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REVIEW

Immune defences of the mammary gland in dairy ruminants

Pierre Germon | Rodrigo Prado Martins

ISP UMR 1282, INRAE, Université de Tours, Nouzilly, France

Correspondence
Rodrigo Prado Martins, ISP UMR 1282, INRAE, Université de Tours, Nouzilly, France.
Email: rodrigo.prado-martins@inrae.fr

Abstract

The mammary gland (MG) of ruminants is essential for assuring the immune protection and nutrition of the suckling youngs. The domestication of these species aimed at increasing milk production for human consumption enhanced udder susceptibility to infections and in this context, a better understanding of the MG immune defences has become a cornerstone for the success of dairy farming. In this review, we explore constitutive and inducible immune mechanisms of the mammary gland and briefly discuss the knowledge gaps that remain to be elucidated for the implementation of strategies focused on boosting mammary immune responses.

KEYWORDS
adaptive immunity, innate immunity, mammary gland, mastitis, ruminants

1 | INTRODUCTION

The mammary gland (MG), an organ that defines mammalian, plays a key role in nutrition of the offspring through the production of milk, as well as being essential in the early moments of life through the production of colostrum.

Over the years, bovine genetic selection for increased production of milk for human consumption has achieved a specialization of the organ, making it the ultimate example of milk secreting gland (Miglior et al., 2017; VandeHaar & St-Pierre, 2006). Yet, this focus on milk production led to increased susceptibility to udder inflammatory diseases known as mastitis (Rupp & Boichard, 1999).

Better knowledge of the immune defence mechanisms of the mammary gland are needed to improve genetic selection schemes and to develop additional preventive measures such as vaccination or immune-stimulation strategies.

We will review the specific immune mechanisms of the dairy ruminants' MG, starting with a presentation of constitutive defences and then emphasizing on the immune response induced by infection.

2 | DESCRIPTION OF THE HEALTHY MAMMARY GLAND ENVIRONMENT

The organization of the MG has been well documented over the years, from macroscopic descriptions to fine ultrastructural studies (Nickerson et al., 1984; Smolenski, 2018; Weber, 1977). Starting from the teat end, access to the lumen of the MG is sealed in between milkings by the streak canal made of multilayered keratinized epithelium. The canal gives access to the teat cisternae where a particular structure called the Furstenberg's rosette is found. The teat cisternae epithelium then evolves to a bi-layered epithelium, which continues into the mammary gland cisterna. From the cisterna, mammary ducts emerge and are connected to multiple alveoli clustered in lobules where milk is produced, forming the mammary parenchyma. Alveoli feature a single layer of mammary epithelial cells, responsible for milk production, surrounded by myoepithelial cells involved in contraction for ejection of milk (Adriance et al., 2005).

The MG fulfils dual function as nutrition and immunological protection that are reminiscent to its origin, the MG either deriving from

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the apocrine gland of a hair follicle or from an integumental skin gland (McClellan et al., 2008; Oftedal, 2013; Vorbach et al., 2006). This dual function of the MG is reflected by the multiple cell types present in the mammary gland: mammary epithelial cells that produce milk along with leukocytes involved in protection (Riollet et al., 2000a).

Plasma cells (PC), involved in immunoglobulin secretion, are found distributed throughout the mammary gland parenchyma with more cells in the rosette and teat, less in the parenchyma and none in the streak canal (Nickerson et al., 1984).

MHCII and CD11c-positive cells, probably macrophages and/or dendritic-like cells, are present in the mammary gland, either in the subepithelial compartment or in the vicinity of ductal epithelium (Fitzpatrick et al., 1992; Lee et al., 1989; Maxymiv et al., 2012). Ductal macrophages were recently characterized by single-cell RNAseq and immunohistochemistry in murine mammary glands (Hassel et al., 2021).

