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Review article

Innate immunity of the bovine mammary gland

Pascal RAINARD*, Céline RIOLLET

Laboratoire de Pathologie Infectieuse et Immunologie, Institut National de la Recherche Agronomique, 37380 Nouzilly, France

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Abstract – Understanding the immune defenses of the mammary gland is instrumental in devising and developing measures to control mastitis, the major illness of dairy ruminants. Innate immunity is an extremely broad field for investigation, and despite decades of research, our present knowledge of the innate defenses of the udder is incomplete. Yet, information is being gained on the recognition of pathogens by the mammary gland, and on several locally inducible defenses. The contribution of mammary epithelial cells to local defenses and to the mobilization of leucocytes is under growing scrutiny. Interactions of mastitis-causing bacteria such as *Escherichia coli* or *Staphylococcus aureus* and the mammary gland represents a suitable model for studies on innate immunity at an epithelium frontier. Powerful new research tools are radically modifying the prospects for the understanding of the interplay between the mammary gland innate defenses and mastitis-causing bacteria: genetic dissection of the immune response, microarray gene technology, transcriptomic methodologies and gene silencing by RNA interference will make possible the discovery of several of the key defense mechanisms which govern the susceptibility/resistance to mastitis at the molecular and genetic levels. It should then be possible to enhance the resistance of dairy ruminants to mastitis through immunomodulation and genetic improvement.

mastitis / cattle / innate immunity / inflammation / milk

Table of contents

* Corresponding author: rainard@tours.inra.fr

1. INTRODUCTION

Among the ailments that affect dairy ruminants, mastitis plays a prominent part. Resulting in most cases from infection of the mammary gland, mastitis induces at least minor but at most fatal illness to the affected animal, causes major economic losses through reduction in milk yield and waste of milk unfit for consumption, entails massive antibiotic use, and is a major cause of premature culling. The prevention and treatment of mastitis represent a serious burden to producers and are primary concerns of the dairy industry. In spite of the efforts deployed to control it, the incidence of mastitis continues to be one of the highest of all the cattle diseases, and, as a result of the long-lasting feature of subclinical mastitis, the most common form of the disease, its prevalence in dairy herds remains at the forefront on the international scale.

To remedy this situation, vaccination against mastitis has long been an active field of research, but for the time being, the panoply of mastitis vaccines is neither well-stocked nor very efficient. Another approach to the control of mastitis is the selection of more resistant animals. Actually, it may well be that the genetic selection for increased milk yield and improved composition had resulted in increased susceptibility to mastitis [52, 66]. To correct this unwanted drift, genetic improvement through selection for resistance against mastitis has recently been called upon, first for dairy cows, then for dairy ewes and soon for dairy goats [156]. Innate immunity is a target of choice for selection against infectious diseases. The broad diversity of its mechanisms offers many potentialities for selection, and it can be anticipated that this very ancient part of the immune system has given rise to genetic diversity that can be exploited to the aim of increasing resistance to udder infections. A preliminary to harnessing the innate immune system is a better knowledge of its intricate and diverse pathways than the rudimentary knowledge we presently have. The availability of potent research tools in genomics, transcriptomics and proteomics makes possible the study of mammary gland immunity and mastitis pathogenesis in more detail and on a larger scale than it was previously. Nevertheless, that will be a formidable task, because innate immunity is an extremely broad topic for investigation.

It is not easy to delineate precisely innate immunity because it is intricately enmeshed with adaptive immunity, and both systems share many effector mechanisms. Innate immunity involves defense mechanisms that are not antigen-specific, although innate immunity should not be equated with non-specific immunity, because innate immunity shows molecular specificity [199]. On the contrary to adaptive immunity, innate immunity to a given pathogen pre-exists the encounter with this pathogen, it does not depend on an immune stimulus. In return, it is not augmented by repeated exposure to the same pathogen: innate immunity has no memory, whereas the efficiency of adaptive immunity rests on memory. Nevertheless, many innate defenses are inducible by infectious encounter, often over a period of hours. Innate immunity is clearly related to the processes of acute and chronic inflammation and to other events such as sepsis [51].

The field of innate immunity can be conceived as having two arms, the sensing arm and the effector arm [11]. The former deals with how the host perceives infection, the

latter with how the organism combats infection. Each arm of innate immunity can be subdivided into humoral and cellular components. Another distinction is between resident innate components, already on the scene when pathogens make inroads or which are induced locally in response to microbial intrusion, and those which have to be mobilized by the host to the particular organ where infection develops. The boundary between local and mobilized innate defenses is drawn by inflammation, so that when resident defenses are not sufficient to contain or eradicate infection, systemic defenses are recruited to come to the rescue.

2. RESIDENT DEFENSES

2.1. The teat canal barrier

The teat canal is the first line of defense against mastitis since this is the route by which pathogens gain entrance to the mammary gland. This canal is sealed between milkings, and during the dry period, by a keratin plug derived from the stratified epithelial lining of the canal. Probably the major role of this waxy plug is to achieve a physical barrier preventing the penetration of bacteria. Keratin is able to bind and immobilize most strains of non-encapsulated mastitis-causing bacteria [34]. Additionally, some components of the keratin have microbicidal activity against mastitis-causing bacteria, although the bactericidal efficiency of whole keratin may be limited [36]. The milking is a critical operation in relation to the barrier efficacy of the teat canal. Milk flushes out the keratin plug, and the teat canal is distended by the vacuum and the milk flux. The teat end contains sphincter muscles that maintain tight closure between milkings. After milking, two hours are required for the sphincter to contract and close the teat canal [164].

Machine milking can have a profound influence on the integrity of the teat duct, by inducing mechanical and circulatory impairments in teat tissues [211]. Improper machine milking use or maintenance favors teat end erosion, and in the long term is likely to alter the functioning of the teat sphincter. Machine milking may also modify the immune defenses of the teat duct [211]. A healthy skin condition of the teat reduces the risk of contamination because it reduces the colonization of the teat skin by bacteria such as *Staphylococcus aureus*, which predisposes the cow to new intramammary infections. It was shown that in many cases bacteria are present in the teat canal for weeks before causing intramammary infections, despite regular teat dipping [134]. Long-lasting teat canal colonization shows that mastitis-causing pathogens have adapted to the teat duct milieu, and suggests that the proximal region of the teat duct may be in a position to play a special role in the immune defense of the mammary gland. It may in particular fulfil a role of sensing of and protection against invading bacteria, in relation to the presence of numerous intra-epithelial leucocytes (see Sect. 2.3).

As a result of the usual efficiency of the teat canal barrier, the intramammary lumen is an aseptic milieu. Important consequences for the immune innate defenses are likely to ensue. On the contrary to other epithelia such as the intestinal, buccal, or upper respiratory epithelia, the mammary epithelium is infrequently stimulated by bacterial components, and any bacterium must be taken as an intruder. Peculiarities of the immune equipment of the mammary gland, such as the sub- and intra-epithelial leucocytes, or the repertoire and distribution of sensor receptors on mammary epithelial cells (MEC) are likely to be conditioned by the aseptic character of normal milk. In this respect, the mammary gland resembles more the urinary system than the intestine.

2.2. Humoral defenses

The contribution of the **complement system** to the defense of the bovine mammary gland has recently been reviewed

[142]. Complement is present in milk of healthy uninflamed glands at low but significant concentrations. The classical pathway is not functional due to the lack of C1q, but the alternative pathway can operate, with two consequences: deposition of opsonic C3b and C3bi on bacteria, and generation of the pro-inflammatory fragment C5a [144, 148]. Concentrations of C3 (2.5% of blood serum concentration) are higher than expected on the basis of a passive transudation from blood, and the alternative pathway activation results in the deposition of an amount of C3b/C3bi equivalent to the deposition achieved by about 2% of serum of adult cows [144]. Milk concentrations of C5 vary widely between cows (0.2 –1.9% of blood values), resulting in very different capacities to generate C5a [145]. This may have consequences for the involvement of C5a in the initiation of the inflammatory response of the mammary gland. The chemotactic fragment C5a has been shown to induce the migration of neutrophils through the mammary epithelium in vitro and in vivo [131, 172], but the role of C5a in the initiation of the inflammatory response of the mammary gland remains to be specified. Although increases in C5a milk concentrations have been reported to occur early after inoculation of the mammary gland with *Escherichia coli* or *S. uberis* and concomitantly with the influx of neutrophils, C5a is usually not detected in milk after inoculation with *S. aureus* even though neutrophils are recruited [6, 7, 153, 170]. These observations suggest either that different pathogens elicit preferentially different mediators and pathways of inflammation, or that C5a is not an initiator but only a booster of inflammation. The triggering of the inflammatory response by C5a may depend on the presence of a receptor at the apical face of MEC. The expression of a receptor for C5a (C5aR) has been demonstrated on human bronchial and alveolar epithelial cells [65], but the expression of a C5aR by MEC remains to be established. C5a is a potent stimulator of the phagocytic function of neutrophils [149], which could be of significance for the efficiency of phagocytosis in milk during infection. If the role of complement in the initiation of inflammation remains uncertain, complement is very likely to contribute to the defense of the mammary gland when increases in vascular and epithelial permeability allow complement components to gain access to the tissue and milk compartments where it can be activated by bacteria and leucocytes.

