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Targeting Mucosal Immunity in the Battle to Develop a Mastitis Vaccine

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Abstract The mucosal immune system encounters antigens that enhance and suppress immune function, and serves as a selective barrier against invading pathogens. The mammary gland not only encounters antigens but also produces a nutrient evolved to protect and enhance mucosal development in the neonate. Efforts to manipulate antibody concentrations in milk to prevent mastitis, an infection of the mammary gland, have been hampered both by complexity and variation in target pathogens and limited knowledge of cellular immunity in the gland. Successful vaccination strategies must overcome the natural processes that regulate types and concentrations of milk antibodies for neonatal development, and enhance cellular immunity. Furthermore, the need to overcome dampening of immunity caused by non-pathogenic encounters to successfully prevent establishment of infection is an additional obstacle in vaccine development at mucosal sites. A significant mastitis pathogen, *Staphylococcus aureus*, not only resides as a normal flora on a multitude of species, but also causes clinical disease with limited treatment options. Using the bovine model of *S. aureus* mastitis, researchers can decipher the role of antigen selection and presentation by mammary dendritic cells, enhance development of central and effector memory function, and subsequently target specific memory cells to the mammary gland for successful vaccine development. This brief review provides an overview of adaptive immunity, previous vaccine efforts, current immunological findings relevant to enhancing immune memory, and research technologies that show promise in directing future vaccine efforts to enhance mammary gland immunity and prevent mastitis.

Keywords Antigen presentation · Vaccine · *Staphylococcus aureus* · Mastitis · Bovine · Dendritic cell

Abbreviations

CMI	cell-mediated immunity
TCR	T cell receptor
MHC	major histocompatability complex
APC	antigen presenting cells
T_{REG}	regulatory T cells
MALT	mucosa-associated lymphoid tissue
GALT	gut-associated lymphoid tissue
PMN	polymorphonuclear neutrophils
SCC	somatic cell count
T_{EM}	effector memory T
T_{CM}	central memory T
IL	interleukin
IsdB	iron-regulated surface determinant system protein B
SUAM	*Streptococcus uberis* adhesion molecule
DC	dendritic cells
LC	langerhans cells
CLR	C-type lectin receptor
PRR	pathogen recognition receptor
TLR	toll-like receptor
NOD	nucleotide oligomerization domain
TNF	tumor necrosis factor
MCSF	macrophage colony stimulating factor

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GMCSF	granulocyte-macrophage colony stimulating factor
TACE	TNF-α converting enzyme
CCL2	chemokine ligand 2
MCP-1	monocyte chemotactic protein-1
IFN-γ	interferon-γ
TGF	transforming growth factor
CFU	colony forming units

Overview

The search for successful vaccination strategies against bacterial infections traverses multiple animal and research models. For certain infections, success has been obtained and infections prevented; for others the search for the magic bullet continues. In the case of bacterial infections caused by normal flora of the host, dampening of host immune defenses may complicate the search for a vaccine. This dampening, or suppression, may enhance the capacity of normal flora to cause disease under the radar of the immune response. Bacterial infection of the mammary gland, known as mastitis, is an example of normal flora traversing into pathogenic organisms causing sometimes-deadly disease. Though all lactating mammals are susceptible to mammary gland infections, mastitis in the bovine is not only of significant economic impact to agriculture, but is also a valuable model for study of host and pathogen interactions. Furthermore, the bovine mastitis model, and long history of vaccine research, can provide answers for immunological control of *Staphylococcus aureus*; a pathogen that is acquiring resistance to antibiotics and increasingly responsible for infections beyond mastitis in a multitude of species.