T-lymphocytes have been described in uninfected mammary glands with CD4 lymphocytes in the connective tissue, while CD8 lymphocytes are present both in the connective tissue and in the ductal epithelium (Nickerson & Heald, 1982; Yamaguchi et al., 1999).

Finally, neutrophils are a population of importance for the defence of the MG (Paape et al., 2003). Present in low numbers in the healthy mammary gland, they are recruited in large numbers in the mammary gland upon infection. Recently, two different neutrophil subsets have been described in bovine blood and milk but their precise contribution to the host response deserves further investigation (Rambault et al., 2023).

In the next parts of this review, we will distinguish the constitutive defences of the MG and the inducible defences that are activated upon infection.

3 | 'CONSTITUTIVE' DEFENCES

3.1 | The teat canal as the first barrier

Because of its anatomic position as the entry gate for mammary gland pathogens, the teat canal is the first barrier to prevent infection by bacteria colonizing the teat apex (Hohmann et al., 2020; Newbould & Neave, 1965; Paduch et al., 2012; Zecconi et al., 1992).

The teat canal is not only a physical barrier but also a biochemical one. Keratins that constitute the epithelial surface of the teat canal are associated with lipids showing antibacterial activities for some mastitis pathogens (Hogan et al., 1988; Paulrud, 2005). Other proteins with antibacterial activities, such as S100 calcium-binding proteins, were shown to be present in the teat canal lining (Hibbitt et al., 1969; Smolenski et al., 2015).

3.2 | Soluble defences

Milk is a rich medium in which a number of bacteria can survive and multiply (Roussel et al., 2017); it is also equipped, probably because of the dual nature of the MG evoked previously, with a number of soluble defences that contribute both to the defence of the offspring and to that of the mammary gland itself. The relative contribution of these soluble components to the antibacterial activity of milk, despite being lower than that of cellular components, should not be overlooked (Koshiishi et al., 2017).

These defences include immunoglobulins, complement and a variety of other antimicrobial components. The diversity and function of immunoglobulins in defence of the MG will be dealt with later in this review when adaptive defences are considered.

3.2.1 | The complement system in milk

The complement system plays a peculiar function as it is both involved in antibacterial activities and in the signalling required for an efficient inflammatory response. Activation of the complement system can occur through three different mechanisms: the classical, C1q-dependent and alternate pathways, following spontaneous conversion of C3 to C3b and the lectin pathway, initiated after recognition of carbohydrates motifs. Activation of one of these pathways results in initiation of the complement cascade which ultimately leads to the formation of the membrane-attack complex (MAC) and release of the chemoattractant C5a (Stoermer & Morrison, 2011).

Not all activities of complement are efficient in milk, probably because of variable concentrations of the different cascade components (Rainard, 2003). C1q, required for activation of the classical pathway, is not present in significant amounts in milk. Yet, adding exogeneous C1q to milk allows the deposition of C3 to occur, indicating that the other components of complement are available for the events downstream of C1q activation to occur (Rainard & Poutrel, 1995).

The deposition of C3 fragments and C4 on the surface of pathogens, shown to occur in milk with a reduced speed compared to serum, should promote their ingestion by milk phagocytes (Rainard & Poutrel, 1995). Yet, the relevance of complement-mediated opsonization with regard to phagocytosis is not obvious: C3 deposition has little impact on phagocytosis of *S. aureus* by neutrophils and that of *S. uberis* by macrophages (Barrio et al., 2003; Grant & Finch, 1997).

Deposition of complement also triggers the formation of the MAC with pore-forming activity. However, the contribution of the MAC to the direct clearance of bacteria is limited: Gram-positive bacteria are naturally resistant to complement-mediated lysis and mastitis *E. coli* isolates are able to grow in normal milk (Rautemaa & Meri, 1999; Roussel et al., 2017; Védrine et al., 2018).