Lactoferrin (Lf) is a protein which exerts several functions related to innate immunity. Lf was first known for its iron-chelating properties, the basis of two of its activities, bacteriostasis and protection against oxygen radicals catalyzed by free iron [93]. Citrate, which is a harbinger of lactation, is secreted in milk by MEC. This buffer chelates iron and makes it available to bacteria. In bovine milk and colostrum, the high molar ratio citrate:Lf precludes Lf to exert bacteriostasis [171]. In the involuted gland, reduced citrate and increased bicarbonate concentrations are more favorable to the iron-chelating properties of Lf.

Bovine milk contains very little Lf (20–200 μg/mL) compared with human milk (1–2 mg/mL) or sow's milk (0.5 mg/mL). Bovine colostrum contains higher amounts of Lf (2–5 mg/mL), and the secretions of non-lactating mammary gland can contain very high concentrations of Lf (20–100 mg/mL). The main source of Lf in milk is the MEC. Expression of Lf is inversely related to alveolar development: no expression or low expression of Lf occurs in lactating alveoli. Expression is moderate to high in the epithelium lining the ducts and cisterns, but absent at the proximal end of the teat canal [107]. Neutrophils, which contain Lf in their secondary and large granules, can account for about 5% of Lf found in milk during acute inflammation [62].

Bacteria which have high iron requirements are susceptible to the bacteriostatic activity of Lf. Among mastitis-causing pathogens, *E. coli* are the most susceptible, followed by *S. aureus*, but streptococci are resistant [138]. *S. aureus*, S*treptococcus*

agalactiae, *Streptococcus dysgalactiae* and *Streptococcus uberis* bind Lf at their surface, and express one or several Lf-binding proteins, indicating a more complex interaction between Lf and pathogens than mere iron chelation [50, 110, 129, 140]. The Lf-binding proteins may allow the bacteria to directly acquire iron from Lf, or be the target of antimicrobial activities of Lf. The bacteriostatic activity of Lf can be enhanced by antibodies specific to mastitis-causing bacteria, possibly by interfering with the bacterial iron-acquisition systems [119, 139].

Bacteriostasis is usually a temporary antibacterial effect, which can be counteracted by bacterial pathogens. Bactericidal activity represents another antibacterial activity of Lf, and particularly of N-terminal fragments like lactoferricin [122]. The polar/nonpolar character of lactoferricin makes its structure similar to cationic peptides which exert their antimicrobial activities through membrane disruption [122]. Another bactericidal N-terminal fragment of bovine Lf has recently been described [193]. It can be hypothesized that bioactive fragments of Lf are generated in milk during infection by proteases in milk. During acute mastitis, the fluid phase of milk (cells removed) becomes bactericidal to *E. coli*, an activity reversed by very high concentrations of iron, which suggests the involvement of cationic peptides [137].

Besides antibacterial activity, Lf is endowed with immuno-modulating and anti-inflammatory properties [93]. In particular, anti-inflammatory properties of Lf could come into play in normal milk. Human Lf was found to bind to the lipid A of bacterial lipopolysaccharides (LPS) with high affinity, resulting in the neutralization of LPS by preventing LPS from interacting with the main actors of LPS signaling, like LPS-binding protein (LBP), soluble CD14 (sCD14) and membrane CD14 (mCD14) [93]. Bovine Lf also is able to reduce the endotoxin-induced response of inflammatory cells [102], but the contribution of Lf to the control of inflammation during *E. coli* mastitis remains to be demonstrated.

Lf may be able to operate in synergy with other defense components, such as complement or lysozyme. Bovine Lf has been shown to modulate complement activation: the binding of Lf to *S. agalactiae* activates the classical pathway of complement, resulting in the opsonization of the bacteria [141]. It can be hypothesized that high concentrations of immobilized Lf are achieved at the surface of bacteria expressing Lf-binding surface components, thus creating a special microenvironment. The activation of the classical pathway in full lactation milk can operate only after inflammatory exudation of plasma components, since only the alternative pathway is functional in milk. Interestingly, activation of the alternative pathway, resulting in increased deposition of C3, was demonstrated at the surface of *S. aureus* after incubation with bovine Lf [75].

Bacteriostatic and bactericidal activities of iron-binding proteins are probably of low efficiency during lactation, but are likely to be significant when the MG is fully involuted. During acute mastitis, Lf is likely to participate in the bactericidal activity of cell-free milk, but only when milk composition has grossly changed, milk yield dramatically decreased, and Lf concentrations have considerably augmented. The binding of Lf and its proteolysis fragments to LPS, LTA and CpG may dampen the inflammatory response during acute mastitis, to the host's benefit [20, 200].

Transferrin is another iron-binding protein which is found in milk. On the contrary to the milk of rodents and rabbits, the milk of ruminants contains only low concentrations of transferrin, from 1 mg/mL in colostrum to 0.02–0.04 mg/mL in milk compared to 4–5 mg/mL in serum [147, 159]. Transferrin is not synthesized in the mammary gland of ruminants, on the contrary to mouse, rat and rabbit mammary glands [160]. Transferrin comes from blood serum, by transcytosis in the normal gland [120],

and through exudation of plasma during mastitis, when transferrin concentrations can reach 1 mg/mL during acute *E. coli* mastitis, paralleling the concentrations of serum albumin [137]. Transferrin may provide a first iron-chelating bacteriostatic agent to milk, before Lf concentrations augment.

Lysozyme (N-acetylmuramyl hydrolase) is a bactericidal protein cleaving the peptidoglycans of the cell wall of Gram-positive and Gram-negative bacteria. Only a few bacterial species are killed and lysed by lysozyme, but this enzyme can synergize with antibodies, complement or lactoferrin. For example, the binding of the cationic lactoferrin to the lipoteichoic acid, an anionic surface component of Gram-positive bacteria, makes staphylococci susceptible to lysozyme [96]. Nevertheless, bovine milk contains an average of only 13 µg lysozyme/100 mL, compared to 10 mg/100 mL in human milk [152]. Lysozyme is not considered a significant defense of the bovine mammary gland.

The enzyme **lactoperoxidase**, in the presence of thiocyanate and hydrogen peroxide, inhibits or kills bacteria of many species, including the most common mastitis-causing pathogens. Bovine milk contains about 30 µg peroxidase/mL [152]. Thiocyanate concentrations vary with the feeding regime of the cow, and certain streptococci produce hydrogen peroxide, but overall the low oxygen tension in milk probably limits the effectiveness against mastitis-causing pathogens.

Xanthine oxidase, an enzyme of the membrane of milk fat globules, catalyses the formation of nitric oxide from inorganic nitrite, which under aerobic conditions leads to the generation of peroxynitrite, a powerful bactericidal agent. Bovine milk, which has a noticeable xanthine oxidase activity, was shown to be bacteriostatic to *E. coli* after the addition of nitrite [61]. Low oxygen tension and pH below 7 favor xanthine oxidase activity, and nitrite can be produced by bacteria themselves or by nitric oxide synthase, which makes a protective role of xanthine oxidase plausible. Xanthine oxidase may contribute to the observed longer shelf-life of wholemilk compared to defatted raw milk.

Natural antibodies are another component of innate humoral defenses. As far as mastitis-causing bacteria are concerned, cows have opsonic antibodies (Ab) in their serum. Rather low concentrations (less than 1%) of serum opsonize efficiently *S. aureus* and *E. coli*. Opsonic antibodies belong to the IgG2 and IgM isotypes [60], but much of the opsonic Ab in adult serum and milk of cows are IgM [68, 205]. Early lactation milk contains enough Ab to opsonize *E. coli*, whereas mid-lactation milk may lack Ab to the most resistant, encapsulated strains [68]. Nevertheless, as soon as inflammation develops and plasma exudes in milk, bacteria are opsonized. This holds true of animals without previous history of mastitis. The spontaneous and general occurrence of Ab, particularly in the IgM class, suggests that these Ab are natural Ab. Natural Ab are produced in the complete absence of external antigenic stimulation. They are mainly auto-antibodies directed against self-antigens, and are polyreactive [10, 17]. They provide immediate, early and broad protection against pathogens, before adaptive Ab are developed in the course of infections. Natural Ab can be found in any isotype but are mainly IgM. Polyreactive Ab have been found in cattle serum [157], suggesting that the cow is not different from humans or rodents in this respect.

Besides opsonic Ab, antitoxin neutralising Ab are also present in the serum of most adult cows without history of infection, as reported for antibodies to staphylococcal leukotoxin [99]. Although neglected or taken for granted, natural Ab are likely to play a prominent role in the innate defense of the mammary gland.

