being layered on a density gradient and centrifuged (800 X g) for 30 minutes at 15 C, cells were washed 3 times in HBSS containing 10% fetal bovine serum (FBS, Hy-Clone, Logan, Utah, USA), and resuspended in 5 ml of complete media (RPMI 1640 [Gibco] containing 10% FBS and 5% of a tissue culture cocktail [0.1 mM nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES buffer, and 1X antibiotic-antimycotic mix, Gibco]).

Immunophenotyping of blood and milk leukocytes

Leukocyte phenotypes were determined by flow cytometry with seven primary antibodies (isotype control, CD3, CD2, CD4, CD8, CD45r, and CD11b). Seven million blood or milk leukocytes were resuspended in PAE buffer containing 2% rabbit serum (first wash buffer) and centrifuged at 350 X g for 10 minutes. One million cells were then transferred to each of seven 12 x 75 mm polypropylene tubes and resuspended in 50 µl of 10% rabbit serum in PAE buffer (second wash buffer). After 10 minutes on ice, 50 µl of the appropriate primary antibody was added to each tube, and tubes were incubated for 30 minutes on ice. A negative isotype control antibody (mouse IgG1[catalog # 08-6599, Zymed, South San Francisco, CA, USA] diluted 1:25 in first wash buffer) was also used as a primary antibody. Mouse monoclonal antibodies against either bovine CD3, CD2, CD4, CD8, CD45r or CD11b (all IgG1 isotypes, VMRD Inc., Pullman, Washington, USA) were diluted 1:25 in the same
buffer. After incubation with primary antibodies, cells were washed in first wash buffer and incubated for 30 minutes at 4 C with 100 μl of FITC-conjugated rabbit anti-mouse IgG (heavy and light chains, Zymed) diluted 1:50 in second wash buffer. Cells were then washed 4 times with second wash buffer, fixed in 500 μl of 2% paraformaldehyde in PBS solution containing 0.1% sodium azide, and kept at 4 C until analyzed by use of flow cytometry (FACSCalibur, Becton-Dickinson, San Jose, California, USA). Cells were processed and fixed within 12 hours of collection.

Flow cytometry analysis

Cell types were identified on the basis of forward and side scatter, as described earlier (Rivas et al, 2001a). Backgating was conducted on T cells (CD3+) or non-T lymphocytes (CD3−), and the median fluorescence intensity (MFI) per cell was determined. At least 40 x 10^3 cells were acquired per test to obtain enough cells of the type least represented in each sample. Data were acquired and analyzed, using commercial flow cytometry software (Becton-Dickinson).

Statistical analyses

Six indices or measures were generated: 1) the ratio of percentage of milk phagocytes (PMN cells and macrophages) to percentage of milk lymphocytes (P/L index) (Rivas et al., 2001b), 2) the ratio of the percentage of milk PMN cells to the percentage of milk macrophages (P/M index), 3) the CD11b median fluorescence intensity on blood monocytes (blood MØ MFI), 4) the CD11b MFI on blood polymorphonuclear cells (blood PMN MFI), 5) the milk MØ MFI, and 6) the milk PMN MFI. Three-factor interactions were assessed with lowess-estimated data. Analysis of lymphocyte phenotypes is reported elsewhere (associated manuscript). Medians, correlation and regression coefficients, confidence intervals and lowess-based plots were determined with a software package (Minitab 12.2, Minitab, State College, PA, USA). For all tests, P < 0.05 was considered significant.
To test the null hypothesis of a common model for the relationship between the responses (SCC, CFU) and each of the 6 predictor variables at the 3 post-challenge time points (1 dpi, 1 and 2 wpi) against the alternative hypothesis of differences among the models at the 3 time points, a likelihood test was performed (Mood et al., 1974). This test was based on the difference between fitting a common gamma function and fitting 3 separate gamma functions to the data from the 3 time points. The gamma function was chosen as the appropriate model because of its shape, rising from the origin to its maximum value and then decreasing to response of 0, as the level of the predictor variable increases without bound.