Maybe the most significant contribution of the complement to the defence of the MG is the release of C5a after cleavage of milk C5. Although concentration of C5 is 10 times less than that in serum and is highly variable between cows, it is enough to generate sufficient amount of C5a that plays a significant role in attraction and activation of neutrophils (Rainard et al., 1998; Stevens et al., 2012).

3.2.2 | Antimicrobial components

Several proteins/molecules with antibacterial activities have been detected in normal milk and can be categorized as acting directly

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on the bacteria, producing toxic compounds or acting by interfering with bacterial growth. Although present constitutively in milk, the expression of most of these soluble antibacterial components is often induced upon infection (Carlsson et al., 1989; Kawai et al., 2015; Tsugami et al., 2022).

3.2.2.1 | *Direct antibacterial action—defensins*

Defensins are short positively charged proteins (<10 kDa) expressed by mammary epithelial cells and neutrophils that kill bacteria after binding to their surface and permeabilizing their membrane (Goldammer et al., 2004; Isobe et al., 2009; Selsted & Ouellette, 2005). Among defensins identified in cattle, only a few have so far been detected in milk, in particular, the lingual antimicrobial peptide (LAP), the tracheal antimicrobial peptide (TAP), the bovine neutrophil ß-defensin (BNBD) and the enteric ß-defensin (Isobe, 2017).

3.2.2.2 | *Enzymes involved in production of toxic compounds*

Among the enzymes present in milk, only a few stand out when one considers their antibacterial potential (Shahani et al., 1973).

Lactoperoxydase (LPO) is constitutively present in milk and displays antibacterial activity by producing toxic products when sources of thiocyanate and H_2O_2 are provided (Marshall et al., 1986). LPO activity, highly variable between cows, is increased during mastitis and correlates with the recruitment of somatic cells in milk. This process is probably related to the production of hydrogen peroxide by the recruited neutrophils or by xanthine oxydoreductase, another abundant milk enzyme (Björck & Claesson, 1979; Fonteh et al., 2002; Isobe et al., 2011). Besides its activity in aerobic conditions, which generates hydrogen peroxide, XO shows antibacterial activity against *E. coli* through the production of nitric oxide in the presence of nitrite (Hancock et al., 2002).

The presence of lysozyme in bovine milk was discovered early in the twentieth century (Fleming, 1932). Its antibacterial activity relies on its ability to break ß1-4 bonds between peptidoglycan subunits in the envelope of bacteria. However, the antibacterial potential of lysozyme in healthy bovine udder is hampered by its low concentration in milk (Seyfert, 1999).

3.2.2.3 | *Iron chelation or 'nutritional immunity'*

Lactoferrin is an iron-binding glycoprotein that has the ability to interfere with the growth of bacteria in the presence of bicarbonate (Masson & Heremans, 1968). However, during lactation, this chelation activity has limited impact because iron is chelated by citrate rather than lactoferrin and certain mastitis causing bacteria, for instance *E. coli*, have the ability to acquire iron by capture of this iron-citrate complex (Goldstone et al., 2016; Rainard, 1983). As a consequence, the antibacterial activity of lactoferrin is significant only when large quantities of lactoferrin are present and when citrate concentrations are low, that is, during involution or mastitis (Hurley & Rejman, 1993; Isobe et al., 2011; Oliver & Bushe, 1987).

4 | RESPONSE TO INFECTIOUS AGENTS

Although these soluble compounds contribute to the defence of the MG, an efficient defence depends on the recruitment of neutrophils as demonstrated by experiments where bacterial clearance, after experimental intra-mammary inoculation of *Klebsiella aerogenes*, was abolished by neutrophil depletion (Jain et al., 1971). The massive neutrophil recruitment observed in clinical mastitis relies on a series of events that start with the recognition of the bacteria by the host-innate immune system followed by the production of pro-inflammatory mediators that ultimately lead to the recruitment of neutrophils in the MG. Return to a normal non-inflammatory status is then controlled by anti-inflammatory mediators.

