BOVINE MAMMARY CELLULAR

IMMUNE RESPONSES TO

STAPHYLOCOCCUS AUREUS

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ABSTRACT

Mastitis is a syndrome manifested by mammary gland inflammation which is thought to cause between $300 and $400 million in annual losses to the Canadian Dairy Industry. Studies have indicated that *S. aureus* may cause the production of anti-inflammatory cytokines which may enhance its survival within the bovine mammary gland. However, other studies have reported differing results following *S. aureus* intramammary infection (IMI). This thesis tested the hypothesis that *S. aureus* generated anti-inflammatory cytokine responses at the site of infection. In the first objective, different *S. aureus* isolates were screened for their effects on cytokine production (IFN-γ, TNF-α, IL-4 and IL-10) by bovine peripheral blood mononuclear cells (PBMCs) *in vitro*. Nine *S. aureus* isolates were co-cultured with PBMCs from lactating dairy cattle. Cattle used in the study had recall immune responses to *S. aureus*. The majority (6/9) of *S. aureus* isolates had minor effectors on cytokine production. The three remaining isolates generated large cytokine responses with both pro-inflammatory (IFN-γ and TNF-α) and anti-inflammatory (IL-4 and IL-10) characteristics. Two of these three isolates were tested *in vivo* by experimentally infecting lactating ewes. Cytokine production was characterized in the teat end, the mammary parenchyma and the supramammary lymph nodes (SMLNs). One isolate generated anti-inflammatory responses *in vivo* (IL-4 and IL-10) whilst the other generated both pro-inflammatory (IFN-γ) and anti-inflammatory (IL-10) responses *in vivo*. Given that some studies have suggested a role of staphylococcal enterotoxin C (*sec*) in the generation of anti-inflammatory responses, the role of *sec* was also investigated using bovine PBMCs. When purified SEC protein was co-cultured with PBMCs from beef steers, anti-inflammatory cytokines were produced. However, a *S. aureus* strain which was transformed for the *sec* gene did not affect cytokine production when co-cultured with PBMCs from lactating dairy cattle. The results of this thesis suggest that *S. aureus* infection can cause anti-inflammatory cytokine production but the response depends on the isolate causing the infection. Furthermore, the role of *sec* appears to be minimal.

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LIST OF ABBREVIATIONS

CD	Cluster of differentiation
cDNA	Complementary-strand DNA
cfu	Colony forming unit
CP	Capsular polysaccharide
CpG	Cytosine-guanine rich area of DNA
CTLA	Cytotoxic T-lymphocyte antigen
CXCL	Chemokine C-X-C motif ligand
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
Fc	Fragment crystallisable region of antibody
g	Gravitational force
Gap	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte macrophage colony stimulating factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IMI	Intramammary infection
iNOS	Inducible nitric oxide synthase
LB	Luria-Bertani
MCP	Monocyte chemoattractant protein
MEM	Minimum essential media
MHC	Major histocompatibility complex
ml	millilitres
OVA	Chicken egg ovalbumin

PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pfu	Plaque forming unit
RNA	Ribonucleic acid
ROI	Reactive oxygen intermediate
s	Seconds
SCC	Somatic cell count
SCV	Small colony variant
SDS	Sodium dodecyl sulphate
SEC	Staphylococcal enterotoxin C
SMLN	Supramammary lymph node
SOC	Superoptimal broth with catabolite repression
TAP	Tracheal antimicrobial peptide
TBS	Tris buffered saline
TGF	Transforming growth factor
T_H	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TSA	Tryptic soy agar
TSB	Tryptic soy broth
VIDO	Vaccine and Infectious Disease Organization

1. INTRODUCTION

Staphylococcus aureus is one of the most common mastitis pathogens in Canadian Dairy Cattle. Mastitis as a disease syndrome manifests as inflammation of the mammary gland which causes annual losses of $300 - $400 million to Canadian dairies [1]. A survey of bulk tank milk in Prince Edward Island reported that *S. aureus* was present in 74% of the bulk tanks sampled [2]. A cohort study of 84 cows in Prince Edward Island reported that up to 5.8% of cows had a detectable *S. aureus* intramammary infection (IMI) during lactation [3]. Currently, no vaccine is available which reliably prevents *S. aureus* IMI. Traditional control measures such as dairy cow hygiene, management and antimicrobial treatment do not always reliably prevent IMI with *S. aureus*.

One potential reason for the ineffectiveness of current vaccines is that *S. aureus* IMI induces poor immune responses in the bovine mammary gland. Immune responses can be classified according to whether they promote a pro-inflammatory environment or an anti-inflammatory environment. Pro-inflammatory responses are characterized by the presence of the cytokines IFN-γ and IL-12. Anti-inflammatory responses are characterized by the presence of the cytokines IL-4, IL-10 and TGF-β. Studies have suggested that *S. aureus* induces ineffective immune responses in the mammary gland [4-6]. Specifically, *S. aureus* has been reported to induce anti-inflammatory responses in the gland. Given that *S. aureus* may become intracellular, a pro-inflammatory response would be appropriate to clear *S. aureus* intramammary infection. Other evidence suggests that the anti-inflammatory responses may be mediated by SEC [7, 8]. However, many studies report differing effects of *S. aureus* on mammary cellular immunity. A potential explanation for these observations is that studies use different *S. aureus* which have differing effects on cellular immunity. Furthermore, the majority of studies have evaluated cellular immune responses to *S. aureus* in cells from blood and mammary secretions. Due to the major differences between blood and milk, and the fact that immune effectors in milk are rapidly removed from the mammary gland during the milking process, studies of the site of infection should provide more relevant information about the effect of *S. aureus* on mammary cellular immunity. Finally, no studies have focused on the role of dendritic cells in *S. aureus* intramammary infection. Dendritic cells are of interest as they play a major role in directing immune responses hence may be a major target of *S. aureus* during IMI.

2. HYPOTHESIS AND OBJECTIVES

The hypothesis to be tested is that *S. aureus* IMI induced by wild type isolates from dairy cattle results in the induction of anti-inflammatory responses in the supramammary lymph nodes (SMLNs), mammary parenchyma and mammary teat ends of ewes.

1. Screen bovine mammary *S. aureus* isolates and determine whether they alter cytokine production by bovine peripheral blood mononuclear cells (PBMCs) *in vitro*. The strains that alter cytokine production will be selected for use in the subsequent objectives.
2. Establish a model of ovine intramammary infection with *S. aureus*.
3. Characterize T lymphocyte populations, DC populations and cytokine production in the supramammary lymph nodes, mammary parenchymal tissue and teat ends of ewes following intramammary infection with the selected *S. aureus* strains.

3. LITERATURE REVIEW

3.1 Bovine *Staphylococcus aureus* mastitis

Mastitis is a syndrome manifest by inflammation of the mammary gland. As a syndrome, mastitis is thought to cause $300-$400 million annual losses to the Canadian dairy industry [1]. The major causes of mastitis in dairy cattle are bacterial [9]. Bacterial mastitis pathogens are divided according to their reservoir of infection and manner of transmission. Contagious mastitis pathogens have a reservoir within the mammary gland and are transmitted at the time of milking. Examples of contagious pathogens include *Staphylococcus aureus, Streptococcus agalactiae, Corynebacterium bovis*, *Mycoplasma spp*. and *S. dysgalactiae* [10]. Environmental mastitis pathogens have reservoir in the environment of the cow and are transmitted between milkings. Examples of environmental pathogens include *Escherichia coli* and *S. uberis* [11].

Staphylococcus aureus is considered to be the most common contagious mastitis pathogen [2]. It is a Gram positive, spore-forming, facultative anaerobic coccus [12]. A survey of bulk tank milk in Prince Edward Island reported that *S. aureus* was present in 74% of the bulk tanks sampled [2]. A cohort study of 84 cows in Prince Edward Island reported that up to 5.8% of cows had a detectable *S. aureus* intramammary infection (IMI) during lactation [3]. *Staphylococcus aureus* IMI usually results in subclinical mastitis causing decreased milk production and increased milk somatic cell count (SCC) [13-15]. Milk with low SCC has increased shelf life and greater cheese yield per unit weight therefore a premium is paid for low SCC milk [16, 17].

Given that *S. aureus* has a major detrimental effect on the dairy industry, several treatment and control strategies have been developed. This chapter will discuss these strategies and their efficacy. The host responses to *S. aureus* and the strategies by which *S. aureus* evades these host responses will also be discussed and the need for a better understanding of host responses to *S. aureus* will be explained.

3.2 Treatment of bovine *Staphylococcus aureus* mastitis

3.2.1 Antimicrobial therapy

As *S. aureus* mastitis is a bacterial infection, antimicrobials have been central to treatment strategies. Antimicrobial agents are administered either during lactation or at the beginning of the dry period. The route of administration can be either parenteral or intramammary. Cure rates of *S. aureus* mastitis are highly variable. For example, cure rates as low as 9.1% [18] and as high as 100% [19] have been reported.

Several host, pathogen and treatment factors have been associated with cure rates during lactation [20, 21]. The major risk factors were all host factors including a lactation number greater than two, SCC greater than 10^6 cells/ml, infection in the hind quarters and stage of lactation greater than 200 days. Antimicrobial resistance is a pathogen factor which may decrease the cure rate of *S. aureus* mastitis during lactation. A study which assessed parenteral and local treatment of *S. aureus* IMI with procaine penicillin G or clavulanic acid potentiated amoxicillin reported that cure rates of penicillin-resistant *S. aureus* strains were significantly lower than those of penicillin-susceptible strains regardless of route of administration or antimicrobial used [22].

Dry cow therapy is considered to be a major part of mastitis control programs [23]. Sol and coworkers studied the association of host factors with bacteriologic cure of *S. aureus* following dry cow treatment [24]. The major risk factors were high SCC, increased age of cattle and infections in multiple quarters. Generally cure rates for antimicrobial therapy at dry off range from 50-80% [25]. Cows harbouring *S. aureus* that remain in the herd during the dry period represent a reservoir for infection during the next lactation.

Whilst all these factors are important in predicting whether antimicrobial treatment may be effective, it is important to consider that antimicrobial treatment is a major part of the "gold standard" mastitis control program [23]. However, the evidence discussed shows that antimicrobial treatment does not completely cure *S. aureus* mastitis. This may be due to the increasing risk of *S. aureus* mastitis with parity and stage of lactation [26]. Animals that are

more likely to possess chronic infections are less likely to be cured and act as a reservoir in the herd. This realization has led a search for alternative treatments.

3.2.2 Other proposed treatments

The use of antimicrobial therapy in combination with vaccines is a relatively recent development. A small study showed no enhancement of *S. aureus* cure rates when a *S. aureus* bacterin was used alongside extended pirlimycin therapy compared with extended pirlimycin therapy alone [27]. A larger study showed significantly increased cure rates in animals treated with the same *S. aureus* bacterin alongside extended pirlimycin therapy [28]. Unfortunately, this study used untreated animals as a control group rather than pirlimycin treated animals therefore the effect of the bacterin cannot be assessed. Larger scale, better designed studies are required before conclusions can be drawn.

Bacteriophages have been the subject of investigation as an alternative treatment to many bacterial diseases [29]. Bacteriophages isolated from farmyard slurry [30] and from sewage entering a water treatment plant [31] are able to lyse *S. aureus* in vitro. Lytic activity has been attributed to the endolysin gene [32]. Administration of bacteriophages to healthy animals has varying effects with one study reporting no increase in SCC following administration [30] and a different study reporting large increases in SCC following administration [33]. Bacteriophages have not been demonstrated to clear *S. aureus* mastitis. Administration of 10^{11} pfu bacteriophage K from *S. aureus* strain ATCC 19685 did not result in a significant increase in *S. aureus* mastitis cure rates when compared with saline treatment [33]. A further study reported that bacteriophage K was unable to replicate in raw milk [34]. Adsorption of milk whey proteins to the surface of *S. aureus* inhibits lytic infection with bacteriophage K [29]. If bacteriophage therapy is to be of use in the treatment of bovine mastitis, these problems will have to be overcome whilst ensuring that the treatment is affordable to the producer.

Bacteriocins are short peptide antimicrobials which are produced by bacteria [35]. Early studies reported that nisin can inhibit *S. aureus* growth *in vitro* [35] and reduce *S. aureus* concentrations on teat ends [36]. Treatment of subclinical mastitis with nisin, a product of *Lactococcus lactis* [37], resulted in a 50% cure rate which was significantly greater than untreated controls [38].

This study did not compare nisin with conventional antimicrobials. Lacticin 3147, which is also produced by *Lactococcus lactis*, was incorporated into a bismuth-based teat seal and reduced shedding of *S. aureus* both before and after *S. aureus* challenge [39]. Whilst this therapy shows promise, cure rates were not determined therefore a comparison of the effects of lacticin with conventional therapies cannot be made. Further studies should focus on cure rates and comparisons with conventional antimicrobial therapy.

Lactoferrin is an iron binding protein primarily occurring in neutrophil granules [40]. It appears to have a variable effect on *S. aureus* in either milk whey or culture broth [40, 41]. These results may be strain-dependent [40]. Due to the variable effect of lactoferrin when used in isolation, studies have evaluated the ability of lactoferrin to enhance cure rates of antimicrobial therapy. A combination of lactoferrin and penicillin G significantly increased chronic *S. aureus* mastitis cure rates 15 days after intramammary infusion of *S. aureus* in late lactation cows when compared with no treatment, lactoferrin alone or penicillin G alone. Cure rates for all treated groups were not significantly higher in animals that were infected in late lactation then treated following parturition [42]. Further studies on naturally occurring *S. aureus* infections need to be performed before lactoferrin can be recommended as a treatment option.

Whilst cows transgenic for antimicrobial agents have been developed [43], this is a preventive rather than treatment strategy therefore will not be considered here. Attempts have been made to transfer the lysostaphin gene into the mammary gland. Lysostaphin is a protein that is effective at lysing *S. aureus* and can be transferred into the goat mammary gland by a human adenoviral vector [44]. Unfortunately, a strong immune response occurred to both the adenovirus and lysostaphin [44]. Use of eukaryotic expression vectors may overcome this problem [45]. This method allowed for the expression of the antimicrobial peptides bovine lactoferricin and bovine tracheal antimicrobial peptide in goats for up to 6 days following intramammary infusion with a vector encoding these peptides [45]. Genomic therapies appear promising but efficacy has not been determined in bovine mammary glands and the potential cost to the producer may be prohibitive and may lead to the selection of resistant *S. aureus* strains.

Cytokines have been studied as a potential therapy for *S. aureus* mastitis due to the ability of IL-1β pretreatment to enhance killing of *S. aureus* by neutrophils *in vitro* [46] and the ability of TNF-α pretreatment to enhance killing of *S. aureus* in the mouse mammary gland [47]. Use of recombinant bovine IL-2 or IL-1β resulted in cure rates for chronic *S. aureus* mastitis in early to mid-lactation cattle of 52% and 75% respectively [48]. Recombinant bovine IL-2 has also been studied as an adjunct to conventional antimicrobial therapy. Cure rates following administration of IL-2, cephapirin, and IL-2 combined with cephapirin were 31%, 42% and 85%, respectively in early to mid-lactation cattle [49]. Combination therapy with IL-2 and cephapirin significantly increased cure rates compared with IL-2 therapy alone. A further study demonstrated significantly decreased bacterial shedding from mammary glands following addition of IL-2 to cephapirin although cure rates were not reported [50].

Egg yolk immunoglobulin (IgY) specific for bovine *S. aureus* has been reported to enhance phagocytosis of *S. aureus* by macrophages [51]. A comparison of IgY and penicillin in the treatment of clinical or experimental *S. aureus* mastitis showed that IgY performed significantly better than penicillin with cure rates for clinical and experimental mastitis being 50% and 83.3%, respectively [52]. This therapy needs to be tested in subclinical, chronic mastitis. A 50% cure rate of clinical mastitis remains insufficient to eliminate *S. aureus* IMI from the herd.

3.3 Control of bovine *Staphylococcus aureus* mastitis

3.3.1 Management-based control measures

Management-based control measures include milking hygiene, post-milking teat disinfection, culling of chronically infected cattle and appropriate use of antibiotic therapy. The use of antibiotic therapy has already been discussed, therefore milking hygiene and culling of chronically infected cattle will be focused on here.

Neave and co-workers [53] studied hygiene programs for mastitis control in 15 dairy herds. Two levels of hygiene were studied, full and partial. Full hygiene consisted of single use udder towels for cleaning the udder before milking, wearing of rubber gloves by milkers, sterilization of teat cups before milking each cow and post-milking teat disinfection. Partial hygiene consisted of all

the above measures with the exception of teat cup pasteurization. Full hygiene reduced the incidence of new *S. aureus* IMI over an 18-month period by 62% and partial hygiene reduced the incidence of new *S. aureus* IMI by 55% when both were compared with no hygiene at the time of milking. Additional control procedures were studied in a herd practicing full hygiene that had suffered an outbreak of *S. aureus* mastitis [54, 55]. These included segregation of cattle with *S. aureus* IMI, intensified culling of animals harboring *S. aureus* IMI, and permanently ceasing lactation in *S. aureus* infected quarters of cattle with *S. aureus* IMI in a single mammary quarter. These practices in combination with full hygiene reduced the incidence of *S. aureus* IMI from 3.4 cases/100 cow months to 0.35 cases/100 cow months and reduced *S. aureus* IMI prevalence from 22% to 8%. A similar study in nine Italian dairy herds [56] which instituted single-use cloth towels to prepare teats before milking, practiced forestripping before milking unit attachment, used a post-milking germicidal teat disinfectant, segregated *S. aureus* infected cattle from the rest of the herd, milked these cattle last, and used blanket dry cow antibiotic therapy. Using these practices, there was a reduction in the incidence of *S. aureus* IMI from as many as 40 cases/100 cow-months to $\leq$ 1 case/100 cow-months.

Given that the reservoir of *S. aureus* IMI is the mammary gland of infected cattle, addition of infected cattle into a herd would clearly increase the risk of *S. aureus* IMI in a dairy herd. Screening of replacement animals has been proposed [57].

From this evidence, it appears that a significant degree of control of *S. aureus* mastitis can be achieved at a management level. However, given the continued prevalence of the disease in the dairy industry, additional control measures are required. Given that vaccination has been used to control many veterinary infectious diseases, many attempts have been made to vaccinate cattle against *S. aureus* IMI.

3.3.2 Vaccination

Attempts to vaccinate against *S. aureus* are summarized in table 3.1. The earliest commercial attempt at vaccination was made in 1966 [58]. The vaccine contained whole cell lysates of five *S. aureus* strains in an aluminum hydroxide adjuvant. The vaccine was studied in cows with naturally-occurring *S. aureus* IMI in three dairy herds over a six-month period. Following

Table 3.1. Summary of previous *S. aureus* vaccine studies

Reference	[58]	[59]	[60]	[61]	[62]	[64]
Year	1966	1975	1985	2006	1975	1980
Vaccine type	Bacterin	Bacterin	Bacterin	Bacterin	Bacterin	Bacterin
Trial type	Field	Chall.	Chall.	Chall.	Chall.	Chall.
Serum antibody	↑IgG	N/E	N/E	N/E	NC	NC
Milk antibody	↑IgG	N/E	N/E	N/E	NC	↑IgA/M/G_2
Effect on new IMI	↓	↓	NC	NC	↓	NC

Reference	[66]	[67]	[68]	[69]	[71]	[74]
Year	1984	2003	1994	2005	1997	2004
Vaccine type	Live	Protein A	CP5	CP5/8/336	CP5/α-toxin	FnBP
Trial type	Chall.	N/A	N/A	N/A	N/A	Chall.
Serum antibody	↑$IgG_1/_2$	↑IgG	↑IgG_2	↑$IgG_1/_2$	↑IgG	N/E
Milk antibody	N/E	N/E	N/E	N/E	↑IgG	N/E
Effect on new IMI	↓	N/A	N/A	N/A	N/A	↓

Reference	[75]	[76]	[77]	[78]	[84]
Year Published	1992	1993	1996	1994	2006
Vaccine type	Bacterin	Bacterin	Bacterin	Bacterin	GapC/B
Trial type	Chall.	Chall.	Field	Field	N/A
Serum antibody	↑IgG_2	↑$IgG_1/_2$	N/E	N/E	↑IgG
Milk antibody	↑IgG_2	N/E	N/E	↑IgG_2	↑IgG
Effect on IMI	NC	↓	↓	↓	N/A

Reference	[85]	[87]	[88]	[90]	[91]
Year Published	2008	1997	1997	2003	2003
Vaccine type	SEC	Bacterin	Bacterin	Bacterin	Bacterin
Trial type	Chall.	Field	Field	Chall.	Field
Serum antibody	N/E	N/E	N/E	↑IgG	N/E
Milk antibody	N/E	N/E	N/E	↑IgG	N/E
Effect on IMI	↓	↓	↓	↓	NC

Chall. Denotes challenge trial
N/A denotes not applicable
N/E denotes not evaluated
NC denoted unchanged

vaccination, significantly fewer vaccinates than controls developed a *S. aureus* IMI. Vaccinates also had increased serum and milk whey total immunoglobulin titers against staphylococci thirty days after the second vaccination. This vaccine is currently marketed as Lysigin (Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO, USA). Lysigin was also tested using an experimental intramammary challenge model [59]. Vaccinated cattle showed a reduced incidence of *S. aureus* IMI, reduced clinical signs and reduced SCC after challenge. However, the challenge model used in this study was inconsistent between cattle, making analysis of the results problematic. Pankey and co-workers [60] also tested Lysigin using an experimental challenge model. Cattle were challenged by immersing teat ends in a suspension of *S. aureus* (ATCC 29740) immediately after milking five days per week for six months during lactation. These procedures were repeated in the same animals for six-month periods during three consecutive lactations. The incidence of *S. aureus* infected mammary quarters immediately after challenge did not vary between groups although vaccinates had a significantly decreased incidence of chronically infected quarters, increased spontaneous quarter cure rate and decreased SCC when compared with non-vaccinated controls. The spontaneous cure rate for animals vaccinated with Lysigin was 73% compared with 47% for control animals. The efficacy of different vaccine formulations (2 experimental formulations and commercially available Lysigin) in the prevention of *S. aureus* IMI in dairy heifers was evaluated using an intramammary challenge model [61]. Cattle vaccinated with commercially available Lysigin had a significantly lower mean duration of clinical mastitis and lower total mastitis score post-challenge than controls. There was no evidence of a difference between any vaccinated group and controls with regard to *S. aureus* clearance rates post-challenge.

Given the inability of Lysigin to consistently protect against *S. aureus* IMI, several other vaccination strategies have been attempted. Vaccination with a killed vaccine made up of one of three *S. aureus* strains (Mexi, BB and 3528) did not increase IgG_1, IgG_2, IgM or IgA titers in serum or milk [62]. Cattle vaccinated with strain 3528 were resistant to infection with the low-virulence (based on clinical severity of disease) strain Mexi but were not resistant to the high virulence strain BB. The authors concluded that vaccination was unlikely to prevent infection of the bovine mammary gland by *S. aureus*. A different heat-killed *S. aureus* bacterin containing Freund's incomplete adjuvant did not alter bacterial growth rates in milk from vaccinates when

compared with controls [63]. Guidry and co-workers [64] attempted local immunization with heat-killed *S. aureus* in four late lactation dairy cows. The vaccine was administered to two quarters of each cow. One immunized and one control quarter per cow was challenged with *S. aureus.* All but one challenge quarter became infected although significant increases in IgA, IgM and IgG_2 were detected in immunized quarters when compared with control quarters. These immunoglobulins were able to opsonize *S. aureus* for phagocytosis by neutrophils.

Live vaccines have been used as another strategy to immunize cattle against *S. aureus* IMI. A live attenuated vaccine should stimulate cell-mediated immunity as well as humoral immunity. Due to the ability of *S. aureus* to become intracellular [65], a vaccine that stimulates cell mediated immunity may have increased efficacy in clearing *S. aureus* IMI. Watson [66] tested a live attenuated *S. aureus* vaccine using dairy heifers in an experimental challenge model. Animals were vaccinated subcutaneously at eight and five weeks prepartum. Vaccinates had increased IgG_1 and IgG_2 titers in serum following the first vaccination and immediately prior to challenge. No vaccinates shed *S. aureus* in the milk postchallenge whereas two control animals shed *S. aureus* in the milk 24 hours postchallenge. One of the control heifers remained infected for the duration of the study. Milk production in controls decreased after challenge whereas milk production in vaccinates remained unchanged. Although this vaccine appears promising, the study was carried out in only 7 animals so few conclusions can be drawn.

Several studies have focused on immunization against *S. aureus* virulence factors. Vaccination using protein A has been studied [67]. Three groups of five animals were studied: 1) unvaccinated controls, 2) immunization with plasmids containing green fluorescent protein (GFP) and protein A and a saline adjuvant, and 3) immunization with plasmids containing GFP and protein A in an aluminum phosphate adjuvant. Cattle vaccinated with either vaccine had significantly higher serum IgG levels against protein A than unvaccinated controls. There was no significant difference between the two vaccinated groups and controls. Six of the 15 animals (3 vaccinates and 3 controls) received a booster of protein A five months into lactation. Both vaccinates and controls had a serum IgG response to the booster and the difference between groups was not significant. Neither the effect of the vaccine on mammary immunity nor the ability of the vaccine to prevent *S. aureus* IMI was studied.

Vaccination with either purified *S. aureus* capsular polysaccharide 5 (CP5) or purified CP5 conjugated to ovalbumin in Freund's incomplete adjuvant has been attempted [68]. Purified CP5 alone did not elicit an antibody response in serum. Vaccination with a CP5-ovalbumin conjugate elicited an IgG_2 response that lasted for 4 weeks after vaccination. Cattle vaccinated with the CP5-ovalbumin conjugate received a booster of the conjugate 3 months after first vaccination that resulted in an IgG_2 response in serum of greater magnitude than after the first vaccination. Milk antibody responses and the ability of the vaccine to prevent *S. aureus* IMI were not studied. A recent study into CP used a trivalent vaccine consisting of three *S. aureus* strains expressing either CPs 5, 8 or 336 [69]. A survey of *S. aureus* isolates from bovine mastitis cases reported that 100% of isolates from the USA and 98% of isolates from Europe expressed either CP5, 8 or 336 [70]. Several formulations were studied: 1) trivalent vaccine alone, 2) trivalent vaccine precipitated in Freund's incomplete adjuvant, 3) trivalent vaccine emulsified in aluminum hydroxide or 4) Freund's incomplete adjuvant alone. When compared with Freund's incomplete adjuvant alone, animals from the other groups had increased serum IgG_1 and IgG_2 against CPs 5, 8 and 336 following vaccination. Furthermore, vaccinated cattle had increased percentages of total CD4+ and CD8+ lymphocytes in peripheral blood following vaccination when compared with animals administered Freund's incomplete adjuvant alone. Unfortunately, the ability of this vaccine to prevent *S. aureus* IMI was not tested.

A different approach was to use staphylococcal α-toxin in conjunction with CP5 [71]. Three vaccines were evaluated in 10 cows: 1) unconjugated α-toxin with Freund's incomplete adjuvant (n = 2), 2) unconjugated α-toxin and unconjugated CP5 with Freund's incomplete adjuvant (n = 2) or 3) CP5-alpha toxin conjugate with Freund's incomplete adjuvant (n = 2). Controls received Freund's incomplete adjuvant alone (n = 4). All vaccinated cattle had increased serum and milk anti-α-toxin antibody titers in response to vaccination. No inflammatory cell recruitment into the mammary gland occurred in cows vaccinated with the CP5-alpha toxin conjugate after experimental intramammary challenge with α-toxin. A different study [72] immunized cattle with *S. aureus* α-toxin alone. When challenged with α-toxin, immunized cattle exhibited an early and large-scale recruitment of neutrophils as well as CD8+ and CD4+ lymphocytes into the mammary gland. However, no data was presented regarding protection against *S. aureus* IMI in this study.

Attempts have been made to vaccinate cattle against fibronectin-binding protein (FnBP). Mamo and co-workers [73] evaluated four vaccines: 1) FnBP conjugated to two IgG binding domains of protein A (ZZ-FnBP), 2) FnBP conjugated to β-galactosidase (Gal-FnBP), 3) FnBP conjugated to 2 albumin binding domains of protein G (BB-FnBP) and 4) ZZ-FnBP-α-toxin conjugate. The vaccines were evaluated in a murine experimental intramammary challenge model. All vaccinated mice had significantly reduced severity of clinical mastitis after challenge when compared with unvaccinated controls. Animals vaccinated with ZZ-FnBP had significantly reduced shedding of *S. aureus* from infected glands after challenge. All vaccinates also had increased IgG levels against FnBP after challenge but the data were not statistically analyzed. Shkreta and coworkers [74] tested a DNA vaccine encoding FnBP and clumping factor A (ClfA), a putative *S. aureus* virulence factor. Four heifers in their seventh month of gestation were vaccinated twice with the DNA vaccine followed by a booster with recombinant FnBP and ClfA. Four heifers served as unvaccinated controls. Three weeks after calving, three mammary quarters of each study animal were challenged with 900 cfu of *S. aureus* (Newbould 305 strain). Vaccinates showed decreased serum haptoglobin levels (an acute phase protein), heart rate and body temperature between 24 and 72 hours after challenge when compared with control. Also, vaccinates had significantly fewer *S. aureus* infected mammary quarters 21 days after challenge. A bacterin containing killed *S. aureus* cultured under conditions promoting formation of a pseudocapsule was combined with a staphylococcal toxoid and a mineral oil and dextran adjuvant. The vaccine was tested in Friesian and Australian Illawarra Shorthorn heifers [75]. The dextran adjuvant was included to induce an IgG_2 response. Infection rates after experimental intramammary challenge were not significantly different between vaccinates and controls for all experiments. However, if the vaccine was administered at 21 and 35 days postpartum, vaccinates had significantly increased milk production during the 25 days after challenge compared with controls. Also, if the vaccine was administered once two months before calving with a booster dose three months later, vaccinates showed significantly increased serum and milk IgG_2 against *S. aureus* following the booster vaccination and before challenge. Vaccinates immunized once two months before calving with a booster dose three months later also had significantly increased milk production during the 21 days after challenge when compared with controls. Nickerson and co-workers [76] evaluated this vaccine in primiparous Jersey heifers (n = 12). Vaccinates received the vaccine either intramuscularly (n = 4) or in the

region of the SMLN (n = 4). Vaccinates had significantly increased IgG, IgG_1 and IgG_2 against *S. aureus* in serum following vaccination compared with controls. However, titers were not presented for cattle that were vaccinated in either site. Following experimental intramammary challenge with *S. aureus*, 11/12 challenged quarters in control cows, 4/11 challenged quarters in cows vaccinated intramuscularly and 6/10 challenged quarters in cows vaccinated in the region of the SMLN became infected with *S. aureus*. Finally, Watson and coworkers [77] evaluated this vaccine in seven commercial dairy herds in Australia. Cattle were vaccinated at the beginning of the third trimester of pregnancy (heifers) and at dry off (lactating cows). The only significant difference was in the herd with the highest prevalence of clinical *S. aureus* mastitis (37.7%). In this herd, the cumulative prevalence of *S. aureus* IMI during the seven months after vaccination was significantly lower for vaccinates.

Nordhaug and co-workers [78, 79] developed a bacterin consisting of whole killed *S. aureus* with pseudocapsule and α and β toxoids in a mineral oil adjuvant. In a field study, Holstein cattle were vaccinated (n = 58) at 8 and 2 weeks prior to calving and compared with unvaccinated controls (n = 50). The relative risk of *S. aureus* IMI in the subsequent lactation was significantly lower for vaccinates than controls and vaccinated cattle had an 8.6% prevalence of *S. aureus* IMI during the subsequent lactation compared with 16.0% for controls. Vaccinates had significantly higher IgG_2 in milk against pseudocapsule during the 120 days after second vaccination. Anti-α-toxin IgG in milk was significantly higher for vaccinates than controls throughout lactation. Anti-β-toxin IgG in milk was only significantly higher for vaccinates compared with controls during late lactation.

