Induction of IgA secretion in the bovine mammary gland

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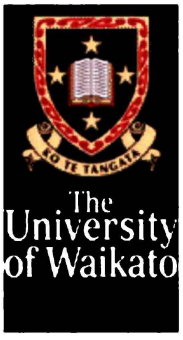

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Abstract

This thesis investigates molecular and cellular mechanisms involved in IgA secretion in the bovine mammary gland. Our previous research showed that multi-site immunisation during late-pregnancy, involving priming by intra-peritoneal and intra-muscular routes followed by intra-mammary immunisation, enhanced IgA secretion in milk and this effect was localised to immunised glands. This thesis addresses the hypothesis that increased IgA is produced by localised IgA plasma cells of mucosal origin that are recruited to the mammary gland by changes induced by intra-mammary immunisation.

Recruitment mechanisms for lymphocytes were studied by examining expression of adhesion molecules MAdCAM-1, VCAM-1 and PNAd in mammary tissues of untreated animals, using immunohistochemical and molecular techniques. No immunoreactive MAdCAM-1 protein was found in venules and very low levels of MAdCAM-1 mRNA were found in tissues. By contrast, VCAM-1 protein was observed on larger venules. PNAd protein was only detected in supramammary lymph nodes, suggestive of a role for recruiting naïve lymphocytes.

Cows were immunised with only one udder-half receiving intra-mammary immunisation to investigate effects on immunised glands. Animals with increased levels of IgA in milk had significantly more IgA plasma cells and T cells in immunised glands compared with non-immunised glands. Again no detectable MAdCAM-1 protein and only very low levels of MAdCAM-1 mRNA were found. No differences were observed in VCAM-1 protein expression between immunised and non-immunised mammary tissues. Together these studies suggest that MAdCAM-1 and VCAM-1 are not involved in lymphocyte recruitment to the bovine mammary gland, which contrasts to mice.

In another study, cows categorised as either high- or low-responders to the multi-site immunisation were re-immunised to compare responses. Changes to lymphocytes in mammary secretions collected at intervals following two intra-mammary immunisations were monitored by flow cytometry. The percentage of T cells was found to decrease in both groups, although CD4 cells increased relative to CD8. The percentage of IgA-positive cells increased in a biphasic manner with high-responding animals showing an earlier and more sustained increase in IgA-positive cells compared with low-responders. The strong correlation observed between

percentages of IgA-positive cells and IgA antibody in secretions suggests high levels of IgA require greater numbers of IgA-producing cells.

Molecular analysis of known key cytokines found higher levels of IL-8 and IFN-γ mRNA in immunised mammary tissues compared with non-immunised glands. In cells from mammary secretions, IL-8 mRNA increased shortly after immunisation while IFN-γ and TGF-β mRNA increased at a later stage. However, no discernible differences were observed between high- and low-responding animals. TNF-α, IL-2, IL-6 and IL-10 mRNA were found to be low in both mammary tissues and secretion cells. IgA mRNA was increased in immunised tissues although no correlation was observed between IgA mRNA and mRNA levels of the polyimmunoglobulin receptor. These analyses indicate that innate and cell-mediated responses were induced along with an antibody response to immunisation.

Together these results support the hypothesis and indicate that intra-mammary immunisation increases IgA-producing cells in the mammary gland accompanied by changes in expression profiles of a range of cytokine genes. However, in contrast to mice, the data suggest little involvement of known adhesion molecules in recruiting lymphocytes to the gland.

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List of Abbreviations

Standard International Unit abbreviations were used throughout this thesis

Ab	antibody
AID	activation-induced cytidine deaminase
BAFF	B-cell activating factor of the tumour necrosis family
BALT	bronchial-associated lymphoid tissue
BLIMP-1	B-lymphocyte induced maturation protein
BLyS	B-lymphocyte stimulator
Bov	bovine
bp	base pair
BSA	bovine serum albumin
C. albicans	*Candida albicans*
CD	cell-differentiation
cDNA	complementary deoxyribonucleic acid
CN	cycle number
DAB	3,3'-diaminobenzidine hydrochloride
DEPC	diethyl pyrocarbonate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
D-PBS	Dulbecco A phosphate buffered saline
DTT	dithiothreitol
E	efficiency
E. coli	*Escherichia coli*
EDTA	disodium ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
EST	expressed sequence tag
FBS	fetal bovine serum
FCA	Freund's complete adjuvant
FICA	Freund's incomplete adjuvant
FITC	fluorescein isothiocyanate
FSC	forward scatter
g	relative centrifugal force
GALT	gut-associated lymphoid tissue
HEV	high endothelium venules

H+L	heavy and light chains of the immunoglobulin molecule
HRP	horse radish peroxidase
ICAM	intercellular cell-adhesion molecule
Ig	immunoglobulin
IHC	immunohistochemistry
IL	interleukin
IM	intra-muscular
IMM	intra-mammary
IP	intra-peritoneal
kb	kilobase
kDa	kilodalton
LFA	leukocyte functional antigen
log	logarithm
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAdCAM-1	mucosal addressin cell-adhesion molecule-1
MALT	mucosal-associated lymphoid tissue
MHC	major histocompatibility complex
MQ-H_2O	deionised water
mRNA	messenger ribonucleic acid
Mu	mouse
NALT	nasal-associated lymphoid tissue
NK	natural killer
O.C.T.	optimal cutting temperature
PBS	phosphate buffered saline
PBS-T	1% Tween® 20 in PBS
PCR	polymerase chain reaction
PE	phycoerythrin
pIgR	polymeric immunoglobulin receptor
PNAd	peripheral node addressin
PP	Peyer's patch
R	receptor
Rb	rabbit
RNA	ribonucleic acid
RNase	ribonuclease
RPMI	Roswell Park Memorial Institute medium
RT-PCR	reverse transcription-polymerase chain reaction
S. aureus	*Staphylococcus aureus*

SC	secretory component
SEM	standard error of the mean
SMLN	supramammary lymph node
SSC	side scatter
S. uberis	*Staphylococcus uberis*
TAE	tris-acetic-EDTA buffer
TALL-1	TNF- and APOL-related leukocyte expressed ligand
TBS	Tris-HCl buffered saline
TBS-T	1% Tween® 20 in TBS
TE	Tris-EDTA
TGF-β	transforming growth factor-beta
TLR	toll-like receptor
TMB	3,3',5,5'-tetramethylbenzidine
TNF-α	tumour necrosis factor-alpha
Tris	tris(hydroxymethyl)aminomethane
TU	titre units
U	units of enzyme
VCAM-1	vascular adhesion molecule-1
VLA	very late activation antigen
v/v	volume per volume
vWF	von Willebrand Factor
w/v	weight per volume

Abbreviations for commercial suppliers

Ajax Chemicals	Ajax Chemicals Ltd, Sydney, Australia
Amersham Biosciences	Amersham Biosciences (NZ), Auckland, NZ
Andrew Industrial	Andrew Industrial Ltd., Auckland, NZ
Applied Biosystems	Applied Biosystems, Scoresby, Vic, Australia
Barnstead	Barnstead International, Dubuque, Iowa, USA
Baxter Healthcare	Baxter Healthcare Pty. Ltd., Old Toongabbie, NSW, Australia
Bayer Diagnostics	Bayer Diagnostics, Auckland, NZ
BDH	BDH Laboratory Supplies, Poole, Dorset, UK
BD	BD (NZ), Auckland, NZ
Bethyl	Bethyl Laboratories, Montgomery, TX, USA
Biolab Scientific	Biolab Scientific, Auckland, NZ
BioRad	Bio-Rad Laboratories Pty. Ltd., Auckland, NZ
Biotech Australia	Biotech Australia, Roseville, NSW, Australia
Bio-Tek	Bio-Tek Instruments, Winooski, VT, USA
Bomac Laboratories	Bomac Laboratories, Auckland, NZ
Cambridge Instruments	Cambridge Instruments GmbH, Heidelberg, Germany
Chemicon	Chemicon Australia Pty. Ltd., Boronia, Vic, Australia
Dako	DakoCytomation, DK-2600, Glostrup, Denmark
Eppendorf	Eppendorf AG, Hamburg, Germany
GraphPad	GraphPad Software Inc., San Diego, CA, USA
IKA-Werke	IKA-Werke GmbH & Co. KG, Staufen, Germany
Invitrogen	Invitrogen (NZ), Auckland, NZ
Jackson	Jackson Immunoresearch Laboratories, West Grove, PA, USA
Leitz	Ernst Leitz GmbH, Wetzlar, Germany
Olympus	Olympus Europa GmbH, Hamburg, Germany
Nunc	Nalge Nunc International, DK-4000, Roskilde, Denmark
Pharmingen	Pharmingen, San Diego, CA, USA
Qiagen	Qiagen GmbH, Hilden, Germany
Roche	Roche Diagnostics NZ Ltd., Auckland, NZ
SciTech	SciTech Pty. Ltd., Preston, Australia
Sigma	Sigma Chemical Co., St Louis, MO, USA
Shoof	Shoof International Ltd., Cambridge, NZ
VMRD	VMRD, Pullman, WA, USA

Chapter 1

Introduction and literature review

1.1 General introduction

This thesis investigates the induction of local immunoglobulin A (IgA) secretion in the bovine mammary gland. Prior to this study, our group at AgResearch had developed a successful immunisation protocol for production of IgA in ruminant milk (Hodgkinson and Hodgkinson, 2003). The concept being that milk antibodies directed against human gut pathogens would provide valuable protection as a human therapeutic agent or bioactive food ingredient. The immunisation development programme specifically targeted IgA antibodies, rather than other immunoglobulin classes, based on the knowledge that IgA is the natural immunoglobulin of mucosal surfaces. For example, IgA is the antibody class normally secreted at the mucosal surface of the gastrointestinal tract. Consequently IgA is structurally designed to resist enzymatic attack in these environments and has a major role in preventing colonization of pathogens at mucosal surfaces and eliminating these microorganisms. Therefore, IgA would logically qualify as an effective and efficient human therapeutic/ prophylactic antibody for providing protection of mucosal surfaces.

Although milk is a rich source of naturally occurring antibodies, in the ruminant the predominant lacteal immunoglobulin is IgG_1, in contrast to humans where IgA is the major immunoglobulin of both colostrum and milk (Butler, 1969). In the human, the origin of IgA in mammary secretions is well defined by the concept of the gut-mammary axis, where precursors of antibody-producing cells traffic between the two mucosal sites (Brandtzaeg, 1983). By contrast, this axis does not appear to be particularly well developed in the ruminant (Watson, 1981).

To induce an IgA response in cows' milk our own empirical experiments have shown that the most successful regimen is to treat pregnant non-lactating animals with injections of antigen by combined intra-peritoneal, intra-muscular and intra-mammary routes, with booster doses of antigen to all three sites given subsequently. The method was successful in raising high levels of antigen-specific and total IgA antibodies but the underlying immunology was not well understood. Additionally, there was a large variation in response to the immunisation regimen. Whereas some animals responded with high-titre IgA antibodies, other individual animals did not respond at all. To ensure the immunisation protocol was economically viable a good response from each animal was important. The fundamental molecular and cellular mechanisms underlying our immunisation regimen required investigation to provide

essential knowledge for further development and optimisation of the immunisation procedure. Development of this understanding is the objective of this thesis.

Chapter One reviews the current literature for the mucosal immune system. This is not an exhaustive review but concentrates on those aspects that pertain to our immunisation regimen. The review focuses on the induction of a B-cell immune response in the gut and follows through the process of homing the cell to the effector site and its terminal differentiation into an antibody-secreting plasma cell. The structure of IgA is described along with its unique characteristics and functions. Essential literature on the immunology of the ruminant mammary gland is reviewed together with the literature backgrounding current know-how on inducing an antibody response in mammary secretions in the cow.

While ruminant immunology has contributed novel findings to the field of immunology as a whole, most research to date has been in rodent and human systems; therefore, the general information reviewed in this chapter is largely based on research in these species. Specific data on the ruminant immune systems is centred on knowledge of the cow (*Bos taurus*) and the sheep (*Ovis aries*) as these domesticated *bovidae* have been the most extensively studied of the ruminants.

1.2 The mucosal immune system

The immune system acts to protect the body from the invasion of pathogens by two main systems; the innate and the adaptive systems, which share many common mechanisms. The innate system is the first line of defence and acts to clear the infection or contain it until the adaptive response develops. Adaptive immunity specifically recognises pathogens and provides protection against re-infection by producing an immunological memory of the antigen. The adaptive immune response can be artificially induced by vaccination, for example, by introducing antigen to the body by injection. The key cells for the adaptive immune response are the lymphocytes, derived from hematopoietic stem cells in the bone marrow. B lymphocytes (B cells) differentiate into plasma cells that secrete antibody. T lymphocytes (T cells) are involved in the activation of B cells and can be further defined into subsets, dependent on their effector role.

In contrast to other leukocytes, lymphocytes continuously recirculate from peripheral blood to efferent lymph through secondary lymphoid tissues. This recirculation increases the chance that a naïve lymphocyte will encounter its specific antigen and is also important for the dissemination of activated cells to their effector sites.

Anatomically the adaptive immune system consists of organised lymphoid tissue that can be separated into primary lymphoid organs, where lymphocytes are generated, and secondary lymphoid organs, where adaptive immune responses are initiated and lymphocytes are maintained. The adaptive system can be divided into two major systems, which in many ways are independent with respect to regulation and function. The first is the systemic lymphoid system which utilises the spleen and peripheral lymph nodes and responds to antigen that has entered the body through tissues or blood. A systemic immune response is characterised by production of serum antibodies of the IgG, IgM and IgE classes. The second major system, the mucosal immune system, consists of mucosal lymphoid tissue, mucosal-associated lymph nodes and secretory epithelium. The response tends to be local and is typified by the IgA class of antibody, produced in external secretions such as saliva, tears, milk and mucus. One exception of note is that of the ruminant mammary gland, where IgG predominates in the mammary secretions.

The mucosal immune system provides protection from foreign antigens and pathogens encountered at mucosal surfaces including the oral and nasal cavities and the respiratory, gastrointestinal, and urogential tracts. Organised lymphoid tissues

throughout the body facilitate the uptake of these potential threats to the host. In the gastrointestinal tract these comprise of the gut-associated lymphoid tissues (GALT). In the respiratory tract and the nasal/oral cavity the organised lymphoid tissues are termed the bronchial-associated lymphoid tissues and the nasal-associated lymphoid tissues, respectively. Collectively, the lymphoid tissues protecting the mucosa are known as the mucosal-associated lymphoid tissues. The GALT region is the largest of the mucosal immune system and at least 80% of all immunoglobulin-producing plasma cells in the body are found in the GALT effector site, the lamina propria of the gut (Conley and Delacroix, 1987).

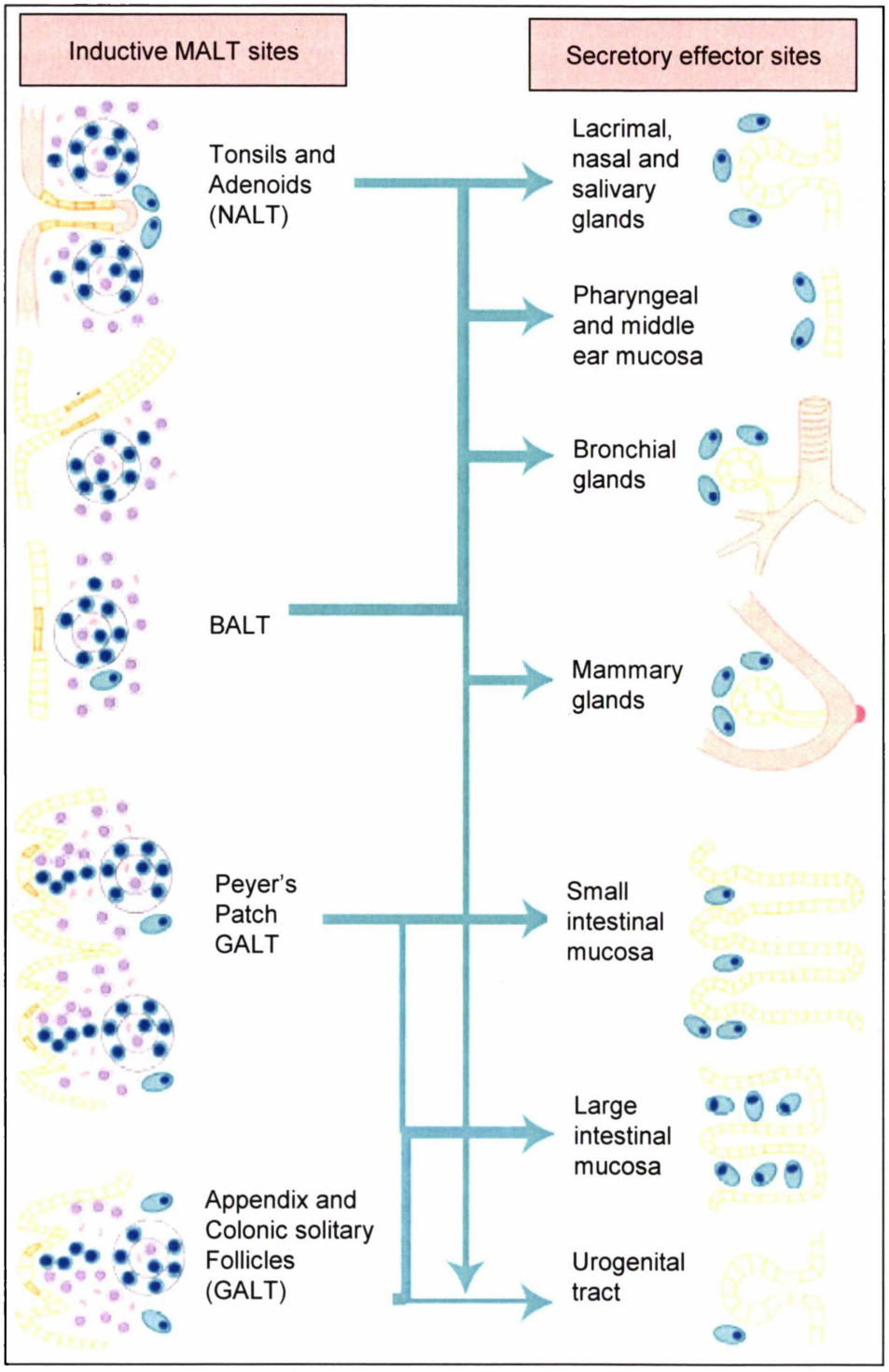
1.2.1 *The common mucosal immune system*

The mucosal immune system provides an integrated network of lymphoid organs that operate, to a large extent, independently of the systemic immune system. The concept of the 'common mucosal immune system' was proposed by researchers as early as the 1970's (McDermott and Bienenstock, 1979; McDermott *et al.,* 1980). Adoptive transfer studies showed that lymphocytes from the GALT populate many mucosal effector sites including intestine, urogenital tract, mammary glands, salivary glands and respiratory tract (Husband and Gowans, 1978; Rudzik *et al.,* 1975; Weisz-Carrington *et al.,* 1979). The concept is that the antigenic experience at one mucosal surface can be mirrored in immune effector responses at a distant mucosal tissue. This rationale has been used as a basis for the design of mucosal vaccines (Iijima *et al.,* 2001; Mestecky, 1987; Staats and McGhee, 1996). We used this principle to develop our immunisation protocol for inducing IgA in ruminant milk (Hodgkinson and Hodgkinson, 2003). More recent studies have indicated that the common mucosal immune system may be more compartmentalised than originally thought (Butcher *et al.,* 1999; Csencsits *et al.,* 2001) and that there may be preferential homing to some locations dependent on the site of induction (Quiding-Jarbrink *et al.,* 1997).

In the remainder of Section 1.2, the course of events that occur in the common mucosal response is described in detail. In the mucosal response, T cells and B cells circulating between inductive and distant effector sites are responsible for the IgA production in external secretions. Figure 1.1 shows the integration of mucosal and effector sites in the common mucosal immune system. Antigen or pathogen is sampled from the external mucosal surface by specialised antigen-presenting cells and transported to inductive sites in lymphoid tissue enriched with T cells and B cells (for example, Peyer's patches in the GALT).

Figure 1.1 A schematic representation of the network of inductive and effector sites in the common mucosal immune system

The immune cells have been colour coded: B cells and plasma cells are green, T cells are purple, and antigen-presenting cells are pink. (Diagram adapted from Brandzaeg *et al.*, 1999b)



MALT - mucosal-associated lymphoid tissue
NALT - nasal-associated lymphoid tissue
BALT - bronchial-associated lymphoid tissue
GALT - gut-associated lymphoid tissue

Antigen presentation induces lymphocyte activation and migration. Effector lymphocytes enter the lymphatic system and are transported into the blood from where they may repopulate the site of initial activation or distant sites in the common mucosal immune system. Preferential migration and extravasation at the effector site is directed by differential expression of adhesion molecule and chemokine receptors on the cell surface of lymphocytes. At the effector sites, local antigen-driven signals are thought to provide stimulus for retention, expansion and terminal differentiation of B cells into plasma cells.

1.2.2 *Induction of the mucosal response*

1.2.2.1 Antigen uptake across epithelial barriers

To initiate an immune response it is essential to first have capture of antigens and pathogens and then presentation to and recognition by the appropriate immune cells. On mucosal surfaces the pathogens and immune cells are separated from one another by epithelial barriers. Epithelium differs structurally at different mucosal sites in the body and the strategy for antigen sampling varies according to the nature of this epithelium (Neutra *et al.,* 1996b). In the stratified epithelium of the tonsils and vagina, motile intra-epithelial dendritic cells, similar to Langerhans cells in the skin, act as surveillance cells (Miller *et al.,* 1992; Okato *et al.,* 1989). These cells capture antigens and then migrate to local or distant lymphoid tissues.

The gastrointestinal tract and airways have a simple epithelium sealed by tight junctions. Antigen is sampled by two distinct methods. One mechanism involves a network of intra-epithelial dendritic cells (Holt *et al.,* 1990; Maric *et al.,* 1996). In the gut, *in vitro* and *in vivo* studies have shown that dendritic cells are able to sample the luminal space via tight junctions in the epithelium. Dendritic cells open the tight junction, extend dendrites outside the epithelium and directly sample bacteria (Rescigno *et al.,* 2001). The integrity of the epithelial layer is preserved because dendritic cells have the ability to express tight junction proteins such as occludin, claudin 1 and zona occludens 1. Dendritic cells then present antigen locally to organised lymphoid tissue or migrate to regional lymph nodes.

An alternative sampling method involves a specialised area in the epithelium, termed the follicle-associated epithelium which overlies mucosal lymphoid follicles. The structure of these follicles differs in different sites in the body. Aggregates of lymphoid follicles, termed Peyer's patches, are found only in the small intestine while

isolated lymphoid follicles are more widespread and occur in the large and small intestine (Hamada *et al.,* 2002) as well as the nasal cavities and the bronchi (Bienenstock and Clancy, 1994). The specialised follicle-associated epithelium contains microfold cells, a sub-epithelial-dome rich in dendritic cells, and B-cell follicles that include germinal centres (Kato and Owen, 1999). In Peyer's patches, there are specific inter-follicular areas containing T-cell-rich areas and specialised post-capillary venules, termed high endothelial venules which serve as entry and exit points for migrating cells (Girard and Springer, 1995).

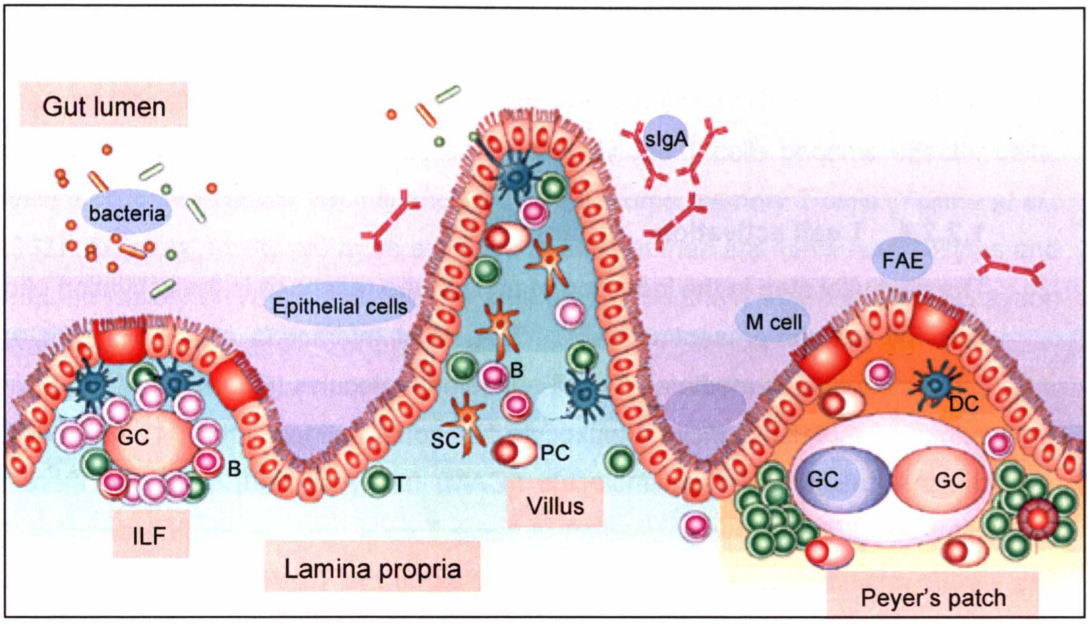
1.2.2.2 Unique features of the follicle-associated epithelium

The follicle-associated epithelium of the small intestine is the best described. It contains unique epithelial M cells that transport foreign macromolecules, particles and microorganisms to antigen-presenting cells within and under the epithelial layer of the mucosa (Hathaway and Kraehenbuhl, 2000; Neutra and Kraehenbuhl, 1992). M cells lack brush border microvilli, do not secrete mucus and have a large invagination of the basolateral membrane that forms a pocket containing lymphocytes. In the human M-cell pocket, it has been shown that there are equal numbers of IgM-producing B cells and memory helper T cells (Farstad *et al.,* 1994). It is postulated that the M-cell pocket may be the site for initial antigen encounter by lymphocytes and may play a part in oral tolerance in the gut (Brandtzaeg *et al.,* 1999a). However, the specific role of these lymphocytes at this site has not been elucidated (Neutra *et al.,* 2001).

The deep invagination of the M cell acts to bring the basolateral surface into close proximity of the apical membrane, so that transcytotic vesicles have only a short distance to travel. Secretory IgA in the lumen can bind to M-cell apical membranes and be taken up into the M-cell pocket. It is postulated that this mechanism allows for the reprocessing of antigen, delivered by the secretory IgA to the mucosal immune inductive sites, and further promotes local contact of intact antigens and pathogens with the follicle-associated epithelial surface (Weltzin *et al.,* 1989). Certain pathogenic bacteria and viruses exploit the characteristics of M-cell adherence and transport mechanisms, to cross the epithelial barrier and invade the host (Neutra *et al.,* 1996a; Neutra and Kraehenbuhl, 1992). The strategies employed by these organisms have in turn been exploited in vaccine design by using attenuated pathogens to deliver antigen to mucosal surfaces (Neutra *et al.,* 2001). A diagrammatic representation of the gut-associated lymphoid tissues with the organised lymphoid structures is shown in Figure 1.2.

Figure 1.2 A schematic representation of the gut-associated lymphoid tissues with the organised lymphoid structures

Peyer's patches and isolated lymphoid follicles (ILF) - and the diffuse tissue of the epithelium and the lamina propria are shown. Beneath the follicle-associated epithelium (FAE) containing M cells, are areas containing dendritic cells (DC, teal) and B-cell follicles with germinal centres (GC) surrounded by T cells (green). The diffuse tissues of the lamina propria contain large numbers of plasma cells (PC, red), T and B cells (pink), stromal cells (SC, brown) and DC. Secreted IgA is transported across the epithelium into the gut lumen. (Diagram adapted from Fagarasan and Honjo, 2003)



1.2.2.3 Presentation of antigen

Antigen-presenting cells are specialised cells that take up antigen and pathogens and process them into peptide fragments by intra-cellular mechanisms. These fragments are presented on the cell surface of the antigen-presenting cells by a specialised group of proteins termed the major histocompatibility complex (MHC) (Germain, 1994). These peptide binding-proteins are of two types, MHC class I, recognised by T-cell receptors on cytotoxic T cells (CD8) and MHC class II recognised by helper T cells (CD4) (Schwartz, 1984; Zinkernagel and Doherty, 1979). Antigens derived from different types of pathogens are displayed on a particular class of MHC molecule (Morrison *et al.*, 1986). The most effective of the antigen-presenting cells is the dendritic cell but macrophages and B cells can also activate naïve CD4 T cells (Kupfer *et al.*, 1986; Underhill *et al.*, 1999). Dendritic cells are dedicated antigen-presenting cells as their only known function is the presentation of antigen to T cells (Banchereau and Steinman, 1998).

Dendritic cells are central to the induction of the adaptive immune response. Recognition of antigen occurs through signalling via the Toll-like receptors (TLR), proteins which are a crucial link between the innate and adaptive immune system (Akira *et al.,* 2001). Binding of antigen by specific receptors on dendritic cells initiates and modulates dendritic cell maturation, and different antigens result in functionally different effector subsets of dendritic cells (Iwasaki and Kelsall, 1999; Iwasaki and Kelsall, 2000). In addition to antigen, signals from inflammatory tissue play a role in dendritic cell activation and migration to T-cell-rich areas. These activated, mature dendritic cells are no longer phagocytotic but display a repertoire of molecules, including co-stimulatory molecules and cell-adhesion molecules and they secrete a range of cytokines (Banchereau and Steinman, 1998).

1.2.2.4 T cell activation

The next vital step in the induction of an immune response is the activation of naïve T cells by antigen-presenting cells. The initial interaction of T cells with antigen-presenting cells is mediated by cell-adhesion molecules (Gunzer *et al.,* 2000). These adhesion molecules include leukocyte functional antigen-1 (LFA-1) and members of the intercellular adhesion molecule (ICAM) family and are described more fully in Section 1.2.3.4 below. Activation of naïve T cells requires two independent signals: firstly, binding of the antigen-MHC complex by the T-cell receptor and secondly, T-cell binding to co-stimulatory molecules CD40 and B7, also expressed on antigen-presenting cells (Wang *et al.,* 2000a). Initial T-cell activation is indicated by expression of interleukin-2 (IL-2) and its receptor, IL-2R, which does not occur in the absence of the co-stimulatory signal. Production of IL-2 by T cells is essential for the proliferation and differentiation of naïve T cells and the cytokine acts in an autocrine manner (Minami *et al.,* 1993).

Naïve CD8 cells differentiate into cytotoxic cells that function in the cell-mediated immune response (Mosmann *et al.,* 1997). The differentiation of CD4 cells is more complex and at this stage they can differentiate into either a T helper (Th) 1 cell, secreting IL-2, interferon-gamma (IFN-γ) and lymphotoxin or Th2 cell, secreting IL-4, IL-5, IL-6 and IL-10 (Mosmann, 1992). The Th type that the T cell develops into is determined by many factors including the subset of dendritic cells presenting antigen and the cytokine profile at the site of T-cell activation. IL-12 promotes Th1 cells and activates cell-mediated immunity, while IL-4 induces Th2 cells and an antibody response. The production of one Th cell type directly inhibits the other, for example IFN-γ is an autocrine agonist for Th1 and antagonist for Th2 cells. In contrast, IL-4

enhances Th2 cell differentiation while IL-10 inhibits Th1 cells (Mosmann and Sad, 1996).

The activation of naïve T cells by antigen-presenting cells leads to their proliferation and differentiation of their progeny into effector cells. Th1 cells function predominantly in cell-mediated immunity but also induce B cells (via IFN-γ) to produce opsonising IgG_2 isotype antibody (Snapper *et al.,* 1988). T cells with a Th2 cytokine profile are required for the induction of B cells to produce IgA, IgG_1 and IgE (Croft and Swain, 1991).

The overall effect of presentation of antigen by antigen-presenting cells is the activation and proliferation of T cells. Most activated T cells become effector cells, while a proportion under the influence of IL-15 become memory T cells (Yajima *et al.,* 2002). Memory T cells are more sensitive to antigen than are naïve lymphocytes and respond rapidly on re-exposure to the antigen that originally induced them (Grayson *et al.,* 2002; Rogers *et al.,* 2000).

1.2.2.5 B-cell activation

The first requirement for B-cell activation is the binding of antigen by B-cell surface immunoglobulin, the B-cell receptor. The antigen is processed within the B cell and presented on the cell surface in association with MHC molecules. Effector CD4 T cells interact with the antigen-presenting cells and are stimulated to produce IL-4 and the ligand for CD40 (CD40L or CD154), a co-stimulatory signal for B cells. These factors in turn stimulate the B cells to proliferate and differentiate (Grewal and Flavell, 1996). The initial immune response produces IgM antibody of low affinity that can bind the target antigen and serve as an immediate protection for the infected host.

In the next stage of the immune response, the B cells undergo proliferation and a number of important modifications, including class-switch, somatic hypermutation and clonal selection. This occurs in the germinal centres which form around follicular dendritic cell networks and provide a specialized microenvironment when activated B cells migrate into lymphoid follicles. Germinal centres can be divided by morphology into a dark zone, rich in proliferating B cells and a light zone, which contains the dendritic cells and B cells undergoing somatic hypermutation (MacLennan and Gray, 1986). At the periphery of the germinal centres are the CD4 T cells.

1.2.2.6 B-cell class-switch

In a mucosal response, after the initial first reaction there is a switch in production from the IgM isotype antibody to IgA. Class-switch recombination occurs in the germinal centres, knowledge of this process coming from *in vitro* studies using mice (Snapper *et al.,* 1995). Class-switch recombination involves looping and deletion of DNA segments and the immunoglobulin gene heavy-chain constant-region is changed from IgM type to IgA. Activation-induced cytidine deaminase (AID), an RNA-editing enzyme, has been recently identified for its essential involvement in this process (Okazaki *et al.,* 2002). AID-deficient mice accumulate a large number of IgM B cells and IgM plasma cells in their lamina propria (Fagarasan *et al.,* 2001). While general antigenic specificity of the new immunoglobulin isotype does not change, the new immunoglobulin isotype has different biological properties.

The class-switch to the IgA isotype is under the influence of helper T cells and cytokines (Husband *et al.,* 1999; McIntyre and Strober, 1999). Experiments using B cells have identified several factors that direct or enhance class switching to IgA, with transforming growth factor-beta (TGF-β) produced by T cells shown to be the most potent (Kim and Kagnoff, 1990; Lebman and Edmiston, 1999). Cells are required to be in the activated state before TGF-β has its effect (Lebman *et al.,* 1990). The drive for class switch to IgA is more pronounced in the gut-associated lymphoid tissues than other mucosal-associated lymphoid tissue sites and is reflected in the predominance of IgA plasma cells in the gut-associated lymphoid tissues over other isotypes. This may reflect the microenvironment of cytokine profiles and accessory cells (Brandtzaeg *et al.,* 1999b).

1.2.2.7 B-cell somatic hypermutation and selection

A process to change the affinity as well as increase the specificity of the immunoglobulin also occurs in the germinal centres. Somatic hypermutation of the immunoglobulin variable region results in the generation of a family of variant immunoglobulin (Neuberger and Milstein, 1995). The variable region of the immunoglobulin gene undergoes a high rate of point mutations that creates additional diversity within the expanding B-cell clone. Similar to class-switch recombination, somatic hypermutation is dependent on the enzyme AID (Honjo *et al.,* 2002; Kinoshita and Honjo, 2001). Iterative cycles of somatic hypermutation and proliferation form populations of B-cell clones. Clonal expansion of plasmablasts is influenced by cytokines including IL-2, IL-5 and IL-10 (Burdin *et al.,* 1995; Collins and Oldham, 1995; Harriman *et al.,* 1988). To ensure B cells with higher affinity for

antigen are selected and those with lower affinity are not, signals for survival are required by the B cell. This process requires simultaneous cross linking of the high-affinity B-cell receptor to antigen on follicular dendritic cells, and of CD40 with CD40L (CD154) on CD4 T cells (Han *et al.,* 1995). Therefore, CD40L (CD154) not only provides a powerful stimulus to initiate B-cell activation but also plays a role in the final steps of the process.

Recent studies have shown that a member of the tumour necrosis factor (TNF) super-family, B-lymphocyte stimulator (BLyS), and one of its three receptors, B-cell-activating factor receptor (BAFF-R) expressed by B cells, are essential components for prolonging survival of mature B cells (Schneider *et al.,* 1999; Schneider *et al.,* 2001). This has been demonstrated experimentally with BLyS-deficient mice that have an almost complete loss of follicular B cells (Schiemann *et al.,* 2001). BLyS was discovered by several groups looking for TNF homologues in the human genome and can also be found in the literature under several other names including BAFF (B-cell-activating factor belonging to the TNF family), THANK (TNF homologue that activates apoptosis, nuclear factor-κβ and c-Jun NH_2-terminal kinase) and TALL-1 (TNF-and ApoL-related leukocyte-expressed ligand) (Moore *et al.,* 1999; Mukhopadhyay *et al.,* 1999; Schneider *et al.,* 1999; Shu *et al.,* 1999).

The end of the somatic hypermutation and selection process results in a large number of high affinity IgA plasmablasts destined to secrete antibodies at the effector site. A population of memory B cells is also generated that provides immunological memory for secondary infection or re-immunisation (Berek *et al.,* 1987).

1.2.3 *Homing of effector cells*

1.2.3.1 From inductor site to effector site

In the gut-associated lymphoid tissues, precursors of the IgA plasma cell leave the mucosal inductive sites and migrate to mesenteric lymph nodes where they continue to divide and differentiate. Finally they exit the lymph nodes and drain with the efferent lymph into the peripheral blood circulation via the thoracic duct where the majority track back to the lamina propria in the gut (McDermott and Bienenstock, 1979). The local antigen repertoire appears to play a role in the site-specific accumulation of plasmablasts and may explain the preference of effector cells from the gut-associated lymphoid tissue to traffic back to the lamina propria, to the site where the antigen introduction occurred. Immunisation studies in animals have

illustrated that the antibody response in local secretions (for example, milk) corresponds to local antigen exposure (Chang *et al.,* 1981; Husband and Gowans, 1978; Lascelles *et al.,* 1981).

Confirmation of this intestinal homing pattern of effector B cells, derived from gut-associated lymphoid tissues, has been shown in several studies. Sequences of immunoglobulin variable heavy-chain region genes of B cells from human Peyer's patch germinal centres were found to be clonally related to those from plasma cells of ileal lamina propria (Dunn-Walters *et al.,* 1997). In the intestinal lamina propria the plasmablasts complete their differentiation into mature IgA-secreting plasma cells and begin the generation of antibody (Iijima *et al.,* 2001). At this stage of terminal differentiation, IgA plasmablasts are unresponsive to receptor binding of antigen but require CD40 stimuli provided by CD4 T cells expressing CD40L (CD154) (McIntyre and Strober, 1999). In the mouse, IL-5 and IL-6 cytokines have been shown to be important for IgA terminal differentiation (Goodrich and McGee, 1999; Yan *et al.,* 1997), whereas IL-10 appears to be important for this process in humans (Burdin *et al.,* 1995; Defrance *et al.,* 1992). These cytokines are all produced by Th2 type T cells.

1.2.3.2 Regulation of trafficking

The immunoglobulin isotype ultimately expressed by plasma cells is influenced by the site in the body where antigen is presented, the type of antigen and the site at which plasma cell differentiation occurs (Kantele *et al.,* 1997). The migration of immune cells is controlled by complex interactions between lymphocyte cell-surface receptors, the expression of receptor ligands on the vascular endothelium of target tissues and chemical cascades produced by chemokines. Studies have shown that the regional specificity of lymphocyte trafficking is governed by specific sets of interacting local vascular endothelial receptors and their respective counter-receptors on circulating lymphocytes (Butcher *et al.,* 1999; Picker, 1994; Radi *et al.,* 2001).