Immunity and Mastitis

An ideal vaccine enhances host immune defenses to prevent establishment of infection. Living organisms are constantly exposed to microbes that are present in their environment and need to deal with the invasion of these microbes into the body. A healthy immune system combats invading microbes due to an ability to distinguish between self and non-self. Immunity can be divided into innate, providing the first line of defense against pathogens, and acquired immunity, often a slow process but specifically mediated through T and B cells [1]. Adaptive immunity displays the remarkable property of memory. Recently, it is considered that the innate immune system also possesses specificity and ability to discriminate between antigens [2]. Adaptive immunity is further classified into cell- mediated immunity (CMI) and humoral immunity. CMI is largely mediated by T cells and humoral immunity by B cells. T cells possess a unique antigen binding receptor molecule on the cell surface called a T cell receptor (TCR). The majority of TCRs recognize antigenic peptides bound to major histocompatibility complex (MHC) derived molecules. T Helper cells ($CD4^+$ cells) recognize processed foreign peptide complexed with MHC class II molecules on the surface of antigen presenting cells (APC) and elicit immune response by secreting cytokines that stimulate CD8 T cells and B cells [3]. Cytotoxic or killer T cells ($CD8^+$ cells) engage with other cells that carry processed foreign peptide complexed with MHC class I molecules on their surface [4]. CMI also includes regulatory T cells (T_{REG}) which downregulate production of CD8 T cells and the memory T cells at completion of an immune response. Humoral immunity is mediated through immunoglobulins secreted by the stimulated B cells differentiated into plasma cells [5]. Many pathogens dysregulate immune responses by impacting function of T and B cells or increasing T_{REG} activity to induce immune suppression [6–9]. Current vaccines should enhance both CMI and/or humoral immunity to ensure that bacteria invading mucosal sites are eliminated prior to disease onset.

The mucosal immune system consists of intestinal, respiratory, urogenital mucosa, and exocrine glands. In the pig the predominant isotype produced at mucosal sites is IgA whereas in the bovine it is IgG1 (reviewed in [90]). Antigen specific immune responses in Peyer's patches dictate mucosal immune development through cytokine production subsequent to immune cell interactions. Recent studies have focused on the cytokine and immune cell content of milk and subsequent impact on mucosal immune development in neonates [90]. Initial contact with epithelial barrier in the gut (oral included) is key to development of oral tolerance (reviewed in [91]). This process prevents reactivity to foods and other daily encountered antigens as well as commensal bacteria. The interactions of intestinal microbial with epithelial cells are also a means of developing tolerance and are modulated through receptor signaling. Mucosa-associated lymphoid tissue (MALT) contains B cell follicles, APC, and T cells similar to other lymphoid organs but lacks afferent lymphatics. The role of M cells found in the epithelial barrier of mucosal tissue, provides the delivery of antigen from mucosal sites to MALT. The gut-associated lymphoid tissue (GALT) and Peyer's Patches in intestinal locations serve as lymph nodes at these sites. All three of these tissues sample antigen directly from mucosal sites through M cells thereby differentiating them from lymph nodes [91]. The sampling of antigen in the mammary gland is focused on identification of invading bacteria to allow for initiation of an immune response. The full spectra of antigen sampling cells, for example the antigen sampling capacity of

mammary epithelial cells, in the mammary gland remains to be defined.

Clearance of bacterial mastitis involves immune responses from resident cells of the mammary gland and cells migrating into the gland from the periphery. The cellular defenses of the mammary gland include: phagocytic cells such as macrophages and polymorphonuclear neutrophils (PMN), as well as natural killer cells, T cells, and B cells. A baseline presence of immune and epithelial cells in the milk, better know as the somatic cell count (SCC), is critical to prevent the establishment of bacteria in the mammary gland and reduce incidence of infection [10]. In addition, an increase in infiltrating cells during infection is important for the successful elimination of bacteria. Epithelial cells and immune cells secrete chemokines in response to bacterial infection, which result in an influx of immune cells from the blood to the mammary gland, thereby resulting in an increase in milk SCC. The SCC of infected milk mainly consists of PMN that respond to the increased concentration of chemokines and is used as an indicator of mastitis. PMN are the predominant cell type responsible for clearance of mastitis pathogens. However, prevention of mastitis may require the recruitment of additional immune cells; mainly T cells and B cells that are capable of either eliminating pathogens through direct cytotoxicity, production of antibodies, or recruitment of PMN. Recent literature indicates that a presence of effector memory T (T_{EM}) cells plays a significant role in enhancing PMN function and protecting vaccinated animals [11–14]. Both T_{EM} and central memory T (T_{CM}) cells mount immune responses more quickly than naive cells but are derived from different T cell lineages. The T_{EM} population, unlike T_{CM}, expresses low levels of migratory and adhesion molecules (L-selectin and CCR7) and produces interleukin (IL)-4 instead of IL-2 (reviewed in [15]). The ability to manipulate memory cell development, survival, and migration are key to successful mucosal vaccination. Understanding of the mechanisms that recruit protective T_{EM} to the mammary gland is necessary for development of cellular based vaccines and other novel methods needed for preventing and treating chronic, subclinical mastitis.