Attempts have been made to vaccinate cattle against the GapC and GapB surface proteins of *S. aureus*. These proteins have a conserved structure among multiple bovine mammary *S. aureus* isolates [80]. Studies of streptococcal mastitis have revealed that immunization with recombinant GapC from *Streptococcus uberis* decreased the severity of streptococcal mastitis following experimental *S. uberis* challenge [81]. Similar results were observed when animals were immunized with recombinant GapC from *Streptococcus dysgalactiae* and challenged with *S. dysgalactiae* [82]. Vaccination of mice with a GapC/B chimera resulted in strong humoral and cell mediated immune responses in blood [83]. An attempt was made to immunize cattle

with a DNA vaccine encoding the bovine GapC/B chimera or with purified recombinant GapC/B protein with or without a boost with recombinant protein [84]. The DNA vaccines alone were unable to generate significant humoral or cell mediated immune responses against the recombinant antigens but significant responses were observed in animals either immunized with recombinant protein or immunized with DNA and boosted with recombinant proteins.

An attempt has been made to immunize cattle against *S. aureus* by immunizing against SEC. Cattle were vaccinated with a *S. aureus* bacterin containing a SEC mutant protein and then subjected to experimental intramammary challenge with a SEC secreting *S. aureus* strain. Vaccinated cattle did not develop *S. aureus* IMI whereas 75% of unvaccinated controls developed *S. aureus* IMI [85]. Given that 10% of bovine mammary *S. aureus* isolates express SEC [86], this vaccine may not provide broad-based protection against *S. aureus*.

A multivalent mastitis vaccine has been evaluated in heifers in Argentina [87]. The vaccine was comprised of four different *S. aureus* strains, one *S. agalactiae* strain and one *S. uberis* strain in an aluminum hydroxide adjuvant. Vaccinates were immunized at 8 and 4 weeks pre-calving or at 1 and 5 weeks post-calving and controls received a placebo at 8 and 4 weeks pre-calving. Cumulative incidence of *S. aureus* quarter infection in the pre-calving and post-calving vaccinated groups were 32.5% and 27.5%, respectively, compared with 75% in the control group ($P < 0.0001$). A further study [88] evaluated the same vaccine in two commercial Argentine dairy herds consisting of 90 cows with a 16% prevalence of *S. aureus* IMI (herd A) and 85 cows with a 15% prevalence of *S. aureus* IMI (herd B). Vaccinates (dairy A, $n = 42$; dairy B, $n = 40$) received the vaccine twice four weeks apart. During the four months after vaccination, the prevalence of *S. aureus* IMI in herd A was 9.9% for vaccinates and 15.7% for controls ($P < 0.02$). The results for herd B were 4.9% for vaccinates and 8.0% for controls ($P < 0.03$). Animals that were culled from the herds during the study were not considered in the calculation of *S. aureus* IMI prevalence. Hence, this vaccine significantly reduced the prevalence of *S. aureus* IMI within these dairy herds.

Recently, a commercial vaccine has been developed in Israel (Mastivac, National Mastitis Reference Center, Bet Dagan, Israel). This vaccine consists of components of three bovine

mammary *S. aureus* strains (VLVL8407, ZO3984 and BS449) in Freund's incomplete adjuvant [89]. Leitner and co-workers [90] tested this vaccine in an experimental bovine intramammary challenge trial using cattle from two herds. Nineteen non-pregnant first and second lactation Holstein cattle free of *S. aureus* IMI were enrolled. In the first herd, five cattle received two doses of Mastivac 37 days apart and five cattle served as controls. Cattle enrolled in the study had no detectable antibodies to *S. aureus*. All cattle were challenged with 1,000 cfu *S. aureus* (VLVL8409) by intramammary infusion into two mammary quarters 28 days after the second vaccination. In the second herd four cattle were vaccinated with Mastivac on days 0, 36 and 56 of the trial and five cattle served as controls. Cattle enrolled in the study had no detectable serum antibodies against *S. aureus*. All cattle in the second trial were challenged with 1,000 cfu *S. aureus* (VLVL8409) by intramammary infusion in two mammary quarters 21 days after the final vaccination. Vaccinates in both herds had a significantly decreased incidence of *S. aureus* infected mammary quarters 2 weeks after challenge and increased IgG titers against *S. aureus* in both serum and milk after vaccination when compared with controls. However, the authors did not report cow cure rates nor did they evaluate different IgG subclass titers against *S. aureus* in milk and serum. A further study [91] evaluated Mastivac in a field setting involving seven herds and 452 pre-partum heifers and found that 3 of 228 vaccinates developed *S. aureus* IMI during the subsequent lactation whereas 6 of 224 unvaccinated controls developed *S. aureus* IMI during the same period. This change was not statistically significant. A better model may have been to study the vaccine in a herd with a high incidence of new *S. aureus* IMI.

A commercial combination *S. aureus* and *E. coli* bacterin has been developed in Spain (Startvac, Laboratorias Hipra, S.A., Girona, Spain). This vaccine does not prevent IMI following experimental intramammary challenge with *S. aureus*, but does decrease bacterial shedding following challenge [92]. Studies into this vaccine under field conditions would establish whether it may be a useful tool in the control of *S. aureus* mastitis.

Despite many attempts at vaccination, an effective vaccine against *S. aureus* has not been developed. An effective vaccine is one which prevents *S. aureus* IMI, prevents disease as a result of *S. aureus* IMI or prevents shedding of *S. aureus* from an infected animal. It has been argued that phagocytosis of *S. aureus* by neutrophils is central to an effective immune response

[93]. Three stages are involved: neutrophil recruitment [94], opsonisation of *S. aureus* by immunoglobulins and complement [95] and ingestion and killing of *S. aureus*. One might therefore assume that developing a strategy to enhance each of these stages would ensure prevention of *S. aureus* IMI. Several disadvantages to this approach exist. Bulk milk SCC is used as a monitor of udder health and milk quality [16]. Increases in SCC result in producers incurring financial penalties. An increase in neutrophil recruitment would inevitably lead to increased SCC. Opsonization of *S. aureus* is inefficient in the mammary gland. In normal milk, IgG_1 predominates due to active transport into the mammary gland but the bovine neutrophil does not possess an IgG_1 receptor [96]. Other antibody isotypes only enter the gland in higher amounts when the blood-milk barrier breaks down, such as in cases of mastitis [93]. Neutrophils in milk are less able to phagocytose and kill pathogens than those in serum due to large numbers of fat globules [97]. Given the lack of an effective *S. aureus* vaccine, several novel control measures have been proposed.

3.3.3 Alternate control measures

Therapeutic cessation of lactation is a method by which a mammary quarter is chemically treated to prevent any further milk production and bacterial shedding from that quarter. If a *S. aureus* infected quarter is no longer lactating, it is no longer a reservoir of infection in the dairy herd. Chlorhexidine and povidone iodine were compared in a study of mid- to late lactation cattle with single quarter *S. aureus* IMI [98]. Lactation ceased permanently in all seven of the quarters treated with povidone iodine whereas five of the seven quarters treated with chlorhexidine produced milk during the subsequent lactation. A subsequent study [99] reported that chlorhexidine is able to be recovered from milk up to 42 days following treatment and was therefore not considered a practical treatment. No study on the elimination kinetics of povidone iodine was performed.

Staphylococcus aureus may enter the herd in dairy heifers that have not been milked [100]. The source of these infections may be flies since herds that practise fly control had a dramatically lower prevalence of *S. aureus* IMI than those which did not [100]. Owens and coworkers [101] reported that horn flies (*Haematobia irritans*) were able to become colonized with *S. aureus* and remain colonized for 96 hours following exposure to teats of dairy heifers with *S. aureus* IMI.

Given that heifers harbouring a *S. aureus* IMI may have *S. aureus* present at other body sites [102], fly control may be a method of reducing *S. aureus* IMI in the dairy herd.

Intramammary devices are coiled polyethylene devices that are inserted into the lactiferous sinus of the mammary gland [103]. The presence of an intramammary device significantly reduced the incidence of *S. aureus* IMI but increased milk SCC following experimental intramammary challenge in one study [103]. A different study [104] reported that, if the quarter SCC following administration of the intramammary device was less than 10^6/ml, *S. aureus* IMI was not prevented but if the quarter SCC was greater than 10^6, only 17% of challenged quarters developed *S. aureus* IMI. However, given that producers are paid premiums for milk of low SCC, the intramammary device has little potential as a control measure in a commercial situation.

Attempts have been made to prevent *S. aureus* IMI by generating animals that are transgenic for lysostaphin. The initial work focused on generating mice that secreted an active form of lysostaphin into milk. Lysostaphin is normally produced by *S. simulans* and becomes glycosylated when produced by eukaryotic cells. Therefore, a bioactive lysostaphin variant (Gln (125,232)-lysostaphin) was generated, was active in milk and did not affect milk protein [105]. Jersey cattle transgenic for lysostaphin were generated and were significantly more resistant to infection than non-transgenic animals [43]. Milk protein was unaffected by the presence of lysostaphin.

3.4 Bovine mammary responses to *Staphylococcus aureus*

3.4.1 Physical barriers to infection

The first physical barrier that a mammary pathogen will encounter is the teat canal. Given that the teat canal may be colonized by a number of different organisms [106], the ability of the teat canal to prevent entry of bacteria into the mammary parenchyma appears critical in preventing *S. aureus* IMI. Opening of the teat canal before calving has been reported as an important development of mastitis [107]. Closure of the teat canal when the animal is not milking is maintained by a muscular sphincter and a keratin plug [108]. Increased milk flow rate through the teat canal and decreased teat canal length are associated with increased risk of IMI [109].

Damage to the teat canal orifice increases the risk of IMI with *S. aureus* [110]. If cows were milked without pulsation, the risk of IMI was significantly increased as was teat canal diameter [111]. Teat canal keratin contains myristic, palmitoleic, and linoleic acids that inhibit bacterial colonization of the teat canal [112]. The importance of these fatty acids was demonstrated by Capuco and coworkers [113] who demonstrated increased susceptibility of lactating Jersey cows to IMI with the contagious pathogen *S. agalactiae* when teat canal keratin was removed.

3.4.2 Innate immune responses

Complement is a major humoral innate factor that is involved in the innate immune responses to pathogens. The complement system consists of two pathways, classical and alternative. Studies on *S. agalactiae* have revealed that the alternative pathway predominates in the mammary gland whilst the classical pathway is not active due to the absence of C1q [114]. The alternative pathway is dependent on a constitutive level of C3 hydrolysis which provides C3b which is able to bind the surface of pathogens and subsequently complex with factors B and D to form a C3 convertase. This leads to a positive feedback loop and increased C3 hydrolysis. The level of C3 in milk is approximately 2.5% that of serum [114]. However, C3 does not appear to enhance the opsonisation and phagocytosis of *S. aureus* by neutrophils [115]. The complement factor C5a is involved in chemotaxis of neutrophils to the site of inflammation [94]. However, IMI with *S. aureus* results in low levels of C5a in the mammary gland [116], limiting the migration of neutrophils into the gland. Based on this evidence, complement alone does not appear to be important in bovine mammary responses to *S. aureus*.

Lactoferrin has already been discussed as a novel treatment strategy for *S. aureus*. Increased expression of the lactoferrin gene from bovine mammary epithelial cells is associated with a decreased susceptibility to mastitis [117]. Lactoferrin expression is increased 24 hours after IMI with *S. aureus* [118] with lactoferrin concentration appearing to correlate with milk SCC [119]. The susceptibility of *S. aureus* to lactoferrin is strain dependent therefore lactoferrin may be effective in some cases but not in others [41, 120]. The interaction between *S. aureus* and lactoferrin is dependent on specific cell surface receptors. Given that a subset of *S. aureus* isolates do not express lactoferrin receptors [121], the strain dependent effects may be due to the presence or absence of these receptors. Lactoferrin may also affect mammary immune responses

through the augmentation of other immune processes. Specifically, lactoferrin appears to permit the deposition of C3 on the surface of *S. aureus* and enhances subsequent complement-mediated killing [122].

Transferrin is another iron-binding protein which is present in milk in low concentrations although the concentration is higher in colostrum or in mastitic milk which suggests transudation of transferrin from blood into the mammary gland [123]. This is supported by evidence which shows that transferrin is not synthesized in the mammary gland of sheep whereas it is synthesized in the mammary glands of other nonruminant species [124]. Given the lack of synthesis in sheep, it is likely that transferrin is not synthesized in the bovine mammary gland. Also, the low transferrin concentrations suggest that it is not an important defence against *S. aureus*.

Lysozyme is a bactericidal peptide which targets bacterial cell wall peptidoglycans. It is able to potentiate the ability of lactoferrin to kill *S. aureus* [125]. In sheep, high milk lactoferrin and lysozyme concentrations are associated with decreased recovery of *S. aureus* from the mammary gland following experimental IMI [126]. In cattle, lysozyme levels appear to drop during *S. aureus* IMI [127] suggesting that it can be overwhelmed in the face of an active IMI.

Natural antibodies are produced in response to normal environmental antigens rather than specifically against foreign pathogens [128]. Natural antibodies against melibiose are present in cattle and appear able to react with the contagious mastitis pathogen *S. agalactiae* [129] suggesting that they may have a protective role in bovine mastitis. Unfortunately, the ability of these antibodies to recognize *S. aureus* has not been investigated.

Toll-like receptors are a class of receptors that recognize pathogen-associated molecular patterns (PAMPs). They appear to have a threefold role. They allow for specific recognition of pathogens by the innate immune system, they act as a bridge between innate and adaptive immunity and they may provide a mechanism for pathogens to avoid protective host responses [130]. Toll-like receptors that are involved in the recognition of *S. aureus* include TLR2 which binds bacterial peptidoglycan and TLR9 which binds bacterial CpG DNA [108]. The roles of

TLRs as a bridge between innate and adaptive immunity as well as the mechanisms by which they may be used by pathogens to avoid protective host responses will be discussed in the section on adaptive immunity.

Few neutrophils are present in the uninfected mammary gland [131]. Neutrophils traffic to the mammary gland in response to cytokines released by macrophages and T lymphocytes [94]. Neutrophils exert antibacterial effects through phagocytosis, production of superoxide radicals [132] and small antimicrobial peptides called defensins [133]. Mammary neutrophils have impaired phagocytic and pathogen killing ability when compared with those in blood [134] due to the presence of large amounts of fat and casein and low amounts of glucose in milk [97, 135]. Despite all of these factors, the ability of neutrophils in the mammary gland to prevent *S. aureus* IMI has been discussed. However, the presence of large numbers of neutrophils in the gland does increase milk SCC and is therefore unacceptable to the producer.

Macrophages are the major cell type in the noninfected or nonlactating mammary gland [136]. They are phagocytic cells which can ingest and kill pathogens although their phagocytic activity is decreased in mammary gland compared to blood [137] and is decreased compared to mammary neutrophils [137]. Their major role is likely to be as part of the adaptive immune response and they will be reviewed in detail in that section.

Mammary epithelial cells are an important cell type in the response to mastitis as they are in direct contact with invading pathogens. Mammary epithelial cells express TLR2 and TLR4 and the expression of these genes appears to be unaffected by exposure to their respective ligands, lipoteichoic acid and lipopolysaccharide [138]. Mammary epithelial cells are able to secrete chemokines. Exposure of primary mammary epithelial cells to *S. aureus* resulted in a dose and time dependent stimulation of IL-8, IL-1β, TNF-α, TGF-β1 and IL-10 [139, 140]. The responses also appeared to be strain dependent [140]. Expression of inflammatory proteins is dependent on the transcription factor NF-κB. Increased NF-κB has been reported in cattle with chronic IMI [141]. A study using the bovine mammary epithelial cell line, MAC-T, revealed that prolactin can promote cytokine expression by MAC-T cells by means of an increase in NF-κB [142]. Other evidence suggests that *S. aureus* fails to activate Nf-κB in mammary epithelial cells

despite activation of TLR-2 and TLR-4 although one might question the mechanism by which *S. aureus* activates TLR-4 in this model [143]. It is clear that mammary epithelial cells can both recognize and respond to pathogens as part of the innate immune response.

Inducible nitric oxide synthase (iNOS) is an enzyme that is produced by bovine mammary epithelial cells and bovine leucocytes in response to inflammatory stimuli [144]. It has been proposed to be a scavenger of ROIs such as peroxynitrite and thus prevents excessive damage to tissues as a result of neutrophil oxidative burst [145]. Inhibition of iNOS during inflammatory responses *in vitro* did not affect neutrophil migration or ROI production [146]. This evidence suggests that iNOS does not have a major role in the innate response to IMI.

Antimicrobial peptides are components of the innate immune system that have antimicrobial activity against pathogens as well as serving as chemoattractants for immune cells. Early work revealed that the bovine tracheal antimicrobial peptide (TAP), a member of the β-defensin class, is active against many pathogens including *S. aureus* [147]. It has been shown that many β-defensin genes are expressed in the mammary gland [148] and β-defensin expression in the mammary gland is increased during mastitis [149]. This is contrasted by the lack of upregulation of cathelicidin expression *in vivo* during mastitis although cathelicidin expression is increased when bovine neutrophils are exposed to LPS [150]. Furthermore, cathelicidins possess antibacterial activity against *S. aureus* [150]. A recent study has characterized antimicrobial peptide expression in the entire bovine mammary gland [151]. Constitutive expression of lingual and tracheal antimicrobial peptides was present in the region of the SMLN, β-defensin 1 expression was localized to the cisternal epithelium and the Rosette of Furstenburg and the expression of psoriasin was located at the teat canal [151].

Serum amyloid A (SAA) is an acute phase protein that was first isolated from the milk of a cow suffering from acute mastitis [152]. Further studies revealed that it was present in milk in increased concentrations during mastitis, including mastitis that was caused by *S. aureus* [153-155]. More recent work has characterized expression of mRNA encoding the SAA3 protein. The protein is expressed by mammary epithelial cells at moderate levels during late pregnancy, at low levels during lactation, and moderate to high levels following dry-off and at high levels

during mastitis [156]. Given that SAA is able to enhance the antimicrobial activity of neutrophils [157], it may play an important role in the innate response to *S. aureus*.

3.4.3 Adaptive immune responses

Antigen recognition is the initial stage of the adaptive immune response. Pathogens are recognised by antigen presenting cells such as mammary macrophages [158] and B cells [159]. Dendritic cells are likely to be involved in antigen presentation from the mammary gland but this has not been studied. In fact, only one study has reported the presence of DCs in the bovine mammary gland [160]. Based on evidence in other species, immature DCs efficiently take up antigen and monitor the environment for danger signals [161]. Endogenous protein antigens are ubiquitinated, degraded by the proteasome into small antigenic peptides which are loaded onto MHC class I in the endoplasmic reticulum which is then displayed at the cell surface [162]. Antigens presented on the cell surface can then be recognised by CD8+ T cells. Exogenous protein antigens are taken up using receptor mediated endocytosis [161]. Once antigens have been internalized, they can either be processed for binding to either MHCII or MHCI. Antigens internalized for loading onto MHCII are degraded in endosomes and lysozomes into small peptides which are then loaded onto the MHCII molecule [150]. Antigens internalized for loading onto MHCI undergo a process known as cross-presentation. Cross-presentation takes place in the endoplasmic reticulum, allowing MHCI loading with antigenic peptides [163].

Antigen presentation alone is insufficient for DC maturation and migration to the local lymph node. A danger signal should also be present. Danger signals include products of inflammation as well as pathogen-associated molecular patterns (PAMPs). Products of inflammation that act as danger signals include CD40L, TNF-α, IL-1, IL-6 and IFN-α [163]. These activate TLRs which enable DC maturation, their migration to the draining lymph node and the can affect the bias of T cell responses [163-166].

The major controllers of adaptive immunity are T lymphocytes. Traditionally, T cell responses can be classified according to the bias of CD4+ T cells. Cell mediated immunity is driven by T_H1 cells that secrete IFN-γ and humoral immunity is driven by T_H2 cells that secrete IL-4 and IL-5 [167]. Furthermore, IFN-γ appears to drive the production of IgG_2 whilst IL-4 appears to

drive the production of IgG_1 [168]. A T_H1 response is thought to be more important in the elimination of intracellular pathogens [169, 170] whilst a T_H2 response is thought to be more important in the elimination of extracellular pathogens [169]. Historically, the two types of response have been considered to be mutually exclusive [171]. However, more recent work suggests that a spectrum of immune responses occurs with T_H1 and T_H2 responses at both poles. For example, effective immunity to the pathogenic stage of the malarial life cycle in mice requires both T_H1 and T_H2 responses [172]. The heterogeneity of bovine responses is illustrated by reports that up to 94% of T cell clones isolated from cattle infected with *F. hepatica* expressed both IL-4 and IFN-γ [173]. Hence, studies of the CD4+ T cell response to infection must determine the contribution of both T_H1 and T_H2 responses.

A third type of T cell is known as the regulatory T cell. Regulatory or suppressor T cell populations have been reported as far back as the 1970s [174]. Since then, several populations of T cells with regulatory capacities have been described including CD4+ CD25+ Foxp3+, CD3+ CD4- CD8- (DN), CD8+ CD28- and type 1 (Tr1) regulatory T cells as well as Th3 and NKT cells [175]. The CD4+ CD25+ Foxp3+ regulatory T cells are the best understood subset. These have been known as naturally-occurring regulatory T cells as they were first described as an endogenous population involved in peripheral immunologic tolerance based on reports that mutations in the Foxp3 gene in humans and mice resulted in autoimmune disease [176, 177]. For example, removal of the thymus in 3-7 day old mice results in autoimmune disease that can be prevented by the adoptive transfer of CD4+ CD25+ T cells [178], suggesting that they develop in the thymus early in life. Regulatory T cells may also be induced in response to inflammation and regulate the magnitude of the inflammatory response. These are known as adaptive regulatory T cells [179]. Adaptive regulatory T cells include all the classes described above, including CD4+ CD25+ Foxp3+ T cells [180]. Adaptive regulatory T cells can circulate through lymphoid tissues and can traffic to a site of inflammation [181]. They may be generated from other classes of T cell [182].

The mechanism of regulatory T cell action is poorly understood. It does appear that blocking of CTLA-4 partially blocks immune tolerance [183] and that anti-TGFβ treatment or the use of IL-10(-/-) mice removed the suppressive effects of naturally-occurring regulatory T cells on airway

hypersensitivity in a murine model [184]. These data suggest that naturally-occurring regulatory T cells act by means of direct cellular interactions mediated by CTLA-4 and by the cytokines TGFβ and IL-10. Adaptive regulatory T cells appear to act in a more cytokine-dependent manner [179].

To date, reports on regulatory T cell populations in the bovine mammary gland have been limited. Their presence has been proposed as mammary immunity has been reported to be less efficient than that of other body systems. Decreased mammary immunity is manifest as decreased activity of mammary neutrophils compared to peripheral blood neutrophils [185], poor antigen presentation in the mammary gland [7] and a poor blastogenic response of milk lymphocytes to stimulation [186]. The poor blastogenic response of milk lymphocytes is particularly apparent during the postpartum period [187].

Studies have focused on mechanisms behind the poor blastogenic response of milk lymphocytes. Park and co-workers [188] reported that cows that were susceptible to mastitis had CD4:CD8 T cell ratios of less than one in mammary secretions and peripheral blood, suggesting that these cell populations play a role in susceptibility to bovine mastitis. The increase in the proportion of mammary CD8+ T cells was attributed to the presence of CD8+ ACT2+ T cells [189]. Shafer-Weaver and Sordillo noted that CD8+ T cells isolated from the mammary gland during the immediate post-partum period had no cytotoxic activity despite a high level of activation [187]. Enrichment of cell cultures with these CD8+ T cells decreased the proliferative activity of CD8+ cells in the cultures. They concluded that these were suppressor T cells. Hence, a population of regulatory T cells appears to be present in the uninfected mammary gland and these may have a role in the pathogenesis of IMI.

Dendritic cells are thought to be crucial in the development of the bias of T cell responses. For example, IL-12 secreted from DCs is able to induce a T_H1 phenotype in CD4+ cells [190]. The action of IL-12 appears to be enhanced by the presence of IL-18 [191] and ICAM-1 [192]. Induction of a T_H2 phenotype is less well understood. Interleukin-4 is required for generation of T_H2 responses [193]. Furthermore, secretion of IL-4 by murine T cells is enhanced by the presence of MCP-1 [194] and the OX40 ligand [195].

Whilst naturally-occurring regulatory T cells are generated in the thymus in the immediate postpartum period and are involved in self-tolerance [176, 177], DCs appear to be central in the induction of adaptive regulatory T cells. The first evidence of this came from a report that exposure of CD4+ T cells to IL-10 resulted in generation of cells with a regulatory phenotype [196]. Recent evidence suggests that stimulation of T cell clones by immature DCs results in T cell tolerance, most likely as a result of T cell anergy caused by a lack of co-stimulation [197]. Dendritic cells can also secrete cytokines such as IL-10 and TGFβ and may express members of the B7 family and members of the Notch signalling pathways to induce a regulatory T cell phenotype [198].

The bias of DCs themselves is mediated by several factors. Exposure to moderate amounts of antigen leads to T_H1 responses whereas exposure to high or low amounts of antigen leads to T_H2 responses [199]. A high DC to T cell ratio also leads to mixed T_H1 and T_H2 responses whereas a low DC to T cell ratio leads to T_H2 responses [200]. Dendritic cell mediated T cell polarization can be altered by the presence of a pathogen. For example, presentation of *Mycobacterium bovis* antigens by DCs of immunologically naïve calves resulted in the induction of a T_H1 response [201]. Furthermore, infection of DCs by *M. bovis* resulted in the secretion of IL-12 and IL-18 [164]. Pathogens such as *Bordetella pertussis* can, in humans, induce the generation of adaptive regulatory T cells [202]. These effects may be mediated by activation of TLRs on DCs. Many TLRs are involved and different DCs can respond differently to TLR stimulation. However, it is generally considered that TLR-3, -4, -5, -7 and -9 are involved in the induction of T_H1 responses whilst TLR-1, -2 and -6 are involved in the induction of T_H2 responses [165].

Dendritic cells can be isolated from the afferent lymph of cattle based on the selective expression of the DEC-205 surface antigen [203] and are able to be separated into one of two populations based on expression of the SIRPα surface molecule [204, 205]. It has been suggested that, as SIRPα+ cells secrete high levels of IL-10 and variable levels of IL-12 and SIRPα- cells secreted higher levels of IL-12, SIRPα+ cells may induce a balanced T cell response whilst SIRPα- cells may induce a predominantly T_H1 response [164]. Hence, DCs from the afferent lymph of dairy cattle may be able to polarize the T cell response. However, no work has been performed on

mammary DCs so far. Studying DCs and their role in bovine mastitis is an interesting and important area.

Several studies have reported the effects of *S. aureus* IMI on cellular immune responses. Studies that report the effects of superantigens are discussed in section 3.5.7. Soltys and Quin reported that numbers of CD4+ T cells in milk increased significantly in response to *S. aureus* IMI [206]. Gronlund and coworkers reported that the proportions of CD4+ and CD8+ T cells in milk decreased during the acute phase of *S. aureus* IMI but recovered during the chronic phase. The proportion of B cells increased at all stages of *S. aureus* IMI, leading the authors to propose that *S. aureus* causes humoral immune responses to occur in the mammary gland [207]. When cattle were experimentally infected with *S. aureus*, the number of CD4+ T cells in milk increased in the early stages of infection and was negatively correlated with bacterial recovery from milk [208]. The same group also reported a decrease in CD3+ cells and an increase in CD11b+ cells in milk following experimental *S. aureus* IMI [209]. However, the study reported percentages of cells identified by flow cytometry so these results probably reflect an influx of neutrophils into the mammary gland following infection. Somewhat paradoxically, the same group reported that the number of CD3+, CD4+ and CD8+ cells decreased in the milk of animals following experimental *S. aureus* IMI [210]. Park and coworkers reported that proliferative responses of milk CD4+ T cells from cattle with *S. aureus* IMI was decreased when compared with uninfected animals. When a population of CD8+ T cells expressing the ACT2 activation marker was removed from cell cultures, proliferative responses in infected animals were increased. Restoration of CD8+ ACT2+ T cells to cell cultures decreased proliferative responses in a dose-dependent manner [7]. This is evidence for the existence of regulatory T cell populations being induced in response to *S. aureus* IMI.

Leitner and coworkers reported that the proportions of CD4+ and CD8+ T cells increased in the milk of animals with chronic but not acute *S. aureus* IMI [211]. A follow-up study from the same group reported that T cell populations in the SMLN did not vary between animals with *S. aureus* IMI and controls. In uninfected mammary parenchyma, the predominant T cell population was CD8+ and this was enhanced following *S. aureus* IMI [212].

Riollet and coworkers studied cell populations and cytokine expression in milk following *S. aureus* IMI [6]. They reported that CD8+ T cells and B cells were recruited into the milk following infection, suggesting a balanced immune response. Furthermore, infection resulted in increased IL-1α, IL-1β, IL-6, TNF-α, IL-10 and IL-12 expression in cells isolated from the milk of infected mammary glands whilst IL-2 and IL-4 mRNA was not present. This evidence supports a T_H1 and a regulatory T cell response in milk. Lee and coworkers studied the expression of IL-6, IL-8, IL-12, GM-CSF, TNF-α and IFN-γ in cells from the milk of animals infected with either *S. aureus* or *E. coli* [213]. They reported that large variation existed between animals. Increases in all cytokines were reported for both infections with the exception of IFN-γ following *S. aureus* IMI. However, the magnitude and speed of onset of the response to *S. aureus* was diminished compared with that for *E. coli*. The authors therefore suggested that the decreased responses to *S. aureus* may explain the chronic nature of *S. aureus* IMI. These results are supported by evidence which showed that IL-1β, IFN-γ, IL-12, and IL-10 in milk were upregulated in the acute phases of *E. coli* and *S. aureus* IMI but the magnitude of the increases was lower for *S. aureus* [4]. A different study reported that milk IFN-γ, IL-12, TGF-α and TGF-β all increase during the first 24-36 hours following *S. aureus* IMI [5]. A recent study reported that cytokine gene expression is induced as a result of *S. aureus* IMI in dairy goats. Specifically, IL-1α, IL-2 and CXCL6 were upregulated during the acute phase of infection [214]. Work in cattle has shown that, during chronic *S. aureus* IMI, expression of IL-2, IL-12, IL-15 and IL-18 is upregulated in PBMCs and expression of IL-17 is upregulated in milk somatic cells [215].

3.5 *Staphylococcus aureus* virulence factors involved in immune evasion

3.5.1 Capsular polysaccharide

Staphylococcus aureus produces a polysaccharide capsule which may be expressed by up to 100% of bovine mammary isolates [70]. In the same study, almost all of the isolates could be classified as possessing capsular serotypes 5, 8 and 336. Kampen and coworkers [216] constructed isogenic variants of a single *S. aureus* strain expressing no capsule, CP5 or CP8. The presence of the capsule inhibited killing of *S. aureus* by bovine neutrophils in vitro through downregulation of oxidative burst. This effect was reversed by the presence of antibodies against CP5 or CP8. This evidence suggests that capsule is an antiphagocytic factor that prevents killing by neutrophils but does not prevent opsonisation of bacteria. Capsular

polysaccharide is also a T cell independent antigen therefore stimulates immune responses in the absence of antigen presentation and T cell help [217]. This means that responses will only be stimulated by mature B cells that produce antibodies that recognise the CP. If an animal does not possess these B cells in the mammary gland, it is unlikely that a response will be mounted. Therefore capsule disrupts both innate and acquired immune responses.

3.5.2 Fc receptors

Protein A is a virulence factor that can bind to the Fc region of immunoglobulins [218] thus preventing recognition of opsonized bacteria by Fc receptors present on phagocytic cells. However, the role of protein A in the pathogenesis of *S. aureus* mastitis is controversial. Experiments in a murine mastitis model reported that the virulence was not affected by protein A [219]. A recent study used linear regression models to associate the presence or absence of virulence genes with the inflammatory response of the bovine mammary gland [220]. The presence of the protein A gene was associated with chronic subclinical mastitis. This evidence, obtained in dairy cattle, suggests that protein A is indeed a major factor in the evasion of bovine mammary immune responses.