4.1 | C5a production and early response

An early mechanism to contribute to the recruitment of neutrophils is the cleavage of C5 following activation of the complement cascade which leads to release of C5a (Stevens et al., 2012). C5a, similar to TNFα and IL-1β, is released in milk as early as 12 h post-inoculation of a low dose of *E. coli* in the MG and this release coincides with the chemotactic activity of milk (Shuster et al., 1997). The binding of C5a to its receptor C5aR not only results in neutrophil attraction to the site of infection, but it also contributes to their activation and increases their phagocytic and bactericidal activities (Nemali et al., 2008; Rainard et al., 2000).

4.2 | Mammary epithelial cells and macrophages as sentinels for the detection of pathogens

The two other pathways involved in the initiation of inflammation rely on the production of cytokines and lipid-derived mediators or oxylipids. These pathways are activated when MG resident cells, mammary epithelial cells or macrophages, are exposed to invading pathogens (Griesbeck-Zilch et al., 2008; Rainard & Riollet, 2006). Recognition of pathogens is mediated by so-called pattern recognition receptors (PRR) expressed either at the surface or in the cytosol that detect conserved motifs expressed by microbes called microbe-associated molecular patterns (MAMP). Five families of PRR have so far been described in eukaryotes: Toll-like receptors (TLR), C-type lectin receptors (CLR), nucleotide-binding domain leucine-rich repeat (LRR)-containing receptors (NLRs or NOD-like receptors), RIG-I-like receptors (RLRs) and the AIM2-like receptors (ALRs) (Brubaker et al., 2015). Not all these receptors have been described in bovine. Among the 10 bovine TLR genes, only some of them have been demonstrated to be expressed by mammary epithelial cells (MEC) and macrophages (Menzies & Ingham, 2006). TLR2 and TLR4 expression was detected in bovine milk macrophages but the full repertoire of TLR receptors expressed by milk macrophages remains to be determined (Alhussien

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et al., 2021; Werling et al., 2006). In MEC, expression of TLR1, TLR2, TLR4 and TLR6 was detected, either at the gene or protein level (Ibeagha-Awemu et al., 2008; Petzl et al., 2008; Porcherie et al., 2012). Surprisingly, the expression of TLR5 gene was not detected in MEC, consistent with the observation that the MG is not responsive to injection of flagellin, a well-known TLR5 agonist (Porcherie et al., 2012).

MEC are responsive to LPS treatment via the TLR4/MD2/CD14 receptor complex (Boudjellab et al., 1998; Pareek et al., 2005; Porcherie et al., 2012; Strandberg et al., 2005). A significant amount of soluble CD14 is present in milk, which contributes to the efficient recognition and clearance of coliform bacteria (Lee et al., 2003a, 2003b; Lee, Paape, & Zhao, 2003; Reinhardt & Lippolis, 2006; Védrine et al., 2018).

In MEC, lipoproteins, particularly those present in the envelope of bacteria, are recognized by the TLR1/TLR2 or TLR2/TLR6 heterodimers while peptidoglycan subunits are recognized by NOD1 and NOD2 receptors (Porcherie et al., 2012).

The relative contribution of MEC and macrophages to the onset of inflammation is not very well understood. Although MEC are likely to be responsible for the recognition of coliform through the detection of LPS, their role in responding to other pathogens is less obvious. Indeed, recent data suggest that detection of *Streptococcus uberis*, a major mastitis pathogen, is rather accomplished by milk or tissue macrophages (Archer et al., 2020; Gunther et al., 2016).

4.3 | Inflammatory cascade

Upon binding of MAMP to their cognate receptors, signalling pathways are activated leading to the expression of pro-inflammatory mediators and antimicrobial peptides (Kawai et al., 2015; Shuster et al., 1997). MEC switches from milk secretion to inflammatory response with decreased secretion of ß-casein and triglycerides, glucose being preferentially directed towards immune response rather than lactose biosynthesis (Blum et al., 2020; Huang et al., 2019; Tsugami et al., 2021).

