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Review Article

Role of neutrophils in protection of udder from infection in high yielding dairy cows

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Summary

Protection of the mammary gland against mastitis-causing pathogens is mediated by many factors in the gland and blood circulation. The professional phagocytic cells of bovine udder, polymorphonuclear neutrophils (PMN) and macrophages, comprise the first line of defense against invading mastitis pathogens. Most researchers now accept that the PMN is a key factor in the cows' defense against intramammary infections. The PMN are the only leukocytes in the milk compartment that are capable of producing large amounts of reactive oxygen species (ROS) to kill phagocytosed bacteria. In this review, the role of PMN function as an effective defense against intramammary pathogens in dairy cows and physiopathological influencing factors on blood and milk PMN functions are discussed. Apart from playing a crucial role in the first line of defense mechanism, the PMN can also, indirectly, interfere with the complex interactions of second line of defense against pathogens. To minimize mammary tissue damage caused by bacterial toxins and oxidative products released by PMN, elimination of invading bacteria should proceed quickly. This can provide balance among inflammation reactions, bactericidal activity and tissue damage. The good balance between host-pathogen interactions might be affected by the physiological (e.g., stage and number of lactation) and pathological (e.g., local-systemic effect of mastitis) status of dairy cows. Hormones, metabolites and acute phase proteins also influence PMN functions, thereby affecting the outcome of mastitis. This is especially the case around parturition. PMN function in healthy cows after parturition is highly heritable and has been related to the cow's susceptibility to clinical mastitis. Despite advances in veterinary science, nutrition and molecular biology, mastitis is still a very big problem in high yielding dairy cows. The long-term and fundamental solution for mastitis affecting high yielding dairy cows is to strengthen cows' immune systems by means of attainable physio-immunological approaches. This requires a comprehensive study on the immunophysiological alterations throughout lactation and during mastitis. This review focuses on some factors affecting PMN functions during the lactation cycle and mastitis in high yielding dairy cows.

Key words: Dairy cows, Lactation, Mastitis, Neutrophils, Immunity

Introduction

PMN are the primary mobile phagocytes of the immune system. Their importance for non-specific defense of the mammary gland has long been a crucial concern in high yielding dairy cows (Burvenich *et al*., 2003; Mehrzad *et al*., 2004, 2005a). Clearly, PMN chemotaxis, diapedesis, phagocytosis, and eventually microbicidal activity each contributes to the ability of the PMN to provide an effective first line defense for the mammary gland. Any condition that depresses PMN functions adversely affects the udder's resistance to invasive infections (Burvenich *et al*., 2003; Mehrzad *et al*., 2004, 2005a, 2007, 2008a, 2009; Mehrzad and Zhao, 2008b). The PMN ROS production can be quantified following stimulation with soluble agents, e.g. PMA or

with particles e.g. zymosan, bacteria, or latex beads. The most widely used technique to quantify bovine PMN ROS production is chemiluminescence (CL) (Mehrzad *et al*., 2000a, b, 2001a, b, 2002a, b, 2004, 2005a, b, 2009). As phagocytosis-induced and/or non-induced CL reflects intracellular and extracellular oxidation-reduction reactions (Mehrzad *et al*., 2001a, 2005a, 2009), changes might offer some evidence about the cow's susceptibility to early lactation-related infections. The mammary gland is an extremely important organ from an economical, immunological and nutritional point of view and mastitis is one of the crucial lactation-related diseases (Paape *et al*., 2002; Burvenich *et al*., 2003). Impairment of PMN, originating from the bone marrow, is a peculiar feature during the periparturient period (Hoeben *et al*., 2000a; Burvenich *et al*., 2003). This impairment might be cumulative upon influx into the milk (Mehrzad *et al*., 2001a, 2004, 2005a, 2008a, 2009). Generalised PMN impairment can be multifactorial, e.g. due to metabolic (Suriyasathaporn *et al*., 1999) and hormonal (Suriyasathaporn *et al*., 2000) changes. Another crucial aspect of mastitis is the viability of PMN, which is apparently influenced by the pathological conditions of the gland (Mehrzad *et al*., 2001a, 2004, 2005a, b).

Several antimicrobial systems exist in the mammary gland (Mehrzad *et al*., 2001a, 2004, 2005a, b, 2009; Paape *et al*., 2002; Burvenich *et al*., 2003). Nevertheless, the presence of PMN in milk provides a central natural defense for the gland (Mehrzad *et al*., 2001a, 2004, 2005a; Burvenich *et al*., 2003). Under both clinical and experimental conditions, mastitis cows show a large variability in illness and a wide range of pathological responses (Heyneman *et al*., 1990; Mehrzad *et al*., 2001a, 2004, 2005a, b, 2008a, 2009; Mehrzad and Zhao, 2008b). There are many unanswered questions on the impact of host factors on the milk and blood PMN functions. One of the key aspects in milk PMN function is their quality and quantity in the mammary gland. In addition to a sustainably constant hematopoeigenesis and PMN recruitment into the milk, the quality and quantity of PMN in milk greatly rely on the dynamics of hematopoeigenesis and PMN diapedesis into the udder, which are dependent on the physiological and mastitis conditions of dairy cow. However, many issues related to hematopoeigenesis in bone marrow and PMN diapedesis remain unclear, and it is still far from conclusive whether mammary gland PMN functionalities can make a huge difference in response to invading pathogens. This review focuses on some factors affecting PMN functions during the lactation cycle and mastitis in high yielding dairy cows.