3.5.3 Invasion of epithelial cells

Staphylococcus aureus is able to avoid humoral immune responses by becoming intracellular. Electron microscopy revealed that *S. aureus* can enter bovine mammary epithelial cells in primary culture [65]. Invasion was shown to involve F-actin polymerization and therefore the cytoskeleton. Fibronectin-binding protein (FnBP) is involved in this process and is thought to interact with heat shock protein 60 and integrins [221] although work in a mouse model showed that removal of FnBP only delayed invasion [222]. Recent work has shown that internalization of *S. aureus* into bovine endothelial cell is promoted in the presence of TNF-α and IL-1β with the effects being reversed by inhibition of NF-κB nuclear translocation [223]. Internalization may be induced by an inflammatory response and therefore may represent an adaptation of *S. aureus* to avoid inflammatory responses. However, it is important to remember that internalized bacteria still undergo antigen presentation through MHCI and can be cleared by the action of CD8+ T_C cells.

3.5.4 Small colony variants

Small colony variants (SCV) of *S. aureus* are variants which form slow growing pin-point colonies on agar. They were originally identified as dwarf colony variants when isolated from cases of bovine mastitis [224]. Mutation of the *hemB* gene of the *S. aureus* Newbould 305 strain results in the generation of a SCV. This variant possesses the same susceptibility to cephapirin treatment but is less able to colonize murine mammary glands than the wild type variant *in vitro* but is better able to persist in murine mammary glands treated with cephapirin *in vivo* [225]. Small colony variants have been isolated from chronic cases of bovine mastitis [226] and seem to cause a mild, persistent IMI [226, 227] when compared with conventional variants. The decreased susceptibility of SCVs to conventional antimicrobial agents has been demonstrated in cattle and has been associated with an intracellular location of bacteria [228]. The intracellular location may also assist SCVs in avoiding host defences.

3.5.5 Cytotoxins

Staphylococcus aureus secretes a variety of virulence factors that are cytotoxic. For example, α-toxin is a cytolysin which forms transmembrane pores in host cells [229]. However, this can affect all cells and is therefore not specifically involved in immune evasion. Similarly, β-toxin is more involved in damage to mammary parenchyma rather than immune evasion [230]. One virulence factor of interest is leucocidin. It is expressed by over 90% of bovine mammary *S. aureus* isolates [231] and its presence is associated with increased mammary inflammation [232]. It forms transmembrane pores in mammary leucocytes and their subsequent death [233] and is considered the most active leukotoxin for bovine neutrophils [234]. This evidence implicates leucocidin in the direct disruption of bovine neutrophils and so is a factor enabling *S. aureus* to evade innate responses.

3.5.6 Biofilms

Costerton and coworkers defined biofilms as: "A structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface" [235]. This community of cells shows increased resistance to treatment with antibiotics, host defenses and disinfectants [236]. Formation involves the binding of cells to a surface followed by cell multiplication. Cells are attached to one another by extracellular polysaccharides. Staphylococci

may also secrete a glycocalyx layer (slime) [236]. Biofilms appear to be referred to as "pseudocapsule" in several studies of bovine mastitis [237]. Bovine mammary *S. aureus* isolates have been shown to form biofilms when grown *in vitro* in milk [238]. *Staphylococcus aureus* strains obtained from milk were more likely to produce biofilm than those obtained from teat skin or milking equipment [239]. "Pseudocapsule" has been shown to be produced when *S. aureus* strains are grown in the bovine mammary gland [240]. Antibodies against "pseudocapsule" opsonised *S. aureus* for phagocytosis by neutrophils [241]. Biofilms appear to have a major role in *S. aureus* mastitis and, given their previously mentioned effects on host defense, are likely to shield bacteria from all aspects of host responses.

3.5.7 Superantigens

Up to 21 different staphylococcal enterotoxins have been characterized [242]. Whilst it is impractical to discuss each toxin individually, mutational analysis reveals that staphylococcal enterotoxins may act as either direct gastrointestinal toxins or as superantigens [243]. The direct gastrointestinal effects are poorly understood. However, *S. aureus* is able to interact directly with epithelial cells. A study using renal tubule epithelial cells revealed that proximal tubule epithelial cells undergo apoptosis following treatment with staphylococcal enterotoxin B [244]. Whether enterotoxin has similar effects on intestinal cells is unclear.

The superantigen effects of *S. aureus* are far better understood. The enterotoxin forms a complex with the MHCII molecule. The complex binds to the variable region of the T cell receptor β chain [245]. The need for a specific peptide antigen to activate T cells is therefore removed, allowing massive T cell stimulation [246]. The stimulated T cells secrete many cytokines at toxic levels with the three most important being IL-2, IFN-γ and TNF-α [247]. The high cytokine levels cause severe systemic clinical signs consistent with a toxic shock syndrome. Evidence also exists that staphylococcal enterotoxins can stimulate bovine γδ T cells in a CD86 and IL-2 dependent manner [248] although the relevance of this remains to be determined. Staphylococcal enterotoxin C is a superantigen encoded on the SapIbov pathogenicity island of *S. aureus* [249] that can activate T cells by cross-linking MHC with the T cell receptor (TCR) [250]. It is expressed by approximately 10% of bovine mammary *S. aureus* isolates [86] and

despite its relatively low prevalence; it has several biological effects including the induction of T cell anergy [251] and deletion [252], toxic shock syndrome [253] and autoimmune disease [254]. It was reported that exposure of bovine PBMCs in culture to SEC induced a state of partial activation of CD4+ T cells [255] as well as the genesis of a population of CD8+ CD26+ T cells [255, 256]. Furthermore, exposure of bovine PBMCs to SEC results in low levels of nucleic acid synthesis compared to human PBMCs used as controls [257]. More recent work compared cows that were susceptible to mastitis and those which tended to be resistant. It appears that susceptible cows possess a certain MHC haplotype and have a CD4+:CD8+ T cell ratio of less than one [188]. It was hypothesized that the decreased ratio was as a result of the presence of CD8+ ACT2+ T cells which possessed a regulatory function [258]. This hypothesis was investigated further by exposing bovine PBMCs in vitro to SEC. Exposure to SEC was reported to increase the proliferation and decrease the apoptosis of CD8+ T cells [257] although the role of ACT2 was not evaluated. A more recent study involved exposing peripheral blood of dairy cattle to SEC. Exposure to SEC increased the proliferation of CD8+ T cells compared with CD4+ T cells [8]. Furthermore, CD4+ CD25+ Foxp3+ T cells which transcribed IL-10 and TGFβ were induced in response to SEC [8]. Stimulation of bovine PBMCs with SEC can also cause IL-10 secretion [259], further supporting the hypothesis that SEC is involved in the generation of regulatory T cells. However, the study [259] did not evaluate the specific cellular source of IL-10.

Despite the aforementioned evidence, the relevance of regulatory T cells in the mammary gland is controversial. Ebling and coworkers studied three groups of cattle, those infected with *S. aureus* expressing SEC, those infected with *S. aureus* that did not express SEC and uninfected controls [260]. No difference existed between any of the groups regarding the CD4+:CD8+ T cell ratio. Also, the number of CD8+ ACT2+ T cells increased following IMI with the *S. aureus* strain that did not express SEC but did not change following IMI with the *S. aureus* strain expressing SEC [260]. One potential explanation for the difference between the studies may be that Ebling and coworkers [260] studied cellular immunity in milk. Cellular immunity in mammary tissue and the regional lymph nodes may be different. Based on this apparent contradictory evidence, the role of SEC in the mammary gland needs to be explored further.

Since *S. aureus* is able to enter bovine mammary epithelial cells [222], a T_H1 response would be likely to clear *S. aureus* IMI. Previous work suggests that an IgG_2 response in milk may protect against *S. aureus* IMI [95, 261, 262], supporting the hypothesis that a T_H1 response would be protective. However, culture of bovine PBMCs in the presence of SEC leads to sustained production of IL-4 and IL-13, but only transient production of IFN-γ, IL-2 and IL-12 [257, 259], suggesting that exposure to SEC induces a T_H2 response in the mammary gland.

Despite its biological effects, the prevalence of *S. aureus* strains expressing SEC is relatively low in the bovine mammary gland. However, evidence exists that *S. aureus* strains vary in their ability to cause disease and avoid mastitis control procedures [55, 263]. The presence of SEC may therefore explain why some strains are better able to cause disease and avoid mastitis control. Despite the current evidence, the effects of SEC on bovine mammary immunity are not entirely clear. Specifically, the majority of studies have been carried out in mammary secretions or using cultured PBMCs. Future studies should focus on the effects that *S. aureus* and SEC have *in vivo* and focus on responses in mammary tissue and in the regional lymph nodes.

4. MATERIALS AND METHODS

4.1 Animals and treatments

Animals used in all experiments were housed in the animal care facility at VIDO. They were housed in accordance with good animal care and husbandry practises as defined by the Canadian Council on Animal Care and were provided with full veterinary care. Furthermore, experimental protocols were approved by the University of Saskatchewan Animal Care Committee.

4.1.1 Cattle as peripheral blood mononuclear cell donors

For studies involving responses to SEC, four castrated male mixed breed beef cattle were sourced from the VIDO research farm. Animals were selected based on low serum titres against *S. aureus* and staphylococcal enterotoxin C (SEC) (table 4.1). Serum titres represent humoral immunity to *S. aureus* and SEC. For studies examining cytokine production by peripheral blood mononuclear cells (PBMCs) in response to *S. aureus in vitro*, dairy cows were sourced from the University of Saskatchewan Greenbrae Herd. Lactating dairy cows with recall responses to *S. aureus* were selected as they were presumed to possess memory T cells specific for *S. aureus*. The existence of recall responses was based on high serum antibody titres against *S. aureus* and large proliferative responses to staphylococcal alpha toxin (stimulation index greater than 100) (table 4.2). Animals also had a current or previous *S. aureus* IMI confirmed by milk culture. To perform milk cultures, milk was aseptically collected from each udder half. Milk was plated onto TSA and incubated for 48 hours at 37°C in the presence of 5% CO_2. If no bacterial growth was observed following 48 hours of incubation, the culture was considered to be negative. If colonies were observed, they were subjected to Gram staining and their Gram staining characteristics recorded.

Blood samples for serum titres were collected into serum separator vacutainer collection tubes (BD Vacutainer SST ®, Franklin Lakes, NJ, USA), allowed to coagulate and centrifuged at 2000g for 10 minutes. Serum was harvested and stored at -20°C until antibody ELISAs were performed. Ninety-six-well microtitre plates (Immunolon 2 HB, Dynex Technologies Inc., Chantilly, VA, USA) were coated with whole cell suspensions of the *S. aureus* Wood 46 strain. The Wood-46 strain was used as it lacks the immunoglobulin binding protein, protein A, and has

Table 4.1. Serum antibody titres specific for *S. aureus* and SEC from beef steers used as blood donors. Animals were used for experiments determining responses to SEC. Serum titres were calculated using a regression model with a positive titre being defined as values exceeding the mean plus two standard deviations of the negative control.

Cow ID	*S. aureus* titre	SEC titre
1	11143	4369
2	3349	800
3	10794	3733
4	794	700

Table 4.2. Serum titres against *S. aureus* and stimulation indices against staphylococcal alpha toxin from dairy cattle used as blood donors. Animals were used for experiments determining responses to *S. aureus*. Serum titres were determined as in table 4.1. Stimulation index was calculated by dividing counts obtained with stimulated cells by those obtained with unstimulated controls.

Cow ID	*S. aureus* titre	Stimulation index
552	1214680	397.68
581	300057	332.67
609	1082091	368.10
620	961299	115.54
656	264882	249.78
660	602003	149.30
666	852887	495.79
667	864319	275.30

been demonstrated to specifically bind bovine antibodies against *S. aureus* in an ELISA [264]. An overnight culture of the Wood 46 strain was added to trypic soy broth (TSB) (BD Biosciences, Mississauga, ON) at a 1:100 dilution and incubated at 37°C with shaking until the mid-exponential growth phase. Bacteria were then centrifuged at 6000g for 10 minutes at 4°C then washed once in PBS (0.1M, pH 7.2). Bacteria were then suspended in PBS to give an absorbance of 0.2 at 600nm and 100µl of this suspension was added to each well of the microtitre plate. Plates were incubated overnight at 4°C then washed five times with 200µl Tris-buffered saline (1M Tris-HCl, 1.8M NaCl, pH 7.5) containing 0.05% Tween-20 (TBS-T). Serum samples were added to each well beginning with 100µl of a 1:100 dilution in TBS-T containing 0.5% gelatin (TBS-TG). Serial fourfold dilutions were made such that seven serial dilutions were made in TBS-TG. Plates were incubated at room temperature for one hour then washed as before. Following the wash, 100µl of a mouse anti-bovine IgG (H+L) antibody conjugated to alkaline phosphatase (Bethyl Laboratories, Montgomery, TX, USA) was added in TBS-TG to each well. Plates were incubated at room temperature for one hour then washed as before. Following the wash, 100µl of a 1mg/ml p-nitrophenyl phosphate (Sigma-Aldrich, Oakville, ON) in substrate buffer (0.1M Diethanolamine, 0.5mM $MgCl_2$, pH 9.8) was added to each well. Plates were incubated at room temperature for 30 minutes and read using a microtitre plate reader with absorbance set at 405nm and reference set at 490nm. Titres were calculated using a regression model with a positive titre being defined as the optical density exceeding mean plus two standard deviations of the negative control.

Blood samples for proliferation assays were collected into vacutainer tubes containing EDTA as an anticoagulant. Peripheral blood mononuclear cells were isolated and purified according to the protocol in section 4.2.1. Following isolation and purification, 100µl of a $3.5x10^6$ cells/ml PBMC suspension in minimum essential media (Invitrogen Canada Inc., Burlington, ON) containing 10% fetal bovine serum, 50µM 2-mercaptoethanol, 1% sodium pyruvate, 1% non-essential amino acids and 1% HEPES (MEM-plus) with 50µg/ml gentamycin was added to each well of a 96 well tissue culture plate (Corning Inc., Corning, NY, USA). Antigens were prepared and 100µl of antigen solution in MEM-plus was added to each plate. Final antigen concentration was 0.5µg/ml staphylococcal alpha toxin (Sigma Aldrich, Oakville, ON) or 1µg/ml concanavalin A (Sigma) as a positive control. Plates were incubated at 37°C in 5% CO_2 for 72 hours then

20µl of a 0.4µCi/ml solution of tritiated thymidine was added to each well. Plates were incubated for a further 18 hours and frozen until harvest. Following harvest, incorporation of tritiated thymidine was determined using a liquid scintillation counter (TopCount NXT, Perkin Elmer Inc., Waltham, MA, USA). Stimulation index was calculated by dividing counts obtained with stimulated cells by those obtained with unstimulated controls.

4.1.2 Sheep for in vivo studies

To establish a model of ovine IMI, nonlactating Suffolk ewes were purchased from the University of Saskatchewan Sheep Farm. Ewes were nonlactating and negative for *S. aureus* based on three sequential negative lacteal secretion cultures. Following screening, nine ewes possessing a total of 14 uninfected udder halves were identified. Details of which udder halves were challenged with which *S. aureus* isolate are in table 4.3. Lacteal secretion samples for culture and SCC were collected on days 3, 6, 9, 12 and 15 following challenge. Clinical examinations were performed on all animals from days 1-9, 12 and 15 following challenge. Specifically, animals were checked for clinical mastitis, systemic disease and rectal temperature. Animals were humanely euthanized 15 days following challenge. Clinical mastitis was defined as abnormal milk alongside mammary gland inflammation.

Milk SCCs were determined according to International Dairy Federation Standard 148A:1995 (Enumeration of Somatic Cells). Milk samples were mixed with a fixative liquid (0.02% eosin in 3.5% formaldehyde solution) at a 50:1 ratio. Somatic cells were fixed at 30°C for 18 hours then cooled to room temperature. Samples were then added to an emulsifier electrolyte mixture (12.5% ethanol, 2% TritonX-100 in 0.9% sodium chloride solution) at a 1:100 ratio and heated to 80°C for 10 minutes. Samples were cooled to room temperature and somatic cells were counted using a Coulter counter (Beckman Coulter, Mississauga, ON).

To determine the effects of *S. aureus* isolates on ovine mammary immune responses *in vivo*, lactating Suffolk ewes were purchased from the University of Saskatchewan Sheep Farm. Ewes were negative for *S. aureus* based on three sequential negative milk cultures. Ewes had high antibody responses to *S. aureus* and high proliferative responses to staphylococcal alpha toxin

(table 4.4). Following screening, twelve ewes possessing a total of 24 uninfected udder halves were identified. All animals were challenged with 50cfu of a *S. aureus* isolate in 2ml sterile,

Table 4.3. Animals and udder halves challenged with *S. aureus* in order to establish a model of ovine *S. aureus* IMI. Challenge doses were administered in 2ml sterile, endotoxin-free PBS. Animal 10-11 possessed an IMI in the left udder half, animals 10-15 and 10-16 possessed an IMI in the right udder half therefore these udder halves were not challenged.

Ewe ID	Udder half	Challenge isolate	Challenge dose (cfu)
08-60	L	SA8	10
	R	SA10	10
10-10	L	SA8	10
	R	SA10	10
10-11	R	SA10	10
10-12	R	SA8	10
10-13	L	SA8	50
	R	SA10	50
08-225	L	SA8	50
	R	SA10	50
10-14	L	SA8	200
	R	SA10	200
10-15	L	SA8	200
10-16	L	SA10	200

Table 4.4. Serum antibody titres against *S. aureus* and PBMC proliferative responses to staphylococcal alpha toxin from ewes. Ewes were used to determine the effects of *S. aureus* isolates on ovine mammary immune responses *in vivo*. Serum antibody titres and stimulation indices were determined as in tables 4.1 and 4.2, respectively.

Ewe ID	*S. aureus* titre	Stimulation index
10-34	312774	290.73
10-35	77071	48.05
10-36	72641	163.61
10-37	254736	291.66
08-38	301114	111.64
10-39	240416	98.38
10-40	203037	66.30
10-41	239047	145.62
10-42	109335	44.18
10-43	296189	26.28
10-44	227603	89.51
08-64	257326	25.24

endotoxin-free PBS. Six animals were humanely euthanized on day 3 following challenge to characterize acute responses. The remaining six animals were humanely euthanized on day 15 following challenge to characterize chronic responses (table 4.5). Milk samples for culture and SCC were collected on days 1, 3, 6, 9, 12 and 15 following challenge. Clinical examinations were performed on all animals as per the above protocol. Following euthanasia, SMLN samples were collected into MEM-plus for FACS analysis. Samples of SMLN, mammary parenchyma and teat ends were collected into a commercial RNA preservative (RNAlater, Ambion Inc.,

Austin, TX, USA) for future RNA extraction and qRT-PCR analysis. Samples of SMLN, mammary parenchyma and teat ends were collected into a commercial cryopreservative (Shandon Cryomatrix, Fisher Scientific, Nepean, ON) for immunohistochemical assays.

Table 4.5. Animals and udder halves challenged with *S. aureus* to determine the effects of *S. aureus* isolates on ovine mammary immune responses *in vivo*.

Ewe ID	Udder half	Challenge isolate	Day euthanized
10-34	R	SA10	3
10-35	L	SA8	3
10-36	L	SA8	15
10-37	L	SA10	3
08-38	L	SA8	15
10-39	R	SA10	15
10-40	R	SA10	15
10-41	R	SA8	3
10-42	R	SA8	3
10-43	L	SA10	15
10-44	R	SA10	3
08-64	R	SA8	15

4.1.3 Preparation of Staphylococcus aureus challenge suspensions

Challenge suspensions were prepared by inoculating 5ml of sterile tryptic soy broth (TSB) with the appropriate *S. aureus* isolate. Isolates were taken from the Vaccine and Infectious Disease Organization culture collection. Isolates SA8, SA10, SA12, SA23 and SA45 possessed the *sec* gene. Cultures were incubated overnight at 37°C with shaking. The overnight culture was added to sterile TSB at a 1:100 concentration and incubated at 37°C with shaking until the optical density at 600nm had reached 0.35. Cultures were centrifuged at 3000 g for 10 minutes at 4°C then washed twice with 25ml sterile PBS. Cultures were then suspended in 10ml sterile PBS and serial tenfold dilutions were made. Bacterial counts were enumerated by plating on TSA and incubating at 37°C overnight with 5% CO_2. During this time, the bacteria were stored in PBS at 4°C. Based on the bacterial counts, bacteria were diluted in PBS such that the appropriate bacterial count in the challenge suspension was obtained. Bacterial counts in the challenge suspension were confirmed by plating on TSA and incubating overnight.

4.2 Peripheral blood mononuclear cell assays

4.2.1 Isolation and purification of peripheral blood mononuclear cells

Blood samples were collected into vacutainers containing EDTA as an anticoagulant. Vacutainers were centrifuged at 1350g for 20 minutes at room temperature. The buffy coats were removed and suspended in 8ml of PBSA containing 2.7mM EDTA (PBSA/EDTA), layered over 5ml Ficoll (GE Healthcare, Uppsala, Sweden) and centrifuged at 1940g for 30 minutes at room temperature. Peripheral blood mononuclear cells were removed from the interface between PBSA/EDTA and Ficoll, resuspended in 10ml PBSA/EDTA and centrifuged at 311 g for 10 minutes at room temperature. Cells were washed twice more in 10ml PBSA/EDTA then resuspended in 4ml MEM-plus. Cell counts were determined using a Coulter counter and resuspended to the concentration required for the assay.

4.2.2 Preparation of Staphylococcus aureus suspensions

Suspensions of *S. aureus* were prepared by inoculating 5ml of MEM-plus with the appropriate stock *S. aureus* isolate. Cultures were incubated overnight at 37°C with shaking. The overnight culture was added to sterile MEM-plus at a 1:100 concentration and incubated at 37°C with

shaking until a mid-log growth phase was reached. The resultant bacterial suspension was centrifuged at 3000g for 10 minutes at 4°C then washed twice with 25ml sterile PBS. Cultures were suspended in 10ml sterile PBS and bacterial counts were enumerated using a haemocytometer. Bacterial counts were confirmed by plating serial tenfold dilutions on TSA and incubating overnight.

4.2.3 Co-culture of peripheral blood mononuclear cells with Staphylococcus aureus

Isolated PBMCs were added to tissue culture plates at a concentration of 10^6 cells/ml. If the cells were to be used for fluorescent activated cell sorting (FACS), 6 well tissue culture plates (Corning) were used with a total volume of 4ml/well. If the cells were to be used for cytokine quantification by ELISA or qRT-PCR, 12 well tissue culture plates (Corning) were used with a total volume of 1ml/well. *Staphylococcus aureus* was added to each well such that the final concentration was 10^4 cfu/ml. To prevent overgrowth of *S. aureus* in the culture medium, a final concentration of 1µg/ml tetracycline was added to all culture media. This concentration was determined by incubating *S. aureus* isolates in MEM-plus containing various concentrations of tetracycline until a concentration of tetracycline which resulted in the number of viable *S. aureus* in the media to remain constant over 72 hours was identified. The PBMCs and *S. aureus* were co-cultured for 24, 48 and 72 hours.

4.3 Quantitative real-time PCR assays

4.3.1 Isolation, purification and quantification of RNA

To isolate RNA from PBMCs, tissue culture plates were centrifuged at 220g for 10 minutes at 4°C to pellet the cells. Culture supernatants were removed and 1ml Trizol ® (Invitrogen Canada Inc., Burlington, ON) was added to each well. Plates were stored at -80°C until RNA isolation was performed. Plates were thawed at 56°C for 1 minute and samples were mixed and placed into RNase-free microcentrifuge tubes. Samples were incubated at room temperature for five minutes then 200µl chloroform was added to each tube. Samples were thoroughly mixed and incubated at room temperature for two minutes then centrifuged at 12,000g for 15 minutes at 4°C. Following centrifugation, the aqueous phase was removed from the sample and transferred to a clean RNase-free microcentrifuge tube. To precipitate the RNA, 500µl isopropanol was

added to each sample then samples were mixed by inversion and incubated at room temperature for 10 minutes. Samples were then centrifuged at 12,000g for 10 minutes at 4°C. Supernatants were removed; samples were suspended in 1ml of 75% ethanol and centrifuged at 7,600g for five minutes at 4°C. Supernatants were removed and samples were air dried for 15 minutes at room temperature. Samples were resuspended in 40µl RNase-free water followed by incubation at 56°C for five minutes, mixed and incubated at 56°C for a further five minutes to fully suspend RNA.

To isolate RNA from tissue samples, tissues were collected into RNAlater (Invitrogen) and frozen at -20°C until RNA isolation was performed. Samples were thawed on ice, a 3mm cubic section was cut, placed in Trizol ® and homogenized using zirconia beads. Chloroform (200µl) was added to each sample; samples were thoroughly mixed and centrifuged at 12,000g for 15 minutes at 4°C. Following centrifugation, 200µl of the aqueous phase was added to a RNase-free microcentrifuge tube. This was then added to an RNase cleanup column (RNeasy Mini Kit, QIAGEN Inc., Mississauga, ON) which allows for the further isolation and purification of RNA from the sample. Briefly, ethanol was added to the sample to allow binding of RNA to the column membrane. Total RNA was bound to the membrane then the membrane was washed to remove contaminants. The RNA was eluted from the membrane using RNase-free water. Extracted RNA was stored at -80°C until reverse transcription. Concentration of extracted RNA was determined by spectrophotometer.

4.3.2 Reverse transcription to make cDNA

Isolated RNA was thawed at 56°C. Sample concentration was adjusted such that 500ng RNA in 10µl RNase free water (Invitrogen) was present in a 200µl RNase free tube and 1µl of a 100U/ml DNaseI solution (Invitrogen) was added to each sample. Samples were incubated for 20 minutes at room temperature then 1µl of 25mM EDTA (Invitrogen) was added to each sample and samples were incubated at 65°C for 10 minutes to inactivate DNaseI. Reverse transcription was achieved by reverse-transcription PCR using random hexamer primers and superscript II reverse transcriptase (Invitrogen). The PCR reaction was carried out using a thermocycler with samples held at 42°C for 20 minutes then 95°C for 10 minutes. Samples were stored at -20°C until quantitative real-time PCR was performed.

4.3.3 Quantitative real-time PCR

Quantitative real-time PCR was performed using SYBR Green (Invitrogen) on a Bio-Rad iCycler (Bio-Rad Laboratories, Mississauga, ON) according to the manufacturer's protocol. Primers for sense and anti-sense strands of the β-actin, IFN-γ, IL-4 and IL-10 genes were designed based on published sequences of the gene of interest. Products from PCR were run on a 1% agarose gel to ensure that a single product of the appropriate length was generated. Products from PCR were also sequenced and compared with the reference sequence to ensure that the appropriate gene was amplified. Details of primers are in table 4.6. The qRT-PCR analysis was performed in duplicate using the housekeeping gene β-actin as a reference. Cytokine gene expression was normalized to β-actin and expressed as relative expression by comparison with untreated controls using the comparative threshold cycle ($\Delta\Delta C_t$) method.

Table 4.6. Primers used to amplify cytokine and housekeeping gene mRNA transcripts. Primer sequence and product length are reported

Gene	Primer direction	Primer sequence	Product length (bp)
β-actin	Forward	5´-AGGCATCCTGACCCTCAAGTA-3´	95
	Reverse	5´-GCTCGTTGTAGAAGGTGTGGT-3´	
Bovine IL-4	Forward	5´-ACGCTGAACATCCTCACAAC-3´	125
	Reverse	5´-CGCCTAAGCTCAATTCCAAC-3´	
Ovine IFN-γ	Forward	5´-TCCAGCGCAAAGCCATCAATGAAC-3´	105
	Reverse	5´-TCCGGCCTCGAAAGAGATTCTGAC-3´	
Ovine IL-4	Forward	5´-ACGCCGAACATCCTCACATC-3´	172
	Reverse	5´-CAAGTCCGCCCAGGAATTTG-3´	
Ovine IL-10	Forward	5´-GATGCCACAGGCTGAGAACC-3´	53
	Reverse	5´-GCGAGTTCACGTGCTCCTTG-3´	

4.4 Fluorescence-activated cell sorting assays

4.4.1 Isolation, purification and quantification of cells

For assays involving PBMCs, cells were isolated according to the protocol in section 4.1.1 and incubated in MEM-plus for the appropriate time. If the cells were to be used for intracellular cytokine staining, a protein transport inhibitor containing monensin (GolgiStop, BD Biosciences) was added to each well according to manufacturer's directions for the final ten hours of incubation. This treatment allows proteins to accumulate in the cells and enhances staining of intracellular cytokines. Following incubation, cells were removed from wells, placed in a polypropylene tube and centrifuged for 311g at 4°C. Cell pellets were washed once with PBSA. The pellet was suspended in FACOLA staining media (PBSA supplemented with 1% fetal calf serum, 0.09% sodium azide, pH 7.5) such that the cell concentration was 2×10^7 cells/ml.

For assays involving cells isolated from lymph nodes, the distal third of the lymph node was collected within 10 minutes of euthanasia and immediately placed in a tube containing ice cold culture media and transported on ice for processing. They were then placed in a Petri dish containing culture media and cut into 2-3mm diameter fragments. Tissue fragments were placed in a 40µm cell strainer (BD Biosciences) and filtered into a fresh tube using culture media. For cell surface marker staining, cells were centrifuged at 325g for 8 minutes at 4°C and washed once in PBSA. Cells were resuspended in FACOLA such that the cell concentration was 2×10^7 cells/ml. For intracellular cytokine staining, 4ml of a 10^6 cells/ml suspension was placed into wells of a 6 well plate. Cells were then incubated for 12 hours in the presence of GolgiStop. Following incubation, cells were removed from wells, placed in a polypropylene tube and centrifuged for 311g at 4°C. Cell pellets were washed once with PBSA. The pellet was resuspended in FACOLA staining media (PBSA supplemented with 1% fetal calf serum, 0.09% sodium azide, pH 7.5) such that the cell concentration was 2×10^7 cells/ml.

4.4.2 Fluorescent antibody staining

Following recovery of cells, 50µl of the 2×10^7 cells/ml suspension was added to wells of a 96 well microtiter plate (Corning Inc., Corning, NY, USA) and 50µl of an appropriate murine anti-bovine lineage specific monoclonal antibody was added. Details of antibodies used in

fluorescent antibody staining are contained in table 4.7. Isotype controls were used to allow for accurate quantification of background binding. One well was left unstained to allow for quantification of cell autofluorescence. Cells were incubated on ice for 30 minutes in the dark then washed by adding 100µl FACOLA to each well, centrifuging at 349g for 2 minutes at 4°C then adding 200µl FACOLA and repeating the centrifugation. The procedure was repeated for a total of three washes. The cell pellets were suspended in 50µl FACOLA and 50µl of an appropriate isotype-specific fluorochrome-conjugated secondary antibody was added to each well. Cells were incubated on ice for 30 minutes in the dark then washed as above. If cell surface staining alone was to be performed, cells were fixed by resuspending in 200µl of 2% formaldehyde (Sigma) and the plates were stored in the dark at 4°C for a maximum of 7 days before analysis.

If intracellular staining was to be performed, cells were resuspended in 100µl of a commercially-available fixation/permeabilization solution (BD Cytofix/Cytoperm, BD Biosciences) and incubated for 20 minutes at 4°C in the dark. Cells were washed twice in a commercial permeabilization washing buffer (BD Perm/Wash Buffer, BD Biosciences). Centrifugation during the wash steps was at 863g for 5 minutes at 4°C. The increased centrifugal force was used as cell buoyancy increases following permeabilization. Cells were resuspended in 50µl buffer containing an appropriate concentration of either a fluorochrome or biotin-conjugated anti-cytokine antibody. Cells were incubated for 30 minutes in the dark at 4°C. If a fluorochrome-conjugated anti-cytokine antibody was used, cells were washed as before and resuspended in 200µl FACOLA and the plates were stored in the dark at 4°C for a maximum of 7 days before analysis. If a biotin-conjugated anti-cytokine antibody was used, cells were resuspended in 50µl buffer containing 0.25µg/ml strepavidin conjugated to fluorescin isothiocyanate (FITC) (Serotec, Raleigh, NC, USA). Cells were incubated for 30 minutes in the dark at 4°C, washed as before, resuspended in 200µl FACOLA and stored as for the other assays.