Initial homing properties of mature naïve lymphocytes to secondary lymph organs are determined developmentally before, or in association with, emigration from the thymus or bone marrow (Butcher, 1999). When naïve lymphocytes are activated by contact with antigen, they differentiate into memory or effector cells that are reprogrammed with different homing properties. In this way, the distribution and trafficking of systemic and mucosal lymphocytes, both naïve and memory/effector cells, demonstrate quite different recirculation properties. This may provide a

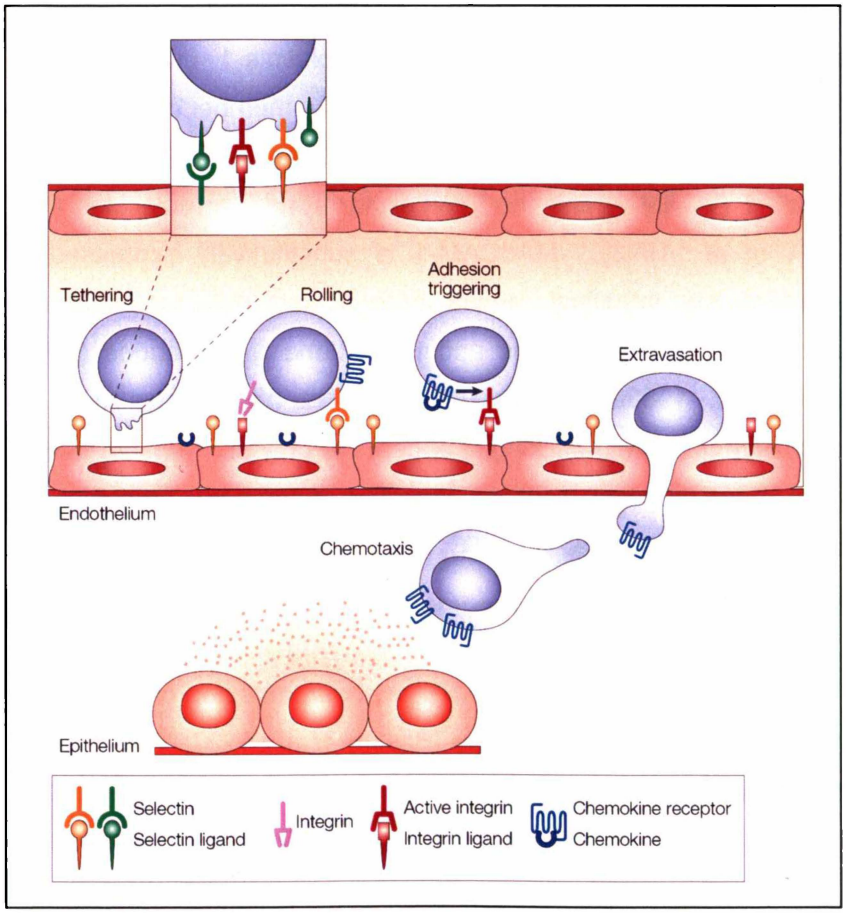
mechanism for segregating the specialised immune responses and contribute to the efficiency of immune responses (Butcher *et al.*, 1999). For example, a memory T cell for intestinal antigens displaying gut-specific homing receptor would circulate preferentially to the gut where it would most likely re-encounter its specific antigen.

1.2.3.3 The multi-step model of lymphocyte recruitment

The recruitment of circulating lymphocytes from the blood into tissues begins with interactions between the lymphocytes and the vascular endothelium. The mechanism involves a multi-step process in which specialised cell-adhesion and signalling molecules contribute to, and mediate, a series of essential steps (Butcher *et al.*, 1999; Picker, 1994). A diagram of the multi-step process of lymphocyte recruitment is shown in Figure 1.3.

Figure 1.3 A schematic representation of the multi-step process of lymphocyte recruitment

Circulating lymphocytes can tether to, and roll on, the vascular endothelium through interactions between selectins and integrins and their complementary receptors displayed on cell surfaces. Adherent lymphocytes migrate across the endothelium into the extravascular space directed by a cascade of chemokines. (Diagram adapted from Kunkel and Butcher, 2003)



The process begins when active adhesion receptors projecting from the microvilli of circulating lymphocytes tether to the vascular endothelium and initiate rolling of the cell along the vessel wall. This primary adhesion slows the transit of the lymphocytes and allows sufficient time for the lymphocyte to sample the endothelium surface for signals such as chemokines. These signals activate integrins on the lymphocyte surface which in turn trigger the firm adhesion of the lymphocyte to the endothelium. The process is also reversible. If, for example, the cell does not receive additional signals within a short time, the adherent cell will begin to roll again and return into the blood circulation. Alternatively, if the correct signalling does occur, the adherent lymphocyte will undergo the process of passage through the tight junctions of the endothelium into the extravascular space. From there, the lymphocyte is directed by gradients of chemokines towards specific targets such as sites of inflamed tissue.

1.2.3.4 Adhesion molecules involved in mucosal homing

The interplay of adhesion molecules and their ligands directs the circulation of lymphocytes between inductive and effector sites. There are several families of adhesion molecules including the immunoglobulin super-family members, integrins and selectins. A list of cell-adhesion molecules, their distribution and ligands are shown in Table 1.1.

The mucosal addressin cell-adhesion molecule-1 (MAdCAM-1) is the primary adhesion molecule for intestinal tissues. It is a member of the immunoglobulin super-family, related to other vascular adhesion molecules including vascular cell-adhesion molecule-1 (VCAM-1) and the intercellular adhesion molecules (ICAM-1, 2, and 3) (Briskin *et al.,* 1993). MAdCAM-1 is constitutively expressed by post-capillary venules in the intestinal lamina propria and gut-associated lymphoid tissues, especially in Peyer's patches and mesenteric lymph nodes (Briskin *et al.,* 1997; Streeter *et al.,* 1988a). While MAdCAM-1 has been shown to be very important for intestinal lymphocyte trafficking, it appears to play only a minor role in their homing to several other mucosal locations including pulmonary, bronchial and oral vascular endothelium (Abitorabi *et al.,* 1996; Briskin *et al.,* 1997; Csencsits *et al.,* 2002; Picker *et al.,* 1994).

Increased expression of MAdCAM-1 has been detected on inflamed intestinal lamina propria in both humans and mice (Briskin *et al.,* 1997; Viney *et al.,* 1996) and has been implicated in the pathogenesis of the gut mucosa in diseases such as colitis (Kato *et al.,* 2000). It has also been demonstrated on the high endothelial venules of

the mouse mammary gland (Streeter *et al.*, 1988a; Tanneau *et al.*, 1999; Van der Feltz *et al.*, 2001). MAdCAM-1 is displayed by follicular dendritic cells of mucosal but not peripheral lymph nodes (Szabo *et al.*, 1997) and is thought to have a role in selecting and binding lymphocytes at this site.

Table 1.1 Cell-adhesion molecules, their distribution and ligands

Adhesion molecule	Distribution	Ligand
Ig super-family		
MAdCAM-1	Mucosal endothelial cells	$\alpha4\beta7$
VCAM-1	Activated non-intestinal endothelial cells	$\alpha4\beta1$
ICAM-1	Activated endothelial cells	$\alpha L\beta2$
ICAM-2	Resting endothelial cells	$\alpha L\beta2$
ICAM-3	Naïve T cells	DC-SIGN
Selectins		
L-selectin	Lymphocytes, monocytes	PNAd
E-selectin	Activated endothelial cells	Sialyl-Lewis^x
P-selectin	Activated endothelial cells	PSGL-1
Integrins		
$\alpha4\beta1$ (VLA-4)	Lymphocytes, monocytes, dendritic cells	VCAM-1
$\alpha4\beta7$ (LPAM-1)	Lymphocytes	MAdCAM-1
$\alpha L\beta2$ (LFA -1)	Lymphocytes, monocytes, macrophages	ICAM-1, 2
CD2 (LFA-2)	T cells	LFA-3
Other		
PNAd	Peripheral lymph nodes	L-selectin

Reviewed - Butcher *et al.*, 1999
- Picker, 1994

The receptor for MAdCAM-1 is the integrin $\alpha4\beta7$ (Berlin *et al.*, 1993). The integrins are a large group of heterodimeric transmembrane glycoproteins (Poupon and Cerf-Bensussan, 1999). The β subunits can associate with several different α subunits, giving sub-groups of integrins. The $\beta1$, $\beta2$, and $\beta7$ integrins are the main β chains expressed by lymphocytes. Similar to other adhesion receptors, $\alpha4\beta7$ is expressed on the microvilli projections of lymphocytes (Berlin *et al.*, 1995). The molecule is

expressed in relatively inactive or low-avidity states on most naïve lymphocytes. However, high expression can be induced on subsets of circulating memory B and T cells, in particular by chemoattractant receptors on the high endothelial venules during the multi-step process of extravasation (Butcher *et al.,* 1999; Campbell *et al.,* 1998). The chemokine receptors differ for T cells and B cells and will be discussed more fully in Section 1.2.3.5 below.

An *in vitro* study has shown that $\alpha 4\beta 7$ also binds to VCAM-1 (Andrew *et al.,* 1994), although another study has not been able to demonstrate this (Tidswell *et al.,* 1997). VCAM-1 primarily binds to the integrin $\alpha 4\beta 1$ (also termed very late activation antigen-4, VLA-4) which is expressed on most lymphocytes (Osborn *et al.,* 1989). Current data suggest that interactions between $\alpha 4\beta 7$ and MAdCAM-1, and between $\alpha 4\beta 1$ and VCAM-1 are largely independent of each other and play a major role in the differential trafficking of intestinal versus non-intestinal memory and effector populations *in vivo* (Butcher, 1999). VCAM-1 is predominantly displayed in the skin, heart, central nervous system and joints, and expression is increased during inflammation (Butcher, 1999; Mackay *et al.,* 1992; Osborn *et al.,* 1989). It is only weakly expressed on intestinal endothelial cells and inflamed mucosa.

The $\beta 7$ chain also associates with the αE chain to form $\alpha E\beta 7$. This integrin is expressed on > 90% of epithelial-associated lymphocytes in the intestine and lung. T cells infiltrating inflamed epithelium in the epidermis, mammary gland and salivary glands have been shown to express $\alpha E\beta 7$ (Cerf-Bensussan *et al.,* 1988; Kruschwitz *et al.,* 1991).

Another important molecule involved in lymphocyte homing is L-selectin (CD62L), a member of the sub-family of lectins including P-selectin (CD62P) and E-selectin (CD62E). L-selectin was first described as a peripheral lymph node homing receptor for the peripheral lymph node addressin (PNAd) (Streeter *et al.,* 1988b). L-selectin is expressed by most circulating lymphocytes, including all naïve lymphocytes of the adaptive immune system and a major fraction of memory T cells and B cells (Picker *et al.,* 1993). In addition to peripheral lymph node homing, L-selectin has been shown to play a role in homing activated lymphocytes to both intestinal and non-intestinal mucosal sites (Bargatze *et al.,* 1995; Csencsits and Pascual, 2002). P-selectin and E-selectin are displayed on activated endothelium during inflammation and are involved in the extravasation process of neutrophils and monocytes by tethering them to the vascular surface (Kansas, 1996).

The leukocyte functional antigen-1 (LFA-1) is a β2 family integrin, αLβ2. It is expressed by most circulating lymphocytes and, similar to α4β7, its expression can be triggered by lymphocyte activation through chemoattractant signals. It is not found on the projected microvilli but on the planar body of the cell (Bargatze *et al.*, 1995). This may explain its function at the activation-triggered arrest stage of the multi-step endothelial interaction, rather that the initial contact. Its receptors are members of the ICAM family. ICAM-1 and ICAM-2 are largely expressed on endothelial cells activated by inflammatory cytokines and ICAM-3 is constitutively expressed on T cells (Radi *et al.*, 2001). The high-affinity binding of ICAM-3 to the lectin DC-SIGN is unique to the T-cell interaction with dendritic cells (Geijtenbeek *et al.*, 2000).

In summary, migration and homing of immune cells is largely orchestrated by tissue-specific adhesion molecules expressed by the vascular endothelium. Lymphocytes recognize and differentially adhere to endothelium in different sites in the body by binding through their counter-receptors. Cells can be directed to specific locations because lymphocytes display a different array of receptors, dependent on their cell phenotype (see Table 1.2) while vascular endothelium displays a range of adhesion molecules dependent on their location and the physiological state of the tissues (see Table 1.1).

Table 1.2 Lymphocyte homing receptors and their counter-receptors on endothelial cells in different tissues

(Adapted from Butcher *et al.*, 1999)

Lymphocytes		**Endothelial cells**	
$\alpha4\beta1^{lo}$ $\alpha4\beta7^{lo}$ L-selectin^{++} LFA-1^{++}	Naïve B or T cells	Peyer's patch	MAdCAM-1
$\alpha4\beta7^{hi}$ $\alpha4\beta1^{lo}$ L-selectin$^{\pm}$	Gut-homing blasts or memory cells	Lamina propria	MAdCAM-1
$\alpha4\beta1^{lo}$ $\alpha4\beta7^{lo}$ L-selectin^{++} LFA-1^{++}	Naïve B or T cells	Peripheral lymph node	PNAd
$\alpha4\beta1^{++}$ LFA-1^{++} $\beta7^{-}$ L-selectin^{+}	Skin-homing memory cells	Skin	E-selectin VCAM-1, ICAMs
$\alpha4\beta1^{hi}$ LFA-1^{++} $\beta7^{-}$ L-selectin$^{\pm}$	Non-mucosal blasts or memory cells	Inflamed CNS, bone marrow, other sites	VCAM-1

1.2.3.5 Trafficking of IgA plasmablasts: the influence of chemokines

As activated B cells differentiate into plasma cells, they complete a final series of migration steps that take them to sites where they can most efficiently carry out their effector functions, *i.e.* secreting antibodies. Until recently, only part of the mechanism for the homing of plasmablasts was understood. Regional specificity was known to be governed by adhesion molecules and their counter-receptors on circulating lymphocytes, but it had been proposed some years ago that a factor derived from mucosal and/or exocrine epithelium may be also involved in selectively attracting mucosal plasma cells (Czinn and Lamm, 1986). Recent work has now established a key role for chemokines in directing plasmablast movement within the secondary lymphoid organs where they are generated (Bowman *et al.,* 2000; Okada *et al.,* 2002), as well as guiding them to their effector sites (Bowman *et al.,* 2002; Hauser *et al.,* 2002).

Chemokines are small, mostly secreted, chemoattractant cytokines first characterised for their role in helping to recruit inflammatory cells to sites of inflammation and priming these cells for full activation (Cyster, 1999). To date, about 40 chemokines have been identified. The first chemokine to be identified was IL-8 (Yoshimura *et al.,* 1987), now renamed CXCL8. Its receptor CXCR1 was the first chemokine receptor to be cloned (Holmes *et al.,* 1991). Chemokines have been reclassified according to the configuration of cysteine residues in primary sequences near the amino-terminus (Murphy *et al.,* 2000). There are four major groups: CC, CXC, C and CX_3C. As knowledge of the family has grown, it has emerged that different chemokines help recruit appropriate subsets of cells for a particular type of inflammatory response and others are constitutively expressed in lymphoid tissues to guide the movement of circulating lymphocytes. As a consequence, the fundamental role of chemokines and their receptors is now recognised as controlling immune cell distribution (Rossi and Zlotnik, 2000; Sallusto *et al.,* 2000). The key chemokines involved in the trafficking of T cells and B cells are listed in Table 1.3, along with their distribution and receptors.

B cells first develop responsiveness to chemokines during their maturation in the bone marrow (Bowman *et al.,* 2000). During an immune response to antigen, the recruitment of mature B cells into lymphoid follicles involves signalling through the CXC-chemokine receptor 5 (CXCR5) and its ligand CXC-chemokine ligand 13 (CXCL13). CXCL13 is made by stromal cells within the B-cell zones in spleen, lymph nodes and Peyer's patches (Cyster *et al.,* 2000; Okada *et al.,* 2002). CCL19 and CCL21 are made by stromal cells in the T-cell zone and their receptor CCR7 is required by T cells and dendritic cells for migration to this region in lymphoid tissue

(Forster *et al.*, 1999; Luther *et al.*, 2002). Within a few hours of antigen encounter, B cells increase their expression of CCR7 and in response to CCL19 and CCL21, re-localise to the boundary of follicles and T-cell zones (Reif *et al.*, 2002). It is characteristically in this zone that the first plasmablasts are formed (Toellner *et al.*, 1996).

Table 1.3 The key chemokines involved in the trafficking of T cells and B cells, their distribution and receptors

Chemokine	Tissue distribution	Receptor	Reference
CCL19	T zone in spleen, lymph nodes, PP	CCR7	Cyster, 1999; Luther *et al.*, 2000
CCL21	T zone in spleen, lymph nodes, PP and HEV	CCR7	Cyster, 1999; Luther *et al.*, 2000
CCL25	Small intestine epithelium and endothelium	CCR9	Kunkel *et al.*, 2000; Zabel *et al.*, 1999
CCL28	Salivary glands, trachea and bronchi, large and small intestine, mammary gland and bone marrow	CCR10	Pan *et al.*, 2000; Wang *et al.*, 2000
CXCL9, 10, 11	Inflamed tissue	CXCR3	Qin *et al.*, 1998
CXCL12	Bone marrow and gut epithelium	CXCR4	Ma *et al.*, 1999; Kawabata *et al.*, 1999
CXCL13	B zone in spleen, lymph nodes, PP	CXCR5	Cyster *et al.*, 2000; Okada *et al.*, 2002

HEV - High endothelial venules
PP - Peyer's patches

Systemically-produced IgG and IgM plasmablasts preferentially travel through the blood to the bone marrow (Hauser *et al.*, 2002). Stromal cells in the bone marrow produce the chemokine CXCL12 where it has a major role in the development and retention of precursor B cells (Bleul *et al.*, 1996). This ligand is also the attractant for IgG plasmablasts which display its receptor, CXCR4. Studies have shown that mice deficient in CXCR4 accumulate fewer IgG plasmablasts in the bone marrow following immunisation and consequently have higher circulating levels of IgG plasmablasts (Hargreaves *et al.*, 2001). CXCL12 is also expressed in the spleen and secondary lymph nodes (Kawabata *et al.*, 1999; Ma *et al.*, 1999). Interaction between this

ligand and its receptor on plasmablasts in these organs may play a role in IgG plasmablasts exiting from these organs but this has yet to be fully established (Cyster, 2003; Wehrli *et al.,* 2001).

Mucosal-derived IgA plasmablasts can up regulate the expression of two different chemokine receptors, CCR9 and CCR10. CCR9 expression appears to have a narrow role and is largely associated with IgA plasmablasts but not IgM or IgG plasmablasts (Bowman *et al.,* 2002). In both the mouse (Lazarus *et al.,* 2003) and the human (Kunkel *et al.,* 2003), CCR9 is expressed by IgA plasmablasts from mesenteric lymph nodes and Peyer's patches but not by those from non-intestinal mucosal lymphoid tissues. Similarly, the CCR9 ligand, CCL25 is expressed at high levels in the small intestine but shows low or zero expression at other mucosal sites (Kunkel *et al.,* 2000). CCR9 and CCL25 also mediate the homing of a subset of memory T cells to the small intestine (Kunkel *et al.,* 2000).

In contrast, almost all IgA plasmablasts express CCR10 and respond to its ligand CCL28 (Kunkel *et al.,* 2000; Lazarus *et al.,* 2003). Few IgG plasmablasts display this receptor, highlighting the unique association of CCR10 with IgA plasmablasts (Kunkel *et al.,* 2003). CCL28, originally named as mucosal-associated epithelial chemokine (MEC), is expressed by many human mucosal sites including the stomach, small intestine, colon, salivary gland, mammary gland, and trachea (Pan *et al.,* 2000; Wang *et al.,* 2000b).

Another chemokine receptor, CXCR3, is involved in homing plasmablasts (Hauser *et al.,* 2002) and effector T cells (Qin *et al.,* 1998) to sites of inflammation. This receptor binds to three different ligands, CCL9, CCL10 and CCL11 which are all strongly expressed in response to IFN-γ, an inflammatory cytokine (Amichay *et al.,* 1996).

Chemokines have been shown to be important in the multi-step extravasation of lymphocytes from the blood into tissues. The unique patterns of expression of CCR9 and CCR10 may allow for the different patterns of dissemination of IgA plasmablasts observed following mucosa immunisation by different routes. CCL25, expressed by endothelial cells in the gut-associated lymphoid tissues and its receptor, CCR9, expressed on mucosal lymphocytes, has been suggested to play a role, along with $\alpha 4\beta 7$, in homing lymphocytes to mucosal surfaces (Zabel *et al.,* 1999). CXCL12 has also been shown to promote cell-adhesion via $\alpha 1\beta 7$and VCAM-1 (Sanz-Rodriguez *et al.,* 2001).

There is now strong evidence that chemokines and their receptors play key roles, together with adhesion molecules, in directing plasmablast trafficking within secondary lymphoid organs, to the bone marrow and to mucosal effector sites.

1.2.3.6 Plasma cell differentiation and longevity

Plasma cells represent the terminal stage of differentiation for all antigen-activated B cells. Antigen deposition at the site of differentiation is thought to have an effect on this process by inducing T-cell activation, with these cells then providing important cytokine signals (Brandtzaeg *et al.,* 1999b). IL-6 is essential in the first phase (Morse *et al.,* 1997). This has been illustrated using an IL-6 knockout mouse model in which significantly reduced numbers of intestinal plasma cells and poorly developed IgA responses to mucosal immunisation were observed (Ramsay *et al.,* 1994a). Plasmablast differentiation itself can be tracked by the sequential expression and silencing of transcriptional factors with a central role for B-lymphocyte-induced maturation protein (BLIMP-1) (Turner *et al.,* 1994).

Bone marrow plasma cells are characterised by the down-regulation of several cell surfaces markers; MHC II, CD45, B220, and B-cell receptor. In contrast, cell-surface markers expressed by plasma cells include the chemokine receptor CXCR4, the integrins VLA-4 and LFA-1, an adhesion/growth factor receptor (syndecan-1) and the receptor for hyaluronic acid (CD44) which is involved in binding cells to the extracellular matrix (Calame, 2001; Cassese *et al.,* 2003). These factors may be involved in the retention and/or longevity of these cells along with as yet unknown factors produced in their microenvironment (Cassese *et al.,* 2003).

There has been debate about the lifespan of plasma cells and until recently the immunological dogma held that plasma cells were short-lived with a half-life of a few days (Slifka *et al.,* 1998). In humans, antibody titres to viral pathogens have been shown to persist for decades (Manz *et al.,* 2002), although it has been suggested that this sustained antibody titre may be due to antigen-independent polyclonal activation of memory B cells resulting in a new population of plasma cells rather than long-lived plasma cells (Bernasconi *et al.,* 2002). Using specific antibody titres as an indicator of plasma cell survival, a recent study in the mouse demonstrated the lifespan of plasma cells to be at least a year (Slifka *et al.,* 1998). Others have shown the plasma cells can be both short- and long-lived dependent on their precursor cell type (O'Connor *et al.,* 2002).

Recent *in vitro* studies using mice plasma cells derived from bone marrow have shown that IL-5, IL-6, TNF-α, CXCL12 and CD44 are all specific signals required for the survival of plasma cells and they act in combination synergistically (Cassese *et al.,* 2003). That the cells only survived approximately five days in culture indicates that other unidentified factors may be involved in plasma-cell longevity (Cassese *et al.,* 2003). This supports the hypothesis that plasma-cell survival is not intrinsic but dependent on stimuli provided by their niche microenvironment (Manz and Radbruch, 2002).

The sole function of plasma cells is to secrete soluble immunoglobulin molecules. The nucleus shows a characteristic pattern of peripheral chromatin condensation. There is a marked increase in the steady-state amounts of immunoglobulin heavy and light chain mRNA, and for IgA secretion, J chain mRNA (Mestecky and McGhee, 1987). Plasma cells have an increased cytoplasm-to-nuclear ratio and prominent amounts of endoplasmic reticulum and secretory granules. Immunoglobulin makes up 10 - 20% of all the protein synthesised by the cell (Janeway *et al.,* 2001d).

1.3 IgA

IgA, produced by plasma cells, is the principal immunoglobulin of the mucosal system. It is present in most mucosal secretions in greater concentrations than any other immunoglobulin isotype, although there are species differences. For example, in the ruminant mammary secretions IgG_1 predominates, not IgA (Butler, 1969). Mucosal surfaces of the body provide a large interface between the outside world and the interior of the body. The mucosal surfaces are colonized by commensal bacteria and the gut is constantly exposed to food antigens and occasionally potentially pathogenic microorganisms and viruses. IgA antibodies provide a first line of defence against pathogens by helping prevent their adherence and penetration of the mucosal epithelium (Lamm, 1997). On average, a human produces 5 – 15 g of IgA at mucosal surfaces per day (Mestecky and McGhee, 1987).

1.3.1 *Structure and function*

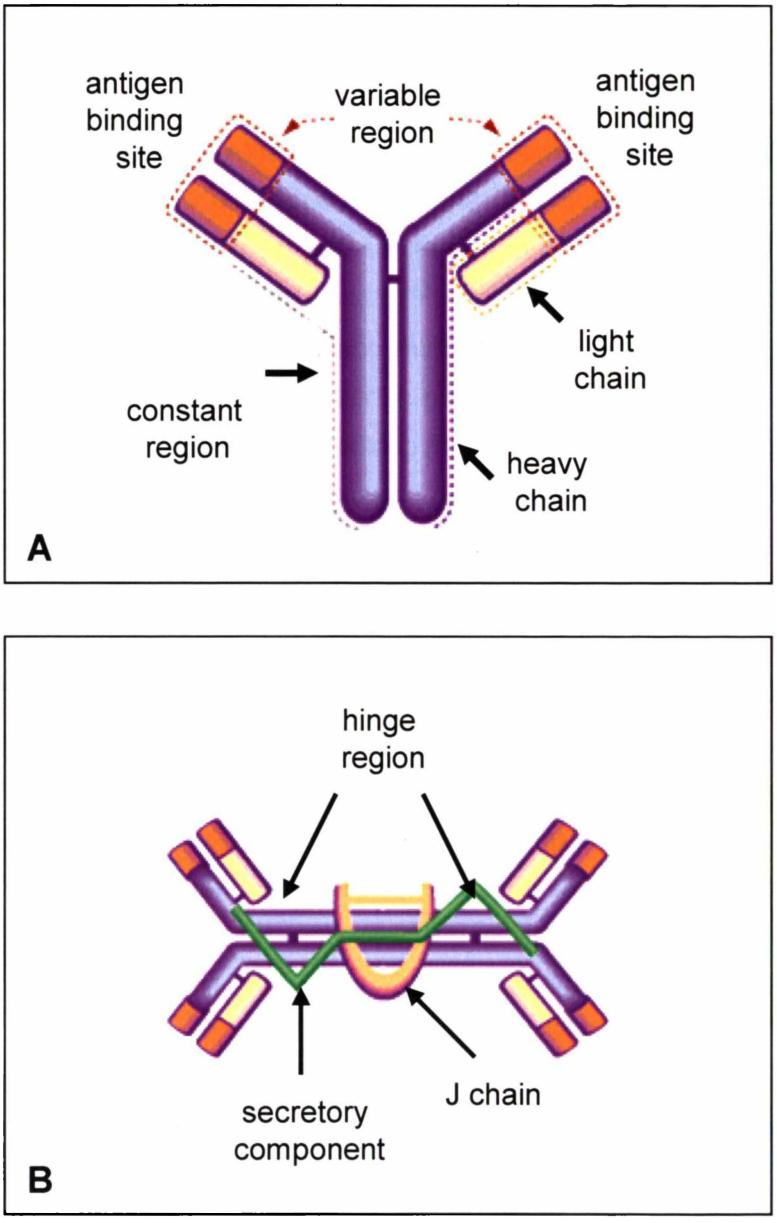
The basic immunoglobulin structure consists of two light-chains (25 kDa) and two heavy-chains (50 - 80 kDa) giving a total molecular mass of approximately 150 kDa (Janeway *et al.,* 2001c). The light chains are of two types, kappa and lambda, which are present in all immunoglobulin classes. In contrast, the heavy chain is unique to each isotype of immunoglobulin. There are constant and variable regions on the chains. The variable (hypervariable) regions are the site of antigen-binding. Disulphide bonds link the heavy and light chains and the two heavy chains. The molecule is flexible at the hinge region and this is the site where proteolytic enzymes act, such as pepsin and trypsin. Pepsin can cleave the immunoglobulin molecule into two fragments termed Fab, containing the antigen-binding hypervariable region, and Fc, containing the effector region of the heavy chains. The immunoglobulins are members of the immunoglobulin super-gene family and related molecules are present on the surface of immune cells, for example CD4 and CD8, (Barclay *et al.,* 1993; Classon *et al.,* 1992).

IgA consists of two basic immunoglobulin units (330 kDa) which are connected in a tail-to-tail formation by a J chain, a glycoprotein (15 kDa), covalently bound to the IgA dimer (Koshland, 1975). J chain has been shown to be a requirement for IgA polymerisation (Della Corte and Parkhouse, 1973). A second glycoprotein, secretory component (80 kDa), is bound to the IgA dimer heavy chain region by covalently linking to the heavy chain region of one IgA monomer (Beale, 1987). The complex of IgA dimer, J chain and secretory component is termed secretory IgA (SIgA) and has

a molecular weight of approximately 420 kDa. A diagrammatic representation of secretory IgA is shown in Figure 1.4.

Figure 1.4 *A schematic representation of the structure of secretory IgA*

A: Showing the basic immunoglobulin unit with the configuration of the heavy and light chains, the constant and variable regions and the site of antigen binding. **B**: Showing the configuration of SIgA with two immunoglobulin units, J chain and secretory component. The hinge regions are indicated. (Diagram adapted from the website - www.biotech.ubc.ca/Biomedicine/ mucosal immunity)



Secretory component is an enzymatically cleaved portion of the polymeric immunoglobulin receptor (pIgR) that is involved in the transfer of IgA into secretions (Beale, 1987; Crago *et al.,* 1978). Secretory component binds only to polymeric immunoglobulin and the ability of IgA to bind secretory component is dependent on its J chain content (Johansen *et al.,* 2001), although secretory component and J chain are not mutually linked. Secretory component can also be found in a free form in external secretions (Porter and Noakes, 1970). Secretory component, bound to IgA, interacts to stabilize the quaternary structure of IgA and importantly, may increase the resistance of IgA to proteolytic enzymes (Crottet and Corthesy, 1998; Lindh, 1975; Mestecky and McGhee, 1987; Renegar *et al.,* 1998). The IgA protein has a greater resistance than IgG to proteases and this is thought to be due in part to differences in the hinge region composition (Crottet and Corthesy, 1998). The resistance of secretory IgA to proteolytic attack is very important as IgA must function in enzymatically hostile conditions. Secretory IgA is more resistant to proteolytic attack by trypsin, chymotrypsin, and pepsin than monomeric or dimeric IgA (Brown *et al.,* 1970; Renegar *et al.,* 1998; Shuster, 1971) and more resistant to digestion by intestinal secretions than IgG and IgM (Brock *et al.,* 1977; Butler and Kennedy, 1978; Horsfall *et al.,* 1978).

In primates there are two forms of the alpha heavy chain, giving rise to different IgA_1 and IgA_2 isoforms. The two isoforms appear to have few distinct biological properties but are distinguished by their different susceptibility to bacterial IgA_1 proteases (Kilian *et al.,* 1983; Plaut, 1983). Also unique to primates, circulating IgA in serum is monomeric. Unlike the general functions of secretory IgA, the *in vivo* functions of serum IgA are poorly understood (Macpherson *et al.,* 2001; Russell *et al.,* 1999).

IgA provides a specialised defence at the interface between mucosal surfaces and the external environment. The primary function of IgA at mucosal surfaces is to inhibit entry of antigens and eliminate antigens which do gain access to the body. This is achieved by two main mechanisms: Firstly, by specifically binding mucosal pathogens, IgA provides protection from invasion against a variety of bacteria, parasites and viruses. Human *in vivo* studies have demonstrated the protective effects of IgA after immunisation with antigen by both oral and intranasal routes (Jertborn *et al.,* 1998; Jertborn *et al.,* 1986; Onorato *et al.,* 1991; Smith *et al.,* 2001; Tomoda *et al.,* 1995). Passive immunisation using monoclonal IgA antibodies secreted by 'back-pack' hybridomas have shown protective effects in mice challenged with viruses (Kraehenbuhl and Neutra, 1992; Ruggeri *et al.,* 1998) and bacteria (Neutra *et al.,* 1991; Winner *et al.,* 1991).

In the intestine, antibody binding can occur in the mucosal lamina propria before secretion into the intestinal lumen. Immune complexes formed in the lamina propria can then be eliminated locally by phagocytic cells or secreted via the pIgR-mediated transport of IgA into the mucosal lumen (Kaetzel *et al.,* 1991). Equally, IgA immune complexes can be translocated from the lumen into the lamina propria via M cells for further immune processing of pathogen (Weltzin *et al.,* 1989). During its normal transport process, IgA has also been shown to be able to bind viruses that are migrating through the cell to the lamina propria thereby neutralising them before they can cause damage (Mazanec *et al.,* 1992).

The second mechanism by which IgA exerts its protective properties is related to its heavy chain, Fc structure and not due to the specific binding of antigens by the hypervariable region. As already described above, one of the most important structural properties of secretory IgA, the linkage with secretory component, imparts resistance to proteolytic degradation and therefore allows it to function in the enzymatically hostile environment of mucosal surfaces.

The adherence of microorganisms to a mucosal surface is the first step in colonisation; therefore, inhibition of adherence by IgA is a major protective function. The hinge region of IgA is richly glycosalated with O-linked oligosaccharides and these have been shown to interact with bacterial attachment receptors, thereby inhibiting adhesion of bacteria to epithelial surfaces (Schroten *et al.,* 1998). Other studies have illustrated that IgA prevents bacterial colonisation of intestinal and oral epithelia and to tooth enamel (Dickinson *et al.,* 1998; Hajishengallis *et al.,* 1992; Vudhichamnong *et al.,* 1982). IgA also prevents pathogenic bacteria and virus adherence by altering the physicochemical properties of the microbial surface. Studies have shown that the Fc-secretory component region of IgA is hydrophilic (Magnusson and Stjernstrom, 1982), a characteristic that allows it to associate with mucus. Microorganisms bound by hydrophilic IgA are more readily entrapped by the mucus layer and eliminated by dynamic processes such as peristalsis in the gastrointestinal tract and mucociliary movement in the airways (Phalipon *et al.,* 2002).

Compared with IgG, IgA is a poor opsonin and a weak activator of both the classical and alternative complement pathways (Russell *et al.,* 1999). However, as IgA acts in an environment where macrophages and complement are not usually present, IgA does not normally have the opportunity to activate these systems. The advantage of not activating complement systems is that elimination activities occur in the absence

of an inflammatory response, in contrast to the action of IgG. IgA in conjunction with secretory component prevents the influx of inflammatory cells and the release of immunological effectors that could increase membrane permeability due to tissue damage (Marshall *et al.,* 2001; Motegi *et al.,* 2000). This property is especially useful in mucous membranes where the immune system continuously interacts with foreign substances and toxins.

1.3.2 *Transport into secretions*

IgA is transported into external secretions by the polymeric immunoglobulin receptor (pIgR) (Kaetzel *et al.,* 1991). A transmembrane glycoprotein, pIgR is selectively expressed on mucosal and secretory epithelial cell surfaces. The primary structure of pIgR has been determined for several species by molecular cloning of complementary DNA and shows several highly conserved features (Piskurich *et al.,* 1995). PIgR is a member of the immunoglobulin super-family and the extracellular ligand-binding region contains five immunoglobulin domains. The pIgR is a sacrificial receptor and is only used once for transport of IgA. For every molecule of IgA secreted, the epithelial cell produces one molecule of pIgR.

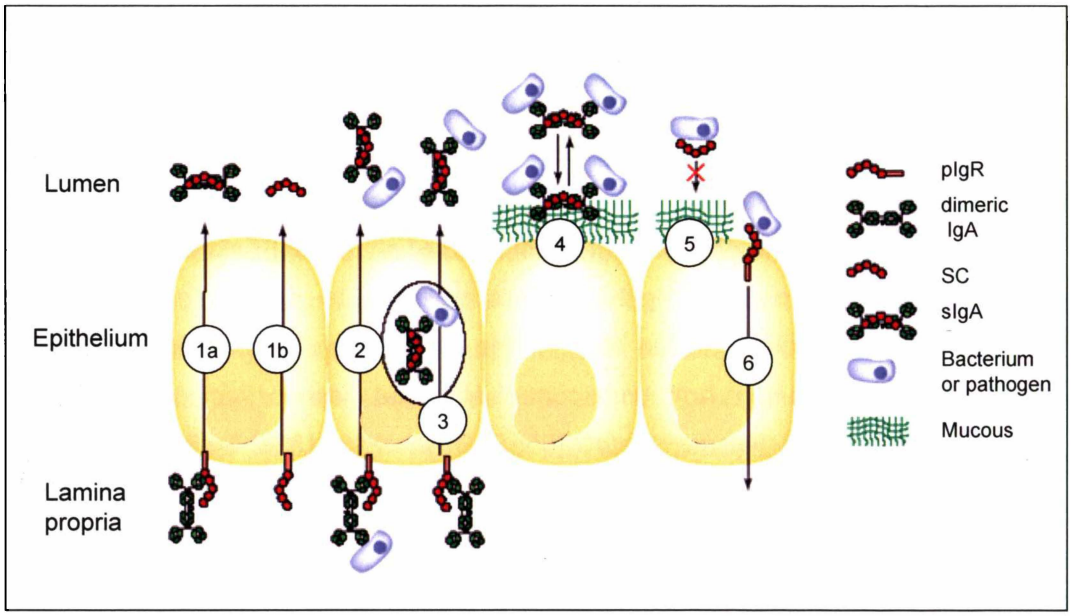
It has been suggested that the production of pIgR may be limiting to the amount of IgA transported into secretions (Scicchitano *et al.,* 1986; Sheldrake *et al.,* 1984). The expression of pIgR in epithelial cells appears to depend on the physiological conditions and on the tissue itself. In the rat and rabbit, expression of pIgR has been shown to be under hormonal control in some tissues, including the uterus and mammary gland (Rosato *et al.,* 1995; Sullivan *et al.,* 1983; Sullivan and Wira, 1983). Research using transgenic mice that over-express pIgR in the mammary gland has shown that IgA concentrations in the milk are 1.5 – 2.0 times higher than those in non-transgenic mice (de Groot *et al.,* 2000).

The receptor-mediated transport of IgA begins with the synthesis of pIgR in the endoplasmic reticulum. Following exit from the Golgi, pIgR is delivered directly to the basolateral membrane where it binds dimeric IgA. This binding is dependent on the J chain (Johansen *et al.,* 2001), therefore, pIgR can also transport IgM. However, its binding affinity for IgA is greater (Brandtzaeg, 1985). The pIgR-IgA complex is endocytosed and transported through a series of endosomal compartments across the cell to the apical membrane (Apodaca *et al.,* 1994). At some stage during the epithelial translocation, IgA becomes covalently bound to pIgR (this does not occur with IgM) by disulphide links (Fallgreen-Gebauer *et al.,* 1993). At the apical

membrane, pIgR is proteolytically cleaved (Musil and Baenziger, 1987) and the complex, including the extra-cellular binding domain of the receptor and dimeric IgA, is released into the mucosal secretions as secretory IgA. Figure 1.5 illustrates the multiple mechanisms for immune exclusion of pathogens involving IgA, pIgR and secretory component.

Figure 1.5 The multiple mechanisms for immune exclusion of pathogens involving IgA, pIgR and secretory component

1a: Transcytosis of IgA across the epithelial cell by pIgR with release of SIgA into the lumen. **1b**: Transport of free secretory component into the lumen. **2**: Transcytosis of IgA bound to antigen across the epithelial cell by pIgR with release of the complex into the lumen. **3**: During transcytosis, IgA binds antigen present in the cell. **4**: Association of IgA with mucus via secretory component provides a barrier for pathogens. **5**: Free secretory component acts as a non-specific microbial scavenger. **6**: pIgR may be exploited by pathogens to translocate them across the epithelial barrier into the lamina propria. (Diagram adapted from Phalipon and Corthesy, 2003)



pIgR - polymeric immunoglobulin receptor
SC - secretory component

As described above, secretory component bound to dimeric IgA acts to protect the complex from proteolytic degradation. In addition, secretory component has a direct effect on the ability of IgA to localise to mucus (Phalipon *et al.*, 2002) and is thereby crucial for one of the protective mechanisms of IgA at mucosal surfaces. Free pIgR, unbound by immunoglobulin, also translocates to the apical surface and secretory component is released in a free form into secretions. Free secretory component can

act as a non-specific microbial scavenger and it has been shown to reduce the effects of toxins (Dallas and Rolfe, 1998) as well as bacteria by binding to fimbrial colonisation factors (de Oliveira *et al.*, 2001). Free secretory component may also have a role in homeostasis as it binds the cytokine IL-8 thereby modifying the pro-inflammatory effects of this cytokine (Marshall *et al.*, 2001). Recent studies have shown that human pIgR can be co-opted by bacteria to translocate them from the lumen across the respiratory epithelium in a receptor-dependant manner which may lead to colonisation by the bacteria and result in disease (Zhang *et al.*, 2000). This phenomenon may be limited to specific strains and cell types (Brock *et al.*, 2002).

1.4 Bovine mammary gland immunology

1.4.1 *Bovine mammary gland*

A cow's udder consists of two separate halves each containing two mammary glands (quarters). The glands are made up of two main tissue types, secretory and ductal, which together are termed the parenchyma. Other components, skin, connective tissue, adipose tissue, blood and lymph vessels and nervous tissue, are collectively called the stroma.

