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Molecular Aspects of Neutrophils as Pivotal Circulating Cellular Innate Immune Systems to Protect Mammary Gland from Pathogens

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1. Introduction

As a pivotally cellular and molecular arms of circulating innate immune system, polymorphonuclear cells (PMNs) are the most vital primary mobile phagocytes in the body of mammals; their appropriate function is very essential to enhance animals' and humans' health performance. As the first type of innate immune cells arriving at the site of infection, neutrophils play a key role in initiating an innate, inflammatory, and specific immune responses; their importance for protection of organs in the body from pathogens has long been a crucial concern (Burvenich et al., 1994; Paape et al., 1996; Reeves et al., 2002; Burvenich et al., 2003; Mehrzad et al., 2004; 2005a; Letiember, et al., 2005; Liu et al., 2005; Borregaard et al., 2007; Stevens, et al., 2011a; 2011b; Bruhn et al., 2011). The proof of vital roles of neutrophils is that the neutropenic animals/humans are always highly susceptible to many pathogens. Clearly, the complex phenomenon of PMN chemotaxis, diapedesis, phagocytosis, and eventually microbicidal activity each contributes to the ability of PMN to provide an effective first line defense for the body and organs like udder (Burvenich et al., 2003; Mehrzad et al., 2000; 2001a; 2001b; 2002a; 2002b; 2004; 2005a; 2007; 2008a; 2008b; 2009; Mayadas & Cullere, 2005; Borregaard et al., 2008). In this concept many powerful afferent (sensing) and efferent (effector) arms of the neutrophils inside and outside of the cytoplasm are involved; the most common arms are enzymes, granules, free radicals or reactive oxygen species (ROS) and reactive nitrogen species (RNS) and in phagolysosome, into which microbicidal agents are released, neutrophil extracellular traps (NETs), neutrophils' membrane receptors like pattern recognition receptors (PRRs), opsonin receptors etc. that sense, bind and efficiently kill invading microbes, destroy virulence factors, or prevent them from spreading.

Oscillation and/or impairment of neutrophils' functions, originating from the bone marrow, is a peculiar feature during the physiological and environmental stresses; this impairment might be cumulative upon diapedesis/extravasation of neutrophils (Mehrzad et al., 2001a; 2002a; 2005a, 2004; 2007; 2008a; 2008b; Van Oostveldt et al., 2002a; 2002b; Burvenich et al., 2003). Generalised PMN impairment can be multifactorial, e.g. due to metabolic (Suriyasathaporn et al., 1999) and hormonal (Gray et al., 1982; Alexandrova, 2009; Lai &

Gallo, 2009) changes. The inevitable occurring of general and local immunocompromised conditions in biologically pivotal organ, mammals' udder, (Burvenich et al., 1994; Hoeben et al., 2000a; Burvenich et al., 2003; Mehrzad et al., 2001a; 2004; 2005a; 2008a; 2009) leads to udder infection and/or inflammation and breast disorders, affecting adult mammals, especially high yielding dairy cows, thereby causing neonatal infections, critical public health damage and economic losses to bovine, meat, dairy and food industries and overall human food chains.

Most researchers see the immunocompromised condition in animals and human as a result of neutrophils' dysfunction; this neutrophils' dysfunctional status can be cumulative upon neutrophils' influx into the udder (Mehrzad et al., 2001a; 2004; 2005a; 2008a; 2009). Despite all progresses and advances in the efferent and afferent branches of molecular aspects of neutrophils, the molecular basis of neutrophils interactions with other immune and non-immune cells in the udder is incompletely understood.

This chapter presents the cellular and acellular branches of neutrophils-pathogens interactions and factors affecting the effectiveness of particularly neutrophils' efferent arms of innate molecules in bovine. I chose bovine as a model to update our knowledge and to incorporate new observations that broaden our understanding of 1) overall neutrophils' involvement in innate immunity 2) how neutrophils engulf and kill invading pathogens and 3) how some key mechanisms of neutrophil's oscillatory events occur in bovine model. I discuss various aspects of bovine neutrophils that are absolutely relevant to their pivotal roles in an efficient innate immune response against pathogens. Aspects such as cellular and molecular innate immunity and host-pathogen interactions, biology, biochemistry and biophysics of bovine neutrophils as well as immunotoxicology, especially environmental immunotoxins (Mehrzad et al., 2011) and some nutritional immunology (Ibeagha et al., 2009), applying basic techniques like luminometry and flow cytometry will be schematically addressed to gain more insight into the static and dynamics branches of circulating and post-diapedetic neutrophils' functions. Although it is hardly possible in this chapter to address the breadth of information available on efferent arms of neutrophils in healthy and diseased animals and humans, our readers are therefore referred to many more recent detailed references of the many aspects of neutrophils in healthy and diseased hosts and their impacts on udders' health and performance as well.

2. Fine structure of neutrophils

As the primary and pivotal cells providing innate host defence against pathogens, neutrophils are characterized by their multi-lobed or sometimes picnotic dark-bluish stained nuclei (see figure 1) with the plenty of membrane receptors. The fine structure of bovine PMN has been exclusively demonstrated in classic studies (Paape et al., 2002; Mehrzad et al., 2001a; 2005b). The cell is delineated by a plasma membrane that has a number of functionally important receptors. These include L-selectin and β_2-integrin adhesion molecules associated with the binding of PMN to endothelial cells that are important for migration into sites of infection (Stevens et al., 2011a; 2011b; Pezeshki et al., 2011). Membrane receptors for the Fc component of the IgG_2 and IgM classes of immunoglobulins and C3b are necessary for mediating effective phagocytosis of invading microbes (Paape et al., 1996). Dying or apoptotic PMN express receptors that mark them for quick disposal by macrophages.

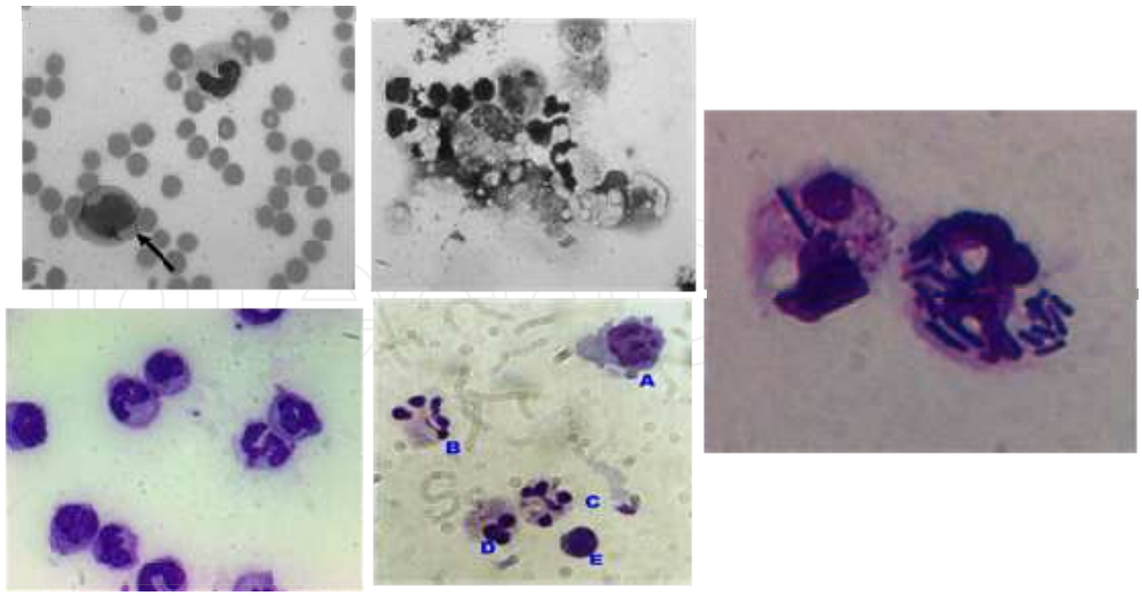


Fig. 1. Upper: Whole blood smear (left), immature neutrophil (arrow); milk neutrohils after first step of centrifugation (right), which is almost indistinguishable, with the techniques established in routine laboratories they can easily be distinguishable. A typical phagocytosed microbes by milk neutrophils (far right); these engulfed microbes must be destroyed effectively to limit infection. Lower: Isolated bovine blood neutrohils from healthy animal (left), which is pure, and postdiapedetic udder (right) neutrohils (B, C and D) macrophage (A) and lymphocyte (E).

The most prominent characteristic of the PMN is the multilobulated nucleus (see figures 1 and 2). The multilobulated nucleus is important because it allows the PMN to line up its nuclear lobes in a thin line, permitting rapid migration between endothelial cells. Macrophages on the other hand have a large horseshoe shaped nucleus that makes migration between endothelial cells more difficult. Thus, the PMN is the first newly migrated phagocytic cell to arrive at an infection site. Their surface microvilli are also pivotal for their functionality (see figures 2 and 3). Within the cytoplasm there are isles of glycogen that make up 20% of the cell on a dry weight basis and numerous bactericidal granules that are used by the cell for bactericidal activity. Generally, human neutrophils have two predominantly distinct granule populations; azurophilic (primary) granules which are large and appear as electron dense granules on electron microscopy, and specific (secondary) granules which are small and appear as light staining granules on electron microscopy. Azurophilic granules are more abundant in immature lineage of neutrophils than specific granules (Borregaard et al., 2007). Similarly, bovine PMN contains azurophilic and specific granules (figure 2). They also contain a third novel granule that is larger, denser and more numerous than the other two granules. These granules contain lactoferrin, which is also found in secondary granules, but they do not contain constituents common to azurophilic granules. Instead, they contain a group of highly cationic proteins and are the exclusive store of powerful oxygen-independent bactericidal compounds (Gennaro et al., 1983). The most important antibacterial mechanism derived from azurophilic granules is the MPO-H_2O_2-halide system (Heyneman et al., 1990; Heyneman & Burvenich, 1992; Mehrzad et al., 2001a; 2002a; 2009). MPO in the presence of hydrogen peroxide (H_2O_2) and halide ions kills bacteria. The functionality of these ROS producing

granules might be altered during physiological and pathological conditions of animals, and should therefore be further investigated.

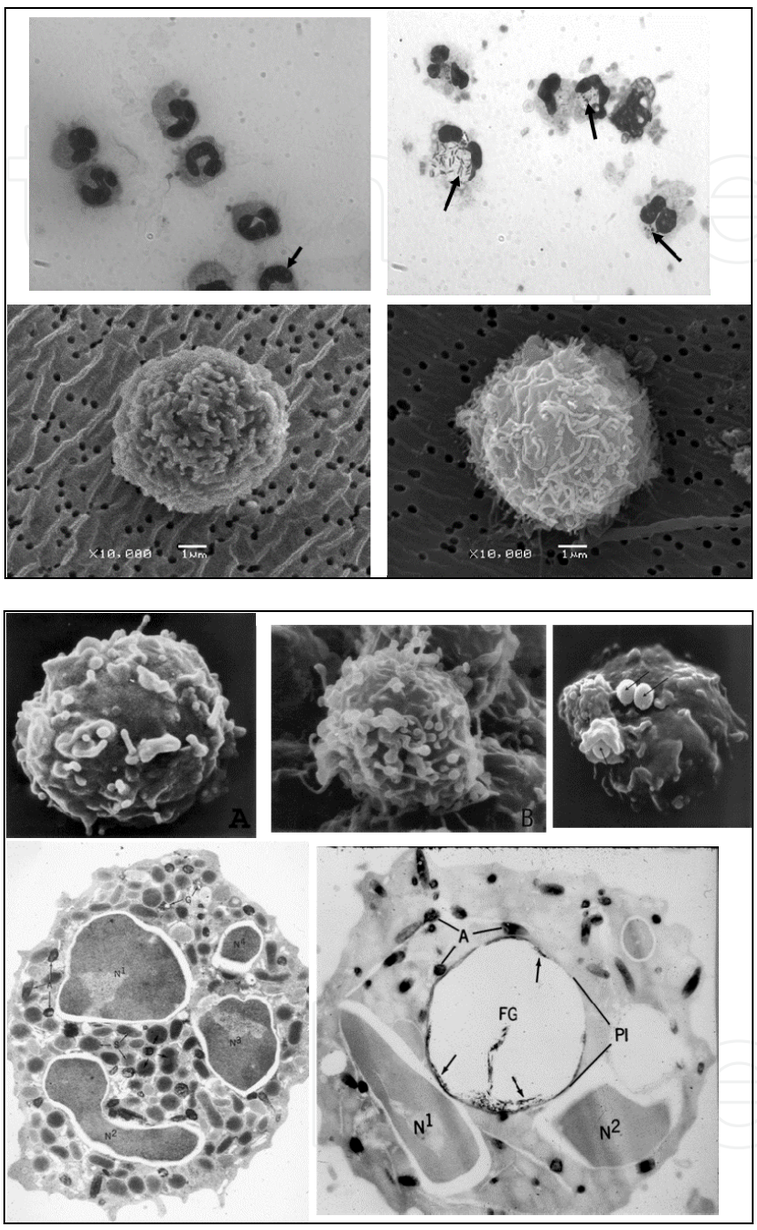


Fig. 2. Upper panel: upper: Isolated neutrophils from blood (left), in which the presence of immature neutrophil (arrow) is distinguishable and post-diapedetic neutrophils in lactating udder (right), in which phagocytosed bacteria (arrows) are visible. Lower: Scanning electron micrograph of PMN isolated from blood (left) and milk (right). Protruding pseudopods needed for phagocytosis in PMN, which is distinguishable in blood and post-diapedetic neutrophils. The blood PMN has higher convulsed cell membrane that forms protruding pseudopods. This might be due to phagocytosis of milk fat globules and casein miscelles. Samples were taken from cows suffering from *E. coli* mastitis. Lower panel: (A) Scanning