Cells in normal milk/mammary gland

Mastitis researchers have long recognized the different types of cells found in bovine milk. Cells in normal mammary gland include PMN, lymphocyte, macrophage, epithelial cells and sometimes eosinophil. The PMN percentage in normal milk varies from 10 to 100%, depending on the gland status (Suriyasathaporn *et al*., 2000; Paape *et al*., 2002; Burvenich *et al*., 2003; Dosogne *et al*., 2003). Total milk SCC for uninfected mammary quarters are almost always less than 200×10^3/ml. Somatic cells/ml include PMN (12%), lymphocytes (28%), macrophage (58%) and epithelial cells (2%) (Paape *et al*., 2002; Burvenich *et al*., 2003). Some have reported counts consistently less than 100×10^3 cells/ml with some counts as low as 10×10^3/ml (Mehrzad *et al*., 2005a, b). Phagocytes, consisting of PMN (short-living; on average a few hours) and macrophages (long-living; on average two months), ingest and kill mastitis pathogens. Macrophages are the predominant cells in normal bovine milk and constitute between 30 to 74% of the total cell population in milk from uninfected quarters (Mehrzad *et al*., 2001a, b; Burvenich *et al*., 2003). The high variation in the concentration of this cell type is probably related to the definitions of macrophage and monocyte that are used in literature by different authors (Burvenich *et al*., 2003; Dosogne *et al*., 2003). Resident macrophages are defined as cells present in the mammary gland in the absence of any inflammation. In contrast, elicited

macrophages migrate into the tissue upon inflammatory stimulus and, in general, it is known that their number can be significantly elevated during a long period when recovering from an inflammatory reaction. Armed with many pattern recognition receptors (PPR), the macrophages act as sentinel cells against invading microbes, releasing chemoattractants that cause a rapid influx of PMN. Resident macrophages are also capable of differentiation into an activated macrophage which is able to kill many intracellular pathogens. Interferon-γ and tumor necrosis factor-α (TNF-α) are both macrophage activating factors. Macrophages may host intracellular pathogens such as *Staphylococci*. In uninfected mammary quarters PMN constitute 12 to 26% of the total cell population. In infected mammary quarters the PMN are the predominant cell type; their numbers can account for 98% of the cell population in acutely infected quarters (Mehrzad *et al*., 2001b, 2004, 2005a; Vangroenweghe *et al*., 2001, 2002; Burvenich *et al*., 2003; Dosogne *et al*., 2003). Because PMN and macrophages are eager ingestors of milk fat globules and casein, their morphology in milk is quite striking in contrast to their appearance in blood (Mehrzad *et al*., 2001a, b, 2004, 2005a), and PMN are highly effective at recognizing, ingesting, and killing microorganisms.

The continuous migration of phagocytes into mammary tissue provides a continuous renewal of the cellular part of the first immunological line of defense against bacterial invasion. They respond to inflammation signals to enter sites of infection and are professional killers, which are generally not a host for intracellular pathogens. Lymphocytes are crucial to the host defense of the mammary gland (Concha *et al*., 1980; Burvenich *et al*., 2003; Mehrzad *et al*., 2008a; Mehrzad and Zhao, 2008b), and maintain a steady state of induction versus suppression of the immune response in the mammary gland (Mehrzad *et al*., 2008a). Concha *et al*. (1980) found 20% B cells and 47% T cells in normal milk and 28% B cells and 47% T cells in dry secretions. During late lactation the percentage of PMN tends to increase while the percentage of lymphocytes decreases (Dosogne *et al*., 2003). Milk also contains expelled mammary epithelial cells, and anti-human cytokeratin can be used as a marker of these cells in milk (Paape *et al*., 2002; Burvenich *et al*., 2003). Recently, reproducible standard flow cytometric procedures were developed to determine differential leukocyte count of bovine milk (Mehrzad *et al*., 2001a, b, 2008a; Dosogne *et al*., 2003).

In milk with low SCC, four major leukocyte populations could be identified: lymphocytes and monocytes, PMN, mature macrophages, and cells with apoptotic features. PMN and macrophage percentages were the lowest and lymphocytes percentage were the highest in early lactation. The percentage of cells with apoptotic features was higher in early lactation than in mid and late lactation (Mehrzad *et al*., 2001a, b; Dosogne *et al*., 2003). Quarters with SCC>$\log_{10}$ 5.4 were found to have higher mononuclear cells and PMN count, and were more often culture positive compared with quarters with SCC<$\log_{10}$ 5.4. Quarters that were bacteriologically positive on the three test occasions had a higher proportion of PMN (33 to 49%) compared with quarters that were culture negative on all three test occasions (17 to 25%). Differential inflammatory cell count in milk has the potential to represent a new technique for evaluation of udder health status (Mehrzad *et al*., 2001a, 2008a; Paape *et al*., 2002; Burvenich *et al*., 2003; Dosogne *et al*., 2003).

Since PMN are the predominant cells in mastitis milk, regarding their functions and structure, there are plenty of fundamental studies available in the literature (Paape and Guidry, 1977; Paape *et al*., 2002; Mehrzad *et al*., 2002a, 2004, 2005a, b, 2009; Burvenich *et al*., 2003). The cell is delineated by a plasma membrane with surface microvilli that has a number of functionally important receptors such as L-selectin and β_2-integrin adhesion molecules, Toll-like receptors, etc. (Dosogne *et al*., 1997; Lee and Kehrli, 1998; Paape *et al*., 2002; Burvenich *et al*., 2003; Diez-Fraille *et al*., 2004; Sohn *et al*., 2007a, b). The most prominent characteristic of the PMN is the multilobulated nucleus; this structure is

important because it allows the PMN to line up its nuclear lobes in a thin line, allowing rapid diapedesis (Paape *et al*., 2002; Burvenich *et al*., 2003). Within the cytoplasm there are isles of glycogen with azurophilic (primary) and specific (secondary) granules as well as tertiary or dense (large) granules. PMN granules also contain β-defensins and most of the cellular antimicrobial protein activity (Mehrzad *et al*., 2005b), termed bacterecins, i.e. members of the cathelicidin family that are antimicrobial peptides that undergo proteolytic processing during phagocytosis. The most important antibacterial mechanism derived from azurophilic granules is the MPO-hydrogen peroxide-halide system (Mehrzad *et al*., 2001a, b, c; Burvenich *et al*., 2003). These cytoplasmic granules together with ROS kill bacteria. PMN also promote tissue injury and disturb mammary function via 1) uncontrolled ROS generation, and 2) granular enzyme release (degranulation) (Mehrzad *et al*., 2004). All of those functions and structures might be altered during the physiological and pathological conditions of the mammary gland in high yielding dairy cows.