RESULTS

Expression of CD11b phagocyte surface density in blood and milk

The density of CD11b (MFI) on milk phagocytes was greater at 1 dpi than before challenge, although not reaching statistical significance. Blood cells appeared to differ in terms of CD11b density. While blood monocytes showed increased density by 1 dpi, blood PMN did so by 1 wpi (Fig. 1). Post-infusion blood CD11b MFI was positively correlated with milk CD11b MFI in both phagocytes ($r > .75, P<0.002$). At least one animal showed values (both in blood and milk cells) beyond the upper limit of the 95% confidence interval for the mean (Tables 1 and 2).

Identification of mastitic vs. non-mastitic animals and estimation of inflammatory phase

The phagocyte/lymphocyte index (P/L I) showed earlier evidence of post-infusion immune/inflammatory changes than the SCC. The P/L I showed 2 significantly higher values at 1 dpi than before challenge ($P<0.004$), while the SCC revealed a similar increase in later observations (Fig. 2). While significant ($P=0.05$), the post-challenge correlation between SCC and CFU showed only a moderate relationship ($r=0.35$). When results were analyzed on individual time points, only one of three post-challenge CFU-SCC correlations (1 wpi) was statistically significant (not shown).
The P/L and P/M indices and those that estimated phagocyte activation (blood and milk CD11b MFI) provided cut-off points with 95% confidence intervals that differentiated mastitic from healthy animals (Table 2). Unlike the SCC, the six indices generated in this study distinguished mastitic (P/L I > 0.56) from healthy cows (P/LI < 0.55), identified early mastitis (those between 1 dpi and 1 wpi) when P/LI > 9.53 and P/M I > 1.85, while older mastitis (i.e., 2 wpi) could be suspected if P/L I was > 0.56 but < 9.52. Phagocyte CD11b MFI identified non-mastitic cows when blood MØ CD11b MFI < 94 and/or milk PMN CD11b MFI < 105.7.

One-day old mastitis was characterized by blood MØ MFI > 192.2, milk MØ MFI > 164.3, and/or milk PMN MFI < 203.5. When blood MØ MFI > 101.3, milk MØ MFI > 16.4, and/or milk PMN MFI > 105.7, early mastitis (1 dpi-1 wpi) was diagnosed. If blood PMN MFI > 129.7, the inflammation was estimated to be one-week old. When CD11b surface density values fell within the range of non-mastitic or 2 week-old mastitis, the P/L I provided the defining diagnostic (if > 0.55, a 2-week old mastitis would be diagnosed).

Estimation of immune cut-off points differentiating mastitic from non-mastitic animals

An exploratory, 2-dimensional data analysis involving all post-infusion observations suggested threshold-like immune responses. Neither bacterial growth nor SCC greater than 500,000 cells/ml were found in milk cultures from cows showing CD11b density per cell greater than certain critical value (vertical broken line, Fig. 3). The threshold cut-off point was the same for both outcome indicators (bacterial counts [CFU] and leukocyte counts [SCC]). Similar or identical inflexion points (where a quasi-horizontal line approximated 0 bacterial counts) were observed both by curves that directly connected those points and those estimated by lowess (Fig. 4).

Based on this validation, three-factor relationships (including pairs of predictor [immune] factors and one outcome factor) were explored with lowess-adjusted estimates (Fig. 5). Below certain critical value (the "immune threshold"), infection predominated (i.e., bacteria were isolated from milk...
cultures), which was expressed as a peak above the plot surface. At greater values, protection predominated (no bacterial counts or leukocyte counts <500,000 cells/ml), which was expressed as a flat plot surface or depression under that surface. Thresholds were further characterized by 2 inflexion points: i) the early point at which the outcome initially observed (i.e., infection) began to reverse into the alternative outcome; and ii) the final inflexion point at which the complete effect of the alternative outcome was shown (i.e., total protection, expressed as no bacterial counts [Fig. 5A]). The same threshold seen in relation to CFU at 1 dpi was observed in relation to SCC, although displaying the opposite outcome profile (i.e., decreases in CFU were associated with increases in SCC, Figs. 5A, B).