Overcoming Traditional Limitations of Mastitis Vaccines

Most common bovine mastitis pathogens, including *Escherichia coli*, *Klebsiella* spp., *Streptococcal* spp., and *Staphylococcal* spp. [10, 16, 17], produce a milieu of proteins and toxins that impact host immune responses. Successful antibody based vaccines against the core antigen lipopolysaccharide component of *E. coli* [18–20] and recently other endotoxin producing mastitis pathogens have been developed but only go so far as to control clinical symptoms, rather than prevent onset of infection. The changing profiles of toxin production by pathogens, especially Gram-positive, at various stages of infections make using only single bacterial proteins in development of vaccines that prevent bacterial establishment a challenge. This may be an underlying cause for failure of human Phase II clinical trials of *S. aureus* capsular polysaccharide (type 5 and 8) based vaccines [21]. Capsular polysaccharide based vaccines are suggested to work against other pathogens but not *S. aureus* due to virulent strains that are capsule negative or express capsule only during stationary growth [22]. Mastitis vaccines developed using multiple bacterial proteins, such as *S. aureus* pseudocapsule and α and β toxins [23], have undergone field trials and shown increased antibody titers in serum and milk but provide no significant protection to vaccinated animals [24]. Similar negative results or unreported results have been obtained with vaccines based on cell-wall anchored iron-regulated surface determinant system protein B (IsdB), clumping factor A, ATP-binding cassette transporter, surface-associated antigens, adhesion proteins [25–27], exotoxins, and superantigens, to name a few. Although a multi-component antibody based vaccine may overcome the lack of success with single component vaccines, further understanding of the pathogen's influence on the host immune response is necessary [22].

During mastitis, whole bacteria produce a large repertoire of toxins at varying concentrations depending on stage of infection against which the immune system can mount antibodies. Recent strategies focused on regulation of toxin production in vaccine strains have shown limited success [28]. It remains unknown how the non-specific lymphocyte activating effects of toxins can be overcome by vaccination to provide a protective immune response. Failure of many antibody based *S. aureus* vaccines can be due to the binding of antibody by protein A [21]. Veronate, a hyperimmune IgG preparation directed against clumping factor A did not achieve substantial protection in Phase II trials [21]. Field trials with the MASTIVACS I [29, 30] and StaphVAX [22] vaccine indicated limited or no protection. Interestingly, human carriers of *S. aureus* possess greater antibody titers against recombinant staphylococcal proteins than non-carriers [31]. However these antibodies are not believed to be protective against subsequent infections. Similarly, antibody titers in the mammary gland are insufficient to protect against new infections following vaccination with lysogen, a *S. aureus* mastitis bacterin [32].

Recent studies use bacterial mutants to identify proteins required for adherence and intracellular survival of bacteria in mammary epithelial cells, such as the *Streptococcus uberis* adhesion molecule (SUAM). [33]. These vaccination strategies continue to rely on antibody

based humoral immune responses and therefore are unlikely to work against *S. aureus* mastitis. However, recent identification of *S. uberis* specific milk memory T cells opens a new avenue for development of cellular based vaccination strategies against mastitis [34]. The generation of a cellular based vaccine that can enhance innate immune function and overcome the nonspecific activation of T cell populations by bacterial toxins will be of benefit against certain mastitis infections.

Evaluating Pathogen Antigenic Profiles

Previous research studies have characterized differential protein expression by commensal and pathogenic strains of *S. epidermidis* [35] and protein induction following changes in environmental growth condition of *Aspergillus fumigatus* [36]. The diversity in protein expression of pathogenic bacterial strains is a major obstacle to successful vaccine development. Moreover, commensal and environmental strains, to which animals are commonly exposed, may cause immune dampening that enables subsequent infections by pathogenic strains of the same bacteria. Novel efforts are needed to identify the immune dampening proteins expressed by commensal strains and the disease causing proteins expressed by pathogenic strains in order to determine their role in disease progression and as immune antigens in vaccine development. Study of strains associated with nasal carriage indicated a differential expression of proteins in carrier strains as compared to strains not associated with nasal carriage [37]. Recent reviews on this area of research, with a specific focus on *S. aureus*, cover the topics of both human carriers [38] and bacterial proteome [39].