electron micrograph of a neutrohil isolated from post-diapedesis in udder. As a result of the cell ingesting its membrane material during phagocytosis of milk fat globules and casein miscelles, the neutrophils lost the protruding pseudopods required for phagocytosis (×20,000). (B) Scanning electron micrograph of a neutrohil isolated from blood. It has a highly convoluted cell membrane that forms protruding pseudopods (×15,000). Far right: Scanning electron micrograph of a PMN isolated from milk with three Staphylococcus aureus (arrows) are present on the surface of the neutrophil; the bacterium at lower left is partially engulfed by a pseudopod (×20,000). Lower left, transmission electron micrograph of bovine neutrophils isolated from blood. The neutrohil, which is limited by the plasma membrane, contains portions of a multilobed nucleus (N1 to N4), glycogen granules (G), specific granules (S), azurophilic granules (A), and large electron dense granules (D). Azurophilic granules are stained more intensely than the specific and large electron dense granules because the neutrophils were incubated with diaminobenzidine and hydrogen peroxide. As a result, an electron-dense product, indicative of peroxidatic activity, has formed in azurophilic granules (×22,000). Lower right, transmission electron micrograph of bovine neutrophils isolated from lactating udder. Deposition of an electron-dense product performed on neutrophils, which was not stained, electron dense product corresponds to areas that are high in peroxidatic activity. These areas represent the azurophilic granules and periphery (arrows) of phagolysosomes containing a milk fat globule. Nuclear lobes (N1, N2), azurophilic granules (A), phagolysosome (PI), fat globule (FG) (×25,000). Partially adapted from (Paape M., Mehrzad j., et al., 2002).

3. Movement of neutrophils from bone marrow to the mammary glands

The issue of life span, mechanomics and biophysics of this dynamically mobile cell, neutrophil, is very pivotal issue in biomedical research. The neutrophilic PMN leukocytes of the blood circulation are specialized terminally differenciated with a short life-span. All blood and immune cells originate from a self-renewing small population of pluripotent stem cells (CFU-S) that can replicate themselves, or can become committed to a particular development pathway. Neutrophils are the major class of leukocytes in peripheral blood of human and domestic animals. The circulation of a healthy human adult contains 4,500-10,000 leucocytes/μl with approximately 60% or more being neutrophils (Nathan, 2006). A healthy adult human produces ~10^{11} neutrophils each day each of which survives about 6-8 hours in the circulation. Almost similar pattern on leukocyte and neutrophils quantities existed in bovine leukocyte and neutrophils (see e.g., Mehrzad et al., 2001a; 200ab; 2001c; 2002a; 2004; 2005a and plenty more). These vital circulating innate immune cells, neutrophils, are formed through the multi-step process of granulopoiesis, from the colony-forming unit of granulocytes (CFU-G) through myeloblasts, promyelocytes, myelocytes, metamyelocytes, 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 microbial killing (Smit et al., 1996; Van Merris et al., 2001a; 2001b; 2002; Burvenich et al., 2003; Mehrzad et al., 2001a; 2001c).

The efficiency of PMN against invading pathogens was previously shown to be highly dependent on the rate of diapedesis into the infection site (Heyneman et al., 1990; Heyneman & Burvenich, 1992; Mehrzad et al., 2001b; 2004; 2005a; 2005b) and on the ability of these PMN to generate ROS (Heyneman & Burvenich, 1992) and Mehrzad et al., 2001a;

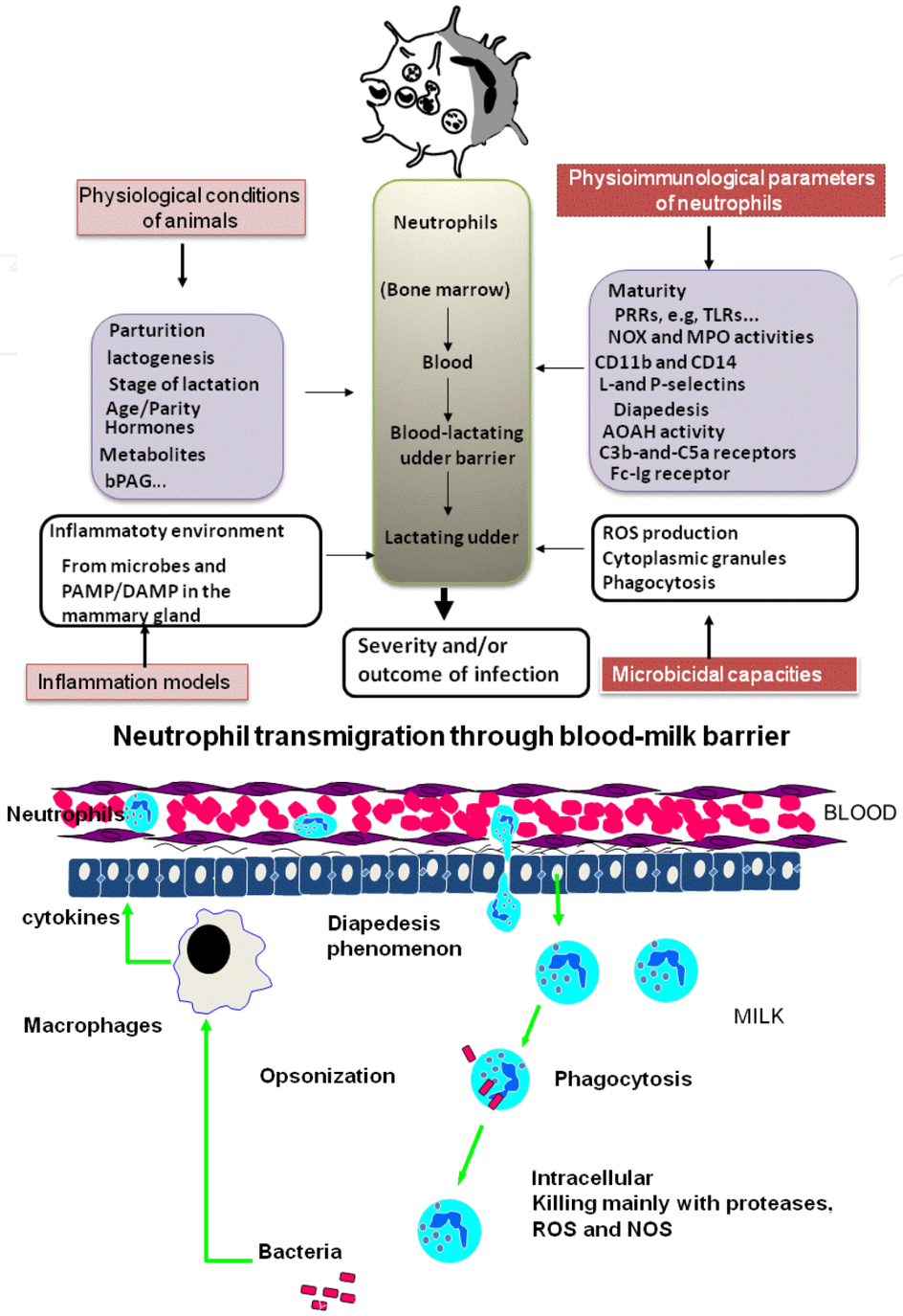


Fig. 3. Upper panel: A schematic representation of the major contributing factors such as some physiological conditions (lactation, parity or age and status of inflammation) to microbicidal capacity of neutrophils in the blood and mammary gland, and the strong link of these conditions to some key immunophysiological parameters of innate host defence in bovine model. This interrelated link happens not only in the blood, but also in the bone marrow, affecting on the most pivotal efferent branches of neutrophil functions in the organs and body, thereby contributing to the outcome or severity of infection. Because

neutrophils are the first cells recruited to the site of infection, their capacity and functionality make this pivotal cell of the circulating innate immune system as one of the cornerstones of the induction and shaping of adaptive immunity in body and organs. Microbicidal activity of neutrophils occurs mainly intracellularly during phagocytosis, with the contribution of many soluble and insoluble proteins, enzymes and ROS 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 (PRRs) (e.g. via Toll-like receptors or TLRs). The activation elicits classic neutrophil functions such as chemotaxis, adherence, ingestion and eventually killing of phagocytosed microbes. Some mediators, cytokines, hormones and metabolites suppress neutrophils' functions throughout the body. Like blood PMN, post-diapedetic PMN functional impairment occurs immediately around parturition; this is coincided with oscillatory events in the cellular and molecular parts of post-diapedetic PMN like viability and killing capacity in the udder. These impairments are more pronounced immediately after parturition and beginning of lactogenesis, which is far more pronounced in older animals. This diagram is based on the author's own studies on bovine PMN functions (see some references appeared in the reference section). The scheme observed in animal model of innate immune system is a fundamental consideration in human, and many molecular aspects of this diagram remains to be further studied in the area of innate immunology in animals and human. Lower panel: Simple scheme of the complex blood-milk barrier showing enterance of invading microbes, which is almost always ascendingly, via teat canal, in the udder cisterna. When microbes enter the gland the triger of innate immune cells in the gland, mainly macrophages and neutrophils, producing veriety of cytokines, chemokins and plenty more immunogenic molecules, creating cell-cell signaling, activating epithelial and endothelial cells, thereby resulting in a masive recruitment of neutrophils in the mammary gland. When real professional phagocytes, neutrophils, reach the site of infection they capture and ingest microbes by phagocytosis and eventually destroy the pathogenic microbes with their microbicidal arsenal, mainly ROS and proteases and RNS (reactive nitrogen species). This final step of first line defence mechanism is the main focus of the future resaerch in the area of molecular immunobiology.

2001c; 2005a; 2005b; 2009). Although the immature neutrophils expressed already the membrane adhesion molecule CD (cluster of differentiation) 11b, they were not capable of rapidly migrating to the infected organs to efficiently ingest and kill the invading pathogens. 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 neutrophils will appear in the blood circulation. These findings support the hypothesis postulated by (Heyneman & Burvenich, 1992; Mehrzad et al, 2001a; 2005a; 2005b; 2009) namely that the presence of myelocytes, metamyelocytes and band cells (shift to the left) observed during acute inflammation and sepsis may compromise the animals' resistance by supplying more cells that are morphologically immature and functionally insufficient. Van Werven et al. (1997) and Mehrzad et al. (2001a; 2002a; 2004; 2005a; 2007) demonstrated that the increase of neutrophil functionality was a result of increased enzyme activity per neutrophil, rather than an increase of the number of neutrophils. Therefore, the enhanced enzymatic activity in neutrophils after onset of infection/inflammation was believed to be

induced by granulocyte-moncyte colony-stimulating factors (GM-CSF), reflecting an increased proliferation and differentiation of bone marrow granulocytes (Heyneman et al., 1990; Heyneman & Burvenich, 1992; Mehrzad et al., 2001a; 2001b; 2004; 2005a). It has been postulated that myeloid stem cells leave the bone marrow almost by a pipeline mechanism, the older cells being released first (Heyneman & Burvenich, et al., 1992). The molecular mechanisms that control the release of mature PMN from the bone marrow into the circulating pool and then extravasation are very complex and poorly understood (see figures 3 and 4).

Neutrophils circulate in the blood until recruited to sites of infection by chemical signals. This migration begins when neutrophils interact with activated endothelial cells. Inflammatory cytokines like tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interferon-γ (IFN-γ) produced by activated macrophages at sites of infection activate endothelial cells and induce expression of receptors like E-selectin (CD62E) and P-selectin (CD62P) and ligands like Sialyl-Lewis X on glycan-bearing cell adhesion molecule-1 (GlyCAM-1) and P-selectin glycoprotein ligand-1 [PSGL-1]) which interact with neutrophil receptors like L-selectin (CD62L) (Diez-Fraille et al., 2004; Sohn et al., 2007a; 2007b ; Zarbock A & Ley, 2008). Selectin-ligand interactions are of low affinity leading to only "rolling" and slowing of neutrophils on the endothelial cell surface in post capillary venules to enable stronger associations.