Overall, the milk of healthy mammary glands does not preclude the growth of mastitis-causing bacteria. There are differences in growth rate of pathogen in milks of individual cows, but there is no established

relationship between growth rate and resistance to mastitis. For example, the in vitro growth of *E. coli* during the early phase of infection differed widely but was not related to the severity of mastitis [85]. The in vivo growth measured as the bacterial concentration in milk at 6 h post-infection correlated with the severity of infection, but is likely to result from the interaction of bacteria not only with the fluid phase of milk but also with the cellular environment, milk leucocytes and MEC [85].

2.3. Cellular defenses

Innate immune cells comprise neutrophils, macrophages, natural killer cells (NK) and dendritic cells. To these leucocytes, another cell type should be added, the MEC, which is at the interface between the body and its environment. The cells which contribute to the innate defense of the udder are either tissue cells or milk cells. Milk from a perfectly healthy bovine udder should contain very few cells, since the gland is not a holocrine secretory organ [161]. Milk cell concentrations vary widely as a function of the lactation cycle. In full lactation, very few leucocytes should migrate in milk in the absence of inflammation, and long ago it was reported that whenever the SCC in milk was above 20 000/mL there was always histological evidence of inflammation in the udder [161]. In most uninfected uninflamed quarters, SCC is appreciably less than 100 000/mL, with a low proportion of neutrophils. As lactation progresses, there is usually an increase in total cell concentration, and in the proportion of neutrophils which can reach 40% near drying-off [32]. At cessation of milking, the udder tissue undergoes intense physiological changes. In the early dry period, cell concentrations augment for the first seven days to reach about $2–5 \times 10^6$/mL, then they decrease to stabilise during much of the period at $1–3 \times 10^6$/mL [73, 103]. At parturition SCC are usually higher than 1 million/mL, and decrease to 10^5/mL in the 7–10 days after calving [73, 104].

For many years, it was considered that the main cell type found in the milk of healthy quarters was epithelial in origin [161]. However, in 1980, it was reported that no secretory epithelial cells and very few ductal epithelial cells were present in udder secretions at any stage, and that the predominant cell type was the macrophage in dry and lactating cows, or the neutrophils in colostrum [89]. Lymphocytes accounted for 10–27% of cells during lactation, but no plasma cells were found. The identification of cells rested mainly on electron microscopy. This report confirmed an earlier observation that the milk cells usually designated as epithelial cells were capable of phagocytosis and were probably macrophages [72]. In the following years, several papers confirmed that epithelial cells are rarely found in cow milk, and that macrophages are the major cell type in secretions of the dry gland, colostrum and milk [32, 73, 103, 104].

Concentrations of lymphocytes are high in the secretion of involuted udders but decrease to very low numbers during the week preceding calving [104] or at calving [73]. In normal udder secretions, at least twice as many T-lymphocytes express the $\alpha\beta$ receptor than the $\gamma\delta$ receptor, are predominantly CD8+, and display the phenotype of memory cells [130, 175, 188].

Recently, several groups reappraised the predominance of macrophages in normal milk. Epithelial cells were reported to be the major cell type, on the basis of absence of labeling with anti-CD11a/CD18 antibodies, in the milk of healthy cows [97, 98]. Others found a majority of lymphocytes in milk by flow cytometry, particularly in early lactation [47]. The differential leukocyte count in low SCC milk samples is difficult, and the methods used differ widely. A possible source of divergence stems from the proportion of cells recovered by centrifugation of milk, which is seldom checked in the experiments aiming at defining the composition of milk cell populations. It can be quite low, 7 to 26% for example after

centrifugation of untreated milk at 400 × *g* for 15 min at 2 °C [109]. This may introduce a bias in the cell population composition if certain cell types are more prone than others to centrifugal selection. Treating milk before centrifugation is advocated to improve cell recovery. Addition of 2% EDTA to disrupt casein micelles and clumps of fat globules resulted in the recovery of 14 to 54% of milk cells [109]. Dilution of milk with isotonic buffer may also favor cell recovery.

The reference method to estimate the number of cells in milk is the time consuming and fastidious milk film microscope counting [161]. Now, the task is conveniently performed with electronic counting devices, so that every report on the composition of milk cells should include information on the yield of cells obtained before analysis. For flow cytometry, reports should also include information on the viability of cells, since dead or damaged cells may take Ab in a non specific way.

Macrophages are a major cell type in milk, secretion of the involuted udder, and mammary tissue [73, 104, 178]. Milk macrophages are phagocytic cells which can ingest the common mastitis pathogens [72]. They are less active than milk neutrophils at phagocytosis, and both milk cell types are less efficient than their blood counterparts [108]. Although a direct defensive role of the milk macrophage is doubted, they are potential antigen-presenting cells, and they are implicated in the detection of invading pathogens and the initiation of the inflammatory response, which may represent their essential function as effectors of innate immunity (see Sects. 3.2 and 5.1.1). The functional capabilities of mammary gland macrophages decrease markedly during the periparturient period, and this alteration has been linked with increased mastitis incidence [177, 202].

The contribution of **neutrophils** present in normal milk to the defense against mastitis is not clear. On the one hand, their concentration is too low for an efficient phagocytosis in suspension [95]. Moreover, part of them are nonviable or in the process of apoptosis, in particular in cisternal milk, and are not in an activated state [22, 196, 197]. On the other hand, the presence of neutrophils in the milk of healthy glands seems to correlate inversely with a risk of intramammary infections [22]. Preinfection milk neutrophil viability is also correlated with severity of coliform mastitis [106]. An alternative to the direct role of residual neutrophils in resistance to infection would be that the baseline concentration of neutrophils results from unidentified mechanisms related to the capacity of the gland to react against bacterial intrusion. It would be useful to answer this question to know whether the straying neutrophils in normal milk are recruited by a latent inflammation, i.e. resulting from the stimuli of suckling or milking, or by a completely physiological mechanism.

Natural killer (NK) cells are large granular lymphocytes that have cytotoxic activity independent of MHC through antibody-dependent cell-mediated cytotoxicity. Although neutrophils and macrophages are well equipped to seek out and eliminate extracellular pathogens, NK cells are critical to the removal of intracellular pathogens. NK cells are also capable of killing bacteria by releasing bactericidal proteins belonging to the saposin-like protein family upon stimulation. It has been shown that cytokine-stimulated bovine mammary gland lymphocytes (Lc) possess antibacterial activity in vitro that is not major histocompatibility complex restricted [180]. Bovine NK-like cells ($CD2^+$ $CD3^-$ T Lc) express bactericidal activity against *S. aureus* upon stimulation with IL-2 and possess genes encoding lysin homologous to the saposin-like protein family [182]. These findings prompt further studies to delineate the contribution of NK cells to the innate defense of the mammary gland.

In addition to the cells found in udder secretions, it is also important to consider the cells infiltrating the mammary tissue. The tissue of healthy mammary glands

hosts few leucocytes. Most of them are T lymphocytes, CD4+ mainly in the interalveolar tissue, CD8+ mainly around the alveoli, and a few macrophages and B cells are also found [98]. A few dendritic-like cells can be seen [98, 186]. NK-like cells have not been identified, probably for lack of a marker, but they are likely to occur in the mammary tissue because they can be recovered by tissue digestion [180]. Lymphocytes were the most common infiltrating cell type observed within the two-layer epithelium lining the teat cistern, along with monocytes/macrophages cells in lower numbers [113]. Leucocytes are more abundant in the tissue at the distal end of the teat cistern, at the junction with the teat canal (Furstenberg's rosette) [113]. Lymphocytes and monocytes are the major cell types in the epithelial lining as in the teat cistern, however, the underlying connective tissue contains many plasma cells [31, 113, 114].

3. AFFERENT ARM OF INNATE IMMUNITY: SENSING THE PATHOGEN

3.1. Receptors involved in the recognition of pathogens

Kinetic studies of experimental clinical mastitis induced by the intramammary infusion of *S. aureus* or *E. coli* showed that the inflammatory response is not initiated until bacterial concentrations reach a certain level in milk [88, 169]. It is likely that bacterial growth is accompanied by the release of microbial products that can be recognized by the host as a danger signal. Sensing the presence of bacteria in the mammary gland is an important component of the innate immunity. A sensitive cellular machinery has been designed to fulfil this function. **Toll-like receptors** (**TLR**) have been identified as a major class of receptors recognizing conserved bacterial structures called pathogen-associated molecular patterns (PAMP). They are crucial for recognition of microbes by the innate-immune system and for bridging the innate and acquired immune responses [111]. The specificity of TLR recognition for important PAMP has been identified. For example, the main bacterial ligands for TLR2 are peptidoglycan and lipoteichoic acid (LTA) of Gram-positive bacteria, and TLR4 recognizes lipopolysacharides (LPS) of Gram-negative bacteria and LTA, TLR5 bacterial flagellin, and TLR9 bacterial CpG DNA [191]. Other accessory molecules are involved in the recognition of pathogens. The binding of LPS by TLR4 is greatly improved by CD14, a glycosylphosphatidylinositol-linked receptor that lacks a transmembrane domain, thus probably incapable of signal transduction. This receptor is mainly expressed on monocytes/macrophages and to a lesser extent by neutrophils [207]. The binding of LPS to membrane CD14 (mCD14) is facilitated by an acute phase protein, the LPS-binding protein (LBP), and the mCD14-LPS-LBP complex is recognized by TLR4. The complex peptidoglycan-CD14 is recognized by TLR2, without the contribution of LBP. For cells that do not express mCD14 (e.g. endothelial and epithelial cells), the soluble ectodomain of CD14 (sCD14) can efficiently present LPS to TLR4 or peptidoglycan to TLR2 [191]. Transmembrane signaling is mediated by TLR, then cascades of activation are set in motion, leading to the activation of nuclear factors NF-kB and activated protein-1 (AP-1). This results in the expression of several pro-inflammatory cytokines and chemokines. Other accessory activation pathways are involved, according to the TLR, which would give some specificity to the responses [111].