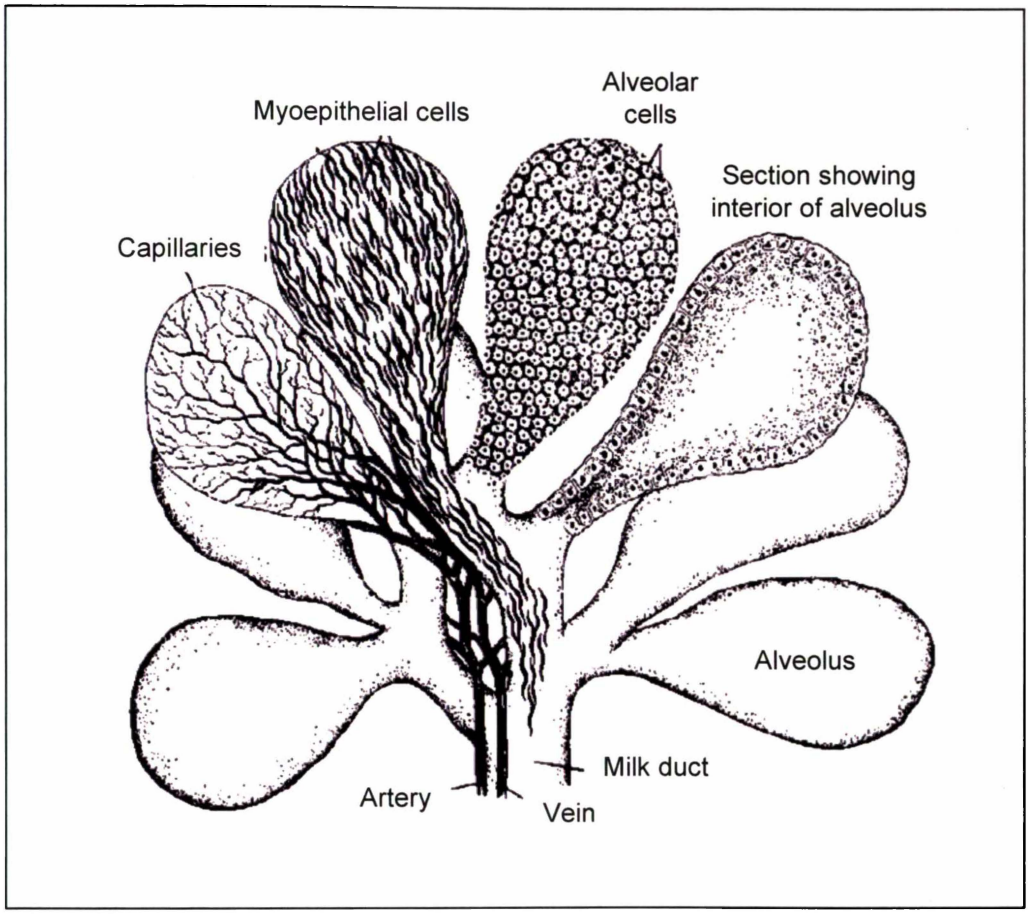
The secretory tissue has been described by Holmes *et al.* (1984b) as being composed of alveoli. Alveoli are formed by groups of secretory epithelial cells arranged in a single layer lining a hollow lumen with myoepithelial cells stretched over their outer surface. The mammary secretory epithelium is probably unique in that fat, protein and carbohydrate are secreted by a single type of cell. Groups of alveoli are encapsulated by connective tissue to form lobules and groups of lobules form lobes. Secretions produced by the alveoli drain consecutively into ductules, larger ducts and finally into a gland cistern at the base of each quarter. Mammary secretions accumulate in the cistern until released via the teat (Holmes *et al.,* 1984b). The mammary gland is richly supplied with blood vessels, with 400 – 500 l of blood flowing through the udder for every litre of milk produced (Holmes *et al.,* 1984b). In addition, tissue fluid, similar to the volume of milk produced, is drained from interstitial areas by lymphatic vessels to two major supramammary lymph nodes situated on the dorsal surface of the udder above the rear teats (Holmes *et al.,* 1984b). Efferent lymph from these nodes eventually drains via the thoracic duct into the blood system.

The main function of the mammary gland is the production of milk. This is a phasic process with growth and development of the gland occurring during puberty and pregnancies (mammogenesis), initiation of milk secretion at parturition (lactogenesis), maintenance of lactation (galactopoiesis) and cessation of milk production (involution) at weaning (Mepham, 1987). In most species, including ruminants, the major lobuloalveolar growth occurs during pregnancy. A marked change occurs in the morphology of the gland as first the ducts and then the lobules grow into the stroma, displacing adipose tissue. By parturition, the growth of the gland is essentially complete and during the lactation period there is a gradual progressive regression of the mammary gland (Holmes *et al.,* 1984a). For the dairy cow, involution is initiated when milking is abruptly stopped. The accumulation of milk causes alveoli tight junctions to break down, the alveolar cells undergo apoptosis and

there is an infiltration of phagocytic cells that remove casein, fat and cellular debris. Progressively the parenchyma regresses and is replaced with connective and adipose tissue (Sordillo and Nickerson, 1988a). A schematic diagram illustrating the structure of a group of alveoli in the bovine mammary gland is shown in Figure 1.6.

Figure 1.6 A schematic representation of the structure of a group of alveoli in the bovine mammary gland

(Diagram adapted from Cowie and Tindal, 1971)



Mammary secretions produced by the gland vary markedly in composition, depending on the stage of the lactation cycle. Secretions can be obtained from a cow as early as mid-pregnancy. Six weeks prior to parturition the volume in the gland increases as colostrum, the mammary secretion immediately *post-partum,* is formed. Colostrum contains more protein and fat and less lactose than later milk. The higher protein content is largely due to a high immunoglobulin concentration, with levels up to 60 g/l. The transition from colostrum to mature milk takes 3 – 4 days *post-partum* (Mepham, 1987).

Milk is a complex mixture of water, fat, carbohydrate, protein, salts and trace elements and provides the sole source of nutrients for the mammalian neonate. Many of the constituents are unique to milk, being synthesised in the secretory epithelium of the mammary gland from nutrients delivered in arterial blood. Cows' milk, perhaps the best characterised of mammary secretions, contains at mid-lactation approximately 3.0 – 6.0% fat, 4.4 – 5.3% lactose and 3.0 – 4.8% protein (Holmes *et al.,* 1984a). The major milk protein, casein, can be readily precipitated at acid pH, thereby forming the characteristic milk curd and leaving an aqueous fraction termed whey. Milk whey contains the soluble milk proteins, including immunoglobulins, β-lactoglobulin, α-lactalbumin, and serum albumin. Other proteins present in lesser amounts are lactoferrin and transferrin, the growth factors and a large number of different enzymes (Mepham, 1987).

1.4.2 *Ruminant mammary gland immunity*

The mammary gland has two distinct immunological roles. First milk plays a crucial function in protecting the neonate from disease and infection, in addition to providing its sole source of nutrients. By far the most significant protective effects for the neonate are provided by the transfer of maternal immunoglobulin and immune type cells via mammary secretions. The second role is providing immunological protection for the gland itself.

1.4.2.1 Immune protection for the neonate

The neonatal ruminant is singularly dependent for survival on passive immunity provided by lacteal factors, principally immunoglobulin and immune cells. In the ruminant, there is no placental transport of maternal immunoglobulin *in utero* and neonates are born agammaglobulinaemic (Brambell, 1970; Hanson and Johansson, 1970). This differs from the human where transfer of IgG does occur via the placenta (Brambell, 1970; Hanson and Johansson, 1970). Prior to gut closure at 24 – 36 h *post-partum*, the ruminant neonate absorbs ingested maternal immunoglobulin directly across the intestinal epithelium into the blood circulation (Kruse, 1970). Studies have shown that all immunoglobulin are absorbed with relatively equal efficiency and since IgG_1 is the predominant immunoglobulin in colostrum, it is absorbed in the greatest amounts (Brandon and Lascelles, 1971). After gut closure, as in the human, the major function of milk immunoglobulin in the ruminant is believed to be a protective role in the lumen of the intestine (Butler, 1981).

In addition to maternal immunoglobulin, milk contains other immune components that provide passive transfer of specific cellular immunity by way of maternal leukocytes. Mammary secretions contain approximately 2.0 x 10^6 cells/ml just prior to parturition, consisting of macrophages, lymphocytes and polymorphonuclear leukocytes (Concha, 1986; Duhamel *et al.,* 1987; Park *et al.,* 1992). Studies have demonstrated that colostral leukocytes can cross the intestine of the ruminant neonate and modulate host defence mechanisms (Liebler-Tenorio *et al.,* 2002; Riedel Caspari, 1993; Sheldrake and Husband, 1985). Once lactation is established, cell counts in the milk from a healthy animal fall to < 10^5 cells/ml (Dosogne *et al.,* 2003).

Other soluble milk components provide non-specific defence against environmental pathogens. For example, lactoferrin is thought to exercise an inhibitory effect on microbial growth in the newborn intestine by binding essential iron required for growth by many microorganisms (Outteridge and Lee, 1988); lactoperoxidase and xanthine oxidase, two enzymes associated with the milk-fat globule membrane, inhibit bacterial metabolism by oxidative processes (Outteridge and Lee, 1988).

1.4.2.2 Immune protection of the mammary gland

Mammary gland defence mechanisms provide protection against a variety of contagious and environmental pathogens including mastitis-causing bacteria such as *Escherichia coli (E. coli)* and *Staphylococcus aureus (S. aureus)*. Features of the physical, biochemical and cellular defence components of the bovine mammary gland have been described in several reviews (Kehrli and Harp, 2001; Mallard *et al.,* 1998; Nickerson, 1989; Sordillo *et al.,* 1997; Sordillo and Streicher, 2002; Watson, 1980). The gland utilises both innate and adaptive immune mechanisms for protection. Innate defences are mediated by the physical barrier of the teat and by neutrophils, macrophages and Natural Killer cells, and by soluble factors, including lactoferrin and lysozyme. The adaptive immune system is activated when the pathogen evades or is not completely eliminated by the innate system, or with a protective vaccination.

The teat is the first line of defence for the mammary gland, acting as a physical barrier to invading pathogens (Nickerson, 1987). Keratin, a waxy protein in the teat canal, provides not only a physical obstruction during the non-lactating period but also contains antimicrobial substances. Microorganisms that breach this barrier initiate an innate immune response. This response is characterised by a large

increase in the number of neutrophils in the gland (> 10^6 cells/ml) compared with the relatively low numbers (< 10^5 cells/ml) in the healthy cow (Leitner *et al.,* 2000b).

Neutrophils circulating in the blood are recruited to the site of infection by changes in the expression of adhesion molecules on vascular endothelium cell walls. These changes are induced by pro-inflammatory molecules released by pathogens or macrophage-derived cytokines and chemokines. During the extravasation process, neutrophils shed the CD62L receptor and up-regulate the Cd11a/Cd18 receptor (LFA-1) (Jutila *et al.,* 1989a; Jutila *et al.,* 1989b; Radi *et al.,* 2001). Under the influence of pro-inflammatory cytokines, neutrophils are activated to express specialised receptors, including those for lipopolysaccharide (LPS), complement and Fc, to facilitate pathogen recognition and phagocytosis (Burton and Erskine, 2003). Their bactericidal effects are mediated through a respiratory burst that produces hydroxyl and oxygen radicals (Heyneman *et al.,* 1990).

In cattle, neutrophils express a specific high-affinity Fc receptor for IgG_2. IgG_2 acts as an opsonin for neutrophils by specifically binding antigens on bacterial-cell surfaces. This is an important mode of defence against mastitis, as demonstrated by the high frequency of mastitis in Danish Red cows, animals with a genetic defect for IgG_2 production (Nansen, 1972). Interestingly, there appears to be evidence of generalised immune suppression in the cow during the week before and after parturition, as evidenced by reduced IFN-γ and IL-2 levels in mammary secretions (Sordillo *et al.,* 1991b) and susceptibility to infection (Kehrli *et al.,* 1989; Mallard *et al.,* 1998). In part, this is thought to be due to a reduced infiltration of neutrophils into the mammary gland due to a decreased expression of the neutrophil adhesion molecule, L-selectin (Monfardini *et al.,* 2002). The overall mechanisms of periparturient immunosuppression remain unknown and may involve pregnancy-associated hormones and metabolites (Mallard *et al.,* 1998; Paape *et al.,* 2002a).

Resident macrophages are prevalent in the milk and tissues of healthy cows (Outteridge and Lee, 1981). Similar to neutrophils, macrophages destroy bacteria by phagocytosis. In addition, macrophages secrete cytokines and chemokines involved in the extravasation and chemotaxis of activated neutrophils (Pighetti and Sordillo, 1994). Macrophages also have a role in the specific immune response through antigen processing and presentation (Fitzpatrick *et al.,* 1992).

Lymphocyte populations in normal non-infected ruminant mammary glands have been well characterised in secretions (Asai *et al.,* 2000; Dosogne *et al.,* 2003;

Duhamel *et al.,* 1987; Jensen and Eberhart, 1981; Leitner *et al.,* 2000a; Park *et al.,* 1992; Wilson *et al.,* 1986; Yang *et al.,* 1997) and tissue samples (Lee *et al.,* 1989; Nickerson *et al.,* 1984; Shafer-Weaver *et al.,* 1996; Sordillo and Nickerson, 1988b; Tatarczuch *et al.,* 2000; Yamaguchi *et al.,* 1999; Yang *et al.,* 1988). Determination of cell populations in mammary secretions at different physiological stages of the mammary gland, using flow cytometry analysis, has shown that cows have a greater percentage of CD4 than CD8 T cells during the dry period. The proportion of CD4 T cells decreases at parturition and remains lower throughout lactation in an inversely-related manner to that of CD8 T cells. At drying off, CD4 T cells increase again (Dosogne *et al.,* 2003; Yang *et al.,* 1997). The T cells have been shown to be predominantly memory cells (Taylor *et al.,* 1994). Overall, about 80 – 90% of the lymphocytes have been found to be T cells, with the percentage of B cells low (5%) or negligible (Duhamel *et al.,* 1987; Leitner *et al.,* 2000a; Yang *et al.,* 1997). Just prior to parturition, B cells in secretions show an increase to 25% of the lymphocytes (Park *et al.,* 1992). Although $\gamma\delta$ T cells have been shown to be highly represented in ruminant immunity, compared with humans and mice (Hein and Mackay, 1991), this phenotype was found to be extremely low in cow's milk throughout lactation (Asai *et al.,* 2000; Yang *et al.,* 1997). The percentage of macrophages and polymorphonuclear leukocytes were lower in early lactation compared with middle and late lactation (Dosogne *et al.,* 2003).

Early studies on pregnant and non-pregnant sheep tissues have found that the mammary gland is heavily infiltrated with lymphocytes and macrophages (Lee and Lascelles, 1969a; Lee and Lascelles, 1969b; Lee and Lascelles, 1970). The majority of lymphocytes were later shown to be CD8 T cells located in the alveolar and ductal epithelium (Lee *et al.,* 1989; Tatarczuch *et al.,* 2000; Yamaguchi *et al.,* 1999). B cells were present in lower numbers and located mainly in the periductal and intra-lobular connective tissues. T cells were also observed congregated around venules. In tissues examined at involution, the predominant leukocytes in the alveolar and ductal lumen were neutrophils. CD8 T cells were present in the alveolar and ductal epithelium, while CD4 T cells predominated in the inter-alveolar and periductal areas (Tatarczuch *et al.,* 2000). Dendritic cells have recently been described in the mammary gland, identified by their characteristic extensive cytoplasmic processes (Tatarczuch *et al.,* 2000).

The adaptive immune response in the mammary gland has been best characterised by vaccination studies to induce antibodies in milk for increased passive protection for the neonate. These are described in Section 1.5.1 below.

1.4.3 *Bovine milk immunoglobulins*

1.4.3.1 IgG

IgG, a monomer of the basic structural immunoglobulin unit (~ 150 kDa), is the main immunoglobulin of ruminant colostrum and milk (~ 70 – 90% of total, Table 1.4). Two major subclasses have been characterized, IgG_1 and IgG_2. A minor third class has been identified, termed either IgG_{2b} (Butler *et al.,* 1987) or IgG_3 (Knight *et al.,* 1988), that is thought to be an allotype of IgG_2 (Butler *et al.,* 1987). IgG is derived directly from blood into ruminant colostrum and milk (Dixon *et al.,* 1961; Newby and Bourne, 1977), and although the two major subclasses occur in relatively equal amounts in serum, IgG_1 comprises > 90% of the total IgG in ruminant mammary secretions (Butler, 1981). The selective transport of IgG_1 into milk appears to involve IgG_1-specific heavy-chain Fc receptors on the membranes of alveolar cells (Kemler *et al.,* 1975). In the cow, as much as 500 g of IgG_1 per week is transported into the mammary gland in the three weeks *pre-partum* (Brandon *et al.,* 1971). Colostral IgG_1 averages 48 g/l but levels decrease to approximately 0.5 g/l in established lactation. Comparative data for concentration and relative percent of immunoglobulin in serum and mammary secretions of the human and cow are shown in Table 1.4.

Antigenic and physicochemical differences between the IgG_1 and IgG_2 of ruminants are more pronounced than in other species and they have distinctly different biological activities as well as having some activities in common (Butler, 1983; Butler, 1986). Both IgG_1 and IgG_2 fix bovine complement and have excellent agglutination properties. Bovine macrophages have membrane receptors for both IgG_1 and IgG_2 but the IgG_2 subclass is more efficient at inducing phagocytosis by neutrophils. It is of interest to note that in cattle, IgG_1 is more resistant than IgG_2 to trypsin and chymotrypsin but not papain and pepsin (Brock *et al.,* 1977; Butler and Kennedy, 1978).

It has been suggested that IgG_1 may function as a secretory immunoglobulin in the ruminant (Chang *et al.,* 1981; Morgan *et al.,* 1981). This was based largely on the observation that ruminants have high concentrations of IgG_1 in lacteal secretions, achieved by a unique selective transport mechanism involving the mammary epithelium.

Table 1.4 Comparison of the amount of the various classes of immunoglobulins in serum and mammary secretions for humans and cows

Species	Ig	Concentration (g/l)			% of total Ig		
		Serum	Colostrum	Milk	Serum	Colostrum	Milk
Human[a]	IgG	12.10	0.43	0.04	78.0	2.0	3.0
	IgA	2.50	17.35	1.00	16.0	90.0	87.0
	IgM	0.93	1.59	0.10	6.0	8.0	10.0
Cow[b]	IgG_1	11.20	46.40	0.58	47.0	75.5	71.6
	IgG_2	9.20	2.87	0.06	38.6	4.7	7.4
	IgA	0.37	5.36	0.08	1.6	8.8	9.9
	IgM	3.05	6.77	0.09	12.8	11.0	11.1

Ig - immunoglobulin
a (Butler, 1974)
b (Butler, 1983)

1.4.3.2 IgA

The structure of IgA in ruminants is similar to that of other species, as previously described in Section 1.3.1. The main difference is that, in ruminants, serum IgA exists as a dimer in contrast to humans where serum IgA is a monomer (Mestecky and McGhee, 1987).

Similar to other mammals, in the ruminant, IgA is the primary immunoglobulin of mucosal secretions with the exception of mammary secretions. In colostrum the level of IgA is 5.4 g/l and in milk 0.1 g/l. This represents approximately 9% of the total immunoglobulins in comparison to human mammary secretions where IgA represents approximately 90% (Table 1.4). IgA plasma cells are the most numerous in the gut-associated lymphoid tissues (Lee and Lascelles, 1970) and IgA is the predominant immunoglobulin in intestinal secretions (Cripps *et al.*, 1974), in contrast to IgG and IgM which are present in low concentrations. A similar distribution of immunoglobulin isotypes is seen in ruminant saliva (Cripps and Lascelles, 1976). IgA is the most abundant immunoglobulin in the upper respiratory tract but IgG is found in higher concentrations in the lower respiratory tract (Gorin *et al.*, 1979). Studies have shown indirectly that following antigen stimulation, IgA plasma cells from the intestine travel to the respiratory tract, demonstrating the common mucosal immune system in the

ruminant (Scicchitano *et al.,* 1984). Effector functions of ruminant IgA are similar to other species, as described in Section 1.3.1.

In ruminant mammary secretions, the function of IgA is less well defined. During the initial suckling period it is likely that all classes of immunoglobulin provide local protective immunity in the gastrointestinal tract of the young ruminant but later IgA may be the most effective. This concept is based on the known functional activities of IgA, and on the increase in the relative occurrence of IgA compared with IgG_1 when concentration data in mammary gland secretions is expressed relative to albumin and serum levels of immunoglobulin (Butler, 1994).

1.4.3.3 IgM

IgM is a large polymeric immunoglobulin (1000 kDa) formed from five units of the basic immunoglobulin unit cross linked by disulphide bonds. Similarly to IgA, IgM has additional glycoprotein associated with it. A single J chain is covalently bound to each pentameric IgM molecule and appears to play a role in the polymerisation of the molecule (Della Corte and Parkhouse, 1973). Polymeric IgM is thought to be transported through the mucosal epithelium via pIgR by a similar mechanism to IgA (Brandtzaeg, 1985; Eskeland and Brandtzaeg, 1974).

IgM is a minor lacteal immunoglobulin and concentrations in ruminant colostrum and milk are similar to that of humans (see Table 1.4). Levels of IgM in cows' colostrum are approximately 6.8 g/l, declining to 0.09 g/l in mature milk.

IgM in ruminant mammary secretions is believed to be of both serum and local origin (Newby and Bourne, 1977). In ruminants, as in other mammals, IgM antibodies are produced at an early stage in response to antigenic challenge. IgM antibodies are of low affinity but the molecule has the ability to bind antigen with up to ten binding sites. The major function of IgM is fixation of complement, being 10 – 20 times as effective as IgG, and IgM also has effective agglutination and neutralization characteristics (Butler, 1983; Butler, 1986).

1.4.4 *Bovine pIgR*

The transport of polymeric immunoglobulin into ruminant mammary secretions is via the polymeric immunoglobulin receptor (pIgR), as in other species. It has been suggested that the low levels of IgA in ruminant milk may be due to there being low

levels of pIgR expression (Scicchitano *et al.,* 1986; Sheldrake *et al.,* 1984). The bovine pIgR was cloned several years ago (Kulseth *et al.,* 1995; Verbeet *et al.,* 1995) and the gene was found to be expressed in the lactating bovine mammary gland (Verbeet *et al.,* 1995), however, expression levels at other stages of the mammary gland development are unknown. Recent work in the sheep during pregnancy and lactation has shown that pIgR expression increases in the third stage of pregnancy and reaches its highest levels during established lactation (Rincheval-Arnold *et al.,* 2002). Further investigation using hormones suggested that the expression of pIgR is under the control of prolactin and glucocorticoids (Rincheval-Arnold *et al.,* 2002).

Bovine secretory component, the extracelluar component of pIgR, is well studied and was the first secretory component to be isolated in free form in any species (Groves and Gordon, 1967). The first 11 amino acids residues of bovine secretory component are homologous with secretory component from various species (Labib *et al.,* 1976).

1.5 Induction of bovine milk antibodies

1.5.1 *Milk antibodies as health treatments*

The majority of bacterial and viral pathogens enter the body through, or remain localized on, mucosal surfaces in the intestinal tract, lung or nose. Enteric infections are characterized by diarrhoea and dehydration caused by the sloughing off of the absorptive cells in the intestine. These diseases are increasing due to a number of factors including a rise in global travel, HIV-immunocompromisation and the continuing malnutrition in poorer areas of world. Current treatments or prophylaxis is often ineffective or results in further health problems. In addition, widespread antibiotic use is resulting in development of resistant strains of pathogens.

Hyper-immune milk products derived from the milk or colostrum of cows have been used for treatment, or prevention of enteric diseases in both animals and humans. To obtain the products, cows have been hyper-immunised during the dry period of their pregnancy with pathogenic antigens in order to induce specific antibodies in their colostrum and milk. These products provide protection through passive immunisation and an increasing number of studies have demonstrated that immune milk products can be effective against diseases caused by enteropathogenic organisms. These have been reviewed recently (Hosseini *et al.,* 2001; Korhonen *et al.,* 2000; Ruiz, 1994; Weiner *et al.,* 1999).

It has been shown in animal studies that colostrum or milk from cows hyper-immunised with rotavirus can provide protection against challenge by the virus in newborn calves (Castrucci *et al.,* 1984; Fernandez *et al.,* 1998; Saif *et al.,* 1983; Snodgrass *et al.,* 1980) and lambs (Fahey *et al.,* 1981). Similarly ruminant neonates fed milk from mothers immunised with *E. coli* were protected from subsequent challenge with the pathogen (Altmann and Mukkur, 1983; Snodgrass *et al.,* 1982). Some researchers noted that the degree of passive immunity achieved by young animals was variable. In some studies complete protection was achieved while in others there was only partial or no protection. The variability in results was thought to be attributable to antibody titre and volume consumed, and the timing of the feeding may have been important also (Fahey *et al.,* 1981; Snodgrass *et al.,* 1980).

There have now been a large number of human studies trialling treatments with therapeutic milk antibodies. In the main, these have focussed on the diseases caused by pathogenic *E. coli*, rotavirus and *Cryptosporidium parvum*. Controlled

clinical trials suggest that the oral administration of immunoglobulin preparations containing high titres of specific antibodies can provide effective prophylactic protection and, to some extent, may also be of therapeutic value against gastrointestinal infections in humans. Immune preparations have also been found to be protective and to some extent of value to treat rotavirus infection in children (Davidson *et al.,* 1989; Ebina *et al.,* 1992; Ebina *et al.,* 1983; Hilpert *et al.,* 1987; Sarker *et al.,* 1998).

A protective or therapeutic effect of immune milk has been demonstrated in humans against enteropathogenic or enterotoxigenic *E. coli* infections in some trials (Freedman *et al.,* 1998; Mietens *et al.,* 1979; Tacket *et al.,* 1988) but it was not always effective (Brunser *et al.,* 1992; Casswall *et al.,* 2000). In treatment of immuno-suppressed patients, hyper-immune milk containing specific antibodies against *Cryptosporidium parvum* has shown promise (Greenberg and Cello, 1996; Nord *et al.,* 1990; Tzipori *et al.,* 1987). Other hyper-immune milk products target other pathogens including *Helicobacter pylori* (Casswall *et al.,* 1998; Korhonen *et al.,* 1994), *Clostridium difficile* (Kelly *et al.,* 1996), *Vibrio cholerae* (Boesman-Finkelstein *et al.,* 1989), *Streptococci mutans* (Loimaranta *et al.,* 1999; Loimaranta *et al.,* 1997), *Candida albicans (Tollemar et al., 1999)* and *Shigella flexneri* (Tacket *et al.,* 1992).

Overall, where protection was poor or not achieved, the low concentration of antibody in the preparation was thought to be the cause. Based on the immunisation regimen used to induce the antibody response, the predominant antibody in these bovine milk products was almost certainly IgG, not IgA. Studies have shown that the IgG antibody isotype is not as stable as IgA in the enzymatically hostile environment of the gut (Brown *et al.,* 1970; Butler and Kennedy, 1978; Lindh, 1975) and this may also account for the poor protection achieved using some of the hyper-immune milks.

1.5.2 *Immunisation*

Ruminant milk is a rich source of naturally occurring antibodies and the concept of transferring passive immunity via lacteal antibodies dates back to the 1950s (Lascelles, 1963). Various immunisation regimens have been employed to induce antibody responses in ruminant mammary secretions, both for use in the ruminant neonate and as a therapeutic agent for treatment of human enteric disease.

Immunisation has been used to boost maternal antibody levels in domestic livestock to induce a more comprehensive passive immunity in offspring. The most commonly

targeted pathogens have been rotavirus and pathogenic *E. coli,* as these are a major cause of neonatal disease in ruminant livestock. The immunisation regimen was generally multiple intramuscular (IM) injection (Altmann and Mukkur, 1983; Crouch *et al.,* 2001; Fahey *et al.,* 1981; Snodgrass *et al.,* 1982) or IM injection at involution followed by intra-mammary (IMM) immunisation at milk formation (Saif *et al.,* 1984; Schaller *et al.,* 1992). The antibody produced was largely of the IgG class, with relatively little IgA, although the antibody class was not always characterized but assumed to be IgG_1 (based on the knowledge that ruminants selectively transfer IgG_1 from serum into milk). The poor IgA response of the ruminant mammary gland was thought in some cases to be attributable to local environmental factors perhaps influenced by the lactational status of the gland (Lascelles *et al.,* 1986). In rats and mice, sequestration of IgA plasma cells to the mammary gland has been shown to be under hormonal control (Weisz-Carrington *et al.,* 1978).

In view of the demonstrated potential for cells generated in gut-associated lymphoid tissues to populate the mammary gland in other species, elevating IgA levels in ruminant mammary secretions by priming the intestinal mucosal immune system using intra-peritoneal (IP) immunisation was studied by some investigators. IP injection prior to IMM infusion was shown to enhance the antigen-specific antibody response in mammary secretions, compared with IP or IMM immunisation alone (Sheldrake *et al.,* 1985b), but these antigen-specific antibodies were predominantly of the IgG_1 class. Although in that study intestinal IgA plasma cells were substantially increased in the sheep immunised by the IP route, there was no evidence of relocation of antigen-specific IgA plasma cells to the mammary gland. Antigen-specific IgA antibody did appear in mammary secretions but the origin of this antibody was unclear. It is of interest to note that non-lactating ewes immunised by the IMM route alone had a significant number of antigen-specific IgA plasma cells in jejunal tissues and these cells were shown to be of gut-associated lymphoid tissues rather than mammary origin (Sheldrake *et al.,* 1985a). This appears to suggest that the gut-mammary axis does function in the ruminant at least in the reverse direction, *i.e.* from the mammary gland to the gut.

Other groups have immunised cows to produce a variety of products for humans, including immune milk for infants, or patients with immunodeficiency disorders (Casswall *et al.,* 2000; Davidson *et al.,* 1989; Ebina *et al.,* 1992; Ebina *et al.,* 1983; Tzipori *et al.,* 1987). Similar to the production of such items for animal applications, cows were immunised by the IM route (Davidson *et al.,* 1989; Ebina *et al.,* 1983), or the combination of the IM and IMM routes (Hilpert *et al.,* 1987; Mietens *et al.,* 1979).

The resulting antibody was often not classified, as only neutralization tests were performed to determine antibody activity in the milk. When antibody class was determined, IgG_1 was the predominant isotype.

In addition to the route and timing of immunisation, other factors were also found to influence titre and isotype of ruminant milk antibody responses. These included the immunogenic properties of the antigen, the antigen dose and the adjuvant used. The differences in response between soluble and particulate antigens have been compared (Nashar *et al.,* 1991). IMM immunisation using soluble antigen was shown to induce an IgG response in serum, but there was little evidence of a response in the mammary gland. In contrast, the particulate antigen induced a sustained IgA response in the milk. Studies using attenuated live bacteria as a treatment for mastitis infection were found to be more effective than killed bacteria (Watson, 1981; Watson, 1984).

Freund's adjuvant, either complete or incomplete, or a combination of both was the most commonly used adjuvant for IM and IP immunisation (Crouch, 1985; Fernandez *et al.,* 1996; Opdebeeck and Norcross, 1984; Snodgrass *et al.,* 1980). Other adjuvants, including aluminium hydroxide gel and bordetella pertussis (Opdebeeck and Norcross, 1984; Snodgrass *et al.,* 1982) have been tried, but the oil-based adjuvants were generally thought to be more effective (Crouch *et al.,* 2000). This was especially so for IP immunisation where Freund's adjuvant was the most effective for eliciting intestinal responses. This was thought to be due to the adjuvant increasing the permeability of the serosa allowing greater penetration of the antigen into Peyer's patches (Lascelles *et al.,* 1986).

Adjuvants have usually not been employed for the IMM route. Antigen has generally been administered up the teat in a sterile solution to minimize the risk of mammary infection detrimental to subsequent milk production (Lascelles *et al.,* 1981; Sheldrake *et al.,* 1988). In IMM immunisation studies where immunogen was emulsified in Freund's incomplete adjuvant, no adverse effects were noted (Fernandez *et al.,* 1996; Saif *et al.,* 1984). In another study, immunogen emulsified in Freund's incomplete adjuvant and injected directly into the supramammary lymph node resulted in high antibody titres. Adverse reactions to this procedure were noted in some animals (Opdebeeck and Norcross, 1984).

More recent immunisation strategies for general mucosal vaccines have focused on new adjuvants and delivery modes (Iijima *et al.,* 2001; McGhee *et al.,* 1999).

Mucosal tolerance to oral immunisation has been circumvented with the use of cholera toxin subunit B or *E. coli* subunit B as adjuvants. These subunits of enterotoxins induce an IgA response together with the co-administered antigens (Nashar *et al.,* 1996; Nashar *et al.,* 1998). Another new adjuvant approach has been to employ the use of cytokines in their intrinsic roles, either using recombinant protein (Bao *et al.,* 1998; Pighetti and Sordillo, 1995; Pighetti and Sordillo, 1996) or introducing the cytokine by way of a viral DNA vector (Ramsay *et al.,* 1994b). Other alternative strategies have modified the physical form of the antigen, for example by microencapsulation using immune-stimulating complex matrix (ISCOM), liposomes or microspheres (Bowersock *et al.,* 1998; De Haan *et al.,* 1995; Mutwiri *et al.,* 2002; Sjolander *et al.,* 2001), or have presented the unmodified antigen via genetically engineered mucosa-binding viral or bacterial vectors (Mestecky *et al.,* 1997).

1.5.3 *The AgResearch multi-site immunisation regimen*

1.5.3.1 Rationale

At AgResearch, we have developed an immunisation protocol for induction of IgA in bovine milk for use as a therapeutic agent or bioactive food ingredient for human applications. The rationale for this strategy is as follows: firstly, the body of available evidence from the literature appears to indicate that bovine IgA, rather than IgG, would be a functionally superior preventative treatment for enteric diseases in the human. In human colostrum, antibodies of the IgA class predominate and in response to mucosal infections, humans normally elicit IgA responses as opposed to the IgG responses of systemic infections (Janeway *et al.,* 2001a). Secondly, due to its array of effector functions, IgA is known to play a major role in preventing colonization of pathogens at mucosal surfaces and aiding their elimination. And thirdly, mucosal IgA is known to be more resistant to the enzymatic conditions in the gastrointestinal tract than other classes of immunoglobulin (Butler and Kennedy, 1978; Lindh, 1975).

Overall, the above points strongly suggest that antibodies of the IgA class would be a more efficacious choice for treatment and/or protection against pathogenic infection of the human gastrointestinal tract. However, IgA class antibodies are normally at relatively low concentrations in bovine colostrum and milk, compared with IgG. In order for IgA treatments to be viable, there was a clearly a need to develop technologies to increase both antigen-specific and total IgA concentrations in

mammary secretions of the cow. This was the rationale for our original decision to develop an IgA immunisation regimen for the ruminant.

1.5.3.2 Development of the multi-site immunisation regimen

As described in Section 1.5.2 above, a number of investigators have used a variety of immunisation strategies in attempts to induce specific antibodies in cows' milk. These included multiple intra-muscular (IM) approaches, intra-mammary immunisation (IMM) alone, and IM or intra-peritoneal (IP) treatments followed by IMM immunisation. However, when taken together, it was apparent that no one had combined all three routes in an immunisation regimen in order to target IgA production. In the work of others the timing of treatments was also varied, with some investigators choosing to treat at involution, others during later gestation and finally, still others, during lactation itself.

As a consequence, we hypothesised that a protocol involving multiple immunisations by all three routes during the *pre-partum* period of colostrum formation would be an effective strategy for inducing IgA in mammary secretions. We proposed a protocol to treat pregnant non-lactating animals using injection of antigen by combined IP and IM routes followed later by IMM immunisation with booster doses of antigen to all three sites being given subsequently. The protocol is outlined in Table 1.5 with the first immunisation by the IP/IM routes being administered eight to ten weeks prior to parturition.

Table 1.5 Routes and timing for the multi-site immunisation regimen

Immunisation	Route	Weeks
I	IP/IM	0
II	IP/IM/IMM	4
III	IMM	6
IV	IP/IM	7

IP - intra-peritoneal
IM - intra-muscular
IMM - intra-mammary

In 1993, we undertook a trial to test the hypothesis using pregnant sheep and *E. coli* as a model antigen. Early studies showed a marked increase in antigen-specific IgA antibody in sheep colostrum, with lower but sustained levels in established milk

(Hodgkinson and Hodgkinson, 2003). We also found that if one side of the udder was immunised but not the other, then antibody was only produced in the side that had been immunised. This suggested that the IgA was being produced locally in the mammary gland and not transported into secretions from blood in the manner of IgG_1. Antigen was found to be a key component of the intra-mammary immunogen, because adjuvant alone did not elicit an IgA response. Subsequent trialling in sheep showed that the regimen could be applied to other antigens including proteins and yeasts. In 1997, we successfully tested the immunisation regimen in cows. A key finding from the early trialling in the cow was that total IgA was elevated in addition to the induction of antigen-specific IgA. Through a series of refinement trials to the present time, the protocol has been used to treat approximately 2 500 animals under close ethical and veterinary scrutiny and no significant deleterious effects of the treatment have been observed.

The body of data from this early investigative work confirmed the hypothesis that the multi-site immunisation regimen could be used to elevate IgA in ruminant mammary secretions. However, the method has been developed empirically without a clear understanding of the molecular and cellular mechanisms underlying the IgA response. A greater understanding of our IgA multi-site immunisation regimen is required to optimise and enhance the procedure for wide spread application in the dairy industry.

Development of this understanding is the justification for the programme of research undertaken in this thesis.

In the next section, I propose a hypothesis of the molecular and cellular mechanisms that may be involved in our IgA multi-site immunisation regimen. It combines the available evidence from the literature based on research findings from several different species, because many details of the process in the ruminant are not yet known, and extrapolates the available evidence to our immunisation regimen.

1.5.3.3 Proposed mechanism of IgA production

The proposed procedure begins with an initial priming dose of antigen emulsified in a standard oil-based adjuvant (Freund's incomplete adjuvant). This is administered via the intra-peritoneal (IP) route. Recognition of antigen as 'foreign' initiates the production of factors such as Toll-like receptors and inflammatory cytokines. These factors activate dendritic cells to take up the antigen and transport it to the numerous

lymph nodes in the abdomen and thoracic cavities. The transport occurs via the extensive network of sub-serosal lymphatics located on the external surface of the abdominal viscera. Dendritic cells degrade the antigen and present fragments on their cell surface in association with the MHC class peptide-binding proteins. Macrophages can also act as antigen-presenting cells.

In the mucosal gut-associated lymphoid tissues, antigens presented by dendritic cells activate naïve T lymphocytes that in turn prime naïve B lymphocytes to become memory or effector B cells. These cells can be distinguished by the different homing receptors that they express on their cell surface. The homing receptors bind adhesion molecules (vascular addressins) which are expressed on vascular endothelial cells of the post-capillary venules. The initial effector B cells differentiate into short-lived plasma cells that are mainly responsible for the IgM primary response.

A second dose of antigen is administered, again via the IP route. This acts to stimulate the memory B cells to undergo clonal expansion, proliferation and differentiation into plasmablasts. These processes occur under the influence of certain cytokines (for example, IL-2, IL-5, IL-6, TGF-β) secreted by helper T cells and antigen-presenting cells. The plasmablasts migrate via lymph vessels to the thoracic duct, then into the blood to circulate throughout the body including distant secretory effector sites, for example the mammary gland.

At the same time as the second dose to the peritoneum is administered, the mammary gland is exposed to antigen with an intra-mammary immunisation (IMM). Resident dendritic cells and/or macrophages are activated by antigen to secrete cytokines and in turn activate other immune cells. It is thought that this signalling process is required to recruit the circulating plasmablasts, presumably by adhesion molecule receptors and/or chemokine receptors on the lymphocytes and adhesion molecule and chemokine expression on the vascular endothelium in the gland. In the mammary gland the plasmablasts are terminally differentiated into IgA-producing plasma cells by the cytokine microenvironment produced by helper T cells, antigen-presenting cells and stromal cells.

At the same time as the IP immunisations, the animal is also given antigen via the intra-muscular (IM) route. Our experiments have shown that this increases the magnitude of the response and also the proportion of animals responding, but the

reason for this is unclear. In the literature, there is evidence to suggest that the systemic and mucosal immune systems are not totally segregated.

The plasma cells produce IgA and J chain, which form a complex of two IgA molecules and a J chain. The polymeric immunoglobulin receptor (pIgR) binds this complex at the basolateral surface of the mammary secretory epithelium and it is translocated to the lumen by endocytosis and transcytosis. A portion of the pIgR becomes covalently linked to the IgA molecule during the transport process and remains bound to IgA as secretory component when secretory IgA is released by cleavage at the apical surface into mammary secretions.