Proteomic and genomic technologies are methods for comparison of bacterial protein and gene expression and identification of immune antigens. These techniques have recently been implemented to compare the host expression of proteins during mastitis [40–42]. Recent studies are using proteomic analysis to characterize pathogenic *S. aureus* isolates and identify virulence factors. Study of bovine *S. aureus* isolates indicated different results when microarray data was compared to proteome analyses. DNA microarray analysis of 17 isolates found 43 conserved genes. However MALDI-TOF MS/MS analysis found only one conserved protein and 11 proteins expressed by 80% of the same isolates [43]. This significant variability in virulence gene transcription and translation is yet another obstacle in successful antibody based vaccine development. Successful identification of antigens differentially expressed by commensal and pathogenic strains, or upregulated upon entry of pathogens to mucosal sites of the host will enhance vaccination efforts. The comparison of antigenic profiles using proteomic technologies is a new area of research, especially when focused on mastitis strains and *S. aureus*. Though there is no doubt that some of the proteins identified will corroborate previous findings, a lot of hope remains that novel antigens to be used in vaccine development will be found.

Antigen Presentation, Delivery, and Subsequent Cellular Immunity

Dendritic cells (DC) and epithelial cells are considered the sentinels of immune system. DC were initially described in 1973 [44] and are considered the professionals of APC because of their unique characteristic of stimulating naïve T cells [45]. Specifically, DC bridge the innate and adaptive immune responses by serving to prime naïve T cells based on microbial stimuli and drive T cell differentiation thus controlling the magnitude and the quality of the adaptive immune response [46]. As such, a true understanding of DC antigen uptake and presentation is key to development of CMI based vaccines.

The DC lineages are derived from both lymphoid and myeloid progenitors. DC subsets perform specialized immune functions depending upon their location [47] and are differentiated by the expression of specific surface receptors. Distinct DC subsets respond to antigens differently, depending on surface receptor expression, tissue location, and antigen sampling and thereby result in differing responses from T and B cells [46]. For example, $CD11b^{+}$ DC migrate to dermal sites and induce cytokine production from both T_{EM} and T_{REG} cells [48]. Migratory DC residing in an immature state at the sites of potential pathogen entry constantly patrol the environment for invading pathogens. Immature DC residing in tissues (e.g. Langerhans cells (LC) of the epidermis) or in peripheral blood mature once they encounter an antigen. The mature DC migrate towards the regional lymph node and present the antigenic peptide through MHC to functional T cells to elicit immune responses [49]. On the other hand, the lymphoid tissue resident DC such as in thymus and spleen do not migrate, and, instead, sample and present antigens in their resident tissue [50]. Research must still define how resident mammary DC uptake antigen and migrate to regional lymph nodes.

Immature DC express a wide variety of receptors on their surface [51] including phagocytic receptors, C-type lectin receptors (CLRs), pathogen recognition receptors (PRRs) and scavenger receptors. Several reports indicate that targeting antigens to DC receptors increases antigen presentation to CD4 and CD8 T cells in vivo [52–55].

DEC205/CD205 is a CLR that can function as an endocytic receptor [56]; however, the ligand for this receptor remains unidentified. The CLRs are Ca^{++} dependent glycan-binding proteins that internalize their ligands through clathrin coated pits resulting in the delivery of ligands to lysosomes or late MHC II rich endosomes [57]. Targeting the endocytic receptor DEC205, improves the efficiency of T cell vaccination and increases antigen presentation on MHC I and II [57, 58]. Though directing peptide antigen to DEC205 receptors results in an initial increase in CD4 and CD8 T cell proliferation in vivo, this may be followed by a state of tolerance in the absence of DC maturation [58, 59]. However, in the presence of maturation stimuli, targeting protein antigens to DEC205 improves T cell vaccination [58]. For vaccine development, not only must the proper DC targeting antigen be selected, but also proper choice of adjuvant to mature DC and induce proper T cell responses should be considered.