Kinetics analyses clearly showed a progressive response of MG to stimuli, with MEC from teat cisternae and gland cisternae as early responders, while response of MEC from the parenchyma occurs later (Petzl et al., 2016; Rinaldi et al., 2010). IL-1ß, IL-6 and TNFα are detected 10–12 h post-inoculation in the MG, whereas CXCL8 is detected later (Bannerman et al., 2004; Rinaldi et al., 2010; Riollet et al., 2000b; Shuster et al., 1997).

Another early event that occurs upon recognition of pathogens is the release of oxylipids (Hayashi et al., 2019; Sordillo, 2018). Platelet-activation factor (PAF) and leukotriene B4 were suggested to play a role in endotoxin-induced mastitis (Persson et al., 1993; Waller, 1997). Interestingly, PAF is released by MEC very quickly after stimulation with LPS (Corl et al., 2008).

Later in the process, the response is characterized by the secretion of anti-inflammatory mediators, such as IL-10 and TGFß or certain oxylipids, which permits a return to the initial non-inflammatory status (Chockalingam et al., 2005; Rinaldi et al., 2010).

4.4 | Adaptive immunity of the mammary gland

In addition to the above-mentioned innate mechanisms, the MG is armed of a second line of defence relying on soluble and cellular components of the adaptive immune system. Over the innate response, the adaptive immune system confers specificity and memory to the host immune response to infection.

4.4.1 | Humoral adaptive response

The activity and antigen specificity of humoral adaptive immune system is assured by more than 10^8 possible antibody or immunoglobulin (Ig) clonotypes, produced by the random recombination of genes encoding for their light chain variable (V), diversity (D) and joining (J) regions or by somatic hypermutation. Upon recognition of specific soluble extracellular antigen through their B-cell receptors (BCR), B cells are activated; they proliferate and mature into plasma cell. Plasma cells secrete antibodies recognizing the same antigen as their BCR and long-term survival of subsets of activated B cells confers immunological memory, allowing a rapid reactivation of humoral responses on reexposure to antigen (Harrison, 2016). Upon clonal expansion and differentiation, plasma cells can reside in their secondary lymphoid tissue of origin or traffic through the efferent lymph to the blood to populate distant sites (Kunkel & Butcher, 2003). In mice, the efficient migration of IgA antibody secreting cells (ASC) to the lactating MG depends on the vascular adhesion molecule VCAM-1 and the lymphocyte-expressed integrin α4, as well as on the chemokine CCL28 and CCR10. Previous evidence suggest that MAdCAM-1, VCAM-1 and peripheral node addressin (PNAd) are not involved in the homing of lymphocytes to the bovine mammary gland (Hodgkinson et al., 2009). To date, the molecules mediating this process in ruminant species are not known (Hine et al., 2019).

In ungulates, Igs found in the mammary secretion are determinant for the transfer of passive immunity from mother to young. This transfer is particularly critical for the survival of young ruminants, since in these species, newborns are agammaglobulinaemic or hypogammaglobulinaemic (Watson, 1980). The colostrum-forming MG accumulates IgG1, IgG2, IgA, IgE and IgM from the blood serum or produced in situ by intramammary plasma cells, transported by transcytosis from the interstitial fluid into the lumen of alveoli (Hine et al., 2010; Stelwagen et al., 2009; Watson, 1980). This active and highly selective process, mediated by the neonatal Fc receptor (FcRN), leads to the production of an IgG1-rich secretion, which is available for ingestion by the newborn immediately after birth (Brandon et al., 1971; Hine et al., 2019).