1.6 Aims of this thesis

The overall aim of this thesis is to develop a greater understanding of the molecular and cellular immunological mechanisms that are involved in the local induction of IgA secretion in the ruminant mammary gland. Cows immunised using our regimen to induce high levels of IgA in mammary secretions were utilised to study these mechanisms. The focus of the investigation was on key elements of the response that are identified in the proposed mechanism of action of the multi-site immunisation regimen outlined above in Section 1.5.3.3 and that are, as yet, unknown for the cow.

It is hypothesised that production of IgA in the bovine mammary gland is dependent on recruitment of primed lymphocytes of mucosal origin that are induced by the intra-peritoneal and intra-muscular immunisations. This recruitment may be mediated by changes in the microenvironment of the mammary gland due to exposure to antigen by the intra-mammary immunisation. Within the mammary gland, lymphocyte trafficking is regulated by expression of adhesion molecules on vascular endothelium, with selection determined by adhesion molecule receptor expression patterns on lymphocytes. The adhesion molecules responsible for the homing of lymphocytes to the bovine mammary gland have not been classified. One objective was to identify which vascular addressins are present in the untreated bovine mammary gland at four different physiological stages of the gland.

The immune cells that are involved in the local induction of IgA secretion in the bovine mammary gland have not been characterised. Another objective was to study the cellular changes induced in the mammary gland by intra-mammary immunisation following an initial priming of the animal via the intra-peritoneal and intra-muscular routes. To achieve this, the tissues of immunised bovine mammary glands were compared with non-immunised glands and differences in the immune cell types and their numbers were identified. This work also investigated whether or not the IgA immunisation regimen induced the expression of the mucosal vascular addressin, MAdCAM-1, in the bovine mammary gland.

The rate of immune cells trafficking into the bovine mammary gland in response to the intra-mammary immunisation is unknown. Another objective was to determine the kinetics of the cellular response induced in mammary secretions over an extended time course before and after the intra-mammary immunisations. The main focus was to look at changes induced in the T- and B-lymphocyte populations and to compare the differences between animals that produce high and low levels of IgA.

The cytokines involved in inducing local secretion of IgA in the bovine mammary gland have not been identified. Another objective was to determine which key cytokines were being expressed by mammary tissues of untreated animals and compare this with the gene expression profiles in mammary tissues from immunised animals. In addition, cytokine expression profiles were determined in cells isolated from secretions collected over an extended time course, before and after intra-mammary immunisations. This knowledge of the changes in gene regulation would help give a better understanding of the immune response that is being initiated by the intra-mammary immunisation, for example, a Th1 or Th2 type response.

Development of analytical methods to undertake some of this work was required.

The overall aim of the investigation into the different molecular and cellular changes induced by the intra-mammary immunisation was to gain insight into the mechanisms of the process. This insight was expected to point to ways that our immunisation regimen may be further developed and enhanced to achieve consistent, elevated IgA antibody production in individual animals and to make the IgA immunisation and resulting human treatments commercial realities.

Chapter 2

General materials and methods

This chapter lists the source of materials and describes the general methodologies used for the studies described in Chapters Three, Four, Five and Six. Any developmental work of these methodologies is described in the relevant chapter.

2.1 Materials

Common laboratory chemicals and reagents were obtained from Ajax Chemicals, BDH, Invitrogen, and Sigma. Specialised reagents, equipment and commercially sourced kits that were used in this study are detailed in the relevant Materials and Methods sections.

2.1.1 *Solutions*

Common solutions are listed in Table 2.1 and were made using Milli Q (Barnstead) purified water (MQ-H_2O).

Table 2.1 Common solutions

Solution	Composition
Buffers	
Acetate buffer	80 mmol/l sodium acetate pH adjusted to 5.4 with acetic acid
D-PBS	10 Dulbecco A phosphate-buffered saline (PBS) tablets (Oxoid) dissolved in 1000 ml MQ-H_2O then autoclaved 121°C, 30 min
D-PBS-FBS	1% (v/v) fetal bovine serum (FBS; Gibco™, Invitrogen) in D-PBS
ELISA coating buffer	16 mmol/l sodium carbonate 35 mmol/l sodium hydrogen carbonate
Lysis buffer	17 mmol/l tris(hydroxymethyl)aminomethane pH adjusted to 7.65 78 mmol/l ammonium chloride

Solution	Composition
PBS	1.9 mmol/l sodium dihydrogen orthophosphate 1-hydrate 8.4 mmol/l disodium hydrogen orthophosphate 2-hydrate 150 mmol/l sodium chloride
PBS-T	0.05% (v/v) Tween 20 in PBS
TAE (50X)	242 g tris(hydroxymethyl)aminomethane 57.1 ml acetic acid 100 ml 0.5 mol/l ethylenediaminetetraacetic acid disodium salt 2-hydrate (pH 8.0) adjusted to 1000 ml
TAE (10X)	TAE (50X) diluted 5-fold
TAE (1X)	TAE (50X) diluted 50-fold
TBS	17 mmol/l tris(hydroxymethyl)aminomethane 3 mmol/l tris(hydroxymethyl)aminomethane hydrochloride 150 mmol/l sodium chloride
TBS-T	1% (v/v) Tween® 20 in TBS
TE	1.21 g tris(hydroxymethyl)aminomethane 2ml 0.5 mol/l EDTA (pH 8.0) pH adjusted to 7.5 adjusted to 1000 ml
Other solutions	
DAB	3,3'-diaminobenzidine hydrochloride (*FAST* DAB peroxidase substrate tablet set; 1ml tablets dissolved in MQ-H_2O; D4168, Sigma)
DEPC-H_2O	0.1% (v/v) diethyl procarbonate in MQ-H_2O mixed overnight, then autoclaved 121°C, 30 min

Solution	Composition
DNA loading dye	40% (v/v) glycerol TAE (10X) 0.25% bromophenol Blue
ELISA stopping solution	2 mol/l sulphuric acid
ELISA substrate solution	250 µl 10% 3,3',5,5'-tetramethylbenzidine in dimethylsulphoxide 250 µl 0.45% hydrogen peroxide in 25 ml acetate buffer
Mayer's haematoxylin	0.1% (w/v) haematoxylin (C.I. 75290, BDH) 5.0% (w/v) aluminium potassium sulphate 0.02% (w/v) sodium iodate
4% paraformaldehyde	22 mmol/l sodium dihydrogen orthophosphate 1-hydrate 78 mmol/l disodium hydrogen orthophosphate 2-hydrate 4% (w/v) paraformaldehyde
RPMI	RPMI-medium 1640 (1X; Invitrogen) 31 mmol/l sodium hydrogen carbonate 1% Penicillin/Streptomycin (15140-148; Gibco™, Invitrogen) 5% FBS pH adjusted to 7.2, sterile filtered

2.1.2 *Antibodies*

Antibodies used in this study were obtained from Amersham Biosciences, Bethyl, Dako, Jackson, Pharmingen and VMRD, as listed in the Materials section of Chapters Three, Four and Five in which they were used.

Anti-human MAdCAM-1 monoclonal antibody was a gift from Dr M. Briskin, Millennium Pharmaceuticals, Cambridge, MA, USA. Anti-human VCAM-1

monoclonal antibody was a gift from Dr T. Tedder, Duke University, Durham, NC, USA. Anti-mouse β7 subunit monoclonal antibody was a gift from Dr E. Butcher, Stanford University, Stanford, CA, USA.

2.1.3 *Animals*

Healthy Friesian breed heifers were used for samples of untreated mammary tissues at different physiological stages. The animals were selected at four different physiological stages of the mammary gland: 40 – 50 days *pre-partum* (late pregnancy), two days *post-partum* (colostral phase), 90 days *post-partum* (lactation phase) and three days after the cessation of milking (involution phase). Animals were managed using standard farm practices.

For immunisation trials, multiparous, pregnant cows of mixed breed and age were selected based on general health, udder palpation and recent somatic cell count history. Throughout the trials, farm management practices continued as normal and the health of the animals was monitored by farm staff.

All animal use and experimental protocols for this thesis were approved by the Ruakura Animal Ethics Committee.

2.1.4 *Normal sera*

Normal sera were used in immunohistochemical protocols to reduce non-specific binding of secondary antibodies. The species of normal sera used in each protocol was the same species as the animal source of the secondary antibody, *i.e.* if the secondary antibody was raised in goats the normal serum used in that assay would be goat serum. Normal goat serum was obtained from Gibco™ (Invitrogen). Normal donkey serum was sourced 'in house'.

2.2 Methods

2.2.1 *Immunisation procedure*

Animals were immunised using our multi-site regimen (Hodgkinson and Hodgkinson, 2003) at three different sites; intra-muscular (IM), intra-peritoneal (IP) and intra-mammary (IMM), according to the schedule outlined in Table 2.2. For IM immunisations, 4 ml of immunogen was injected into the anterior neck muscle using

an 18 gauge needle. For IP immunisations, 4 ml of immunogen was injected into the peritoneal cavity, at a location 5 cm in front of the hip bone and 5 cm below the small ribs on the left hand side of the cow, using an 18 gauge needle. For IMM immunisations, 2 ml of immunogen was injected up each of the teat canals using a Bovi-Vet teat infusion syringe tip (Shoof). Prior to injection, IM and IP sites were cleaned and swabbed with 70% ethanol, and teats were cleaned using ethanol wipes. After immunisation, sites were sprayed with Stock iodine (Bomac). Cows were monitored for adverse reactions to the immunisation using standard animal husbandry practices.

Table.2.2 Routes and timing for the multi-site immunisation regimen

Immunisation	Route	Weeks
I	IP/IM	0
II	IP/IM/IMM	4
III	IMM	6
IV	IP/IM	7

IP - intra-peritoneal
IM - intra-muscular
IMM - intra-mammary

The antigen used for immunisations and the antigen-specific IgA ELISA (see Section 2.2.6.1) was a heat-killed whole-cell preparation of *Candida albicans* (*C. albicans*, ATCC 10231) prepared from a fermentation culture (batch CDA/008) by Biotech Australia. The protein concentration of the stock antigen was measured using the BioRad DC™ Protein Assay Kit (see Section 2.2.7). The antigen was then diluted to 500 μg protein/ml sterile saline (0.9% sodium chloride; Baxter) and emulsified with Freund's incomplete adjuvant (one part aqueous: three parts oil; Sigma) using a homogeniser (Ultra Turrax T-25, IKA-Werke).

The initial immunisation was 10 – 20 weeks before the group's averaged estimated calving date. In some experiments, cows were immunised in one side of the udder only. This allowed for the other side of the udder to act as a non-immunised control for that animal.

2.2.2 *Sample collection and preparation*

2.2.2.1 Tissues

Cows were slaughtered at the Ruakura abattoir and mammary glands excised for dissection. Tissue samples were collected from the alveolar and cisternal areas of the mammary gland, and the supramammary lymph nodes. Peyer's patch tissues and mesenteric lymph nodes were also obtained to represent typical gut-associated lymphoid tissue.

Frozen tissues for total RNA extraction: Tissue samples were cut into approximately 50 x 20 x 20 mm pieces, wrapped in tinfoil and snap-frozen in liquid nitrogen. Frozen tissues were ground under liquid nitrogen, by mortar and pestle, and stored at -80°C.

Frozen tissues for immunohistochemistry: Tissue samples were cut into 10 x 10 x 5 mm pieces, placed into tinfoil boats that had been partially filled with Tissue Tek O.C.T. compound (Bayer Diagnostics) and then completely covered with O.C.T. compound. The tissues were then snap-frozen in liquid nitrogen and stored at -80°C.

Ethanol-fixed tissues: Tissue samples were cut into 10 x 10 x 2-3 mm pieces, placed into Tissue-Tek Unicassettes (Bayer Diagnostics), and submerged in PBS (Table 2.1) containing 0.01% (w/v) sodium azide, with gentle mixing for 24 h at 4°C. Cassettes were then transferred to a solution of 95% (v/v) ethanol for 24 h at 4°C. The fixed tissues were processed overnight in a Leica JungTP1050 fully enclosed vacuum tissue processor (Cambridge Instruments) following the protocol in Table 2.3.

Table.2.3 Protocol for paraffin treatment

Solution	Time	Temperature
70% (v/v) ethanol, twice	60 min	Room-temperature
95% (v/v) ethanol, twice	60 min	Room-temperature
100% ethanol, twice	45 min	Room-temperature
toluene, twice	60 min	Room-temperature
paraffin	60 min	60°C
paraffin, twice	120 min	60°C

At the completion of the process, the tissues were embedded in blocks of paraffin wax (Paramat pastillated; BDH), using a Thermolyne Histo-Centre II-N embedding machine (Barnstead). Embedded tissues were stored at room-temperature.

2.2.2.2 Colostrum and milk

Colostrum samples were manually obtained from the udder while the animals were in the dairy on the milking platform. For milk, animals were machine-milked in the dairy on the milking platform. Samples were kept refrigerated for up to 2 h, until centrifuged (1 650 *g*, 10 min, 4°C). The fat layer was removed and the defatted colostrum/milk was recentrifuged (11 000 *g*, 60 min, 4°C) to pellet the casein and the whey supernatant was removed for storage at -20°C.

2.2.2.3 Non-lactating mammary secretions and cells

Mammary secretions collected from pregnant, non-lactating (dry) animals were sampled in the dairy on the milking platform. Teats to be sampled were cleaned using ethanol wipes and the keratin plug from each teat was manually expelled using a milking action. Sterile saline (25 ml) was injected up the teat canal, using a Bovi-Vet teat infusion syringe tip. The udder quarter was gently massaged then 25 ml of sample was collected into a 50 ml test tube by hand milking. Stock iodine was applied to the teat when finished. In the first few days following intra-mammary immunisations the infusion of saline was not required due to oedema of the gland.

The samples collected were centrifuged (350 *g*, 10 min, 4°C) and then the fat layer was aspirated from the top and a sample of supernatant removed for storage at -20°C. The remaining supernatant was aspirated to waste. The cell pellet was resuspended in 10 ml D-PBS (Table 2.1) and centrifuged (350 *g*, 10 min, 4°C). The supernatant was aspirated to waste and the cell pellet resuspended in 5 ml D-PBS then filtered through 100 μm mesh. An aliquot of cell suspension was diluted (1:20) in 0.4% Trypan Blue Stain (Gibco™, Invitrogen) and viable (*i.e.* unstained) cells were counted using a haemocytometer. The remainder of the cell suspension was centrifuged (350 *g*, 10 min, 4°C) and the cells resuspended in D-PBS to a concentration of 5 x 10^6 cells per ml. Cells required for flow cytometry were used immediately. Cells for total RNA extraction were frozen at -80°C, 2 ml of suspension per tube.

Non-lactating mammary secretions containing red blood cells: Mammary secretion samples that contained red blood cells were treated with lysis buffer (Table 2.1) prior to the step of resuspending in 5 ml of D-PBS and filtering. This treatment involved resuspending the cell pellet in 10 ml of lysis buffer and incubating for 10 min at 37°C prior to centrifugation (180 *g*, 3 min, room-temperature). The cell pellet was then washed twice by resuspending in 10 ml D-PBS and recentrifuging (180 *g*, 3 min, room-temperature). The pellet was then resuspended in 5 ml D-PBS then filtered, counted and resuspended at 5 x 10^6 cells per ml D-PBS as for normal samples.

Non-lactating mammary secretions containing gelatinous precipitate: Some mammary secretion samples contained a gelatinous precipitate, especially during the first few days after intra-mammary immunisation. These samples were treated differently from samples that were clear. Mammary secretions were centrifuged (350 *g*, 10 min, 4°C). The fat layer was aspirated from the top and then a sample of supernatant was removed for storage at -20°C. The remaining supernatant was aspirated to waste. D-PBS (20 ml) was added, the sample mixed, then centrifuged (75 *g*, 10 min, 4°C). The supernatant containing the cells was transferred to a clean 50 ml test tube. D-PBS (20 ml) was mixed with the remaining sample and the sample recentrifuged (75 *g*, 10 min, 4°C). The supernatant was removed and mixed with the supernatant from the previous centrifuging. The combined supernatants were centrifuged (350 *g*, 10 min, 4°C) and the supernatant discarded. The cell pellet was resuspended in 5 ml of D-PBS then filtered, counted and resuspended at 5 x 10^6 cells per ml D-PBS as for normal samples.

2.2.3 *Immunohistochemistry*

2.2.3.1 Frozen sections

Frozen tissues embedded in Tissue Tek O.C.T. compound were equilibrated to -20°C and 6 μm serial sections cut using a Leica Cryocut 1800 (Cambridge Instruments). Sections were mounted (three per slide) on Esco Polysine™ slides (Biolab Scientific), and left to dry at room-temperature overnight. Slides were stored in sealed boxes at -80°C. Directly prior to immunostaining, the sealed boxes were re-equilibrated to room-temperature before the slides were removed, to prevent condensation forming on the sections. Slides were then fixed in ice-cold ethanol for 10 min and air dried for 1 h.

2.2.3.2 Ethanol-fixed sections

Ethanol-fixed, paraffin-embedded tissue blocks were cut into 6 μm ribbon sections on a Leitz Type 1212 microtome. A ribbon of four sections was floated onto 42°C water and collected on an Esco Polysine™ slide and left to dry on a heating plate for 2 h at 60°C. Mounted sections were stored in boxes at room-temperature. Prior to immunostaining, the slides were deparaffinised and rehydrated as outlined in Table 2.4.

Table.2.4 Protocol for removing paraffin and rehydrating paraffin sections

Procedure	Time
xylene, twice	5 min
100% ethanol, twice	2 min
95% (v/v) ethanol	2 min
70% (v/v) ethanol	2 min
50% (v/v) ethanol	2 min
MQ-H_2O	10 min

2.2.3.3 Immunostaining

Immunostaining enabled the detection of target cells by utilising a primary antibody directed against chosen cell-surface antigens then the sequential application of biotinylated secondary link antibody, an avidin-enzyme complex and a chromogenic substrate. The end product allowed for visualisation of the target cells *in situ*. The procedure was based on a described method (Hsu *et al.*, 1981) and utilised an avidin-biotin-peroxidase complex and 3,3'-diaminobenzidine hydrochloride (DAB) as the detection method. Specific details of the antibodies used for immunostaining are described in the Materials and Methods section of Chapters Three and Four.

All incubations were performed at room-temperature in a humidity box, unless otherwise stated. Between incubation steps, slides were transferred to a glass slide-staining dish for washing (3 times, 10 min) with TBS-T (Table 2.1), unless otherwise stated. Individual tissue sections on slides were circled with a hydrophobic solution using a PAP pen (Dako) to contain assay reagents (50 μl).

Slides were first equilibrated with TBS (Table 2.1), and then endogenous peroxidase was inactivated with 3% H_2O_2 for 5 min. Slides were washed (3 times, 10 min) in TBS then endogenous biotin was blocked using the Dako Biotin Blocking Kit, incubating with reagents pre-diluted to 20% in TBS-T, for 20 min. After the slides were washed, non-specific background was reduced by incubation with blocking buffer (3% normal serum in TBS-T) for 30 min. The species of normal serum used in the assay was the same species as the animal source of the secondary antibody. Excess blocking buffer was tapped off prior to the addition of primary antibody, diluted to optimised concentrations in reagent buffer (1.5% normal serum in TBS-T), and the slides then incubated overnight at 4°C. For each tissue sample, a control was run to check for non-specific binding, adding reagent buffer only for the primary antibody step. The following day, slides were re-equilibrated to room-temperature and washed (6 times, 10 min). Biotinylated secondary antibody, diluted to optimised concentrations in reagent buffer, was incubated with sections for 120 min. Slides were washed (6 times, 10 min) and sections were then incubated for 30 min with streptavidin biotinylated horse radish peroxidase (HRP) complex (Amersham Biosciences). In the next step, DAB (Table 2.1) was incubated with the sections for 5 min, to form an insoluble brown end-product with the HRP. Slides were washed in TBS (3 times, 10 min) to stop the reaction. Finally, the sections were counterstained with Mayer's haematoxylin (Table 2.1), dehydrated with ethanol, cleared with xylene (Andrew Industrial) and coverslips mounted with D.P.X. mountant (BDH). Sections were viewed using an Olympus BX50 microscope and images captured using a Spot colour camera (SciTech).

Immunostaining to detect IgG cell-surface antigen utilized a primary antibody directly conjugated to HRP and therefore, a reduced protocol was required. After equilibration with TBS, blocking with H_2O_2, avidin and normal serum, slides were then incubated for 120 min with primary antibody conjugated to HRP and washed (6 times, 10min). Following this, sections were reacted with DAB, washed, counterstained, dehydrated, cleared and coverslips mounted as described in the protocol above.

2.2.4 *Molecular Biology*

2.2.4.1 RNA extraction and purification

Total ribonucleic acid (RNA) was extracted from tissues or cells using TriZOL (Invitrogen) according to the manufacturer's protocol.

Tissue RNA extraction: For tissue, 150 mg of frozen pulverized sample was homogenised with 1.5 ml TriZOL using an Ultra Turrax T-25 homogeniser (IKA-Werke). After 5 min incubation at room-temperature, the mixture was centrifuged (11 000 *g*, 10 min, room-temperature). The supernatant was transferred to a clean tube, 300 µl chloroform was added and the tube shaken for 15 s. Following incubation at room-temperature for a further 3 min, the sample was centrifuged again (11 000 *g*, 15 min, room-temperature). Approximately 700 µl of the upper aqueous phase was transferred to another clean tube and total RNA precipitated with 750 µl isopropanol. After standing for 10 min, total RNA was pelleted by centrifugation (11 000 *g*, 10 min, room-temperature) and the supernatant removed. The pellet was washed by resuspending in 750 µl 75% (v/v) ethanol and recentrifuging (11 000 *g*, 10 min, room-temperature). The pellet was then redissolved in 50 µl DEPC-H_2O (Table 2.1). An aliquot of the RNA was diluted 200-fold with MQ-H_2O and the absorbance measured at 260 nm using a spectrophotometer. The concentration was calculated using the formula:

Concentration (µg/ml) = A_{260} x 40 x 200 (inverse of dilution of sample).

Samples were stored at -80°C.

Cell RNA extraction: Frozen cells (10^7 per tube) were lysed using 1 ml TriZOL. Lysates were pipetted several times to shear genomic deoxyribonucleic acid (DNA). After 5 min incubation at room-temperature, 200 µl chloroform was added and the tube shaken for 15 s. Following incubation at room-temperature for a further 3 min, the sample was centrifuged (11 000 *g*, 15 min, room-temperature). Approximately 500 µl of the upper aqueous phase was transferred to a clean tube and total RNA precipitated with 500 µl isopropanol. After standing for 10 min, total RNA was pelleted by centrifugation (11 000 *g*, 10 min, room-temperature) and the supernatant removed. The pellet was washed by resuspending in 750 µl 75% (v/v) ethanol and recentrifuging (11 000 *g*, 10 min, room-temperature). The pellet was then redissolved in 50 µl DEPC-H_2O. The concentration of RNA was determined as described in the section above. Samples were stored at -80°C.

RNA purification: Deoxyribonuclease I (DNase I, Amplification grade; Invitrogen) was used according to the manufacturer's kit protocol to digest any contaminating DNA in the extracted RNA samples. For each µg total RNA, 1 µl DNase I and 1 µl DNase I buffer (10X) was added to the RNA, plus a volume of DEPC-H_2O to bring the total volume to 10 µl. After incubating for 15 min at room-temperature, the DNase I was deactivated by the addition of 2.5 mmol/l EDTA and heating for 10 min at 65°C.

Tubes were placed on ice and DEPC-H_2O added to give a total volume of 100 µl. An RNeasy® Mini kit (Qiagen) was then used to clean up the digestion mixture, according to the manufacturer's protocol. Buffer containing guanidine isothiocyanate (350 µl) and ethanol (250 µl) were added to the DNased RNA sample to ensure optimal binding of the RNA to the silica-gel membrane. The mixture was applied to the RNeasy® column and the RNA bound to the column allowing the contaminants to be washed away using Wash buffer. RNA was finally eluted from the column with 30 – 50 µl DEPC-H_2O. Samples were stored at -80°C.

2.2.4.2 First-strand cDNA synthesis

Synthesis of first-strand complementary DNA (cDNA) for subsequent polymerase chain reaction (PCR) used Superscript™ II Reverse Transcriptase (Invitrogen). Using a Superscript First-Strand Synthesis System for RT-PCR kit (Invitrogen), reverse transcriptase reactions were performed according to the manufacturer's protocol. For the reaction, 0.5 µg oligo $(dT)_{12-18}$ was annealed to 1 – 5 µg total purified RNA in a 10 µl reaction containing 1 mmol/l dNTP mix. The annealing reaction was incubated at 65°C for 5 min then tubes were chilled on ice. A second reaction mixture containing reverse transcriptase buffer (1X), 10 mmol/l magnesium chloride, 20 mmol/l dithiothreitol, and 40 U RNaseOUT was prepared and 9 µl was added to the first reaction mixture. The resultant mixture was incubated at 42°C for 2 min then 50 U Superscript II reverse transcriptase was added and the complete mixture incubated at 42°C for 50 min, followed by incubation at 70°C for 20 min. Finally, RNase H (2 U) was added and the mixture was incubated at 37°C for 20 min. The cDNA was stored at -20°C.

2.2.4.3 PCR with Platinum® *Taq*

The PCR process uses multiple cycles of template denaturation, primer annealing, and primer elongation to amplify DNA sequences. Platinum® *Taq* DNA Polymerase (Invitrogen) was used for PCR amplifications. This recombinant *Taq* polymerase is complexed to an antibody that blocks polymerase activity until after the first 94°C denaturation step in PCR cycling. Using a Superscript First-Strand Synthesis System for RT-PCR kit (Invitrogen), amplifications were performed according to the manufacturer's protocol. PCR reactions (20 µl) contained 0.2 mmol/l dNTPs, 200 nmol/l forward primer, 200 nmol/l reverse primer, 0.4 U Platinum® *Taq* DNA Polymerase, PCR buffer (1X), 1.5 mmol/l magnesium chloride and 0.8 µl cDNA from

the reverse transcriptase reaction (Section 2.2.4.2). A GeneAmp PCR system 9700 (Applied Biosystems) or an Eppendorf Mastercycler (Eppendorf) was used for PCR thermo-cycling. Following initial denaturation at 94°C for 2 min, the target DNA was amplified with 35 cycles, each cycle consisting of a denaturation step at 94°C for 30 s, annealing for 30 s and extension at 72°C for 30 s. The last cycle was terminated at 72°C for 5 min. Specific temperatures for annealing were primer dependent and are described in the Methods section of the relevant chapters along with the primer pairs.

2.2.4.4 Electrophoresis of DNA

Separation of DNA products following PCR reactions was performed using agarose gel electrophoresis. Agarose gels, 1 – 1.5% (w/v) agarose, depending on the size of DNA fragments to be separated, were prepared using TAE (1X) buffer (Table 2.1) containing ethidium bromide (400 ng/ml). DNA samples were premixed with DNA loading dye (Table 2.1) prior to loading on the gel. A 1kb Plus DNA ladder (Invitrogen) was loaded on each gel to enable the base pair size of the DNA products to be determined. Electrophoresis was carried out in Horizon tanks (Invitrogen) containing TAE (1X) buffer, at 80 – 100 V until the required separation was achieved. DNA was illuminated and photographed using the Gel Doc system (Bio-Rad).

2.2.4.5 Purification of DNA

DNA samples that had been separated using agarose gel electrophoresis (see Section 2.2.4.4) were recovered and purified using an Agarose Gel DNA Extraction Kit (Roche) according to the manufacturer's protocol. A slice of agarose gel containing the DNA fragment of interest was cut from the gel using a scalpel blade and incubated with 300 µl Agarose Solubilization buffer per 100 mg gel, plus 10 µl Silica Suspension, for 10 min at 60°C. The silica was pelleted by centrifugation (11 000 *g*, 30 s, room-temperature), the supernatant removed and the silica-bound DNA incubated briefly with Nucleic Binding buffer. After centrifugation (11 000 *g*, 30 s, room-temperature), the silica-bound DNA was washed twice by resuspending in Washing buffer and recentrifuging (11 000 *g*, 30 s, room-temperature). DNA was eluted from the silica by resuspending in two separate volumes (15 µl) of TE buffer (Table 2.1) and incubating for 10 min at room-temperature, followed each time by centrifugation (11 000 *g*, 30 s, room-temperature). Purified DNA was stored at -20°C.

2.2.4.6 Quantitative real-time PCR

Real-time PCR was used to determine the relative quantity of mRNA template of the target gene in samples. This was achieved by monitoring the amplification of DNA in real-time utilising a fluorescent dye that only emits a signal when bound to double-stranded DNA (Higuchi *et al.,* 1993; Wilhelm and Pingoud, 2003). The amount of target DNA generated by a given number of PCR cycles is dependent on the initial amount of target gene and the efficiency of the amplification of the sequence. (The maximum efficiency possible in PCR is two, where every PCR product is replicated every cycle.) This can be expressed in an equation (Rasmussen, 2001):

$$T_n = T_0(E)^n$$

where T_n is the amount of target sequence at cycle n, T_0 is the initial amount of target and E is the efficiency of the reaction.

Real-time (quantitative) PCR utilised a LightCycler instrument (Roche) to perform the PCR reaction, including induction and detection of the fluorescent signals. A ready-to-use hot start reaction master mix (Roche) containing FastStart Taq DNA Polymerase, reaction buffer, $MgCl_2$, SYBER green 1 fluorescent dye and dNTP mix was used. The real-time PCR reaction mixture (10 μl) contained 400 nmol/l forward primer, 400 nmol/l reverse primer, 2 μl Lightcycler FastStart DNA Master^{Plus} SYBER Green I (3515869, Roche) and 5 ng reverse-transcribed RNA. The reactions were performed in glass capillary tubes (1909339, Roche).

The LightCycler protocol consisted of pre-incubation for 10 min at 94°C followed by amplification of target cDNA with 45 cycles, each cycle consisting of a denaturation step at 94°C for 10 s, annealing for 5 s and extension at 72°C for 10 s. Specific temperatures for annealing were primer dependent and described in the Methods section of the relevant chapters along with the primer pairs. The specificity of the reaction was checked by melting curve analysis at the conclusion of the amplification cycles. In addition, an end-product sample of DNA from the real-time PCR reaction for each gene was analysed by gel electrophoresis (see Section 2.2.4.4) to confirm the expected size of the product and verify the presence of a single band.

LightCycler software calculated the cycle number at the crossing point for each PCR reaction, *i.e.* the point where the fluorescent signal generated by the reaction is visible above the background fluorescence of the sample. The cycle where each reaction first rises above background is dependent on the amount of target template present at the beginning of the real-time PCR reaction. The smaller the cycle

number at the crossing point the larger the amount of target template in the original sample.

To determine the efficiency of the reaction for each primer pair, five serial dilutions (10-fold) of a purified PCR product (see Section 2.2.4.3, Section 2.2.4.4 and Section 2.2.4.5) generated by RT-PCR using the primer pair, were measured by real-time PCR. From the data, the cycle number at the crossing point was plotted versus the log concentration of the dilution of PCR product. Using the slope of the line and the equation (Rasmussen, 2001):

$$E = 10^{(-1/slope)}$$

the Lightcycler software was able to calculate the value for the efficiency of the reaction. An equation that correlates the cycle number at the crossing point and the efficiency of the real-time PCR reaction was used to calculate the relative amounts of mRNA in unknown samples (Wilkening and Bader, 2004).

$$\text{The relative concentration} = 1.12 \times 10^{10}/E^{CN}$$

where E is the efficiency of the reaction and CN is the cycle number at the crossing point.

Samples of interest were analysed for the target gene and an endogenous control gene, β-actin. For each sample, the expression levels of the target gene were reported relative to the expression levels of β-actin. This adjusted for any variation in the quantity of total mRNA used for each reaction.

2.2.5 *Flow cytometry*

Using a panel of antibodies specific for lymphocyte cell-surface antigens, cells were indirectly labelled with fluorescent dyes then measured using a flow cytometer. Thus percentages of T and B cells were calculated. Double labelling of cells using antibodies coupled to two different dyes allowed for the further distinction of sub-populations of cells. Specific details of antibodies used for immunostaining and negative isotype control sera are described in the Materials and Methods section of Chapter Five.

Fresh cells isolated from mammary secretions, processed and resuspended in D-PBS at 5 X 10^6 cells per ml (see Section 2.2.2.3), were dispensed (200 μl) into a V bottom 96 well plate (MicroWell™ plates; Nunc). The plate was centrifuged (200 *g*, 2 min, room-temperature) and the supernatant flicked off the cells into a waste

container. Cells were resuspended with 50 µl of primary antibodies directed against lymphocyte cell-surface antigens, diluted at optimal concentration with RPMI (Table 2.1), and incubated for 30 min at 4°C in the dark. Cells were then washed using 100 µl D-PBS-FBS (Table 2.1) per well, the plate centrifuged (200 *g*, 2 min, room-temperature) and the supernatant flicked off the cells. Cells were then rewashed twice by resuspending in 200 µl D-PBS-FBS, recentrifuging the plate (200 *g*, 2 min, room-temperature) and flicking the supernatant off the cells. Cells were then resuspended with 50 µl of secondary antibodies (directed against the primary antibody isotype) conjugated to fluorescent dyes, diluted to optimal concentration in RPMI, and incubated for 30 min at 4°C, in the dark. Cells were washed three times as described above. Following the washes, 200 µl D-PBS-FBS was added to each well, the cells resuspended and filtered through 100 µm mesh into Falcon FACS tubes (352052; BD) containing 200 µl D-PBS-FBS. Cells were fixed with 100 µl 4% paraformaldehyde (Table 2.1) and stored at 4°C, in the dark, until analysed by the flow cytometer. Analysis took place either that day or the following day.

Non-specific binding of antibodies to cells was determined by the addition of RPMI only or negative isotype control serum for the primary antibody step. This percentage of non-specific binding was subtracted from the total to give a corrected value for each sample.

Cells were analysed using a FACScan™ flow cytometer (BD). The machine was calibrated using CaliBRITE™ 3 beads (BD) to set the forward scatter (FSC) and side scatter (SSC) parameters and compensation for the two fluorescent channels (FL1 and FL2). The compensation for FL1 and FL2 was corrected using cells separately labelled with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) dyes, respectively. A test sample was used to identify and gate the lymphocyte population of cells. Dead cells and debris were excluded from the count by setting the threshold at 150. Data was acquired for 10 000 events and analysed using the Cellquest™ software (BD).

2.2.6 *Enzyme-linked immunoassay*

2.2.6.1 ELISA for specific anti-*C. albicans* IgA

Specific anti-*C. albicans* IgA was measured using a non-competitive indirect enzyme-linked immunoassay (ELISA) protocol based on described methods (Clark and Engvall, 1980; Hodgkinson *et al.,* 1995). All washes were carried out by an

automated plate washer (ELP-35; Bio-Tek) using PBS-T (Table 2.1), all samples and reagents were diluted with PBS-T containing 1% w/v bovine serum albumin (BSA; A-7906, Sigma) and all reagent addition volumes were 100 µl, unless otherwise stated.

ELISA plates (Maxisorp F-96 immunoplates; Nunc) were coated with stock antigen as used for immunogen (see Section 2.2.1), diluted to 10 µg/ml in ELISA coating buffer (Table 2.1), incubated overnight at 4°C and washed three times. Remaining activated sites on immunoplates were blocked by adding 250 µl PBS-T containing 1% w/v BSA to each well and incubating the plate for 2 h at room-temperature. After washing the plates twice, four 10-fold serial dilutions of test sample (primary antibody: 1:100; 1:1 000; 1:10 000; 1:100 000) were added to duplicate wells. Plates were incubated for 2 h at room-temperature then washed three times and the secondary antibody consisting of rabbit anti-bovine IgA heavy-chain specific (diluted 1:70 000; Bethyl) was added. Plates were incubated overnight at 4°C, then washed three times prior to the addition of antibody-enzyme conjugate, goat anti-rabbit immunoglobulin conjugated to HRP (diluted 1:12 000; Dako). After incubation for 2 h at room-temperature, the plates were washed twice with PBS-T then twice with PBS containing no Tween® 20, and then freshly prepared ELISA substrate solution (Table 2.1) was added to each well. Following 30 min incubation at room-temperature on an automatic shaker (IKA-Werke), 50 µl of ELISA stopping solution (Table 2.1) was added and the optical density measured at 450 nm using an automated plate reader (ELP-35; BioTek).

All ELISA plates were run with positive and negative control samples (FBS, diluted 1:1000). The median optical density between the maximum value for the positive control and the value for the negative control was used as a reference point (reference optical density) to determine test sample antibody titres. The reciprocal of the test sample dilution equivalent to the reference optical density was given as the antibody titre. Values were calculated using Prism software (GraphPad). The titre value for the positive control sample, run with each assay, was used to monitor inter-assay variation.

2.2.6.2 ELISA for total bovine IgA

Total bovine IgA was measured using a non-competitive sandwich ELISA method with kit reagents from Bethyl used according to the manufacturer's protocol. All standards, samples and reagents were diluted with PBS-T containing 1% w/v BSA (A-7906; Sigma), all reagent addition volumes were 100 µl and all washes were

carried out by an automated plate washer (ELP-35; BioTek) using PBS-T, unless otherwise stated.

ELISA plates (Maxisorp F-96 immunoplates; Nunc) were coated with sheep anti-bovine IgA heavy-chain specific (Bethyl) diluted 1:200 in ELISA coating buffer. After incubation overnight at 4°C, the plates were washed three times. Remaining activated sites on immunoplates were blocked by adding 250 µl PBS-T containing 1% w/v BSA to each well and incubating the plate for 30 min at room-temperature. After washing the plates three times, standards (range 88 – 1 000 ng/ml, Bethyl) and test samples (colostrum diluted 1:5 000 and 1:10 000; milk diluted 1:500 and 1:1 000; non-lactating mammary secretions diluted 1: 1 000, 1: 5 000 and 1: 20 000) were added to duplicate wells. Plates were incubated for 60 min at room-temperature then washed three times and sheep anti-bovine IgA heavy-chain specific conjugated to HRP (diluted 1:85 000; Bethyl) was added. After incubation for 60 min at room-temperature, the plates were washed twice with PBS-T, then twice with PBS containing no Tween® 20 and then freshly prepared ELISA substrate solution was added. Following 30 min incubation at room-temperature on an automatic shaker (IKA-Werke), 50 µl of stopping solution was added and the optical density measured at 450 nm by an automated plate reader (EL311s, Bio-Tek).

A standard curve was generated using Prism software (Graphpad) and values of unknown samples were determined. A quality control sample was run with each assay to monitor inter-assay variation.

2.2.7 *Protein assay*

Total protein concentrations were measured using the Bio-Rad DC™ protein assay based on the method of Lowry (Lowry *et al.,* 1951). The assay was performed according to the manufacturer's Microplate Assay protocol.

Six standards (range 0.0625 - 2.0 mg/ml) were prepared using BSA (A-7906; Sigma) diluted in PBS. If required, samples were also diluted in PBS. In a microtitre plate, 5 µl standard or sample dilutions, 25 µl Reagent A (alkaline copper tartrate solution) and 200 µl Reagent B (dilute Folin reagent) were added to duplicate wells. The plates were incubated for 15 min on an automatic shaker (IKA-Werke) and then the optical density was measured at 750 nm by an automated plate reader (EL311s, Bio-

Tek). OD values for standards were used to generate a standard curve using Prism software (Graphpad) and values of unknown samples were determined.

2.2.8 *Statistical analysis*

Values, where appropriate, were expressed as mean ± standard error of the mean (SEM). A two tailed t-test was used to determine significant differences between two data sets, the test parameters depending on the data set to be analysed. For example, when the two data sets were compared from the same group of animals at different time points then a paired t-test was used. When the two data sets were compared from two different groups of animals at the same time point then a t-test with equal variance was used.

Chapter 3

Expression of vascular addressins in the untreated bovine mammary gland

3.1 Introduction

The overall objective of this thesis is to investigate the molecular and cellular mechanisms involved in the local induction of IgA secretion in the bovine mammary gland. Based on findings in the literature, it is hypothesised that production of IgA in the bovine mammary gland (resulting from our multi-site immunisation regimen), is dependent on recruitment of primed lymphocytes of mucosal origin arising from the intra-peritoneal (IP) and intra-muscular (IM) immunisations. This recruitment may be mediated by changes in the microenvironment of the mammary gland due to exposure to antigen by the intra-mammary (IMM) immunisation.

The homing of lymphocytes in blood and lymph to specific tissues is an important part of the immune response and is required for an efficient and targeted response to antigenic challenge. Homing is not a random process but regulated by the interplay of cell-surface adhesion molecules on lymphocytes and complementary receptors (vascular addressins) expressed on the vascular endothelium (see Section 1.2.3.4). Trafficking of lymphocytes to particular regions is directed by the specific sets of local vascular addressins and their ligands on circulating lymphocytes (Butcher *et al.,* 1999; Picker, 1994; Radi *et al.,* 2001).