DC activation is characterized by the induction of cytokines and costimulatory molecules upon recognition of pathogenic stimuli. DC activation induces the expression of chemokine receptors such as CCR7 enabling them to migrate to the draining lymph nodes to elicit T cell response [60]. The chemokine receptor CCR7 plays a major role in the localization of antigen specific T cells and antigen loaded DC in the lymph nodes [61]. CCR7 is expressed primarily on naïve T cells, T_{CM} cells, mature DC, and mature B cells which frequently migrate to secondary lymphoid tissues [62]. Absence of CCR7 results in the absence of a T cell response [63]. Tissue DC, such as LC, migrate to the local lymph node during the process of maturation and present antigen to naïve T cells. Since naïve T cells do not traffic to peripheral tissues such as skin, migration of DC is very important for the proper immune response. The expression of CCR7 on matured DC facilitates the migration of DC to lymph nodes [60] to allow for presentation of antigen collected at peripheral mucosal sites to T cells in local lymph nodes.

Immature DC constantly patrol for invading pathogens at the common sites of potential pathogen entry [51, 64, 65]. Circulating monocytes, tissue macrophages, and DC recognize pathogen associated molecular patterns (PAMPs) from various pathogens through PRRs, such as Toll-like receptors (TLRs), nucleotide oligomerization domains (NODs) and CLRs resulting in their activation [66, 67]. Activation through TLRs trigger DC maturation and thereby modulates the adaptive immune responses. Depending upon the stimuli received from the pathogen DC undergo maturation to induce immunity, tolerance or become sessile whereas immature DC induce only tolerance [68]. This program of maturation of DC brings about the up- regulation of MHC class II [69] and co-stimulatory molecules CD80 and CD86 [70], and expression of CCR7 [71]. The mature DC become more efficient in antigen presentation, while less efficient in phagocytosis [69]. Activation of phagocytic cells induces secretion of proinflammatory cytokines, growth factors, and chemokines that recruit additional inflammatory cells to the site of infection resulting in pathogen clearance [72]. Important proinflammatory cytokines include tumor necrosis factor (TNF)-α, IL-1β and IL-6. The cytokine, TNF-α suppresses expression of the macrophage colony stimulating factor (MCSF) receptor and directs the differentiation of monocytes into DC rather than to macrophages [73]. The growth factor, granulocyte-macrophage colony stimulating factor (GMCSF) in combination with IL-4 strongly up-regulate TNF-α convertase enzyme (TACE) expression and activity in monocytes [74]. TACE is a type 1 transmembrane metalloproteinase that is required for the activation of pro-TNF-α [75]. The function of TACE is to shed ectodomains of membrane-bound proteins such as cytokines, chemokines, growth factors, receptors or adhesion molecules [75, 76]. The chemokine ligand 2 (CCL2) or monocyte chemotactic protein-1 (MCP-1) is secreted on stimulation by monocytes and other innate cells [77] . In response to CCR2-CCL2 interaction, monocytes traffic to the sites of microbial infection [78]. There, monocytes differentiate into macrophages or DC to curtail the infection by phagocytizing and killing the pathogens. Thus, monocytes along with neutrophils form an integral part of innate immune system and play a key role in early containment of infections such as mastitis. Targeting innate and adaptive immunity either directly, or indirectly through T cell function, would enhance current mucosal vaccine efforts.

Monocytes originate from myeloid progenitors, and upon extravasation to tissue from blood can serve as precursors for tissue macrophages and DC [79] depending on stimuli and the cytokines present at the site of infection [80]. Monocytes ($CD14^{+}$) and early hematopoetic progenitor cells ($CD34^{+}$) have the potential to differentiate into DC when cultured with GMCSF, but the exact regulatory mechanisms of this differentiation is not known [81, 82]. Several studies have shown that mice deficient in MCSF are also deficient in monocytes and skin Langerhans cells. This information points to the fact that migratory DC could be derived from monocytes stimulated with GMCSF and IL-4 and these cytokines may be key in enhancing antigen presentation at mucosal sites such as the mammary gland. Bovine monocyte derived DC are characterized by increased expression of MHC II, CD11c, co-stimulatory molecules CD80 and CD86 and decreased expression of CD14, and CD21 surface markers [83]. The relative expressions of various markers on monocyte and monocyte derived DC are depicted in Table 1. CD80 and

Table 1 Relative expression of markers on monocytes and dendritic cells (DC)

Markers	Monocytes	Monocyte derived Mature DC
CD14	+++	+ (Low level of expression)
MHCII	+++	+++ (MFI moderate-high)
CD11c	+	+++ (MFI moderate-high)
CD11b	+++	++ (High on inflammatory DC)
CD205	+	++ (less on monocyte derived DC)
CD80	+	+++ (relatively high on DC)
CD86	++	+++ (High expression)

Marker expression (mean fluorescent intensity (MFI) on cell surface is indicated as follows: Low– '+', Moderate– '++', High '+++'.