Table 4.7. Antibodies used in fluorescent antibody staining of cells for flow cytometric analysis.

Antibody	Isotype	Supplier and clone	Working dilution (μg/ml)
Mouse anti-bovine CD4	IgG2a	VMRD IL-A11	10
Mouse anti-bovine CD8	IgM	VMRD BAQ111A	6.7
Mouse anti-bovine TcR-N24 (γδ T lymphocytes)	IgG2b	VMRD GB21A	2.5
Mouse anti-ovine CD4	IgM	VMRD GC50A1	10
Mouse anti-ovine CD8	IgG1	VMRD CACT80C	10
Mouse anti-ovine TcR-N6 (γδ T lymphocytes)	IgM	VMRD CACTB6A	10
Mouse anti-ovine CD11c	IgM	VMRD BAQ153A	10
Mouse anti-bovine IFN-γ FITC	IgG1	Serotec MCA1783F	10
Mouse anti-bovine IL4 Biotin	IgG2b	Serotec MCA2372B	5
Mouse anti-bovine IL10 Biotin	IgG1	Serotec MCA2111B	20
Goat anti-mouse IgM PE		Invitrogen M31504	5
Goat anti-mouse IgG1 PE		Invitrogen M32017	5
Goat anti-mouse IgG2a PE		Invitrogen M32204	5
Goat anti-mouse IgG2b PE		Invitrogen M32404	2.5

4.4.3 Flow cytometry

The purposes of the flow cytometry experiments were to determine changes in cell surface marker expression on PBMC and lymph node cells and to determine the cellular source of cytokine production. Variables in the experiment were the proportion of cells staining for each cell surface marker or cytokine.

Specimens were taken from bovine PBMCs cultured in the presence of staphylococcal enterotoxin C, co-cultures of bovine PBMCs and *S. aureus*, freshly isolated cells from ovine supramammary lymph nodes (SMLNs) or from co-cultures of SMLN cells and *S. aureus*. Cell surface markers and cytokines were detected by staining with the appropriate primary and secondary antibodies as detailed in section 4.4.2. Secondary antibodies were conjugated to either the phycoerythrin (PE) or fluorescein isothiocyanate (FITC) fluorophores. These were detected by stimulation with a laser of an appropriate frequency using a FACScan flow cytometer (BD Biosciences). Data were analyzed with the CellQuest program (BD Biosciences). Cells were characterized according to cell size (forward scatter), cell granularity (side scatter) and fluorescent staining. Thresholds for fluorescent staining were determined by using appropriate isotype control antibodies. The number of cells staining for each marker was determined by multiplying the proportion of cells stained by the proportion of viable cells following culture.

Compensation was achieved by using known positive cells stained for each fluorophore. Cells stained singly for each fluorophore and cells stained for both were compared and compensation was adjusted such that minimal interference between the two fluorophores occurred. If live cells were used in experiments, gating was based on propidium iodide staining to differentiate live from dead cells. If fixed cells were used in experiments, gating was based on previous propidium iodide results alongside the forward and side scatter characteristics of the cells. This allows gating of the cells of interest. For staining of cell surface markers, 10,000 gated events were collected. For intracellular staining of cytokines, 50,000 gated events were collected due to the low proportions of cells staining positive for each cytokine.

4.5 Enzyme-linked immunosorbent assays

Detection of IFN-γ, IL-10 and TNF-α into cell culture supernatants was achieved by ELISA. For all procedures, 96 well plates were coated with an anti-bovine cytokine monoclonal antibody diluted in carbonate coating buffer (pH 9.6). Details of antibodies used in the ELISAs are contained in table 4.8.

Plates used for each assay were as follows: IFN-γ and TNF-α: Immunolon 2 (Fisher); IL-10: Maxisorp (Fisher). Diluted antibody was added to plates at a volume of 100µl per well. Plates were incubated overnight at 4°C. Following incubation, plates were washed four times in TBS-T. Samples were applied to plates at 100µl per well at the following dilutions: undiluted, 1:5 and 1:25. Dilutions were carried out in TBS-TG. Standard recombinant bovine cytokines were diluted to 1ng/ml (IFN-γ), 150 units/ml (IL-10) or 5ng/ml (TNF-α) in TBS-TG and two-fold dilutions were made to form a standard curve. Plates were incubated for two hours at room temperature. Following incubation, plates were washed four times in TBS-T and an appropriate secondary antibody was added at 100µl per well. Plates were incubated for one hour at room temperature then washed four times in TBS-T. If the secondary antibody was not biotinylated, a biotinylated goat anti-rabbit IgG (Invitrogen) was added at a 1:10,000 dilution in TBS-TG at 100µl per well. Plates were incubated for one hour at room temperature then washed four times in TBST. Streptavidin alkaline phosphatase (Cedarlane Laboratories, Burlington, ON) was added at a 1:5,000 dilution in TBST-G at 100µl per well. Plates were incubated for one hour at room temperature then washed four times in TBST. Following the wash, 100µl of a 1mg/ml p-nitrophenyl phosphate in substrate buffer was added to each well. Plates were incubated at room temperature for 30 minutes and read using a microtitre plate reader with absorbance set at 405nm and reference set at 490nm. Reactions were not stopped before reading. Cytokine concentrations were calculated using a regression model which compared the sample absorbance with the standard curve.

Table 4.8. Antibodies used in ELISA assays for the detection of cytokines in cell culture supernatants.

Antibody	Supplier	Clone	Working dilution (µg/ml)
Mouse anti-bovine IFN-γ	VIDO	2-2-1A	0.125
Rabbit anti-bovine IFN-γ	VIDO	92-131	0.4
Mouse anti-bovine TNF-α	VIDO	1D11-13	1
Rabbit anti-bovine TNF-α	VIDO	Pool 88	0.67
Mouse anti-bovine IL-10	Serotec	CC318	0.5
Mouse anti-bovine IL-10 biotin	Serotec	CC320	0.125

4.6 Immunohistochemistry assays

Tissues for immunohistochemistry (IHC) were harvested immediately following humane euthanasia of animals, placed in a commercial fixative (Shandon Cryomatrix), frozen on dry ice and stored at -80°C until cutting. Sections of tissue with a thickness of 6µm were cut on a microtome at -20°C, placed on electrostatically charged microscope slides (Superfrost Plus, VWR International, Mississauga, ON) and immediately fixed with 100% acetone for 8 minutes. Slides were air dried and stored at 4°C for up to two weeks. For staining, slides were dried at room temperature for 20 minutes and washed 3 times with PBSA. Non-specific protein binding was blocked by incubating for 20 minutes in PBSA with 1% goat serum. Slides were washed once in PBSA and non-specific avidin and biotin binding were blocked by adding a commercial avidin D blocking solution (Vector Laboratories, Burlington, ON), incubating for 15 minutes, washing once with PBSA and then adding a commercial biotin blocking solution (Vector) and incubating for a further 15 minutes. Slides were washed three times in PBSA and incubated for 60 minutes with 100µl of an appropriate murine anti-bovine lineage specific monoclonal antibody. Slides were washed three times in PBSA and incubated for 60 minutes with 100µl of an appropriate biotinylated secondary antibody. Primary and secondary antibodies used and working concentrations are summarized in table 4.9. Slides were washed three times in PBSA and endogenous peroxidase production was blocked by incubating with PBSA containing 0.1%

sodium azide and 0.3% hydrogen peroxide for 7 minutes. Slides were washed twice in PBSA and incubated in a commercial avidin-biotinylated peroxidase solution (ABC kit, Vector) for 30 minutes. Slides were washed three times in PBSA and a commercial diaminobenzidine peroxidase substrate solution (DAB peroxidase substrate kit, Vector) was added for 5 minutes. Slides were washed twice in PBSA and fixed for 10 minutes with absolute methanol. Staining was intensified by incubating for 10 minutes in 0.5M copper sulphate. Slides were washed once in distilled water and counterstained for 120 seconds with 5% Giemsa stain in distilled water (Sigma). Slides were washed twice in distilled water, dried overnight and coverslipped using a commercial, toluene-based mounting medium (Shurmount, Triangle Biomedical Sciences, Durham, NC, USA).

Table 4.9. Antibodies used in immunohistochemical assays for the detection of cell surface markers**.**

Antibody	Isotype	Supplier and clone	Working dilution (µg/ml)
Mouse anti-ovine CD4	IgM	VMRD GC50A1	10
Mouse anti-ovine CD8	IgG1	VMRD CACT80C	10
Mouse anti-ovine TcR-N6 (γδ T lymphocytes)	IgM	VMRD CACTB6A	10
Mouse anti-ovine CD11c	IgM	VMRD BAQ153A	10
Mouse anti-ovine CD205	IgG2b	Serotec CC98	10
Goat anti-mouse IgM biotin		Invitrogen M31515	20
Goat anti-mouse IgG1 biotin		Invitrogen A10519	20
Goat anti-mouse IgG2b biotin		Invitrogen M32515	20

4.7 Bacterial DNA manipulations

4.7.1 Genomic and plasmid DNA extraction

To extract genomic DNA from *S. aureus* isolates, bacteria were grown overnight in TSB at 37°C. Bacteria were centrifuged at 1940 g for 10 minutes, supernatants were removed and bacteria were suspended in 2ml lysis buffer (20mM Tris HCl, 2mM EDTA, 1% Triton-X, 100µg/ml lysozyme, 5 units/ml lysostaphin and 0.1mg/ml RNaseI). Bacterial suspensions were incubated at 37°C for 30 minutes, bacterial lysis was confirmed visually by observing the presence of gelatinous material and 25µl of a 25mg/ml proteinase K solution was added to each suspension. Suspensions were incubated at 56°C for 60 minutes. Following incubation, 20µl sodium acetate (3M, pH 5.2) and 5ml ethanol (100%, -20°C) was added to each isolate to precipitate DNA. Precipitated DNA was removed on a glass spool, rinsed three times in 70% ethanol and dissolved overnight in distilled water at 4°C.

To extract plasmid DNA from *E. coli* isolates, one of two procedures was performed. If small quantities of plasmid DNA were required, bacteria were grown overnight in LB broth at 37°C. Aliquots of the bacterial suspensions (1.5ml) were centrifuged at 20,000g for 3 minutes, supernatants were removed and bacteria were suspended in 100µl of cold solution I (50mM glucose, 25mM TrisHCl, 10mM EDTA) and incubated on ice for 5 minutes. Following incubation, 200µl solution II (0.2N NaOH, 0.5% SDS) was added to each suspension and incubated on ice for 5 minutes. Following incubation, 150µl potassium acetate (3M, pH 5.3) was added to each suspension and incubated at -20°C for 10 minutes. Suspensions were centrifuged at 20,000g for 15 minutes, 400µl of the supernatant was transferred to a clean microcentrifuge tube and 240µl isopropanol (100%, -20°C) was added to each sample. Samples were incubated for 15 minutes at room temperature then centrifuged at 20,000g for 15 minutes. Supernatants were removed; samples were washed three times in 500µl 70% ethanol and dried at 42°C. Once dry, DNA was resuspended in 50µl RNase (5µg/ml) and incubated for 15 minutes at 37°C.

If plasmid DNA of high purity was required for transformation of bacterial species, a commercial DNA purification column was used according to the manufacturer's instructions (QIAGEN Plasmid Midi Kit, QIAGEN Inc.).

4.7.2 PCR procedures

To amplify the *sec* gene for expression of SEC protein, a *S. aureus* strain possessing the *sec* gene was obtained. The presence of the *sec* gene encoding SEC was demonstrated using PCR primers based on published sequences of the gene and designed to amplify the segment of the gene encoding the mature protein (table 4.10). To amplify the *sec* gene for transforming *S. aureus*, primers were designed to include the promoter sequence for *sec* (table 4.10). The template DNA was added to a reaction mixture containing a commercial reaction buffer (GE Healthcare), 3mM dNTPs, 1.6µM of each primer and 0.1 units of Taq DNA polymerase (Illustra rTaq DNA polymerase, GE Healthcare). Conditions for the PCR reaction were as follows: 1 cycle of 2 minutes at 94°C; 30 cycles of 1 minute at 94°C, 1 minute at 47°C, 1 minute at 72°C and a final cycle of 5 minutes at 72°C. Aliquots of the sample were analysed by running on a 1% agarose gel, resolving the bands by electrophoresis, staining the gel with ethidium bromide and photographing the gel under UV light conditions.

Table 4.10. Primers used for the amplification of DNA sequence to be used in cloning experiments.

Gene	Direction	Primer sequence
SEC	Forward	5´-AAAAAGGATCCGAGAGCCAACCAGACCCTACG-3´
	Reverse	5´-AAAAACCCGGGTTATCCATTCTTTGTTGTAAGG-3´
SEC with promoter sequence	Forward	5´-AAAAAGAATTCTTGAATATTTAAGATTATAAGATATATTTAAAG-3´
	Reverse	5´-AAAAACCCGGGTTATCCATTCTTTGTTGTAAGG-3´

4.7.3 Restriction digestion and DNA ligation procedures

For expression of the SEC protein, the PCR product and pQE-30 expression vector were digested with the *BamH*I and *Sma*I restriction enzymes according to the manufacturer's instructions (New England BioLabs (NEB), Pickering, ON). The *sec* gene was ligated into the pQE-30 expression vector using a commercial DNA ligase enzyme according to the manufacturer's instructions (NEB). The gene was sequenced and BLAST analysis of the gene sequence revealed a 92%

homology with the published sequence. BLAST analysis of the protein sequence revealed a 100% homology with the published sequence.

For transformation of *S. aureus* with *sec*, the PCR product and pAW11 shuttle vector were digested with the *EcoR*I and *Sma*I restriction enzymes according to the manufacturer's instructions (NEB). The *sec* gene was ligated into the pAW11 vector using a commercial DNA ligase enzyme according to the manufacturer's instructions (T4 DNA ligase, NEB).

4.7.4 Transformation of Escherichia coli and Staphylococcus aureus strains with DNA

Electrotransformation was used to transform bacterial strains with DNA. *Escherichia coli* cells for use in electrotransformation procedures were prepared according to the following procedure. Fresh LB broth was inoculated with a 1/100 volume of a fresh overnight *E. coli* culture. Bacteria were grown at 37°C with shaking until the optical density at a wavelength of 600nm was 0.3-0.4. Cells were chilled on ice for 15 to 30 minutes then centrifuged at 4,000g for 15 minutes at 4°C. Supernatants were removed and cell pellets were resuspended in an identical volume of cold water. Cells were centrifuged as before and resuspended in 50% of the volume of cold water. Cells were centrifuged as before and resuspended in 4% of the original volume of 10% glycerol. Cells were centrifuged a final time and resuspended in 10% glycerol such that the final cell concentration was 3×10^{10} cells/ml. Cells were frozen in 50µl aliquots on dry ice and stored at -70°C. *Staphylococcus aureus* cells for use in electrotransformation procedures were prepared according to a similar procedure except cells were cultured in TS broth and washes and resuspensions were performed in 500mM sucrose.

Electroporation was performed by adding between 1µg and 5µg DNA to an aliquot of electrocompetent bacterial cells. If *E. coli* was to be transformed, the mixture was added to a 1mm or 2mm electroporation cuvette. For 1mm cuvettes, electroporation was carried out at 1.8kV with a 25µF capacitor and the pulse controller unit set to 200Ω. For 2mm cuvettes, the electroporation was carried out at 2.5kV with no change to the other parameters. If *S. aureus* was to be transformed, the mixture was added to a 2mm electroporation cuvette and electroporation was carried out at 2.5kV with a 25µF capacitor and the pulse controller unit set to 100Ω. Immediately following electroporation, transformed *E. coli* were placed in SOC media

(2% bacto-tryptone, 0.5% w/v bacto-yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 20mM glucose) for 1 hour at 37°C for non-temperature sensitive plasmids or 2 hours at 30°C for temperature sensitive plasmids. Transformed *S. aureus* were placed in TS broth for 4 hours at 37°C for non-temperature sensitive plasmids or 6 hours at 30°C for temperature sensitive plasmids. Following recovery, bacteria were plated on either LB agar (*E. coli*) or TS agar (*S. aureus*) containing an appropriate antibiotic concentration for the plasmid used. Details of antibiotic concentrations for each plasmid are included in table 4.11.

Table 4.11 Antibiotic concentrations used for each plasmid during cloning experiments

Plasmid	Bacterial species	Antibiotic	Concentration (μg/ml)
pQE-30	*E. coli*	Carbenicillin	50
pAW11	*E. coli*	Erythromycin	300
pAW11	*S. aureus*	Erythromycin	5

4.7.5 Protein expression, purification and quantification

To express SEC protein, an overnight culture *E. coli* transformed with pQE-30 expressing the *sec* gene was added at a 1:100 dilution to LB broth (Difco) and incubated at 30°C with shaking until mid-log phase. Following incubation, 0.2mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to initiate transcription of the *lac* operon thus inducting protein expression. Cultures were incubated for 2 hours at 37°C with shaking then centrifuged at 3000g for 10 minutes. Supernatants were discarded and pellets were suspended in 0.9% sodium chloride and frozen overnight. Cell pellets were thawed on ice and resuspended in lysis buffer (50mM sodium phosphate, 300mM sodium chloride, 10mM imidazole, pH 8) and 1mg/ml lysozyme was added. The mixture was sonicated (40% amplitude, 10s burst, 10s cooling for 10 cycles on ice) then the lysate was centrifuged at 11,500g for 30 minutes at 4°C. The supernatant was saved for protein purification.

Use of the pQE-30 plasmid for cloning of the *sec* gene causes a 6x histidine tag to be present at the N-terminus of the SEC protein. This allows for protein purification using nickel chromatography. The supernatant was added to nickel-NTA resin and mixed at 4°C for 60 minutes. The mixture was added to a column, washed three times in wash buffer (50mM sodium phosphate, 300mM sodium chloride, 20mM imidazole, pH 8) then protein was eluted with three washes in elution buffer (50mM sodium phosphate, 300mM sodium chloride, 250mM imidazole, pH 8). Protein concentration was determined using a modified Lowry technique. Protein purity was determined by running on a 12% SDS-PAGE gel and densitometry.

4.8 Statistical analysis

To compare the effects of different *S. aureus* isolates on PBMC cytokine secretion *in vitro*, a one way repeated measures analysis of variance (ANOVA) was performed with isolate as the independent variable and time as the repeated measure. Cytokine secretion data in the current studies was not parametrically distributed therefore data were ranked for analysis in accordance with previous studies [265-268]. To compare the effects of different isolates on cytokine gene expression by PBMCs *in vivo*, a one way repeated measures ANOVA was performed with isolate as the independent variable and time as the repeated measure. Previous work has identified data from similar experiments as being non-parametrically distributed [269-271]. An attempt was made to make a logarithmic transformation of the data [271] but this was unsuccessful therefore data were ranked for analysis [269, 270].

To compare bacterial shedding with different *S. aureus* isolates, a one way ANOVA was performed with isolate as the independent variable and time as the repeated measure. To determine the effects of different *S. aureus* isolates on milk somatic cell counts, a two way repeated measures ANOVA was performed with isolate and quarter challenge status as the independent variables and time as the repeated measure. The data from control udder halves were subtracted from the data from challenged udder halves to correct for pairing. Somatic cell count data were transformed using a $\log_{10}$ transformation before analysis [4, 6, 213].

To compare the effects of different *S. aureus* isolates on leukocyte lineage specific marker and intracellular cytokine staining, multiple t-tests were used to compare infected and uninfected

udder halves. To compare the effect of different *S. aureus* isolates and duration of infection, the data from control udder halves were subtracted from the data from challenged udder halves to correct for pairing. These data were then analysed using a two-way ANOVA in which isolate and duration of infection were set as the independent variables. Given that previous work has reported that qRT-PCR data regarding cytokine expression in the mammary gland is parametrically distributed [206-211], these data were not transformed.

To compare the effects of different *S. aureus* isolates on cytokine expression in tissues, multiple t-tests were used to compare infected and uninfected udder halves. To compare the effect of different *S. aureus* isolates and duration of infection, the data from control udder halves were subtracted from the data from challenged udder halves to correct for pairing. These data were then analysed using a two-way ANOVA in which isolate and duration of infection were set as the independent variables. Given that previous work has reported that qRT-PCR data regarding cytokine expression in the mammary gland is parametrically distributed [6, 213], these data were not transformed.

All analyses were performed using commercially available statistical analysis software (GraphPad Prism, GraphPad Software Inc., La Jolla, CA, USA). Significance level was set at $P<0.05$. However, given the low numbers of animals present in each treatment group for *in vivo* studies, differences with $P<0.10$ may also be truly significant. Use of increased numbers of animals would be able to establish whether differences are truly significant.

5. RESULTS

5.1 In *vitro* effects of *Staphylococcus aureus* isolates on bovine peripheral blood mononuclear cells

5.1.1 Screening of Staphylococcus aureus isolates

Eight dairy cows with recall responses to *S. aureus* were sourced from the University of Saskatchewan dairy facility and used as blood donors for PBMC isolation. Nine wild type *S. aureus* isolates were incubated with PBMCs for 24, 48 and 72 hours. Secretion of IFN-γ (Table 5.1), IL-10 (Table 5.2) and TNF-α (Table 5.3) into cell culture supernatants was determined by ELISA. Due to the consumption of IL-4 by PBMCs in cell culture [272], IL-4 was assayed by determining gene expression using qRT-PCR (Table 5.4).

Table 5.1. Summary of the effect of different *S. aureus* isolates on IFN-γ secretion by bovine PBMCs. Median IFN-γ concentrations (pg/ml) are reported. * represents a significant increase in concentration when compared with media alone.

	Time (h)		
Isolate	24	48	72
SA8	5175*	16724*	10592*
SA10	2178*	2776*	2579*
SA12	502	871	657
SA23	1817*	15518*	18645*
SA25	273	923	710
SA31	718*	943*	1020*
SA34	671*	1208*	1165*
SA37	637	926*	929
SA45	952*	1342*	3861*

Table 5.2. Summary of the effect of different *S. aureus* isolates on IL-10 secretion by bovine PBMCs. Median IL-10 concentrations (units/ml) are reported. * represents a significant increase in concentration when compared with media alone.

	Time (h)		
Isolate	24	48	72
SA8	358*	394*	428*
SA10	594*	524*	489*
SA12	280	226	247
SA23	59*	103*	195*
SA25	60*	61*	87
SA31	164	233	182
SA34	236*	268*	259*
SA37	210*	225*	242*
SA45	196*	163	231*

Table 5.3. Summary of the effect of different *S. aureus* isolates on TNF-α secretion by bovine PBMCs. Median TNF-α concentrations (pg/ml) are reported. * represents a significant increase in concentration when compared with media alone.

	Time (h)		
Strain	24	48	72
SA8	4418*	4471*	3983*
SA10	7545*	6120*	4490*
SA12	709	591	694
SA23	605*	1792*	3264*
SA25	282	649	1096
SA31	1172	1249	963
SA34	1598*	1709*	1599*
SA37	189	150	277
SA45	243	1244*	603*

Table 5.4. Summary of the effect of different *S. aureus* isolates on IL-4 mRNA transcription by bovine PBMCs. Median relative expression values are reported. * represents a significant change in relative expression when compared with media alone.

	Time (h)		
Strain	24	48	72
SA8	22.5	0.12	26.5
SA10	0.40	3.78	0.64
SA12	0.33	0.06	8.8
SA23	3.8	0.65	1.12
SA25	0.60	0.22*	1.04
SA31	0.99	0.36	1.36
SA34	2.82	0.61	0.83
SA37	0.59*	0.20*	3.74*
SA45	0.33*	0.21*	1.24

Six isolates (SA8, SA10, SA23, SA31, SA34 and SA45) significantly increased IFN-γ secretion at all time points with one isolate (SA37) significantly increasing IFN-γ secretion following 48 hours of incubation. Two isolates (SA8 and SA23) caused dramatic increases in IFN-γ secretion compared to other *S. aureus* isolates. Two isolates (SA10 and SA45) caused more moderate increases in secretion compared to other isolates. Five isolates (SA8, SA10, SA23, SA34 and SA37) significantly increased IL-10 secretion at all time points with one isolate significantly increasing IL-10 secretion following 24 and 72 hours of incubation (SA45). Two isolates (SA8 and SA10) caused dramatic increases in IL-10 secretion compared to other *S. aureus* isolates. Four isolates (SA8, SA10, SA23 and SA34) significantly increased TNF-α secretion at all time

points with one isolate (SA45) significantly increasing TNF-α secretion following 48 and 72 hours of incubation. Three isolates (SA8, SA10 and SA23) dramatically increased TNF-α secretion compared to other *S. aureus* isolates. Transcription of IL-4 mRNA was not consistently affected by *S. aureus*. Generally, a decrease in transcription was observed. Two isolates (SA37 and SA45) decreased IL-4 transcription at 24 and 48 hours of incubation and one isolate (SA25) decreased IL-4 transcription at 48 hours. One isolate (SA37) increased transcription at 72 hours of incubation.

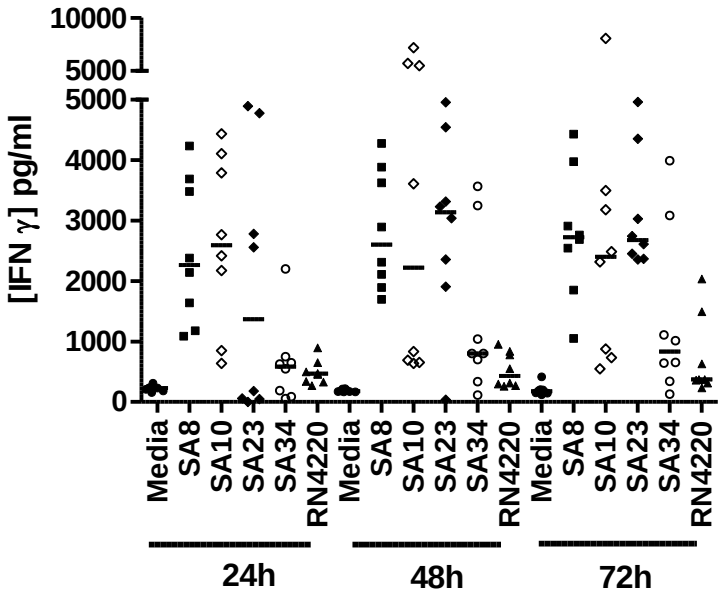
Based on these data, isolate variation appears to exist in the cytokine response of bovine PBMCs to *S. aureus*. Interestingly, the most dramatic responses appear to be because of isolates which possess the *sec* gene. However, isolates that possess the *sec* gene had varying effects. For example, the SA8 and SA23 isolates caused dramatic increases in TNF-α, IFN-γ and IL-10. This suggests that these isolates induce both pro-inflammatory (indicated by IFN-γ and TNF-α) and anti-inflammatory (indicated by IL-10 responses). The SA10 isolate induced a moderate increase in IFN-γ but dramatic increases in IL-10 and TNF-α. This suggests that this isolate induces both pro-inflammatory and anti-inflammatory response. Whilst these isolates possess the *sec* gene, the role of *sec* in these responses is unclear. As *S. aureus* RN4220 expressing SEC had a minimal effect, it appears that SEC does not have major effects alone.

One major weakness in these data is that, due to the number of cells required for each assay, it was impossible to assay all isolates at the same time therefore different isolates were assayed on different days. The experimental model for screening did not allow for variations in PBMC responses based on the day the experiment was run. Therefore, it was decided to re-run the isolates that had the largest effects on PBMC cytokine production against a consistent control strain of *S. aureus*. The RN4220 strain was chosen as the control strain.

5.1.2 Comparison of select Staphylococcus aureus isolates with a control strain

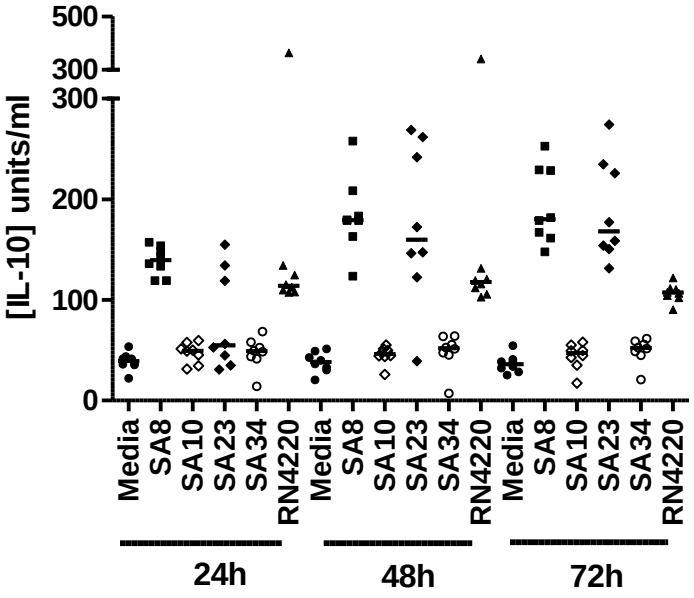
The SA8, SA10 and SA23 isolates were compared against RN4220 due to the large effects that they had on PBMC cytokine secretion. The SA34 isolate was used as it appeared to be representative of the remaining screened isolates. Responses to the RN4220 isolate were consistent among assays therefore representative data for RN4220 are presented. Concentrations of IFN-γ were significantly increased by *S. aureus* SA8 at all timepoints when compared with media alone or RN4220. Concentrations of IFN-γ were significantly increased by *S. aureus* SA10 at all timepoints when compared with media alone and at 24 hours when compared with RN4220. Concentrations of IFN-γ were significantly increased by *S. aureus* SA23 at 48 and 72 hours when compared with media alone or RN4220. Concentrations of IFN-γ were significantly increased by *S. aureus* SA34 at all timepoints when compared with media alone (Figure 5.1). The SA8, SA10 and SA23 isolates dramatically increased IFN-γ concentrations compared with RN4220.

Figure 5.1. Effect of *S. aureus* on IFN-γ secretion by bovine PBMCs at 24, 48 and 72 hours incubation when compared with RN4220. Bars represent median values. All isolates significantly increased IFN-γ secretion compared with media. No isolate differed significantly with RN4220.



Concentrations of IL-10 were significantly increased by *S. aureus* SA8 at 48 and 72 hours when compared with media alone and at 72 hours when compared with RN4220. Concentrations of IL-10 were significantly increased by *S. aureus* SA10 at all timepoints when compared with media alone. Concentrations of IL-10 were significantly increased by *S. aureus* SA23 at 48 and 72 hours when compared with media alone and at 24 hours when compared with RN4220. Concentrations of IL-10 were significantly increased by *S. aureus* SA34 at 48 and 72 hours when compared with media alone and at 24 hours when compared with RN4220 (Figure 5.2). Interestingly, the IL-10 concentrations generated by the SA10 isolate appeared smaller than those generated when this isolate was run previously. Given that no positive control was run previously, I believe the SA10 data reported here to be representative of the normal behaviour of SA10.

Figure 5.2. Effect of *S. aureus* on IL-10 secretion by bovine PBMCs at 24, 48 and 72 hours of incubation compared with RN4220. Bars represent median values. All isolates significantly increased IL-10 secretion compared with media. No isolate differed significantly with RN4220.



Concentrations of TNF-α were significantly increased by *S. aureus* SA8 at all timepoints when compared with media alone or RN4220. Concentrations of TNF-α were significantly increased by *S. aureus* SA10 at all timepoints when compared with media alone. Concentrations of TNF-α were significantly increased by *S. aureus* SA23 at 48 and 72 hours when compared with media alone or RN4220. Concentrations of TNF-α were significantly increased by *S. aureus* SA34 at all timepoints when compared with media alone (Figure 5.3). The SA10 and SA23 isolates dramatically increased TNF-α concentrations compared with RN4220. Expression of IL-4 was significantly increased compared with media alone by SA8 at 48 hours, by SA10 at 24 and 48 hours and by SA23 and 48 and 72 hours. Expression was significantly increased compared with RN4220 by SA8 at 24 and 48 hours, by SA10 at 24 hours and by SA23 at 24, 48 and 72 hours (Figure 5.4). Expression of IL-4 was unaffected by the SA34 isolate.