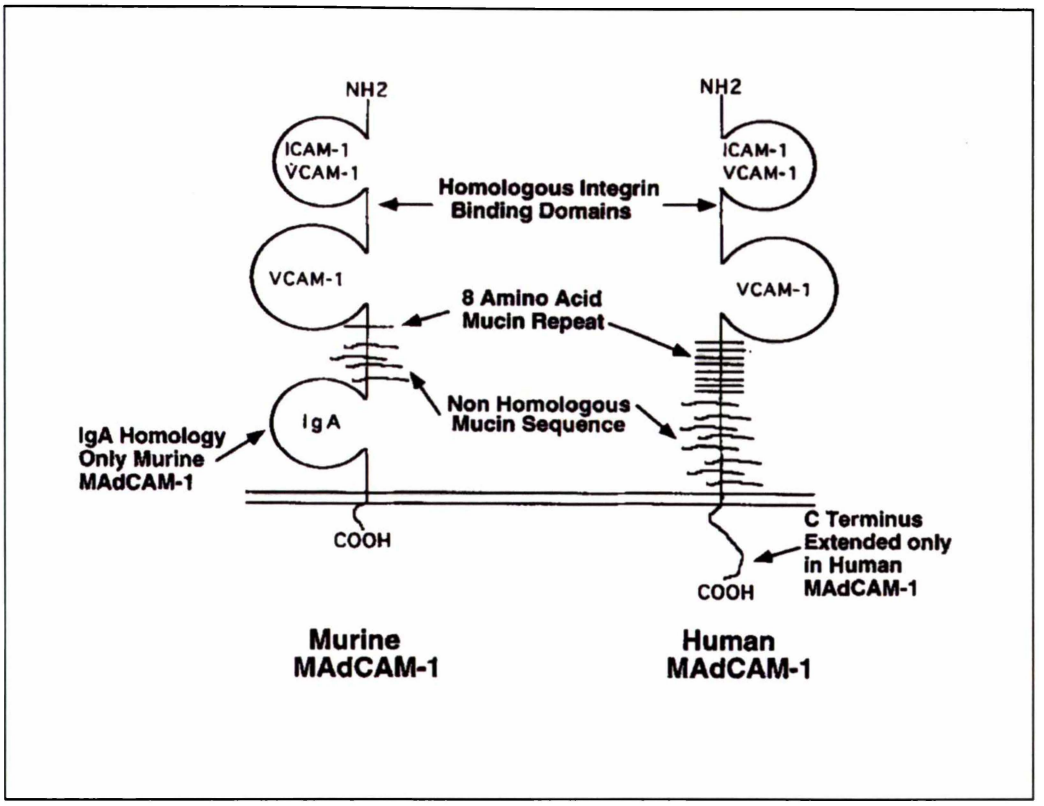
The mammary gland requires lymphocytes not only for immune protection from invading environmental pathogens but also to generate antibodies for secretion into milk. In addition, immune cells are also transferred into colostrum that are of benefit to the neonate (Liebler-Tenorio *et al.,* 2002). Lymphocyte populations in the ruminant mammary gland have been well characterised (see Section 1.4.2.2) but little is known about the vascular addressins that are involved in their recruitment. In contrast to this, as early as 1988 a report was published describing the expression of the mucosal addressin cell-adhesion molecule-1 (MAdCAM-1), in the lactating gland of the mouse (Streeter *et al.,* 1988a).

MAdCAM-1 is a 58 – 66 kDa glycoprotein adhesion receptor that belongs to the immunoglobulin super-family. It has a unique sequence whose only significant homology is with two other protein family members VCAM-1 and ICAM-1, as shown in Figure 3.1 (Shyjan *et al.,* 1996). DNA sequence homology for mouse and human MAdCAM-1 is poor (42%), while the sequence homology for the expressed protein is even weaker at 39% (Briskin *et al.,* 1993; Shyjan *et al.,* 1996). While MAdCAM-1 is commonly believed to be the mucosal cell-adhesion molecule, its expression in mucosal tissues varies widely. It has little or no role to play in homing lymphocytes to

bronchial or oral mucosal tissues (Abitorabi *et al.*, 1996) and is expressed in combination with VCAM-1 in genitourinary tracts (Kelly and Rank, 1997). VCAM-1 predominates in homing lymphocytes to inflamed tissues (Butcher *et al.*, 1999; Mikulowska-Mennis *et al.*, 2001; Neumann *et al.*, 1997).

Figure 3.1 Comparison of murine and human MAdCAM-1 protein structures

Areas of homology with family members VCAM-1 and ICAM-1, are shown. (Schematic adapted from Shyan *et al.*, 1996)



Studies on homing mechanisms in the mammary gland have largely focused on the mouse. In this species, it has been shown that mammary gland B cells predominantly originated from gut-associated lymphoid tissues (GALT) and they began to traffic into the gland as IgA plasmablasts around parturition, followed by a major rise in middle and late lactation (Tanneau *et al.*, 1999; Weisz-Carrington *et al.*, 1977). This was reflected in the milk IgA concentration which increased over the lactation period (Van der Feltz *et al.*, 2001).

In the mouse, T-cell migration to the mammary gland displayed an inverse pattern, with a rise during late pregnancy and fall during lactation. This corresponded to the amount of MAdCAM-1 expressed on the mammary gland vascular endothelium

(Tanneau *et al.,* 1999). In contrast, no relationship was observed between levels of MAdCAM-1 expression and numbers of IgA plasmablasts (Tanneau *et al.,* 1999) although binding of B lymphocytes to the murine mammary gland during mid-lactation was inhibited by antibodies to MAdCAM-1 (Van der Feltz *et al.,* 2001). A factor in milk but not serum has been shown to have chemoattractant properties for GALT-derived IgA B cells (Czinn and Lamm, 1986) and it has been postulated that in addition to MAdCAM-1, chemoattractants may play a role in IgA plasmablast recruitment (Tanneau *et al.,* 1999). VCAM-1 was also detected in the lactating mammary gland but associated with larger blood vessels, not high endothelial venules where the diapedesis of lymphocytes takes place (Tanneau *et al.,* 1999).

A separate murine study was unable to demonstrate the expression of MAdCAM-1 in the mammary gland, but found strong evidence for a role for VCAM-1 in the recruitment of IgA plasmablasts to the mammary gland (Finke and Acha-Orbea, 2001). This study transferred activated B and T cells from mice infected with mammary tumour virus into naïve mice and monitored the movement of the lymphocytes. The T cells migrated to secondary lymph nodes and to the large intestine whereas the B cells migrated to the mammary gland, lung and liver. The B cells expressed the integrin $\alpha 4\beta 1$: its counter receptor VCAM-1, was detected in the mammary gland (Finke and Acha-Orbea, 2001).

Current literature suggests that the profile of addressins in ruminants is similar to that of other species, with MAdCAM-1 predominating in Peyer's patches and mesenteric lymph nodes, and with PNAd the main vascular addressin in peripheral lymph nodes. Based on expression of $\alpha 4\beta 7$ and L-selectin, studies have shown that subsets of ovine T cells migrate preferentially to their corresponding cell-adhesion molecule MAdCAM-1 and PNAd, respectively (Mackay et al., 1996; Premier and Meeusen, 1998). Experiments on sheep demonstrated that lymphocytes homing to the lung expressed low levels of $\alpha 4\beta 7$ and L-selectin and it was suggested that other (unidentified) molecules may be involved in migration to the lung and associated lymphoid tissues (Abitorabi *et al.,* 1996). Work by Rebelatto *et al.* (2000) in the cow has shown that nasal-associated lymphoid tissue and palatine and pharyngeal tonsils are similar to Peyer's patches in their distribution of B and T cells but there is dual expression of intermediate levels of MAdCAM-1 and PNAd. This combination of mucosal and peripheral phenotype in the cow is similar to that in the mouse (Csencsits *et al.,* 2002).

Expression of vascular addressins in the ruminant mammary tissues has not been described, but a small number of investigations have demonstrated the expression of homing receptors on lymphocytes in bovine milk. In one study, the expression of L-selectin and αLβ2 (LFA-1) on T-cell subsets was examined (Van Kampen *et al.,* 1999). These molecules were shown to be expressed at high levels on naïve lymphocytes. While LFA-1 did not change, the L-selectin expression decreased over the lactation period and it was suggested that there was a possible role for this molecule in T-cell trafficking to the mammary gland (Van Kampen *et al.,* 1999). Other experiments have shown αLβ2 to be important in the diapedesis of bovine neutrophils from blood into milk (Smits *et al.,* 2000; Smits *et al.,* 1998).

Very recently a study has compared the expression of the adhesion molecules, L-selectin, αLβ2 (LFA-1), α4β7 (LPAM-1), and CD44 on T-cell subsets in milk and blood samples collected from dairy cattle infected with Johne's disease (Harp *et al.,* 2004). L-selectin, αLβ2 and α4β7 were expressed on a significantly higher percentage of all T-cell subsets in milk than in blood at various times after parturition. It was suggested that these adhesion molecules are involved in selectively trafficking lymphocytes from blood into the mammary gland.

3.2 Aim and approach

The aim of the work in this chapter was to characterise the vascular addressins expressed in the bovine mammary gland of untreated animals. The main focus was to identify, at four different physiological stages of the mammary gland, the distribution and abundance of MAdCAM-1, VCAM-1 and PNAd in alveolar and cisternal tissues and the supramammary lymph node (SMLN). The aim was also to define the vascular addressin receptors expressed on T and B cells present in the mammary tissues collected at the four different physiological stages.

The first objective of this work was to develop immunohistochemical methodology for the molecules of interest:

- MAdCAM-1, VCAM-1 and PNAd – vascular addressins
- von Willebrand factor (vWf) – expressed on vascular endothelial cells
- CD3 – pan T cells
- CD4, CD8 – T-cell subpopulations
- B-B2 – pan B cells
- CD62L and $\alpha 4\beta 7$ - vascular addressin receptors expressed on lymphocytes

The second objective was to collect alveolar, cisternal and SMLN tissue samples from untreated cows at each of four different physiological stages of the mammary gland:

- Late pregnancy
- Colostral phase
- Lactation phase
- Involution phase

and to analyse these mammary tissues by immunohistochemical methods for expression of vascular addressins on endothelial cells and vascular addressin receptor expression on lymphocytes.

In addition, to verify and quantify the expression of MAdCAM-1 mRNA in the mammary gland, alveolar tissues were further analysed using RT-PCR and real-time PCR analyses.

3.3 Materials

3.3.1 *Antibodies*

Primary antibodies used in the optimised immunohistochemistry methods are listed in Table 3.1. The cell-differentiation (CD) antigen for the monoclonal antibody BAQ44A does not have a known human equivalent, unlike the other CD antigens used in this study. The antigen for BAQ44A is expressed by the majority of mature B cells in the blood (Letesson *et al.*, 1991; Mukwedeya *et al.*, 1996).

Table 3.1 Primary antibodies for immunohistochemistry

Clone	Target antigen	Cellular Expression	Iso-type	Host	Supplier
MM1A	Bovine CD3	Pan T cell – associated with T-cell antigen receptor	IgG_1	Mu	VMRD
ILA-11A	Bovine CD4	MHC class II T cell	IgG_{2a}	Mu	WSU
CACT80C	Bovine CD8	MHC class I T cell	IgM	Mu	VMRD
BAQ44A	Bovine BB2	Pan B cell (Non-Ig marker)	IgM	Mu	VMRD
BAQ92A	Bovine CD62L (L-selectin)	B and T cells monocytes, NK cells	IgG_1	Mu	VMRD
Fib 30	Mouse β7 integrin	B and T cells	IgG_{2a}	Rat	Dr Butcher
MECA-79	Mouse PNAd	Endothelium	IgM	Rat	Pharmingen
7G11	Human MAdCAM-1	Endothelium	IgG	Mu	Dr Briskin
Hae 2a	Human VCAM-1	Endothelium	IgG	Mu	Dr Tedder
-	Human vWf (factor VIII-related antigen)	Endothelium	Poly-clonal	Rb	Dako

Mu - mouse
Rb - rabbit
NK - natural killer
Ig -immunoglobulin

There are few antibody reagents for measuring bovine cell-adhesion molecules and therefore, antibodies directed against mouse and human antigens, known to cross-react with the cow, were used (Rebelatto *et al.*, 2000; Van Kampen and Mallard, 2001).

Reagents used for the detection of the primary antibodies in the immunohisto-chemistry methods are listed in Table 3.2.

Table 3.2 Immunohistochemistry detection reagents

Binding Reactivity	Conjugate	Dilution	Host	Supplier
2° Reagents				
Mouse IgG & IgM (H+L)	Biotinylated	1:500	Goat	Jackson
Rat IgG & IgM (H+L)	Biotinylated	1:500	Goat	Jackson
Rabbit immunoglobulins	Biotinylated	1:500	Donkey	Amersham Biosciences
3° Reagents				
Biotin	Streptavidin biotinylated-HRP	1:200	-	Amersham Biosciences

H+L - heavy and light chains of the immunoglobulin molecule

3.3.2 *Primers*

Primers used in RT-PCR and real-time PCR for detection of MAdCAM-1 and β-actin mRNA expression in mammary alveolar tissues are listed in Table 3.3. The primer set for MAdCAM-1 was developed 'in house' (see Section 3.4.3.1). The primer set for β-actin was from a published bovine gene database (Coussens and Nobis, 2002).

Table 3.3 Oligonucleotide primers for RT-PCR and real-time PCR

Target	Primer sequence (5' – 3')	Product Size (bp)	Accession number
MAdCAM-1	F:GAGGTCGCCTGCACGGCCCACAA	250	BE808468
	R: GCCGGGCAGCCTGATGGT		
β-actin	F: CAGAAGGACTCGTACGTGGG	200	AF191490
	R: TTGGCCTTAGGGTTCAGGG		

bp - base pair
F - forward primer
R - reverse primer

3.4 Methods

3.4.1 *Sample collection and preparation*

Healthy untreated pregnant Friesian heifers were used to obtain samples of mammary tissues. Four animals were selected at each of four different physiological stages of the mammary gland: 40 – 50 days *pre-partum* (late pregnancy), two days *post-partum* (colostral phase), 90 days *post-partum* (lactation phase) and three days after the cessation of milking (involution phase). These cows were part of a larger trial that was conducted to obtain tissues from the mammary gland for gene expression analysis.

Cows were slaughtered at the Ruakura abattoir and mammary glands excised for dissection. Tissue samples were collected from the alveolar and cisternal areas of the mammary gland, and from the SMLN. Peyer's patch tissues and mesenteric lymph nodes were also obtained to represent typical gut-associated lymphoid tissue.

Tissue samples for immunohistochemical analysis were processed and frozen as per the protocol outlined in Section 2.2.2.1 and serial sections were cut from these tissue samples as described in Section 2.2.3.1. Tissues samples for RT-PCR and real-time PCR were processed and frozen as per the protocol outlined in Section 2.2.2.1.

3.4.2 *Immunohistochemistry*

3.4.2.1 Immunohistochemical protocol development

Immunohistochemical methods for the detection of lymphocyte subsets and cell-adhesion molecules in mammary tissues were not available 'in house' and development work was required. The immunohistochemical scientific and manufacturers' literature for detecting lymphocyte subsets and cell-adhesion molecules and 'in house' immunohistochemical procedures for other tissue-specific antigens were adapted and modified.

The general procedure was:

- Freeze tissue samples in Tissue Tek O.C.T. compound
- Cut 6 μm frozen serial sections from the tissue samples and mount on Esco Polysine™ slides
- Dry sections and fix
- Block endogenous peroxidase and biotin activity

- Immunostain with primary antibody directed against the antigen of interest
- React primary antibody with a secondary link antibody conjugated to biotin
- Detect secondary antibody with enzyme-avidin complex and chromogenic substrate
- Counterstain, dehydrate, and place coverslips on slides
- Observe cellular staining using a light microscope

A variety of reagents was trialled and tested in order to determine optimal concentrations and incubation times. Some parameters were not tested but kept the same as 'in house' immunohistochemical standard protocols, including cryostat sectioning, immunohistochemical buffers (TBS and TBS-T), incubation temperatures, washing procedures between different reagent applications and counterstaining with haematoxylin.

For the detection of cell-surface membrane antigens on lymphocytes using monoclonal antibodies, preservation of tissue samples by snap-freezing in liquid nitrogen is considered preferable to preserving tissues in formalin (Gutierrez *et al.,* 1999; Pollard *et al.,* 1987). The procedure used for these studies involved immersing tissue samples in Tissue Tek O.C.T. compound (Bayer) and freezing rapidly in liquid nitrogen. There is very often a 'trade off' in the quality of the morphology of the tissue section using frozen tissues, this being poorer than in those tissues preserved with formalin and paraffin-embedded. The advantage is that the cell-surface antigens are not masked by cross-linked protein aggregates or damaged by harsh processes. Therefore, antigens retain their reactivity with antibodies (Boenisch, 2001).

Tissue fixing for frozen tissue samples was performed after sectioning. A variety of fixatives for the mounted frozen sections have been used by different laboratories and a selection of these was trialled: methanol (room-temperature), ethanol (ice-cold), acetone (ice-cold), 1% paraformaldehyde (room-temperature) and 4% paraformaldehyde (room-temperature). The fixatives were compared in the immunohistochemical protocol using the CD3 primary antibody, an antibody that gave strong positive staining of cells. Ice-cold ethanol gave the best quality morphology with retention of strong positive staining of cells and was used for the new protocols.

Primary antibodies were titrated to determine the optimal concentration for use in the immunohistochemistry protocols (Section 2.2.3.3). The optimal concentration was

judged as the dilution of antibody that gave the strongest staining of cell-surface antigen with minimal non-specific background staining. Frozen sections of Peyer's patch and SMLN tissues were used for these tests. A dilution series of 1:100, 1:300, 1:1 000, 1:3 000, and 1:10 000 was tested for each individual antibody. The exception to this was the antibody Fib 30, which had a very low antibody concentration and was tested neat and at dilutions of 1:3, 1:10, 1:30 and 1:100. The optimal antibody concentrations found are listed in Table 3.4. Detection reagents were used at the manufacturers' recommended dilutions (see Table 3.2). Non-specific binding of detection reagents was determined by substituting buffer for the primary antibody.

Table 3.4 Optimal concentrations for primary antibodies used for immuno-histochemical staining

Clone	Target antigen	Optimal concentration
MM1A	bovine CD3	1:1 000
ILA-11A	bovine CD4	1:100
CACT80C	bovine CD8	1:5 000
BAQ44A	bovine BB2	1:3 000
BAQ92A	bovine CD62L	1:100
Fib 30	mouse β7 integrin	1:3
MECA-79	mouse PNAd	1:1 000
7G11	human MAdCAM-1	1:100
Hae 2a	human VCAM-1	1:500
Rabbit polyclonal	human vWf	1:30 000

Protocols for CD4 and CD62L proved difficult to develop because no positive staining of cells could be achieved. After exhaustive testing of other parameters in the assays, it was decided that the problems were largely due to the primary antibody. For CD4, five other monoclonal antibodies were trialled and of these, one worked for a time. The reason for this variation was not able to be ascertained and the protocol for this antigen was never fully validated. A second antibody was tested for CD62L and this gave good staining of lymphocytes but tended to also have some non-specific background staining in mammary gland tissues.

An immunohistochemical protocol for MAdCAM-1 also proved difficult to develop. The initial antibody used to develop the protocol was a commercially sourced antibody; clone MECA-367, from Pharmingen. This antibody was raised in rats against endothelial cells isolated from BALB/c mouse mesenteric lymph nodes.

Trials with this antibody to stain MAdCAM-1 in bovine Peyer's patch tissue proved unsuccessful with no positive staining achieved. Although the antibody did not work in bovine tissues, it did positively stain endothelial venules in mouse Peyer's patches. The conclusion was that the antibody worked in the protocol but did not cross react with bovine MAdCAM-1, in agreement with other studies in sheep (Premier and Meeusen, 1998). A second antibody raised in mice against human MAdCAM-1 (7G11; gifted by Dr Briskin) successfully stained endothelial venules in bovine Peyer's patches. This antibody was used in the studies in this thesis. It had been used previously in one other bovine study detecting MAdCAM-1 in nasal-associated lymphoid tissues (Rebelatto *et al.,* 2000).

Attempts to set up immunohistochemical protocols to detect IgA-, IgG- and IgM-positive B cells were unsuccessful due to background staining of soluble immunoglobulin in the frozen tissue samples. A solution to this problem was found after searching the literature and discussing options with other researchers in their field of expertise. Details of this solution are described in Chapter Four.

3.4.2.2 Immunohistochemical analysis

Serial sections of frozen alveolar, cisternal and SMLN tissues from four untreated cows at each of four different physiological stages of the mammary gland were immunostained with antibodies specific for vascular addressins to identify the presence and distribution of the addressins in the mammary gland. All vascular endothelial cells were identified by immunostaining with an antibody for von Willebrand factor (vWf), an antigen expressed on vascular endothelium. Samples and analyses were:

- serial sections of alveolar, cisternal and SMLN tissues
- four untreated animals per stage
- four physiological stages of the mammary gland – late pregnancy, colostral phase, lactation phase, involution phase
- vascular addressins – MAdCAM-1, VCAM-1, PNAd
- control for vascular endothelial cells – vWf

A semi-quantitative subjective measure of vascular addressin expression was made by a visual comparison of the vascular endothelium in serial sections of tissue that were stained by the addressins and the vascular endothelium stained by vWf. The level of expression of vascular addressins was expressed as the proportion of venules stained for vWf that also stained for addressins. Expression was graded from 0 – 4: 0 = no addressin expression; 1 = 1 – 25% venules stained with addressin that stained with vWf; 2 = 25 – 50%; 3 = 50 – 75%; 4 = 75 – 100%.

Serial sections of frozen alveolar, cisternal and SMLN tissues from four cows at each of four physiological stages of the mammary gland were also immunostained with the following antibodies to identify the lymphocyte subsets present in the mammary gland.

- CD3 – pan T-cell population
- BB2 – pan B-cell population
- CD62L – counter receptor expressed on lymphocytes for PNAd
- β7 – counter receptor expressed on lymphocytes for MAdCAM-1
  (NB: β7 detection does not distinguish between α4β7 and αEβ7)

3.4.3 *MAdCAM-1 RT-PCR*

3.4.3.1 Primer development for MAdCAM-1 RT-PCR

For the amplification of mRNA by RT-PCR, oligonucleotide primers are required based on the DNA sequence of the target gene. Although the human and mouse sequences for MAdCAM-1 are available in the public domain, there is no published sequence for the bovine homologue. RT-PCR primers for use in these studies were first designed based on the human sequence using a bioinformatics software programme, Vector NTI (Informax, Invitrogen). Using mRNA extracted from Peyer's patch tissues and mesenteric lymph nodes as described in section 2.2.4.1, these primers were used for RT-PCR amplification of the MAdCAM-1 DNA sequence using the protocol outlined in Section 2.2.4.2 and Section 2.2.4.3. The resultant DNA products were separated using agarose gel electrophoresis using the protocol outlined in Section 2.2.4.4. However, no detectable bands were seen at the expected base pair size (526 bp).

A variety of conditions were tested to try and improve the annealing of primer to template. The PCR reaction was run at a range of different annealing temperatures (52 – 68°C) with variable concentrations of magnesium chloride (1.0 mmol/l, 1.5

mmol/l, 2.0 mmol/l, and 3.0 mmol/l) and with the absence or addition of 2.5 µl dimethylsulphoxide (DMSO), but there was no improvement and no DNA products were detected at the expected base pair size (526 bp).

A line-up comparison of the MAdCAM-1 amino acid sequences for human, macaque and mouse (Figure 3.2) demonstrated similarities in some domains but divergence in others (Shyjan *et al.,* 1996). A second set of primers was designed using areas of amino acid sequence with greater homology. These primers also failed to produce any product when used for RT-PCR amplification of mRNA from Peyer's patch tissues and mesenteric lymph nodes. A variety of conditions were tested to try and improve the annealing of primer to template, as for the first set of primers, but again no products were detected at the expected base pair size (440 bp).

Failure of primers to detect bovine MAdCAM-1 DNA based on the human MAdCAM-1 DNA sequence may not be unexpected. Species differences between the mouse and human MAdCAM-1 DNA have been well demonstrated (Shyjan *et al.,* 1996). Therefore, there are very probably differences between the bovine and human sequences.

In an attempt to locate a partial bovine MAdCAM-1 DNA sequence, the human MAdCAM-1 sequence (1608 base pairs, NCBI reference - NM_130761) was blasted against The Institute for Genomic Research (TIGR) database for cattle. This resulted in a hit for an expressed sequence tag (EST) DNA sequence (526 base pairs, TIGR reference – BE808468). There was 65% sequence similarity between this EST and the human sequence, with a score of 829, P = 3.8e-32. Figure 3.3 shows the line-up of the two DNA sequences.

A third set of primers was designed using areas of DNA sequence with greatest agreement between the bovine TIGR EST (BE808468) and the sequence for human MAdCAM-1 (Figure 3.3). It should be noted that although there was an exact match between the bovine and human amino acid sequences for the primers, there were some differences in the corresponding nucleotide sequences. RT-PCR amplification of mRNA from Peyer's patch tissues and mesenteric lymph nodes using this third set of primers produced a DNA product at the expected base pair size (250 bp).

Figure 3.2 Comparison of the mouse, macaque and human MAdCAM-1 amino acid sequences

Amino acid homology is shown with a greyed background. The location of the primer pairs designed from the human sequence are shown in red: the first primer pair designed are designated F:I & R:I; the second primer pair designed are designated F:II and R:II. (Figure adapted from Shyjan *et al.* 1996)

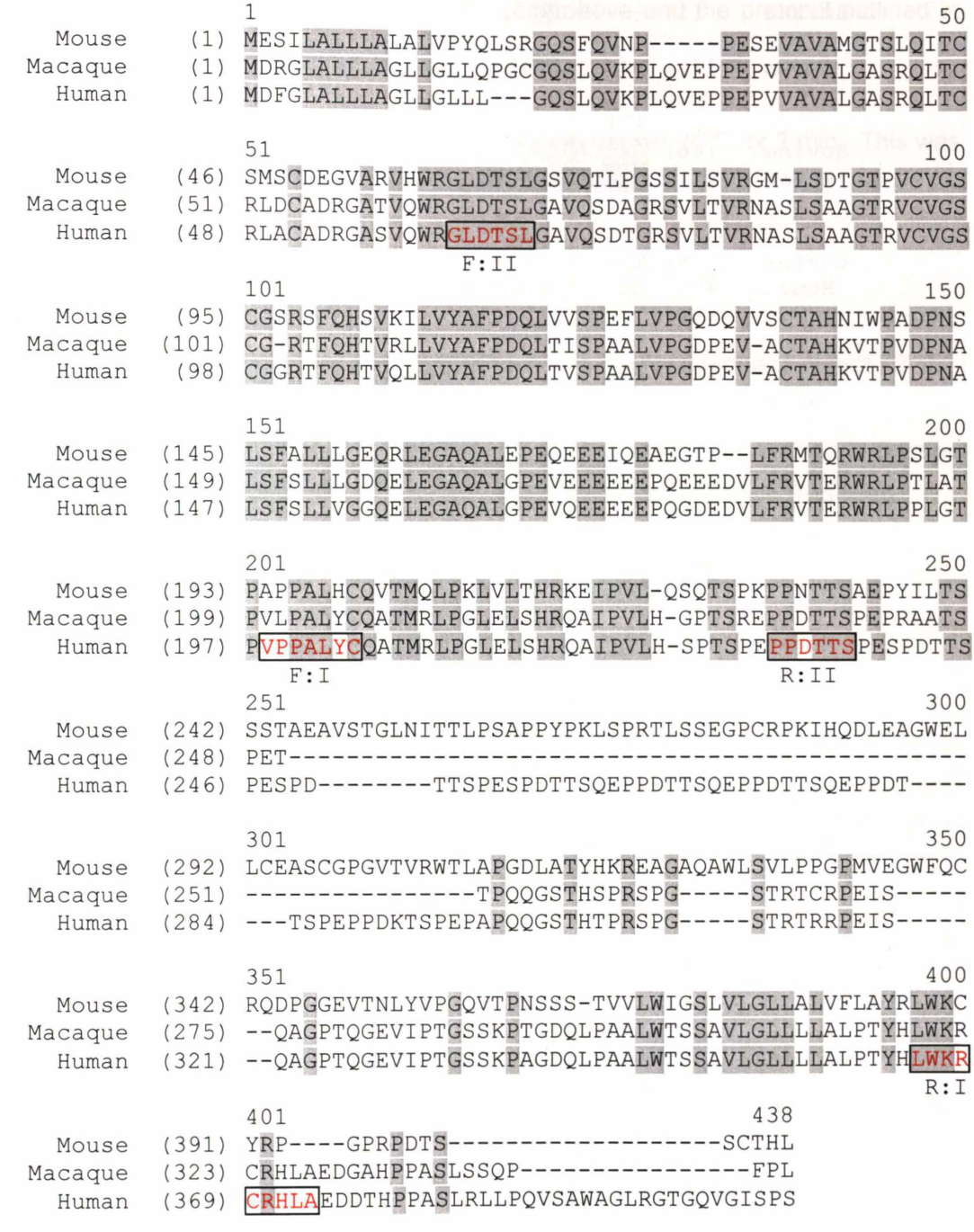


Figure 3.3 Comparison of bovine TIGR EST (BE808468) and human MAdCAM-1 DNA sequences

Identical DNA sequence is shown with a greyed background. The location of the primer pair designed from the bovine sequence and the amino acid sequences for the primers are shown in red.

```
                371                                                 420
Bovine     (1)  -----GGCCGTTGTGCCAGGGAGGGACCAGGAGGTGGCCTGCACGGCCCA
                                                   E  V  A  C  T  A  H
Human    (371)  TCCCCAGCAGCCCTGGTGCCTGGTGACCCGGAGGTGGCCTGTACGGCCCA

                421                                                 470
Bovine    (46)  CAACGTCACACCTCCTGGCCCTGACACCCTCTCCATGTCCCTGCTCCTGG
Human    (421)  CAAAGTCACGCCCGTGGACCCCAACGCGCTCTCCTTCTCCCTGCTCGTCG

                471                                                 520
Bovine    (96)  GGGATCGGGAACTGGAGGGAGTGGAAGCCCT---CCCGAACGTGACTGAG
Human    (471)  GGGGCCAGGAACTGGAGGGGGCGCAAGCCCTGGGCCCGGAGGTGCAGGAG

                521                                                 570
Bovine   (143)  GAG---------CCCCAGGAAGGCGAGGATTCGCTGTTCCAAGTGACCCA
Human    (521)  GAGGAGGAGGAGCCCCAGGGGGACGAGGACGTGCTGTTCAGGGTGACAGA

                571                                                 620
Bovine   (184)  GCGCTGGCTGCTGCCCACCTCAGAGACCTCCAGCCTGCGCACCCTCCACT
Human    (571)  GCGCTGGCGGCTGCCGCCCCTGGGGACCCCTGTCCCGCCCGCCCTCTACT

                621                                                 670
Bovine   (234)  GCCAGGTGACCATGAGGCTGCCCGGCCGGGAGCTGACCCACCACCGGACC
                          T  M  R  L  P  G
Human    (621)  GCCAGGCCACGATGAGGCTGCCTGGCTTGGAGCTCAGCCACCGCCAGGCC

                671                                                 720
Bovine   (284)  ATTCCAGTCCTGCAGGGCCTGACGTCCCCGGAGCCCCCCAGCATCACCTC
Human    (671)  ATCCCCGTCCTGCACAGCCCGACCTCCCCGGAGCCTCCCGACACCACCTC

                721                                                 770
Bovine   (334)  CTCAGAGCTGCCCACCATGACCCCTGCAAAGCCCAGCATCACAGCGTCCC
Human    (721)  CCCGGAGTCTCCCGACACCACCTCCCCGGAGTCTCCCGACACCACCTCCC

                771                                                 820
Bovine   (384)  CGGAGCCCACTGATACAACCACCCCAGAGTCTTTTGTCATGAAGCCCCCG
Human    (771)  CGGAGTCTCCCGACACCACCTCCCAGGAGCCTCCCGACACCACCTCCCAG

                821                                                 870
Bovine   (434)  AAGCCTCCCATCACTACCTCCCCCGAGCTGGCCTCCACCTACAGCCCCGC
Human    (821)  GAGCCTCCCGACACCACCTCCCAGGAGCCTCCCGACACCACCTCCCCGGA

                871                                                 920
Bovine   (484)  GAGTCCTGGCCCCGCAAGTCCTGGCGCCACGTCCAGCAACAGCTCCACCA
Human    (871)  GCCTCCCGACAAGACCTCCCCGGAGCCCGCCCCCAGCAGGGCTCCACAC

                921                         949
Bovine   (534)  GGCCGTGCCTCCC----------------
Human    (921)  ACACCCCCAGGAGCCCAGGCTCCACCAGG
```

3.4.3.2 MAdCAM-1 RT-PCR analysis

Extraction and purification of mRNA from the alveolar mammary tissues of four untreated cows at each of four different physiological stages of the mammary gland was performed using the protocol outlined in Section 2.2.4.1. Peyer's patch tissues were included as positive control samples. RT-PCR was performed using the third primer set designed as described in the section above and the protocol outlined in Sections 2.2.4.2 and 2.2.4.3.

The PCR reaction began with an initial denaturation step at 94°C for 2 min. This was followed by 35 thermo-cycling steps using the conditions:

- Denaturation for 30 s at 94°C
- Annealing for 30 s at 65°C
- Extension for 30 s at 72°C

At the end of the last cycle, the extension phase was run for a further 5 min at 72°C.

DNA products were separated by agarose gel electrophoresis as outlined in Section 2.2.4.4, then illuminated and photographed using the Gel Doc system (Bio-Rad).

3.4.4 *MAdCAM-1 real-time PCR*

3.4.4.1 Real-time PCR development for MAdCAM-1

The MAdCAM-1 primers designed using the EST DNA sequence (BE808468) from the TIGR cattle database were validated for real-time PCR using the protocol outlined in Section 2.2.4.6. Annealing temperatures for real-time PCR for MAdCAM-1 and the endogenous control gene β-actin were optimal at 65°C and 60°C, respectively.

In order to determine the efficiency of the real-time PCR reaction, RT-PCR products for MAdCAM-1 and β-actin were prepared using the protocol in Section 2.2.4.3 and annealing temperatures of 65°C and 60°C for MAdCAM-1 and β-actin, respectively. Extracted mRNA from alveolar mammary tissue was used for β-actin sequence amplification and extracted mRNA from Peyer's patch tissue was used for MAdCAM-1 sequence amplification. The products were isolated on agarose gel (see Section 2.2.4.4) and purified (see Section 2.2.4.5) and five 10-fold serial dilutions prepared.

3.4.4.2 Real-time PCR analysis of MAdCAM-1

Extracted, purified and reverse transcribed mRNA from the alveolar mammary tissues of four untreated cows at each of four different physiological stages of the mammary gland, used in the RT-PCR reactions (see Section 3.4.3.2), were analysed using real-time PCR. In addition four different samples of bovine Peyer's patch tissue were also analysed. Using the protocol outlined in Section 2.2.4.6 and the primers and annealing temperatures validated in Section 3.4.4.1, the mRNA levels of MAdCAM-1 and β-actin was determined for all the samples. Five 10-fold serial dilutions of the PCR product for both MAdCAM-1 and β-actin (see section 3.4.4.1) were also analysed by real-time PCR to determine the efficiency of the reaction and to calculate the relative quantity of mRNA in unknown samples, as described in Section 2.2.4.6. The mRNA levels of MAdCAM-1 in unknown samples were reported relative to the mRNA levels of β-actin. This adjusted for any variation in the quantity of total mRNA used for each reaction. The relative expression levels of MAdCAM-1 in different alveolar mammary tissues and Peyer's patch tissues were compared.

3.5 Results

3.5.1 *Immunohistochemical analysis of vascular addressin expression in the untreated mammary gland*

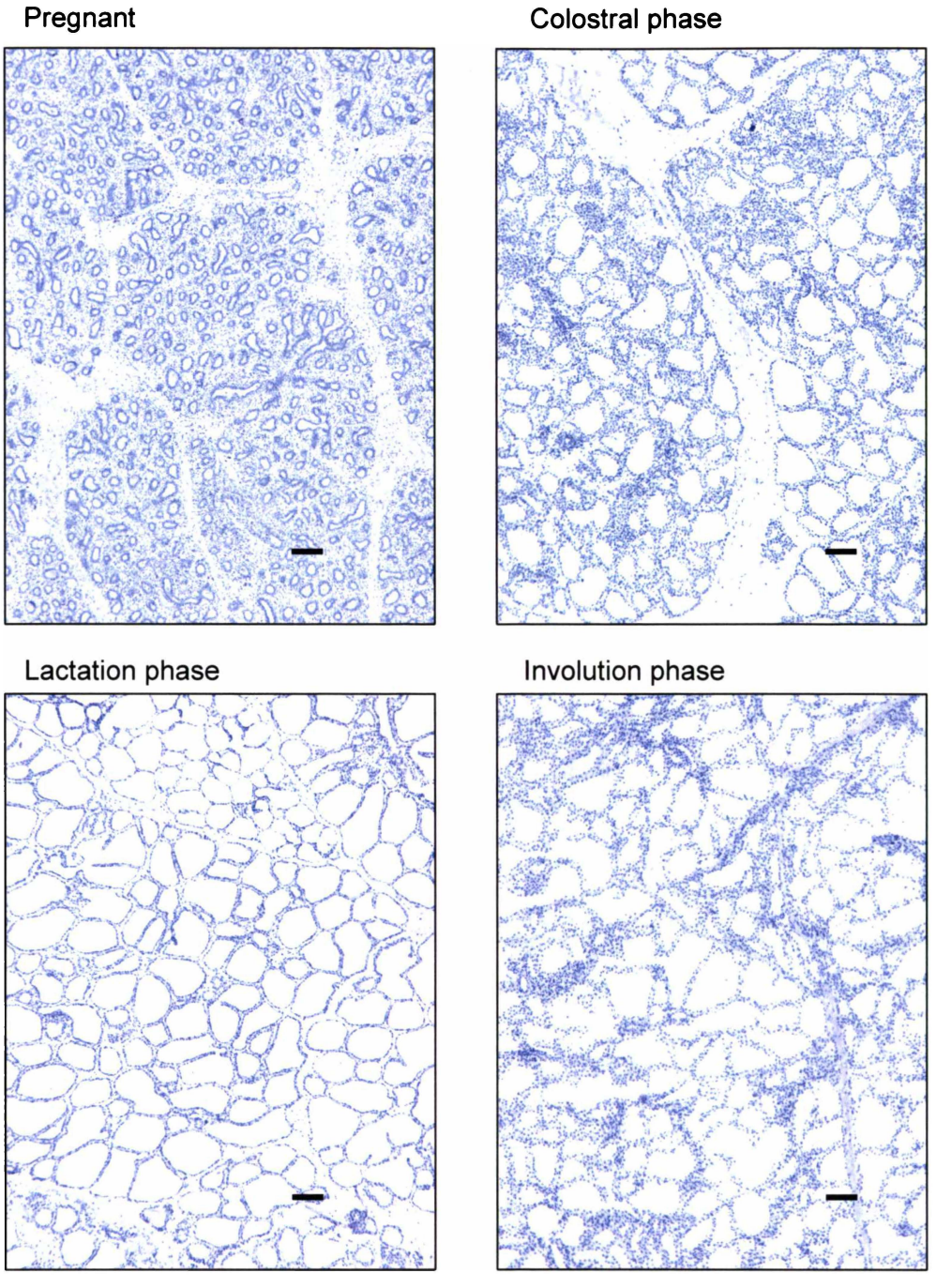
Illustration of the characteristic architecture of alveolar tissues collected from the untreated mammary gland at four different physiological stages and stained with haematoxylin is shown in Figure 3.4. The mammary gland during the stage of late pregnancy (40 – 50 days *pre-partum*) already had well developed alveoli interspersed within the stromal tissue. Lobes and ducts were also well defined. Two days *post-partum*, in the colostral phase, the alveoli had increased in number compared with late pregnancy but there were still interstitial spaces between the alveoli. By 90 days *post-partum*, the gland was in established lactation. At this stage, the alveoli had enlarged such that the epithelial walls of the individual alveoli were touching their neighbour within lobes. Three days after the cessation of milking, the involuting tissue from the mammary gland showed the beginnings of the breakdown of the integrity of the alveoli. Compared with lactation tissue the size of the alveoli had also diminished. There were no apparent differences in the characteristic architecture of the supramammary lymph node (SMLN) sampled from the four different physiological stages (data not shown).

The results of the immunohistochemical characterisation of MAdCAM-1, VCAM-1 and PNAd expression in mammary gland tissues of untreated cows are described in the following sections. Illustrations of bovine Peyer's patch tissues, used as positive control tissues in the immunohistochemical protocols, stained with MAdCAM-1, VCAM-1 and vWf are shown in Figure 3.5.