Interactions between leukocytes

Positive linear relationships were observed at 1 dpi and 1 wpi between the macrophage CD11b density and that of PMN. No linear relationship was observed between these cells at 2 wpi (not shown). Immune thresholds were also suggested by interactions between phagocyte and lymphocyte markers (raw data reported in the accompanying article). The density of CD11b on milk lymphocytes and macrophages showed a critical value at 1dpi (approximately 400 MFI units for macrophages and 120 MFI units for lymphocytes) above which no bacterial counts were observed (Fig. 6 B), value lower than that seen in relation to CD3 (Fig. 6 A). No protective thresholds were noted with CD4 and CD45r (not shown). At 1 dpi, macrophages showed opposite patterns than PMN in relation to CD2 and CD8. While PMN showed a low threshold in relation to CD2 (Fig. 6 G), MØ displayed protective effects at a higher threshold when interacted with the same marker (Fig. 6 C). In relation to CD8, MØ induced protection while PMN did not (Figs. 6 D, H).

Interactions between bacterial and leukocyte counts

All interactions involving milk phagocytes and lymphocytes showed opposite patterns in relation to CFU and SCC at 1 dpi (i.e., Fig. 5). In contrast, at 1 wpi, identical profiles were showed by bacterial
and leukocyte counts, as shown in Fig. 7 A, B, E, F. Opposite slopes (although not linearly related) were observed between bacterial and leukocyte counts at 2 wpi (Fig. 7 C, D, G, H).

Longitudinal thresholds

The relationship between the 6 predictors and the 2 responses was modeled as a curve with the shape of a gamma function. The null hypothesis of a common model at the 3 post-infusion times (i.e., no change in threshold over time) was tested against the alternative hypothesis of differences at the 3 observation times (i.e., different threshold values over time), using a likelihood ratio test. The null hypothesis was rejected in 9 of the 12 tests. This indicated that decreased thresholds were observed as the time since the immune response began, progressed (i.e., over time, less marker density expression was required to achieve protective effects). Both milk phagocytes displayed significantly lower thresholds at 1 or 2 wpi than at 1 dpi ($P<0.01$, Table 3).

Decreased protective thresholds were observed over time. The analysis of curves generated by fitted data at 1 dpi, 1 and 2 wpi showed lower immune thresholds over time. Both milk phagocytes displayed significantly lower 1 or 2 wpi thresholds than the 1 dpi threshold ($P<0.01$, Table 3).

Three-factor assessments (which included interactions between phagocytes and lymphocytes) suggested additional threshold reductions over time. For example, the interaction between macrophage CD11b MFI and that of lymphocyte CD3 MFI resulted, at 1 dpi, in a protective threshold at, approximately, 600 MFI units (for MO) and 180 MFI units for lymphocytes (Fig. 6 A), whereas, one week later, a threshold was shown at around 150 and 70 MFI units (Fig. 7 A) and, at 2 wpi, an even lower threshold was seen (Fig. C). A linear relationship among longitudinal thresholds was suggested by milk lymphocyte CD3 MFI and milk macrophage CD11b MFI interactions ($R^2$ adjusted$= .93, P< 0.001$, Fig. 8 A), as well as between PMN CD11b MFI and lymphocyte markers (not shown). They indicated that the involvement of phagocyte and lymphocyte markers may decrease even further the threshold required to achieve full protection. For instance, a protective threshold
observed at 1 dpi at, approximately, 200 CD11b MFI units (for PMN) and 80 CD4 MFI units (for lymphocytes) (Fig. 5A), one week later was reduced to 150 and 70 MFI units, respectively (Fig. 7 E). However, no further decreases were observed later. The 2 wpi PMN threshold was not lower than that seen at 1 wpi (Fig. 7 G).