CD86 are the co-stimulatory molecules present on APC and interact with the CD28 (stimulatory) and CTLA-4 (inhibitory) receptors of the T cell. The absence of CD80 and CD86 results in lack of co-stimulatory signal delivery to T cells and leads to clonal anergy and lack of proper T cell response [84]. CTLA-4 interacts with B7 molecules [85] to inhibit T cell activation and induce T cell anergy by competitive antagonism of CD28:B7 mediated costimulation; however, the complete absence of CTLA-4 results in unrestricted activation of T cells [86]. Regulation of costimulatory molecule expression and activation by the pathogen or through vaccination can inhibit or encourage development of T_{EM} cells that will be effective in the mammary gland.

Naïve T cell activation requires signals from the co-stimulatory molecules and cytokines provided by the mature DC [87]. There are three phases in T cell priming following contact with a DC-MHC II-antigenic peptide complex [88]. Phase one is the contact of T cells with antigen loaded DC and subsequent up-regulation of its activation marker, CD69, depending on the threshold of antigen. Phase two is characterized by further activation of CD69 and onset of IL-2 and interferon-γ (IFN-γ) secretion. In phase three, transient DC-T cell interaction occurs followed by the induction of T cell proliferation. DC tightly regulate their ability to induce effector T cells by secreting cytokines. In vaccine development, immune adjuvants are required to activate APC through PPR resulting in upregulation of costimulatory molecules (CD40, CD80, and CD86) and chemokine receptors [89]. The immunological synapse is the rearrangement and interactions of cell surface molecules following peptide presentation by MHC to T cell receptors. A minimum of 2 h is required for proper interactions and promotion of T cell proliferation [89] and following immunological synapse, antigen-specific signaling occurs within 10 h. These processes are taken advantage of in tetramer technologies that use antibodies to study antigen specific responses by targeting cellular receptors using an antigen bridge. Antigen primed T cells can interact with B cells to allow for effector cell development and antibody production. APC also can directly interact with B cells in a similar fashion to T cell interaction (CD40–CD40L) resulting in reorganization of surface cell receptors (B cell receptors) and subsequent antigen specific intracellular signaling. Similar to tetramer technology, superantigens also bridge MHC molecules with T and/or B cell receptors but result in intracellular signaling leading to differentiation, proliferation, and antibody production. The functional outcomes of DC-T and B cell interactions are critical in the differentiation of an effective T cell memory pool and providing protective immunity.

Th17 Based Vaccination

An integral defense in mucosal immunity is the Th17 cell, a lineage characterized by production of IL-17, IL-22, and IL-26. Production of these cytokines during infection controls dissemination from mucosal sites. Mice deficient in any of these Th17 cytokines succumb to bacterial infections as a result of dissemination from the original infection site (reviewed in [92]).

Recent findings highlight the importance of T cell dependent immune activation in clearance of *S. aureus* in mouse models. The T cell-derived cytokines are responsible for both protection and chronic status of certain infections. Production of IFNγ and recently identified IL-17 are required for cell mediated protection against certain viral and bacterial infections. During bacterial infections, production of IL-17 is required for abscess formation [12] but like IFNγ [93], this cytokine is an indicator of antigen-specific immune responses against BCG vaccination against tuberculosis [94]. Mice that cannot produce IL-17, due to a deficiency in γδ T cells, are more susceptible to intradermal *S. aureus* infection due to a decrease in PMN infiltration. Both IL-17 and IL-22, can induce production of chemokines (keratinocyte chemoattractant (KC:CXCL-1) and macrophage inflammatory protein (MIP-2:CXLC2/CXCL3) by epithelial cells and fibroblasts as well as induce PMN recruitment. It appears that presence of T cell at the site of infection may be critical for the production of chemokines, regulation of PMN infiltration, and resolution of infection [95]. Other studies have identified human $CD4^+$ memory ($CD45R0^+$) T cells, but not naïve cells, that secrete IL-17 following *S. aureus* stimulation [96]. Though initial findings implicate a role for IL-17 in clearance of *S. aureus* infections, the role in protection against mastitis remains to be elucidated. The potential of involving IL-17 immunity to enhance PMN infiltration into mammary tissue is a current focus of our research for design of a mucosal vaccine against *S. aureus*.