3.5.1.1 MAdCAM-1

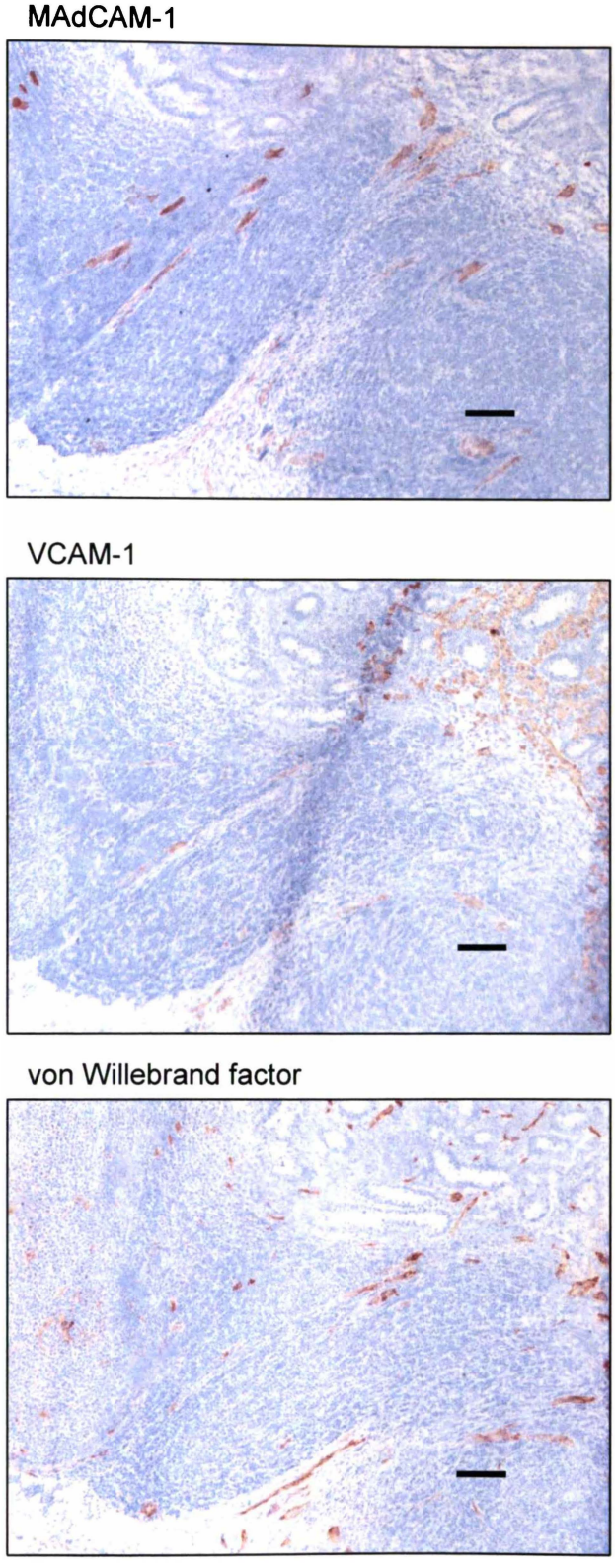
Expression of MAdCAM-1 was detected on the vascular endothelium of bovine Peyer's patch tissue (see Figure 3.5). However, no MAdCAM-1 expression was detected on the vascular endothelium in any of the mammary gland tissue samples tested. This included the alveolar, cisternal and SMLN tissue samples collected from all four physiological stages of the mammary gland. A comparison of MAdCAM-1, VCAM-1 and vWf staining in serial sections of alveolar tissue from the colostral phase is illustrated in Figure 3.6.

Figure 3.4 Representative illustration of haematoxylin stained frozen alveolar tissue from different physiological stages of the bovine mammary gland of untreated animals



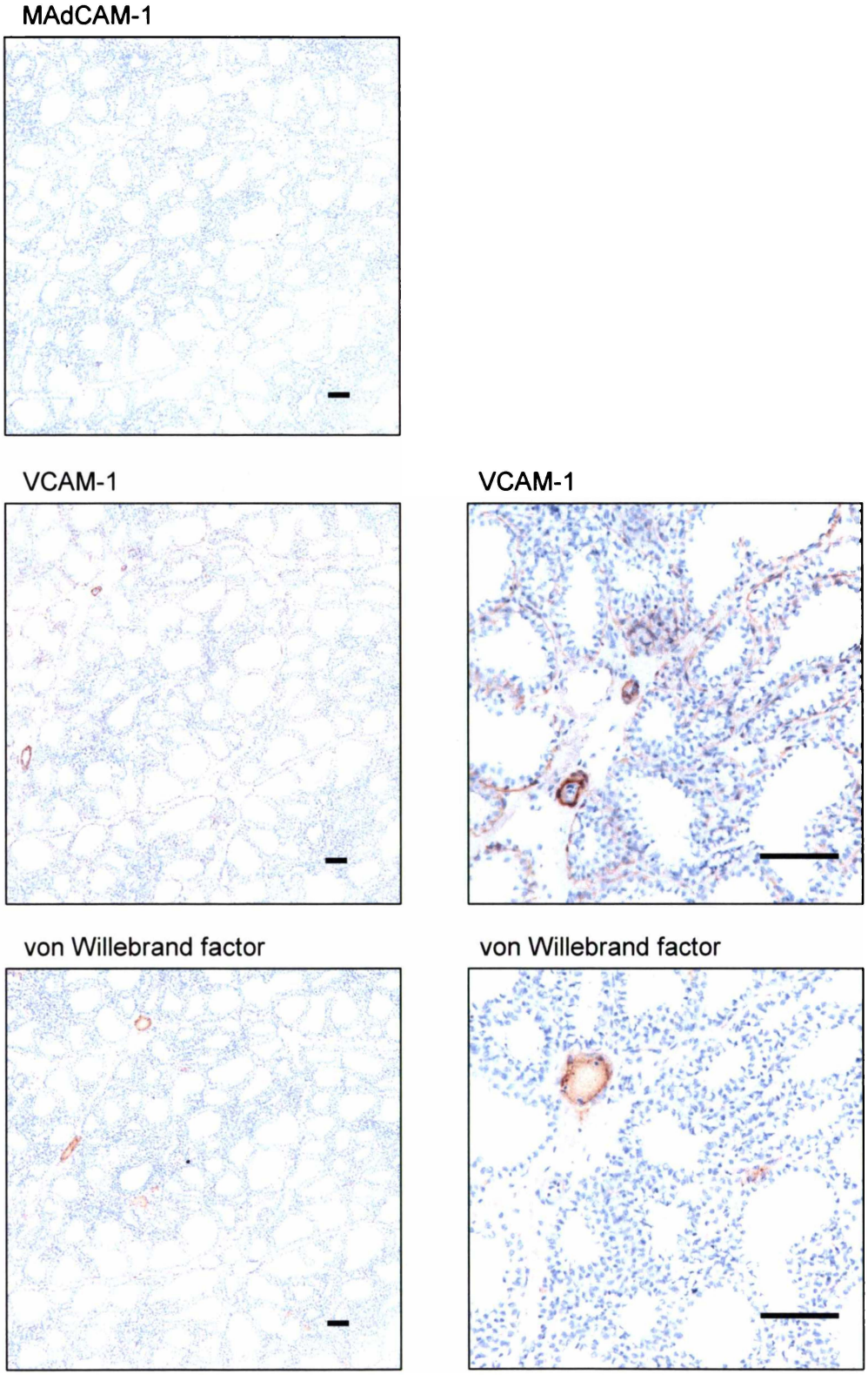
Black solid bar is 100μm

Figure 3.5 Representative illustration of MAdCAM-1, VCAM-1 and vWf immunohistochemical staining on vascular endothelium in frozen serial sections of bovine Peyer's patch tissue



Black solid bar is 100μm

Figure 3.6 Representative illustration of MAdCAM-1, VCAM-1 and vWf immunohistochemical staining on vascular endothelium in frozen alveolar serial sections from the colostral phase of the bovine mammary gland



Black solid bar is 100μm

3.5.1.2 VCAM-1

The expression of VCAM-1 in mammary gland tissues from the four physiological stages was compared. The positive staining of VCAM-1 was most pronounced in the tissue samples from the colostral phase with expression in all four individual animals at this physiological stage. There was VCAM-1 expression detected in alveolar tissues of three individual animals from the lactation phase. There was little or no expression of VCAM-1 in tissues collected at other stages. Table 3.5 shows the number of untreated animals found to express VCAM-1 in the mammary gland at various physiological stages.

Table 3.5 *VCAM-1 expression in the mammary gland of untreated animals*

The number of animals where expression of *VCAM-1* was detected in alveolar, cisternal and SMLN tissues at different physiological stages of the mammary gland is shown.

Physiological stage of the mammary gland	**Proportion of animals per group with VCAM-1 expression detected**		
	Alveolar	*Cisternal*	*SMLN*
Pregnant	0/4	0/4	1/4
Colostral	4/4	4/4	4/4
Lactating	3/4	0/4	1/4
Involuting	0/4	0/4	1/4

SMLN - supramammary lymph node

Positive staining in the alveolar and cisternal tissues was detected on larger venules that were also positively stained by vWf. The expression of VCAM-1 detected in alveolar tissue samples from the colostral phase of the mammary gland is illustrated in Figure 3.6. In alveolar tissues at this physiological stage, positive staining for VCAM-1 was also visible around a number of the alveoli (Figure 3.6). This area did not stain positively for vWf.

In the colostral alveolar and cisternal tissue samples, VCAM-1 expression was seen on 50 – 100% of the vascular endothelium that was also stained positively with vWf, as shown in Table 3.6. The staining in the lactation tissue samples was less prevalent than that detected in the colostral stage, with VCAM-1 expression being seen on 0 – 50% of the vascular endothelium that was also stained with vWf. Expression was graded from 0 – 4: 0 = no VCAM-1 expression; 1 = 1 – 25% venules

stained with VCAM-1 that stained with vWf; 2 = 25 – 50%; 3 = 50 – 75%; 4 = 75 – 100% (see section 3.4.2.2).

Illustration of positive staining of VCAM-1 in the SMLN is shown in Figure 3.7. VCAM-1 expression was detected in SMLN tissue samples on both large and small venules that were stained with vWf (Figure 3.7). In SMLN tissue samples, VCAM-1 was expressed on 0 – 50% of the vascular endothelium that also stained positively with vWf (see Table 3.6).

Table 3.6 Proportion of endothelial venules in the tissues of the mammary gland of untreated animals that stained positive for both VCAM-1 and vWf

Alveolar, cisternal and supramammary lymph nodes tissues were collected from untreated animals at each of four physiological stages of the mammary gland. Expression was graded from 0 - 4: 0 = no VCAM-1 expression; 1 = 1 - 25% venules stained with VCAM-1 that stained with vWf; 2 = 25 - 50%; 3 = 50 - 75%; 4 = 75 - 100%. (Group average ± SEM, n = 4). Different lowercase letters in superscript indicate statistically significant differences for the tissue at different physiological stages ($p < 0.05$).

Physiological stage of the mammary gland	**Proportion of vascular endothelium stained positive for VCAM-1 that also stained positive for vWf**		
	Alveolar	*Cisternal*	*SMLN*
Pregnant	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.8 ± 0.8^{a}
Colostral	$4.0 \pm 0.0^{b,}$	3.7 ± 0.3^{b}	2.5 ± 0.5^{b}
Lactating	1.8 ± 0.6^{c}	0.0 ± 0.0^{a}	0.8 ± 0.8^{a}
Involuting	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	$2.0 \pm 1.4^{a, b}$

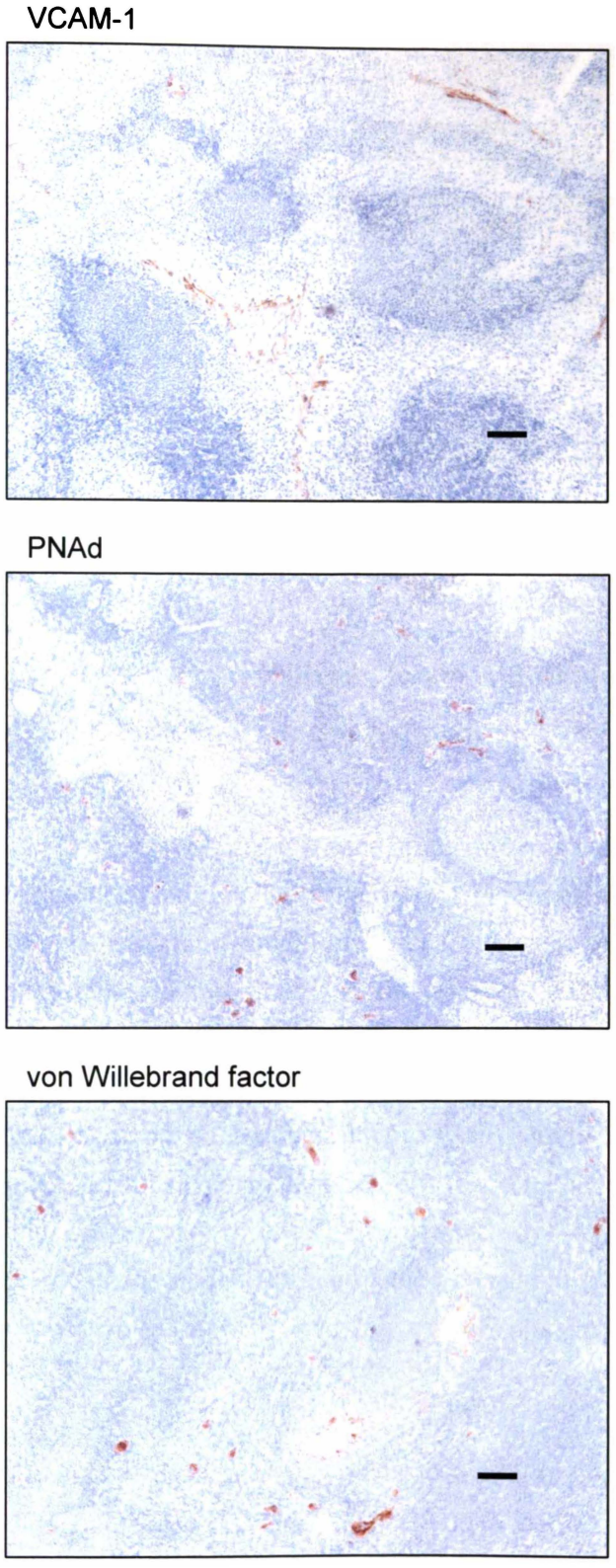
SMLN - supramammary lymph node
vWf - von Willebrand factor

3.5.1.3 PNAd

The expression of PNAd detected in the SMLN is illustrated in Figure 3.7. Positive staining was seen in the SMLN samples of all animals, at all physiological stages of the mammary gland. PNAd expression was detected in 25 – 100% of the endothelial venules that were also stained with vWf. The proportion of PNAd staining compared with vWf staining for different physiological stages of the mammary gland is shown in Table 3.7.

No PNAd was detectable in alveolar or cisternal mammary tissue samples from the pregnant, colostral, lactation or involution stages.

Figure 3.7 Representative illustration of VCAM-1, PNAd and vWf immuno-histochemical staining on vascular endothelium in frozen bovine supramammary lymph node tissues from the colostral phase



Black solid bar is 100μm

Table 3.7 Proportion of endothelial venules in the supramammary lymph nodes of untreated animals that stained positive for both PNAd and vWf

Supramammary lymph nodes were collected from untreated animals at each of four physiological stages of the mammary gland. Expression was graded from 0 - 4: 0 = no PNAd expression; 1 = 1 - 25% venules stained with PNAd that stained with vWf; 2 = 25 - 50%; 3 = 50 - 75%; 4 = 75 - 100%. (Group average ± SEM, n = 4). Different lowercase letters in superscript indicate statistically significant differences at various physiological stages ($p < 0.05$).

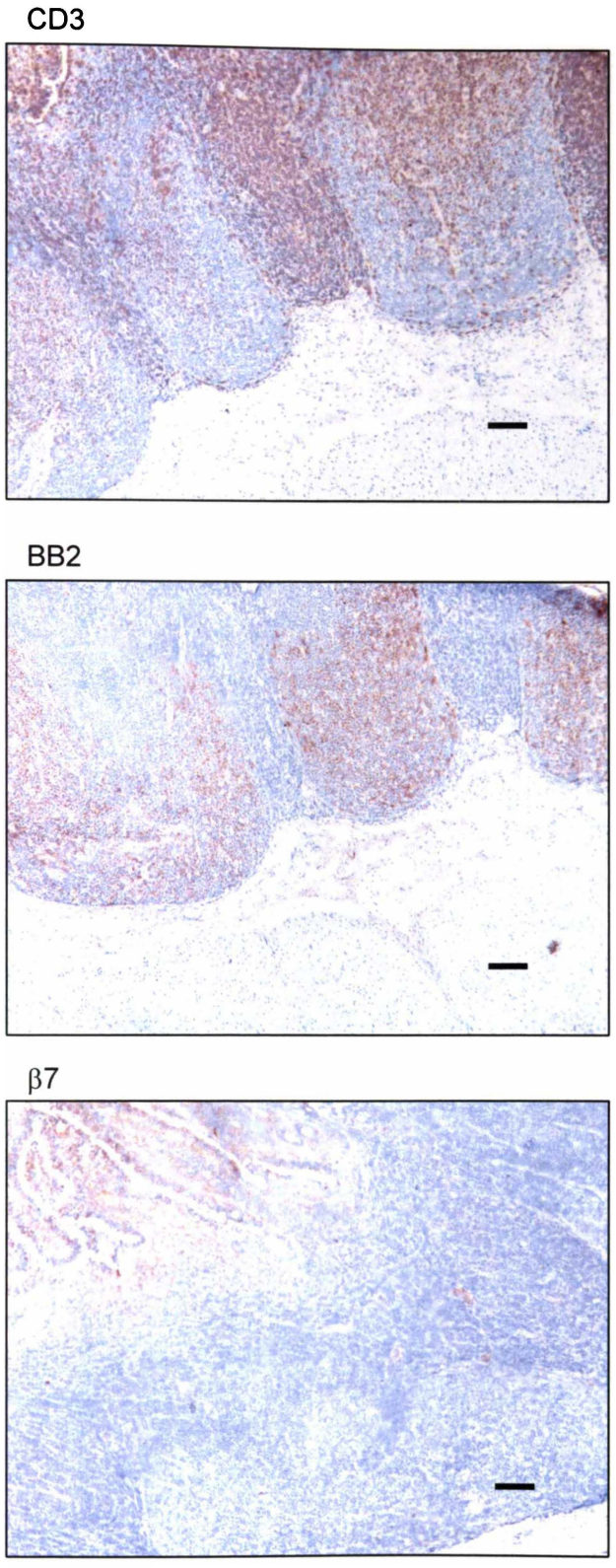
Physiological stage of the mammary gland	Proportion of vascular endothelium stained positive for PNAd that also stained positive for vWf
Pregnant	2.3 ± 0.5^{a}
Colostral	2.0 ± 0.7^{a}
Lactating	4.0 ± 0.0^{b}
Involuting	$3.5 \pm 0.3^{a, b}$

3.5.2 *Immunohistochemical characterisation of vascular addressin receptor expression on lymphocytes in the untreated mammary gland*

Lymphocytes in the mammary gland were detected by immunohistochemical analysis using pan T cell (MM1A) and pan B cell (BB2) monoclonal antibodies. T cells were found to be widely distributed throughout the interstitial spaces of alveolar and cisternal regions, with no large variation in numbers apparent for the different physiological stages of the mammary gland. In contrast, no B cells were detected in alveolar or cisternal tissue samples at any physiological stage. In the SMLN, both T and B cells were evident. Similar to characteristic aggregation patterns described in other studies of lymph nodes and Peyer's patch tissue, B cells were isolated in follicular regions with T cells observed mostly at the periphery of follicles and also scattered throughout the tissue. Illustrations of immunohistochemical staining of pan T cells and pan B cells in Peyer's patches and the SMLN are shown in Figure 3.8 and Figure 3.9, respectively.

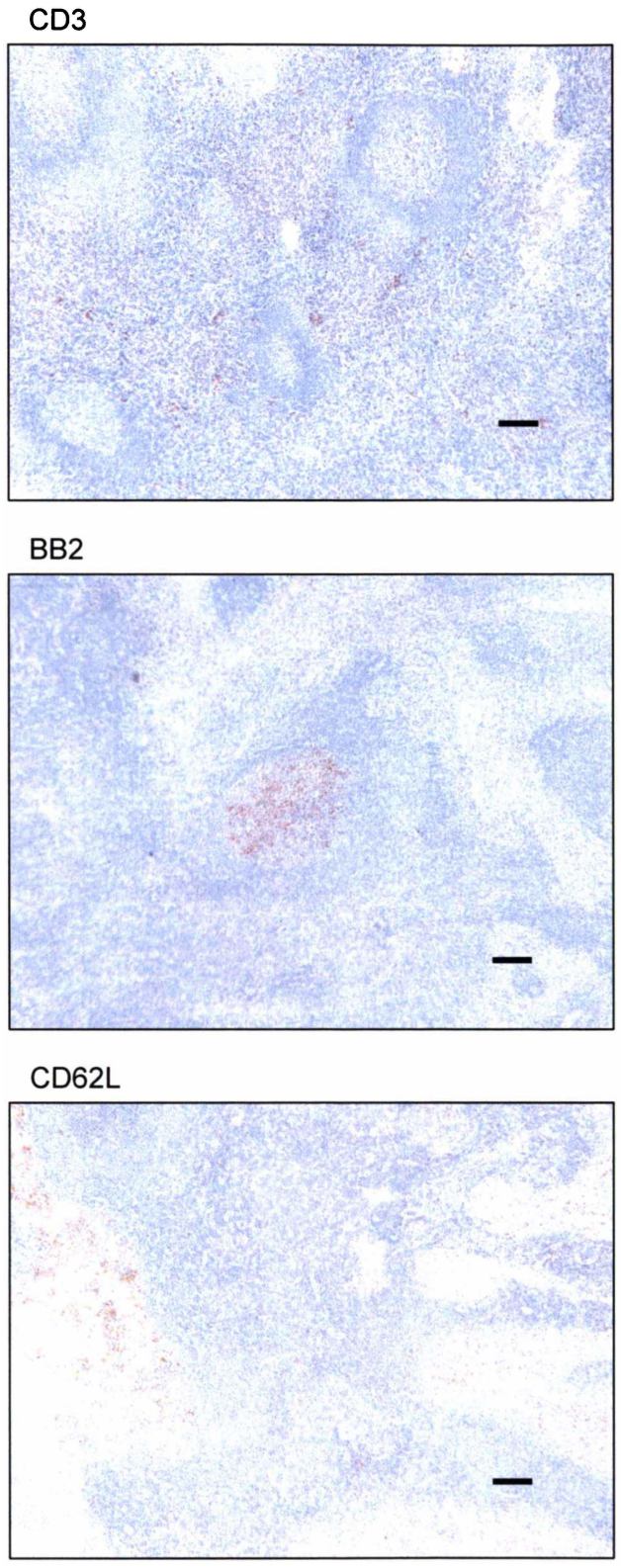
Immunohistochemical analysis of alveolar and cisternal tissues and SMLN tissues revealed no lymphocytes expressing β7, the cell-surface receptor for MAdCAM-1. There was no evidence of these cells in the tissues at any physiological stage of the

Figure 3.8 Representative illustration of immunohistochemical staining of CD3-, BB2- and β7-positive cells in bovine Peyer's patch tissues



Black solid bar is 100μm

Figure 3.9 Representative illustration of immunohistochemical staining of CD3-, BB2- and CD62L-positive cells in bovine supramammary lymph node



Black solid bar is 100μm

mammary gland. However, the β7 monoclonal antibody did stain cells in bovine Peyer's patch tissues (see Figure 3.8).

Lymphocytes expressing CD62L (L-selectin) were detected in the SMLN (see Figure 3.9) and expression did not appear to vary with the different physiological stages of the mammary gland. Difficulties were encountered assessing L-selectin positive staining in alveolar and cisternal mammary gland tissues due to non-specific staining encountered with this monoclonal antibody in these tissues.

3.5.3 *MAdCAM-1 mRNA expression in the untreated mammary gland*

3.5.3.1 Detection of MAdCAM-1 mRNA in the untreated mammary gland using RT-PCR

A purified DNA product, amplified by RT-PCR using the primers designed from the TIGR EST DNA sequence of bovine MAdCAM-1 and mRNA extracted from bovine Peyer's patch tissue, was sequenced. A perfect match was achieved between this DNA sequence and the TIGR EST DNA sequence, establishing that the primers were amplifying the correct sequence.

Initially, when expression of MAdCAM-1 mRNA was assessed in alveolar mammary tissue samples from four cows at each of four different physiological stages of the mammary gland, results showed significant levels of MAdCAM-1 mRNA in pregnant and colostral phase tissue samples, with lower levels of mRNA in tissue samples from the lactation and involution phase. However, expression was also detected in control samples that lacked the reverse transcriptase and thus had no cDNA formed from mRNA. This result suggested that the mRNA samples were contaminated with genomic DNA. To eliminate this contamination, samples were treated with DNase and re-purified. Following this treatment, weak but discernible levels of MAdCAM-1 mRNA were still detectable in some samples for mammary alveolar tissue samples from all physiological stages.

3.5.3.2 Quantitative analysis of MAdCAM-1 mRNA in the untreated mammary gland

The efficiencies of the real-time PCR reactions for MAdCAM-1 and β-actin were calculated by the Lightcycler software (Roche) to be 1.848 and 1.814, respectively

(Figures 3.10 and 3.11). To assess the accuracy and reproducibility of the real-time PCR intra-assay precision was determined in five repeats of one sample within a Lightcycler run and inter-assay variation was determined using one sample in five different experimental runs performed on five days. The coefficient of variation for the cycle number value within assay was 0.61% and between assays was 1.60%.

Levels of MAdCAM-1 mRNA in the alveolar mammary tissue samples from four cows at each of four different physiological stages of the mammary gland were compared with levels of mRNA in four different samples of Peyer's patch tissue. Data reported as the level of MAdCAM-1 mRNA relative to the level of β-actin mRNA are shown in Table 3.8. The average value for the mammary tissues at each of the four physiological stages was divided by the average value for the Peyer's patch tissues to obtain a gene expression level of MAdCAM-1 for mammary gland tissues relative to Peyer's patch tissues. The results obtained indicate that alveolar mammary gland tissue contained 4.7 – 9.1 x 10^3-fold lower MAdCAM-1 mRNA than Peyer's patch tissues (Table 3.8). This difference was highly significant ($p < 0.001$). There was no significant difference for levels of MAdCAM-1 mRNA between any of the different physiological stages of the mammary gland.

Table 3.8 *MAdCAM-1 gene expression in the mammary gland of untreated cows compared with that in bovine Peyer's patch tissues*

Level of MAdCAM-1 mRNA (relative to β-actin) in bovine Peyer's patch tissue compared with alveolar tissue samples from the bovine mammary gland of pregnant, colostral, lactation, and involution phases (n = 4).

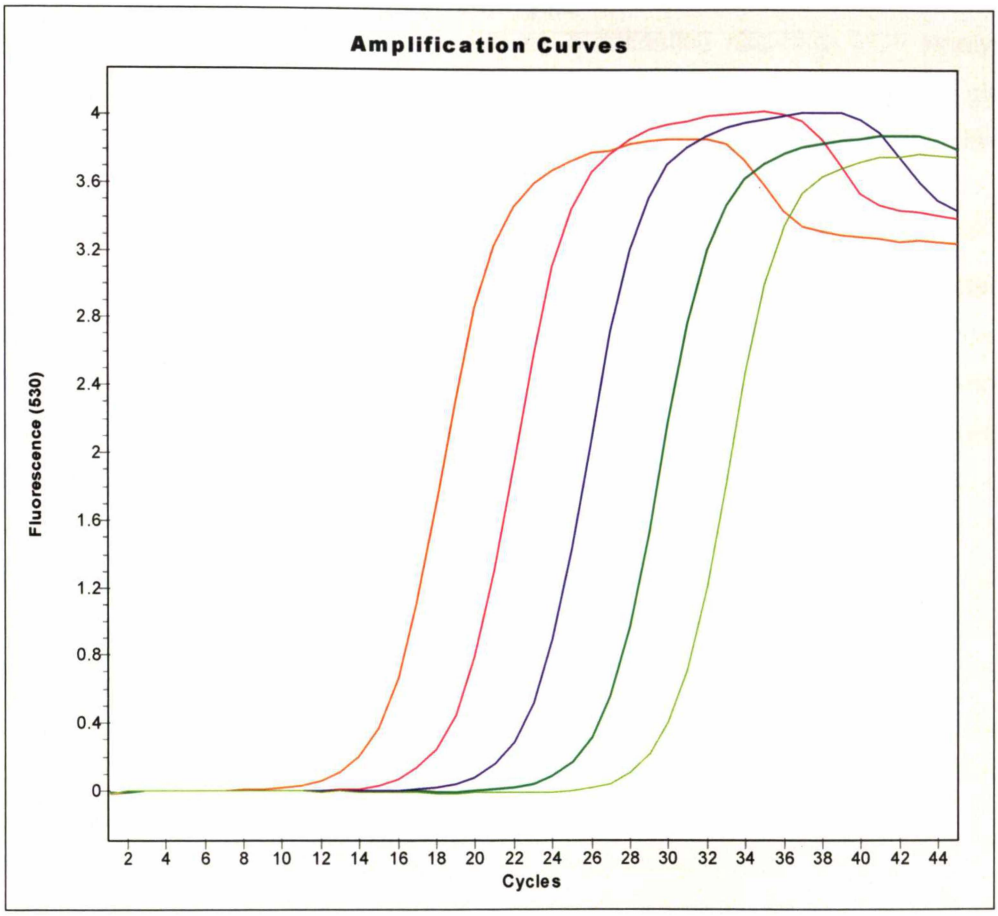
Tissue	**MAdCAM-1 mRNA relative to β-actin mRNA[a]**	**Mammary gland compared to Peyer's patches[b]**
Peyer's patch	70.380 ± 8.022	100.00
*Mammary gland**		
Pregnant	0.008 ± 0.001	0.011
Colostral	0.012 ± 0.008	0.017
Lactation	0.018 ± 0.006	0.026
Involution	0.015 ± 0.003	0.021

a. The level of MAdCAM-1 mRNA is relative to the level of β-actin mRNA. The results are reported as the group average ± SEM.
b. The comparison of the expression of MAdCAM-1 mRNA in the mammary gland compared with Peyer's patch is obtained by dividing the group average relative level of MAdCAM-1 mRNA in the mammary gland by the average relative level of MAdCAM-1 mRNA in the Peyer's patch samples.
* alveolar tissue from the mammary gland

Figure 3.10 Amplification curves and standard curve for MAdCAM-1 real-time PCR

A, Real-time PCR amplification curves for five 10-fold serial dilutions of MAdCAM-1 cDNA; cycle number plotted against fluorescence (530 nm) **B**, Representative standard curve of MAdCAM-1 used to determine the efficiency of the real-time PCR reaction required for the relative quantification of mRNA in unknown samples. Log concentration of the dilution of MAdCAM-1 cDNA is plotted against the cycle number at the crossing point.

A



B

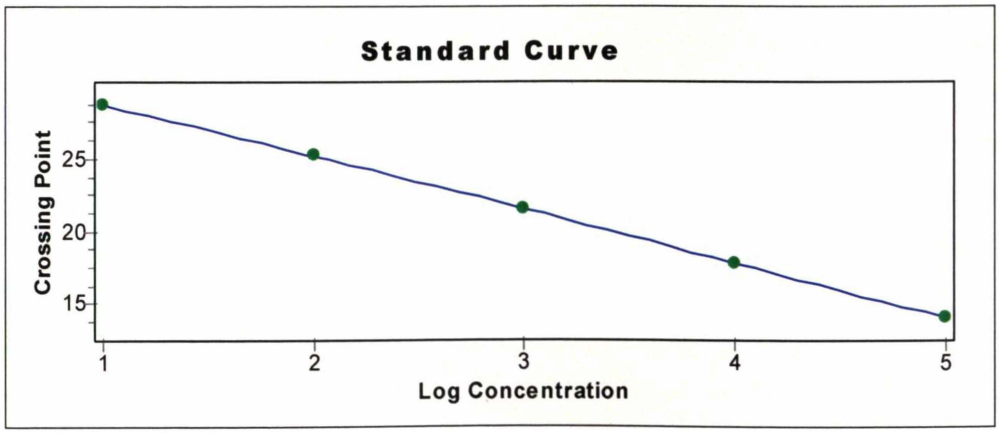
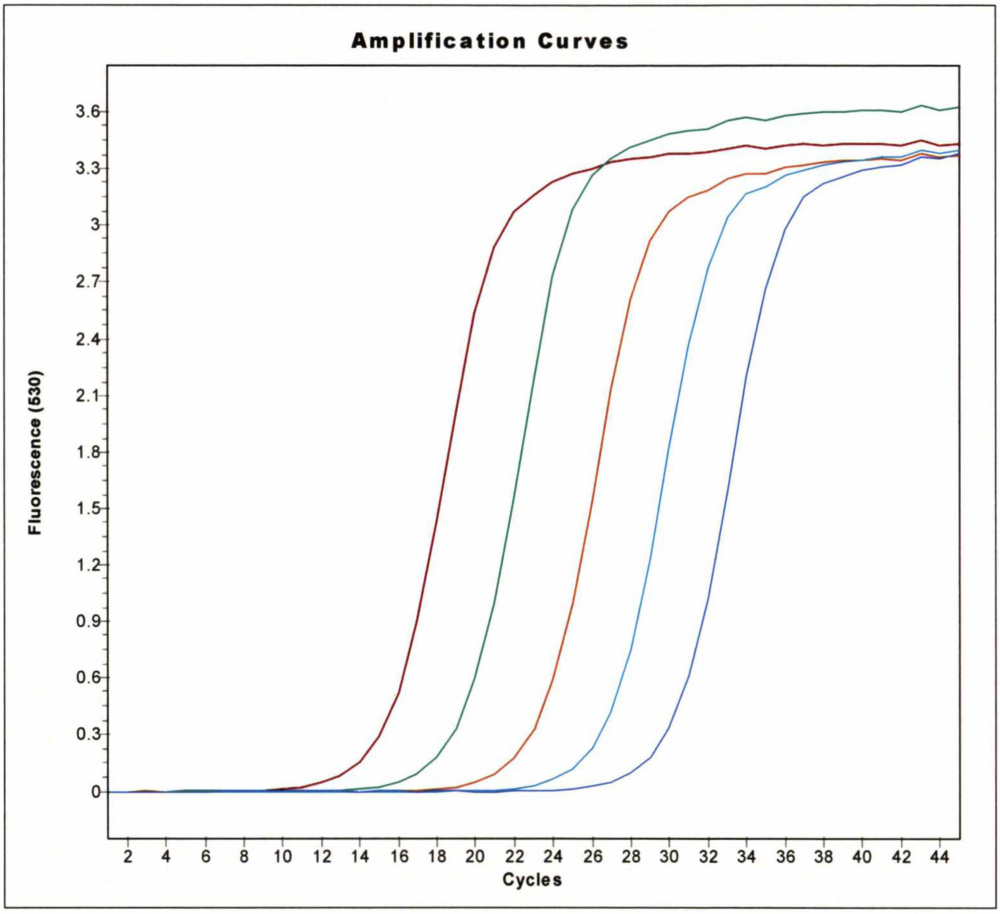


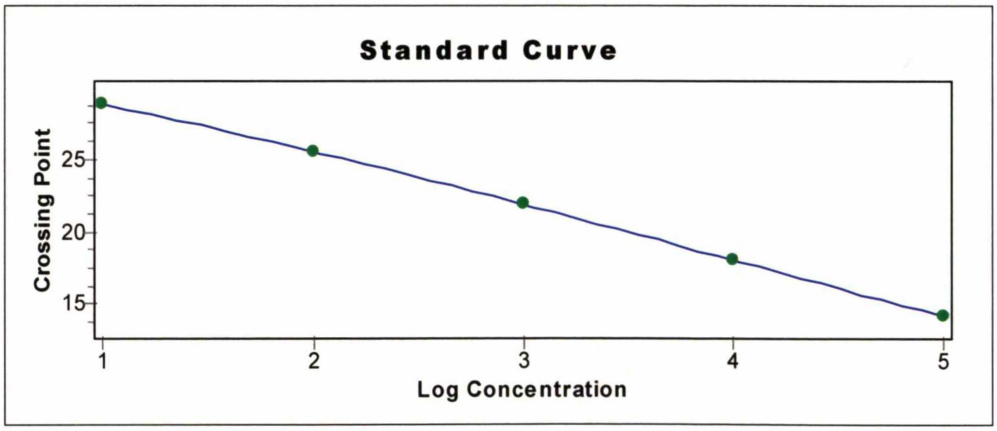
Figure 3.11 Amplification curves and standard curve for β-actin real-time PCR

A, Real-time PCR amplification curves for five 10-fold serial dilutions of β-actin cDNA, cycle number plotted against fluorescence (530 nm); **B**, Representative standard curve of β-actin used to determine the efficiency of the real-time PCR reaction required for the relative quantification of mRNA in unknown samples. Log concentration of the dilution of β-actin cDNA is plotted against the cycle number at the crossing point.

A



B



3.6 Discussion

The results presented in this chapter indicate that the vascular addressins involved in trafficking lymphocytes into the bovine mammary gland may differ from those described in the literature for the mouse. In contrast to the studies in the mouse where MAdCAM-1 expression was reported in the pregnant and lactating mammary gland (Tanneau *et al.,* 1999; Van der Feltz *et al.,* 2001), in the present study no MAdCAM-1 was detected in bovine mammary gland tissues by immunohistochemical analysis. This finding was substantiated by quantitative real-time PCR analysis. Although levels of MAdCAM-1 mRNA were detectable in the bovine mammary gland tissues, these levels were very low especially when compared with mRNA levels detected in bovine Peyer's patch tissues.

There may be several explanations as to why no MAdCAM-1 protein was detected in the bovine mammary gland tissues. The simple explanation is that there may be no MAdCAM-1 protein expressed in the bovine mammary gland. Although the ruminant vascular addressin profile is similar in most respects to other species (Abitorabi *et al.,* 1996; Rebelatto *et al.,* 2000), the mammary gland may diverge from this pattern. The ruminant mammary gland is unique in that IgG_1 is the predominant immunoglobulin in mammary secretions and IgA levels are low, whereas in other species, IgA is a predominant immunoglobulin in early milk (Butler, 1974). Therefore, it is feasible that the low levels of IgA observed in bovine mammary secretions may be because the cow does not have, or does not activate, the homing mechanisms, including the mucosal addressin MAdCAM-1, required to attract lymphocytes of mucosal origin to the gland.

Although MAdCAM-1 protein expression has been found in the mouse mammary gland in several studies (Streeter *et al.,* 1988a; Tanneau *et al.,* 1999; Van der Feltz *et al.,* 2001) another study failed to detect this addressin (Finke and Acha-Orbea, 2001). The Finke and Acha-Orbea study also investigated lymphocyte homing, however, there were major differences compared with the other murine studies: lymphocytes were isolated from the draining mammary lymph nodes of one animal and adoptively transferred to naïve recipients and their progress tracked. No reference was made to the physiological phase of the mammary gland in the recipients, although the histology shown suggests that the animals were in the lactation phase. In two of the other studies (Tanneau *et al.,* 1999; Van der Feltz *et al.,* 2001), it was noted that the MAdCAM-1 expression in the mammary gland decreased as lactation advanced, although it was still detectable, so this would not

fully explain the lack of detection in the Finke and Acha-Orbea study. One explanation for the differences observed for MAdCAM-1 expression between the murine studies may be due to the different primary antibodies the authors used to detect MAdCAM-1. MECA- 367, the monoclonal antibody used in the Tanneau *et al.* (1999) and Van der Feltz *et al.* (2001) studies is reported to bind the first domain of MAdCAM-1 and block MAdCAM-1-dependent binding *in vitro* (Streeter *et al.,* 1988a). MECA-89, the monoclonal antibody used in the Finke and Acha-Orbea (2001) study is reported to bind the second domain of MAdCAM-1 and does not block MAdCAM-1 dependent binding *in vitro* (Streeter *et al.,* 1988a).

In the experiments described in this chapter, the primary antibody chosen to detect MAdCAM-1 was shown to be very important. No positive binding to bovine Peyer's patch endothelial cells was observed using the monoclonal antibody specific for mouse MAdCAM-1 (MECA-367) and MECA-367 was shown subsequently to not cross-react with bovine MAdCAM-1. The monoclonal antibody for human MAdCAM-1 (7G11) positively stained endothelial cells in bovine Peyer's patches and mesenteric lymph nodes, but not in bovine mammary gland tissues. In humans, MAdCAM-1 has several isoforms (Leung *et al.,* 1997; Leung *et al.,* 1996; Sampaio *et al.,* 1995; Schiffer *et al.,* 1995) and this may also be the case in the cow. An antibody that recognises MAdCAM-1 in Peyer's patches and mesenteric lymph nodes may not necessarily detect MAdCAM-1 in the mammary gland if the proteins are expressed as different variants in the different tissues. On the other hand, in the mouse, MECA-367 was used to detect MAdCAM-1 in both the Peyer's patch and the mammary gland (Abitorabi *et al.,* 1996). In a bovine study, the human MAdCAM-1 monoclonal antibody 7G11 positively detected MAdCAM-1 in Peyer's patches, mesenteric lymph nodes and nasal/pharyngeal regions (Rebelatto *et al.,* 2000). Therefore, overall it would seem reasonable to assume that 7G11 would detect MAdCAM-1 in the bovine mammary gland if it were present.