Maturation and life span of PMN: from bone marrow to the mammary gland

All blood and immune cells originate from a self-renewing small population of pluripotent stem cells that can replicate themselves, or can become committed to a particular development pathway. PMN are formed through the multi-step process of granulopoiesis, from the colony-forming unit of granulocytes through myeloblasts, promyelocytes, myelocytes, metamyelo-cytes, and band cells. Precursor cells undergo substantial morphologic, biochemical and functional changes during granulocytic maturation. These changes are associated with significant changes in cell size and nuclear shape, and with the development of stage-specific proteins essential for phagocytosis and bacterial killing (Smits *et al*., 1996; Mehrzad *et al*., 2001a, c; Van Merris *et al*., 2001a, b, 2002; Burvenich *et al*., 2003). The efficiency of PMN against mastitis-causing pathogens was previously shown to be highly dependent on the rate of diapedesis into the infection site (Heyneman *et al*., 1990; Heyneman and Burvenich, 1992) and on the ability of these PMN to generate ROS (Heyneman and Burvenich, 1992; Mehrzad *et al*., 2001a, b, c, 2005a, b, 2009). Although the immature cells had already expressed the membrane adhesion molecule CD11b, they were not capable of rapidly migrating to the infected mammary gland and efficiently ingesting and killing the invading bacteria. The impairment of ROS production was attributed to the absence of membrane-bound NADPH-oxidase activity, as myeloperoxidase was already present in the rare azurophilic granules at the promyelocytic stage (Van Merris *et al*., 2002). Thus, when maturation is impaired due to an increased proliferation rate, a higher number of immature cells will appear in the circulation. These findings support the hypothesis postulated by (Heyneman and Burvenich, 1992; Mehrzad *et al*., 2001a, 2005a, b, 2009), namely that the presence of myelocytes, metamyelocytes and band cells (left-shift) observed during acute coliform mastitis in early lactation may compromise the cows' resistance by supplying more cells that are morphologically immature and functionally insufficient. It was demonstrated that the increase of neutrophil functionality was a result of increased enzyme activity per neutrophil, rather than an increase in the number of neutrophils. Therefore, the enhanced enzymatic activity in neutrophils after the onset of mastitis was believed to be induced by granulocyte colony-stimulating factors, reflecting an increased proliferation and differentiation of bone marrow granulocytes (Heyneman *et al*., 1990; Heyneman and Burvenich, 1992). It has been postulated that cells leave the bone marrow more-or-less by a pipeline mechanism; in this mechanism the older cells are released first (Heyneman and Burvenich, 1992). The mechanisms that control the release of mature PMN from the bone marrow into the circulating pool are poorly understood.

A potential role of L-selectin in the release of PMN from the bone marrow is very critical, because L-selectin is highly

expressed on mature PMN in the post-mitotic pool in the bone marrow and in the circulation (Diez-Fraille *et al*., 2004). The process of granulopoiesis is strictly controlled by regulatory growth factors, comprising cytokines and colony-stimulating factors, which have pleiotropic effects on proliferation, differentiation and a functional activation of precursor cells (Burvenich *et al*., 2003). Using an optimised cell culture assay for the bovine (Smits *et al*., 1996; Van Merris *et al*., 2001a, b), it was demonstrated that physiological concentrations of β-hydroxybutyric acid and acetoacetic acid induced significant inhibitory effects on the proliferation of hematopoietic cells (Hoeben *et al*., 1999). Bovine pregnancy-associated glycoproteins (bPAG) also reduced the proliferative activity of bovine progenitor cells (Hoeben *et al*., 1999). Therefore, the circulating pool is largely dependent on the proliferative capacity of the bone marrow. After having exerted their role in immune function, PMN die by senescence (Van Merris *et al*., 2002; Burvenich *et al*., 2003). Aged PMN undergo spontaneous apoptosis in the absence of pro-inflammatory agents prior to their removal by macrophages (Paape *et al*., 2002; Burvenich *et al*., 2003), thus preventing the release of their cytotoxic content. Inflammation and infection do increase the rate of PMN production, shortening the maturation time, and thereby leading to the release of immature immune cells in the circulation.

Recently, advances in mammary gland immunology have provided insights into the mechanisms responsible for the defense of the mammary gland against infection. PMN has a pivotal role in the protection of the gland from infections (Mehrzad *et al*., 2001a, b, c, 2002a, 2004, 2005a, b, 2009; Paape *et al*., 2002; Burvenich *et al*., 2003). The life cycle of the bovine PMN is short. Formed in the bone marrow, PMN require 10 to 14 days to mature (Bainton *et al*., 1971; Burvenich *et al*., 2003). After maturation, PMN may be stored for a few additional days. Mature PMN leave the hematopoietic compartment of the bone marrow and enter the vascular sinus by travelling in migration channels through endothelial cells. The PMN circulate in the blood stream briefly (mean half-life of about 9 h) (Carlson and Kaneko, 1975), leave the blood stream by diapedesis, and enter tissues where they function as phagocytes for 1 to 2 days. In healthy dairy cows, production and destruction of PMN is tightly regulated, which keeps their number in blood, milk, and tissue almost constant (Heyneman *et al*., 1990; Heyneman and Burvenich, 1992; Mehrzad *et al*., 2002a; Paape *et al*., 2002; Burvenich *et al*., 2003). Continuous influx of PMN into the mammary gland is an essential element of their role in the first line of immune defense. This influx is orchestrated by the local accumulation of chemotactic factors, which may be of endogenous or exogenous origin. Examples of the former include complement derived factors (e.g., C5a), lipid-derived mediators (e.g., leukotriene B4, platelet-activating factor or PAF) and tissue-derived chemokines (in particular interleukin (IL)-8). The dynamic of PMN diapedesis through the blood/milk barrier helps to explain the observed PMN activity fluctuations in milk (Smits *et al*., 1996, 1999; Van Oostveldt *et al*., 2002).