Targeting Antigen Specific Memory Cells in Vaccine Development

Depending on the type of infection and antigen presented, T cells will develop into immune phenotypes that are characterized by their transcription factors, cytokine profiles, and effector functions. The αβ T cells differentiate into either $CD4^+$ Th cells of the Th1 (IFNγ and TNFα producing), Th2 (IL-4, IL-5 and IL-13 producing), T_{REG} (IL-10 and GMCSF) and transforming growth factor (TGFβ) producing), and Th17 (IL-17 and IL-22 producing) phenotype or CD8+ cytotoxic (Tc) (perforin and granzyme producing) cells (Fig. 1). The differentiation of γδ T cells during infection is not as clearly defined but can result in production of the same cytokines as αβ cells [97]. All of these cell lineages can be of the memory phenotype ($CD45RO^+$). The presence of $CD45RO^{high}$ and $CD62L^{low}$ T cells among proliferating lymphocytes confirms an effector memory phenotype in previously infected cows. Several studies have reported that the bovine memory CD4 and CD8 T cells express CD45RO and CD62L [98, 99]. Challenge induced CD8 T cells fall into either T_{CM} or T_{EM} cell group and they differ in their functional ability and phenotypic marker expression [100]. Whether a CD8 mediated immune response is itself sufficient to prevent mastitis infection has yet to be determined.

The many toxins produced by *S. aureus* stimulate proliferation of T cell expressing a Vβ region in the T cell receptor in a non-specific manner [101]. For example, staphylococcal enterotoxin (SE) C stimulates bovine lymphocytes to express suppressor [102] and Th2 biased phenotypes [103]. Specific T cell stimulating bacterial toxins include *S. aureus* toxic shock syndrome 1, SE-A, -B, -C, -D, etc., and Group A streptococcal pyrogenic exotoxin serotypes A, C, etc. and superantigens [104]. Depending on which SE is used to stimulate cells collected from blood, there are significant differences in the responses and antigens presented by APC [105–107], subsequent Th polarization of T cells [108], expression of adhesion molecules, and cytokine production [107]. These toxin-induced immune responses elicit little long-term protection, if any, and are the cause of toxic shock syndrome and chronic mastitis [109]. It remains unknown how the non-specific lymphocyte activating effects of toxins can be overcome by vaccination to provide a protective memory immune response. However, a successful vaccine against *S. aureus* must also consider the capacity of staphylococcal enterotoxins to non-specifically bind MHC II molecules and result in T cell (CD4) activation [110] and modulation of mucosal immunity.

Significant research conducted in the study of tuberculosis vaccines in bovine models indicates success in eliciting cellular mucosal immunity. Vaccination of 6 month old cows with *M. bovis* BCG strain administered subcutaneously elicited IFNγ producing, $CD8^+$, $CD45RO^+$ memory cells in the periphery [98]. These peripheral memory cells produced perforin and exhibited antigen-specific lysis of BCG infected macrophages. Similarly, T_{CM} cells were identified from lymph nodes of cattle infected with *Mycoplasma mycoides* subspecies *mycoides* small colony. These T_{CM} cells were defined as $CD62L^+$ and proliferated in response to antigen stimulation [99]. Since antigen specific memory cells have been identified, characterized, and targeted for prevention of tuberculosis, it stands to