Predictions on future outcomes to bacterial exposure

As indicated by a linear relationship, milk macrophage CD11b MFI could be predicted, at 1 dpi, from blood monocyte thresholds (Fig. 9 A). Consequently, the pre-infusion value of this marker, on blood monocytes, was compared to that displayed at 1 dpi. It showed a positive and statistically significant correlation ($r = 0.985, P < 0.001$, Fig. 9 B), which was negatively associated with bacterial counts. This indicated that animals showing the greatest pre-infusion CD11b surface density on blood mononuclear cells were the same ones showing the greatest MFI after challenge. Above a critical value (which, under the conditions of this protocol, corresponded to approximately, 70 MFI units per blood monocyte in non-challenged animals), this measure predicted bacterial clearance at 1 dpi.

DISCUSSION

This study provided: i) measures that identified non-inflammed from inflammed animals, ii) indices that distinguished early from late inflammatory processes, and iii) cut-off values that differentiated protective from non-protective responses (thresholds). Leukocyte counts (SCC) indicated a threshold-like response around 500,000 cells/ml (Fig. 3). SCC greater than half a million cells/ml are indicative of infectious mastitis (O'Sullivan et al., 1992). While these conclusions were supported on analysis of 2-factor interactions (Figs. 1-4 and Table 3), lowess-based modeling of the data provided 3-factor, explanatory approaches such that pairs of predictor (immune factors) and outcome factors (i.e., bacterial counts) could be assessed at individual time points. As shown in this study (Fig. 4), locally
weighted robust regression (lowess) results in nearly unbiased parameter estimates and improved statistical power (Borkowf et al., 2003).

Identical phagocyte thresholds were observed at 1 dpi in relation to both outcome indicators. Previous studies have indicated identical thresholds regardless of the variable being measured (i.e., cytokine production or cell proliferation) (Viola & Lanzavecchia, 1996). While those reports measured T cells, this study expanded those findings to phagocytes as well.

Although both phagocytes showed no or very high thresholds in relation to lymphocyte CD3 (Fig. 6 A, E), lower thresholds resulted from interactions involving lymphocyte CD11b (Fig. 6 D, F), as well as other non-CD3 lymphocyte markers (Fig. 6 D). These findings are consistent with immune "tunable thresholds" mediated by non-CD3 markers (Grossman et al., 2001; Tanchot et al., 2001), being CD11b a critical facilitator of lymphocyte adhesion to endothelia and migration, which may lead to lower threshold for antigen recognition (Randriamampita et al., 2003).

These data also showed positive correlations between PMN CD11b MFI and lymphocyte CD4 and CD2 MFI (Figs. 5 A, 6 G) that resulted in very low thresholds. This suggests that PMN activation may influence early (1 dpi-1 wpi) T cell responses. In other species, PMN enzymes have been reported to modulate immune responses (Bank & Ansorge, 2003).

Lymphocyte CD8 (at 1 dpi) appeared to interact more with MØ than PMN, as indicated by a protective threshold (observed with the former but not seen with the latter, Fig. 6 D, H). Collaborative associations between CD8+ lymphocytes and macrophages have recently been reported (Gurlo & von Grafenstein, 2003). The brief linear relationship between lymphocyte CD11b and PMN CD11b (not observed at 2 wpi [Fig. 8 C, E]) is consistent with the short lifespan shown by activated PMN. The initial recruitment of PMN to the inflammatory site is followed by new waves of neutrophils of less mature ability (Lanzavecchia & Sallusto, 2001).