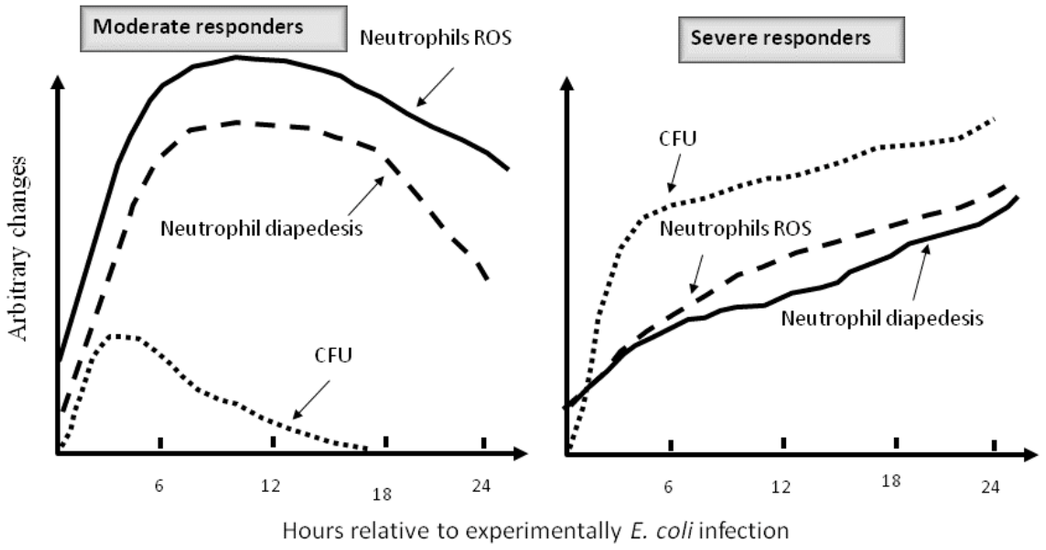


Fig. 4. This figure shows the overall disparities of neutrophils' diapedesis rate, post-diapedetic neutrophil ROS production capacity and *E. coli* CFU dynamics in modertate and severe responders of animal infection model; it is based on the study of the kinetics of chemiluminescence and intramammary infection/inflammation (Mehrzad et al., 2001b, 2004; 2005a). Moderate responders' neutrophils are functioning much more appropriately than severs'. The fast increase in neutrophils' diapedesis rate and post-diapedetic neutrophil ROS production capacity during acute infection of mammary gland lags exponential growth of *E. coli* in the gland. Compared with severe responders, the fast-strong local response in moderate responders facilitated recovery of acute infection and inflammation. The bacterial growth in the lactating udder is exceeded to the neutrophils' diapedesis and ROS

production rates in sever responders. This is very important and basic cellular part of innate immunity of mammary gland. This different response is mainly due to the far stronger pre-infection blood and post-daipedetic neutrophils' functions, especially, ROS production as well as the neutrophils' ROS production during the "early phase" of infection/inflammation. So, the capacity of PMN ROS production (especially intracellular) and quality of pos-diapedetic neutrophils both before and during early infection, is crucial for the severity of the diseases, and leads to a faster elimination of pathogens, becuae the fast-strong local response in moderate responders facilitated recovery of infection/inflammation.

Besides increased expression of selectins, TNF- α and IL-1 also enhance endothelial cell expression of vascular cell adhesion moledule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) and ICAM-2, which are ligands for the neutrophil integrins very late antigen-4 (VLA-4) and leukocyte function antigen-1 (LFA-1 or CD11a/18 or $\alpha_L\beta_2$) and macrophage-1 antigen (Mac-1, CD11b/18 or $\alpha_m\beta_2$), respectively. Interactions of neutrophils with these molecules results in firm attachment to the endothelium, followed by their activation, and spreading. Interactions of neutrophil receptors CXCR1 and CXCR2 down a concentration gradient of their ligand chemokine CXCL8 result in transmigration of neutrophils between endothelial cell spaces to the infectious site (Paulsson et al., 2010). Neutrophil LFA-1-endothelial ICAM-1 and CD177-CD31 (platelet endothelial cell adhesion molecule-1 [PECAM-1]) interactions facilitate this migration (Sachs et al., 2007).

A potential role of adhesion molecules like L-selectin in the release of neutrophils 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-Fraile 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 functional activation of precursor cells (Burvenich et al., 2003). Using an optimised cell culture assay for the bovine (Smit et al., 1996; Van Merris et al., 2001a; 2001b), it was demonstrated that physiological concentrations of β-hydroxybutyric acid and acetoacetic acid induced remarkable suppression on the proliferation of hematopoietic cells (van Werven et al., 1997; Hoeben et al., 1999). Bovine pregnancy-associated glycoprotein also reduced the proliferative activity of bovine progenitor cells (Hoeben et al., 1999). Therefore, the neutrophil circulating pool is largely depending on the proliferative capacity of the bone marrow. After having exerted their role in immune function, PMN die by senescence (Mehrzad et al., 2001a; 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 hugely increase the rate of PMN production, shortening the maturation time, thereby leading to the release of immature neutrophils in the circulation pool.

Advances in mammary gland immunology of recent decades 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 (Paape et al., 2002; Burvenich et al., 2003; Mehrzad et al., 2001a; 2001b; 2001c; 2002; 2004; 2005a; 2005b; 2009). 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 enters the vascular sinus by travelling in migration channels through endothelial cells. Normally, mature neutrophils circulate in the blood stream briefly (half-life of ~9 hours), then leave the blood stream by diapedesis and enter tissues where they function as strong phagocytes for 1 to 2 days. In healthy animals, production and destruction of PMN is tightly regulated, which keeps their number in blood, milk, and tissue almost constant (Heyneman et al., 1990; Heyneman & Burvenich, 1992; Paape et al., 2002; Burvenich et al., 2003; Mehrzad et al., 2002a) and this is an essential element of their role in the first line of immune defense. Continuous influx of PMN 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 IL-8). The dynamic of PMN diapedesis through blood/milk barrier helps to explain the observed PMN activity fluctuations in milk (Smits et al., 1999; Mayadas & Cullere 2005; Pezeshki et al., 2011).

Transendothelial/epithelial concentrations of neutrophils is very critical to effectively kill invading microbes (Li et al., 2002; Mehrzad et al., 2005a; 2005b). The issue of blood and post-diapedetic PMN functions and concentrations in different physiological and pathological conditions remains the focus of most concern. Although the presence of strong chemotactic factors in non-inflamed udder is the subject of debate, their presence in inflammatory environment of udder and milk is indisputable (Manlongat et al., 1998; Rainard, 2002; Stevens et al., 2011a; 2011b; Pezeshki et al., 2011). 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 simply 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 (Gray et al., 1982). These chemoattractants bind to specific receptors on the PMN plasma membrane.

When the completely equipped neutrophils reach the site of infection/inflammation, they with macrophages capture and ingest microbes by phagocytosis and destroy them with their microbicidal arsenal. Neutrophils engage microorganisms through extracellular membrane receptors like PRRs, e.g., TLRs, C-type lectins (mannose receptor), scavenger receptors like CD36, Nod-like receptors (NLRs), and N-formyl Met-Leu-Phe (f- MLP) receptors (Nathan., 2006; Diez-Fraille et al., 2004; Bellocchio et al., 2004; Sohn et al., 2007a; 2007b ; Zarbock A & Ley, 2008; Rainard, 2002; Stevens et al., 2011a; 2011b). Neutrophils also bind to pathogens coated with various opsonins like IgG, complement components (e.g. iC3b), and lectins. Activated neutrophils express the high affinity Fc-receptor, FcγR1, which binds to IgG-Fc of antibody-coated microbes, and β_2 integrins on the neutrophil surface can capture pathogens coated with the iC3b. Bound pathogens are then surrounded by neutrophil membrane projections and engulfed in the cellular cytoplasm inside a phagosome. The phagosome fuses with a lysosome to produce a phagolysosome where the captured pathogens are destroyed.

One of the critically hottest topics in the molecular aspects of inflammation, infection and sepsis in relation to neutrophils in animals and human would be the cross-talk between C5a

and neutrophils' surface receptors, e.g., C5a-C5aR, C5aR-TLR4 interactions. Normally, C5aR is up-regulated in inflammatory environment (Bruhn et al., 2011; Stevens et al., 2011a; 2011b). There is considerable evidence for the participation of C5aR in the harmful consequences of experimental infection, sepsis and cancer in human and animals. Therefore, interception of either C5a or C5aR dramatically improves the exaggerated inflammatory reactions which can be good direction to treat and remodel tissue injuries and damages. This evidence would open a new door to the molecular aspects of controlling and treating of inflammation, infection and sepsis. For example, *in vivo* blockade of C5aR resulted in greatly improved survival of animals after sepsis. Similar phenamena could be observed and would be applied for other key molecules like IL-1-IL-1R interactions as well as TLRs antagonists/agonists in the inflammatory environment.

Extravasation of activated PMN occurs after their adhesion to the endothelial surface. This is accomplished by the expression of specific membrane adhesion molecules. The essential role of the CD11/CD18 family of adhesion molecules in bovine PMN-surface adhesion is well-documented (Diez-Fraille et al., 2004). These molecules bind to ICAM-1, 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). Inflammatory environment of the udder induces 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); in such an environment, blood PMN number, and effective adhesion, migration, opsonization, phagocytosis and killing are of crucial importance to the outcome of the infection and the severity of the disease (Gray et al., 1982; Burvenich et al., 1999; 2003; Mehrzad et al., 2001a; 2001b; 2005a; 2005b; 2009). The impact of fast PMN diapedesis during udder infection/inflammation on PMN quality and their ROS production capability could cause dissimilarities between post-diapedetic PMN from inflamed and non-inflamed quarters (Mehrzad et al., 2001b; 2005a; 2005b; 2009) (see later figures of this chapter). The underlying cellular and molecular mechanisms of this disparity would be pivotal for further investigation in the area of animals and human mammary gland immunobiology and neoplasia.

The source of host and/or pathogen-derived cytokines in udder secretions and their impact on udder 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; Mehrzad et al., 2001b; 2004; 2005a; 2005b; Rainard, 2002; Stevens et al., 2011a; 2011b; Pezeshki et al., 2011). These cytokines influence PMN function. For example, the IL-8 is involved in the recruitment of PMN and T lymphocytes into the gland (Barber et al., 1999). Proinflammatory cytokines, like TNF- α, IL-1β, and LPS suppress the gene expression of cytochrome P-450 1A1 (*cyp1a1*), 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; 2008b). From bone marrow to the blood stream and the extravasation, the on time PMN influx to the site of inflammation is important in limiting injury and promoting recovery of severe inflammation (Carey et al., 1997; Mehrzad et al., 2001b; 2004; 2005a; 2005b).

4. Location of microbicidal weaponry in circulating and post-diapedetic neutrophils

Armed with an array of highly microbicidal weapons, such as enzymes that hydrolyze and destroy proteins, lipids and sugars of pathogens, the weaponry is mainly stored in, at least, three different kinds of granules in the cytoplasm as well as outside the cytoplasm. Additionally, neutrophils have powerful systems for generation of large amounts of free radicals or ROS. Microbial pathogens are taken up into an intracellular compartment, called phagolysosome, into which microbicidal agents are released. There are also many new forms of innate effector molecules like neutrophil extracellular traps (NETs), PRRs, opsonin receptors and plenty more that sense, bind and efficiently kill invading microbes, destroy virulence factors, or prevent them from spreading.

Following adherence of opsonized bacteria to surface receptors on the PMN, phagocytosis, respiratory burst and degranulation are triggered. The process of opsonization, though not essential for phagocytosis, certainly promotes the uptake of bacteria by PMN. The phagocytosis process is energy-dependent and requires the presence of a functional cytoskeleton. The cytoskeleton machinery, when sequentially activated following receptor stimulation, is thought to envelope the microorganism in a "zipper mechanism" (Griffin et al., 1975). Immunological recognition is mainly accomplished by specific antibodies (IgG_2 and IgM) which recognize the bacterium through Fab-regions and bind to PMN via Fc-receptors on the PMN plasma membrane (Paape et al., 1996). There is a synergy between the Fcγ and C3b receptors activity and neutrophils' ROS production (Newman & Johnston, 1979).

Neutrophils produce several proteolytic enzymes that degrade and destroy microbes in the phagolysosome. Two important proteases produced by neutrophils are the serine protease, elastase, and cathepsin G (Reeves et al., 2002; Mehrzad et al., 2005b). More importantly, neutrophils also destroy ingested microorganisms with ROS produced by the "phagocyte oxidase system (phox)," or "NADPH oxidase or NOX," which reduces molecular oxygen into ROS in the phagolysosome (Mehrzad et al., 2001a; 2001c; 2005a; Reeves et al., 2002). NOX pumps electrons from the oxidation of NADPH to NADP in the neutrophil cytosol, across the phagolysosome membrane via flavocytochrome b558 (the core component of phox) and onto molecular oxygen (O_2) in the phagolysosome reducing it to superoxide anion ($O_2^{\cdot-}$), and cascade reaction of respiratory burst starts. NOX activity and K^+ flux are important for provision of acidic pH in the phagolysosome for effective killing of engulfed microbes (Reeves et al., 2002). Normally, during intracellular killing pH of the phagolysosome (initially neutral) rapidly drops to ~4 within <10 min; this change in pH is very important for killing of engulfed microbes, because many enzymes and peptides necessary for microbicidal are activated at acidic pH.