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In human and rodents, lactating mammary tissue contains significant numbers of IgA-containing plasma cells and a substantial amount of IgA is observed in colostrum and milk. Nevertheless, IgG1 is the most predominant Ig isotype found in the colostrum and milk of cows and ewes (Watson, 1980). Besides, although Igs make approximately 5% of bovine colostrum content, the cow lactating MG is characterized by a low presence of Ig-containing plasma cells and little amount of Igs (Stelwagen et al., 2009). A 10-fold enhancement of IgG1 levels can be observed in bovine colostrum relative to serum, whereas milk show IgG1 concentrations 10-fold inferior to blood (Baumrucker et al., 2010).

Humoral adaptive mechanisms of the ruminant MG are not only relevant for the establishment of the offspring immune defences, but they also contribute to the protection of the mammary gland itself. IgG1, IgG2 and IgM act as opsonins in milk, leading to the phagocytosis of bacteria by macrophages and neutrophils (Sordillo et al., 1987). Milk opsonizing capacity is clearly weak when compared to serum, but local stimulation due to infections or by vaccination increases the capacity of local antibodies to modulate immune response. Igs show an elevated influx from blood into milk during mastitis due to an impairment of blood–milk barrier (BMB). Consequently, the selective transport of IgG1 into the mammary alveoli is lost, leading to an increase of IgG2 levels which becomes the predominant Ig in milk and plays a critical role in the control of infections by facilitating the activity of migrating neutrophils. Ewes affected by *Streptococcus epidermidis*-caused mastitis, but not healthy controls, show pathogen-specific IgG and IgA in milk (Queiroga, 2018; Wellnitz & Bruckmaier, 2021). *Staphylococcus aureus*-specific IgG1 are also found in mammary quarters of clinically healthy lactating cows and the presence of higher pre-existing IgG1 titres was associated to lower *S. aureus* shedding (Boerhout et al., 2016).

Enhancement of mammary gland humoral adaptive mechanisms also relies on an increase of antibody-producing cells in tissue. Although B-lymphocytes represent a minor portion of milk lymphocytes, the percentage of these cells is significantly increased in the milk of cows affected by chronical *S. aureus* mastitis (Riollet et al., 2001). Vaccination through the intramammary route also proved to elicit an increase of IgA, IgG and IgM-producing cells in the mammary tissue, reinforcing the role of local stimulation in the triggering of adaptive humoral responses in the MG (Hodgkinson et al., 2009; McDowell & Lascelles, 1971). The presence of anticapsular IgG1, IgG2, IgA and IgM has been also reported in the MG dry secretion of cows immunized in the supramammary lymph node with *S. aureus*. These antibodies led to an enhancement of *S. aureus* phagocytosis by neutrophils, with the exception of IgG1, which is not opsonic for bovine neutrophils, but increase macrophages phagocytic capacities and their role in the defence against bacteria (Guidry et al., 1994).

The involvement of epithelial cells adherence/invasion by bacteria in the pathogenesis of mastitis is controversial (Anderson, 1978; Cifrian et al., 1994; Frost et al., 1977). Nevertheless, different reports have shown that vaccination-induced antibodies could inhibit the invasion of a mammary epithelial cell line by pathogens and increase phagocytosis by macrophages (Prado et al., 2011; Renna et al., 2014). Although IgG1 and IgG2 constitute the majority of Ig in serum, IgA has also been reported to hamper the adhesion of invading pathogens to the mammary epithelial surface (Sheldrake & Husband, 1985; Sordillo et al., 1987). Plasma cells residing in the mammary gland produce the IgA found in bovine colostrum and milk. IgA is then transported to mammary alveoli by the polymeric immunoglobulin receptor (pIgR) and is released with a portion of pIgR named secretory component (SC). SC contributes to the defence of MG by enhancing the functionality of IgA and eosinophils as well as by acting as a microbial scavenger (Stelwagen et al., 2009).

Staphylococci can produce toxins, such as staphylococcal enterotoxins (SEs), toxic shock syndrome toxin-1 (TSST-1), leucocidins, haemolysis and exfoliatin to evade host defences and cause diseases, including mastitis (Abril et al., 2020). Interestingly, local antibodies take part in the protection of mammary tissue by neutralizing these toxins (Kuroishi et al., 2003).