The fact that early (1-day to 1-week post-challenge) but not late (2 wpi) cellular responses (as expressed by PMN CD11b MFI-lymphocyte CD11b MFI interactions) showed protective effects,
together with the fact that SCC did not correlate with bacterial counts at 2 of the three post-infusion observations, indicated that measurement of predictor (i.e., immune factors) or response factors (i.e., SCC), whether alone or together, are enough to predict immune outcomes. Estimation of the time/phase of the inflammation (i.e., early vs. late) is also needed. The effect of time on immune outcome could be observed on individual animals. Cows “C” and “D” (Table 1) seemed to be poor responders (anergic), as suggested by the fact that at 1 dpi their milk PMN CD11b MFI was lower than their pre-infusion values. Because lymphocytes may become anergic within a few days after becoming activated (Lanzavecchia & Sallusto, 2001; Knudsen et al., 2002, Schwartz, 2003), a pre- and post-intervention measurement of CD11b surface density (a test that assesses a response over time) may be needed to evaluate anergy/immune responsiveness.

In contrast to immune factors, SCC did not provide estimates for post-challenge time. In addition, SCC did not differentiate between efficient responses (those resulting in bacterial clearance) from inefficient ones (those resulting in mastitis): SCC was not significantly correlated with bacterial counts in 2 of 3 post-challenge observations. The discrepancy observed between SCC and bacterial counts is consistent with three events that take place in host-pathogen interactions. They are: i) bacterial invasion, ii) bacterial multiplication, and iii) bacterial colonization of mammary tissues. No infection (tissue colonization) is expected to occur when the immune response prevents bacterial multiplication. As observed in this and other studies, experimental bacterial challenge may result in no post-challenge isolation of bacteria in milk culture even when the SCC increases (Albenzio et al., 2002; Winter et al., 2003). It is hypothesized that, although cows may encounter some bacteria on daily basis (bacterial invasion), many animals manage to eliminate microbes before they can multiply, becoming infected only those that fail to clear bacteria before microbes multiply.

A linear and significant relationship involving macrophage-associated thresholds (Fig. 8), suggested that phagocyte-associated immune thresholds do not vary at random. Assessment of earlier phases of the immune response predicted later outcomes. Given the high and positive correlation
observed between pre-infusion and 1 day post-infusion blood monocyte CD11b MFI (Fig. 9), it is suggested that testing of blood cells (even in non-challenged animals) may predict post-challenge outcomes. Findings consistent with the hypothesis of efficient protective responses ("high" responders) were also observed. For instance, cows “A” and “B” (Table 1 & 2) showed 2-fold or greater post-challenge (1 dpi) than pre-challenge mononuclear phagocyte CD11b density and no bacterial counts were recorded, which is compatible with (immune-mediated) bacterial clearance completed before bacterial multiplication took place.

These findings represent, to the best of our knowledge, the first evidence of bovine phagocyte immune thresholds. Previous research on immune thresholds, in other species, has focused on antigen concentration, avidity, cytokine synthesis, duration of TCR stimulation, or distribution of participating molecules ("polarization") on the leukocyte surface (Bitmansour et al., 2002; Langenkamp et al., 2002; Štefanová et al., 2002). This study expanded those assessments by focusing on the density of leukocyte cell surface molecules as the predictive variable for immune outcomes. While additional variables (i.e., cytokines) and larger sample sizes would be required to further validate this model, it is concluded that it provides an informative view of the bovine’s ability to respond to bacterial mastitis. If applied to individual animals, it may be used for diagnostic and selection purposes, and, if used collectively, for evaluation of therapies against bovine mastitis.
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Table 1. Leukocyte ratios and cell function in blood and milk phagocytes.