Figure 5.3. Effect of *S. aureus* on TNF-α secretion by bovine PBMCs at 24, 48 and 72 hours of incubation compared with RN4220. Bars represent median values. All isolates significantly increased TNF-α secretion compared with media. No isolate differed significantly with RN4220.

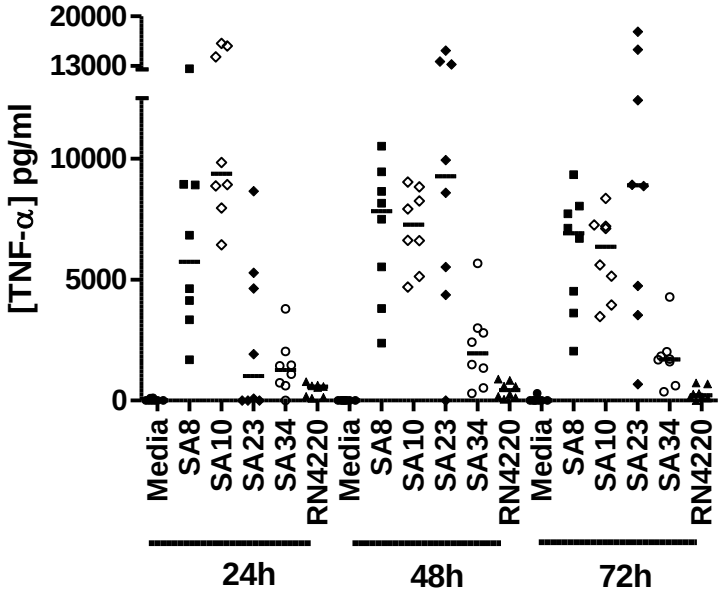
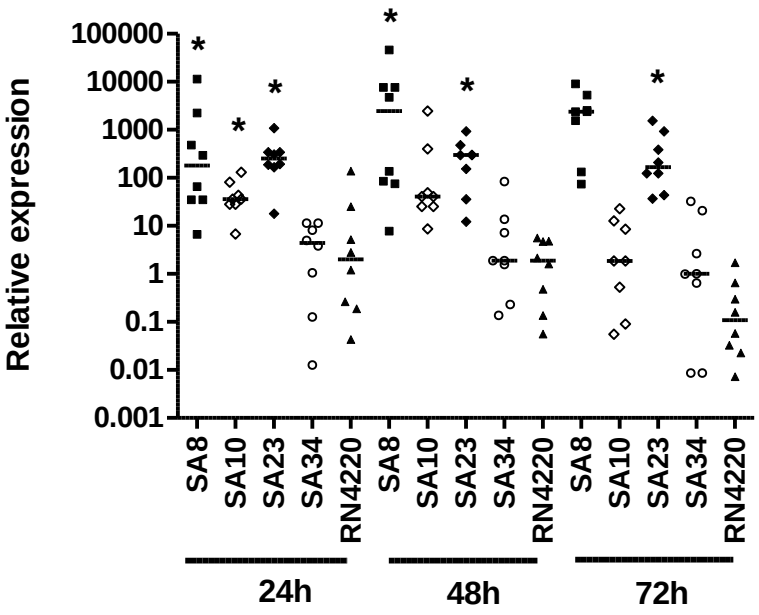


Figure 5.4. Effect of *S. aureus* on IL-4 expression by bovine PBMCs at 24, 48 and 72 hours of incubation compared with RN4220. Bars represent median values. * indicates significantly increased IL-4 expression compared with RN4220.



It is important to identify the cellular source of IL-10 since it may indicate the presence of regulatory T cells. Intracellular staining for IL-10 was carried out on PBMCs exposed to the SA8 isolate. Observation of the flow cytometry profiles suggested that a population of CD4+ IL10+ cells was generated by the SA8 isolate which was not generated by RN4220 (Figure 5.5). Analysis of the number of viable cells of each type revealed that SA8 generated significantly higher numbers of CD4+ IL10+ cells than either RN4220 or media alone (Figure 5.6). The source of IL-10 in cells exposed to RN4220 appears to be CD8 and γδ T cells (Figures 5.5 and 5.6).

Based on these data, the isolate variation observed in the cytokine response of bovine PBMCs to *S. aureus* is consistent with what was reported in section 5.1.1. These data are stronger however as assays were run with a consistent positive control. Generally, *S. aureus* isolates appear to cause increases in IFN-γ, IL-10 and TNF-α secretion and IL-4 expression compared with media alone although differences between isolates are apparent.

Figure 5.5. Representative flow cytometry profiles from cells co-cultured with PBMCs and *S. aureus*. Panel (a) indicates the gated cell population, panel (b) represents CD4+ IL-10+ cells incubated with media alone, panel (c) represents CD4+ IL-10+ cells incubated with SA8 and panel (d) represents CD4+ IL-10+ cells incubated with RN4220.

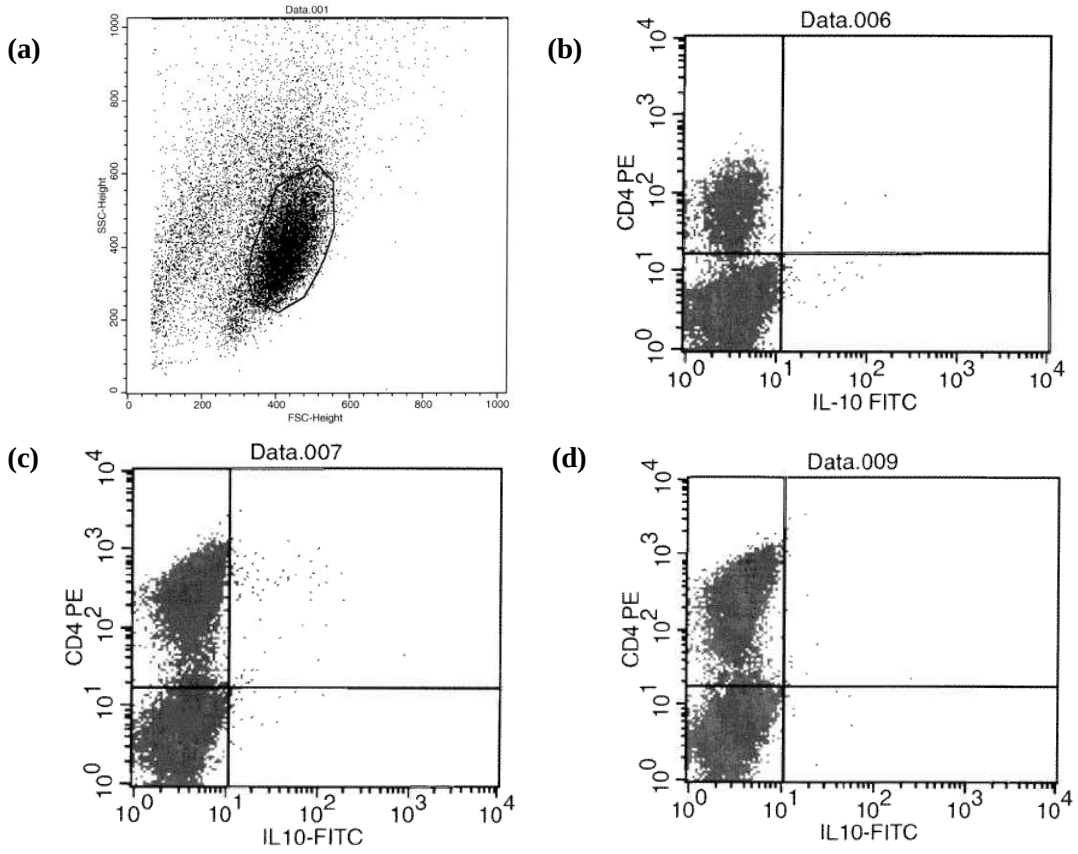
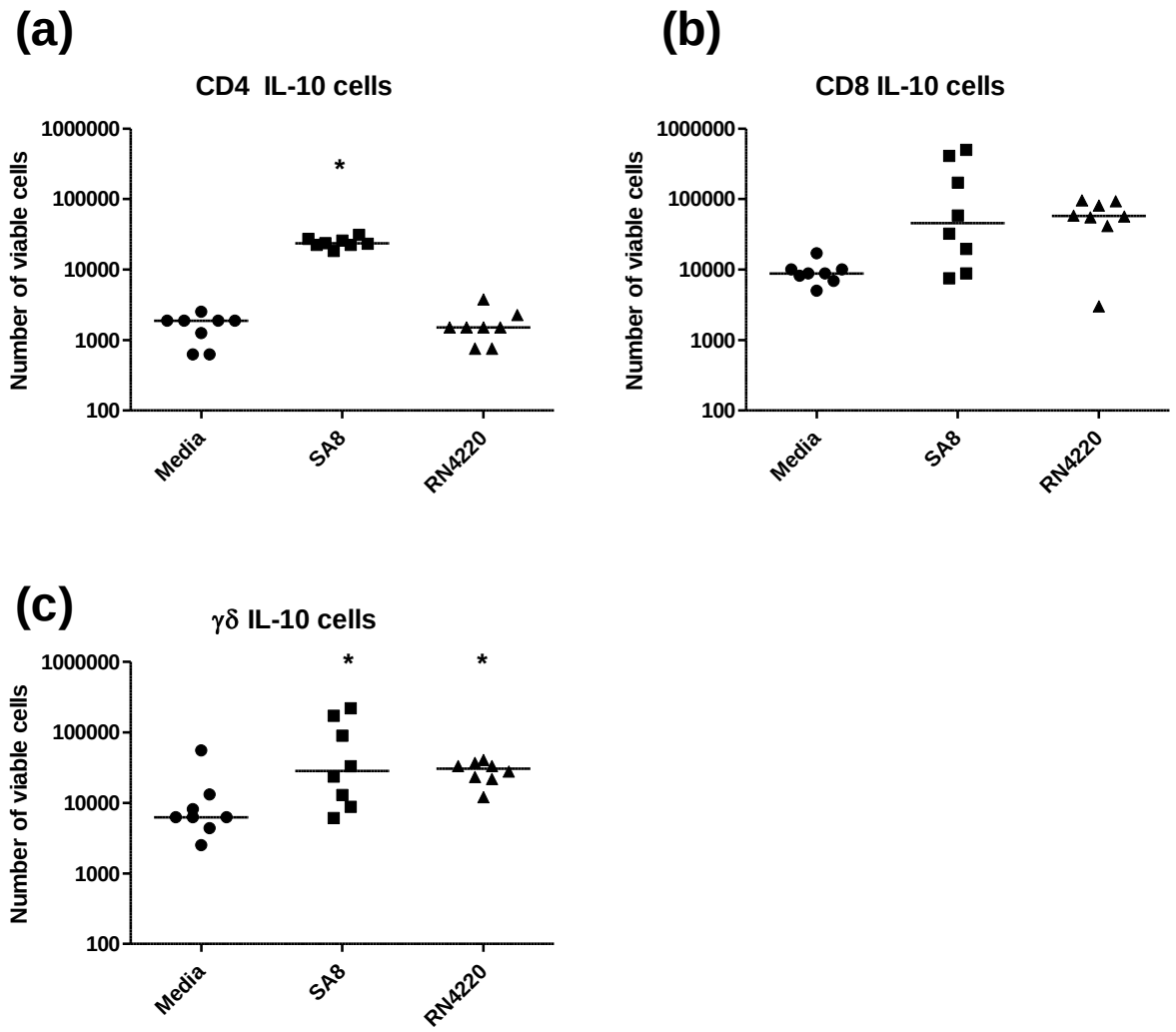


Figure 5.6. Cellular source of IL-10 following incubation of SA8 with bovine PBMCs for 24 hours. Number of viable cells was determined as in section 4.4.3. Bars represent median values. * denotes a significant increase in viable cell numbers compared with RN4220. Panel (a) represents CD4+ IL-10+ cells, panel (b) represents CD8+ IL-10+ cells and panel (c) represents γδ+ IL-10+ cells.



5.2 Effects of staphylococcal enterotoxin C on bovine peripheral blood mononuclear cells *in vitro*

5.2.1 Effect of purified staphylococcal enterotoxin C protein on bovine peripheral blood mononuclear cells

To determine the effect of SEC protein on bovine PBMCs, mixed breed naive beef calves were used as blood donors. Cells were incubated with 10µg/ml SEC protein for 24, 48 and 72 hours. Secretion of IFN-γ and IL-10 into culture supernatants was determined by ELISA and expression of cell surface markers (CD4, CD8, CD25, ACT2) was determined by flow cytometry. Incubation with SEC did not significantly affect concentrations of IFN-γ in cell culture supernatants (Figure 5.7) but did increase IL-10 concentrations at 48 and 72 hours (P = 0.020) (Figure 5.8). Cell surface marker expression did not vary with exposure to SEC protein (Figures 5.9 and 5.10).

Figure 5.7. Concentration of IFN-γ in culture supernatants following incubation of bovine PBMCs in the presence or absence of 10µg/ml SEC for 24, 48 or 72 hours. Bars represent median values.

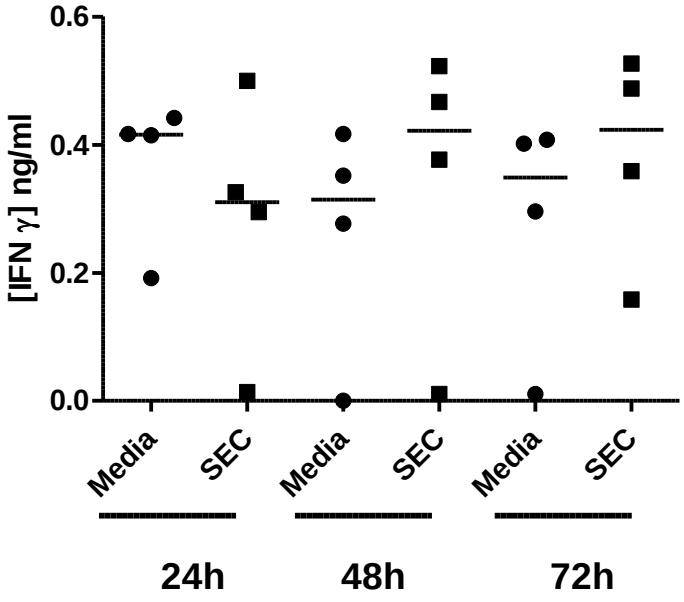


Figure 5.8. Concentration of IL-10 in culture supernatants following incubation of bovine PBMCs in the presence or absence of 10μg/ml SEC for 24, 48 or 72 hours. Bars represent median values. * represents a significant ($P < 0.05$) increase in IL-10 compared with media alone.

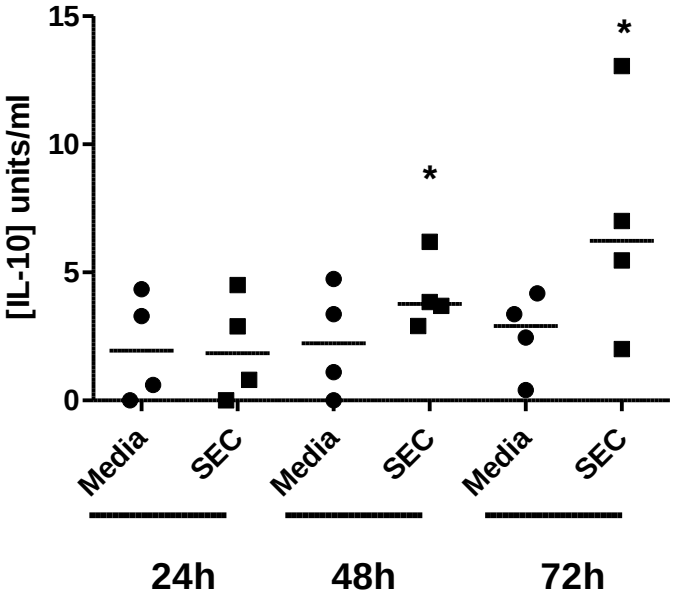


Figure 5.9. Representative flow cytometry profiles from PBMCs co-cultured with media alone (panel a) or 10μg/ml staphylococcal enterotoxin C from 72 hours (panel b). Cells were stained for CD4 and CD25.

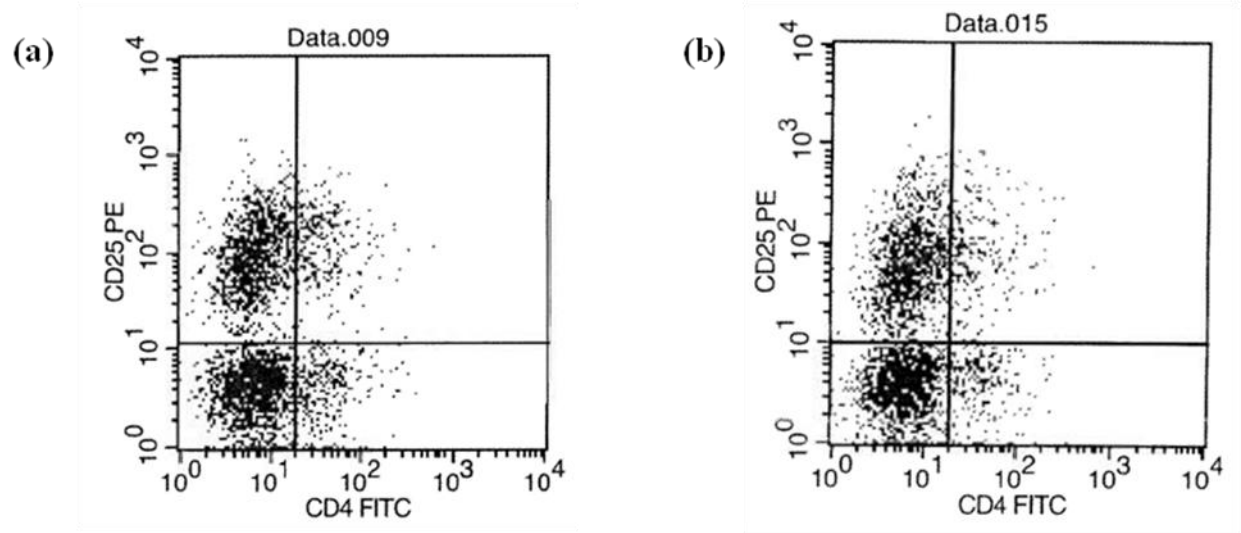
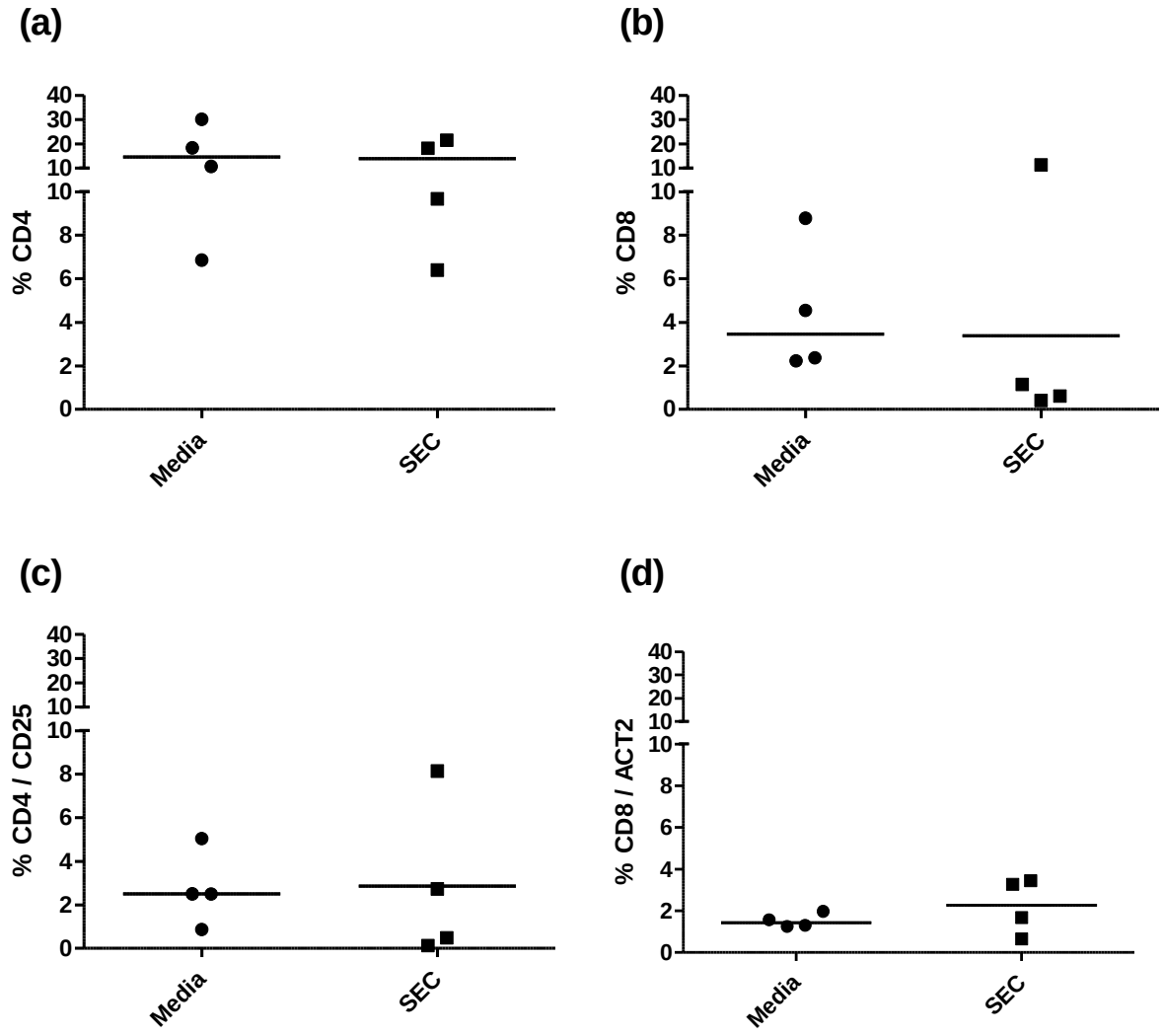


Figure 5.10. Proportions of each cell population following incubation of bovine PBMCs in the presence or absence of 10µg/ml SEC for 72 hours. Bars represent median values. Panel (a): CD4+ cells, (b): CD8+ cells, (c) all CD4+ CD25+ cells and (d) CD8+ ACT2+ cells.



5.2.2 Effect of a Staphylococcus aureus strain transformed for staphylococcal enterotoxin C on bovine peripheral blood mononuclear cells

To determine the effect of a *S. aureus* strain transformed for SEC on bovine PBMCs, we transformed the *S. aureus* RN4220 strain with the *sec* gene. The gene for *sec* was amplified by PCR. Primers were designed so that the promoter for *sec* was incorporated in the PCR product. The product was ligated into the pAW11 vector and was aligned in the opposite orientation to the promoter contained within the vector. The ligation product was used to transform *E. coli* JM109 cells and purified plasmid was obtained. This was used to transform *S. aureus* RN4220 cells. The presence of the *sec* gene in the RN4220 cells was confirmed by PCR. The pAW11 vector alone was used to transform *S. aureus* RN4220 and the resultant strain was used as a control. Secretion of SEC into cell culture supernatants was confirmed by western blot. An attempt was made to make the western blot semi-quantitative using known dilutions of SEC to create a standard curve. Unfortunately, the amount of SEC secreted into cell culture supernatants was insufficient to be compared with the standards by densitometry.

The transformed strain (RN4220-SEC) was incubated with PBMCs for 24, 48 and 72 hours. Secretion of IFN-γ, IL-10 and TNF-α were significantly increased by *S. aureus* RN4220 however transformation with the *sec* gene did not affect cytokine concentrations (Figures 5.11, 5.12 and 5.13). Transcription of IL-4 mRNA was decreased by *S. aureus* RN4220 although the decrease appeared to occur 24 hours sooner when the strain was transformed for the *sec* gene (Figure 5.14). These data suggest that transformation of RN4220 with the *sec* gene causes a transient decrease in anti-inflammatory cytokine release.

Figure 5.11. Effect of *S. aureus* RN4220 transformed with the pAW11 vector containing the *sec* gene on IFN-γ secretion by bovine PBMCs at 24, 48 and 72 hours of incubation compared with RN4220 transformed with vector alone. Bars represent median values. All isolates significantly increased IFN-γ secretion compared with media. Transformation with the *sec* gene did not have a significant effect.

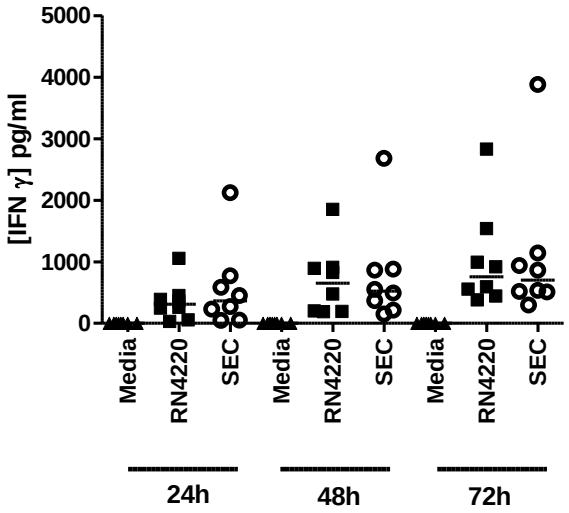


Figure 5.12. Effect of *S. aureus* RN4220 transformed with the pAW11 vector containing the *sec* gene on IL-10 secretion by bovine PBMCs at 24, 48 and 72 hours of incubation compared with RN4220 transformed with vector alone. Bars represent median values. All isolates significantly increased IL-10 secretion compared with media. Transformation with the *sec* gene did not have a significant effect.

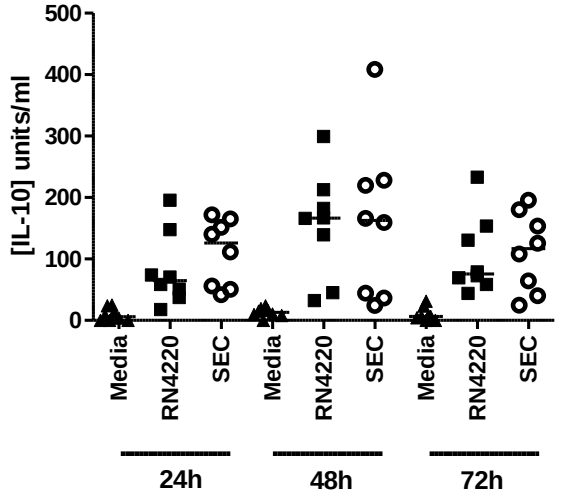


Figure 5.13. Effect of *S. aureus* RN4220 transformed with the pAW11 vector containing the *sec* gene on TNF-α secretion by bovine PBMCs at 24, 48 and 72 hours of incubation compared with RN4220 transformed with vector alone. Bars represent median values. All isolates significantly increased TNF-α secretion compared with media. Transformation with the *sec* gene did not have a significant effect.

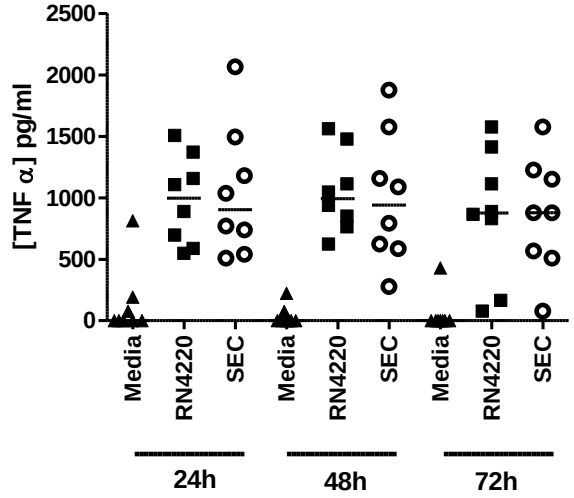
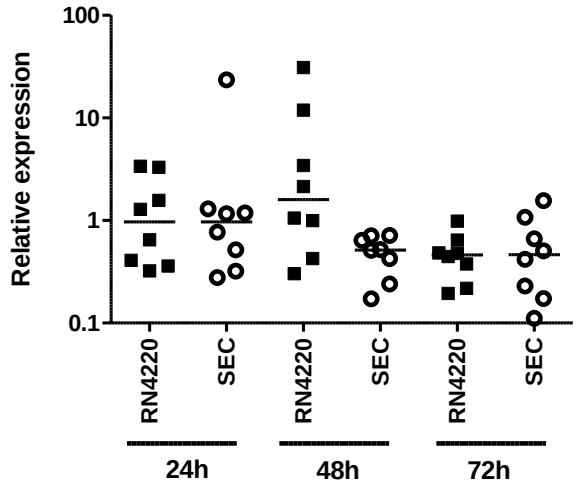


Figure 5.14. Effect of *S. aureus* RN4220 transformed with the pAW11 vector containing the *sec* gene on IL-4 gene expression by bovine PBMCs at 24, 48 and 72 hours of incubation compared with RN4220 transformed with vector alone. Bars represent median values. Expression of IL-4 mRNA was significantly decreased compared with media at 48 and 72 hours for RN4220 transformed with *sec* and at 72 hours for RN4220 transformed with vector alone.



5.3 Establishment of a model of ovine intramammary infection

A reliable model of ovine IMI with *S. aureus* is required before the effects of *S. aureus* on ovine mammary cellular immune responses can be determined. Nine ewes which, between then, possessed 14 uninfected mammary halves were challenged with *S. aureus* isolates SA8 and SA10 as summarized in table 4.3. Due to the limited number of ewes available, some ewes were challenged with a different *S. aureus* isolate in each mammary half. Based on morphological characteristics of recovered colonies, *S. aureus* strains did not move between mammary halves. If a ewe was challenged with more than one *S. aureus* isolate, the same challenge dose was used for each. All experimental intramammary challenges resulted in IMI which persisted for the duration of the study (Figures 5.15 and 5.16). Repeated measures ANOVA revealed that bacterial shedding did not vary significantly for either SA8 ($P = 0.56$) or SA10 ($P = 0.33$). As the animals used were non-lactating, it was not consistently possible to collect sufficient mammary secretion to determine udder half SCCs on each day of the study. However, similar SCCs were observed following challenge in udder halves infected with each challenge dose (Figures 5.17 and 5.18). Transient episodes of clinical mastitis were observed in two animals during the study. Rectal temperature

did not vary more than 0.4°C from the rectal temperature recorded on the day of challenge (Figure 5.19) and no systemic clinical signs were observed. Due to the fact that some ewes were challenged with a different isolate in each udder half, the effect of each isolate on clinical disease could not be assessed.

Given that both isolates generated subclinical disease at all doses and no significant differences were noted between doses, the 50cfu dose was chosen as the challenge dose for studies of the effect of *S. aureus* on mammary cellular immune responses *in vivo*.

Figure 5.15. Bacterial shedding from mammary halves of ewes challenged with 10cfu (circles), 50cfu (squares) or 200cfu (triangles) of *S. aureus* isolate SA8 on days 3, 5, 9, 12 and 15 following challenge. Data are presented as median cfu/ml. Error bars represent range. Bacterial shedding did not vary significantly between groups (P = 0.56) or between day post-challenge (P = 0.12).

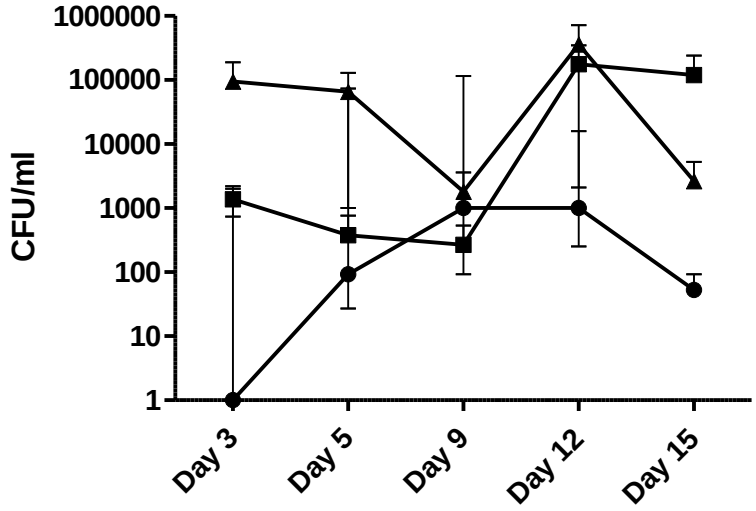


Figure 5.16. Bacterial shedding from mammary halves of ewes challenged with 10cfu (circles), 50cfu (squares) or 200cfu (triangles) of *S. aureus* isolate SA10 on days 3, 5, 9, 12 and 15 following challenge. Data are represented as median cfu/ml. Error bars represent range. Bacterial shedding did not vary significantly between groups (P = 0.33). A significant effect of day post-challenge was observed, independent of challenge dose (P = 0.04).