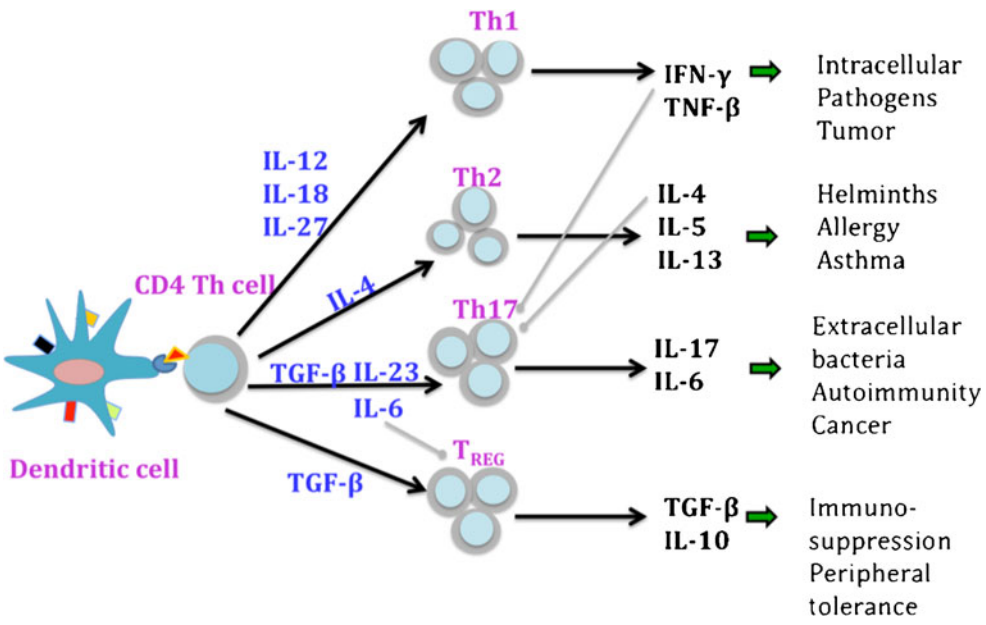

Figure 1 Antigen presenting cells polarize CD4 helper T cells depending on the dose of antigen, route of antigen exposure, and cytokine secretion. Classification of T helper cell polarization induced by dendritic cells based on their cytokine signature, and function. Grey lines indicate ability of cytokines to block polarization

reason that this technique may be beneficial in prevention of mastitis.

Efficacy of mucosal-based vaccines has been tested in both murine and bovine mastitis models. Catagliuolo et al. [111] formulated a multi-component vaccine consisting of multiple *S. aureus* adhesins and administered it as two doses of DNA intranasally. Both specific antibody and T cell responses were augmented in Balb/c mice 5 weeks after initial vaccination. Vaccinated mice had a several log decrease in colony forming units (CFU) of mammary gland tissue at 48 h following *S. aureus* challenge. Mucosal vaccines that enhance T cell function are supported by previous studies that confirm presence of antigen specific cells in mammary secretions. Denis et al. [34] used *S. uberis* antigens to induce proliferation of CD8+ T cells collected from mammary gland secretions of cows with *S. uberis* mastitis. These CD8+ cells released IFNγ and IL-10 upon proliferation and exhibited killing activity against *S. uberis*. Even though the presence of memory T cells in the peripheral circulation is evident in many infections, the migration of these cells to peripheral tissues such as skin, intestines and lungs are limited [112]. Migration of memory T cells depend on the expression of different molecules such as CD45RO, CCR7 and CD62L or specific combination of these molecules [113]. Vaccines that target and enhance mucosal cellular immunity and trafficking of memory cells to mucosal sites will decrease pathology and incidence of mastitis.

Conclusions

In the bovine model, a role for mammary DC in mastitis prevention and during *S. aureus* infection remains to be completely defined. In fact, there are few studies focused on induction of immune responses, to include DC function, T cell responses, and memory development by whole *S. aureus* rather than individual toxins. Though historically, *S. aureus* vaccines focused on antibody responses in the mammary gland have met with limited success, the potential to increase antibody responses in the gland may still translate to mucosal vaccines that reduce incidence of disease. In addition, manipulation of milk antibody type and concentration also can be used to enhance neonatal development of intestinal mucosal barrier and immune function [114]. It is important to remember that antibodies entering milk are generated to protect the young from pathogens in the surrounding environment and that manipulating this system may be more difficult than simply increasing antibody titers in circulation [20, 115]. During infection, or after vaccination, there are only marginal increases of antigen specific antibody's in milk. However, the need to include a cellular response in mucosal vaccination against mastitis that will enhance T cell migration to the gland and potentially improve PMN function is a path that cannot be ignored. A successful vaccine for mammary mucosal sites may well translate to a vaccine that combats *S. aureus* infections at other mucosal sites. A complete understanding of the mechanism associated with immune enhancement is necessary for stimulation of mucosal memory and development of therapeutics for the prevention and/or treatment of *S. aureus*.

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