Timing at which the tissue samples were taken from the bovine mammary gland is one possible explanation for the failure to detect MAdCAM-1. The cows were 33 – 34 weeks pregnant when sampled for the pregnant phase, which is during the last quarter of gestation. However, in comparison, mice sampled on Day 12 approximately half way through their pregnancy had significant MAdCAM-1 expression in the mammary gland (Tanneau *et al.,* 1999; Van der Feltz *et al.,* 2001). Peak MAdCAM-1 expression in the murine mammary gland was just prior to parturition and declined as lactation progressed. Therefore, it could be expected that if MAdCAM-1 was expressed in bovine mammary tissues, it would be apparent in

colostral phase tissues collected two days *post-partum*. However, no MAdCAM-1 was detected in these tissues.

To verify the finding that MAdCAM-1 was absent from the bovine mammary gland, RT-PCR studies were used to look at mRNA expression in these tissues. The methodology was difficult to establish because the DNA sequence for bovine MAdCAM-1 is unknown. The failure of initial primer sets was probably due to species differences in the MAdCAM-1 sequence. MAdCAM-1 homologues are very divergent, although sequence comparisons demonstrate that the first two immunoglobulin-like domains, at 57%, are the most highly conserved regions of these receptors (Briskin *et al.,* 1993). The mucin regions are the most highly divergent of the MAdCAM-1 homologues (Shyjan *et al.,* 1996). Because the monoclonal antibody for human MAdCAM-1 was more successful at recognising bovine MAdCAM-1, the sequence for human MAdCAM-1 was used initially for design of primer sets. These primers did not work, probably due to different species encoding proteins using different DNA bases. A successful primer set was finally designed from an EST DNA sequence located in the TIGR cattle database by blasting the database with the human MAdCAM-1 sequence. However, as these primers did not span intron-exon regions of the DNA sequence, there remained the potential to amplify contaminating genomic DNA in the mRNA samples.

MAdCAM-1 mRNA expression was detected in mammary gland alveolar tissues, although the resulting RT-PCR products only showed weak bands on agarose gel electrophoresis. Quantification by real-time PCR demonstrated that the level of MAdCAM-1 mRNA was 4.7 – 9.1 x 10^3- fold less in bovine mammary gland tissues than in bovine Peyer's patch tissues.

Together the failure to detect MAdCAM-1 in bovine mammary gland tissues by immunohistochemistry and the very low apparent expression of MAdCAM-1 mRNA in these tissues, strongly suggests that MAdCAM-1 is present at only low levels and is not involved in recruitment of lymphocytes to the bovine mammary gland.

In contrast to MAdCAM-1, the vascular addressin VCAM-1 was detected in mammary tissues, staining both larger venules that also stained positively for vWF and also the perimeter of alveoli, an area which did not stain positively for vWF. This finding was similar to that observed in the mouse by Finke and Acha-Orbea (2001). Tanneau *et al.* (1999) also observed staining of larger venules in mammary gland tissues by VCAM-1 but noted its absence on mammary gland capillaries (where extravasation

of lymphocytes occurs) and concluded that VCAM-1 was not involved in lymphocyte recruitment into the mammary gland. The same conclusion must be drawn for the VCAM-1 expression on the larger venules in the bovine mammary gland, that it is not involved in lymphocyte recruitment.

Of interest is the VCAM-1 staining observed on the periphery of the alveoli. This was not seen by Tanneau *et al.* (1999) but a similar observation was reported by Finke and Acha-Orbea (2001). This result suggests that VCAM-1 was expressed on either the epithelial cells or alveoli capillaries. However, no conclusion can be drawn either way from data to hand. The expression of VCAM-1 in these areas was phase dependent. It was evident in colostral tissues where all four individual animals gave positive results, whereas it was absent in the pregnant, lactation and involution phases. The pronounced expression in the colostral phase may suggest that VCAM-1 has a role in the active migration of cells into colostrum and thereby cells are transferred to the neonate for uptake prior to gut closure (Liebler-Tenorio *et al.,* 2002). Overall, the pattern of VCAM-1 expression suggests that the VCAM-1 is not constitutive to the mammary gland but activated by physiological events.

PNAd was not detected in the alveolar or cisternal mammary tissues but was present in the SMLN. This finding is similar to reports in literature reviews (Butcher *et al.,* 1999; Picker, 1994), that PNAd is largely expressed in peripheral lymph nodes, indicative that the role of PNAd recruiting naïve lymphocytes to inductive sites is similar in the ruminant to that of other species. In addition to PNAd, VCAM-1 expression was also found in the SMLN. This was not only on large venules similar to the VCAM-1 expression found in mammary tissues and also on small venules. This suggests that VCAM-1 may have a role in recruiting lymphocytes to the bovine SMLN. Dual expression of cell-adhesion molecules in lymph nodes has been reported by others (Csencsits *et al.,* 2002; Rebelatto *et al.,* 2000).

Analysis of the expression of lymphocyte cell-adhesion molecule receptors was limited to β7 integrin and L-selectin due to reagent availability. The absence of lymphocytes expressing β7 in any of the mammary tissues suggested that there were no lymphocytes present that would bind to MAdCAM-1. This finding was in direct contrast to the paper reporting high levels of this cell-adhesion molecule receptor on milk lymphocytes from dairy cows infected with Johnes disease (Harp *et al.,* 2004). Although this paper claimed to detect α4β7, the monoclonal antibody they used was only directed against the β7 chain of the molecule, similar to the monoclonal antibody

used in the studies in this thesis. Therefore, the lymphocytes in the milk could have been expressing either $\alpha 4\beta 7$ or $\alpha E\beta 7$, as $\alpha 4$ and αE alpha chains both associate with $\beta 7$. The $\alpha E\beta 7$ integrin mediates binding of T cells to epithelial cells via the cell-adhesion molecule, E-cadherin (Cepek *et al.,* 1993). T lymphocytes infiltrating inflamed epithelium, including salivary and mammary glands, express $\alpha E\beta 7$ (Cerf-Bensussan *et al.,* 1988; Kruschwitz *et al.,* 1991). In the mouse study by Tanneau *et al.* (1999), 60% of the T lymphocytes in the mammary gland were characterised as $\alpha E\beta 7$ positive and these were situated in the epithelium. It is very possible that the $\beta 7$-positive lymphocytes detected in the milk of the chronically diseased cows were actually $\alpha E\beta 7$, not $\alpha 4\beta 7$ as reported.

The cow does not naturally secrete high levels of IgA in mammary secretions. As discussed above, the cow may not express MAdCAM-1 under normal conditions, but if a mucosal IgA response is induced, then MAdCAM-1 may be detected in mammary gland tissues. The testing of the hypothesis that differences between the cow and other species for lymphocyte homing to the mammary gland may be due to differences in IgA secretion is described in Chapter Four, using animals immunised with our IgA protocol.

Chapter 4

Effects of intra-mammary immunisation on vascular addressins and lymphocytes in the bovine mammary gland

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4.1 Introduction

The work in Chapter Four examines the cellular changes that are induced in mammary gland tissues by our multi-site immunisation regimen. For the induction of IgA production in bovine mammary secretions using our procedure, pregnant animals are initially primed with antigen by intra-peritoneal (IP) and intra-muscular (IM) immunisation. Subsequently a booster dose is given four weeks later at the IP/IM sites, along with antigenic challenge to the mammary gland via intra-mammary (IMM) immunisation. A second IMM immunisation is given two weeks later. Based on published literature and our own studies, I hypothesised in the proposed mechanism of IgA production (Section 1.5.3.3) that IMM immunisation stimulates the mammary gland to recruit lymphocytes of gut-associated lymphoid tissue (mucosal) origin, generated by the IP/IM priming.

In the ruminant mammary gland the gut-mammary axis, where lymphocytes traffic between mucosal inductor and effector sites, appears to be under-developed (Watson and Kennedy, 1981). This is also indicated by the observation that IgA is a minor immunoglobulin in ruminant mammary secretions. Immunoglobulin secreted by the mammary gland into colostrum and milk can be derived from two sources: either it is transported into the mammary gland via the blood, or the immunoglobulin is locally produced in the gland. For example, in the untreated cow, IgG_1 is derived directly from the blood into the mammary gland by active transport (Dixon *et al.,* 1961). Earlier studies have indicated that the IgA antibody induced by our immunisation regimen is locally produced (Hodgkinson and Hodgkinson, 2003). When one side of the udder was immunised and not the other side, then increased levels of IgA were only found in the colostrum and milk from the immunised gland. This strongly suggests that IMM immunisation activates local mechanisms in the immunised gland to induce this response, for example, by recruitment of lymphocytes to the gland, because to have local IgA production in the mammary gland it is essential to have immune cells in the same locality.

In the mouse mammary gland, there is no requirement for antigenic stimulation to recruit lymphocytes. T-cell migration to the mammary gland has been shown to rise during late pregnancy and fall during lactation while plasma-cell numbers increase during late pregnancy and the rise continues to mid-lactation when it falls (Tanneau *et al.,* 1999; Weisz-Carrington *et al.,* 1977). These plasma cells are predominantly IgA. In the ruminant, studies investigating the lymphocyte populations in non-immunised mammary tissues have shown a variety of findings. The predominant

lymphocytes in the ruminant mammary gland are T cells and these are largely CD8 (Lee *et al.,* 1989). Some authors report low numbers of plasma cells in the ruminant mammary gland (Lee and Lascelles, 1970; Yurchak *et al.,* 1971). Some researchers found numerous plasma cells that increased gradually following involution to peak two weeks prior to parturition (Sordillo and Nickerson, 1988b). Cells positive for the three immunoglobulin isotypes, IgG, IgA and IgM, are all represented in the bovine mammary gland although IgG is the principal isotype (Sordillo and Nickerson, 1988b; Yurchak *et al.,* 1971).

When the ruminant mammary gland is infected by mastitis-inducing microorganisms or challenged with antigen, numbers of plasma cells in mammary tissues change. In infected quarters, plasma-cell numbers tend to be raised compared with non-infected quarters although the differences have only been found to be statistically significant for plasma-cells of the IgM isotype (Doymaz *et al.,* 1988; Sordillo and Nickerson, 1988b). Studies that infused antigen into the mammary gland of sheep demonstrated an increased number of plasma cells in the infused gland compared with the non-infused gland (Lee and Lascelles, 1970; Lee *et al.,* 1992). It was noted that CD8 cells were less numerous in the infused gland and there was an increase in CD4 cells that were closely associated with B cells (Lee *et al.,* 1992). In one study in ovine mammary glands, differential migration of T and B cells was observed in an acute inflammatory response and attributed to different regulation of the lymphocytes recruitment mechanisms (Meeusen *et al.,* 1991).

In the mouse, the recruitment of circulating lymphocytes into the mammary gland has been shown to involve adhesion molecules expressed on vascular endothelium (Tanneau *et al.,* 1999; Van der Feltz *et al.,* 2001). As described in the introduction to Chapter Three, a few studies have examined adhesion molecule receptor expression on bovine milk lymphocytes while expression of vascular addressins in the ruminant mammary gland has not been described. In Chapter Three, lymphocyte homing mechanisms in the bovine mammary gland of untreated animals were investigated. Although there was evidence of VCAM-1 and PNAd protein expression similar to that seen in other species, significantly no MAdCAM-1 protein expression was observed, in marked contrast to findings in the mouse. The work in this chapter builds on that observation and investigates whether there are changes to the protein expression of the vascular adhesion molecules in the bovine mammary gland when an IgA response is induced in the gland using our multi-site immunisation procedure.

4.2 Aim and approach

The aim of the work in this chapter was to characterise the effects induced in the mammary gland by our multi-site immunisation regimen and in particular the intra-mammary (IMM) immunisations. To achieve this, pregnant cows were immunised with only one side of the udder receiving the IMM immunisations. Both short-term and long-term effects in the mammary gland were investigated. The short-term effects being those observed seven days after the 2nd IMM immunisation in the pregnant animal and the long-term effects being those observed approximately 12 weeks after the 2nd IMM immunisation, in the lactating animal. The long-term effects were studied because it had been noted that cows immunised using our regimen continued to secrete elevated levels of IgA for an extended time into lactation (unpublished data), many months after they had received the last immunisation.

The two main areas of investigation were:

- to identify the effects of the IMM immunisation on the expression of the vascular addressins, MAdCAM-1, VCAM-1 and PNAd
- to identify the effects of IMM immunisation on T and B cell populations

The first objective of this work was to immunise eight pregnant cows using our immunisation regimen and collect mammary tissue samples. In these studies only one side of the udder was immunised, with the other non-immunised side acting as a control. Alveolar and cisternal tissues and supramammary lymph nodes (SMLN) were collected at two different time points following the immunisation schedule:

- seven days following the 2nd IMM immunisation, in pregnant cows
- approximately 12 weeks after the 2nd IMM immunisation, in lactating cows

from both the immunised and control sides of the udder. To test the response to immunisation, samples of mammary secretion were also collected from the immunised animals and analysed for titres of antigen-specific antibody.

The second objective of this work was to develop immunohistochemical methodology to measure plasma cells specific for the following immunoglobulin isotypes:

- IgA
- IgG
- IgM

The third objective of this work was to use immunohistochemical methodologies to analyse the collected tissue samples for:

- vascular addressin expression on endothelial cells
- vascular addressin receptor expression on lymphocytes
- numbers of T cells
- numbers of isotype-specific plasma cells

In addition, to verify and quantify the expression of MAdCAM-1 mRNA in the mammary glands, alveolar tissue samples were further analysed by real-time PCR, using methods developed in Chapter Three.

4.3 Materials

4.3.1 *Antibodies*

Primary antibodies used in immunohistochemical methods are listed in Table 3.1 and Table 4.1.

Table 4.1 Primary antibodies for immunohistochemistry

Target antigen	Host	Cellular expression	Product code	Supplier
Bovine IgA	Rabbit	IgA-positive cells	A10-108A	Bethyl
Bovine IgG	Sheep	IgG-positive cells	A10-118P	Bethyl
Bovine IgM	Rabbit	IgM-positive cells	A10-100A7	Bethyl

Reagents used for the detection of the primary antibodies are listed in Table 3.2. The antibody specific for bovine IgG was directly conjugated to horse-radish peroxidase and did not require a secondary antibody.

4.4 Methods

4.4.1 *Immunisation procedure*

Eight healthy multiparous pregnant cows of mixed Friesian and Jersey breeds were immunised using our multi-site regimen (Hodgkinson and Hodgkinson, 2003) as outlined in Section 2.2.1. The immunisation schedule is outlined in Table 4.2.

Table 4.2 Routes and timing for the multi-site immunisation regimen

Immunisation	Route	Weeks
I	IP/IM	0
II	IP/IM/IMM	4
III	IMM	6
IV	IP/IM	7

IP - intra-peritoneal
IM - intra-muscular
IMM - intra-mammary

The initial immunisation was 13 weeks before the group's averaged estimated calving date. For these studies, both quarters of the right side of the udder were immunised. The two quarters on the left side of the udder were not immunised and acted as a control for each individual animal.

4.4.2 *Sample collection and preparation*

Four pregnant cows were sacrificed seven days following the 2nd IMM immunisation, approximately five weeks prior to calving. The four lactating cows were sacrificed approximately 12 weeks after the 2nd IMM immunisation, five weeks following calving. Cows were slaughtered at the Ruakura abattoir and mammary glands excised for dissection. Tissue samples were collected from the immunised side of the udder and the control (non-immunised) side. The tissues were sampled from alveolar and cisternal areas, and the supramammary lymph nodes (SMLN).

Tissue samples for immunohistochemical analysis were processed and frozen as per the protocol outlined in Section 2.2.2.1 and serial sections were cut from these tissue samples as described in Section 2.2.3.1 and Section 2.2.3.2. Alveolar tissue samples for plasma-cell analysis were processed differently, as described in Section 4.4.3.1 below. Tissues samples for real-time PCR were processed as per the protocol outlined in Section 2.2.2.1.

Samples of (dry) mammary secretions were collected from the immunised and control glands of the pregnant cows on the day prior to slaughter, six days after the 2nd IMM immunisation. For the lactating cows, mammary secretions were collected from the whole udder on the first and seventh day after calving. In addition, during established lactation, milk samples were collected separately from the immunised and control quarters of these cows on two occasions, three and five weeks after calving. Samples were processed as per the protocol outlined in Section 2.2.2.2 and stored at -20°C.

4.4.3 *Immunohistochemistry*

4.4.3.1 Development of immunohistochemical protocol for plasma cells

In the Chapter Three the development of protocols for the measurement of plasma cells in frozen tissue sections had been unsuccessful. This was due to the binding of the primary antibody reagent to soluble immunoglobulin present in the extra-cellular

regions of the tissues. This produced a high non-specific background effect. To counteract this problem, alveolar tissue collected from the immunised animals was processed in two different ways and compared by immunohistochemistry to determine the method that produced the lowest non-specific binding effects.

Method one: Tissue samples were cut into 10 x 10 x 2-3 mm pieces, placed into Tissue-Tek Unicassettes (Bayer Diagnostics) and submerged in 95% (v/v) ethanol for 24 h at 4°C.

Method two (Brandtzaeg P, personal communication): Tissue samples were cut into 10 x 10 x 2-3 mm pieces, placed into Tissue-Tek Unicassettes (Bayer Diagnostics) and submerged in phosphate buffered saline (PBS) containing 0.01% (w/v) sodium azide for 24 h at 4°C, with gentle mixing. Cassettes were then transferred to a solution of 95% (v/v) ethanol for 24 h at 4°C.

The fixed tissues from both methods were processed overnight in a Leica JungTP1050 fully enclosed vacuum tissue processor (Cambridge Instruments) following the protocol in Table 2.3. The tissues were then embedded in paraffin blocks, as described in Section 2.2.2.1.

Paraffin-embedded tissues were sectioned and mounted on slides, as described in Section 2.2.3.2. Prior to immunostaining, the slides were deparaffinised and rehydrated, as outlined in Table 2.4. Tissue sections were immunostained according to the general protocol described in Section 2.2.3.3. Primary antibodies specific for bovine IgA, IgG and IgM were titrated to determine the optimal concentration for use, using a dilution series of 1:100, 1:500, 1:1 000, 1:5 000, and 1:10 000. The optimal concentration for IgA- and IgM-specific antibodies was found to be 1:500 and for IgG it was 1:5 000.

A comparison between the two different processing methods determined that the method that washed the alveolar tissue samples in PBS for 24 h prior to fixing in ethanol gave the cleanest result with very little non-specific binding apparent. Tissue samples processed by this method were used for immunohistochemical analysis.

4.4.3.2 Immunohistochemical analysis

Immunostaining was performed on serial sections of frozen alveolar, cisternal and SMLN tissues collected from the immunised and control mammary glands of the four

pregnant cows seven days following the 2nd IMM immunisation and the four lactating cows five weeks after calving. Antibodies specific for the vascular addressins MAdCAM-1, VCAM-1 and PNAd were used to identify their presence and distribution in the mammary gland. Vascular endothelial cells were identified by immunostaining with an antibody specific for von Willebrand factor (vWf), an antigen expressed on vascular endothelium.

A semi-quantitative subjective measure of vascular addressin expression was made by a visual comparison of the vascular endothelium stained by the addressins and the vascular endothelium stained for vWf. The level of expression of vascular addressins was expressed as a proportion of venules stained for vWf that also stained for addressins. Expression was graded from 0 – 4: 0 = no addressin expression; 1 = 1 – 25% venules stained with addressin that stained with vWf; 2 = 25 – 50%; 3 = 50 – 75%; 4 = 75 – 100%.

Serial sections of the alveolar and cisternal frozen tissues analysed for vascular addressins were also immunostained with the following antibodies to identify the lymphocyte subsets present in the mammary gland:

- CD3, CD8 – T-cell subsets
- BB2 – pan B-cell population
- CD62L – counter receptor expressed on lymphocytes for PNAd
- β7 – counter receptor expressed on lymphocytes for MAdCAM-1
  (NB: β7 detection does not distinguish between α4β7 and αEβ7)
- There was no analysis for CD4 because the assay could not be validated

Serial sections of ethanol-fixed paraffin-embedded alveolar tissues collected at both time points from the immunised and control mammary glands of the experimental animals were immunostained with the following antibodies to identify the isotype-specific plasma cells present in the mammary gland:

- IgA
- IgG
- IgM

Data are expressed as the average number of cells (± SEM) counted in ten random microscope fields at 200 times magnification.

4.4.4 *Analysis for specific anti-*C. albicans *IgA in secretions*

Samples of mammary secretions collected from both the pregnant and lactating animals were assayed by ELISA to determine the titres of specific anti-*C. albicans* IgA, as per the protocol outlined in Section 2.2.6.1. Results were reported as titre units x 10^3 (kTU) of antibody.

4.4.5 *MAdCAM-1 quantitative real-time PCR*

The mRNA was extracted and purified from the alveolar tissues of the immunised and control sides of the udder of the four pregnant cows and the four lactating cows following the protocol outlined in Section 2.2.4.1. In addition, four samples of bovine Peyer's patch tissues were similarly treated. First strand synthesis of cDNA from the purified mRNA was performed using the protocol outlined in Section 2.2.4.2.

To quantify the mRNA levels of MAdCAM-1 and β-actin in unknown samples, real-time PCR was then performed using the protocol outlined in Section 2.2.4.6 with the primers listed in Table 3.3 and annealing temperatures of 65°C and 60°C for MAdCAM-1 and β-actin, respectively. Five 10-fold serial dilutions of PCR products for MAdCAM-1 and β-actin (see Section 3.4.4.1) were also analysed by real-time PCR to determine the efficiency of the reaction and to calculate the relative quantity of mRNA in unknown samples, as described in Section 2.2.4.6. The mRNA levels of MAdCAM-1 in unknown samples were reported relative to the mRNA levels of β-actin. This adjusted for any variation in the quantity of total mRNA used for each reaction. The relative expression levels of MAdCAM-1 in the different alveolar mammary tissue samples and Peyer's patch tissues were compared.

4.5 Results

4.5.1 *Immunohistochemical characterisation of vascular addressin expression in the immunised mammary gland*

The immunohistochemical characterisation of MAdCAM-1, VCAM-1 and PNAd protein expression in immunised mammary gland samples is described in the sections below. Alveolar, cisternal and SMLN tissues were sampled from immunised and control sides of the udder of the four pregnant cows (seven days following the 2nd IMM immunisation) and the four lactating cows (five weeks *post-partum*).

4.5.1.1 MAdCAM-1

No MAdCAM-1 expression was detected on the vascular endothelium in any of the mammary tissue samples tested. No difference was observed between the immunised and control mammary glands in the samples from either the pregnant or the lactating animals. Bovine Peyer's patch tissue, included as positive control tissue, always immunostained positively for MAdCAM-1.

4.5.1.2 VCAM-1

There was a variation in the numbers of animals in each of the groups that expressed VCAM-1 in mammary tissues and this ranged from 2/4 – 4/4 (see Table 4.3). However, there was no significant difference between the different types of tissues for the number of animals that expressed VCAM-1.

Table 4.3 *VCAM-1* expression in the mammary gland of immunised cows

The number of immunised cows expressing *VCAM-1* in alveolar, cisternal and supramammary lymph node tissues: comparison of immunised and control tissues collected at two time points following the 2nd intra-mammary immunisation.

Interval after 2nd IMM immunisation	**Mammary gland treatment**	**Proportion of animals per group expressing VCAM-1**		
		Alveolar	*Cisternal*	*SMLN*
7 days	*Immunised*	4/4	3/4	4/4
7 days	*Control*	3/4	3/4	3/4
~ 12 weeks	*Immunised*	4/4	4/4	2/4
~ 12 weeks	*Control*	2/4	2/4	2/4

SMLN - supramammary lymph node

The proportion of VCAM-1 staining compared with vWf staining is shown in Figure 4.1 for all tissues collected seven days after the 2nd IMM immunisation from the pregnant group and five weeks after calving from the lactating group. Expression was graded as described in Section 4.4.3.2.

Positive staining for VCAM-1 in the alveolar and cisternal mammary tissue samples was on larger venules that were also positively stained for vWf. In these samples, VCAM-1 was expressed on 25 – 100% of the vascular endothelium that also stained positive for vWf. The positive staining for VCAM-1 around alveoli, reported in Chapter Three in the colostral phase group of the untreated cows, was not apparent in any of the tissues samples collected from the immunised pregnant cows seven days or the immunised lactating cows, approximately 12 weeks following the 2nd IMM immunisation.

VCAM-1 expression was detected in SMLN tissue samples on both large and small venules that also stained positively for vWf. In SMLN tissue samples, VCAM-1 was expressed on 25 – 75% of the vascular endothelium that also stained positive for vWf.

There was no significant statistical difference in the expression of VCAM-1 for any of the tissue types when the immunised and control mammary glands were compared.

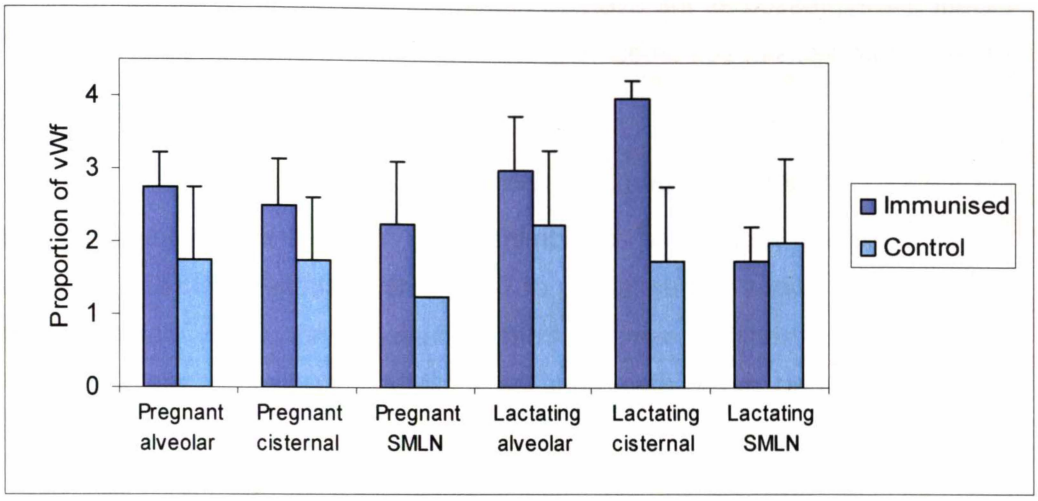
4.5.1.3 PNAd

No PNAd was detectable in alveolar or cisternal tissue samples collected seven days after the 2nd IMM immunisation from the pregnant group and five weeks *post-partum* later from the lactating group, irrespective of whether or not the gland was immunised. This result was similar to that found for the untreated cows at different physiological stages of the mammary gland, in Chapter Three.

Positive staining was seen in the SMLN samples of all the cows from both the immunised and control sides of the udder collected at the two time points. PNAd expression ranged from 25 – 75% of the endothelial venules that were also positively stained for vWf. The proportion of PNAd staining compared with vWf staining is shown in Figure 4.2 for the different groups. Expression was graded as described in Section 4.4.3.2. There was no significant difference for PNAd staining in SMLN tissues between the immunised and control sides of the udder or between the pregnant and lactating groups.

Figure 4.1 Proportion of endothelial venules in the mammary gland tissues of immunised cows that stained positive for both VCAM-1 and vWf

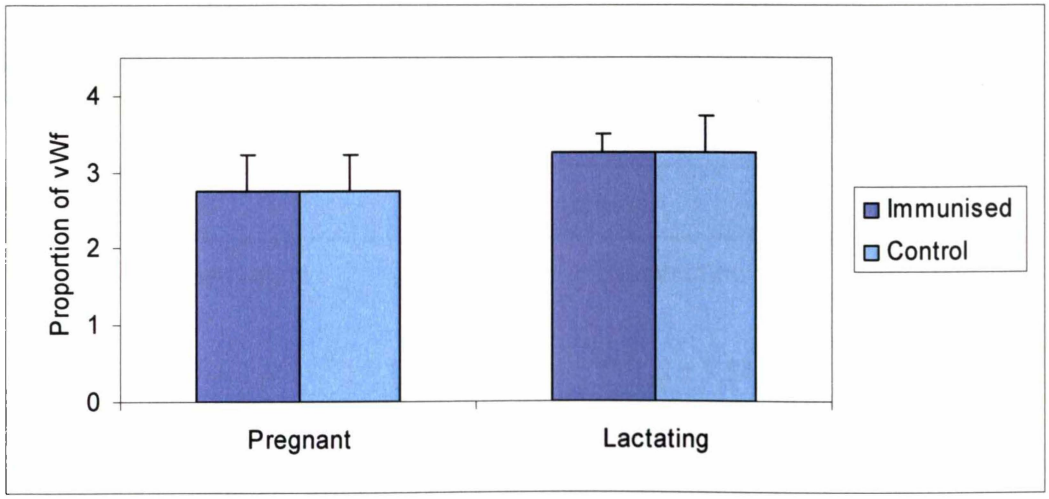
Tissues were collected from the immunised and control mammary glands seven days after the 2nd IMM immunisation from the pregnant animals and five weeks *post-partum* from the lactating animals. Expression was graded from 0 - 4: 0 = no VCAM-1 staining; 1 = 1 - 25% venules stained with VCAM-1 that stained with vWf; 2 = 25 - 50%; 3 = 50 - 75%; 4 = 75 - 100%. (Group average ± SEM, n = 4)



SMLN - supramammary lymph node

Figure 4.2 Proportion of endothelial venules in the supramammary lymph node of immunised cows that stained positive for both PNAd and vWf

Tissues were collected from the immunised and control mammary glands seven days after the 2nd IMM immunisation from the pregnant animals and five weeks *post-partum* from the lactating animals. Expression was graded from 0 - 4: 0 = no PNAd staining; 1 = 1 - 25% venules stained with PNAd that stained with vWf; 2 = 25 - 50%; 3 = 50 - 75%; 4 = 75 - 100%. (Group average ± SEM, n = 4)



4.5.2 *Immunohistochemical characterisation of lymphocytes in the immunised mammary gland*

Alveolar and cisternal tissue samples of the immunised and control mammary glands of the four pregnant cows and the four lactating cows were also analysed by immunohistochemistry for the lymphocytes subsets: CD3, CD8, BB2, CD62L and β7. Data are expressed as the average number of cells (± SEM) counted in ten random microscope fields at 200 times magnification.

4.5.2.1 CD3- and CD8-positive cells

T lymphocytes were found predominantly in the interstitial spaces between the alveoli. Illustrations of the staining of T-lymphocyte subsets CD3 and CD8, in frozen alveolar tissue sections sampled from immunised and control mammary glands of pregnant animals seven days after the 2nd IMM immunisation, are shown in Figure 4.3.

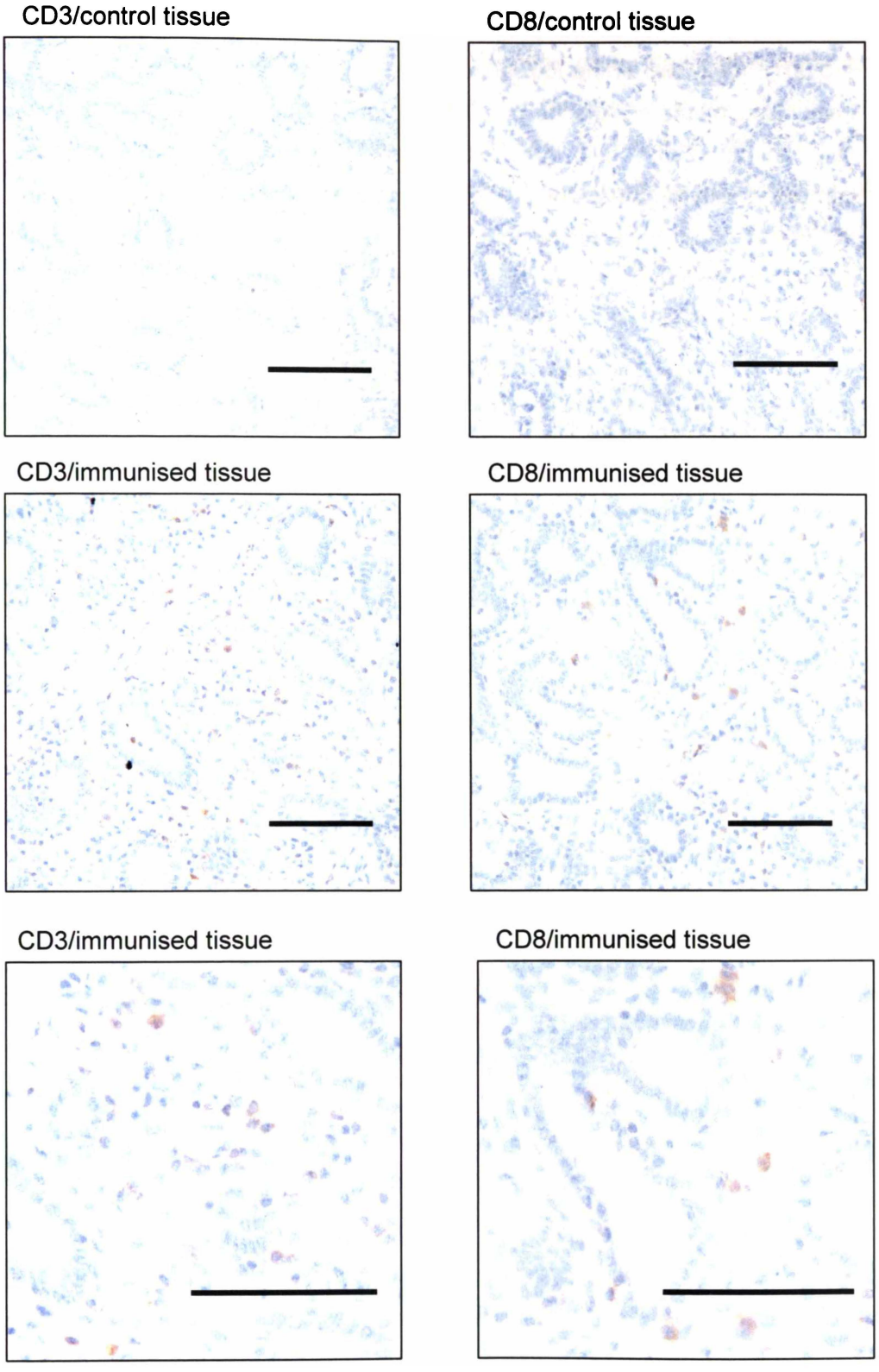
The average numbers of CD3- and CD8-positive cells in the alveolar and cisternal tissues from the immunised and control mammary glands, collected seven days after the 2nd IMM immunisation, are shown in Table 4.4. Group average numbers of both CD3- and CD8–positive cells were higher in the immunised mammary tissues compared with the control tissues except in the cisternal tissues where CD8-positive cell numbers for the control and immunised tissues were similar.

Table 4.4 Comparison of T-cell numbers in immunised and control mammary glands, seven days after the 2nd intra-mammary immunisation

Group T-cell numbers (average ± SEM, n = 4): cell counts from 10 random microscope fields of tissue section from each sample, at x 200 magnification. Significant statistical differences between immunised and control tissues are marked (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

	Alveolar tissue		**Cisternal tissue**	
	Immunised	*Control*	*Immunised*	*Control*
CD3	12.7 ± 2.1***	2.7 ± 0.8	8.9 ± 2.1*	3.7 ± 1.1
CD8	9.8 ± 2.5**	2.5 ± 0.6	2.6 ± 0.7	3.8 ± 0.9

Figure 4.3 Representative illustration of immunohistochemical staining of CD3- and CD8-positive cells in frozen alveolar bovine mammary gland tissues, seven days after the 2nd intra-mammary immunisation

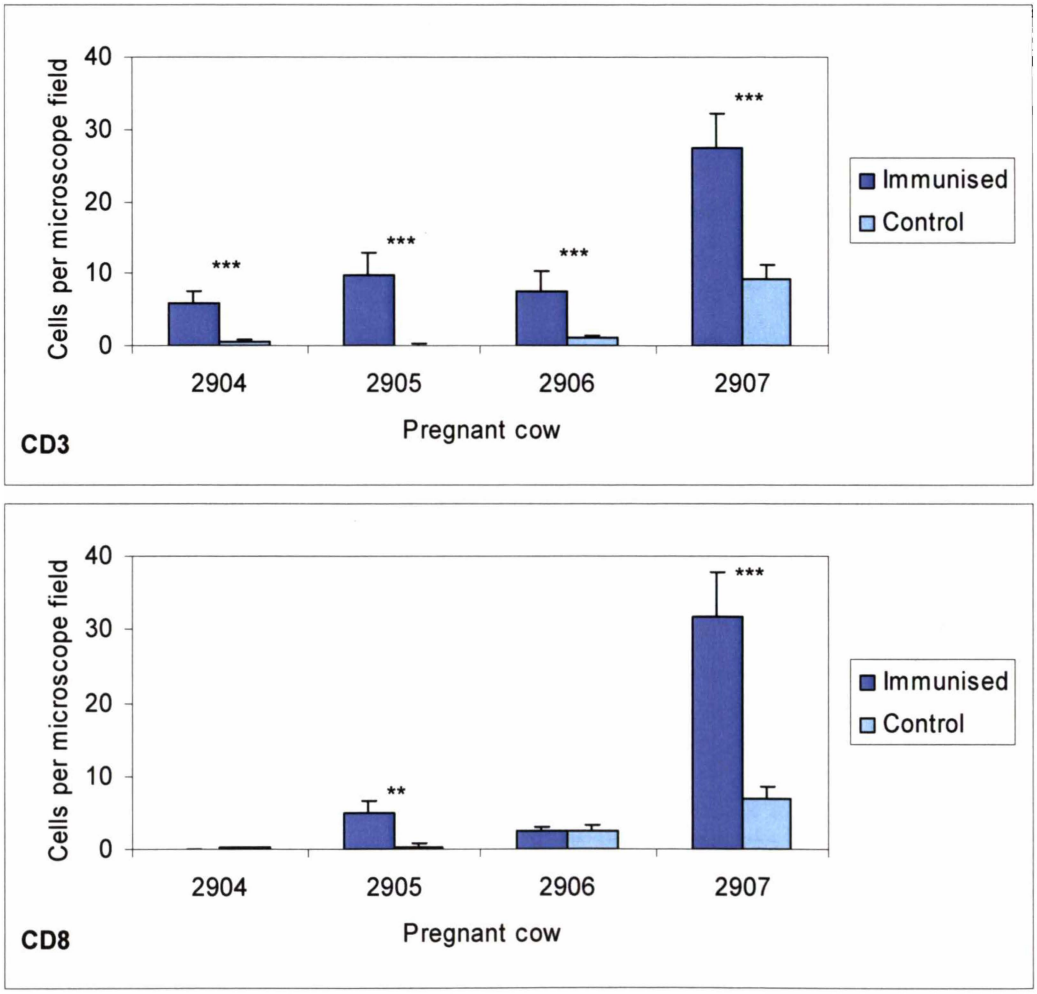


Black solid bar is 100μm

T-cell data for the alveolar mammary tissue samples from individual pregnant animals are shown in Figure 4.4. There were higher numbers of CD3-positive cell numbers observed in the immunised mammary glands compared with the control mammary glands for all four pregnant animals and this was statistically significant ($p < 0.001$). Only two of the four animals had higher numbers of CD8-positive cells in immunised mammary glands compared with control mammary glands ($p < 0.01$). One animal (2907) had three-fold higher T-cell numbers in immunised tissue compared with the other animals in the group and the numbers of T cells in control tissues of 2907 were similar to the numbers in immunised tissues for the other animals. This difference was observed for both CD3- and CD8-positive cells.

Figure 4.4 Analysis of immunohistochemical staining of CD3- and CD8-positive cells in frozen alveolar bovine mammary tissues, seven days after the 2nd intra-mammary immunisation

Cell counts from random microscope fields of tissue sections from individual animals (Average ± SEM; n = 10; x 200 magnification). Significant statistical differences between the immunised and control glands are marked (** $p < 0.01$; *** $p < 0.001$).



The numbers of CD3- and CD8-positive cells in the tissues collected from the lactation group (five weeks *post-partum*) tended to be lower than that found in the pregnant animals. The average was 1 - 2 cells per microscope field in the alveolar and cisternal tissue sections for three of the animals. Similar to the finding in the pregnant group, one animal (2902) in the lactation group had higher T-cell counts than the other animals but this was only in the immunised alveolar tissue sample. For this animal, the average number of cells per microscope field was 13.0 ± 2.1 and 10.6 ± 2.2 for CD3 and CD8, respectively. In this animal, comparison of the immunised and control alveolar tissue sample for CD3 and CD8 showed a significant difference ($p < 0.001$). There was no significant difference between the immunised and control tissue samples for the other animals in the lactation group (data not shown).