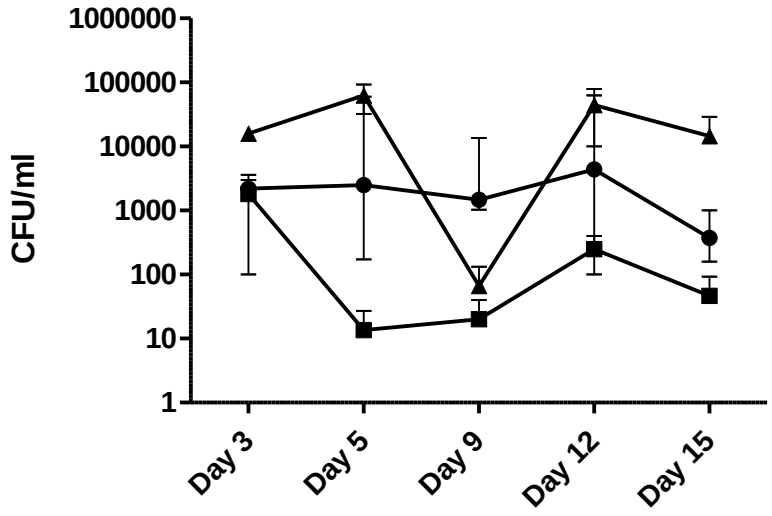


Figure 5.17. Udder half somatic cell counts from ewes challenged with 10cfu (circles), 50cfu (squares) or 200cfu (triangles) of *S. aureus* isolate SA8 on days 3, 5, 9, 12 and 15 following challenge. Data are represented as median cfu/ml. Error bars represent range.

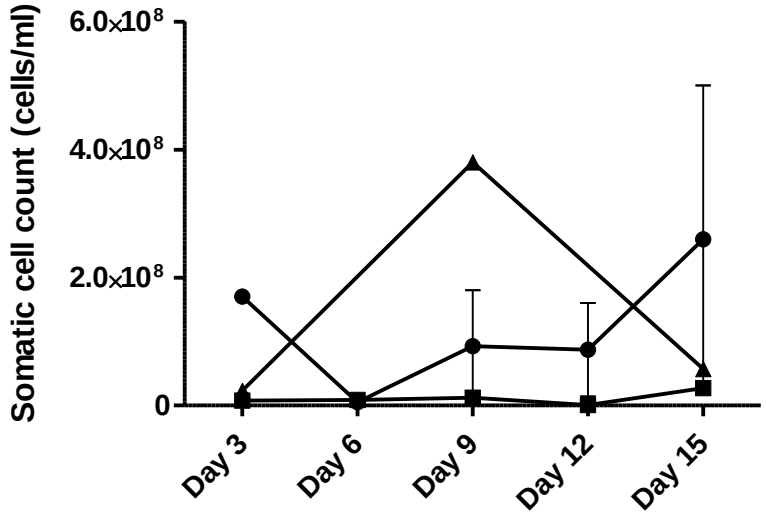


Figure 5.18. Udder half somatic cell counts from ewes challenged with 10cfu (circles), 50cfu (squares) or 200cfu (triangles) of *S. aureus* isolate SA10 on days 3, 5, 9, 12 and 15 following challenge. Data are represented as median cfu/ml. Error bars represent range.

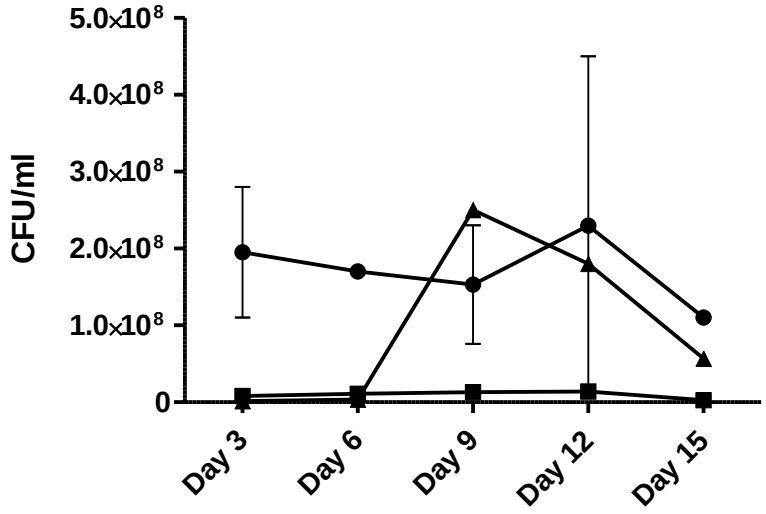
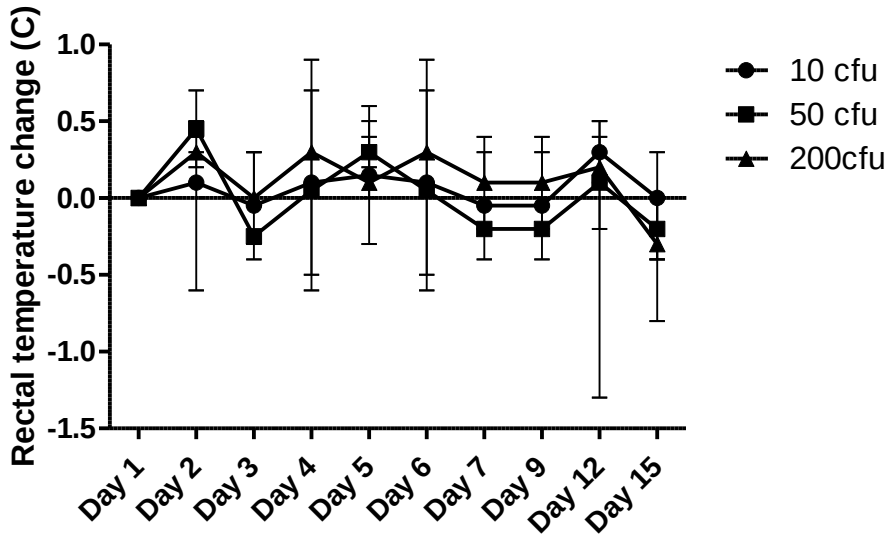


Figure 5.19. Median changes in rectal temperature of ewes challenged with different doses of *S. aureus*. Animals were challenged with either 10cfu (circles), 50cfu (squares) or 200cfu (triangles) *S. aureus*. As some animals were challenged with more than one *S. aureus* isolate, comparisons between isolates could not be obtained.



5.4 Effects of *Staphylococcus aureus* isolates on ovine mammary immune responses *in vivo*

5.4.1 Generation of Staphylococcus aureus intramammary infections

Lactating ewes were challenged with either 50cfu of SA8 or 50cfu of SA10 in a single udder half. The contralateral udder half was used as an uninfected control. Details of animals and udder halves infected are presented in table 4.5. All animals developed a *S. aureus* IMI in the challenge half which persisted for the duration of the study. There was no recovery of *S. aureus* from any unchallenged udder half. Bacterial shedding was significantly lower six days post-challenge with SA8 ($P = 0.04$) (Figures 5.20 and 5.21). Milk SCCs were determined on the day of challenge and all days following challenge. During the acute phase of infection, milk SCC did not vary between isolate, infection status of the mammary quarter or timepoint (Figure 5.22). During the chronic phase of infection, the SA10 isolate significantly increased milk SCC compared with every other treatment. Otherwise, milk SCC did not significantly vary between isolate, infection status of mammary quarter or timepoint (Figure 5.23). Clinical mastitis, characterized by abnormal mammary secretion, was noted on one study day for two infected udder halves. Otherwise, no clinical mastitis was observed. The lack of a significant effect of *S. aureus* IMI on milk SCC may either indicate that these isolates do not significantly increase SCC or that insufficient animals were used to determine statistically significant differences. However, given that *S. aureus* IMIs were established that persisted for the duration of the study, the infection model was successful and allows for the determination of the effects of *S. aureus* IMI on mammary immune responses.

Figure 5.20. Bacterial shedding from ewes challenged with 50cfu *S. aureus* SA8 (open bars) or SA10 (closed bars) and euthanized 3 days following challenge. Data are represented as median cfu/ml. Error bars represent range. Bacterial shedding did not vary significantly between groups (P = 0.25) or between day post-challenge (P = 0.21).

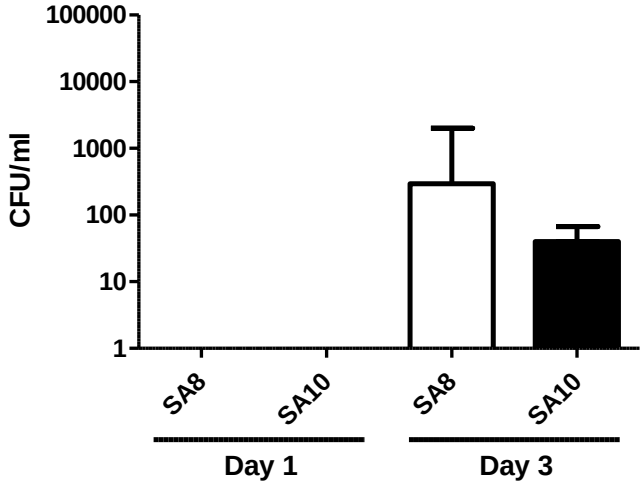


Figure 5.21. Bacterial shedding from ewes challenged with 50cfu *S. aureus* SA8 (open bars) or SA10 (closed bars) and euthanized 15 days following challenge. Data are represented as median cfu/ml. Error bars represent range. Bacterial shedding was significantly lower in animals challenged with *S. aureus* SA8 six days post-challenge (P = 0.04).

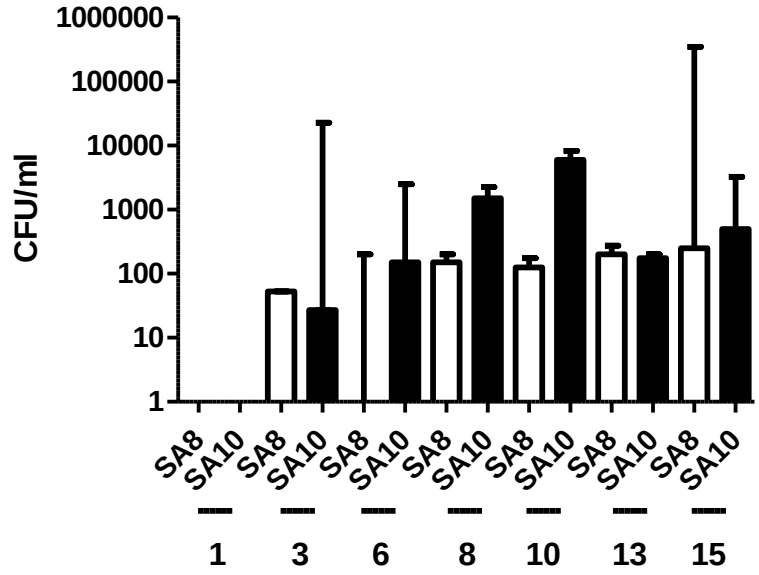


Figure 5.22. Udder half somatic cell counts from ewes challenged with 50cfu *S. aureus* SA8 (open bars) or SA10 (closed bars) and euthanized 3 days following challenge. Data are represented as median cfu/ml. Error bars represent range. Milk SCC did not vary with treatment (P = 0.35) or days post-challenge (P = 0.25).

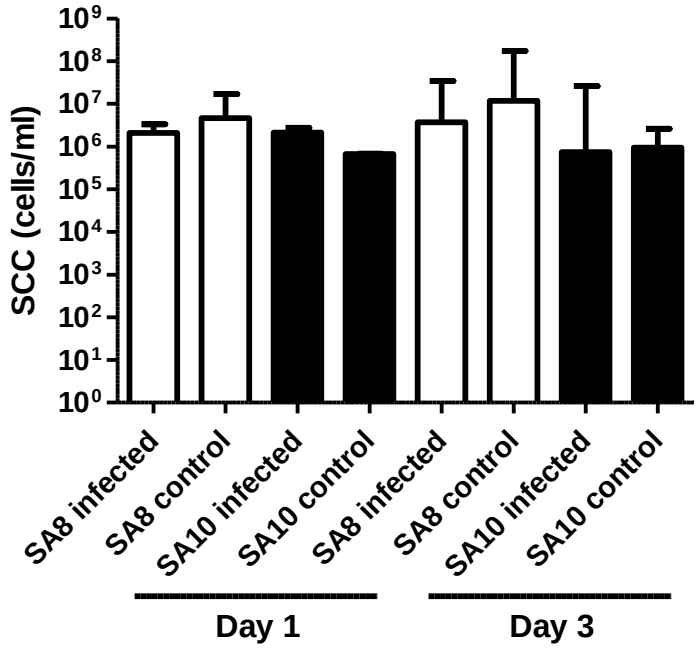
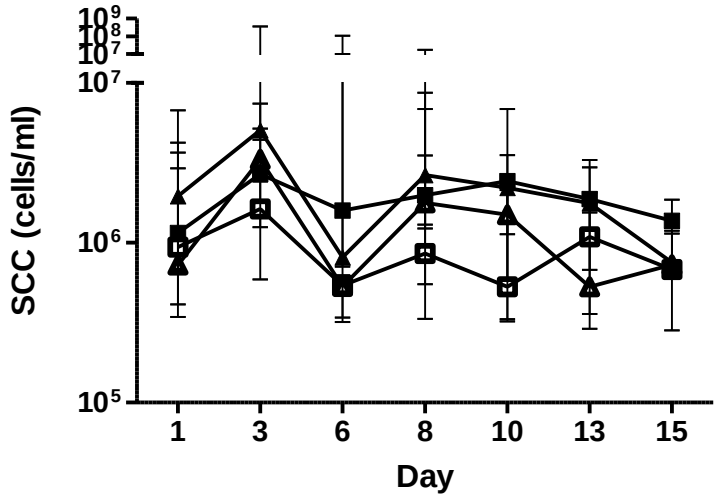


Figure 5.23. Udder half somatic cell counts from ewes challenged with 50cfu *S. aureus* SA8 (squares) or SA10 (triangles) and euthanized 15 days following challenge. Challenged halves are represented with filled symbols, control halves are represented with open symbols. Data are represented as median cfu/ml. Error bars represent range. Milk somatic cell counts from udder halves challenged with *S. aureus* SA10 were significantly higher than those from all other challenges at day 3 post-challenge ($P < 0.01$).



5.4.2 Effect of Staphylococcus aureus isolates on leukocyte populations in supramammary lymph nodes

Leucocyte surface marker expression in the SMLNs was quantified by flow cytometry. Cell surface marker expression in the teat ends, mammary parenchyma and the SMLNs was determined qualitatively using IHC. At euthanasia, it was observed that the control lymph node in one animal challenged with SA10 contained large amounts of purulent material therefore this lymph node was not excluded analysis. Unfortunately, the purulent material was not saved for culture.

The number of CD4+ cells during the acute phase of infection was significantly higher for animals challenged with SA10 than those challenged with SA8 ($P = 0.007$). This effect was independent of infection status of the udder half ($P = 0.80$). When data were analysed with the values for control halves subtracted from those for infected halves, numbers of CD4, CD8, γδ and CD11c positive T cells did not vary significantly between *S. aureus* isolates ($P > 0.14$) or with duration of infection

($P > 0.31$). Furthermore, no differences were observed between challenged and unchallenged halves ($P > 0.43$). Representative flow cytometry profiles are shown in figure 5.24. Data for acute and chronic infection are summarized in figures 5.25 and 5.26, respectively.

At the teat end, IHC revealed large numbers of CD8 positive cells but few CD4 or γδ positive cells (Figure 5.27). No CD11c positive cells were visible at the teat end. No changes were observed as a result of infection, isolate or study day. Results in the mammary parenchyma were variable. Some sections revealed typical mammary parenchymal morphology whilst others revealed significant cellular infiltration into the mammary gland with concurrent disruption of normal morphology (Figure 5.28). These changes appeared to be independent of infection status and may reflect the fact that animals were at the end of lactation and entering the dry period. Generally, the numbers of CD4+ cells were moderate, the numbers of CD8+ cells were high and the numbers of γδ, CD11c and CD205 positive cells varied from low to moderate. Cells tended to be diffusely distributed throughout the mammary parenchyma. However, chronic infection with *S. aureus* SA8 appeared to increase the number of CD4 and CD205 positive cells (Figure 5.29). CD205 is a marker for dendritic cells in the SMLN. In some udder halves, aggregates of lymphoid cells were observed (Figure 5.30). This appeared to be occurring independent of infection status of the udder half. The morphology of SMLNs was as expected with a cortex containing follicles consisting of B cells, paracortex containing large numbers of T and B cells with low numbers of γδ cells and medullary cords containing T and B cells and low numbers of γδ cells (Figure 5.31). As expected given the flow cytometry results, no differences in numbers of cells stained for each cell surface marker were observed between any of the groups.

Figure 5.24. Representative flow cytometry profiles of leukocytes from supramammary lymph nodes in animals euthanized three days following challenge with 50cfu *S. aureus* SA8. Panel (a) represents CD4+ cells, panel (b) represents CD8+ cells, panel (c) represents γδ T cell receptor + cells and panel (d) represents CD11c+ cells.

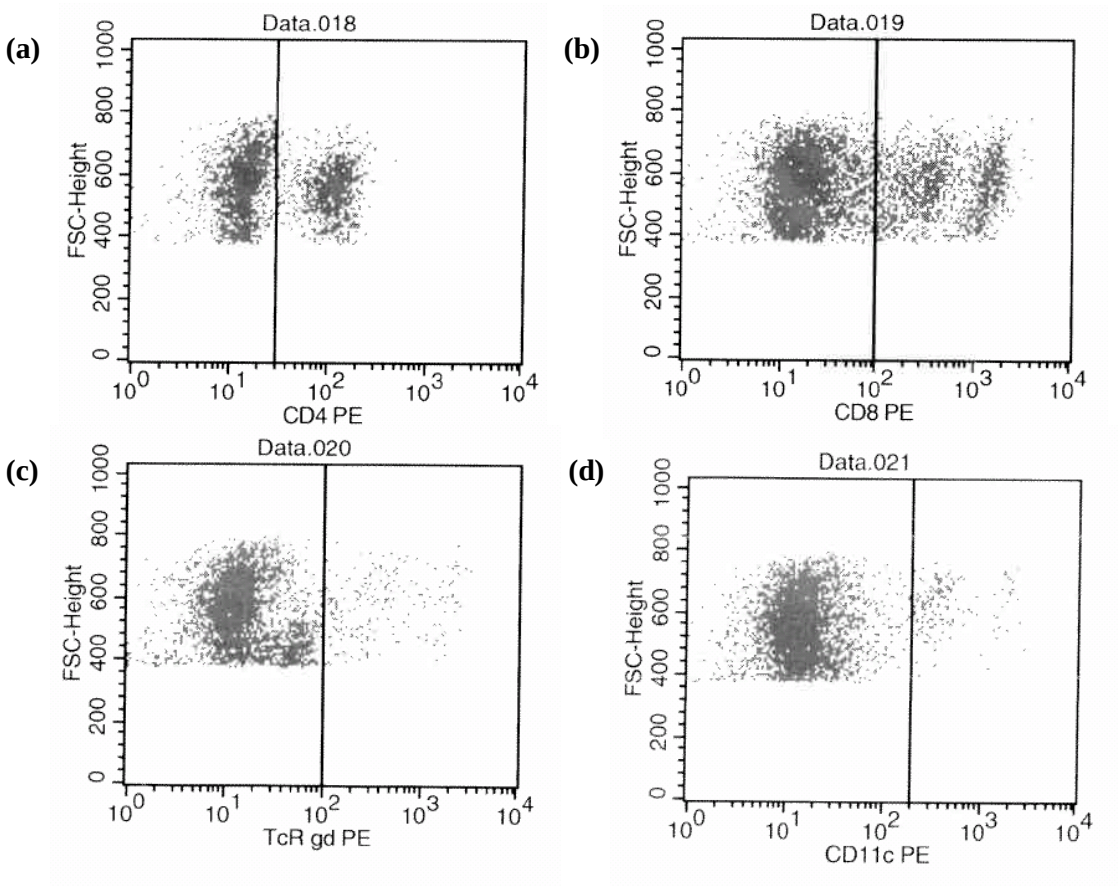


Figure 5.25. Number of cells staining for CD4 (panel a), CD8 (panel b), γδ T cell receptor (panel c) and CD11c (panel d) leukocyte surface markers from the SMLNs of animals euthanized three days following challenge with 50cfu *S. aureus* SA8 (circles) or SA10 (triangles). Challenged halves are represented with filled symbols, control halves are represented with open symbols. The number of CD4 positive cells was significantly higher for animals challenged with SA10, regardless of infection status of the udder half (P = 0.007).

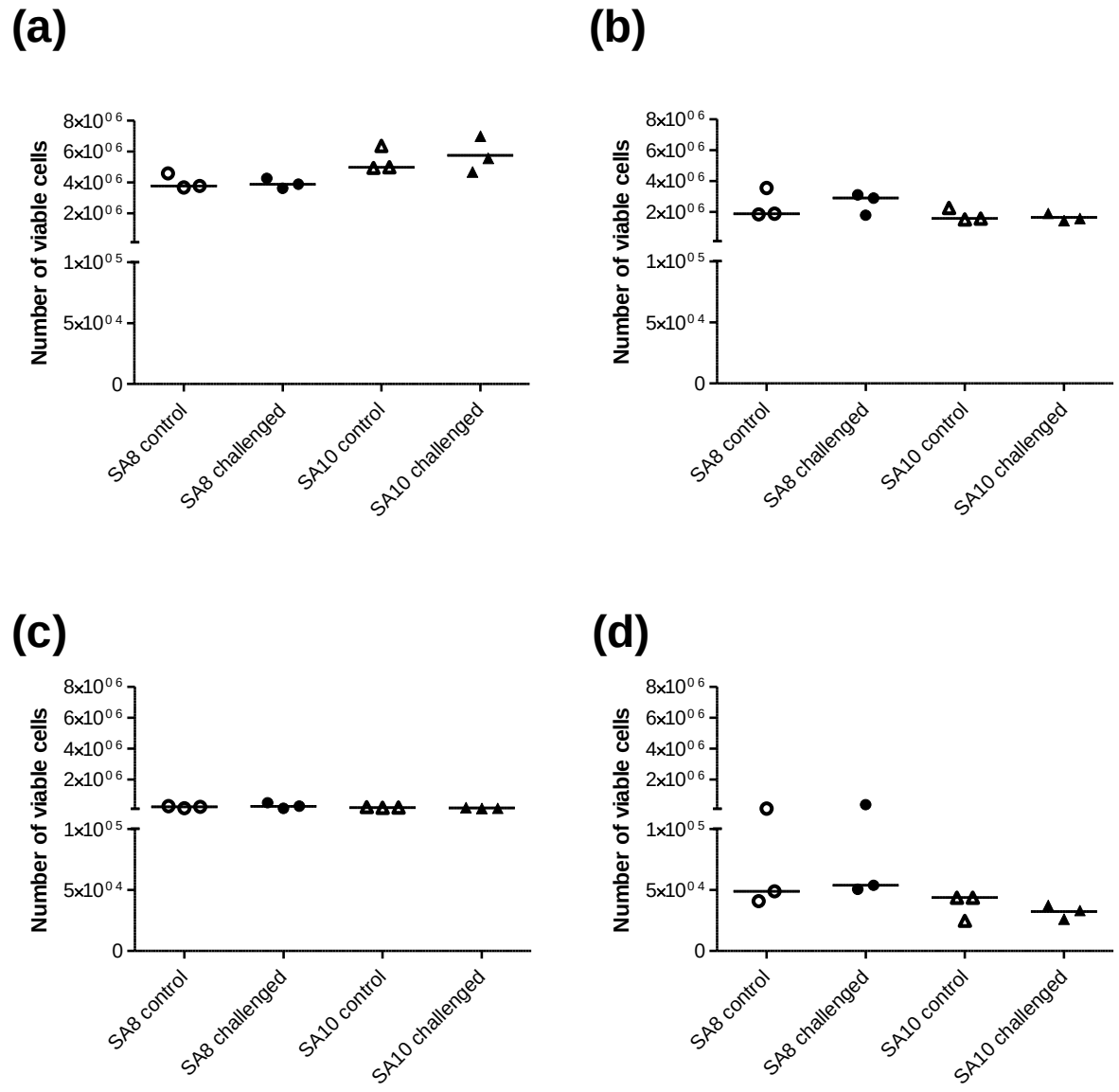


Figure 5.26. Number of cells staining for CD4 (panel a), CD8 (panel b), γδ T cell receptor (panel c) and CD11c (panel d) leucocyte surface markers from the SMLNs of animals euthanized 15 days following challenge with 50cfu *S. aureus* SA8 (circles) or SA10 (triangles). Challenged halves are represented with filled symbols, control halves are represented with open symbols. Numbers of cells did not vary significantly between *S. aureus* isolates ($P > 0.14$) or with duration of infection ($P > 0.31$). No differences were observed between challenged and unchallenged halves ($P > 0.43$).

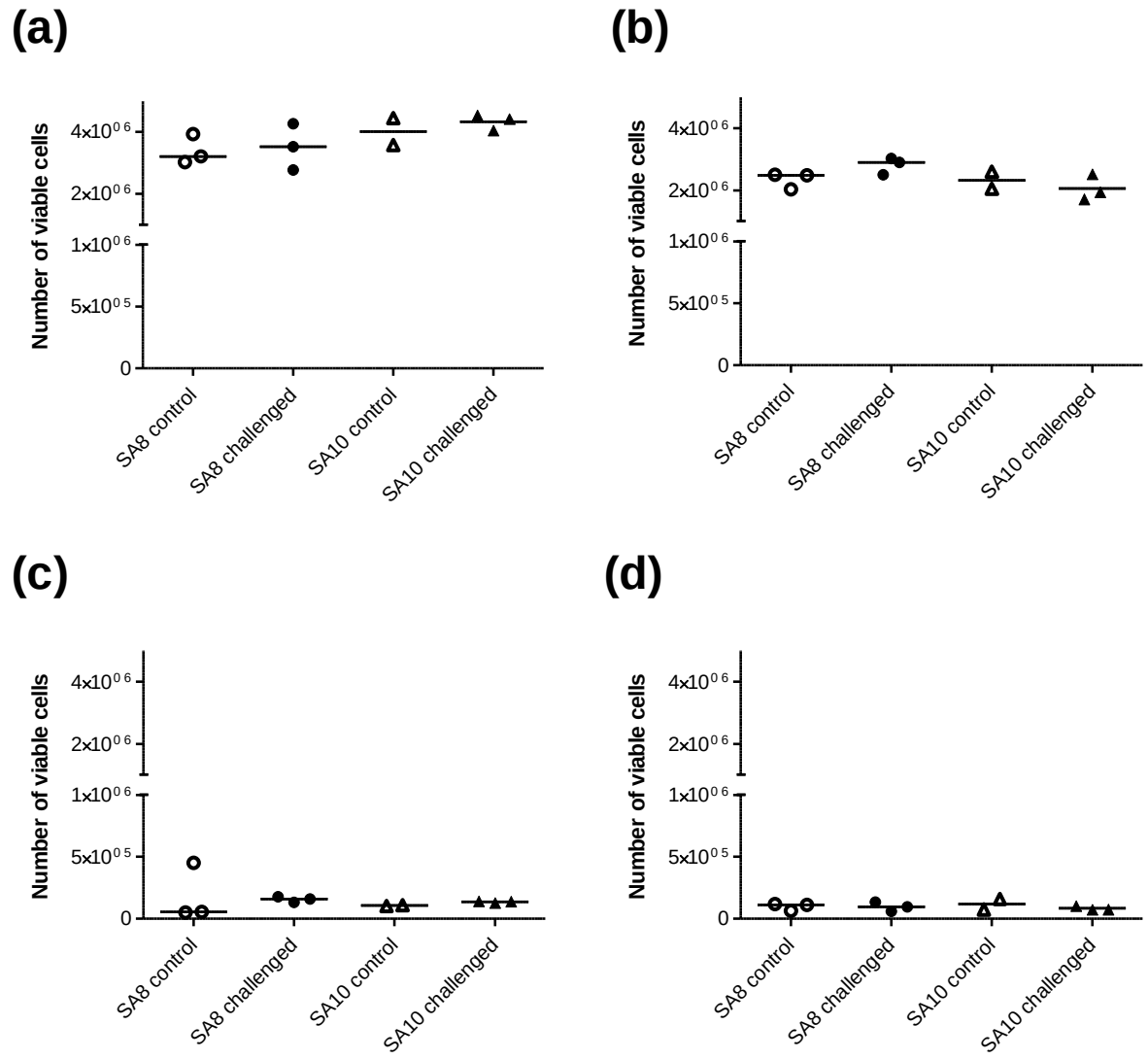


Figure 5.27. Sequential sections through the teat end of a ewe challenged with *S. aureus* isolate SA10. These sections are representative of all the teat ends from animals used in the study. Cells stained were CD4 (panel a), CD8 (panel b) and γδ T cells (panel c). Cells positive for cell surface markers stain dark brown. Few CD4 and γδ positive T cells are observed. Many CD8 positive T cells are observed in cellular nests, connective tissue and invading the keratinizing epithelium of the teat canal.

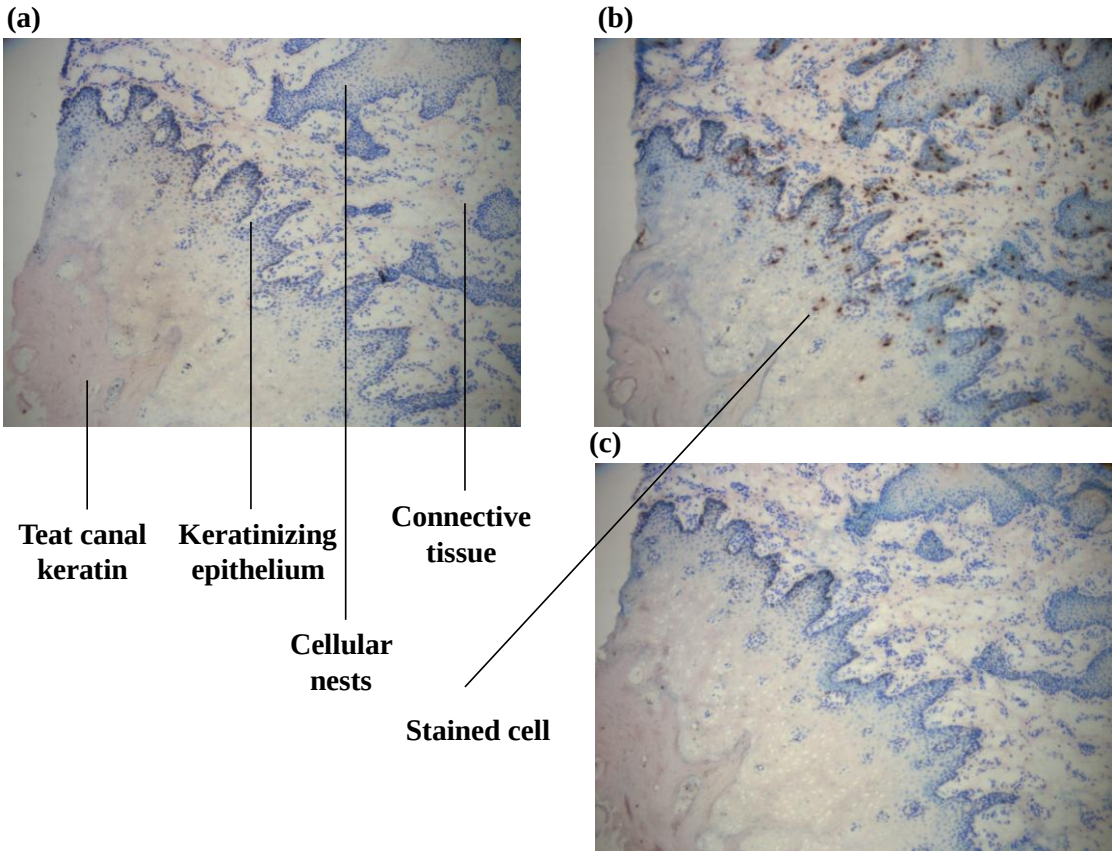


Figure 5.28. Sections from the mammary parenchyma of two different ewes. Panel (a) represents normal secretory tissue. Panel (b) represents mammary tissue with large scale cellular infiltration.