Although the presence of strong chemotactic factors in non-mastitis milk is the subject of debate, their presence in mastitis milk is indisputable (Manlongat *et al*., 1998). Most inflammatory chemoattractants are only induced and released during acute infection. However, a restricted number of chemoattractants can be constitutively present in normal plasma at high concentrations, e.g. Regakine-1 (Struyf *et al*., 2001). During mastitis, inflammatory chemoattractants guide PMN toward infection foci. Potent bovine PMN chemoattractants include C5a, an active cleavage product of the C5 in the complement system, various lipopolysaccharides (LPS), IL-1, IL-2 and IL-8 (Daley *et al*., 1991). These chemoattractants bind to specific receptors on the PMN plasma membrane. Extravasation of activated PMN occurs after adhesion of these cells to the endothelial surface. This is accomplished by the expression of specific membrane adhesion molecules. The essential role of the CD (cluster of differentiation) 11/CD18 family of adhesion molecules in the bovine PMN-

surface is well-documented (Diez-Fraille *et al*., 2004). These molecules bind to endothelial intercellular adhesion molecules (i.e., ICAM-1 and ICAM-2) and endothelial leukocyte adhesion molecules (ELAM-1) on the endothelial surface. After binding to these molecules, PMN leave the circulation and are ready to function at the infection site. Down-regulated CD11/CD18 in circulating PMN can cause a harder and slower PMN recruitment into the mammary gland (Burvenich *et al*., 2003; Diez-Fraille *et al*., 2004). *E. coli* mastitis induces the adherence of circulating PMN to the endothelium by up-regulation of CD11b/CD18 (Diez-Fraille *et al*., 2004), of which activity is crucial to bovine PMN diapedesis across the blood/milk barrier (Smits *et al*., 2000; Van Oostveldt *et al*., 2002). In bovine mastitis, blood PMN number, and effective adhesion, migration, opsonization, phagocytosis and killing are of crucial importance to the outcome of intramammary infection and the severity of the disease (Mehrzad *et al*., 2001a, b, 2005a, b, 2009; Burvenich *et al*., 2003). The impact of fast PMN diapedesis during mastitis on PMN quality and their ROS production capability could cause dissimilarities between milk PMN from inflamed and non-inflamed quarters (Mehrzad *et al*., 2001b, 2005a, b, 2009). The underlying mechanism of this disparity would be pivotal for further investigation.

Figure 1 shows an overview of our recent findings about changes in PMN functions, viability and the dynamic of the PMN structure and maturity in healthy and mastitis dairy cows. Briefly, the overall results concerning the changes in PMN function and morphology changes when they diapedesed in the udder in healthy cows. In a mastitis udder, the pattern of changes differs from a healthy one. This is very important for understanding the pathophysiology of mastitis. This difference might lead to the overall PMN diapedesis rate, bactericidal capacity and the dynamics of CFU in moderate and severe responders.

The source of host and/or pathogen-derived cytokines in milk and their impact on milk PMN function has been a subject of investigation. There is evidence of cytokines secretion by mammary macrophages and epithelial cells during both physiological and pathological conditions of the gland (Boudjellab *et al*., 1998). These cytokines influence PMN function. For example, the IL-8 is involved in the recruitment of PMN and T lymphocytes into milk (Barber *et al*., 1999). Proinflammatory cytokines, like

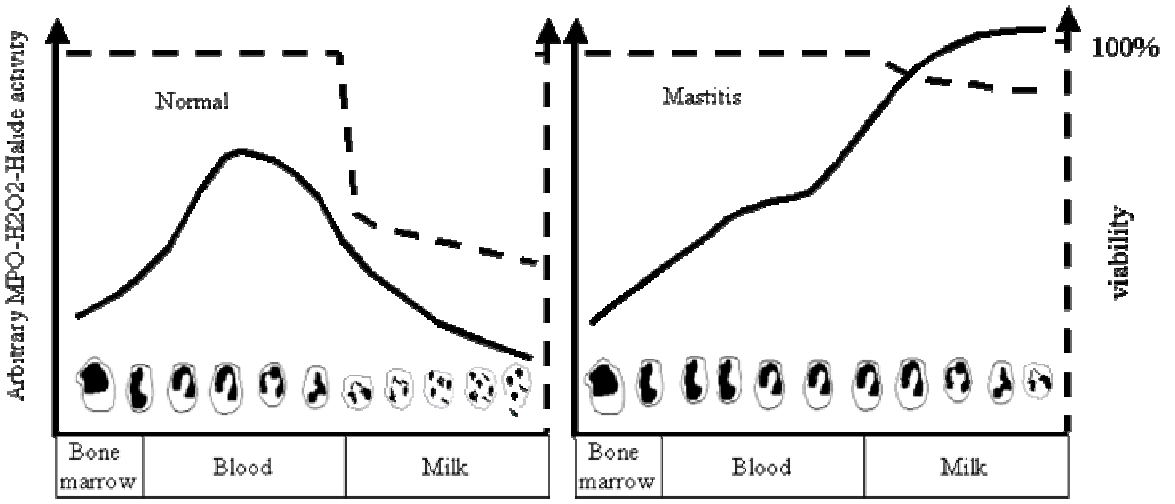

Fig. 1: An schematic overview on our recent findings (together with literatures) about changes in PMN MPO-H_2O_2-Halide system (solid lines), viability (dashed lines) and dynamic of PMN structure and maturity in healthy and mastitis dairy cows. Formed in bone marrow, blood PMN function and structure changed after normal diapedesis in milk. These changes differed during mastitis. The most probable reason for these disparities would be the "rate of diapedesis", which is faster during mastitis; this difference results from the contribution of many soluble and insoluble molecules in the cells and fluids

TNF-α, IL-1β, and LPS suppress the gene expression of cytochrome P-450 1A1, by activating the transcription nuclear factor κB (NF-κB) (Notebaert *et al*., 2005). PMN also play a crucial role in the recruitment of other leukocytes such as $CD4^+$ T lymphocyte and $CD8^+$ T lymphocyte to the inflammation sites (Mehrzad *et al*., 2008a). PMN influx to the site of inflammation is important in limiting injury and promoting recovery of severe inflammation (Burvenich *et al*., 2003; Mehrzad *et al*., 2005a, b).

Milking and PMN function in the mammary gland

Milking removes compromised PMN, which are replaced by newly, blood derived PMN, thus enhancing defense against bacterial infection. Influx of PMN into the mammary gland occurs at a low level for immune surveillance, but increases rapidly in response to bacterial invasion. Under physiological conditions, the nursing or milking stimulus induces directed migration of fresh PMN into mammary tissue (Paape *et al*., 2002; Burvenich *et al*., 2003) and subsequently in milk. In this way, the normal sterile mammary gland is supplied with a constant source of PMN. Furthermore, drainage of newly synthesized milk into milk ducts and cistern, leads to the removal of freshly migrated PMN; this potentially leads to a further exudation of PMN into newly formed milk in the alveoli. However, once in the lumen of alveoli, ingestion of fat and casein causes a loss in phagocytic and bactericidal functions and leads to the death of PMN (Paape *et al*., 2002; Burvenich *et al*., 2003).