After a complicated cascade of release of biological substances and activation of the endothelium, neutrophils migrate into the mammary gland and finally also appear in the fluid of the lactiferous sinus (Burvenich et al., 1994; 2003). Although several antimicrobial systems exist in the mammary gland (Burvenich et al., 1994; Paape et al., 1996; 2002), but, it is the massive influx of neutrophils that will resolve the infection through efficiently killing of the invading bacteria (Mehrzad et al., 2001b; 2005a; 2005b; Burvenich et al., 2003; Paape et al., 2002). Diapedesis will also affect binding of immunoglobulins to the PMN surface. An increased expression of Fc receptors and phagocytosis happens after *in vitro* migration of

bovine PMN through membranes (Berning et al., 1993; and Worku et al., 1994). After *in vivo* migration of PMN into mammary quarters of nulliparous heifers, binding of IgG_1 and IgG_2 increased while binding of IgM decreased. Binding of IgA remained unchanged. The greatest change occurred with the binding of IgM. Seventy-six percent of the blood PMN bound IgM, whereas only 2% of the post-diapedetic PMN bound IgM. Interestingly, phagocytic activity of PMN increased after *in vitro* chemotaxis but not after *in vivo* chemotaxis of neutrophils. Activation of complement also promotes chemotaxis, extravasation, phagocytosis and killing capacities. The C3b and iC3b, generated on the surface of bacteria following antibody union, are recognized respectively by CR1 and CR3 receptors located on the PMN cell membrane. The type of bacteria also affects bovine PMN bactericidal capacity. For example, slime-producing *Staphylococcus aureus* hampers the killing capacity of PMN (Barrio et al., 2000; Mehrzad et al., 2009). The specific interactions between extracellular matrix proteins of *Staphylococcus aureus* and ICAM-1 inhibits further PMN recruitment, boosting anti-inflammatory reactions (Chavakis et al., 2002). This might be counterproductive for the killing activity of PMN. During migration of PMN into milk in response to infection increased binding of C3b was observed (DiCarlo et al., 1996). Thus, PMN are fully armed to confront invading bacteria, resulting in a more rapid ingestion and elimination of the pathogens. Once complement and immunoglobulins bind to receptors on the PMN surface, PMN become activated and generate ROS, such as $O_2^{\cdot-}$, H_2O_2 and halogen reactive species. This process associated with the respiratory burst is called the "oxygen-dependent" or "oxidative" killing, and that associated with neutrophil granules is also called "oxygen-dependent", but "non-oxidative" killing. Killing classified on the basis of these criteria has been explicitly addressed (e.g., Root & Cohen, 1981; Babior, 1984; Spitznagel & Shafer, 1985; Babior, 1994; Reeves et al., 2002; Mehrzad et al., 2001c; 2009; 2011). The pivotal role of ROS in all further events for killing of engulfed microbes by neutrophils is therefore indisputable.

ROS formed by neutrophils are critical microbicidal agents against infection as evidenced by individuals afflicted with Chronic Granulomatous Disease (CGD). Patients with CGD have mutations in key elements of NOX and are profoundly susceptible to bacterial and fungal infections (Heyworth et al., 2003), though this kind of mutation has not been observed in bovine. In addition, a number of proteolytic enzymes including elastase, cathepsin G, myeloperoxidase, gelatinase, and others contained in the azurophilic and specific granules fuse with the phagolysosome and also contribute to the intracellular killing of microbes and degradation of its contents.

Intracellular killing of phagocytosed microorganisms is accomplished by following adherence to the PMN surface, usually, but not necessarily, via specific receptors (Horwitz et al., 1982). Three different mechanisms are involved for intracellular bacterial destruction: 1) an oxygen-dependent mechanism (production of ROS), 2) a nitrogen dependent mechanism (RNS especially nitrogen oxide (NO) derived from L-arginine) and 3) an oxygen-and nitrogen-independent microbicidal mechanism e.g., lyzozyme, lactoferrin, proteases, pH changes and even neutrophil extracellular traps (NETs). Because of their essentiality for killing, here my prime focus is the oxygen-dependent microbicidal mechanisms and how these mechanisms are generated in neutrophils to efficiently destroy pathogens. Though not predominantly, pathogens are trapped and killed extracellularly, e.g., NETs, as well. Impaired neutrophil NETs formation would be considered as a novel innate immune deficiency of animals and human; the NETs are lattices of DNA, histones,

granule enzymes, and antimicrobial proteins that are released by the PMNs in parallel with extrusion of nuclear material. It has been found that defect in NETs formation in neutrophils is substantially rescued by the ROS generation, confirming the broadly essential roles of ROS in NETs-related killing of pathogen. Similarly, NETs-related killing of pathogens and NETs formation occurs via pathways involving both ROS and RNS (Allport et al., 2002). Thus, ROS generation is always the most pivotal and prerequisite for all further events for efficient killing of invading microbes.

Baldridge and Gerard (1933) first reported that an increase in oxygen consumption by neutrophils takes place when phagocytosis is triggered. ROS generated by reduction-oxidation (redox) reactions, have been recognized as one of the major contributors to the killing of pathogens. This phenomenon is accompanied by an increase in oxygen consumption and the hexose monophosphate shunt (HMPS) activity by PMN and has been termed the "respiratory" burst. The oxygen molecule is central for PMN respiratory burst activity (Allen et al., 1972; Babior, 1984; 1994; Reeves et al., 2002); its importance in microbicidal activity of PMN was highlighted by the inefficient PMN bactericidal activity in anaerobic conditions (Mandell, 1974). One example: for each molecule of O_2 consumed 4 $O_2^{\cdot -}$ ions are generated; roughly 0.5 fmol of O_2 is consumed for each bacterium engulfed, resulting in an intravacuolar $O_2^{\cdot -}$ release of about 4 mol.l^{-1} (Reeves et al., 2002). Though still remains inconclusive, the application of ozone gass (O_3) would be further examined in domestic animals for treatment of infections such as mastitis, metritis, arthritis, cancer and increase many cell signaling pathways for tissue remodeling and repair; because it boosts milk PMN ROS production capacity (Ogata & Nagahata, 2000), cleaning pathogen/damage-associated molecular patterns PAMPs/DAMPs from inflamed site (Lai & Gallo., 2009; Alexandrova, 2009).

As shown in figure 5, the first step in the cascade of respiratory burst is the formation of $O_2^{\cdot -}$, requiring NOX, of which substrate (NADPH) is generated by HMPS to act as an electron donor (Rossi and Zatti, 1964; Babior, 1984; Rossi, 1986).

$$2\,O_2 + NADPH \rightarrow 2O_2^{\cdot -} + NADP^+ + H^+$$

Different stimuli (e.g., complement components, immunoglobulin, formyl-methionyl-leucyl-phenylalanine (fMLP), phorbol myristate acetate (PMA) and bacterial peptides) act via different specific receptors and thus have various signal transduction mechanisms to activate the NOX. Extensive research into activation by PMA has followed the identification of protein kinase-c (PK-C) as its cytosolic receptor (Nishizuka, 1984). PMA is a strong NADPH-oxidase and PK-C agonist (Tauber, 1987, Karlsson et al., 2000). Particulate stimuli may also act indirectly via PK-C (Cooke & Hallett, 1985) or via other intermediates such as arachidonic acid and its metabolites and phospholipase-A2 (Tauber, 1987). The next step is the formation of H_2O_2 by dismutation of $O_2^{\cdot -}$, which is mediated by superoxide dismutase (SOD):

$$2\,O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$

The $O_2^{\cdot -}$ and H_2O_2, generated by NADPH-oxidase and SOD, are the precursors of variety of subsequent powerful ROS. Included among these ROS are a variety of oxidized halogens, including hypohalite ions or HOX (Thomas & Fishman, 1986; Weiss et al., 1986) and a variety of chloramines (Thomas et al., 1982) used by PMN as microbicidal agents. These are

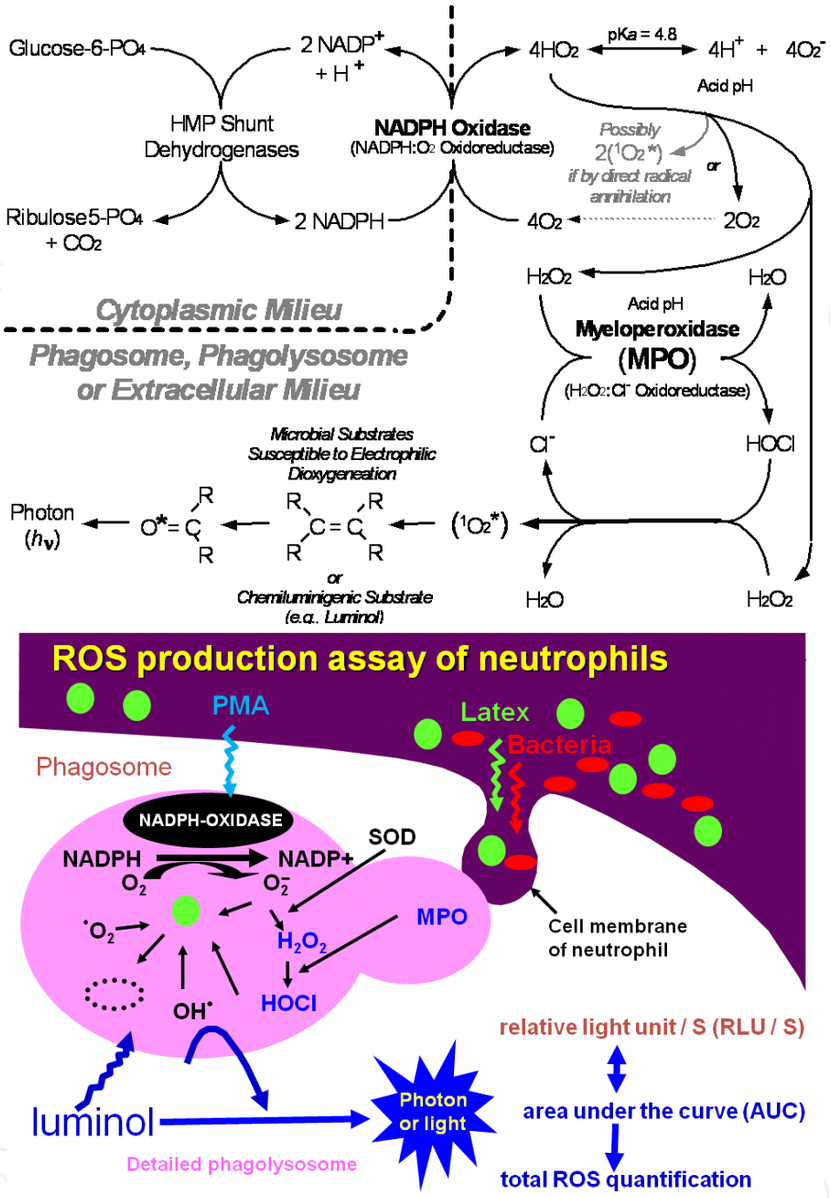


Fig. 5. Upper panel: Diagram depicting the major enzymatic systems responsible for microbicidal metabolism and oxygenation activities and the relationship of these activities to photon emission. In the scheme the activities of the cytoplasmic milieu are separated from those of the phagosome-phagolysosome-extracellular milieu. The superscripted number that precedes each molecular symbol (e.i., ^{1}for singlet, ^{2}for doublet, and ^{3}for triplet multiplicity) depicts the equation: $|2s| + 1$ = multiplicity (n). The diagram adapted from (Allen et al., 2000). Lower panel: Brief scheme of ROS production process by neutrophils during phagocytosis of bacteria. As shown the cell membrane of the neutrophils and bacteria (red). This is non-specefic phagocytosis of bacteria. The bacteria are engulfed by the neutrophils and form phagosome and fussion of phagosome to lysosome and then detialed phagolysosome is highlighted on the scheme. Activation of NADPH-oxidase (NOX) is the triger of neutrophil ROS production, the most fundametally powerful efferent arms of the

innate immune system .When bacteria are attached to the cell membrane ROS production starts and this continues when the pathogen are engulfed. Before engulfment most ROS are produced extracellularly, but afterwards it is produced intracellularly, both of which are pivotal for continuation of innate immune response against pathogens. This is a fundamental concept in the area of ROS-mediated phagocytosis and killing of microbes, in which NOX and MPO are central in this pathway; this is an interestingly big subjetc in innate immunophysiology. These ROS can be measured by chemiluminescence (CL) and this is the main technique that was highlighted in this chapter of the book. To conduct an assay on one of the pivotal efferent arms of neutrophils microbicidal capacity (light or photon produced by ROS), the pure neutrophils are activated artificially with phagocytosis-dependent (latex beads, bacteria etc.) or non-phagocytosis-dependent (PMA...) methods. Researchers routinly use photon enhancer like luminol, isoluminol, lucigenin and plenty more. The metabolites of, e.g., luminol (aminophtalate) is very unstable and can easily and immediately be oxidized by hydrogen peroxide-MPO-halide system and gain to the relaxation state and emit light. This light, which is unequivocal representative of PMN ROS production load, can be easily and precisely quatified by CL assay. The two units, which luminometer gives, are the ROS production in function of time.

generated by the H_2O_2-mediated oxidation of halide ions under catalysis by MPO or eosinophil peroxidase (EPO) and the subsequent oxidation of amines:

$$H_2O_2 + X^- \rightarrow HOX + OH^- \quad (X = Cl^-, Br^-, I^-)$$

$$HOX + R\text{-}NH\text{-}R' \rightarrow R\text{-}NX\text{-}R' + H_2O$$

Though not as crucial as PMN in infection and inflammation, the activity of compound I of EPO to react with H_2O_2 is similar to that of MPO but with substrates like Cl^-, however, it is far higher, yielding more HOX (Arnhold et al., 2001; Mehrzad et al., 2001a, 2005a; 2009; 20011).