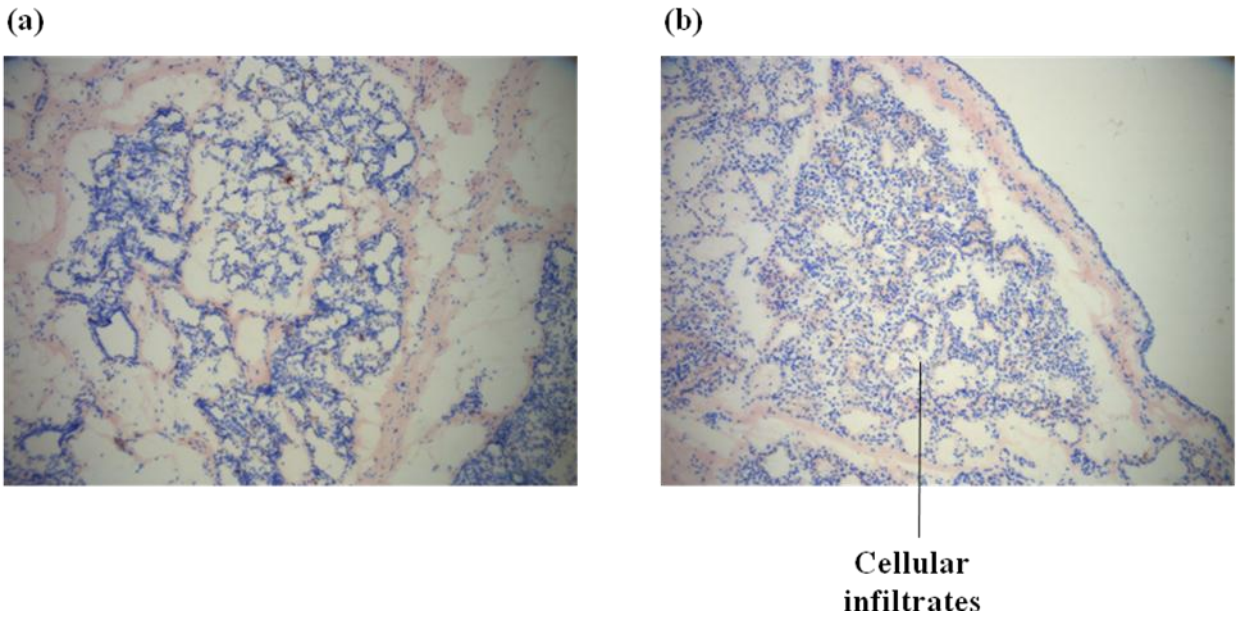


Figure 5.29. Sections from the mammary parenchyma of a ewe challenged with *S. aureus* isolate SA8. Cells stained were CD4 (panels a and b) and CD205 (panels c and d). Panels (a) and (c) represent the control udder half; panels (b) and (d) represent the infected udder half. Cells positive for cell surface markers stain dark brown. Increased numbers of CD4 and CD205 positive cells are observed with chronic infection.

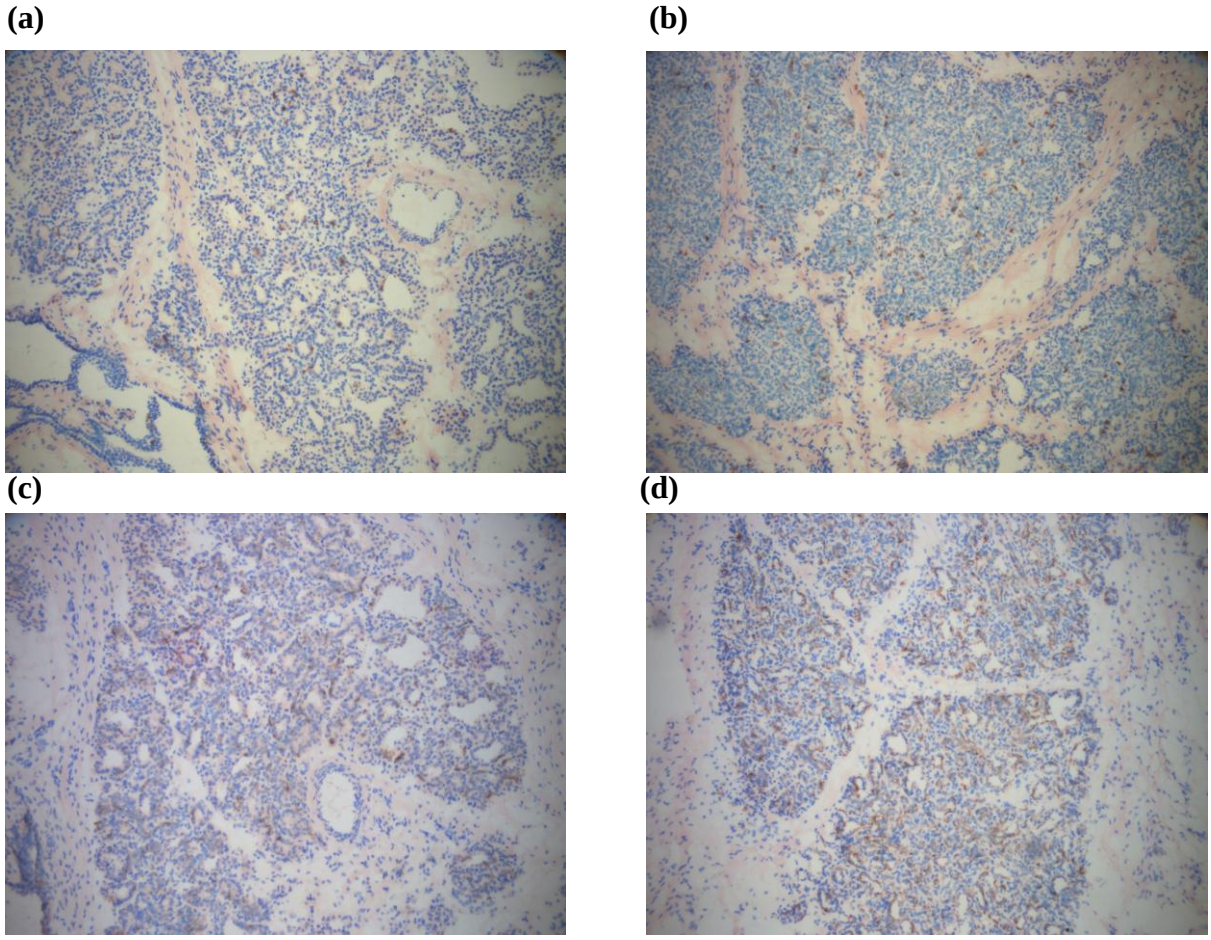


Figure 5.30. Sections from the mammary parenchyma of a ewe illustrating aggregates of lymphoid cells. Cells stained were CD4 (panel a), CD8 (panel b), γδ (panel c) CD11c (panel d). The organization of lymphoid cells occurred independent of infection status of the udder half.

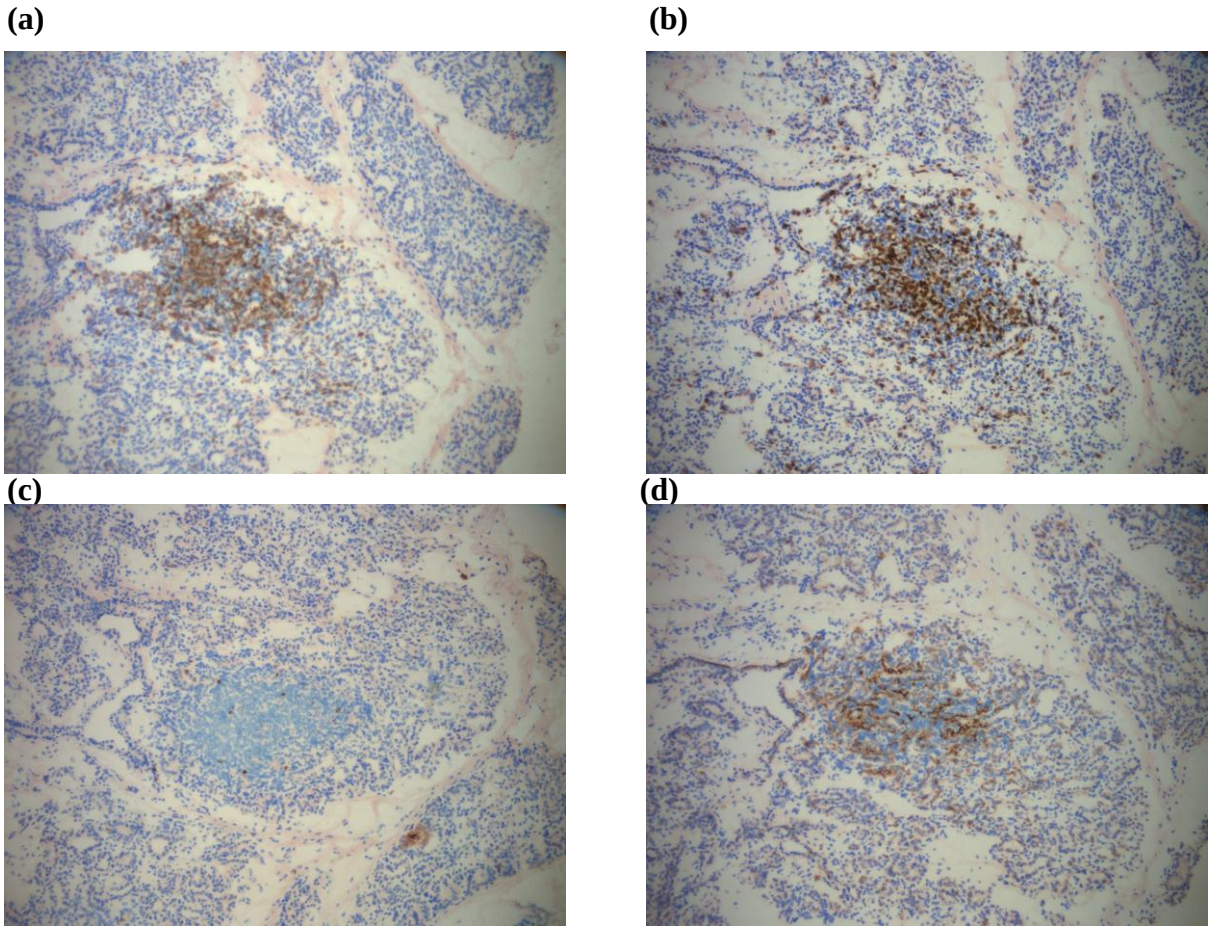
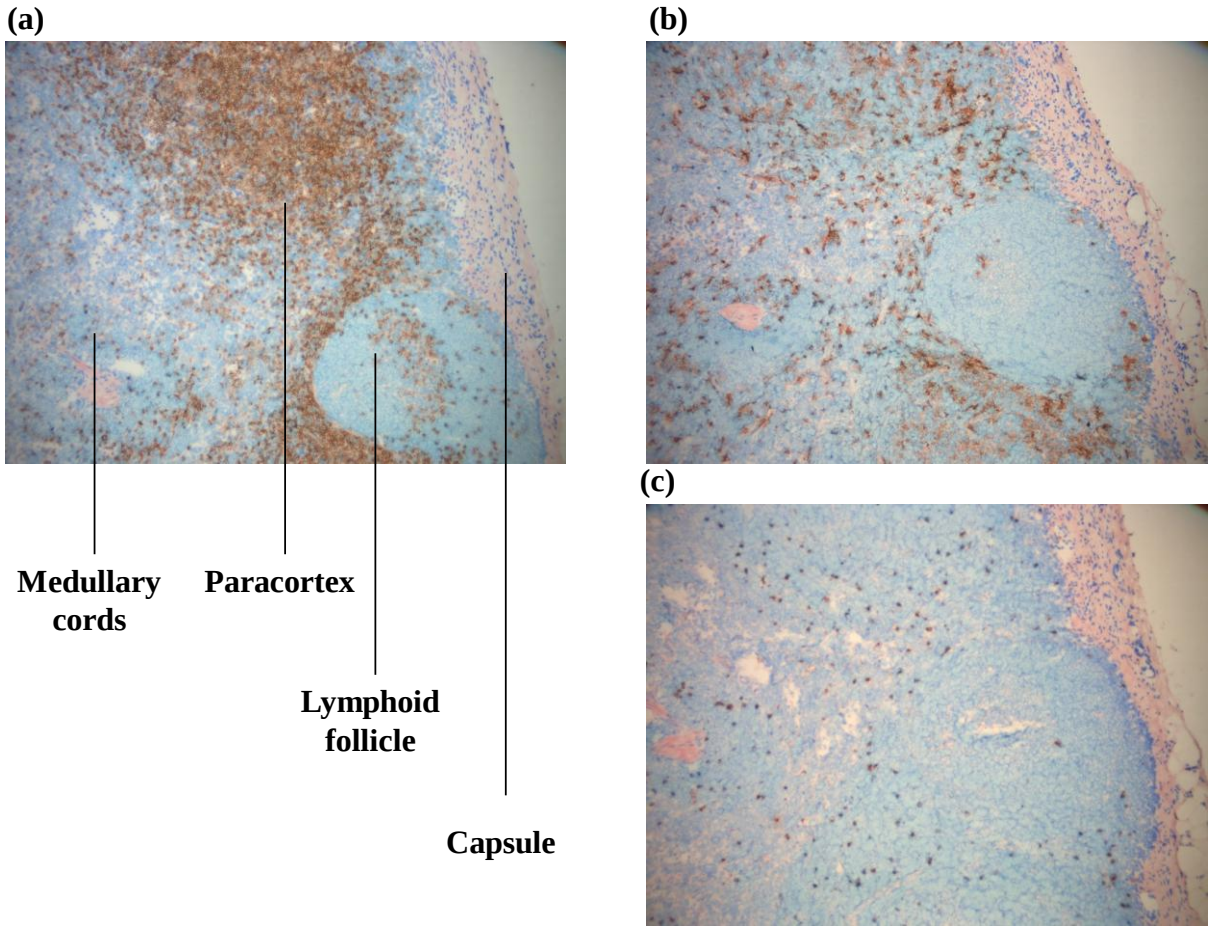


Figure 5.31. Sections from the supramammary lymph node of a ewe illustrating cortical follicles, paracortex and medullary cords. Cells stained were CD4 (panel a), CD8 (panel b) and γδ (panel c). No differences between groups were observed for cellular staining characteristics.



5.4.3 Effect of Staphylococcus aureus on cytokine expression

Cytokine expression at the teat end, in the mammary parenchyma and in the SMLNs was quantified using qRT-PCR. Expression of IFN-γ was not significantly different between infected and uninfected udder halves for animals challenged with SA8 (Figure 5.32). Expression of IFN-γ was significantly higher in the teat ends of animals challenged with SA10 during the acute phase of infection when compared with uninfected controls (P = 0.01) (Figure 5.33). When isolates and duration of infection were compared, no significant differences were noted. However, SA10 caused approximately five fold higher expression of IFN-γ at the teat end than SA8 which was independent of duration of infection but the difference was not statistically significant (P = 0.09).

Figure 5.32. Effect of *S. aureus* SA8 on IFN-γ expression at different sites during acute (3 days following challenge) and chronic (15 days following challenge) infection. Data are expressed as relative expression normalized to the control udder half. A relative expression value of 1 indicates no difference between challenged and control udder halves. Expression of IFN-γ was not significantly different between infected and uninfected udder halves.

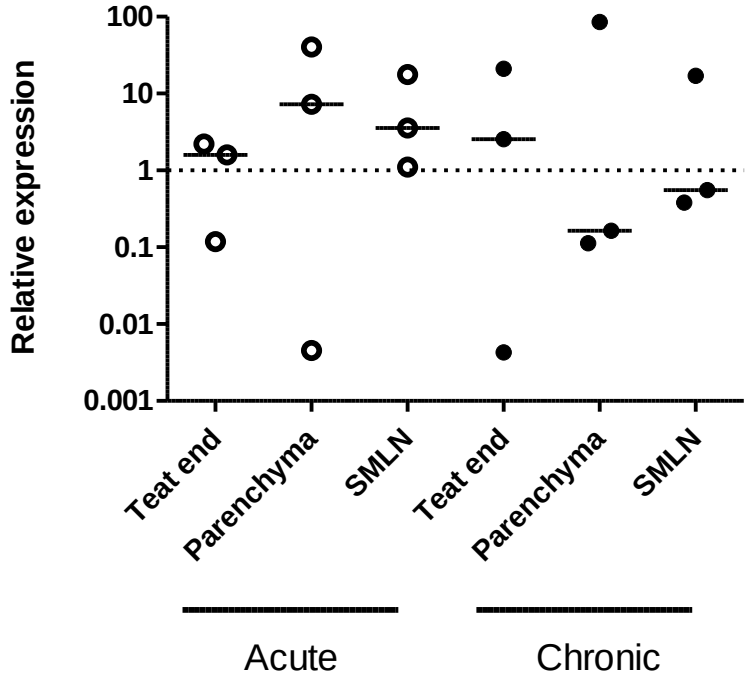
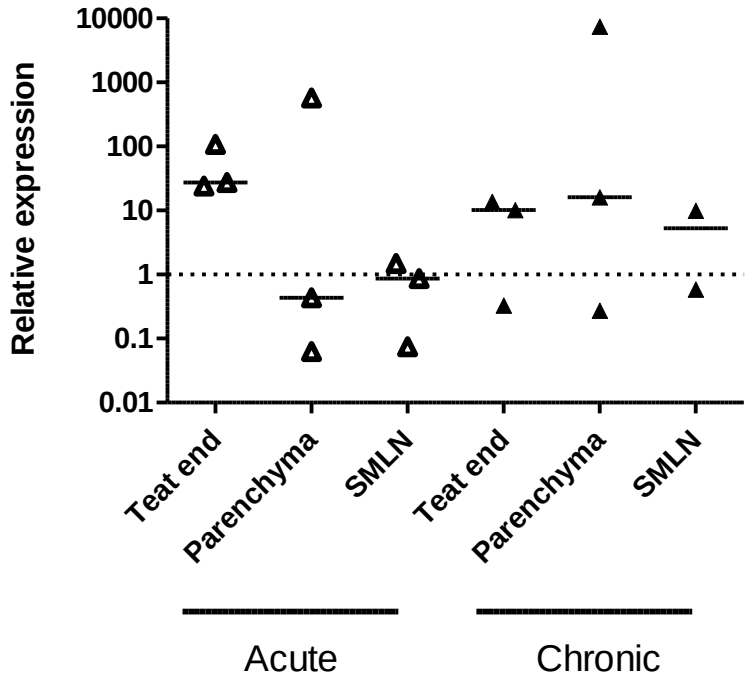


Figure 5.33. Effect of *S. aureus* SA10 on IFN-γ expression at different sites during acute and chronic infection. Data are expressed as relative expression normalized to the control udder half. A relative expression value of 1 indicates no difference between challenged and control udder halves. Expression of IFN-γ was significantly higher at infected teat ends when compared with control teat ends during the acute phase of infection (P = 0.01).



Expression of IL-4 was not significantly different between infected and uninfected udder halves for animals challenged with SA8 or SA10 (Figures 5.34 and 5.35, respectively). When isolates and duration of infection were compared, no significant differences were noted. Expression of IL-10 was significantly lower in the SMLNs of animals challenged with SA8 during the acute phase of infection compared with uninfected controls (P = 0.04) (Figure 5.36). Expression of IL-10 was not significantly different between infected and uninfected udder halves for animals infected with SA10 (Figure 5.37). When isolates and duration of infection were compared, expression of IL-10 was significantly higher in the teat ends of animals chronically infected with SA8 when compared with animals acutely infected with SA8. Expression of IL-10 was also higher in the mammary parenchyma of animals challenged with SA10 compared with those challenged with SA8 (P = 0.09).

Figure 5.34. Effect of *S. aureus* SA8 on IL-4 expression at different sites during acute and chronic infection. Data are expressed as relative expression normalized to the control udder half. A relative expression value of 1 indicates no difference between challenged and control udder halves. Expression of IL-4 was not significantly different between infected and uninfected udder halves.

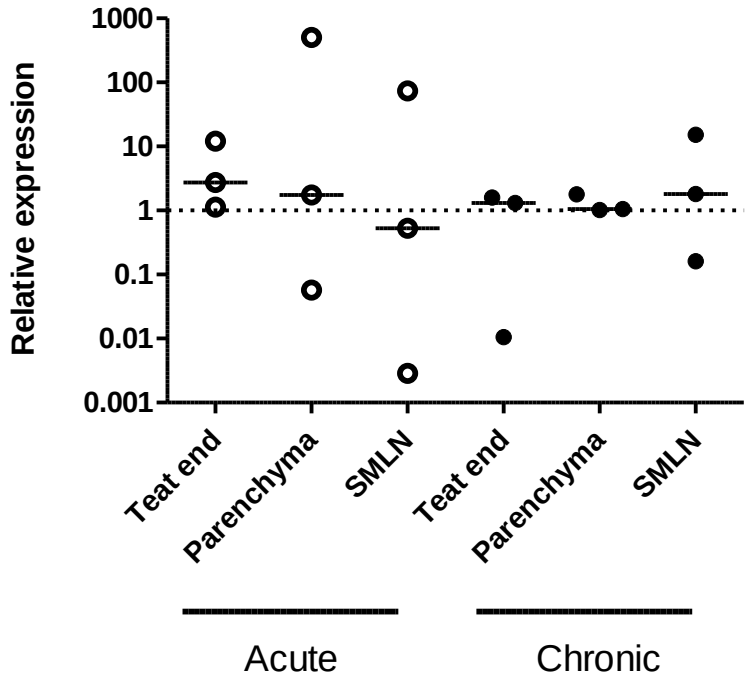


Figure 5.35. Effect of *S. aureus* SA10 on IL-4 expression at different sites during acute and chronic infection. Data are expressed as relative expression normalized to the control udder half. A relative expression value of 1 indicates no difference between challenged and control udder halves. Expression of IL-4 was not significantly different between infected and uninfected udder halves.

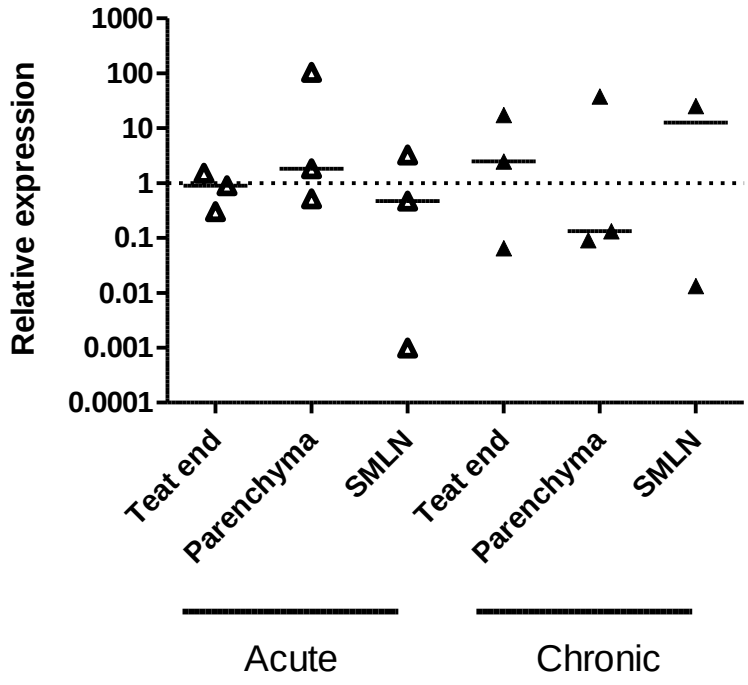


Figure 5.36. Effect of *S. aureus* SA8 on IL-10 expression at different sites during acute and chronic infection. Data are expressed as relative expression normalized to the control udder half. A relative expression value of 1 indicates no difference between challenged and control udder halves. Expression of IL-10 was significantly lower in the SMLNs from infected udder halves when compared with uninfected controls. Expression of IL-10 was significantly higher in chronically infected teat ends than acutely infected teat ends.

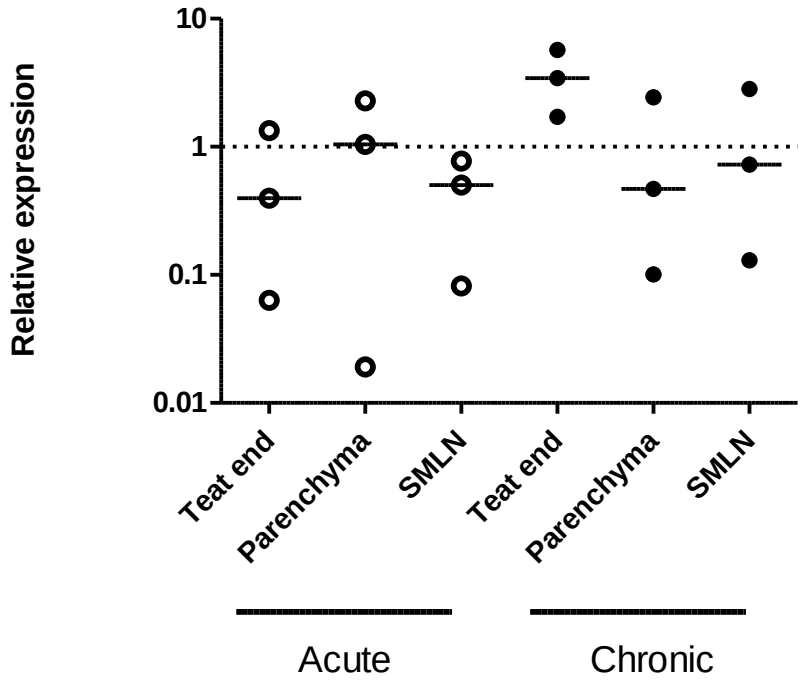
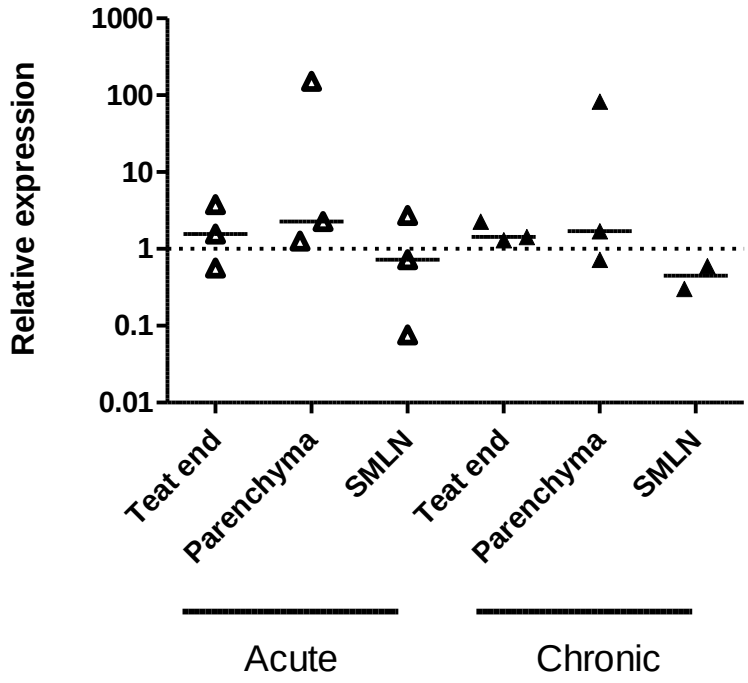


Figure 5.37. Effect of *S. aureus* SA10 on IL-10 expression at different sites during acute and chronic infection. Data are expressed as relative expression normalized to the control udder half. A relative expression value of 1 indicates no difference between challenged and control udder halves. Expression of IL-10 was higher in the mammary parenchyma of animals challenged with SA10 than those challenged with SA8 (P = 0.09).



The source of cytokine expression in SMLNs was determined by flow cytometry using cells stained for cell surface markers and cytokines. Numbers of CD4 positive cells expressing IL-10 were significantly lower in the SMLNs of animals acutely infected with SA10 compared with uninfected controls and significantly higher in the SMLNs of acutely infected animals than chronically infected animals (P = 0.04) (Figure 5.38). When baseline numbers of cells in control udder halves were subtracted, numbers of CD11c positive cells expressing IL-4 were significantly higher in the SMLNs of animals infected with SA8 than those infected with SA10 and in the acute phase of SA8 infection when compared with the chronic phase (Figure 5.39). As noted above, expression of IL-10 was significantly lower in the SMLNs of animals challenged with SA8 during the acute phase of infection compared with uninfected controls. However, no major differences in the number of CD4, γδ or CD11c positive cells expressing IL-10 were observed

(Figure 5.40). Unfortunately, due to an experimental error in staining for flow cytometry, CD8 positive cells expressing IL-10 were not assayed. It remains a possibility that the decrease in IL-10 observed during the acute phase of infection may be attributable to this cell population.

Figure 5.38. Numbers of CD4 positive cells expressing IL-10 from the SMLNs of animals infected with SA10. Numbers of CD4 positive cells expressing IL-10 were significantly lower in animals acutely infected with SA10 compared with uninfected controls and significantly higher in acutely infected animals than chronically infected animals (P = 0.04).

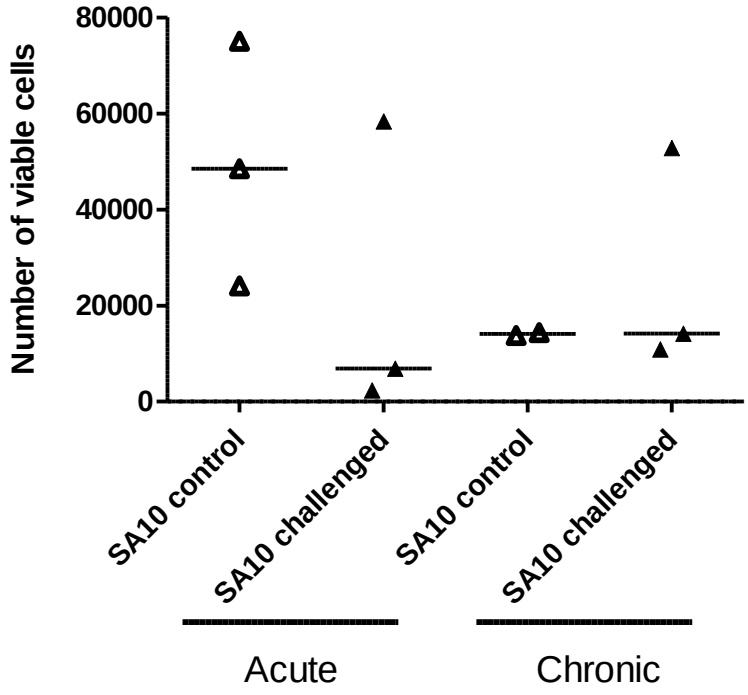


Figure 5.39. Numbers of CD11c positive cells expressing IL-4 from the SMLNs of animals infected with SA8 and SA10 at acute (panel a) and chronic (panel b) stages of infection. When baseline numbers of cells in control udder halves were subtracted, numbers of CD11c positive cells expressing IL-4 were significantly higher in animals infected with SA8 than those infected with SA10 and in the acute phase of SA8 infection when compared with the chronic phase.