The mechanical action of milking enhances PMN diapedesis in milk, which is an important mechanism for PMN surveillance of the mammary gland. Immediately after machine milking, concentrations of PMN in blood from the subcutaneous abdominal vein decrease, while concentrations in mammary lymph and milk increase (Paape and Guidry, 1977). Thus, the normally sterile mammary gland is provided with millions of PMN for defensive purposes. Besides, newly synthesized milk leads to the removal of migrated PMN and further exudation of PMN into the newly formed milk in the mammary gland alveoli (Schalm and Lasmanis, 1976). The ingestion of milk fat globules and casein by PMN, however, results in phagocytic and bactericidal insufficiency and rapid necrosis in PMN (Paape *et al*., 2002; Burvenich *et al*., 2003). Conversely, no adverse effect from milk fat globules on C5a chemotactic activity has been reported (Rainard, 2002). There are plenty of studies on the effect of milking frequency on milk PMN functions, and more frequent milking would be less detrimental for the udder immunity, both in primiparous and in multiparous dairy cows (Moya *et al*., 2008). In summary, milking removes compromised PMN, which are replaced by new PMN, thus enhancing the defense of the mammary gland against bacterial infection.

The LPS-CD14 complex in the mammary gland

Apart from the existence of many bactericidal mechanisms in the bovine mammary gland (Mehrzad *et al*., 2001a, b, c, 2002a, 2004, 2005a, b, 2007, 2008a, 2009; Burvenich *et al*., 2003), there are many other soluble and insoluble proteins on the immune cells in the gland that protect it from invading pathogens. One of them is CD14 molecules. The CD14 receptors, which are commonly found on monocytes and macrophages but not on circulating PMN and lymphocytes, were recently discovered on bovine mammary PMN and macrophages (Sohn *et al*., 2007a). The CD14 receptor binds LPS-protein complexes and induces the synthesis and release of TNF-α (Paape *et al*., 2002; Burvenich *et al*., 2003). TNF-α up-regulates PMN phagocytosis, adherence, chemotaxis and ROS production. It is now recognised that two forms of CD14 exist in bovine phagocytes, a soluble and a membrane form (Sohn *et al*., 2007a, b). The soluble form results from the shedding of membrane CD14 (mCD14). Soluble CD14 (sCD14) can bind LPS directly and prevent it from binding to mCD14, thus preventing over-secretion of TNF-α that could lead to increased severity of clinical symptoms. The

sCD14 has been identified in bovine milk and colostrum as a 46 kDa protein (Wang *et al*., 2002) and may play a role in neutralizing LPS and controlling the clinical symptoms associated with acute coliform mastitis. CD14 has been cloned and the protein has been expressed in a baculovirus insect cell system (Wang *et al*., 2002). *In vitro* incubation of recombinant bovine (rbo) sCD14 with PMN and LPS prevented LPS induced upregulation of CD18 adhesion receptors (Wang *et al*., 2002; Sohn *et al*., 2007a). Intramammary and systemic use of rbosCD14 may provide a means of eliminating the potential damaging effects of LPS during acute coliform mastitis. Also, complexes of CD14 and low concentrations (0.2 μg) of LPS induced an increase in milk SCC (Wang *et al*., 2002; Sohn *et al*., 2007a, b). An increase in SCC was not observed after intramammary injection of either CD14 or LPS. It has been reported that LPS-CD14 complexes will bind to Toll-like receptors on endothelial and epithelial cells and cause the release of IL-8, an important cytokine for the recruitment of bovine PMN (Ulevitch and Tobias, 1999). Transgenic mice carrying the gene for CD14 in their mammary cells are currently being developed by scientists in the Gene Evaluation and Mapping Laboratory at the USDA in Beltsville. Experimental infections with *E. coli* have been conducted to see if the secreted rbosCD14 will complex with the LPS produced by *E. coli* and result in the recruitment of PMN in the mammary gland and elimination of the organisms.

LPS detoxification in the mammary gland

Endotoxins or LPS are released during bacterial growth and lysis of Gram-negative bacteria and have been recognized as important mediators for the treatment and outcome of coliform mastitis (Dosogne *et al*., 2002; Mehrzad *et al*., 2007). The role of the absorption of free LPS into the circulation is controversial (Dosogne *et al*., 2002; Mehrzad *et al*., 2007). In contrast, it is accepted that the amount of released LPS into the mammary gland, its subsequent detoxification and TNF-α production significantly contribute to the outcome of coliform mastitis (Hoeben *et al*., 2000b; Mehrzad *et al*., 2007). Severity of *E. coli* mastitis seems to be related to the enhanced release of secondary induced inflammatory mediators such as TNF-α (Mehrzad *et al*., 2007), as a result of impaired LPS detoxification mechanisms in milk. It has been suggested (Paape *et al*., 2002; Burvenich *et al*., 2003) that local CD14 expression modulates the toxic effects of LPS in the mammary gland. Another detoxification system is acyloxyacyl hydrolase (AOAH), an enzyme produced by PMN that hydrolyses LPS (Mehrzad *et al*., 2007). AOAH is also present in bovine PMN granules (McDermott *et al*., 1991; Mehrzad *et al*., 2007) and hydrolyses two acyl chains of the lipid A of endotoxin; this results in a substantial decreased toxicity of LPS, while retaining much of the immunostimulatory potency of native LPS (Munford and Hall, 1986). Little has been investigated about milk PMN AOAH activity either during physiological or mastitis conditions. Immediately after calving, there is a decreased blood PMN AOAH activity (Dosogne *et al*., 1998) that coincides with the decreased PMN ROS production and number in circulation (Mehrzad *et al*., 2001a, b, c, 2002a). This coincidence could be considered as a risk factor for coliform mastitis during early lactation (Mehrzad *et al*., 2001b, 2004, 2005a). Indeed, intravenous LPS administration to rabbits resulted in a rapid (within 90 min) increase of plasma AOAH activity (Erwin and Munford, 1991). The finding that PMN AOAH activity is increased upon LPS stimulation may indicate the existence of a PMN-dependent self-regulatory protection mechanism against endotoxemia. It is suggested that a decreased AOAH activity in milk PMN can also contribute to the outcome of coliform mastitis (Burvenich *et al*., 2003; Mehrzad *et al*., 2007).