3.2. Role of milk cells and mammary epithelial cells

Sensing the pathogen and reacting by initiating inflammation is a function crucial to the recruitment of neutrophils as bovine neutrophils, which, in contrast to neutrophils of other species, are not attracted directly by bacteria or bacterial products

[21, 28, 57]. Milk macrophages may be the first cells to encounter invading bacteria. Although their bactericidal competence seems limited, they phagocytose actively, which shows that they are able to recognize bacteria. In particular, receptors for opsonins (IgG1, IgG2) have been documented on milk macrophages [42]. Milk macrophages are stimulated by *E. coli* LPS and respond by secreting IL-1 [133]. Bovine macrophages express mCD14 on their surface and are potential sources of sCD14 [126, 174]. "Resident" neutrophils could also contribute to the signaling of bacterial intruders, although this has not been demonstrated. Both cell types can release chemotactic and inflammatory mediators following bacterial encounter and recognition.

Other cells which are in direct contact with invading bacteria are the mammary epithelial cells. Bovine mammary tissue were shown to express mRNA for TLR2, 4 and 9, and the expression of TLR2 and 4 was increased in quarters suffering from subclinical, moderate or severe infections, proportionately to severity [56]. TLR2 and TLR4 gene expression by MEC has been reported [184], but expression at the protein level remains to be documented. Since MEC are able to respond to *E. coli* LPS or to *S. aureus* LTA in culture, membrane expression of several TLR is likely [184].

The detection of Gram-negative bacteria by the bovine mammary gland has recently been investigated, in particular the contribution of CD14 to the recognition of LPS. Bovine MEC apparently do not express mCD14 and were not reported to release sCD14, but sCD14 is present in the milk of healthy glands [203]. The average concentration of sCD14 in milk from uninfected quarters was in the 1 to 6 μg/mL range [4, 6, 92], although a lower concentration (about 120 ng/mL) was also reported [174]. Concentration of LBP in normal milk was reported to be about 6 μg/mL [4]. Human milk contains more sCD14 (about 12 μg/mL) but much less LBP (0.01 μg/mL) [87]. Accordingly, bovine milk appears to provide an adequate medium for the recognition of LPS. The cellular source of sCD14 in human milk was shown to be the mammary epithelial cells, which secrete a slightly smaller form of sCD14 in culture than the sCD14 released by monocytes [87]. In bovine milk the molecular mass of sCD14 has been shown to be 53 and 58 kDa, not different from the forms found in blood plasma [174]. Another possible source would be milk macrophages, but it is dubious that they can account for the bulk of milk sCD14.

The actual source of the increased LBP in the course of the inflammatory response to LPS challenge was not determined. The curve of LBP concentrations in milk does not parallel the curve of BSA concentrations, but parallels the increase of LBP concentrations in plasma, indicating that only a part of milk LBP comes from blood plasma [4]. The possibility exists that this acute phase protein be partly synthesized by MEC. Interestingly, respiratory and intestinal epithelial cells have been demonstrated to produce LBP in response to proinflammatory cytokines such as IL-1, IL-6 and TNF-α [41], cytokines whose concentrations rise markedly during *E. coli*-induced mastitis [169, 170].

The intramammary coadministration of sCD14 with an inoculum of *E. coli* enhanced bacterial clearance and the early influx of cells, although reducing the concentration of TNF-α and of IL-8 in milk [91]. This result suggests that sCD14 activates the innate immune response by contributing to the recognition of *E. coli* and moderates the inflammatory reaction by favoring an early mobilization of phagocytes, and possibly by neutralizing the proinflammatory activity of LPS. This would also suggest that the basal concentration of sCD14 in milk is sub-optimal.

The concentrations of LBP and sCD14 in milk during mastitis have been monitored during the response of the mammary gland to infections induced with *E. coli*, *S. aureus*, *Klebsiella pneumoniae*, *Serratia marcescens*

and *S. uberis* [5–7]. Although the kinetics of concentration changes differed according to the pathogen, in all cases both sCD14 and LBP concentrations increased markedly. These observations are in favor of an important role of these proteins in the innate defense of the mammary gland.

4. INDUCIBLE DEFENSES

The inducible **nitric oxide synthase (iNOS)** is a key element in antimicrobial activity of activated macrophages [100]. This enzyme complex catalyses the conversion of arginine to citrullin and nitric oxide (NO), a highly reactive radical. NO is short-lived and reacts with oxygen to yield nitrite (NO_2) and nitrate (NO_3), or the bactericidal peroxynitrite when interacting with superoxide. Concentration of NO is very low in colostrum or mature milk from healthy cows [12]. Production of NO is transiently increased in milk after *E. coli* LPS infusion, but NO is released in higher amounts and for longer periods in *E. coli* or *S. aureus* infected quarters [12, 13, 84]. Milk leucocytes were shown to release NO upon stimulation with bacterial products like LPS or staphylococcal enterotoxin [13, 84]. Macrophages are likely to contribute the bulk of milk leucocytes NO, because bovine macrophages are good producers of NO upon stimulation with LPS, on the contrary to neutrophils [16, 74]. Bovine macrophages are also able to secrete NO when stimulated with components of Gram-positive bacteria, but they need co-stimulation with cytokines like IFN-γ [74]. The pro-inflammatory cytokine TNF-α is likely to synergize with staphylococcal enterotoxin C for induction of iNOS and NO production by bovine macrophages, possibly through activation of the NF-κB pathway [84]. A marked increase in iNOS mRNA of mammary secretory tissue was reported to occur as soon as 3 h after infusion of LPS through the teat canal [163]. Infiltrating leucocytes may have contributed to this increase. A bovine mammary epithelial cell line (FbE cells but not MAC-T cells) was found to release nitric oxide after exposure to IL-1β but not upon exposure to LPS [16]. This leaves open the question of the contribution of CEM to the production of NO monitored in milk during mastitis. Immunohistochemical studies of the rat mammary gland clearly localized iNOS to the basal layers of alveoli and lactiferous ducts of organ cultures after exposure to LPS [121]. Since the staining pattern differed from the staining of casein, myoepithelial cells are likely to be the producing cells. It remains to be shown if this holds true for bovine epithelial cells.

Apart from its antibacterial activity, NO may be involved in the phase of hyperemia of the mammary gland that follows the fever peak [26]. Nitric oxide may act as a modulator of the arachidonic acid cascade and in the generation of oxygen-active species, in particular by reducing the activity of the enzyme 5-lipoxygenase which catalyses the generation of leukotrienes, an important mediator of inflammation. The increase of iNOS mRNA and the decrease of 5-lipoxygenase mRNA following infusion of LPS in the mammary gland raises the possibility of such an interference during mastitis [163].

The induction of iNOS in mammary tissue stimulated by LPS and TNF-α, and increased concentrations of NO found in mastitic milk suggest that NO plays a part in the pathophysiology of mastitis, but for the moment the contribution of NO to antibacterial protection or mammary tissue damage is not documented.

The regulation of bovine **lactoferrin** expression in the mammary gland appears to be contrary to that of the other milk proteins [162]. Lactoferrin concentration augments at cessation of milking, but also during clinical mastitis when milk yield decreases and secretion of caseins is reduced [171]. The dramatic increase in Lf concentration in milk during acute mastitis is consistent with the idea that Lf is an acute phase response protein in the mammary

gland, in accordance with the presence of acute phase response elements in the Lf gene promoter region [116]. The expression of mRNA in mammary tissue is dramatically augmented during LPS-induced mastitis [163]. Subclinical mastitis is accompanied by a slight (twofold on average) increase in Lf concentration, but during acute mastitis Lf concentrations can reach 5–6 mg/mL [64, 137, 147]. It is noteworthy to remark the delay of 24–48 h of the profile of Lf concentrations compared to serum albumin concentrations in milk [64, 137]. With acute mastitis, Lf is likely to participate in the bacteriostatic activity of cell-free milk, but only when milk composition has grossly changed, milk yield dramatically decreased, and Lf concentrations have considerably augmented [137]. The binding of Lf and its proteolysis fragments to LPS, LTA and CpG may dampen the inflammatory response during acute mastitis, to the host's benefit [20, 200]. Lf inhibition of pro-inflammatory cytokines by monocytes stimulated by LPS may reduce the systemic inflammatory response and down regulate the activation of neutrophils in milk and mammary tissue, contributing to the protection of the MG from neutrophil-induced damages.