Another group of ROS that are produced from $O_2^{\cdot -}$ are the hydroxyl radicals (OH°) generated in a transition metals (Fe or Cu) catalyzed reaction between $O_2^{\cdot -}$ and a hydroxyperoxide (a well-known Haber-Weiss reaction, if R = H):

$$O_2^{\cdot -} + ROOH \rightarrow RO + OH^- + H_2O_2 \qquad (R = H, -C, C(=O))$$

or in a reaction between previously generated oxidizing radical and another compound:

$$OH^\circ + RH \rightarrow R^\circ + H_2O$$

Eventually, singlet oxygen ($^1O_2$) has been found to be produced by neutrophils, and eosinophils (see e.g., Root & Cohen, 1981; Allen et al., 2000), possibly through a reaction between hypohalyte and H_2O_2:

$$HOX + H_2O_2 \rightarrow {}^1O_2 + X^- + H_2O$$

It is evident that the production of large quantities of ROS with a cascade of reactions will provide an environment that is destructive for any microorganims exposed to it, but it is also harmful to the nearby tissues. That is to say that ROS represent a "double-aged sword". Alternatively, ROS also enhance the activity of natural killer cell, T cell and dendritic cells

(DCs), neutralization of PAMPs and proinflammatory cytokines (Suthanthiran et al., 1984; Cemerski et al., 2002; Reth, 2002; Mehrzad et al., 2008a; 2008b; Lai & Gallo, 2009; Alexandrova, 2009), indicating that PMN ROS may not only damage cells and tissues but may also accelerate recovery of inflammation. The above reactions are tightly regulated so that the PMN releases its ROS under appropriate circumstances, depending on the physiological and pathological conditions of animals. What is not yet clear is whether there is a transient PMN ROS production change during physiopathological conditions, and if so, whether this change is or is not beneficial for animals and humans.

Neutrophils are capable of producing a range of ROS following activation of the membrane bound NOX. It is also generally agreed that ROS boost oxygen-independent microbicidal activity, like proteases (Reeves et al., 2002; Mehrzad et al., 2004; 2005a; 2009; 20011). Antimicrobial peptides especially proteases in the udder which is mainly released by neutrophils (Mehrzad et al., 2005b), will potentially promote angiogenesis, activation of macrophages, neutralization of PAMPs and pro-inflammatory cytokines, initiation of T-cell recruitment (Mehrzad et al., 2008a; 2008b) and immature (i)DCs and block of TLRs on iDCs (Lai & Gallo, 2009). Root and Cohen (1981) have suggested several possible direct sites of action for ROS, related to their microbicidal activity; these include: 1) unsaturated carbon bounds that may lead to toxic lipid peroxidation, 2) sulphydryl groups that lead to the destruction of sulphydryl containing enzymes, 3) amino group and possible peptide bound breakage and 4) nucleic acids. *In vitro* studies with $O_2^{\cdot-}$ generating systems in neutrophils such as xanthine oxidase (Rosen & Klebanoff, 1976) suggests that $O_2^{\cdot-}$ are far more toxic to bacteria if they operate in MPO H_2O_2-halide system (Reeves et al., 2002; Mehrzad et al., 2001a; 2001b; 2005a; 2009; 2011), which leads to the production of powerful chlorinated oxidising agents such as ClO^- which have a microbicidal effect by halogenating microbial proteins (see figure 5). Although the microbicidal mechanisms of neutrophils is very hugely broad and complex, but there are plenty mechanisms by which microbes evade and overcome the host's phagocytosis and killing mechanisms to succeed infections in the udder, like avoiding contact with phagocytes and inaccessible to phagocytes, inhibition of inflammatory responses and phagocyte chemotaxis and engulfment, even survival inside of phagocytes.

5. Techniques for neutrophils' ROS quantification versus their quality in the mammary gland

ROS production capacity of neutrophils is the most powerful efferent arms of the innate immune systems in blood stream and interstitial fluid for provision of further cascade of effective innate and adaptive immune responses. Several techniques of PMN ROS quantification are frequently applied. For example, the cytochrome c reduction test, flow cytometry method (Salgar et al., 1991), the scopoletine test (Root & Cohen, 1981) and chemiluminescence (CL) assay (Allen et al., 1972; Hoeben et al., 2000a; Mehrzad et al., 2000; 2001b; 2001c; 2002; 2004; 2005a; 2009; 20011). Also plenty laboratory kits available for precise neutrophils' functional tests both for genomics, proteomics and mechanomics related to the ROS production. The most widely used technique to quantify neutrophils' ROS production is CL (Mehrzad et al., 2000; 2001a; 2001b; 2002a; 2002b; 2004; 2005; 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 animals'/humans' susceptibility to infections.

Whereas many differential leukocyte count methods for blood leukocytes are available, study on the qualitative role of milk leukocytes in healthy and diseased animals and human is rare. The milk leukocytes differentiation also appears difficult. In addition, little attention has been paid to the standardization of particularly sample preparation procedures. To unequivocally evaluate PMN functional assays (from genomics to proteomics, mechanomics and metabolomics) appropriate isolation, differentiation and quantification of neutrophils in original or purified samples are essential. This is more special for neutrophils in non-inflammatory environment of milk/udder; not merely because a variety of cells e.g., PMN, macrophages, lymphocytes and epithelial cells, are existed but because their shapes, size and population could differ, compared to the blood. Milk sample processing varies from the use of centrifuged whole milk samples to dilution with a hypotonic buffer (Mehrzad et al., 2000; 2001a; 2001b; 2001c; 2002a; 2004; 2005a; 2009). Without microscopic confirmation, flow cytometric identification of milk cells based on forward and side scatter is inconclusive because phagocytosis of milk components may alter both size and intracellular granularity. Cellular debris may also interfere with the scatter pattern of normal milk cells. Even for blood leukocytes, their shapes and population changed significantly during mastitis. All of these changes could interfere with the assessment of PMN function. Therefore, developing an isolation, differentiation and enumeration of leukocytes in blood and milk in the laboratory to better assess PMN function is always critical. Nowadays, the problem of breast cancer in human is rising substantially, and particularly deep focus on the cellular and molecular aspects of interstitial fluid of mammary gland is urgently needed.

A functional udder immune system depends on the existence of high quality neutrophils in the interstitial fluid of mammary gland and/or milk, protecting the gland against invading pathogens (Burvenich et al., 1994; 2003; Mehrzad et al 2001a; 2001b; 2001c; 2002; 2005a; 2005b; 2009; 2011). Investigation on PMN viability can provide suitable information about PMN quality and tissue damage. This is more special for milk PMN, which migrate to the apparently unsuitable environment. PMN life span might be affected by many physiological and pathological factors in the gland. Many cellular and acellular signaling pathways are available in blood and mammary tissue for the modulation or inhibition of PMN survival. Till now, little attention has been paid on the neutrophils viability in the interstitial fluid of mammary gland/milk. Accordingly, the contribution of neutrophil enzymes (e.g., activity of NOX, MPO etc.) to the viability is critical (Mayer et al., 1989; Jankowski et al., 2002). This supports the assumption of the existence of a good correlation between PMN viability and CL. To obtain a better insight into the effects of (patho)physiology of mammary gland on non-specific defense mechanisms of the udder, assessment of viability of blood and milk PMN can be pivotal. Milk PMN viability assessment could also be an index for the detection of inflammation of the mammary gland.

To overcome any problem and to simplify PMN functional assay, researchers, who wants to work on this area in animals/human breast physioimmunology, should properly isolate targeted cells from blood and mammary gland for quantitative and qualitative assays (see figures 1-2 and 6); there are plenty references in these topics, and some appeared in the reference list. CL simplifies PMN ROS production measurement, and is a relatively recent technique. Application of CL technique to study PMN function helps to gain more insight into first line of immune defense mechanisms and the pathophysiology of physiological and environmental stresses-related infections and/or inflammations. For CL quantification, we need viable PMN, a PMN activator (e.g. PMA, fMLP, particles, etc.) and a CL substrate (e.g.,

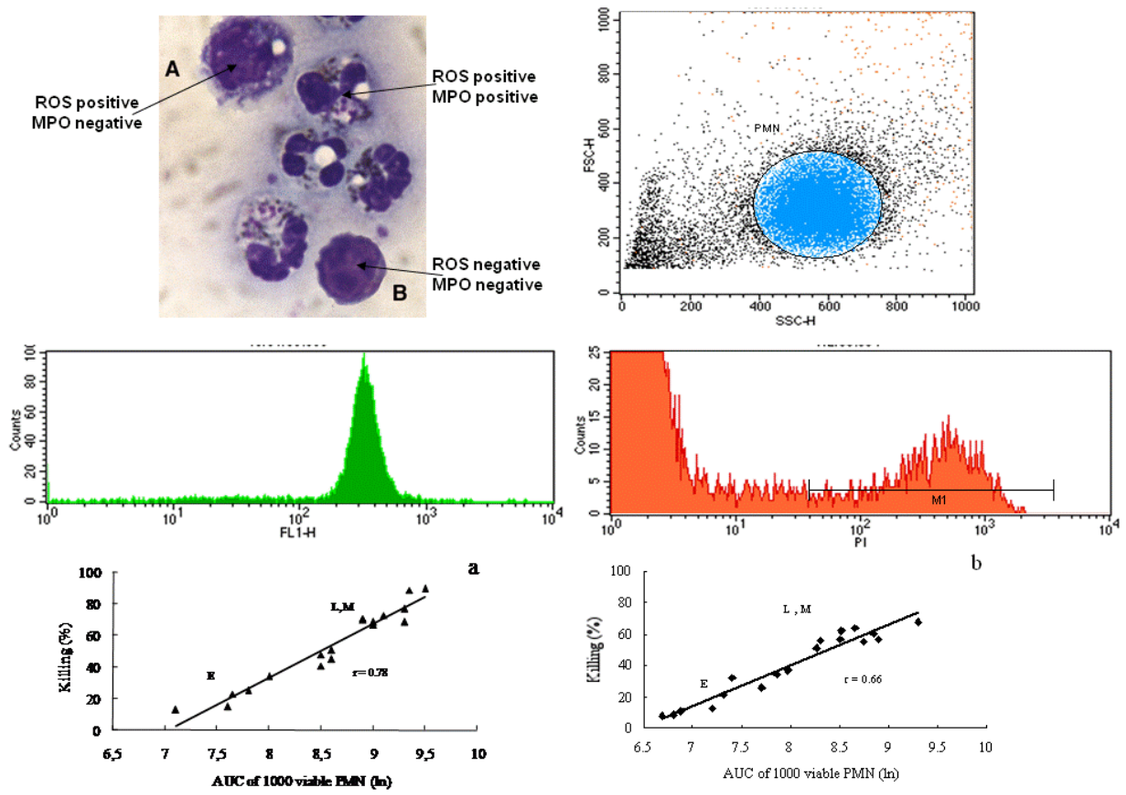


Fig. 6. Upper left: Light microscopic image of PMN (middle), a macrophage (A) and a lymphocyte (B) isolated from non-inflamed lactating udder stained with benzidine dihydrochloride plus H_2O_2 and counterstained with hematoxilin-eosin (× 1000). Because MPO is central to intracellular ROS-associated microbicidal and luminol dependent CL, the contribution of other cells especially macrophages on CL and MPO activity assays should also be considered. Bovine milk macrophages has no MPO activity in our assay, and in case of having mixed cells in the samples their contribution to the CL assay is little. Conversely, bovine post-diapedetic neutrophils has huge MPO activity, and they are main source of CL assay of milk cells. Flow cytometric analysis of isolated bovine milk neutrophils gated in the FS-SS dot plot (upper right). Green fluorescence of PMN labeled with a monoclonal antibody specific against bovine granulocytes and with a secondary FITC-labeled antibody (middle left). Red fluorescence of propidium iodide-incubated PMN selectively gated in the FS-SS dot plot; gate M1 is applied to determine the percentage of dead PMN (for the quantification of viability/quality of post-diapedetic neutrophils) (middle right). Lower panel shows correlation between PMA induced luminol-dependent CL (LDCL) of blood neutrophils (a) and post-diapedetic neutrophils in lactating udder (b) and their effectiveness towards killing of *S. aureus*. E: early lactogenesis period; L, M: late and mid lactogenesis periods, respectively. Partially adapted from (Mehrzad et al., 2001a; 2001c).

luminol, isoluminol, lucigenin etc). Luminol-dependent CL has been described as an appropriate probe for assessment of blood and milk PMN ROS production (Briheim et al., 1984). The PMN metabolic pathways responsible for O_2-dependent bactericidal activity and CL assays are depicted schematically in figure 5.