4.4.2 | Cell-mediated adaptive immunity

The cell-mediated immunity (CMI) relies on the activity of T lymphocytes (or T cells) specifically sensitized by contact with a foreign antigen presented in the context of major histocompatibility complex (MHC) molecules. MHC class I (MHC-I) molecules are present on all nucleated cells, whereas MHC class II (MHC-II) molecules are expressed by professional antigen presenting cells (APCs): dendritic cells (DCs), monocytes, macrophages and B cells. Upon cell infection or pathogen phagocytosis, MHC-I and MHC-II molecules present peptide antigens to T-cell receptors (TCR) of $CD8^+$ and $CD4^+$ lymphocytes respectively (Harrison, 2016).

Cell-mediated immune response can be classified as type 1, 2 or 3 according to the implicated effector cells, their functions and their pathophysiological effects. Type 1 (IFNγ-dependent) and type 3 (IL-17-dependent) responses are the most relevant in the context of bacterial infections. They involve $CD4^+$ and $CD8^+$ T cells producing IFNγ (Th1 and Tc1 respectively) and those producing IL-17 (Th17 and Tc17). Type 3 immunity is characterized by the recruitment of neutrophils and stimulation of epithelial antimicrobial defences against extracellular bacteria and fungi, whereas type 1 immunity provides an effective response against intracellular microbes by boosting the killing capacity of phagocytes and infected cells (Annunziato et al., 2015). A general overview of CMI mechanisms in the MG of ruminants will be provided in this article. For a more comprehensive review, refer to Rainard et al., 2022.

Studies carried out in the early 1980s provide the initial observations associating the CMI to the defence of the MG. *S. aureus* mastitis leads to monocytes migration to the mammary tissue and their maturation into macrophages (Nickerson & Heald, 1982). Besides phagocytosing degenerate neutrophils and milk constituents, macrophages are supposed to enter lymphatics and migrate to lymphoid organs or process antigens for presentation to lymphoid cells located in the interalveolar stroma. These lymphoid cells, accumulated

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in the MG after infections, were initially considered as precursors of antibody-producing plasma cells. However, further research showed that mononuclear cells found in the MG could also induce local neutrophil responses in an antigen-specific way (Colditz & Watson, 1982).

Since then, accumulating evidence highlight a major role of cellular immunity, in particular IL-17 mediated CMI, in the defence of the MG. Mammary antigen-specific reaction marked by a neutrophilic inflammation essential for the clearing of invading organisms has been associated to IL-17A-producing $CD4^+$ T cells (Rainard et al., 2015). Using an immunization schedule exploiting the intramammary route, Cebron et al. (2020) associate a better clearance of *E. coli* intramammary infections to an increased homing and/or proliferation of T-lymphocytes producing IL-17 in the MG. IL17A and IL17F are key cytokines for the recruitment, activation and migration of neutrophils (Annunziato et al., 2015). IL-17A expression, at both mRNA and protein levels, has been described in mammary tissue and milk from cows experimentally infected with *E. coli* (Herry et al., 2017; Roussel et al., 2015) and *S. uberis* (Tassi et al., 2013). Bovine mammary epithelial cells express the two components of the IL-17 receptor and respond to IL-17 by releasing more chemokines and antimicrobial peptides (Bougarn et al., 2011).