Another acute phase protein of the mammary gland is milk **serum amyloid A** (**SAA**). Acute-phase SAA is the archetypal vertebrate major acute phase protein [190]. The SAA family of apolipoproteins comprises a number of differentially expressed members which are synthesized primarily by the liver, but extrahepatic production has also been reported. During mastitis, SAA is found in markedly increased amounts in plasma, associated with high-density lipoproteins [69]. Rapid and large increases in SAA concentrations have been shown in milk from cows suffering from clinical mastitis but also from chronic subclinical mastitis [49, 59, 71, 94, 115]. Highly alkaline isoforms of SAA3 were demonstrated in milk from clinical *E. coli* mastitis, in much higher concentrations than the acidic isoforms of systemic origin [71]. The mRNA expression of SAA by bovine MEC in cultures exposed to *E. coli* LPS or to *S. aureus* is dramatically enhanced, suggesting that the main source of milk SAA during mastitis is MEC [204]. The contributions of SAA to the defense of the mammary gland, which could relate to the pro-inflammatory and regulatory functions of SAA [190] are not defined yet, but its dramatic increase during mastitis is a strong incentive for further studies in this direction. Another potentially immunomodulatory acute phase protein, α1-acid glycoprotein, was found in mammary secretions, mainly colostrum, and gene expression by mammary tissue was documented [29].

Host defense peptides, which are generally cationic and contain both polar and nonpolar domains, constitute a broad family of peptides (between 12 and 50 amino acids long) conserved across plants, animals and insects. The main cellular sources of host defense peptides are granulocytes, monocytes, macrophages, platelets, and epithelial cells. In the bovine, dozens of such peptides have been described, such as defensins in neutrophils (bovine neutrophil β-defensins, BNBD) [165, 210], lingual antimicrobial peptide (LAP) from lingual, intestinal and respiratory epithelia [183] or tracheal antimicrobial peptide (TAP), the first mammalian β-defensin described [46]. Defensins are tridisulfide-containing peptides comprising two families, α and β-defensins, distinguished by their disulfide bonding pattern [210]. Defensins have potent antibacterial activities against *S. aureus* and *E. coli* in vitro [165]. Several β-defensin genes are expressed in the bovine udder, both in healthy mammary tissue (LAP, TAP and BNBD3), likely through constitutive expression, or in infected tissue, in which case the contribution of infiltrating leucocytes cannot be excluded [155]. Staphylococcal mastitis increases mammary mRNA expression of β-defensin 5, and in situ hybridization revealed that most of the expression is by the MEC [56]. Another study showed by in situ hybridization that abundant LAP mRNA expression

is located in the ductal linings of the teat and cistern of mastitic quarters, suggesting that LAP mRNA expression is induced at the site of infection [185]. Expression of the B-defensin gene was reported to be markedly (500 fold) increased by incubation of MEC in primary culture with LPS [184]. These observations show that defensins are expressed in a constitutive but mainly inducible manner by the mammary gland, and they suggest that defensins play a role in the innate immune response of the mammary gland to mastitis.

Initially described as "antimicrobial peptides" owing to their in vitro bacteriostatic or bactericidal activities, host defense peptides have several alternative functions in vivo. In fact, at physiological concentrations, and in the majority of extracellular sites within the body, most human host defense peptides are not antimicrobial [18]. Physiological salt concentrations, in particular of NaCl and Ca and Mg cations, inhibit the antimicrobial activity of host defense peptides [53], and milk may be inhibitory to the antimicrobial activity of cationic peptides [81]. In particular conditions, such as the phagosomes of leukocytes, there is no doubt that host defense peptides contribute to the killing of ingested bacteria, but in extracellular situations, immunomodulatory properties are likely to be of more significance [18]. A number of human antimicrobial peptides have been shown to possess chemotactic activity for monocytes, immature dendritic cells and T-lymphocytes, and they can also induce the secretion of IL-8 by epithelial cells, and proliferation of epithelial cells and fibroblasts [48, 195]. Model peptides devoid of direct antimicrobial activity were found to be protective in animal models of *S. aureus* and *Salmonella* infections, implying that host defense peptides can protect through immunomodulatory properties [19]. In the mammary gland, the protective roles of host defense peptides remain to be documented. This area of research deserves particular attention, in the light of the many and important potential immunomodulatory activities of these peptides, and of the induction of several of these peptides during mastitis.

5. RECRUITED DEFENSES

5.1. Initiation of inflammation

5.1.1. Role of milk cells

In vitro experiments showed that macrophages isolated from the involuted mammary gland ingest killed pre-opsonized *S. aureus* and secrete unidentified mediators that stimulate migration of bovine neutrophils [33]. Another indirect evidence that milk macrophages may be able to set in motion events leading to inflammation is the observation that macrophages adhering to intramammary polyethylene device secrete, upon stimulation by phagocytosis of killed *S. aureus*, mediators that attract bovine neutrophils [37]. The washing fluid of adherent macrophages from an infected quarter, which showed ingested bacteria, was chemotactic for neutrophils, strongly suggesting that macrophages can signal in vivo the presence of bacteria in the mammary gland. The mediators released by phagocytosing milk macrophages were not identified, but they are unlikely to be leukotrienes or prostaglandins, and it is known that production of IL-1β by milk macrophages is limited [35, 132].

Milk macrophages have been shown to have much reduced activities when compared to monocyte or monocyte-derived macrophages. Milk macrophages are less efficient at producing and secreting IL-1 than blood monocytes [133]. Considering the low concentrations of macrophages in normal milk, in particular in the milk of high-producing cows, and their limited activities, one may question the biological significance of their contribution to the defense of the mammary gland in lactation. The detection of invading bacteria in particular, which rests upon the random collision of bacteria and stray macrophages, is

open to question. It must be kept in mind that the direct contribution of milk macrophages to the initiation of inflammation has never been experimentally documented.

5.1.2. Contribution of mammary epithelial cells

Even though the contribution of epithelial cells to leukocyte recruitment has still not been thoroughly studied, it has now been demonstrated that upon interaction with invading bacteria, bovine mammary epithelial cells are able to generate a variety of inflammatory mediators such as cytokines, chemokines, host defense peptides, and arachidonic acid metabolites. The physiological role of these messenger molecules secreted by MEC is still not completely understood but they have modulating abilities by acting in an autocrine or paracrine fashion. They may be involved in the recruitment of neutrophils and lymphocytes into milk.

Bovine MEC are able to synthesize **chemokines**. In vitro studies using MAC-T cells, a bovine epithelial cell line developed from mammary alveolar cells [70], demonstrated that bovine MEC express IL-8 mRNA and secrete IL-8 protein when stimulated with LPS. This response to LPS stimulation is time- and dose-dependent [14]. Recombinant IL-8 can induce the migration of neutrophils through an in vitro model of the endo-epithelial barrier consisting of the bovine aortic endothelial cell line and MAC-T cells [90].

Primary cell cultures of mammary epithelial cells have also been used to study the ability of MEC to secrete IL-8 [204]. Stimulation with *S. aureus* or LPS induced IL-8 secretion in a dose- and time-dependent fashion. IL-8 is not the only chemokine that MEC could produce during infection. Upon stimulation with LPS, primary cultures of MEC showed enhanced expression of CXCL5 (ENA-78), CXCL6 and CCL5 (RANTES), chemokines which target neutrophils and a variety of mononuclear leucocytes, respectively [128, 184].

A comparison of the results obtained with primary cells and MAC-T cells indicate some differences. The response obtained by Wellnitz et al. [204] with primary cells to exposure to LPS was more than the one reported using MAC-T cells [14], and the same trend was confirmed by others [184]. Moreover, primary cells seem to be more sensitive to LPS than MAC-T cells since no response was detected following MAC-T exposure to 1 μg/mL of LPS while a significant response (about 100 pg/mL of IL-8) was observed following primary cell exposure to only 50 ng/mL of LPS.

In addition to the inducible secretion of chemokines by MEC, constitutive secretion of neutrophil chemoattractants has also been observed in culture supernatants of a caprine mammary epithelial cell line [9]. Anti-IL-8 antibodies were able to partially block the chemotactic activity suggesting that IL-8 was not the only chemokine secreted. The biological significance of such a constitutive secretion is still unclear but it could help in the recruitment of cells at a basal level in the normal gland.