<table>
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<th>Cow, day</th>
<th>Ct MFI</th>
<th>MØ MFI</th>
<th>Ct MFI</th>
<th>Pmn MFI</th>
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<th>Pmn %</th>
<th>MØ %</th>
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LØ: lymphocyte. MØ: monocyte/macrophage. PMN: polymorphonuclear cells. Ct: isotype (negative control) antibody. P/L Index: ratio of phagocytes (percentage of milk PMN and mononuclear phagocytes) to percentage of milk lymphocytes. PMN/ MØ Index: milk PMN/ MØ ratio. b: blood. m: milk. SCC: somatic cell counts (1 x 10³ cells/ml). CFU: bacterial counts in milk cultures (colony forming units/ml). Day(s): 0: pre-bacterial infusion; 1 dpi: 1 day post-infusion; 1, 2 wpi: 1, 2 week(s) post-infusion.
Table 2. Time-adjusted means, medians and intervals (95% confidence) of count indices and CD11b molecules (MFI) associated with *S. aureus* mastitis. Post-infusion data are those of 1 day post-infusion (1 dpi), 1 week and 2 weeks post-infusion (1 wpi, 2 wpi).

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<th>Indices</th>
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P/L I < 0.55: non-inflamed. P/L I > 0.56: inflamed. P/L I >9.53: early infection (1 dpi-1 wpi). If P/L I > 0.56 but <9.52: late infection (2 wpi). If blood MØ MFI > 192.2, and/or milk MØ MFI > 164.3, and/or milk PMN MFI <203.5: very early mastitis (1 dpi). If blood MØ MFI > 101.3, and/or milk MØ MFI > 114.5, and/or milk PMN MFI >105.7: early mastitis (1 dpi-1 wpi). If blood PMN MFI > 129.7: 1 wpi.
Table 3. Likelihood ratio test statistics (LR)

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# MØ = monocytes/macrophages; PMN = polymorphonuclear cells.

Hypotheses and critical values: i) Ho: there is a common cubic polynomial model for all three post-infusion (pi) times (1 day pi, 7 days pi, and 14 days pi); ii) Ha: there are differences among the three times in the cubic polynomial model coefficients; and iii) critical values are chi-square (6,.95) = 12.5916, and chi-square (6,.99) = 16.8119.

* Significant at p=0.05

**: Significant at p=0.001
LEGENDS

Fig. 1. Longitudinal expression of CD11b in experimental S. aureus-induced mastitis. Boxplots indicate median fluorescence intensity (MFI) per cell and 25 and 75 percentiles (lower, upper external horizontal lines) and 5 and 95 percentiles (lower, upper vertical lines) in blood monocytes (A), blood polymorphonuclear (PMN) cells (B), milk macrophages (C) and milk PMN (D).

Fig. 2. Longitudinal leukocyte count-related indices in experimental S. aureus-induced mastitis. Somatic cell counts (A), milk phagocyte/lymphocyte index (B) and milk PMN/macrophage index (C). Boxplots indicate median, 25 and 75 percentiles (inside and [lower, upper] external horizontal lines) and 5 and 95 percentiles (lower, upper vertical lines) obtained at pre-infusion, 1 day, 1 week and 2 weeks post-infusion (d/wpi) (n=5).

Fig. 3. Relationships involving post-infusion CD11b expression on blood and milk phagocytes. Fifteen post-infusion observations (one from each of the 5 animals, at each of the 3 time points) indicate a threshold effect between CD11b vs. bacterial counts (colony-forming units per ml or CFU) and SCC (1 x 10^3 cells/ml). CFU-related data are shown in A, C, E, G, and SCC-related data in B, D, F, H.

Fig. 4. Relationship between non-transformed and lowess-estimated data on phagocyte CD11b MFI and bacterial counts (CFU/ml). Curves resulting from connecting non-transformed observations at individual time points (“connect”, A-H) were identical to those generated by lowess at the same time points (not shown) and similar to all post-challenge lowess-fitted curves (“lowess”, I, J).
Fig. 5. Three-dimensional host-pathogen relationships. The graph shows an example of one day post-infusion (dpi) responses measured on 5 cows, which are expressed as bacterial counts (CFU, A) or leukocyte counts (SCC, B). They display: i) a two-factor relationship involving leukocyte surface marker density per cell (expressed as percentage of milk PMN CD11b MFI and milk lymphocyte CD4 MFI), and ii) the net outcome resulting from the overall predictor-outcome factors. In this example, the relationship between the 2 leukocyte markers is positive and linear (increases in one factor [i.e., the surface density of CD11b on milk PMN] result in increases in the other factor [i.e., the surface density of CD4 on milk lymphocytes]). The relationship between immune factors and the outcome factor (i.e., CFU) shows a threshold (vertical line) above which a protective effect is displayed (no bacterial counts are observed) is noticed. The same threshold is observed in relation to both outcome variables (CFU, SCC). The “immune threshold” can be characterized by two inflexion points: i) the point above which the effect reverses (i.e., the early inflexion point indicates where the non-protective effect begins to become protective), and ii) the point at which the final effect is achieved (i.e., when protection [0 bacterial counts] is complete).