Besides AOAH, bovine PMN granules also contain different LPS binding cationic proteins such as lactoferrin, and a huge variety of cationic antimicrobial proteins (Levy *et al*., 1995). These proteins do not degrade the LPS molecule, but binding to LPS results in a decreased LPS bioavailibility and hence may attenuate its

toxicity during Gram-negative bacterial infections. In a recent study, oral lactoferrin administration attenuated spontaneous TNF-α production by peripheral blood cells in human (Zimecki *et al*., 1999). Study on this topic would be very interesting for bovine immunologists.

In recent years several classes of phagocyte-derived antimicrobial peptides have been purified from mammalian phagocytes, and it is now clear that next to their production of ROS, bovine PMN also inactivate microorganisms by exposing them to these antimicrobial peptides and proteins within the phagolysosomal vacuole. Bovine PMN granules contain a group of highly cationic proteins. Beta-defensins, a family represented by 13 cationic, tridisulfide-containing peptides with 38-42 residues, have potent antibacterial activities against both *S. aureus* and *E. coli in vitro*. Similar molecules have also been isolated from specialized epithelia. These polypeptides are structured through disulfide bonds of cysteine but can also be linear and unstructured. They contribute significantly to host defense against the invasion of microorganisms (Raj and Dentino, 2002). Because β-defensins and bacterecins, another antimicrobial peptide, are stored together in the dense granules, it is likely that they are discharged simultaneously during PMN activation. Although co-packaged in the dense granules, cathelicidins, but not β-defensins, are stored as inactive propeptides. Following PMN stimulation with PMA, the cathelicidins Bac5 and Bac7 are cleaved from their respective propeptides and released extracellularly. In contrast, β-defensins exist as fully processed peptides in bovine PMN.

Bovine lactoferrin is doing more than binding iron, and has a considerable inhibitory effect on bacterial growth (Bishop *et al*., 1976). Lysozyme or muramidase can cleave the mucopeptide layer of most non-encapsulated Gram-positive bacterial cell walls, resulting in cytoplasmic blebbing of the bacterial cell through the wall defect. If the osmolarity of the surrounding environment is sufficiently low, direct lysis of the pathogen can occur. Lysozyme also has the capacity to neutralise and strongly interact with *E. coli* LPS; another non-oxidative antimicrobial agent is PMN elastase. Elastase, cathepsin G and other granule-proteases degrade the outer membrane protein A of *E. coli*, which is located on the surface of the bacteria (Belaaouaj *et al*., 1998; Raj and Dentino, 2002). Overall, apart from the role of bovine PMN granules and enzymes, it is widely accepted that PMN ROS production plays a major role in the protection of the udder from infection (Burvenich *et al*., 2003; Mehrzad *et al*., 2004, 2005a).

Periparturient PMN dysfunction

Reduced functional competence of PMN has been associated with decreased immunocompetence, resulting in an increased susceptibility to infection and suppression of host defense mechanisms. A dramatic reduction in random migration, iodination and ROS production of blood PMN were observed during the first week after parturition (Kehrli *et al*., 1989; Heyneman *et al*., 1990; Shuster *et al*., 1996; Hoeben *et al*., 2000a; Dosogne *et al*., 2001; Mehrzad *et al*., 2001a, b, c). It was recently discovered that the adhesion molecule L-selectin is shed from the surface of PMN at parturition (Diez-Fraille *et al*., 2004). Surface expression of L-selectin remains low for several days following parturition and could contribute to the reported defect in bovine PMN chemotaxis during the period immediately following calving (Berning *et al*., 1993; Diez-Fraille *et al*., 2004). Regulation of bovine PMN adhesion molecules during mammary gland infection and possible use of immunomodulators has recently been studied (Diez-Fraille *et al*., 2004). Cumulative deficiencies in opsonin levels (IgG_1 and conglutinin) were observed in periparturient cows, which closely coincided with impaired PMN oxidation-reduction reactions capacity (Detilleux *et al*., 1994; Burvenich *et al*., 2003). The proportion of all cases of clinical coliform mastitis that develop during the first 8 weeks of lactation has been reported to be more than 50% (Burvenich *et al*., 2003). Both in milk and blood, PMN function is substantially decreased around calving

(Dosogne *et al*., 2001; Mehrzad *et al*., 2001a, c, 2002a, 2009; Burvenich *et al*., 2003). At present, the underlying mechanisms involved in periparturient immunosuppression remain unknown. However, metabolites (e.g. β-hydroxybutyrate) (Suriyasathaporn *et al*., 1999) and hormones (e.g. growth hormone, cortisol, bPAG) (Suriyasathaporn *et al*., 2000) have been reported as attributable factors.

Hormones and metabolites versus PMN functions

There are many reports demonstrating that at least some hormones and metabolites contribute to PMN function, targeting both PMN influx to tissue and PMN ROS production capacity (Hoeben *et al*., 1999, 2000a; Suriyasathaporn *et al*., 1999; Lamote *et al*., 2004). As these studies suggest, the link between periparturient immunosuppression and hormonal and metabolic changes is nevertheless apparent; most of which directly/indirectly affect PMN functions. Hormonal and metabolic changes such as glucocorticoids, ketone bodies and bPAG play a causative role in impaired PMN ROS production capacity (Dosogne *et al*., 1998; Hoeben *et al*., 2000a). These hormones and metabolites also inhibit the proliferation of bone marrow cells *in vitro* (Hoeben *et al*., 1999; Van Merris *et al*., 2001a, b, 2002). Our understanding of the precise ways in which the complex cascade of ROS production occurs in blood or milk PMN during physiological and mastitis conditions is still in its early stage. This is especially true for the mechanism of *in vivo* effect of PMN functions by hormones and metabolites. Based on the current understanding of the impact of hormones on PMN function, the membrane, cytosolic and nuclear effects of hormones (e.g. growth hormone, sex hormones, cortisol, bPAG) and metabolites (β-hydroxybutyrate, non-esterified fatty acid) on blood and milk PMN functions are to be more fundamentally investigated.