The secretion of **pro-inflammatory cytokines** by MEC has been studied using cryopreserved primary MEC or MAC-T cells. A constitutive secretion of IL-1 and IL-6 was measured in supernatants of primary MEC using bioactivity tests but the physiological role of such a constitutive secretion is still unknown [117]. The cells secreted IL-1β only under serum-free condition whereas IL-1β bioactivity was not detected in the conditioned medium supplemented with FBS which contained an IL-1 receptor antagonist. On the contrary, IL-6 bioactivity was not demonstrated in cells cultured with unsupplemented medium. These results highlight the importance of the culture conditions when production of cytokines is investigated. In the same study, transcripts for IL-1α, IL-1β, IL-6 and TNF-α were found to be strongly expressed, whereas those for IL-10 and GM-CSF were

weakly expressed. Moreover, expression of cytokine mRNA for IL-2, IL-4 and IFN-τ were not detected. It is noteworthy that mRNA expression does not necessarily correlate with protein secretion and in particular, TNF bioactivity was not detectable in any supernatant.

In vitro, LPS stimulation increased the production of both IL-1 and IL-6 from cryopreserved MEC in a dose-dependent manner [118]. IL-1 and IL-6 are both important mediators of inflammation including stimulation of the acute phase response and in vivo, IL-1β was detected in milk from cows suffering from mastitis [6, 7, 153]. Secretion of IL-1 has also been studied using MAC-T cells after stimulation with LPS [15]. LPS induced IL-1α mRNA synthesis in a dose- and time-dependent manner. The authors also investigated the effect of IL-1β on the release of IL-8 by MAC-T cells. They observed that IL-8 secretion is secondary to the expression of IL-1β and incubation of MAC-T cells with IL-1β increased IL-8 secretion. Moreover, both IL-1ra and anti-IL-1RI inhibited IL-1β-induced IL-8 production in a dose-dependent manner. These data indicate that IL-1 appears to play, at least partially, a role in the generation of IL-8 by MEC.

Furthermore, primary culture of MEC expressed TNF-α mRNA following *S. aureus* infection or LPS exposure [204]. This response was rapidly initiated, being detectable three hours after the start of the treatments, and mRNA expression remained elevated throughout the entire 24 h experiment. TNF-α serves as a rapidly responding central mediator of inflammatory functions and is likely to play an important role in mastitis since its concentrations in vivo increase in milk after intramammary injection of LPS or during *E. coli* mastitis [123, 170].

5.2. Inflammatory cells in milk and mammary tissue

Following bacteria entry into the gland, **neutrophils** are the first cells recruited into the milk and then represent the predominant cell type. This recruitment is the consequence of an inflammatory response most likely initiated by milk macrophages and MEC that are the two main cell types that invading pathogens encounter at first when entering the mammary gland.

The neutrophil migration from the blood into the infected mammary gland is triggered by inflammatory cytokines (TNF-α and interleukins) through several actions. Cytokines activate the endothelial cells to express more E-selectin and P-selectin [136]. These molecules allow neutrophils to bind more tightly in these areas. This binding, as well as stimulation by inflammatory mediators, enhances expression and adhesiveness of another neutrophil adhesion molecule, Mac-1 (also known as CD11b/CD18), which is a member of the β_2-integrin family of leukocyte adhesion molecules. At the same time that Mac-1 expression increases, L-selectin is proteolytically shed from the neutrophil surface [82]. The Mac-1 molecule allows neutrophils to bind tightly to activated endothelium via another endothelial adhesion molecule, ICAM-1. This second adhesive interaction allows neutrophils to migrate along the endothelial surface and into tissues along a concentration gradient of chemoattractants, the most important being complement components C5a and C3a, LPS, IL-1, IL-2 and IL-8 [35, 38, 57, 90, 187]. Milk from both healthy glands and infected glands exhibits chemotactic activity for neutrophils in vitro but this activity is blocked by anti-IL-8 monoclonal antibodies in milk from infected glands. This indicates that IL-8 plays a major role in neutrophil recruitment during mastitis and is not involved in neutrophil recruitment in healthy glands [8].

Once at the site of infection, neutrophils ingest and kill bacteria, exhibiting a respiratory burst that produces hydroxyl and oxygen radicals, which are key components of the oxygen-dependent killing mechanism. Inflammatory cytokines such as IL-1, TNF-α or IFN-γ can enhance neutrophil

phagocytosis and/or their bactericidal activity [158, 176]. It should be noted that milk neutrophils (as well as milk macrophages) tend to be less functional than the homologous circulating cells. The ingestion of milk fat globules and casein by neutrophils causes a loss of cytoplasmic granules which are associated with a reduction in bactericidal activities and leukocyte rounding that eliminates the pseudopods needed for phagocytic capabilities [124, 125]. Moreover, it has been shown in vitro that diapedesis of neutrophils across the mammary epithelium reduces bactericidal activity of neutrophils [173]. In addition to their phagocytic capabilities, neutrophils are a source of anti-microbial proteins (lactoferrin, bactenecins, defensins), which are able to kill a number of pathogens that cause mastitis. In milk from infected glands where oxygen tension is low, oxygen-independent killing mechanisms may be important [127].

The neutrophil recruitment from the circulation to the focus of infection is essential in the defense of the mammary gland against invading bacteria and the promptness of the recruitment and the amount of recruited neutrophils are determining for the outcome of the infection. For instance, a one-hour delay in neutrophil recruitment into an infected gland could result in an 8-fold larger number of *E. coli* to kill and much more endotoxin to detoxify [26].

The neutrophil recruitment varies in intensity and rapidity according to the pathogen and to the cow. *E. coli* and *S. aureus* are with *S. uberis* the three main pathogens accountable for mastitis and the courses of infection they cause are very different. *E. coli* infusion into a healthy gland causes clinical mastitis with severe clinical signs and a loss of milk production. In a few hours, large amounts of the pro-inflammatory cytokines IL-1 and TNF-α and the chemokine IL-8 are detected in milk. Neutrophil concentrations increase rapidly (between 3 and 12 h post-challenge) and can reach more than 10^7 cells/mL of milk. Most of the time, the acute phase response allows the elimination of the bacteria which represent the inflammatory stimuli, within a few days after the beginning of the infection, and then neutrophil recruitment ceases and SCC returns to healthy levels [6, 153, 170]. Similar effects are observed following intrammamary injection of LPS [167].

On the contrary, *S. aureus* mastitis is often less severe and often subclinical. Experimental subclinical *S. aureus* mastitis induces a moderate (under 10^6 cells/mL) and a delayed (between 24 and 48 h post-infection) neutrophil recruitment in the challenged glands, and bacteria survive this first wave of host response [153]. Moreover, neither IL-8 nor TNF-α are detected in milk even during clinical *S. aureus* mastitis [6, 153]. This is likely to favor the establishment of a chronic infection during which the inflammation and the leukocyte migration continues for months. More than two months after the beginning of a staphylococcal infection, neutrophils still represent the majority of cells present in infected glands [154]. Prolonged diapedesis of leukocytes is likely to cause damage to mammary parenchyma tissue, contributing to decreased production of milk [63]. Recently, a study compared two other Gram-positive and Gram-negative bacteria, *S. uberis* and *Serratia marcescens*, in order to determine whether the inflammatory response elicited by *E. coli* and *S. aureus*, respectively, is characteristic of all Gram-negative and Gram-positive intramammary infections [7]. The innate immune response to *S. marcescens* was similar to that reported for *E. coli*. In contrast, the innate immune response to *S. uberis* differed greatly from that reported for *S. aureus*, in accordance with a previous study describing the inflammatory response following *S. uberis* intramammary injection [151]. This indicates that the response to Gram-positive bacteria is a function of the causative organism.

Cow factors are also determining for the outcome of mammary infection. Stage of lactation affects the capability of the cow to

eliminate the invading bacteria. In particular, cows are very susceptible to infection around the periparturient period and more than 50% of all cases of clinical coliform mastitis develop in early lactation [25]. Functional competences of neutrophils are reduced, including a defect in chemotaxis and in bactericidal activity. The underlying mechanisms involved in periparturient immunosuppression are still unclear even if hormonal and metabolic changes associated with pregnancy, parturition and onset of lactation may influence the inflammatory reaction [123]. The cow parity also has an effect on the outcome of the infection and there is an increasing risk for developing mastitis with each subsequent parity. The underlying mechanisms are unknown and few studies have been published regarding this cow factor. It is reported that the degree of periparturient impairment in neutrophil function is more severe for cows in parity ≥ 4 than for younger cows [55]. Van Werven et al. found that the only significant differences between second parity cows and older cows were the number of peripheral circulating leukocytes and expression of CD11b/CD18 and CD11c/CD18 receptors on their surface [194]. Recently, it was shown that primiparous cows react with a moderate inflammatory response following intramammary *E. coli* injection compared to multiparous cows that developed severe inflammation. The rapid influx of neutrophils in the infected glands was associated with fast clearance of bacteria and a rapid recovery in primiparous cows [198]. Moreover, neutrophil functionality was reported to be more efficient in primiparous cows than in multiparous cows [105]. Genetic variability is also very important concerning the defense of the mammary gland.