Fig. 6. Three-factor interactions involving 1 day post-infusion CD11b MFI on milk phagocytes, selected lymphocyte markers and outcome indicators (CFU) (n=5). The protective effect of CD11b is suggested by the fact that interactions involving macrophage CD11b MFI and lymphocyte CD3 MFI (A) showed higher protective thresholds than those involving CD11b MFI on both cell types (B). Macrophages and PMN differed in their effects. Unlike macrophages, PMN CD11b MFI did not show a protective threshold in relation to lymphocyte CD3 MFI (E), but a much lower (although not throughout its entire range) when interacted with lymphocyte CD11b density (F). While the expression of CD11b on macrophage surfaces resulted in a very low threshold when interacted with lymphocyte CD8 (D), PMN CD11b MFI did not show a protective threshold in relation to the same
lymphocyte marker (H). In contrast, the PMN CD11b density displayed a very low threshold in relation to lymphocyte CD2 MFI (G), effect not shown by macrophages (C).

Fig. 7. Three-factor interactions involving 1 and 2 week(s) post-infusion CD11b MFI on milk phagocytes, lymphocyte CD3 MFI and outcome indicators (CFU and SCC) (n=5). Identical outcome profiles are observed at 1 wpi in relation to bacterial and leukocyte counts, both by macrophages and PMN (A, B, E, F). Neither evidence of linear relationships between immune factors nor identical outcome profiles are observed at 2 wpi (C, D, G, H).

Fig. 8. Longitudinal relationships involving immune thresholds. A: Linear relationship generated by milk lymphocyte CD3 MFI and milk macrophage CD11b thresholds (based on data depicted in Figs. 6 & 7). Regression coefficient (R^2 [adjusted]): 0.929 (P<0.001). PMN appeared to show a lower threshold in relation to CD4 at 1 wpi (B) than at 1 dpi (Fig. 5 B), but neither a lower threshold nor a linear relationship involving this marker were observed at 2 wpi (C). Responses involving CD11b both in PMN and lymphocytes did not show lower thresholds at 1 or 2 wpi (D, E) than at 1 dpi (Fig. 5 F).

Fig. 9. Relationship between pre-infusion/post-infusion blood monocyte CD11b MFI and bacterial counts. Positive correlations are observed between post-infusion (1 dpi) blood versus milk mononuclear phagocyte CD11b MFI (A), and between pre-infusion and post-infusion (1dpi) values of blood cells (B). Pre-infusion CD11b MFI on blood cells was negatively associated with bacterial counts reported at 1 dpi. This indicates that measurement of immune thresholds in individual animals (and, consequently, future outcomes after exposure to bacterial invasion), may be predicted from a blood test.
Phagocyte immune thresholds in bovine mastitis

![Graphs showing SCC, Phagocyte/Lymphocyte Index, and PMN/Macrophage Index over time: Pre-infusion, 1 dpi, 1 wpi, 2 wpi.](image-url)
Phagocyte immune thresholds in bovine mastitis