Recombinant bovine somatotropin (bST) has been shown to boost cows' milk production and compositional performance following experimentally induced *E. coli* and *Streptococcus uberis* mastitis (Hoeben *et al*., 1999). Recombinant bST also prevented severe local and general clinical symptoms in cows suffering from *E. coli* mastitis, especially in severe responders. Prolactin, bST, and insulin-like growth factor-I (IGF-I) are thought to be involved in several immune functions (Elvinger *et al*., 1991; Hooghe *et al*., 1993; Adriaens *et al*., 1995; Kooijman *et al*., 1996). The function of bST on PMN can either be directly or indirectly mediated through IGF-I. Plasma and milk concentrations of IGF-I increase after bST administration (Cohick *et al*., 1989; Mielke *et al*., 1990; Vicini *et al*., 1991; Burton *et al*., 1992; Zhao *et al*., 1992). Their concentration differs throughout lactation in milk (Campbell *et al*., 1991; Baumrucker *et al*., 1993). Massart-Leën *et al*. (1990) reported an increased number of circulating leukocytes, band neutrophils, and an enhanced PMN function in cows treated with bST after calving. PMN ROS generation, chemotaxis, random migration, and phagocytosis towards IgG-opsonised micro-organisms are boosted by IGF-I and bST (Kelley, 1989; Fu *et al*., 1991, 1994; Edwards *et al*., 1992; Jin *et al*., 1993; Wiedermann *et al*., 1993; Bjerknes and Aarskog, 1995; Warwick Davies *et al*., 1995). The expression of complement receptors can be upregulated by bST and IGF-I. Increased chemotaxis and random migration (Wiedermann *et al*., 1993), increased numbers of circulating neutrophils (Clark *et al*., 1993), and increased proliferation of granulocyte and monocyte precursors (Scheven and Hamilton, 1991; Merchav *et al*., 1993) have also been observed following bST and IGF-I elevations *in vivo*. Elvinger *et al*. (1991) reported little or no effect on phagocytosis and killing of *E. coli* by circulating PMN or on cytochrome *c* reduction *in vitro* as well as *in vivo*. However, Heyneman and Burvenich (1989) observed an increased PMN ROS production capacity after *in vivo* administration of bST in healthy cows. We observed similar results *in vitro* (Mehrzad *et al*., 2002b). Thus, there is ample evidence that bST and IGF-I can modulate the immune response of PMN. Concentration of some biomolecules like β-lactoglobulin is lowest during early lactation (Caffin *et al*.,

1985), revealing its potential immunostimulatory effects in dairy cows (Mehrzad *et al*., 2000b). Hence the insight into PMN activators and/or inhibitors in milk during physiological and pathological conditions is a crucial concern for the udder's first line defense mechanism in high yielding dairy cow.

Blood and milk PMN ROS production versus lactation, parity and mastitis

Blood and milk PMN have the potential to produce substantial amounts of ROS to kill engulfed bacteria (Hoeben *et al*., 2000a; Mehrzad *et al*., 2001a, c, 2002a, b, 2004, 2005a, b, 2009). ROS production can be measured in resting (non-stimulated) cells and after stimulation with e.g., PMA, zymosan, bacteria, latex beads. Production of ROS is effective in killing engulfed bacteria, especially gram-negatives (Burvenich *et al*., 2003). The most widely used technique to estimate PMN ROS production in dairy cows is the CL assay (Hoeben *et al*., 2000a; Mehrzad *et al*., 2001a, b, c, 2002a, b, 2004, 2005a, 2009). CL results from reactions of ROS with luminescence agents and requires both cytoplasmic membrane-derived NADPH-oxidase and myeloperoxidase in the azurophilic granules of the PMN.

During the last few years little comparison has been made between the CL of PMN in blood and milk. To interpret and assess the responsiveness of PMN to stimulating agents such as PMA, it is necessary to distinguish between stimulated and non-stimulated PMN. This offers information about the activity of protein kinase C and NADPH-oxidase, as PMA is a protein kinase C and NADPH-oxidase agonist (Mehrzad *et al*., 2001a, b, c, 2002a, 2004, 2005a, 2009). Ingestion of milk fat globules and casein micelles affects milk PMN quality (Paape and Guidry, 1977) and subsequent degranulation (Paape *et al*., 2002). No such problem in blood exists. Smits *et al*. (1996, 1999) have shown that diapedesis of PMN across mammary epithelium *in vitro* reduces ROS production of PMN. It has also been shown that milk PMN function differs from their blood counterparts (Paape and Guidry, 1977; Smits *et al*., 2000; Mehrzad *et al*., 2001a, b, c, 2002a, 2009). Some physiological influencing factors such as stage (Mehrzad *et al*., 2001a, c) and number of lactation (Mehrzad *et al*., 2002a, 2009; Mehrzad and Zhao, 2008b) are involved in PMN impairment. Some other contributing factors would be β-lactoglobulin (Mehrzad *et al*., 2000b), of which concentration in milk is minimal during early lactation (Caffin *et al*., 1985). The PMN function impairment generally coincides with cow's susceptibility for environmental mastitis (Shuster *et al*., 1996; Burvenich *et al*., 2003; Mehrzad *et al*., 2004, 2005a). More fundamental research on this topic is needed.

In general, with the knowledge obtained in this study on the blood and milk PMN, it is clear that peripartum high yielding dairy cows are relatively immunosuppressed. Several physiological and mastitis conditions affect the most pivotal cellular part of the innate immune system of the udder, PMN microbicidal capacity, in high yielding dairy cows. Figure 2 gives a schematic overview of the major contributing factors affecting some key immunological parameters and the relation of these parameters to the severity of mastitis in the udder of high yielding dairy cows.