Beside neutrophils, **macrophages** are also important actors of the inflammatory response. As seen previously, they participate in the triggering of the innate immune response by phagocytosing the invading pathogens and secreting inflammatory mediators. Moreover, following bacteria elimination, neutrophil influx subsides and is followed by a predominantly mononuclear influx. Macrophages do not only replace neutrophils but also actively participate in their removal. The rapid elimination of neutrophils by macrophages is essential to minimizing inflammatory-derived injury to the mammary secretory epithelium, which can result in scarring and can lead to a permanent decrease in milk production [26, 127].

Cells of the adaptative immunity (T and B lymphocytes) also contribute to the defense of the mammary gland and lots of interactions exist between the innate and adaptative immune cells. The recruitment of lymphocytes in milk and mammary tissue following infection is not in the scope of this paper.

5.3. Humoral defenses

The epithelium sheet lining the mammary gland separates the milk space from the interstitial space. During lactation, this separation is impermeable to small molecules such as lactose or to ions such as Na^+ and Cl^-. The structure responsible for this is the tight junction, the most apical component of the junctional epithelial complex, which forms a sort of gasket around each epithelial cell [112]. During lactation, the tight junctions form a highly impermeable barrier between the milk and the interstitial fluid, whereas during the dry period, the epithelium is permeable to fairly large molecules, such as serum albumin and immunoglobulins [112]. During mastitis, there is a bi-directional passage of molecules from the milk to the body (e.g. lactose) and from the blood to milk (e.g. serum albumin). This increase in permeability is an important component of the innate defense of the mammary gland since it allows blood and tissue immune components to reach the lumen of the gland. It is likely that two mechanisms contribute to the increased permeability. First, cellular damage by bacterial toxins [30, 54, 189] may result in discontinuity in the epithelial barrier. Second, pro-inflammatory mediators such as

histamine, TNF-α and IFN-γ have been shown to alter epithelial tight junction efficiency [101]. Variations in milk serum albumin concentrations is a frequently used marker of exudation of plasma. A low concentration of BSA, around 0.2 mg/mL compared to the 40–45 mg/mL in plasma, is found in the milk of healthy glands, probably leaking from the blood, although part of it may be secreted by the MEC [166]. The increase in serum albumin concentrations is an early event in case of clinical mastitis, temporally coincident with the influx of cells [7, 153, 170]. Tight junction permeability is under hormone regulation (prolactin, progesterone, glucocorticoids) of growth factors (epithelial growth factor, tranforming growth factor-β). The bovine mammary gland is able to regulate the opening and closure of epithelial tight junctions in response to a number of stimuli of bacterial or indigenous origins and this process has a bearing with several of the components of innate immunity.

Exudation of plasma brings to the infection scene a number of effectors of innate immunity. Among these effectors, natural antibodies and complement are to be mentioned because of their potent opsonic properties. Antibody concentrations in mammary secretions are rather high during the dry period, very high in colostrum, but low in full lactation milk: milk whey contains about 0.60 mg/mL IgG_1, 0.05 mg/mL IgG_2, 0.04 mg/mL IgM and 0.13 mg/mL sIgA [27]. The low concentrations of IgG_2 and particularly of IgM may be limiting factors for the efficient opsonization of pathogens [68], but plasma exudation is likely to convey sufficient opsonic antibodies in most cases. Deposition of the complement cleavage product C3b and its transformation into C3bi on bacteria favors their recognition by phagocytes through binding to the receptors CR1 and CR3, respectively [127]. Exudation of plasma provides the complement components which are absent in normal milk (in particular C1q), making possible the functioning of the classical pathway of activation, and the increase in the concentration of C3 augments the deposition of opsonic C3 fragments [142, 144]. There is a temporally coincident increase in the BSA milk concentration and the generation of C5a in milk of *E. coli* mastitis [6, 170]. C5a contributes to create a stimulating environment for neutrophils, augmenting their phagocytic and bactericidal activities [149].

Exudation of plasma brings into milk a number of blood components that may participate in the innate defenses of the mammary gland. The concentrations of some of them have been shown to rise in milk during mastitis, such as transferrin and acute phase proteins like SAA, haptoglobin and serum amyloid A [49, 58, 59, 115, 137]. Their roles are ill-defined, but several of them are likely to contribute to the control of inflammation and healing of mammary tissue lesions.

6. MODULATION OF INNATE MAMMARY IMMUNITY

6.1. Immunomodulation

Immunomodulation of innate and adaptive immune defenses of the mammary gland has attracted a lot of interest. Many studies, which have been thoroughly reviewed, have reported the effects of the use of recombinant cytokines with a view to prevent or cure mammary infections [2, 78, 177, 181].

The stimulation of leukocytes with cytokines has been given particular attention. The granulocyte colony-stimulating factor (G-CSF), which targets the neutrophils, causes dramatic increases of neutrophil numbers in blood and milk, stimulates the phagocytic and bactericidal activities of neutrophils, and consequently should have been expected to help the cow to combat mastitis. Although a several-week previous treatment with G-CSF protected against experimental challenge with *Klebsiella*, it had no effect on pre-existing *S. aureus* mastitis [77, 79]. Granulocyte-macrophage CSF

(GM-CSF), which targets both neutrophils and monocytes, stimulates the antibacterial efficiency of phagocytes, and affords some protection from subsequent *S. aureus* challenge [39]. The effect of recombinant bovine IL-1β and IL-2 were also tested on mammary gland infections. These cytokines induce an influx of neutrophils into the milk when infused in the lumen of the mammary gland. They have demonstrated some preventive and curative effects on experimental infections, but their effective doses were not far from the toxic doses [39, 179, 181]. Recombinant bovine interferon γ (IFNγ), which potentiates the activities of T lymphocytes, macrophages and neutrophils, has been shown to modulate mammary gland neutrophil functions during the periparturient period [176]. This critical physiological period, when the cow is more susceptible to infections owing to depressed immune functions, represents a target of choice for mastitis prevention through immunomodulation. Nevertheless, as therapeutic agents, recombinant cytokines administered intramammarily were not more active than classical antibiotic treatments [39, 181].

Although intensely studied, the use of recombinant cytokines has not led to commercial application. Probably the present knowledge of the cytokine networks in play in the bovine mammary gland during infection is insufficient for a rational use of such potent biological agents. The complexity of the multiple interactions involved is still compounded by the variability of the pathogenic processes particular to the pathogen responsible for infection. Also, the practicality of recombinant cytokine prevention of therapy depends on the dose required, regimen of administration, total cost and side-effects under field conditions.

Other immunomodifiers have attracted less attention [78, 135, 177]. Since the reported effects were moderate, it is difficult to make a judgement on their efficacy and their usefulness under the conditions of the field.

6.2. Genetic selection

Genetic selection to maximize milk production has probably had a negative correlate: the production of amounts of milk exceeding the needs of the offspring, submits the cow to physiological stress and increases the dilution of cellular and soluble immune defenses. These consequences have potential weakening effects on the resistance of the mammary gland against infections. Indeed, a negative correlation has been found between milk production and resistance to mastitis [45, 52, 67]. This demonstrates that certain alleles or associations of alleles are detrimental to the immune resistance of the udder, and that it must be possible to correct the past unfavorable drift by selecting or creating animals with "restored" udder defenses. Indeed, some genetic variation exists as to resistance or susceptibility to mastitis, as shown by differences of mastitis prevalence among breeds or between individuals within breeds [80, 156].

There are several possible approaches for selective breeding for resistance to udder pathogens. One of them is to search for favorable or disadvantageous alleles of genes which have a major effect on the resistance or susceptibility to mastitis. Although mastitis resistance in cattle is polygenic in nature, single genes with potentially large effects have been and are likely to be discovered. The gene encoding the CD18 subunit of the Mac-1 glycoprotein that plays an important role in leucocyte adhesion and trafficking is an example of single-gene related susceptibility to infectious diseases. Point mutations in an allele of the gene result in defects defining the bovine leucocyte adhesion deficiency (BLAD) syndrome [168]. It makes sense that interference with phagocytic defenses entails susceptibility to pyogenic bacteria. It is sensible to search other major favorable or unfavorable alleles among the genes known to play a part in the proven or supposed resistance to mastitis. Genes associated with neutrophil function are potential

genetic targets for mastitis resistance, since neutrophils are essential for the control of most mastitis pathogens [123]. The ability of neutrophils to migrate into infected tissues is dependent upon the recognition of inflammatory mediators such as (ELR)CXC chemokines (which possess the sequence Glu-Leu-Arg preceding the first two Cys) and the complement fragment C5a through specialized receptors. Recently, it was shown that a single polymorphism nucleotide (SNP) within the CXCR2 gene (one of the two receptors for (ELR)CXC chemokines) was associated with impaired neutrophil migration and correlated with the frequency of subclinical mastitis in Holstein cows [150, 209]. This correlation needs to be validated on a large scale and the individual allele effects on leucocyte migration needs to be measured experimentally, but this kind of finding is promising, since it may herald the advent of effective means of marker-assisted selection for mastitis resistance. Other genes governing the detection of bacteria or the mobilization of leucocytes by the mammary gland are potential research targets.