An increase of IFN-γ expression/abundance in the MG has been reported during mastitis caused by multiple pathogens including *S. aureus*, *E. coli* and *S. uberis*. This cytokine, produced mainly by T-lymphocytes and natural killer cells (NK cells), enhances the microbicidal activity of macrophages and neutrophils and upregulates the expression of MHC molecules, boosting $CD4^+$ and $CD8^+$ T cells activation (Bannerman, 2009). Despite its role in linking the innate and adaptive arms of the immune response, the impact of IFNγ in the pathogenesis of mastitis is still ambiguous, as it varies depending on the causing agent. Herry et al. (2017) observed that immunization with heat-killed *E. coli* generates a marked increase of IFNγ concentrations in milk that is inferred to improve bacterial clearance and enhance resistance to mastitis. Better bactericidal response against *S. uberis* has been also attributed to the induction of IFNγ-mediated mechanisms in non-immunized (Denis et al., 2011a) and immunized cows (Wedlock et al., 2014). Contrary to this, high concentrations of IFN-γ have been reported in milk of animals infected with pathogens causing persistent infections (Schukken et al., 2011). IFN-γ has been additionally related to an enhancement of susceptibility to mastitis by *S. aureus* in bovine (Liu et al., 2020).

Unlike peripheral blood, T cells in healthy mammary glands and milk are mostly $CD8^+$ (Sordillo & Streicher, 2002). However, increased amounts of $CD4^+$ T cells concurrent with a decrease of their $CD8^+$ counterparts is observed in milk during mastitis (Riollet et al., 2001; Soltys & Quinn, 1999; Tucker et al., 2023) indicating that these cells are important mediators of MG immune defences. Nevertheless, the contribution of $CD8^+$ T cells to the protection of mammary tissue should not be neglected. Denis et al. (2011b) demonstrated that a high proportion of *S. uberis*-specific T cells from bovine blood and MG secretion shows a memory $CD8^+$ phenotype and potent bactericidal activity in vitro. Nevertheless, evidence also attributes a suppressive role to CD8+ T cells during mastitis by *S. aureus* (Park et al., 1993) and further research is necessary to clarify the impact of these cells in the establishment of persistent infections.

During mastitis, epithelial cells are the first barrier to infection and pathogens adapted to the mammary gland can eventually penetrate these cells and survive in the intracellular milieu. Since antigen-specific responses by $CD8^+$ T cells are efficiently triggered during bacterial infections (Joffre et al., 2012), it is possible that activated IFNγ-producing cytotoxic T cells fight the bacterial intracellular niche by eliminating infected cells. Additionally, IL-17-producing CD8+ T cells have been identified as relevant sources of IFNγ, TNFα, IL-21 and IL-22 in human (Srenathan et al., 2016). These cells have been recently reported in bovine (Elnaggar et al., 2018) and their functions in the protection of the mammary gland remains to be clarified.

5 | CONCLUDING REMARKS AND KNOWLEDGE GAPS

The different families of immune system mediators present in the ruminant mammary gland are now rather well identified. Nevertheless, the diversity of cell populations and molecules composing these families remains to be deciphered. Current high-throughput techniques would be very valuable to finely characterize the different MG lymphocytes, neutrophils and macrophages sub-populations. To foster the development of new and innovative vaccination strategies, further knowledge of the functionality of milk and tissue lymphocytes is eagerly needed, as well as of the biology of antigen presenting cells in the MG. A better understanding of how MG cell populations interact with each other and what are their respective roles in host response is also necessary.

Although current trends often focus on cytokines, they may not be the earliest signals in the host response to infection. A deeper comprehension of how oxylipids, for example, contribute to the defence of the MG is needed and this is of particular interest given that these molecules derive from polyunsaturated fatty acids, which metabolism could be adjusted through nutrition (Lin et al., 2013). Similarly, the role of C5a in the early steps of inflammation has been suggested and would deserve renewed investigations (Stevens et al., 2012). Finally, one should bear in mind that innate and adaptive immunity go hand in hand and should not be considered separately.

AUTHOR CONTRIBUTIONS

PG and RPM prepared and edited the original draft.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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ORCID

Pierre Germon https://orcid.org/0000-0003-3078-876X
Rodrigo Prado Martins https://orcid.org/0000-0002-8213-4015

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