Many other factors affect PMN functions and the outcome of mastitis; one of the key and interestingly dynamic attributing factors would be the effect of genetics on the PMN function. There is no doubt that inflammation and infections in animals is profoundly controlled by genes, and related to breed (Dietz *et al*., 1997; Radwan *et al*., 2007; Bannerman *et al*., 2008). In dairy cows, the concentration of circulating PMN during early lactation is highly heritable (h^2 = 0.87; Riollet *et al*., 2000). Although heritability is low for mastitis, it is clear that genetic selection for cows' resistant to mastitis could become an important alternative for prophylactic measures of mastitis in the future (Wall *et al*., 2005). Study on genetic and molecular biology may lead to a better insight into the susceptibility for mastitis and provide new direction in research for genetic resistance to diseases. The challenge is to effectively

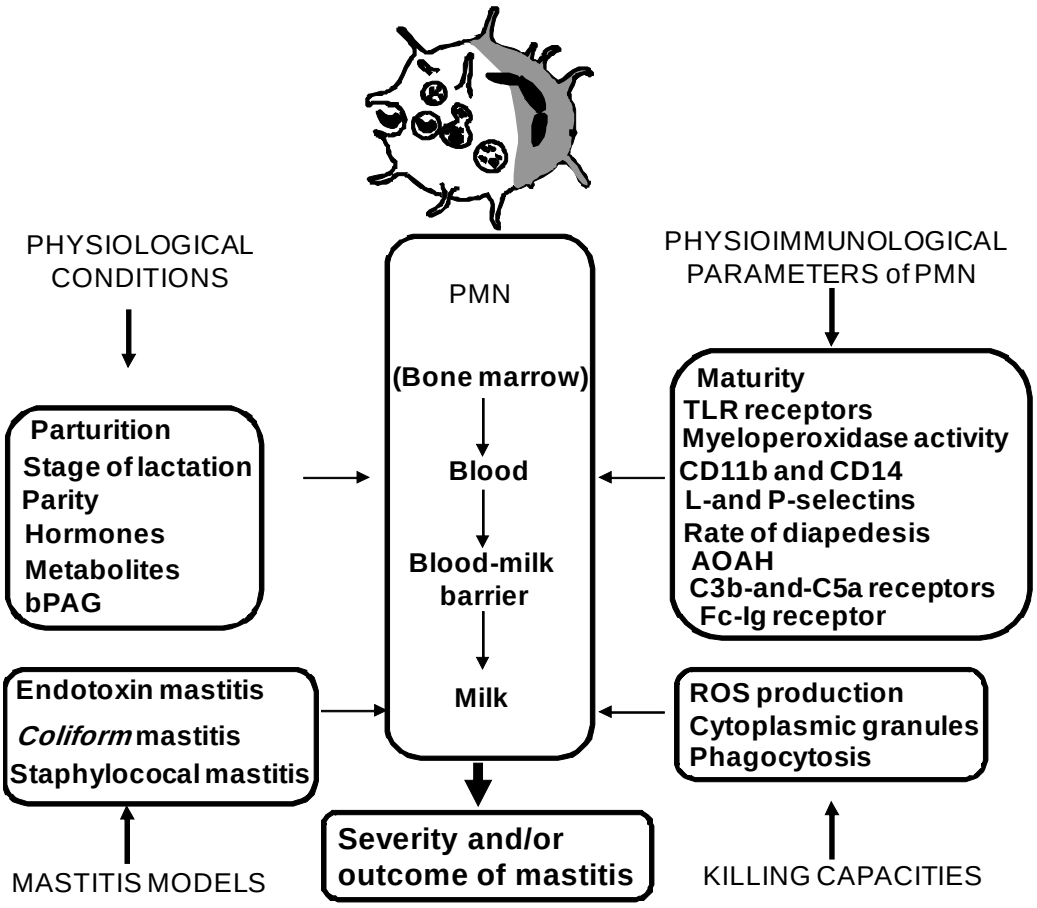

Fig. 2: Diagram depicting the major contributing factors such as some physiological conditions (lactation, parity or age and mastitis) to the microbicidal capacity of PMN (neutrophils) in the blood and udder, and the strong link of these conditions to some key immunophysiological parameters of the innate host defence in high yielding dairy cows. This effect and link happens not only in the blood, but also in the bone marrow, affecting the neutrophil functions in the udder, thereby contributing to the outcome or severity of mastitis. Because neutrophils are the first cells recruited to the inflamed udder, their capacity and functionality make this cell of the innate immune system one of the cornerstones of the induction and shaping of adaptive immunity in the udder. Microbicidal activity of neutrophils occurs mainly intracellularly during phagocytosis, with the contribution of many soluble and insoluble proteins, enzymes and free radicals produced both inside and outside of the neutrophils. Neutrophils are activated by a wide array of compounds, such as inflammatory mediators, cytokines and ligands for many receptors like pattern recognition receptors (e.g. via TLRs or Toll-like receptors). The activation elicits classic neutrophil functions such as chemotaxis, adherence, ingestion and finally digestion or destruction of phagocytosed microbes. Some mediators, cytokines, hormones and metabolites suppress neutrophils' functions throughout the body. Like blood PMN, milk PMN functional impairment occurs immediately around calving, this coincides with the impairment of milk PMN viability and killing capacity in the udder. These impairments are more pronounced in early lactating cows, which are far more pronounced in older dairy cows. This diagram is based on the authors' own studies on bovine PMN functions (refer to references listed in the reference section); the diagram is a fundamental consideration in this review study, and many issues and subjects about this diagram remain to be further studied in the area of bovine immunology

integrate the information from molecular and quantitative genetics into existing breeding programs, and genetic engineering could provide a viable tool for enhancing resistance to mastitis.

In summary, based on what is known so far, it is conceivable that the defence of the mammary gland against mastitis-causing pathogens could be mediated by PMN functions. These important parameters might be affected by physiological (e.g., stage of lactation and lactation number) and

pathological (endotoxin, coliform and staphylococcal mastitis) conditions of high yielding dairy cows. Milk PMN functions at the start of bacterial invasion can be considered as major attributable factors for phagocytosing and intracellular killing of invading bacteria, which influence the outcome of mastitis. These PMN functionality fluctuations and mastitis outcome are multifactorial and can be affected by speed of diapedesis and the complex chemical environment of the milk compartment. Substantial fundamental studies are required on the immuno-physiological status of dairy cows. This review study was performed to gain more insight into the complex events of first line of cellular defence mechanisms of the udder in high yielding dairy cows.

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