The antibacterial functions of phagocytic cells have attracted a lot of attention, probably because of the major role they play in the control of mastitis. Large variation among individual animals have been reported in the ability of mammary gland macrophages and blood neutrophils to kill *S. aureus* [108, 206]. These observations led the authors to suggest that testing neutrophil functions in dairy bulls entering artificial insemination may provide a tool for selection against infectious diseases [206]. Significant sire progeny group differences in in vitro neutrophil migration, ingestion and production of oxygen radicals have been reported [76]. Unfortunately, heritability estimates of bactericidal activity and response to chemotactic factors were found to be low [43, 44]. The antimicrobial potency of milk may also constitute a target for breeding, assuming that present day ruminants express suboptimal levels of antimicrobial substances in milk [83].

Immunoresponsiveness traits are being considered as potential physiologic markers of disease resistance [80, 156]. Several studies have reported associations between immune response measures such as lymphocyte functions or between major histocompatibility complex alleles and mastitis traits, but few studies have checked the genetic correlation with mastitis. At present this research field is rather confusing, and although the approach has its merit and deserves attention, further studies will be necessary before a selection strategy based on defined alleles can be implemented.

The quest for major mastitis-resistance genes may prove to be long and arduous. In the meantime, research on traits resulting from polygenic determinism, each gene associated with small effect, is ongoing. Direct selection against the incidence of clinical mastitis would be a straightforward approach for a successful genetic improvement program. A dairy cattle selection experiment was started in Norway in 1989, which involved two selection groups, one high protein yield (HPY), and one low clinical mastitis (LCM). After three generations, cows from the LCM group had less (8.6%) clinical mastitis, lower circulating neutrophil counts, neutrophil/lymphocyte ratios, and blood cortisol concentrations than cows from the HPY group [66, 86]. The difference was significant at the pre-partum period. These differences could be interpreted as the result of a better resistance to stress by the cows of the LCM group. Lower cortisol concentrations may be the cause of the lower blood neutrophil counts, and may increase the susceptibility to infections, in particular around parturition [23, 24]. Transcriptomic studies of the response of phenotypically different animals (such as the incidence of clinical mastitis) to experimental bacterial exposure could help in identifying the genes involved in the resistance or susceptible phenotypes.

Mastitis-related traits have been recorded on a large scale because of its economic importance and the available infrastructure, which has made the detection of quantitative trait loci (QTL) for mastitis possible. QTL are chromosomal regions responsible for a fraction of the genetic variability of a trait. At present they cover large chromosomal regions involving hundreds or thousands of genes. Several chromosomal regions have been linked to marked effects on mastitis resistance [156]. A fine mapping of the mastitis-associated QTL could make marker-assisted selection for mastitis possible, and eventually facilitate the identification of resistance genes and alleles.

Selection for resistance to mastitis is a promising research field. It has great potential in complementing other control means like hygiene and vaccination. Nevertheless, many questions need to be answered before the variability of the genes governing innate immunity can be harnessed to curb mastitis in dairy ruminants.

6.3. Genetic engineering

Genetic engineering of dairy cows is potentially a potent tool for enhancing innate defenses of the mammary gland. There are a number of potential targets for biological manipulation. A variety of molecular components of the innate immune system can be expressed in the lactating mammary gland under the control of milk protein gene promoters. Among these components, peptides and proteins are the preferred candidates, because glycoconjugates and lipids, which are synthesized by the interplay of enzymatic cascades, would be manipulated with greater difficulty and at a higher cost [83]. Host defense peptides present several advantages: they are natural components of the host if not of the milk, and they usually possess broad antimicrobial activity. Nevertheless, their antimicrobial activity in milk may not be high, and otherwise, they should not interfere with the bacteria involved in the downstream processing of milk (e.g. cheese manufacturing). One of these peptides, the bovine β-defensin related peptide TAP was expressed in the lactating mammary gland of transgenic mice, but the antibacterial activity in milk and the protection afforded were not tested [208].

Transgenic technology has resulted in the generation of cows that secrete human lactoferrin in their milk [192]. Transgenic cows are used in this case as bioreactors for the large scale production of biopharmaceuticals. It can be envisaged to enhance host defense by over-expression of proteins or peptides naturally secreted in bovine milk, but it will be necessary to document the protection afforded, i.e. by pathogen challenge experiments, to evaluate the efficiency of the construction. Such an evaluation has been done for a transgene, encoding the enzyme lysostaphin, whose expression in milk under the regulation of an ovine β-lactoglobulin promoter was shown to protect the transgenic cows from *S. aureus* mastitis [201]. Lysostaphin is a potent peptidoglycan hydrolase, secreted by *S. simulans*, which is bactericidal to *S. aureus* at low concentration. Constitutive secretion of lysostaphin at concentrations of 0.9 to 14 μg/mL prevented the establishment of *S. aureus* in the mammary gland, likely without the intervention of innate defenses [201]. This result demonstrates the feasibility of introducing disease-resistance genes into cattle to confer protection against mastitis. The most difficult issue raised by this work may be that of public acceptance. The constitutive expression in milk of a protein of bacterial origin, antibiotic in nature, for the benefit of the cow and of the milk-producer, is likely to be perceived by the consumers as an unnatural manipulation [143]. Milk producers, too, may be reluctant to endorse a technique that may alter the image of milk, a natural and mother-related product. This issue could be alleviated by inflammation-inducible expression. The manipulation of the expression of an innate defense gene of the host should be more acceptable.

7. PERSPECTIVES AND CONCLUSIONS

There are still numerous gaps in our knowledge of individual components of the innate defense of the mammary gland, and our understanding of their functioning during mastitis is rudimentary, but the mammary gland is a good model to study these topics because of the ease of sampling inflammatory mammary secretion which makes possible the monitoring of the course of the inflammatory and immune responses, and the possibility for in vitro modeling by use of MEC cultures. Another asset is the amount of data collected on a large scale in the field on mastitis incidence and prevalence, directly or indirectly through SCC assessments, in several different cattle breeds and dairy ruminant species. As a result, interactions of mastitis-causing pathogens such as *E. coli* (Gram-negative) and *S. aureus* (Gram-positive) and the ruminant mammary gland represent a suitable model for studies on innate immunity at an epithelium frontier.

There are a number of possible leads to search for mechanisms of resistance which could be harnessed to control mastitis on a large population scale. The time period corresponding to the initial growth and settling of bacteria in the lumen of the mammary gland, followed by the initiation of the inflammatory response and the first wave of leucocytes in mammary tissue and milk, is certainly a target of choice for research. The whole time window lasts less than 24 h in most cases, and, although the subject of many investigations in the past decades, many of its key events remain mysterious, such as the variations in apparent growth rates of bacteria among individuals. The prompt recruitment of neutrophils by the mammary gland following the invasion of the cistern and duct system by bacteria is an important feature of innate defenses. The ability of neutrophils to ingest and kill bacteria is pivotal for the control of infection. Many studies have shown both the importance and the limitations of neutrophil-mediated defense of the mammary gland, and have explored the possibilities of modulation of the phagocytic defense of the udder [26, 123, 127, 146, 177]. Much less is known about the roles of MEC in sensing the pathogens and alerting the immune system, or in direct control of the multiplication of the pathogens through production of antibacterial components (host defense peptides, proteins, lipids or oxygen radicals). Delineating the contributions of MEC to mammary gland innate defenses should constitute a major area of research for the future.

Powerful new research tools are radically modifying the prospects for progress in understanding the interplay between the mammary gland and mastitis-causing pathogens. Microarray gene technology has transformed experimental biology by enabling quantitative analysis of the transcriptome of cells and tissues. Transcriptome profiling of the in vivo and in vitro response to pathogens has the potential to dramatically improve our understanding of the pathophysiology associated with mastitis. Genetic dissection of innate immune response could even be accelerated by combining the QTL approach with gene transcription analysis, which could provide a powerful approach to identifying candidate genes controlling complex traits like resistance to infection [40]. The genetic dissection of complex traits like susceptibility or resistance to mastitis is far from being straightforward, but for several reasons such as large family size and a moderate number of alleles at each locus, domestic species lend themselves rather well to detection of QTL mutations that underlie variation in complex traits [3]. The imminent completion of the draft genome of cattle should soon remove a bottleneck that is hampering several genomics and post-genomics approaches to the genetics of disease resistance in dairy ruminants. Proteomics will be a useful complementary approach to transcriptional analysis. Finally, gene silencing by RNA interference [1] is another methodology whose import in the

mastitis research field should be of great help in defining the functions of the candidate genes identified by the other methodologies.

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