Fig. 3

A

Blood monocytes

CFU

Milk macrophages

B

CD11b MFI

C

Blood monocytes

SCC

Milk macrophages

D

CD11b MFI

E

Blood PMN

CFU

Milk PMN

F

CD11b MFI

G

Blood PMN

SCC

Milk PMN

H

CD11b MFI
Phagocyte immune thresholds in bovine mastitis

Fig 4

A

1 dpi CFU

Connect, 1dpi milk macrophage CD11b MFI

B

1 dpi CFU

Connect, 1dpi Pmn CD11b MFI

C

1 wpi CFU

Connect, 1wpi milk macrophage CD11b MFI

D

1 wpi CFU

Connect, 1wpi milk Pmn CD11b MFI

E

2 wpi CFU

Connect, 2wpi milk macrophage CD11b MFI

F

2 wpi CFU

Connect, 2wpi milk Pmn CD11b MFI

G

Post-challenge CFU

Post-challenge milk macrophage CD11b MFI

H

Post-challenge CFU

Post-challenge milk Pmn CD11b MFI

I

Lowess, post-challenge milk macrophage CD11b MFI

J

Lowess, post-challenge milk Pmn CD11b MFI
Phagocyte immune thresholds in bovine mastitis

Fig 5

Threshold

Protection

Positive linear relationship

Early inflexion point

Final inflexion point

1 dpi CFU

1 dpi milk PMN CD11b MFI

1 dpi milk lymphocyte CD4 MFI

1 dpi milk PMN CD11b MFI

1 dpi milk lymphocyte CD4 MFI

1 dpi SCC
Phagocyte immune thresholds in bovine mastitis

Fig 6

A. 600 MFI (MØ) 180 MFI (LØ)

B. 400 MFI (MØ) 120 MFI (LØ)

C. 100 MFI (MØ) 50 MFI (LØ)

D. 200 MFI (PMN) 50 MFI (LØ)

E. 150 MFI PMN) 70 MFI (LØ)

F. 100 MFI (MØ) 50 MFI (LØ)

G. 150 MFI PMN) 70 MFI (LØ)

H. 150 MFI PMN) 70 MFI (LØ)
Phagocyte immune thresholds in bovine mastitis

Fig 7

A

1 wpi CFU
1 wpi milk macrophage CD11b MFI
1 wpi milk lymphocyte CD3 MFI

B

1 wpi SCC
1 wpi milk macrophage CD11b MFI
1 wpi milk lymphocyte CD3 MFI

C

2 wpi CFU
2 wpi milk macrophage CD11b MFI
2 wpi milk lymphocyte CD3 MFI

D

2 wpi SCC
2 wpi milk macrophage CD11b MFI
2 wpi milk lymphocyte CD3 MFI

E

1 wpi CFU
1 wpi milk PMN CD11b MFI
1 wpi milk lymphocyte CD3 MFI

F

1 wpi SCC
1 wpi milk PMN CD11b MFI
1 wpi milk lymphocyte CD3 MFI

G

2 wpi CFU
2 wpi milk PMN CD11b MFI
2 wpi milk lymphocyte CD3 MFI

H

2 wpi SCC
2 wpi milk PMN CD11b MFI
2 wpi milk lymphocyte CD3 MFI
Phagocyte immune thresholds in bovine mastitis

Fig 8

A

Milk macrophage CD11b MFI

Milk lymphocyte CD3 MFI

1 wpi

1 dpi

2 wpi

B

1 wpi CFU

2 wpi CFU

1 wpi milk PMN

CD11b MFI

1 wpi milk lymphocyte

CD4 MFI

2 wpi milk PMN

CD11b MFI

2 wpi milk lymphocyte

CD4 MFI

D

1 wpi CFU

2 wpi CFU

1 wpi milk PMN

CD11b MFI

2 wpi milk PMN

CD11b MFI
Phagocyte immune thresholds in bovine mastitis

Fig 9

A

1 dpi CFU
1 dpi blood monocyte CD11b MFI
1 dpi milk macrophage CD11b MFI

B

1 dpi CFU
Pre-infusion blood monocyte CD11b MFI