A flow cytometric technique has also been used to detect ROS production, necrosis, apoptosis and many immunological assays on bovine blood and interstitial fluid neutrophils (Mehrzad et al., 2001a; 2001b; 2001c; Dosogne et al., 2002; Vangroenweghe et al., 2001; Van Oostveldt et al., 2001; 2002a; 2002b Mehrzad et al., 2002; 2004; 2005a; 2009; 2011) applying propidium iodide (PI) exclusion method, Anexi V and JC-1 solution (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) (Merhzad et al., 2001a; 2001b; 2001c; 2011; Van Oostveldt et al., 2002a; 2002b) (figures 6). Although many immunological assays can be done with flow cytometry, the assay of neutrophils' ROS production with flow cytometry has revealed some challenging results, compared to luminometry technique; also, with the flow cytometry it is hardly possible to measure the kinetics and the dynamics of the neutrophil-pathogen interaction 'during' neutrophil-pathogen interaction.

Methodological studies reveal a very well correlation between PMN viability and CL activity and killing capacities. Isolated blood and milk PMN can appropriately be identifiable on FS-SS dot plot (see figures 5, 6). Flow cytometry is an accurate and reproducible technique for the rapid quantification of PMN apoptosis and necrosis in physiological and pathological conditions of animals and human (figure 6). This can effectively facilitate further PMN functional assay, hence boosting insight into the first line defense mechanism of the host.

6. Lipopolysaccharide, TLR4, TNF-α and NO levels in inflammatory environment of the mammary gland

The compound of the bacterial outer membrane, peptidoglycan monomers and lipopolysaccharide (LPS) or endotoxins, are unique to all Gram-negative bacterial cell walls. In the case of Gram negative bacteria, the principal stimulator of the innate immune system is the LPS; this LPS evokes several functional responses in these short-lived, bone marrow myeloid-derived cells, neutrophils, to the site of inflammation or infection.

The PRRs are the main sensors of pathogens and danger signals in innate immunity. Though they are mainly highly expressed by macrophages and DCs of different organs, neutrophils also highly express these sensor molecules inside and outside their surface. Toll like receptors/proteins, homologues of the *Drosophila* protein Toll, are the most studied and best characterized PPRs, which are responsible for sensing PAMPs and also products of inflamed tissues, DAMPs. TLRs activation triggers signaling pathways that lead to activation of transcription factors such as NF-κB and the interferon regulatory factors. This, in turn, leads to induction of immune and inflammatory genes, including such important cytokines as TNF-α and type I interferons. The contribution of PRRs to inflammation induced by microbial infection, tissue damage and cancer are a hot topic in immunology, immunopathology and immunotherapy. Much evidence points to the role for PRRs and especially TLRs in immune and inflammatory diseases and increasingly in cancer detection and therapy (Bellocchio et al., 2004; Simons et al., 2008). For example, cancerous cell lines are one of the best models to study the biological roles of PRRs in cancer and tumor biology. Role of neutrophils on those points remained deeply unnoticed. Study also reports how, e.g., TLR2 expression by endothelia is locally upregulated by the action of activated neutrophils via an unprecedented mechanism involving cell-cell interaction and NOX,

emphasizing yet another way in which the primordial innate immune system is remarkably complex.

The interaction of neutrophils in the sites of inflamed/infections with these key elements of the PAMPs, LPS, and bind to PRRs is pivotal to overcome pathogen and limit the severity of infection. The PRRs are present on a variety of defence cells of the body causing them to synthesise and release a variety of cytokines. Synergistically, the LPS interacts with LPS-binding protein and CD14, which in turn promotes the ability of particularily TLR4 on neutrophils and macrophages to respond to the LPS with the release of various pro-inflammatory cytokines and chemokines (Sohn et al., 2007a; 2007b; Rinaldi et al., 2010; Stevens et al., 2011a ; 2011b). These pro-inflammatory molecules bind on target cells via specific receptors and initiate inflammation. TLRs have been detected on the surface of mammalian cells (Beutler, 2002). They are important in the responses of phagocytes to bacterial, viral, and fungal antigens. TLR2 and TLR4 have candidate genes for resistance to several diseases as they recognise broad classes of PAMPs, such as peptidoglycans and LPS (Werling & Jungi, 2003; Bellocchio et al., 2004). Bovine and human neutrophils express substantial amounts of TLR4 and other TLRs, critical for response to PAMPs/DAMPs (Kurt-Jones et al., 2002 ; Hayashi et al., 2003; Werling. & Jungi, 2003; Rinaldi et al., 2010; Stevens et al., 2011a ; 2011b). TLR4 activates the inflammatory gene expression through NF-κB (Hayashi et al., 2003). GM-CSF and and G-CSF dramatically up-regulate TLR2 and CD14 expression (Kurt-Jones et al., 2002). Recent *in vitro* studies have shown that mammary epithelial cells actively participate in the immunoregulation during inflammation via mainly cytokine production (Sohn et al., 2007a; 2007b; Rinaldi et al., 2010; Stevens et al., 2011a; 2011b). A MAC-T cell line was utilised to investigate the expression of many innate immune mRNAs like IL-1 and its subsequent secretion after stimulation with PAMPs showed that these cells secrete IL-1 in response to LPS. IL-1 also appeared to be an important mediator for the release of the IL-8 and many other chemokines (Huynh et al., 1991; Boudjellab et al., 1998; 2000; Pezeshki et al., 2011).

During infection of mammary gland, IL-1, IL-6 and IL-8 (Shuster et al., 1997), platelet-activating factor (PAF), (Pezeshki et al., 2011), prostaglandins (Pezeshki et al., 2011; Shuster et al., 1997), activated complement C5 (C5a; Shuster et al., 1997; Rinaldi et al., 2010) NO (Blum et al., 2000), many ROS (Mehrzad et al., 2001; 2004; 2005a) and proteases (Mehrzad et al., 2005b) are released locally. In milk, an increase in TNF-α, a cytokine of particular interest in the pathogenesis of mastitis, is observed (Blum et al., 2000; Hoeben et al., 2000b; Shuster et al., 1997). The kinetics of cytokines in the inflammatory environment of udder shows that the increase in TNF-α and IL-1, the mother of proinflammatory cytokines, occurs faster during inflammation from endotoxins than from *E. coli* mastitis, though more pronounced in *E. coli* infection (Blum et al., 2000; Hoeben et al., 2000b). Absorption of this cytokine from the udder into the blood circulation is highest during *E. coli* mastitis. The level of these key cytokines in milk correlated well with the pyrexia and the severity of the udder infection (Shuster et al., 1997; Blum et al., 2000; Hoeben et al., 2000b; Pezeshki et al., 2011).

Many of the biological activities of LPS are mediated by TNF-α. LPS and cytokines stimulate the synthesis of NO, which is a vasodilator. Synthesised from L-arginine, this diatomic free radical, NO, is lipid soluble and easily diffuses through the cell membrane. It is short lived and usually reacts and degrades fast. The natural form is a gas that reacts with a variety of innate immune molecules and mediates a large spectrum of immunobiological effects in the

body. An inducible NO synthase (E.C. 1.14.13.39, iNOS) is expressed by a variety of cells, especially phagocytes, as a result of triggering with substances of microbial origin such as LPS and TNF-α. In response to invading pathogens and PAMPs like LPS, bovine phagocytes produce NO (Adler et al., 1995; Stich et al., 1998), functioning as a strong anti-PAMP agent. When the NO is produced in excessive amounts, it can also induce cytotoxic and apoptosis in the cells (Moncada et al., 1991; Anggard, 1994)

Several studies on ruminants have shown a relationship between LPS, TNF-α and production of NO. The increase in TNF-α is followed by a delayed increase in NO_x (NO_2 + NO_3) (Blum et al., 2000; Bouchard et al., 1999). The NO_x production lasts longer in the udder. A causal relationship between TNF-α and NO_x production was observed in studies in which *E. coli* LPS was injected intravenously. It seems that severe forms of udder's inflamed environment are accompanied by the highest increase in blood stream's levels of both TNF-α and NO_x; the increase in NO_x and TNF-α during infection is not inhibited by antibiotics (Blum et al., 2000), supporting the notion that the release of NO_x is PAMP dependent rather than *E. coli*. Initially called endothelium derived factor (EDRF), NO causes many other vital physiological phenomena like vasodilation and subside of inflammation. It is released after the fever peak, and is involved in this delayed phase of hyperemia.

7. Neutrophil AOAH as a potent protector of mammary gland from pathogens

The contribution of circulating and postdiapedetic neutrophils' acyloxyacyl hydrolase (AOAH) to the outcome of infection in animals has recently been highlighted (Dosogne et al., 1998; Mehrzad et al., 2007). Apart from existence of many bactericidal mechanisms in the bovine neutrophils (Barrio et al., 2000; Burvenich et al., 2003; Mehrzad et al., 2001a; 2001b; 2001c; 2002; 2004; 2005a; 2005b; 2007; 2008a; 2009), there are many other soluble and insoluble proteins on the neutrophils in the mammary gland that protect the gland from invading pathogens. One of them is AOAH molecules. Endotoxins or LPS are released during bacterial growth and lysis of Gram-negative bacteria have been recognized as important mediators for the treatment and outcome of coliform mastitis (Pyörälä et al., 1994; 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., 2004; 2005a; 2007). Conversely, 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 infection (Blum et al. 2000; Hoeben et al. 2000b; Mehrzad et al., 2007). Severity of *E. coli* infection seems to be related to the enhanced release of secondary induced inflammatory mediators such as TNF-α (Blum et al., 2000; Mehrzad et al., 2007), as a result of impaired LPS detoxification mechanisms in inflamed organ. It has been suggested (Burvenich et al., 1996; 2003; Paape et al., 1996; 2002) that local CD14 expression alleviates the toxic effects of LPS in the mammary gland. AOAH, an enzyme hugely produced by bovine neutrophils, hydrolyses LPS (McDermott et al., 1991; Mehrzad et al., 2007) and alleviate the inflammation. This neutrophils' arsenal, AOAH, hydrolyses two acyl chains of the lipid A of LPS, leading to substantial decreased toxicity of LPS while retaining much of the immunostimulatory potency of native toxicity of LPS (Munford and Hall, 1986).

Although there are some rare investigation of bovine blood PMN AOAH activity, but little has been done on post-diapedetic neutrophils' AOAH activity either during physiological or pathological conditions. Immediately after parturition, 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., 2001b; 2002a; 2004; 2005a). This coincidence could be considered as a risk factor for Gram negative bacterial infections during especially physiological stress (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 and sepsis. It is suggested that a decreased AOAH activity in post-diapedetic PMN can also contribute to the outcome of organ failure and even death, as we observed in bovine (Mehrzad et al., 2007).

Apart from AOAH, bovine neutrophils granules also contain different LPS binding cationic proteins such as lactoferrin, and a huge variety of cationic antimicrobial proteins (Levy et al., 1995; Mehrzad et al., 2005b). 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 immunobiologists.

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 vacuoles. 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 remarkably contribute to host defense against pathogens. Because many bactericidal peptides like β-defensins etc. are stored in the dense granules of neutrophils, 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.

The 46 kDa soluble CD14 (sCD14), which is shedding of membrane CD14 (mCD14) from phagocyte, is also available in interstitial fluid/milk (Wang et al., 2002; Sohn et al., 2007a; 2007b), and can bind LPS directly and prevent LPS from binding to mCD14, thus preventing over-secretion of TNF-α, thereby silencing the severity of inflammation and clinical symptoms. This potentially plays a role in neutralizing LPS and controlling the clinical symptoms associated with acute infection. *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 infection and sepsis; this approach would also be applicable in human inflammatory and infectious diseases.

Furthermore, iron-binding protein, lactoferrin, is small protein synergizes neutrophils' functions and LPS detoxification. Bovine lactoferrin does far beyond the binding of iron, and has 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 wall, leading to direct bacterial lysis, especially when the osmolarity of the infected microenvironment is sufficiently low. Lysozyme has also the capacity to neutralise and strongly interact with *E. coli* LPS; another non-oxidative antimicrobial agent in PMN, which directly/indirectly enhances LPS degradation, 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). PMN ROS production synergises activity of all above mentioned antimicrobial and anti-PAMP compounds. Overall, apart from the role of bovine PMN granules and enzymes, it is fully accepted that the PMN ROS production plays a major role in protection of udder from Gram negative bacterial infection (Burvenich et al., 2003; Mehrzad et al., 2004; 2005a) and detoxification of LPS.