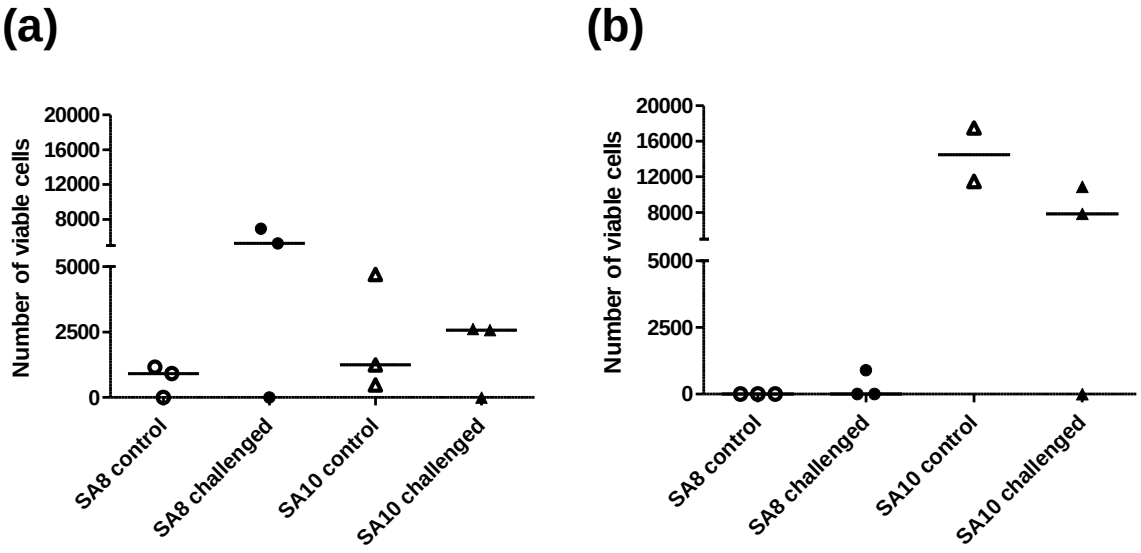
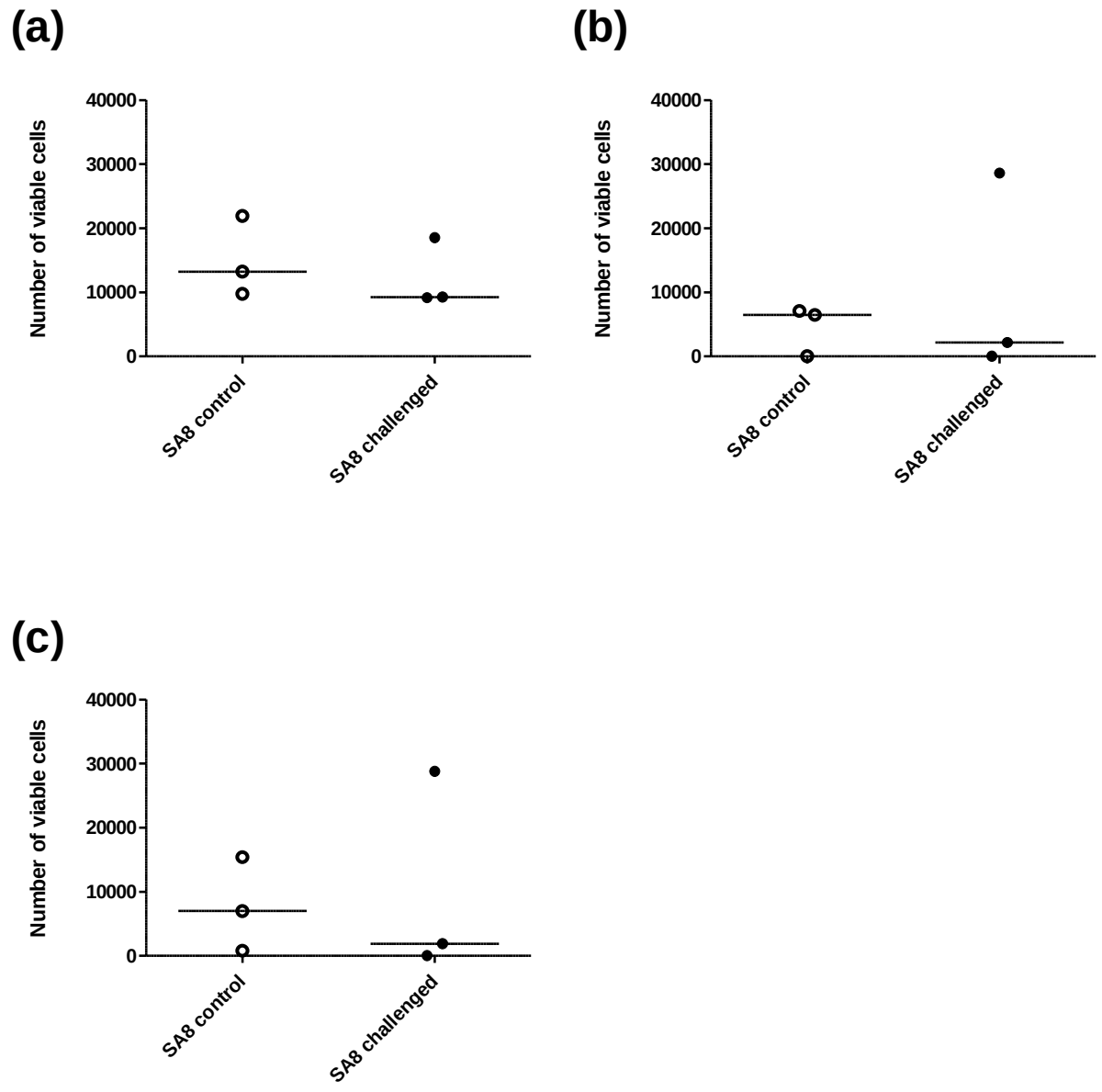


Figure 5.40. Numbers of cells expressing IL-10 from the SMLNs of animals infected with SA8 at the acute stage of infection. Cells assayed were CD4 (panel a), CD8 (panel b) and γδ positive (panel c). No significant differences were observed.



6. DISCUSSION

The first objective of this study was to screen bovine mammary *S. aureus* isolates and determine whether they altered cytokine secretion by bovine PBMCs *in vitro*. Three isolates had large effects on cytokine expression *in vitro*. However, the remaining six isolates had similar responses. This suggests that the majority of *S. aureus* isolates may have similar effects on cellular immune responses. The general effect of these isolates was a moderate increase in IFN-γ and TNF-α secretion, a negligible increase in IL-10 secretion and no change in IL-4 expression when compared with media alone. Of the three remaining isolates, two caused large increases in IFN-γ, TNF-α and IL-10 secretion and the third caused large increases in IFN-γ and TNF-α secretion alongside an increase in IL-4 expression.

Previous studies have reported contradictory results regarding IFN-γ upregulation as a result of *S. aureus* IMI [4-6, 213-215]. These studies used a variety of *difference S. aureus* isolates and show that different isolates induce different increases in IFN-γ secretion. The isolates (Newbould 305, strain DV137, uncharacterized wild type isolates) used were different to those used in our study. Strain differences may explain differences that are observed between studies.

Previous work has reported increases in IL-10 due to *S. aureus* IMI although the increases have been reported to be of a low magnitude [4, 5]. These data show that some *S. aureus* isolates increase IL-10 concentrations to a large degree whilst others cause minimal increases in IL-10 concentrations, suggesting that strain dependence is important. The minimal increases in IL-10 appear to be as a result of secretion from CD8 and γδ positive T cells. The increased IL-10 secretion as a result of the SA8 isolate appears to be as a result of CD4 positive T cells. These data seem to be consistent with those of Seo and coworkers [8] who showed increased CD4+ IL-10 secreting cells *in vitro* when PBMCs were exposed to SEC. Given that SA8 possesses the *sec* gene, this may be a reason for the observed results with SA8. It appears that the low-virulence RN4220 strain causes increased secretion of IL-10 compared to many wild type strains. The RN4220 strain is a laboratory strain that appears to lack enterotoxin genes [273], has minimal haemolysin production [274] and low activity of the virulence regulator *agr* as a result of a frameshift mutation at the 3' end of *agrA* [275]. Several wild type strains may produce virulence

factors under the control of *agr* that result in decreased IL-10 production by CD8+ and γδ T cells.

The role of IL-4 in *S. aureus* IMI has not been a major topic of previous studies. It has been reported that IL-4 mRNA is not present in somatic cells from the milk of cows with *S. aureus* IMI [6]. The fact that IL-4 mRNA was present in the PBMCs of the current study reflects the different cell types assayed. Studies *in vivo* will provide a far better comparison of whether IL-4 mRNA can be isolated from the mammary gland.

The moderate changes in cytokine expression observed due to the majority of *S. aureus* isolates is consistent with previous work which reports that *S. aureus* causes mild and transient increases in cytokine levels in milk [4, 213] when compared with those observed as a result of *E. coli* IMI. It has also been hypothesized that the reason for *S. aureus* IMI being a chronic disease is that pro-inflammatory cytokine levels do not rise sufficiently rapidly or to sufficiently high levels to clear infection. However, certain isolates resulted in large IFN-γ and TNF-α responses which occurred rapidly yet the isolates were recovered from chronic *S. aureus* IMI. It must be remembered that these isolates were recovered from individual animals therefore the behaviour of an isolate in an individual may not reflect its behaviour in an entire population. Furthermore, other explanations may exist for the chronic nature of *S. aureus* IMI including the decreased concentration and function of immune effectors in the mammary gland compared with blood and the ability of *S. aureus* to prevent phagocytosis and killing by neutrophils allowing its localization within microabscesses and its shielding from host responses.

During the *in vitro* studies, the most dramatic responses appeared to be associated with isolates possessing the *sec* gene. However, strains that possess the *sec* gene had varying effects. For example, the SA8 isolate caused increases in IFN-γ, IL-10 and IL-4. The SA23 isolate caused increases in IFN-γ, IL-10, TNF-α and IL-4. This suggests that these isolates seem to induce non-specific stimulation of immune responses. The SA10 isolate induced increases in IFN-γ, TNF-α and IL-4 but not IL-10. This suggests that this isolate induces a balanced T_H1 and T_H2 response. Given that different isolates containing the *sec* gene have varying effects, experiments were carried out to characterize the role of *sec* alone and were detailed in section 5.2.

To characterize the role of *sec*, the first experiment investigated the effect of purified SEC protein on cytokine secretion and cell surface marker expression by PBMCs *in vitro*. An increase in IL-10 secretion was observed from PBMCs of naive cattle exposed to SEC. This evidence suggests that SEC promotes an anti-inflammatory response. This is consistent with the results observed by Ferens and co-workers [259]. However, no change in the proportion of cells expressing specific leukocyte lineage markers (CD4, CD8, CD25, ACT2) was observed. This is in contrast to an increase in CD4+ CD25+ cells following exposure to SEC reported by Seo and co-workers [8]. Experimental conditions in the current study differed to those in the previous study [8] in which PBMCs were stimulated with 5ng/ml SEC for 10 days. Exposure of PBMCs to SEC for 10 days appeared to promote differential levels of proliferation of different cell populations. Exposure to low doses of SEC protein for long periods may be necessary to induce CD4+ CD25+ cells. The fact that the proportion of CD8+ or CD8+ ACT2+ cells did not change following exposure to SEC appears to be inconsistent with previous *in vitro* work using PBMCs [8, 257]. The previous studies used lower concentrations of SEC (0.1μg/ml and 5ng/ml respectively) than the current study therefore low SEC concentrations may also be needed to induce these cell populations.

A fundamental problem with using purified SEC protein to assess the responses of PBMCs to SEC is that the physiological relevance of *in vitro* doses is unknown. This is especially relevant since *in vivo* evidence suggests that the quantities of SEC protein secreted by *S. aureus* do not result in the generation of CD8+ ACT2+ cells [260]. A better approach is to use a *S. aureus* strain transformed for the *sec* gene that secretes SEC and compare it to an isogenic *S. aureus* strain that was not transformed for the *sec* gene therefore does not secrete SEC. This approach was used in section 5.2.2. Furthermore, only four animals were used as PBMC donors in section 5.2.1 and they were beef animals with low antibody titres against *S. aureus* and SEC and the results obtained were probably more reflective of innate responses rather than adaptive immune responses to SEC. The studies described in section 5.2.2 used eight dairy animals with recall immune responses to *S. aureus* allowing for better characterization of adaptive immune responses. Finally, the effects of *sec* on IL-4 and TNF-α were also assayed as the limited

cytokine repertoire analysed using SEC protein prevented analysis of anti-inflammatory responses. This provides a better model for evaluating the effect of SEC on cellular immunity.

The only difference observed between the RN4220 strain and the RN4220 strain transformed for *sec* was a decrease in IL-4 expression which occurred earlier in the transformed strain. This decrease in IL-4 mRNA appears to be contradictory to previous research which reported sustained increases in IL-4 production following exposure of PBMCs to SEC protein [259]. However, given that the RN4220 isolate also decreased IL-4 mRNA expression therefore the effect observed may be a mild effect observed over time rather than a major difference between the isolates.

Use of a *S. aureus* isolate transformed for *sec* should provide a more physiologically relevant model for the study of SEC in *S. aureus* pathogenesis. The only previous study using a transformed *S. aureus* strain was an *in vivo* study which also showed no effect of the *sec* gene on the nature of mammary immune responses when compared to an isogenic *S. aureus* strain which did not contain the gene [260]. Several potential reasons exist for the differences observed between studies which use purified SEC protein and those that use a transformed *S. aureus* strain. Firstly, the transformed strains appear to secrete very low concentrations of SEC. Whilst these concentrations may be too low to cause any changes in milk or cell cultures, local SEC concentrations *in vivo* may rise to sufficiently high levels that biologic effects may occur at discrete sites in the mammary gland. This may lead to the generation of small foci in which *S. aureus* may be evading immune responses.

Staphylococcal enterotoxin C may operate in conjunction with other virulence factors. The RN4220 isolate is a laboratory isolate with minimal virulence and it may lack the expression of virulence factors that enhance the effects of SEC. To test this hypothesis, attempts were made to create a *sec* mutant strain of *S. aureus* from a wild type *S. aureus* isolate. A construct of *sec* was generated in which a 100bp deletion was made and replaced with the kanamycin resistance gene *aphA-3*. This was used to successfully transform *E. coli* DH5α and *S. aureus* RN4220. Transformation of a wild type *S. aureus* isolate possessing the *sec* gene was unsuccessful and

was probably as a result of the restriction modification system in wild type isolates. Therefore, this hypothesis was not tested.

Given that the prevalence of *sec* in *S. aureus* isolates from bovine mastitis appears to be low [86], *sec* may not be an important virulence factor in the pathogenesis of *S. aureus* IMI. This may also explain the apparent lack of an effect of SEC on mammary immune responses *in vivo* and in studies which use a *S. aureus* isolate transformed for *sec*.

Weaknesses of the experiments discussed so far include the fact that isolates were studied *in vitro* and that the role of DCs was not characterized. This work was carried out *in vitro* so that a large number of *S. aureus* isolates could be characterized whilst minimizing the use of animals. Furthermore, the use of PBMCs from the same group of cattle removes variation among individual animals when comparing responses. However, results *in vitro* are not always reflected *in vivo*. As transformation of *S. aureus* for the *sec* gene did not have significant effects *in vitro*, wild type isolates were chosen for the *in vivo* studies. Given that the isolates that caused the most dramatic cytokine responses either caused a non-specific response or a balanced response, we chose an isolate representative of each of these responses to use *in vivo*. These isolates were SA8 and SA10. As has been previously discussed, DCs are central to the induction of different cellular immune responses therefore DC responses were characterized in the *in vivo* studies. Dendritic cell responses were characterized by staining DCs using cell surface markers followed by intracellular cytokine staining.

To characterize responses *in vivo*, a model of *S. aureus* IMI was required. An ideal model of IMI would be to use lactating dairy cattle. However, due to the numbers of animals and associated handling facilities required, the costs associated with such a trial would have been prohibitive. Some previous studies have used mice as experimental models of bovine mastitis. However, it has previously been reported that the roles of cytokines as determined in murine models cannot be extrapolated to bovine responses [168]. Sheep have also been previously used as experimental models for bovine *S. aureus* IMI due to their similarity in immune responses to cattle [276-278]. The increased ease of handling and decreased cost of sheep compared with cattle make them a practical alternative to dairy cattle in studies of *S. aureus* IMI. However, it

must always be remembered that this is a model of IMI and results obtained may not always reflect the situation in the bovine. Specifically, the metabolic requirements of a lactating dairy cow are likely to far exceed those of a lactating ewe.

The original intention in developing the model of ovine *S. aureus* IMI was to challenge three udder halves with each of the three challenge doses of each *S. aureus* (10, 50 and 200 cfu). This would have required 18 uninfected udder halves. Based on the screening work performed, only 14 uninfected udder halves were available from the available population of ewes therefore the 10 cfu doses were administered to three udder halves whilst the 50 and 200 cfu doses were administered to two udder halves. All challenge doses of both *S. aureus* isolates induced an IMI. Even though no significant differences in bacterial shedding were noted between challenge doses, the 10 cfu dose of the SA8 isolate resulted in generally lower bacterial shedding than the other doses. No differences in SCC were noted between challenge doses. Given that only transient episodes of clinical mastitis were noted in two animals and rectal temperature varied by no more than 0.4°C during the study, subclinical mastitis was induced by all challenge doses. As a result of these observations, the 50 cfu dose was chosen as the challenge dose for studies of mammary cellular responses *in vivo*.
When animals were challenged to determine mammary cellular responses, challenge with 50 cfu of either isolate generated a subclinical *S. aureus* IMI which persisted for the duration of the study, demonstrating a persistent IMI was established.

Following arrival of an antigen at a regional lymph node, CD4+ T cells specific for that antigen are trapped in the lymph node [279]. Expansion and differentiation of T cell populations follows such that antigen specific T cells capable of directing the immune response are present. Therefore, one might have expected an increase in both CD4+ and CD8+ T cell populations in the SMLNs following *S. aureus* IMI. Animals challenged with SA10 had significantly higher numbers of CD4+ cells in the SMLNs three days following infection. As this change was observed in both infected and uninfected udder halves, it may reflect a systemic effect of SA10 infection on CD4 positive cell numbers. Fifteen days following infection, the SA8 isolate appeared to increase the numbers of CD4, CD8 and γδ positive T cells in the lymph nodes draining infected udder halves although the changes were not statistically significant. It would be interesting to report this

experiment with more animals to observe if these changes were repeatable. If so, changes may reflect an expected induction of adaptive cellular immune responses at the local lymph node. A similar increase in CD4 positive cells was observed fifteen days following SA10 infection. This may also reflect induction of adaptive responses although the lack of CD8 cell recruitment may indicate that cytotoxic T cells are not being generated. A previous study [212] reported no significant alterations in SMLN leukocyte populations following *S. aureus* IMI. However, this study analysed leukocyte populations using immunohistochemistry and did not quantify changes in cell populations. It is to be expected that the current study is of increased sensitivity due to the use of flow cytometry for accurate quantification of leukocyte populations.

Immunohistochemical staining revealed no major effects of *S. aureus* IMI on local leukocyte populations. However, it must be remembered that IHC is a very subjective method of analysis and is better suited to evaluation of tissue morphology rather than absolute numbers of cells in tissue. The observed increase in CD4 and CD205 positive cells fifteen days following SA8 infection may reflect a local immune response. Leitner and co-workers [212] reported an increase in the numbers of CD8 positive cells in mammary parenchyma as a result of *S. aureus* IMI greater than three months in duration. It has also been reported that the numbers of CD4 and CD8 positive cells in milk increase as a result of *S. aureus* IMI greater than two months in duration [6]. We did not observe an increase in CD8 positive cells. The results presented here are more consistent with recruitment of CD4 positive T helper cells and myeloid cells differentiating into DCs in the mammary gland of animals chronically infected with *S. aureus*. Our studies may not have analysed infections of sufficient duration to observe recruitment of immune effectors into the mammary gland although we may be observing the induction of cellular responses in infected tissue.

The development of lymphoid aggregates in the mammary gland was an interesting and unexpected observation. Organization appeared to occur independent of infection status of the mammary half although only a small proportion of the gland is observed using IHC. A potential explanation for this observation is that the animals in the study all had recall immune responses to *S. aureus* (Table 4.4). Even though animals were not infected with *S. aureus* at the beginning of the study, this does not rule out previous *S. aureus* IMI or IMI with other mastitis pathogens that

resulted in chronic inflammation in mammary parenchyma and establishment of these lymphoid aggregates.

Whilst observation of changes in cell populations is useful in the evaluation of mammary cellular immune responses, it does not give any information regarding cell function and the nature of the immune response generated. Evaluation of changes in cytokine levels in each of these locations and investigation of the cell types that secrete each cytokine enables us to determine this information.

Our data revealed that the SA8 isolate was associated with decreased IL-10 expression in the SMLNs during acute infection. No differences were noted in CD4, γδ or CD11c positive cells expressing IL-10 as a result of SA8 infection. As no data are available regarding CD8 positive cells expressing IL-10, the contribution of this cell type to SA10 levels cannot be determined. Infection with SA8 did result in an increase in CD11c+ IL-4 secreting cells in the SMLN. The SA10 isolate caused increased IFN-γ expression in the teat ends during acute and chronic infection. The SA10 isolate also decreased CD4+ IL-10 expressing cells in SMLNs during acute infection.

Previous studies have reported contradictory results regarding cytokine expression in *S. aureus* IMI. For example, studies have reported that IFN-γ levels in milk may increase [4-6] or remain unchanged following *S. aureus* IMI [213]. Our data may provide an explanation for these differing results in that IFN-γ increased in the teat ends of animals challenged with SA10 but not in those challenged with SA8. However, we observed no changes in IL-10 expression in the mammary parenchyma following infection with either isolate. It is difficult to draw comparisons between cytokine levels at different locations and those in milk but one might expect changes in mammary parenchyma to most closely reflect those in milk. The only previous study of IL-4 levels in milk reported that no IL-4 mRNA was present in milk somatic cells. The data reported in the current study indicates that IL-4 mRNA was isolated from different sites in the mammary gland. The lack of IL-4 mRNA in milk somatic cells may reflect the fact that the majority of milk somatic cells during *S. aureus* IMI are neutrophils therefore are unlikely to express IL-4. No differences in IL-4 expression were observed in any of the sites assayed in the current study.

However, in the SMLNs of animals infected with SA8 we observed an increase in CD11c+ cells expressing IL-4. Even though no changes in expression were observed on qRT-PCR, we might be observing an early shift towards an IL-4 response. If the animals were euthanized at a later date, more evidence for an IL-4 response may have been obtained. Previous studies all report an increase in milk IL-10 following *S. aureus* IMI during the acute (48 hours following challenge) [4] or chronic [6] (greater than three months following challenge) phases of infection. A decrease in IL-10 was observed during the acute phase of infection with SA8 followed by an increase in IL-10 during the chronic phase in the current study. This may reflect differences in the *S. aureus* isolates used since Newbould 305 was used to observe responses 48 hours following challenge and multiple wild type isolates were used to observe responses greater than three months following challenge.

A major limitation of the *in vivo* work is the low numbers of animals in each group. This was due to the very limited availability of lactating ewes that were negative for *S. aureus* on three consecutive milk cultures before the study. The limited number of animals increases the likelihood of type II statistical error in which the hypothesis that no difference exists between the groups is inappropriately retained. Repeating this work with increased numbers of animals would confirm the results that we have observed and may increase the chance of observing significant differences.

During the *in vivo* studies, qRT-PCR was used to determine cytokine expression in tissue. This technique was chosen due to its high sensitivity in detecting mRNA in tissue. A recent study characterized the kinetics of ovine IFN-γ and IL-10 mRNA and protein *in vitro* [280]. Animals used as blood donors had been previously immunized with OVA. When PBMCs collected from these animals were restimulated *in vitro* with OVA, IFN-γ mRNA levels increased for the first 48 hours after restimulation and then decreased slightly until 96 hours. When IFN-γ protein levels were assayed, protein levels remained low over the first 48 hours but then increased up until 96 hours. Following restimulation, IL-10 levels peaked at 6 hours, then decreased until 24 hours and remained at a plateau. Increased IL-10 protein levels began at 6 hours and continued to increase until 96 hours. These data suggest that changes in mRNA levels precede those of protein. While these data appear to support the use of qRT-PCR to determine tissue cytokine expression, it must be remembered that cytokine gene expression and protein production occur at different times

therefore results here may not have been reflective of cytokine protein assays performed at the same timepoints. Also, qRT-PCR does not take cell recruitment into account. Specifically, if increased numbers of cells are secreting cytokine, cytokine protein levels will increase even though gene expression, as assayed by qRT-PCR, will remain unchanged.

In summary, the two *S. aureus* isolates generated significantly different responses. For both isolates, we observed an initial acute inflammatory response at the teat end which was characterized by an increase in IFN-γ levels. This increase persisted to day 15 following SA10 infection. Increases in IFN-γ 15 days following SA10 infection occurred in the mammary parenchyma and SMLNs, suggesting a pro-inflammatory response, although the range of data recorded is large and not statistically significant. No major changes in IL-4 levels occurred as a result of IMI. However, CD11c+ IL-4 expressing cells increased significantly as a result of SA8 IMI which may reflect the early stages of an IL-4 response. A decrease in IL-10 expression was observed at the teat ends and SMLNs during the acute phase of infection. Teat end IL-10 expression increased during the chronic phase. These changes may reflect an initial decrease in IL-10 levels as a result of acute inflammatory responses followed by a regulatory response in the teat end. This would be reflective of the changes observed as a result of SA8 *in vitro*.

Differences also exist between cytokine responses observed *in vitro* and *in vivo*. These differences may result from differences between the experimental models. The first difference is that sheep and cattle were used and care should be taken in directly comparing immune responses between species.

The major difference between *in vitro* and *in vivo* models is the more complete anatomic localization of T cell recruitment *in vivo*. This is especially relevant when antigen presentation is considered. When PBMCs are isolated, the resulting cell mixture consists of a majority of lymphocytes alongside monocytes and monocyte-related cells such as DCs [281]. Dendritic cells in blood tend to be in an immature state with low levels of MHC proteins and costimulatory molecules and are thus far less able to induce T cell responses than are those in tissue [281]. Immature dendritic cells in tissue possess this immature phenotype and have high phagocytic capacity. Acquisition of antigen and migration of DCs to the draining lymph node is associated

with DC maturation and antigen presentation [282]. Given that migration to the regions lymph node cannot occur *in vitro*, antigen presentation is likely to be as a result of monocytes and immature DCs. Antigen presentation will not be anatomically linked therefore will be less efficient. Therefore, the majority of the antigen-specific response *in vitro* will probably by memory T cells in the blood that have not yet trafficked to a lymph node at a site of antigenic stimulation. The animals used for *in vitro* studies had prior exposure to *S. aureus* and memory T cells are thought to retain the cytokine secreting characteristics present in the initial immune response [283]. They appear to be more effective in generating T cell help than cells induced during primary immune responses [284]. This suggests that stimulation of PBMCs in this work will result in the secretion of the same cytokines as those that occurred when animals were initially exposed to *S. aureus* antigens. However, the magnitude of the increases may be exaggerated. Furthermore, the presence of teichoic acid in the staphylococcal cell wall may act as a stimulus for T cell proliferation, further exaggerating the magnitude of changes.

When animals are challenged *in vivo*, antigen presentation can occur by DCs and the DCs themselves can influence T cell responses. Memory T cells will also play a role in our model as the animals had prior exposure to *S. aureus*. It is likely that the acute responses observed *in vivo* reflect innate immunity. The innate responses are reflected in the increases in IFN-γ observed at the teat end in response to each *S. aureus* isolate. As the immune responses move into the chronic phase, both DCs will play a role. We studied the function of DCs in the SMLNs using flow cytometric staining for cytokines. The only significant change noted was an increase in CD11c+ IL-4 expressing cells following SA8 IMI. This increase may reflect stimulation of TLR2 by lipoteichoic acid on the *S. aureus* cell surface. However, as has been discussed, there was no evidence of an overall IL-4 response.

As the overall changes observed could not be explained by the results observed in SMLN DCs, it is likely that memory T cells played a major role. This is the most likely explanation for the increase in IFN-γ observed during the chronic phase of SA10 IMI. The initial exposure of these animals to *S. aureus* may have promoted the formation of DCs with a T_H1 bias and generated a T_H1 response. This response recurred when the animals were challenged with *S. aureus* during the current study.

It is important to discuss how the changes that we have observed may be important in the pathogenesis of *S. aureus* IMI. During the acute phase of IMI, we observed inflammatory responses at the teat end. Results with the SA8 isolate suggest that it may induce an anti-inflammatory response characterized by IL-4 and IL-10 *in vivo* although the responses to SA8 were generally of a low level and not statistically significant. This is similar to the *in vitro* changes observed in PBMC cultures following exposure to the majority of *S. aureus* isolates and may reflect an adaptation of *S. aureus* to prevent the generation of protective cellular immune responses and thus enhance bacterial survival in the mammary gland. Results with the SA10 isolate suggest that it may induce a pro-inflammatory response characterized by IFN-γ *in vivo*. This response is considered to be appropriate for a pathogen such as *S. aureus* which is able to become intracellular. Although the available evidence suggests that SA10 is able to survive in the mammary gland, the evidence is limited to observations in the *in vivo* studies carried out in this study. Studies with increased numbers of animals may reveal that SA10 is less likely to survive within the mammary gland than other *S. aureus* isolates. It must also be remembered, however, that *S. aureus* possesses other virulence factors that may facilitate its survival in the mammary gland. Also, as has been already discussed, immune responses in the mammary gland appear to be less effective than those in blood making the survival of *S. aureus* more likely.

7. CONCLUSIONS

The data presented show that different *S. aureus* isolates have different effects on mammary gland cellular immune responses. However, the same isolates do not have identical effects *in vitro* and *in vivo*. The differences between the *in vitro* and *in vivo* effects may be explained by differences between the models as a result of differences in antigen presentation, the role of memory T cells and the different species of animal used in each model. In summary, SA8 appears to induce pro-inflammatory and anti-inflammatory responses *in vitro* but appears to generate an anti-inflammatory response *in vivo*. The SA8 isolate does increase IFN-γ at the teat end at the acute phase of infection. This may be suggestive of a pro-inflammatory response at the teat end alone. The SA10 isolate appeared to induce pro-inflammatory and anti-inflammatory responses *in vitro* but only generated a pro-inflammatory response *in vivo*.

Staphylococcal enterotoxin C appears to cause a regulatory T cell response when purified protein used to stimulate PBMCs *in vitro*. It did not have any effect when secreted by a transformed bacterial strain therefore no effect of SEC is apparent when it is present at physiologic conditions. Given that the strain transformed was a low virulence strain, SEC may work in conjunction with other virulence factors. Generation of a *sec* mutant *S. aureus* strain would have tested this hypothesis but this strain could not be made. Given that the two *S. aureus* isolates tested *in vivo* both possess the *sec* gene and that the prevalence of the *sec* gene is relatively low, it might be expected that *sec* has minimal effects on mammary gland immune responses.

The results of this work suggest that different *S. aureus* isolates have distinct effects on mammary gland immune responses. One of the isolates (SA8) appears to generate an anti-inflammatory response *in vivo* but both pro-inflammatory and anti-inflammatory responses *in vitro*. A different isolate (SA10) appears to generate both pro-inflammatory and anti-inflammatory responses *in vitro* and *in vivo*. Therefore, the original hypothesis for this work is valid for some *S. aureus* isolates but not others.

The results of these studies illustrate the limitations of *in vitro* studies when determining immune responses to *S. aureus* as *in vivo* and *in vitro* studies reported different effects. Also, the

limitations of investigating immune responses using a single isolate are illustrated. Future studies should focus on investigating immune responses *in vivo* and should characterize responses to different strains. This may have implications for future vaccine strategies in that strains selected to immunize against *S. aureus* should generate protective immunity and should be tested against infection with different *S. aureus* strains. Future studies should also focus on the site of infection and not just assay markers of immune responses in milk.

8. REFERENCES

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