8. Oscillatory events on neutrophils functions

Neutrophil dysfunction in animals and human has been associated with decreased immunocompetence, resulting in the suppression of host defense mechanisms and increased susceptibility to many infectious and non-infectious diseases. Nowadays, potential increasingly environmental stresses and worldwide-food scarcity issues have resulted in unstoppably intensive feed/food and dairy production. The intensive production system leads to unstoppable oscillatory events on innate immune systems, especially neutrophils in bone marrow, blood and udder of high yielding dairy cows, compromising innate defence system in udder, thereby making animals particularly sensitive to infections. Oscillatory events on neutrophils occurs in all stages of maturation and functions (see figures 7 and 8), not only in mature animals but also in neonate, e.g., in bovine (Mehrzad et al., 2001a; 2001c; 2002a; 2008a 2008b; 2008c). Relative magnitude of circulating and post-diapedetic PMN impairment differed from different physiopathological status of animals and humans. A dramatic reduction in random migration, iodination and ROS production of blood PMN were observed during the first week after parturition (Heyneman et al., 1990; Hoeben et al., 2000a; Mehrzad et al., 2001a; 2001b; 2001c). It was recently discovered that the adhesion molecule L-selectin is shed from the surface of bovine PMN at parturition (Diez-Frail 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 parturition (Berning et al., 1993; Diez-Frail et al., 2004). Regulation of bovine PMN adhesion molecules during mammary gland infection and possible use of immunomodulators has recently been studied (Diez-Frail et al., 2004).

Cumulative deficiencies in opsonin levels (IgG_1 and conglutinin) were observed in peak of physiological stress in animals, which closely coincided with impaired PMN oxidation-reduction reactions capacity (Detilleux et al., 1994; Burvenich et al., 2003). The proportion of all cases of infections especially mastitis, metritis, arthritis and laminitis that develop during period at which the animals encounter with maximal stress status; these period is coincided with maximal oscillatory events in circulating and post-diapedetic neutrophils (Burvenich et al., 1994; 2003; Mehrzad et al., 2001a; 2001c; 2002a; 2008b; 2008c).

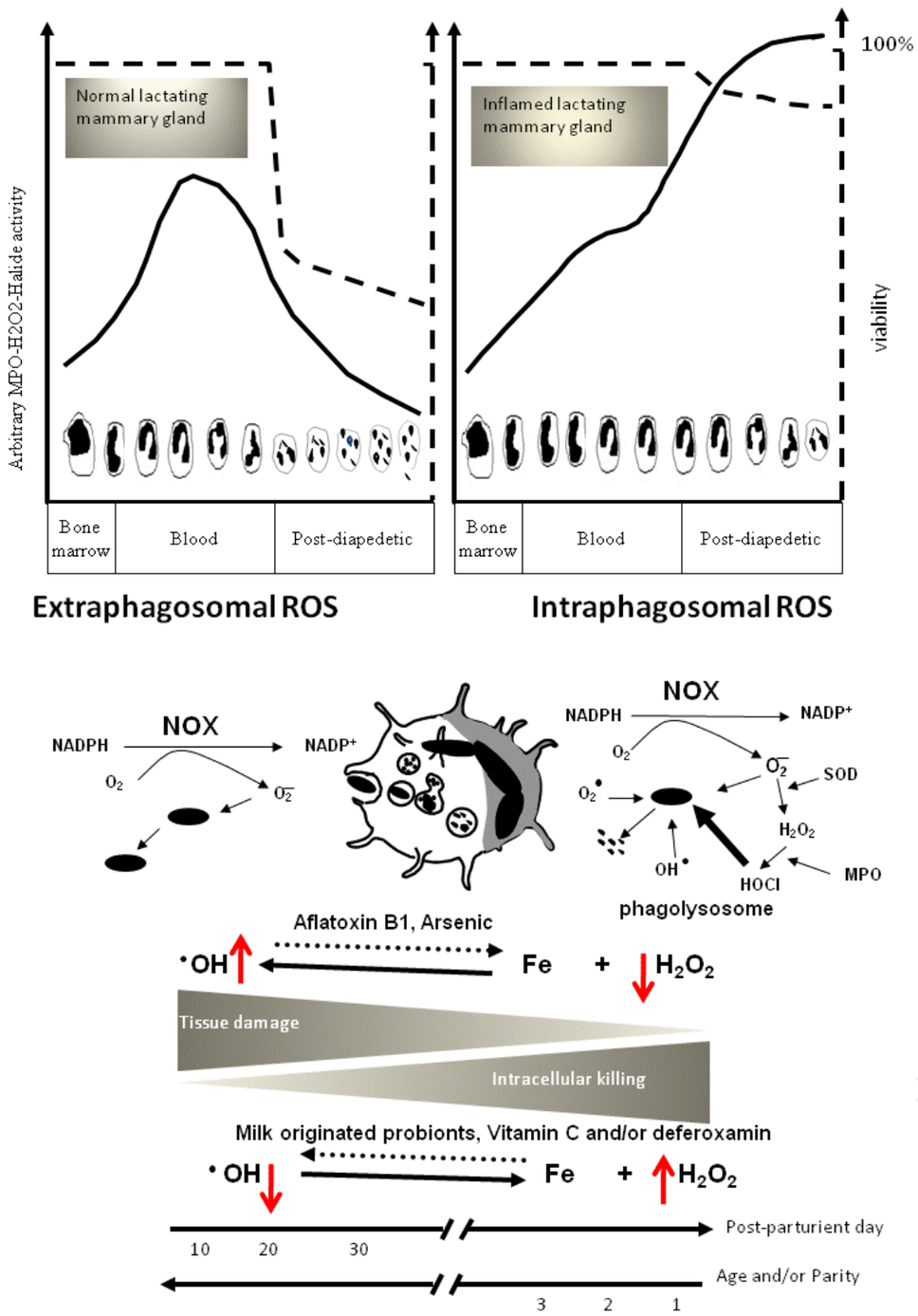


Fig. 7. Upper panel: An schematic overview on author's recent findings (together with literatures) about changes in neutrophils' MPO-H_2O_2-Halide system (solid lines), viability (dashed lines) and dynamics of neutrophils structure and maturity in healthy and mastitis dairy cows. Formed in bone marrow, blood neutrophils function and structure changed after normal extravasation. These changes differed in inflammatory environments. The most probable reason for these disparities would be the "rate of diapedesis", which is faster

during inflammation; pinpointing the aspects of molecular mechanisms of this classically mechanomical phenomenon of neutrophils in different physiological and pathological conditions in animals and humans is very interesting topics for further fundamental research in the area of innate immune system. Lower panel: Diagram illustrating the oscillatory events on neutrophils and some potential nutritional and environmental interventions (Ibeagha et al., 2009; Mehrzad et al., 2008c; 2011) in animals and human to modulate their neutrophils' ultimate functions. These provocative hypotheses address the points that during severe infection/inflammation of the udder extracellular production of ROS by neutrophils may be impaired and could lead to tissue damage in mammary gland. Normally, ROS for bactericidal activity is produced intracellularly for effective destruction of microbes with a minimal tissue damage. The hypothesis is based on the study of the kinetics of chemiluminescence: 1) there is a difference between extracellular and intracellular ROS production of both blood and post-diapedetic neutrophils during different stages of lactogenesis, and 2) in blood and milk, extracellular ROS production by neutrophils is more pronounced in old animals. This may lead to an impaired bactericidal capacity of resident udder neutrophils and boost tissue damage in aged animals (Mehrzad et al., 2001a; 2001b; 2002; 2004; 2005a; 2005b) and partially adapted from (Burvenich et al., 2003). Further, many environmental toxins cause oscillations on neutrophils' ultimate functions (Mehrzad et al., 2011); conversely potential nutritional intervention could reverse the oscillatory events in neutrophils (Ibeagha et al., 2009; Mehrzad et al., 2008c); what happens on the impacts of nanotubes, nanovectors, nanoneedles, nanoparticles and nanoadjuvants on this casecade of events can be very interesting to work. Promising photoredox and antioxidant properties of vitamin C, probionts and deferoxamin with huge quenching capacity mainly on $OH^{\bullet}$ with much less pronounced on H_2O_2 and $O_2^{\bullet -}$ (Mehrzad et al., unpublished data), and dietary supplementation with probiotics and peptides originated from bovine milk augments neutrophils' functions (Gill et al., 2000; 2001a; 2001b; Mehrzad et al., 2002b). Reverse results have been observed with aflatoxin B1 (Mehrzad et al., 2011), Arsenic, Lead (Mehrzad et al., unpublished data) and plenty more with marked oscillatory effects on neutrophils' ultimate functions. Both in animals and humans due to many environmental and physiological stresses influencing on the overall immune system of the body, switching the oxidant-antioxidant systems on the body from antioxidant status to prooxidant ones and excessive extracellular ROS will accumulate in the body. To remove excessive and unwanted ROS, especially $OH^{\bullet}$ in the body some antioxidants like vitamins C, E, A…, deferoxamin and probionts can be promisingly helpful and here immunobiologists are strongly encouraged to focus on those novel topics of nutritional and environmental immunology in animals and humans.

One of the most critically physiological associated stress periods would be periparturient period and early lactogenesis. Both in milk and blood PMN function is substantially decreased around parturition (Van Oostveldt et al., 2001; Mehrzad et al., 2001a; 2001c, 2002; 2009; Burvenich et al., 2003). Up till now 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, pregnancy associated glycoprotein) (Gray et al., 1982; Burvenich et al.,1994; Suriyasathaporn et al., 1999) have been reported as attributable factors.

There are many reports demonstrating that at least some of hormones and metabolites contribute to the oscillatory events in PMN function, targeting both the afferent and efferent

arms of PMN functions (Gray et al., 1982; Burvenich et al., 1994; Suriyasathaporn et al., 1999; Hoeben et al., 1999; 2000a). 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 pregnancy associated glycoproteins play a causative role in oscillatory events on key efferent arms of PMN function, ROS production/microbicidal 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; 2001b 2002).

Our understanding of the precise ways in which the complex cascade of ROS production occurs in blood or milk PMN during physiological and pathological conditions is still in its infancy. This is especially true for the mechanism of in vivo effect of PMN functions by hormones and metabolites, especially the metabolomics aspects of neutrophils' dysfunction. Based on current understanding of impact of hormones on PMN function, the membrane, cytosolic and nuclear effects of hormones (e.g. growth hormone, sex hormones, cortisol, pregnancy associated glycoprotein) and metabolites (β-hydroxybutyrate, non-esterified fatty acid) on blood and post-diapedetic 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* infection (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; 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 (Zhao et al., 1992). Their concentration differs throughout lactation in milk (Campbell et al., 1991). An increased number of circulating leukocytes, band neutrophils, and an enhanced PMN functions in cows treated with bST after stress related to parturition. Also PMN ROS generation, chemotaxis, random migration, phagocytosis towards IgG-opsonised microorganisms is boosted by IGF-I and bST (Fu et al., 1991; Wiedermann et al., 1993; Warwick-Davies et al., 1995; Mehrzad et al., unpublished). The expression of complement receptors on neutrophils 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 (Merchav et al., 1993); *in vivo* bST administration leads to elevation in blood IGF-I lovel. Though *in vivo* might be different from *in vitro,* many recent studies revealed an increased PMN ROS production capacity after *in vivo* administration of bST in healthy animal model; similar results were observed *in vitro* (Mehrzad et al., 2002b). Thus, there is ample evidence that bST and IGF-I can boost neutrophils' functions. Concentration of some biomolecules like β-lactoglobulin in udder secreta is minimal during maximal stress condition of lactaogenssi (Caffin et al., 1984); this means that the β-lactoglobulin can be a potential immunomodulator in the mammary gland and good topic to further focus and apply in human breast and milk physioimmunobiology (Wong et al., 1998; Mehrzad et al., 2000). Hence the insight into PMN activators and/or inhibitors in milk during physiological and

pathological conditions is crucial concern for udder's first line defense mechanism and neonatal passive immunity in animals and humans.

As explained before, blood and post-diapedetic neutrophils have the potential to produce substantial amounts of ROS to kill engulfed bacteria (Hoeben et al., 2000a; Mehrzad et al., 2001a; 2001c; 2002a; 2004; 2005a; 2005b; 2009). ROS production can be measured in resting (non-stimulated) neutrophils and after stimulation with e.g., PMA, zymosan, bacteria and latex beads.

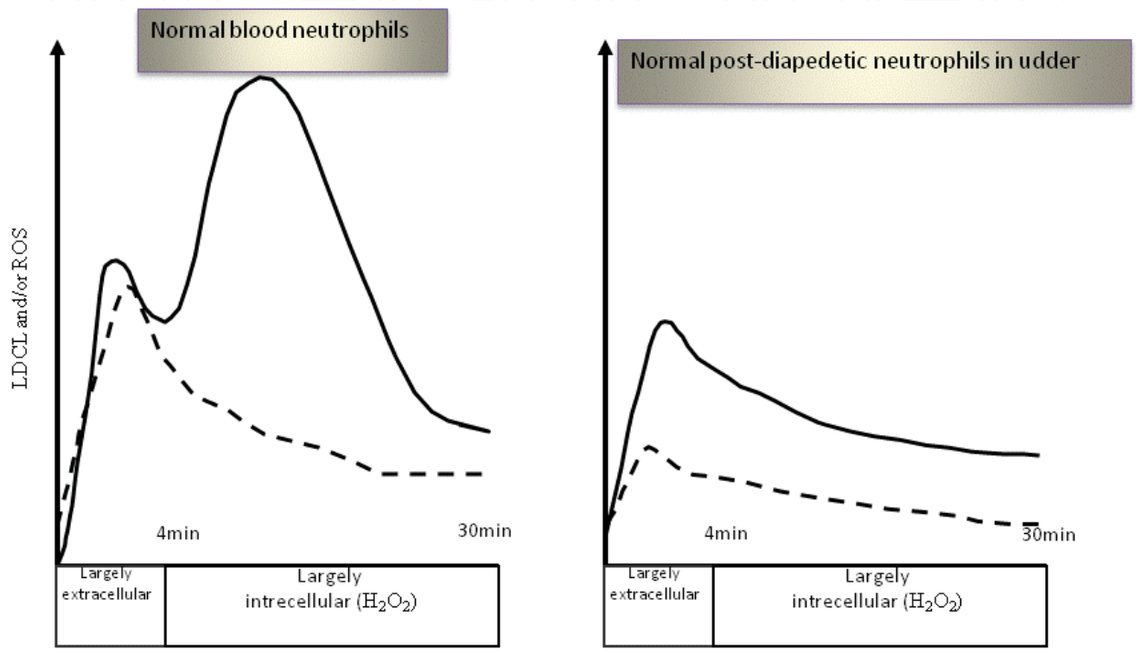


Fig. 8. A comparison between blood and post-diapedetic neutrophils chemiluminescence (CL) profiles of PMA-activated luminol-enhanced neutrophils during different physiological status of animals. The intra-and-extra-cellular ROS production of neutrophils and the concept of biphasic versus monophasic CL pattern of neutgrophils are overviewed. The first peak is the result of mainly initial extracellular reactions and the second peak is mainly a result of subsequent intracellular reactions of the MPO-H_2O_2 system. The Y-axes are the cumulative RLU / s in function of time, and the X-axes are the entire measurement period of CL. Based on the author's previous studies, curves are arbitrarily depicted to show how neutrophils microbicidal capacity changes after diapedesis through blood-milk barrier and during different physiological status. Oscillatory phenomenon observed in the postdiapedetic neutrophils specially the monophasic pattern with minimal ROS production peak. Throughout lactogenesis period of the lactating mammary gland, the kinetics of post-diapedetic PMN CL never exhibited double phase patterns. Further, blood PMN CL kinetics immediately after parturition gave neither double phase nor high intensity as in later phase of lactogenesis period. The plateau and shape of the blood and- post-diapedetic neutrophils' CL curves shows a biphasic pattern of the blood neutrophils CL during minimal stress condition (like later period of lactogenesis). The intensity of ROS production is lower in post-diapedetic neutrophils than in blood neutrophils, the plateau and shape of the milk- and blood CL curves were similar during maximal physiological and environmental

stresses. Thus, the oscillatory phenomenon of milk PMN ROS production/microbicidal capacity during parturition and lactation seems to be directly related to that of the blood PMN. This animal model of ineffectiveness of the oxygen-dependent intracellular killing mechanisms of neutrophils during inevitable physiological and environmental stresses is very interesting topic of efferent arms of neutrophils for further research in animals and human.

Production of ROS is effective in killing engulfed microbes (Burvenich et al., 1994; 2003; Reeves et al., 2002; Mehrzad et al;, 2001a; 2001c; 2002a; 2004; 2005a; 2005b; 2009). Also the most widely used technique to estimate oscillatory events in bovine and human PMN ROS production is the elegantly simple CL assay (Hoeben et al., 2000a; Mehrzad et al., 2001a; 2001b; 2001c; 2002a; 2004; 2005a; 2009; 2011).

Little comparison has been made between CL of circulating and post-diapedetic neutrophils. 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 (Karlsson et al., 2000; Mehrzad et al., 2001a; 2001b; 2001c; 2002; 2004; 2005a; 2009) in the lactating mammary gland. Ingestion of milk fat globules and casein micelles affects milk PMN quality (Mehrzad et al., 2001a; 2001b; 2004; Paape et al., 2002; Burvenich et al., 2003) and subsequent degranulation. No such a problem in blood stream is existed. Smits et al, (1999) have shown that *in vitro* transepithelial/endothelial diapedesis of PMN across mammary gland reduces ROS production of PMN. It has also been shown that the function of post-diapedetic neutrophils in udder differs from their blood counterparts (Smits et al., 2000; Mehrzad et al., 2001a; 2001b; 2001c; 2002a; 2009). Some physiological influencing factors such as lactogenesis (Mehrzad et al., 2001a; 2001c) and ageing (Mehrzad et al., 2002a; 2008b; 2009) are involved in overall PMN impairment. Some other contributing factors would be β-lactoglobulin (Mehrzad et al., 2000b), of which concentration in milk is minimal during maximal stress condition like early lactogenesis (Caffin et al., 1984). The PMN function impairment generally coincides with cow's susceptibility for environmental infection (van Werven et al., 1997; Burvenich et al., 1994; Burvenich et al., 2003; Mehrzad et al., 2004; 2005a). Dynamically, the topic is very important especially for human medicine and further research on this topic of mammary gland innate defence system is deeply needed.

Apart from the involvement of environmental and nutritional factors in hosts' PMN functions and resistance to infection, plenty of many other factors contribute to PMN oscillatory events and the outcome of infection/inflammation in humans and animals; one of the key and interestingly dynamic attributing factors would be the effect of genetics on the quantity and quality of circulating and and post-daipedetic neutrophils (Dietz et al., 1997; Riollet et al., 2000; Wall et al., 2005; Radwan et al., 2007; Rupp et al., 2007; Bannerman et al., 2008). Undoubtedly, all of above mentioned factors could become an important alternative for prophylactic measures of infectious and inflammatory diseases in animals and humans.

Another future increasingly challenging issue in the area of innate immunotoxicity is the environmental and artificial nanoparticles (Karathanasis et al., 2009; Sadikot & Rubinstein, 2009; Gonçalves et al., 2010; Paulsson et al., 2010; and plenty more), which can be both friend

and foe for the innate immune system and cancer development. This can be more special for the oscillatory events on both afferent and efferent arms of blood and post-diapedetic neutrophils. Exposures to airborne nanosized particles have been frequently experienced by animals/humans throughout their evolutionary stages, affecting on, e.g., portals of body's entry (respiratory and gastrointestinal tracts and skin). To highlight potential mechanisms of nanotubes, nanovectors, nanoneedles, nanoparticles and nanoadjuvants and cancer nanomedicine, researchers should always focus on immune systems especially the issue of genomics, proteomics and mechanomics aspects of free radicals production of the phagocytes. Application of those potentially promising idea and hypotheses in relation to the neutrophils' oscillatory events versus neutrophils the medicines, eg, topical wound protectant and antiseptics for the treatment of human/animals blister wounds contaminated with microbes or to kill abnormal cells in the body would be very challengingly promising. The challenge is to integrate effectively all information from cellular to molecular events anthropogenically happening for animals/humans' health and performance may contribute to further oscillatory in neutrophils.

9. Conclusions and future perspectives

The issues on animals and humans' diseases are many; among them is neutrophil function that has been the researchers' past, current and future concern. It is clear that appropriate function of neutrophils in the body of animals and humans is very vital to enhance their health and performance. The concepts of neutrophil recruitment at the site of microbial invasion and the interesting phenomenon of nonspecifically engulfing and killing of microbes by neutrophils is still a complex cascade of many cellular and molecular events; molecularly, afferent (sensing) and efferent (effector and/or highly intracellular-and-extracellular microbicidal compounds) weapons of neutrophils are vital in protection of the hosts. Inevitable occurring of general and local immunocompromised conditions especially on the effector weaponry of neutrophils leads to countless infectious and non-infectious diseases. Most researchers see the immunocompromised condition of the organs like udder as a result of neutrophils' dysfunction in bone marrow, bloodstream and interstitial fluid. This chapter would make the complex oscillatory events happening in neutrophils a little bit more comprehensible.

Despite intense progress in molecular biology, medicine, nutrition, genetics and nanomedicine, animals and humans are still susceptible -more than before- to environmental bacteria; this susceptibility is maximal during stress, of which neutrophils dysfunction can be one of the most central attributable factors. Immunomodulation is still far from assured. The long-term and fundamental solution for the oscillatory event on neutrophils is to strengthen their functions by means of attainable physio-immunological approaches. This requires a comprehensive study on molecular and cellular aspects of physio-immunological alterations throughout gestation, lactation and diseases.

One of the focuses of the chapter was a comparative overview of blood and post-diapedetic neutrophils' functions. To uncover further evidence on neutrophils' oscillatory events during stress conditions the shape of blood and post-diapedetic PMN CL proven one more reason for high susceptibility of animals/humans to infections. On this topic, many more questions remain open for future research. Future research is also necessary to pinpoint the physiopathological influencing factors on post-diapedetic neutrophils' necrosis and apoptosis. The hypotheses of contribution of antioxidants like vitamins C, E, A, GHS and

dynamic of phagolysosomal pH on post-diapedetic neutrophils' quality and first line defense during stress condition could be tested in the future research.

It is conclusive that blood PMN had stronger weaponry than that of post diapedetic PMN, when encountered with pathogens. It is also concluded that the relative magnitude of blood and post-diapedetic neutrohpils' oscillation/impairment differ from different physiopathological status of animals. For example, post-diapedetic PMN quality impairment is more pronounced in older animals/humans. This impairment coincided with the impairment of PMN microbicidal capacity in bloodstream; PMN ineffectiveness against invading pathogens not merely resulted from the quantity of PMN, but, more importantly, from the quality of PMN, which was identified via PMN CL kinetics and PMN viability. In healthy animals the lowest post-diapedetic PMN quality is found during stress conditions, which is more pronounced in older ones, proving one more reason for high susceptibility of aged animals and humans to infections during environmental and physiological stresses. The explanations in this chapter aimed to increase the insight into the first line defense mechanism of organs like mammary gland/breast, could further deepen our understanding at the complex physiopathology of mammary gland infections, cancers and other stress-related infectious and non-infectious diseases. It is conceivable, however, more novel findings and views on these topics remain for future research.

With the existed knowledge, it is clear that stressed animals and humans are relatively immunosuppressed. This could boost their susceptibility to environmental bacterial infections. Nowadays, the overstressed animals/humans are more susceptible to environmental pathogens than before. The main concern now is how to control and enhance these non-specific aspects of immune system. Clearly, the most appropriate treatment for infectious and non-infectious diseases is preventive treatment. The chapter clearly demonstrates that the severity of mammary gland infections is highly related to pre-infection neutrophils' functions, quick recruitment of neutrophils in the gland and their quality after diapedesis. All of these neutrophils' functions impaired during stress conditions. Therefore, preventive measures on animals around stress should be thoroughly performed. The preventive measures should be aimed at lowering stressful conditions and ensuring a high standard of nutrition and hygiene. Both in animals and humans zero stress status must be implemented for the future; this is hardly achievable. The long-term, environmentally friendly ways and fundamental solution for stress-related infectious diseases in animals/humans is "to strengthen their first line defense" by means of attainable physio-immunological approaches. This requires more insight into the first line defense mechanism, which is absolutely crucial. Perhaps one exciting and environmentally acceptable approach for infection/inflammation control would be application of "probionts". Protection of the organ from pathogens with less/non-pathogen bacteria, then further research on host-bacteria interactions would be promising.

As addressed, the protruding psuedopodes in blood differs from those of post-diapedetic neutrophils; it would be worth studying the impact of stress, age and infections on surface morphology of blood and post-diapedetic neutrophils. To further mimic the first line defense mechanism similar study should be conducted on "bone marrow-blood-barrier". Positive role of transient PMN impairments during parturition and early lactation in animals and humans should not be ignored as a good event, because this might be responsible for less damage on biomolecules, cells and tissue during periparturient period,

potentially providing better passive immunity to neonates from invading pathogens. Study should also be focused on molecular mechanisms of higher blood and milk neutrophil's functions (both efferent and afferent arms) in younger animals and humans.

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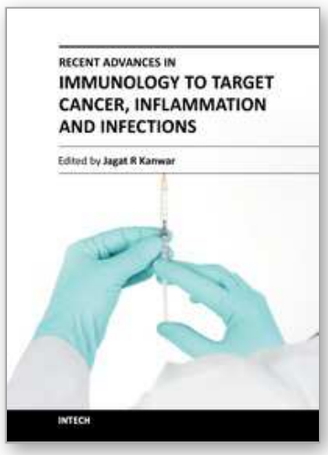
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Immunology is the branch of biomedical sciences to study of the immune system physiology both in healthy and diseased states. Some aspects of autoimmunity draws our attention to the fact that it is not always associated with pathology. For instance, autoimmune reactions are highly useful in clearing off the excess, unwanted or aged tissues from the body. Also, generation of autoimmunity occurs after the exposure to the non-self antigen that is structurally similar to the self, aided by the stimulatory molecules like the cytokines. Thus, a narrow margin differentiates immunity from auto-immunity as already discussed. Hence, finding answers for how the physiologic immunity turns to pathologic autoimmunity always remains a question of intense interest. However, this margin could be cut down only if the physiology of the immune system is better understood. The individual chapters included in this book will cover all the possible aspects of immunology and pathologies associated with it. The authors have taken strenuous effort in elaborating the concepts that are lucid and will be of reader's interest.

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