4.5.2.2 CD62L-positive cells

Table 4.5 shows a comparison of the group average numbers of CD62L-positive cells (± SEM) in immunised and control mammary glands collected from the pregnant cows seven days after the 2nd IMM immunisation and the lactating cows five weeks after calving.

Table 4.5 Comparison of CD62L-cell numbers in immunised and control mammary glands at intervals following the 2nd intra-mammary immunisation

Group CD62L-cell numbers (average ± SEM, n = 4): cell counts from 10 random microscope fields of tissue section from each sample, at x 200 magnification.

Interval after 2nd IMM immunisation	**Alveolar tissue**		**Cisternal tissue**	
	Immunised	*Control*	*Immunised*	*Control*
7 days	1.03 ± 0.37	0.13 ± 0.05	0.60 ± 0.17	0.65 ± 0.38
~ 12 weeks	2.25 ± 0.18	0.83 ± 0.95	0.13 ± 0.20	0.40 ± 0.07

Low numbers of CD62L-positive cells were detected in all alveolar and cisternal mammary gland tissues and there was no significant difference between immunised and control tissues at the two time points sampled.

4.5.2.3 β7-positive cells

There was no evidence of cells staining for β7 in any of the alveolar and cisternal mammary tissue sections at the two different time points, irrespective of whether or not the gland had been immunised. There was positive staining of cells with β7 antibody in all samples of Peyer's patch tissues, used as an assay positive control.

4.5.2.4 BB2-positive cells

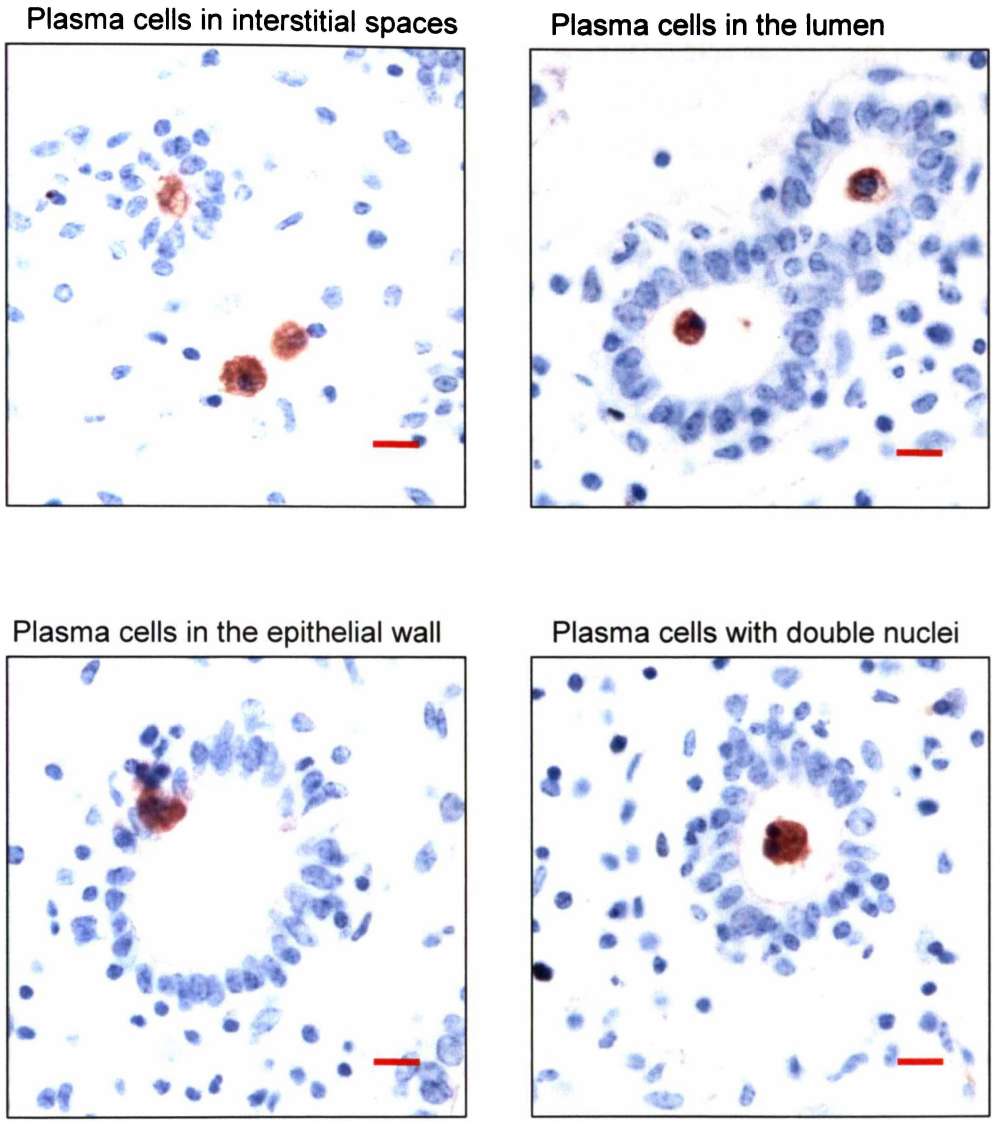
There was no observation of cells that stained positively for BB2 in any of the alveolar and cisternal mammary tissue sections at the two different time points, irrespective of whether or not the gland had been immunised. Cells in Peyer's patch tissues, used as an assay positive control, always stained positive with BB2 antibody.

4.5.3 *Immunohistochemical characterisation of plasma cells in the immunised mammary gland*

Alveolar tissue samples collected from immunised and control mammary glands of the four pregnant cows seven days after the 2nd IMM immunisation and the four lactating cows five weeks *post-partum* were analysed by immunohistochemical methods for the plasma-cell isotypes: IgA, IgM and IgG. Plasma cells tended to be located in the interstitial spaces between alveoli although many were also located in the alveolar or ductal lumen. Positively staining cells were also detected in the alveoli or ductal epithelium wall.

A small number of the plasma cells were observed to have double nuclei. These cells with double nuclei stained predominantly for IgA and IgM. Illustrations of IgA-positive cells in different locations in alveolar tissue of the immunised mammary gland seven days after the 2nd IMM immunisation are shown in Figure 4.5.

Figure 4.5 Representative illustration of immunohistochemical staining of IgA-positive cells (in various locations) in ethanol-fixed paraffin-embedded alveolar bovine mammary gland tissues, seven days after the 2nd intra-mammary immunisation

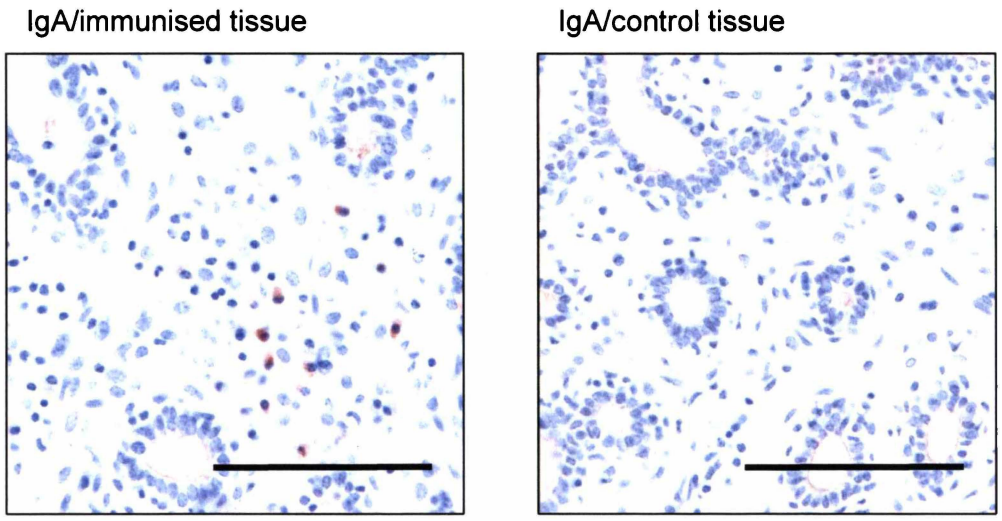


Red solid bar is 10μm

4.5.3.1 IgA-positive plasma cells

IgA-positive cells were detected in the tissues of the immunised mammary glands of the pregnant animals. Illustrations of ethanol-fixed paraffin-embedded alveolar tissue sections from immunised and control mammary glands, stained with specific bovine IgA antibody, are shown are Figure 4.6. The group average for IgA-positive cells per random microscope field (200 x magnification) was 5.2 ± 0.8 in the immunised glands compared with 1.3 ± 0.3 IgA-positive cells in the control glands. The variation for the numbers of IgA-positive cells between the individual animals can be seen in Figure 4.7. The average number of IgA-positive cells in the immunised glands was significantly higher compared with the control glands for three of the four animals in the pregnant group (2907, $p < 0.01$; 2904 and 2905, $p < 0.001$).

Figure 4.6 Representative illustration of immunohistochemical staining of IgA-positive cells in ethanol-fixed paraffin-embedded alveolar bovine tissues from immunised and control mammary glands, seven days after the 2nd intra-mammary immunisation

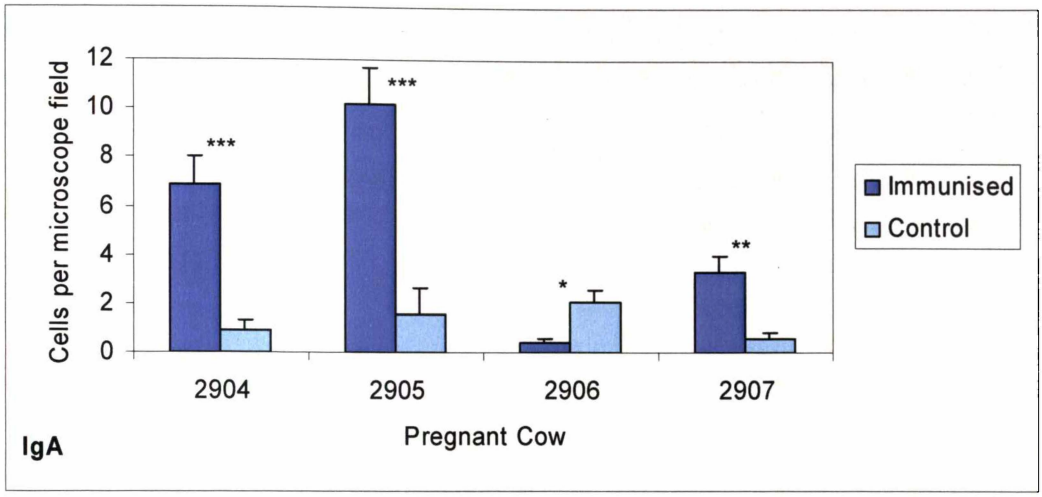


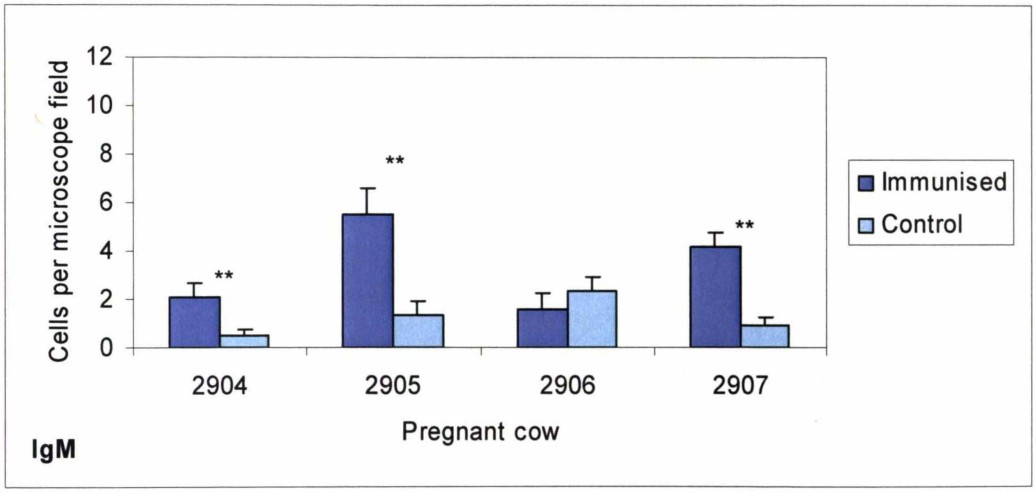
Black solid bar is 100μm

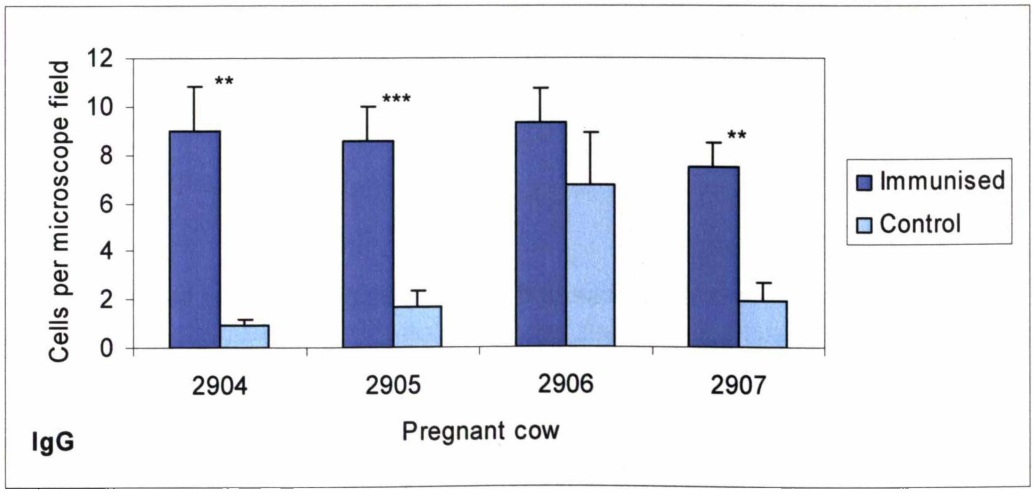
IgA-positive cells in ethanol-fixed paraffin-embedded alveolar tissue sections of the lactation group, collected five weeks *post-partum*, were undetectable in both immunised and control glands.

Figure 4.7 Analysis of immunohistochemical staining of IgA-, IgM- and IgG-positive cells in ethanol-fixed paraffin-embedded alveolar bovine mammary gland tissue, seven days after the 2nd intra-mammary immunisation

Cell counts from random microscope fields for individual animals (Average ± SEM; n = 10; magnification x 200). Statistically significant differences between the immunised and control glands are marked (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)



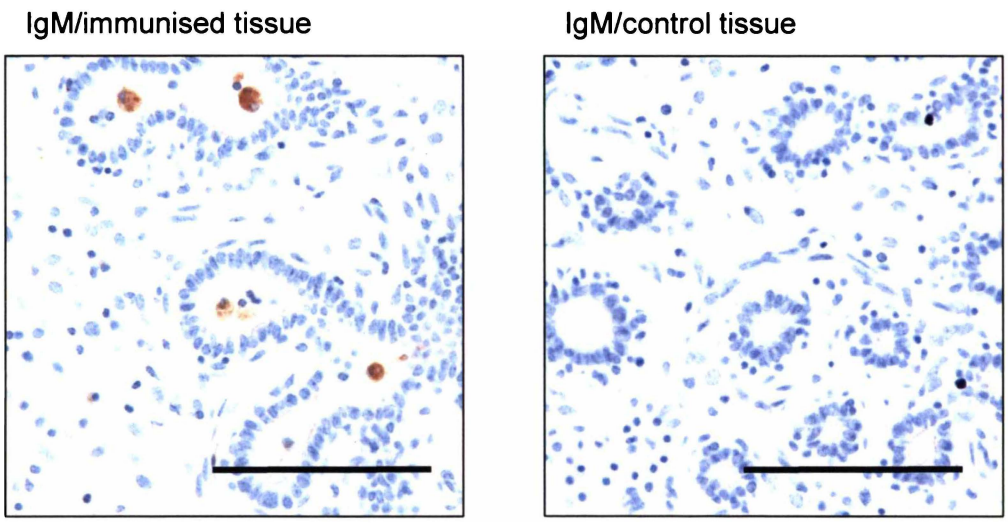




4.5.3.2 IgM-positive plasma cells

Illustrations of ethanol-fixed paraffin-embedded alveolar tissue sections from immunised and control mammary glands of the pregnant animals, stained with specific bovine IgM antibody, are shown in Figure 4.8. In alveolar tissues collected from the four pregnant animals seven days after the 2nd IMM immunisation, the group average IgM-positive cells per random microscope field (200 x magnification) was 3.4 ± 0.4 in the immunised glands and 1.3 ± 0.3 in the control glands. The variation for the individual animals showed a similar pattern to that observed for the IgA-positive cells (Figure 4.7). The average numbers of IgM-positive cells in the immunised glands were significantly higher than control glands for three animals (2904, 2905 and 2907) in the pregnant group ($p < 0.01$).

Figure 4.8 Representative illustration of immunohistochemical staining of IgM-positive cells in ethanol-fixed paraffin-embedded alveolar bovine tissues from immunised and control mammary glands, seven days after the 2nd intra-mammary immunisation



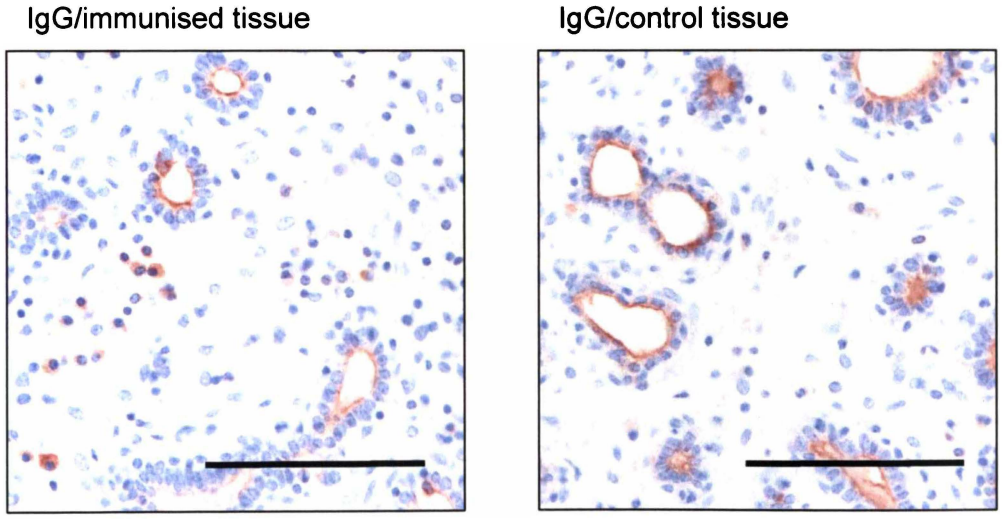
Black solid bar is 100μm

Few IgM-positive cells were observed in the alveolar mammary tissues collected from the lactation group five weeks *post-partum*. One or two cells were detectable in a few of the microscope fields, but generally no IgM-positive cells were visible. There was no apparent difference in the average numbers of IgM-positive cells between the immunised and control glands in the lactation group.

4.5.3.3 IgG-positive plasma cells

In the alveolar tissues collected from the four pregnant animals seven days after the 2nd IMM immunisation, the group average IgG-positive cells per random microscope field (200 x magnification) was 8.6 ± 0.7 for the immunised glands and 2.8 ± 0.7 for the control glands. Illustrations of ethanol-fixed paraffin-embedded alveolar tissue sections from immunised and control mammary glands, stained with specific bovine IgG antibody, are shown in Figure 4.9. There was less variation for the average number of IgG-positive cells between individual animals for the immunised glands, compared with IgA- and IgM-positive cells (Figure 4.7). The average numbers of IgG-positive cells in the immunised glands were significantly higher than control glands for three of the four animals in the pregnant group (2904 and 2907, $p < 0.01$; 2905, $p < 0.001$).

Figure 4.9 Representative illustration of immunohistochemical staining of IgG-positive cells in ethanol-fixed paraffin-embedded alveolar bovine tissues from immunised and control mammary glands, seven days after the 2nd intra-mammary immunisation



Black solid bar is 100μm

Similar to the other isotypes, there were very few IgG-positive cells detected in the tissues of the lactation group collected five weeks *post-partum,* and there was no apparent difference between immunised and control glands.

4.5.3.4 Other cell types

Macrophages and neutrophils that positively stained with isotype-specific antibody were also detected in the ethanol-fixed paraffin-embedded alveolar tissue sections of the pregnant immunised animals. These cells were generally located in the alveolar or ductal lumen. These cells were most prevalent in the immunised tissue samples and animal 2907 had the highest number compared with all the other animals. These cells stained positively both for IgA and IgG.

4.5.4 *Antigen-specific IgA levels in mammary secretions*

Collecting secretion samples from the control gland of pregnant animals proved to be difficult. Secretion volumes from these glands were low and samples very viscous. In contrast, samples from the immunised glands of the pregnant group were copious and watery. No secretions could be collected from either the control or immunised glands of Cow 2906.

Assays for specific anti-*C. albicans* IgA in samples of mammary secretions found Cow 2904 and 2905 had higher titres of antigen-specific antibody compared with Cow 2907 (0.9, 1.6 and 0.01 kTU, respectively). Samples from the control glands of the pregnant animals were not suitable for assay.

Titres of specific anti-*C. albicans* IgA for colostrum and milk samples collected from the lactation group are shown in Table 4.6. Samples for the first and seventh day after calving were collected from the whole udder, although only the one side of the udder had been immunised. Three animals responded to the immunisation regimen with high titres of antigen-specific IgA. One animal (2903) responded poorly to the immunisation process and had low titres of antibody, even on the first day after calving. The milk samples for Week 3 and Week 5 were collected separately for the immunised side of the udder and the control side. Milk collected from the immunised side of the udder had very high titres of antigen-specific antibody compared with the control side and this difference was statistically significant ($p < 0.01$).

Table 4.6 Titres of specific anti-*C. albicans* IgA (kTU) in colostrum/milk samples for individual cows in the lactation group

Samples were collected from the whole udder on days one and seven after calving. In established lactation, samples were collected separately from the immunised and control sides of the udder three and five weeks *post-partum*.

Cow	Day 1	Day 7	Week 3		Week 5	
	Whole udder	*Whole udder*	*Immunised side*	*Control side*	*Immunised side*	*Control side*
2901	8.20	3.60	3.70	0.01	4.70	0.01
2902	19.60	0.90	1.20	0.01	1.40	0.10
2903	0.70	0.20	0.50	0.01	0.04	0.01
2908	13.90	1.90	3.00	0.10	3.00	0.10

kTU – titre units x 10^3

4.5.5 *Quantitative analysis of MAdCAM-1 mRNA in the immunised mammary gland*

The results of the quantitative analysis of mRNA levels of MAdCAM-1 and β-actin by real-time PCR, for alveolar tissues collected from the immunised and control mammary glands of the four pregnant cows seven days after the 2nd IMM immunisation and the four lactating cows five weeks after calving, are described in the sections below.

The efficiency of the real-time PCR reaction for MAdCAM-1 and β-actin was calculated by the Lightcycler software (Roche) to be 1.848 and 1.814, respectively.

The level of MAdCAM-1 mRNA in the alveolar tissues sampled from the immunised and control glands of the pregnant and lactation groups were compared with levels of mRNA in four different samples of bovine Peyer's patch tissue. Data reported as the level of mRNA of MAdCAM-1 relative to the mRNA level of β-actin are shown in Table 4.6. The group average value for the mammary gland tissues was divided by the average value for Peyer's patch tissues to obtain an expression level of MAdCAM-1 mRNA for mammary gland tissues relative to Peyer's patch tissues.

These results indicate that expression of MAdCAM-1 in these bovine mammary alveolar tissues was very low compared with that in bovine Peyer's patch tissues.

The level of MAdCAM-1 mRNA in immunised tissue was approximately four-fold higher when compared with the level in control tissue, for both pregnant and lactation animals. The immunised tissues contained 0.6 x 10^3 – 0.7 x 10^3 fold lower MAdCAM-1 mRNA than Peyer's patch tissues (see Table 4.7), and the alveolar mammary gland control tissue contained 2.7 x 10^3 – 4.3 x 10^3 fold lower MAdCAM-1 mRNA than Peyer's patch tissues (see Table 4.6). The differences between mammary tissues and Peyer's patch tissues were highly significant ($p < 0.001$).

Table 4.7 MAdCAM-1 gene expression in immunised and control mammary glands compared with that in Peyer's patch tissues

Level of MAdCAM-1 mRNA (relative to β-actin) in bovine Peyer's patch tissue compared with immunised and control (non-immunised) alveolar mammary tissue samples collected from pregnant animals seven days after the 2nd intra-mammary immunisation and from lactating animals five weeks *post-partum* (n = 4).

Tissue	MAdCAM-1 mRNA relative to β-actin mRNA[a]	Mammary gland compared to Peyer's patches[b]
Peyer's patches	70.380 ± 8.022	100.00
*Mammary gland**		
Pregnant immunised	0.166 ± 0.030	0.165
Pregnant control	0.037 ± 0.027	0.053
Lactating immunised	0.101 ± 0.049	0.144
Lactating control	0.023 ± 0.014	0.033

a. The level of MAdCAM-1 mRNA is relative to the level of β-actin mRNA in individual samples. The results are reported as the group average ± SEM (n = 4).
b. The comparison of the expression of MAdCAM-1 in the mammary gland compared with Peyer's patches is obtained by dividing the group average relative level of MAdCAM-1 mRNA in the mammary gland by the average relative level of MAdCAM-1 mRNA in the Peyer's patch samples
* Alveolar tissue from the mammary gland

4.6 Discussion

In this chapter the cellular effects induced in the bovine mammary gland by our multi-site immunisation regimen were investigated to gain an understanding of the types of immune responses being activated. Introduction of antigen by infection with pathogenic microorganisms or by vaccination provokes an immune response in the body. This first line of defence is the innate-immune response, with macrophages producing cytokines and chemokines that induce an inflammatory response and infiltration of neutrophils. In addition, the innate-immune response contributes to the activation of adaptive-immunity where the principal cells are lymphocytes (Fearon and Locksley, 1996). Dependant on dendritic cell signalling (Banchereau and Steinman, 1998; Iwasaki and Kelsall, 1999), either a cell-mediated type response is initiated, where CD8 and Th1 CD4 cells have the main role, or a humoral antibody type response is induced where B cells and Th2 CD4 cells predominate. These two immune responses are not exclusive to one another and many infections stimulate both types of responses.

In this study, cows received IMM immunisation only on one side of the udder so that differences between lymphocyte populations of immunised and control glands could be compared. The data indicate that the intra-mammary (IMM) immunisations induced an increase in T cells in the immunised mammary glands. For example, in the pregnant group seven days after the 2nd IMM immunisation, there was a significant increase in CD3-cell numbers in the immunised glands compared with control glands (Group average ± SEM; 12.7 ± 2.1 compared with 2.7 ± 0.8). CD4-positive cells were not directly measured, however, others have reported that the total numbers of T lymphocytes in the mammary gland equate to the sum of CD4 and CD8 cells (Yamaguchi *et al.,* 1999). Therefore, the observation of a similar number of CD3- and CD8-positive cells in control alveolar tissues suggests that the majority of the T cells were CD8. Conversely, in the immunised alveolar tissue a lower number of CD8-positive cells found compared with numbers of CD3-positive cells suggests a greater proportion of CD4-positive cells in the immunised side compared with the control side of the udder. Overall, both CD4- and CD8-cell numbers were increased in the immunised mammary glands seven days following the IMM immunisations, signifying a complex immune response was induced in the glands.

It is of interest to note that the pregnant animal which gave the poorest IgA response (Cow 2907), had a different T-lymphocyte profile from the other animals in that group. Cow 2907 had three-fold higher numbers of CD3- and CD8-positive cells in both the

immunised and control alveolar tissue samples compared with the other animals. This animal also had T-cell numbers in the non-immunised side of the udder equivalent to the T-cell numbers in the immunised side of the other animals in the pregnant group. These T cells were predominantly CD8- not CD4-positive cells, as CD8 numbers were similar to CD3 numbers. The reason for these elevated CD8-positive cells in this animal is unclear but may be indicative of a sub-clinical chronic infection in the udder. Studies investigating mammary glands chronically infected with *S. aureus* have shown an increased number of CD8 cells compared with CD4 (Riollet *et al.,* 2001). Cow 2907 also had increased numbers of macrophages and neutrophils compared with the other animals, which may also be indicative of an infectious state in the gland.

In the lactation group low numbers of T lymphocytes were found in the mammary gland, irrespective of whether or not the glands had been immunised. These animals were sampled 12 weeks after the 2nd IMM immunisation, five weeks after calving, suggesting that the immune response induced by the IMM immunisation had subsided by the time full lactation was established, when these tissue samples were collected.

In addition to the comparison of T-lymphocyte populations in immunised and control mammary tissue samples, plasma-cell populations were also characterised. In agreement with other ruminant studies (Lee and Lascelles, 1970; Yurchak *et al.,* 1971), low numbers of plasma cells were detected in the control mammary gland. In contrast, numbers of plasma cells were generally higher in the immunised mammary gland compared with the control gland, a finding similar to studies in the sheep (Lee and Lascelles, 1970; Lee *et al.,* 1992). Comparison of the different isotypes of plasma cells in immunised tissues of the pregnant group indicated that IgA and IgM plasma-cell numbers varied more for individual animals, whereas the IgG plasma cells showed a more uniform response. This would imply that animal-to-animal variation in IgA response to the multi-site immunisation does not hold true for an IgG response, and therefore suggests that the IgA and IgG responses are activated by different pathways.

In the pregnant group, the two individual cows (2904 and 2905) that had the highest number of IgA-positive plasma cells in their immunised alveolar tissue samples also had the highest titres of antigen-specific IgA. This observation suggests that there is a direct correlation between the number of plasma cells and the resultant IgA

antibody levels in mammary secretions, and warrants further analysis using a larger sample size.

One cow (2906) had a greater number of IgA-positive plasma cells in the control alveolar tissue (2.1 ± 0.2) compared with immunised tissue (0.4 ± 0.5). This cannot be explained. This animal also had a higher count of IgG-positive plasma cells in the control mammary gland when compared with the other animals in the group. Unfortunately, no secretion sample was obtained from this animal for antigen-specific antibody analysis; and therefore, no conclusion could be made about the disparity of the plasma-cell response observed in this animal. The T-lymphocyte profile for this animal (2906) was similar to the two animals that had produced a high antigen-specific antibody response in secretions. However, this does not necessarily indicate that the antibody response was high in this animal. This may simply indicate that T-cell numbers do not necessarily correlate to IgA plasma-cell numbers.

There were low or undetectable numbers of IgA-, IgM- and IgG-positive plasma cells in the mammary gland of the lactation group with no discernible difference between the immunised and control glands. Although the immunised glands maintained a persistent level of IgA secretion into milk during the lactation phase, numbers of IgA-positive plasma cells detected in tissues cannot account for this maintenance of IgA antibody secretion. This is in contrast to the correlation between plasma-cell numbers and antibody responses seen in the pregnant group. One explanation could be that the individual IgA-positive plasma cells remaining in the lactating immunised mammary gland are more efficient and may produce greater amounts of antibody or antibody at an increased rate of production.

In the study in this chapter, the effects of IMM immunisation on vascular addressin expression in the bovine mammary gland were also investigated. Similar to the findings in Chapter Three, VCAM-1 detection in the mammary gland tissues of these experimental animals was on larger venules that also stained positively for vWf. Expression of VCAM-1 was detected in more pregnant animals in the immunised group compared with the untreated pregnant cows analysed in Chapter Three. The increase in VCAM-1 expression was observed in alveolar and cisternal tissue samples as well as the SMLN tissues, although there was no significant difference between the immunised and control glands. The increase in the numbers of immunised pregnant animals expressing VCAM-1, compared with untreated pregnant animals, may reflect changes induced by the immunisation regimen, but a role for this increased VCAM-1 expression could not be elucidated from these experiments.

Another study that investigated effects of antigen challenge to regional lymph nodes and skin reported the presence of VCAM-1 on small venules at these sites and suggested that the role of VCAM-1 was recruiting memory T cells (Mackay *et al.,* 1992). However, no VCAM-1 expression was observed on small venules in the mammary gland in this study, suggesting VCAM-1 does not have a role in lymphocyte extravasation in the mammary gland.

It is possible that the non-detection of VCAM-1 on smaller venules in the immunised mammary gland may be due to the timing of collection of tissue samples from pregnant cows, which occurred seven days after the 2nd IMM immunisation. If the trafficking of lymphocytes to the mammary gland was transient then the expression of VCAM-1 may also have been transient. However, a cellular immune response to immunisation takes time to develop. In the literature, one study in mice demonstrated that an initial plasma-cell response peaked at eight days after immunisation (Slifka *et al.,* 1998). In another study, mice responding to a booster immunisation had peak levels of circulating plasma cells six to eight days after the immunisation (Bernasconi *et al.,* 2002). Therefore, it would seem reasonable to conclude that sampling tissues in the mammary gland seven days after the 2nd IMM immunisation would be an ideal time.

PNAd was not detected in the alveolar or cisternal tissues of the immunised cows, only in the SMLN, similar to the findings in the untreated animals in Chapter Three. Levels of expression of PNAd were similar for immunised and untreated cows with slightly lower levels in the pregnant cows compared with the lactating cows in both cases. There was no significant difference in PNAd expression between the SMLN draining the immunised and control glands. Therefore, immunising cows to induce an IgA response does not appear to change PNAd expression in the mammary gland. This would suggest that the SMLN is not activated to recruit naïve lymphocytes in response to antigen challenge to the mammary gland via the IMM immunisation. However, this observation may also be influenced by the timing of the sample collection.

In the study in Chapter Three, the key result was the absence of MAdCAM-1 expression in mammary gland tissue samples of untreated animals. This is in direct contrast to findings in the mouse (Tanneau *et al.,* 1999; Van der Feltz *et al.,* 2001) but may be explained by the fact that there are major differences between the mouse and the cow in the expression of immunoglobulin isotypes in mammary secretions. The work in this chapter tested whether inducing a local IgA response in the bovine

mammary gland by immunisation would induce MAdCAM-1 expression in the mammary gland. The results presented in this chapter would suggest that MAdCAM-1 is not involved in the trafficking of lymphocytes to the bovine mammary gland. Even when the mammary gland was immunised to induce IgA production, no mammary tissue samples from the immunised animals were found to express MAdCAM-1. Similar to the outcome in Chapter Three, there are several explanations as to why MAdCAM-1 was not detected in this study. The simple explanation is that there is no MAdCAM-1 expressed by the bovine mammary gland and that mucosal lymphocytes are recruited to the gland by other vascular addressins and/or chemokines. The possibility that the absence of MAdCAM-1 in tissues was due to timing of the collection of samples cannot be discounted, although the arguments for timing of collection and VCAM-1 expression discussed above, also hold true for MAdCAM-1.

The eight immunised animals were naïve and had not previously undergone the immunisation regimen. Our earlier studies have shown that not all cows respond to the IgA immunisation protocol and that there is considerable variation in responses between individual animals. If the cows in the pregnant group had not responded to the immunisation then it would not have been possible to test the hypothesis. In this study, secretion samples were collected from the pregnant cows the day preceding the tissue collection. Secretion samples analysed for antigen-specific IgA levels indicated that at least two of the four cows responded to the immunisation protocol. One of the four animals had very low levels of antigen-specific antibody and the remaining cow did not give sufficient sample for testing. Therefore, IgA was induced in at least two of the animals but there was no evidence of MAdCAM-1 expression in their mammary tissues. Thus the accumulating evidence suggests that MAdCAM-1 is not involved in lymphocyte homing to the bovine mammary gland even when the mammary gland is activated by antigen to induce a local IgA response.

The immunohistochemical finding of the absence of MAdCAM-1 in mammary gland tissues was substantiated by the quantitative analysis of tissue for expression of MAdCAM-1 mRNA. Compared with bovine Peyer's patch tissues, levels of mRNA in bovine mammary gland tissues of immunised animals were very low. Further to this, no lymphocytes expressing β7 were found in any of the mammary gland tissues, supporting the evidence of no MAdCAM-1 expression in the immunised mammary gland.

Overall, it can be seen that the IMM immunisations induced many changes in the immunological environment of the mammary gland. The treatment induced marked changes in the lymphocyte population in immunised mammary tissues with higher numbers of T and differentiated B cells in the pregnant animals observed seven days after the 2nd IMM immunisation. These changes were not evident in the long-term study as there was no discernible difference between the immunised and control mammary tissues in the lactating animals. While the IgA-response to the IMM immunisations may be correlated to numbers of IgA-positive plasma cells in the recently immunised gland, the same correlation does not hold true for the lactating animal that had been immunised many weeks previously. The reason for the continued production of IgA in the milk could not be identified from these studies. Although there was no evidence of MAdCAM-1 expression, VCAM-1 levels in the immunised animals were elevated in comparison to the untreated animals. It must be remembered that these findings have been based on a small number of animals. Due to the variability between animals, further experiments with larger numbers of animals are required before any definitive conclusions may be drawn.

Chapter 5

Comparison of immune responses in the mammary gland following immunisation of high- and low-responding cows

5.1 Introduction

The work in Chapter Five investigates the rate of immune cells trafficking into the bovine mammary gland in response to intra-mammary (IMM) immunisation. The work in Chapter Four established that by seven days after treatment with IMM immunisation, there is an increase in lymphocyte numbers in the mammary gland. In this study, bovine mammary secretions are examined for changes in lymphocyte populations and antibody levels over an extended time course following IMM immunisation.

Animals immunised with our multi-site immunisation regimen for the induction of IgA in mammary secretions have a variable IgA response. Earlier studies have found that the variability in antibody response is reproducible for individual animals. An animal that produces a high IgA response in its mammary secretions to the immunisation regimen in one year will respond in a similar way the following year (unpublished data). Similarly, animals that produce a low IgA response in one year will produce a low response to the immunisation in subsequent years. Animals that have been previously immunised can thus be grouped into high- and low-responding animals and thereby provide a unique resource for investigating the differences in their immune responses. In this chapter, animals that have been immunised in the previous two years were reimmunised and their cellular immune responses in mammary secretions compared.

The differences observed in an animal's immune response to our multi-site immunisation regimen may be due to a number of factors, including the priming of lymphocytes by the initial intra-peritoneal and intra-muscular immunisation, differences in the efficiency of recruitment of immune cells to the mammary gland, and/or differences in the proliferation and differentiation of plasmablasts. Earlier studies have indicated that IgA antibody is produced locally in the immunised gland in response to our immunisation regimen (Hodgkinson and Hodgkinson, 2003). Antibody is only produced in the quarter of the udder that has been immunised. For the local production of immunoglobulin to occur, it is hypothesised that immune cells will be required *in situ*. In Chapter Four it was demonstrated that antigenic stimulation by IMM immunisations activates mechanisms for the recruitment of immune cells to the immunised gland. This was shown by increased numbers of lymphocytes, both T cells and B cells, in the tissues of the immunised glands compared with control (non-immunised) glands.

Many studies investigating the cellular response to IMM immunisation in the ruminant have utilised sheep; sacrificing the animals to collect tissue samples to characterise immune cells (Lee and Lascelles, 1970; Lee *et al.,* 1992; Sheldrake *et al.,* 1985a). This approach was also adopted in Chapter Four of this thesis. However, clearly for studies in the cow this limits the number of animals that can be used because of the value of the animals. Many studies looking at bovine mammary health have utilised mammary secretions and flow cytometry methodology to characterise T-cell and B-cell populations, for example, Dosogne *et al.,* (2003), Leitner *et al.,* (2000b) and Rivas *et al.,* (2001). Mammary secretions from unchallenged bovine mammary glands show clear differences in the relative proportions of lymphocyte populations compared with circulating peripheral blood lymphocytes. It has been suggested that this difference may be associated with functional activity in the mammary gland (Park *et al.,* 1992). Differences in the ratio of CD4 and CD8 cells may reflect the type of immune response induced by pathogens in the mammary gland. During the dry (non-lactating) period, there is a higher percentage of CD4-positive cells compared with CD8-positive cells in mammary secretions. This ratio becomes inverted during the lactation phase (Yang *et al.,* 1997), with the majority of CD8 cells being activated (Park *et al.,* 1992). The percentage of B cells in the mammary secretions is higher (25%) in the periparturient stage compared with the lactation phase (where it is < 5%) (Leitner *et al.,* 2000b; Park *et al.,* 1992).

In the lactating mammary gland challenged by pathogenic antigens, the cellular profile of mammary secretions changes and milk leukocyte population patterns have been reported to be indicative of udder infections of different aetiologies (Leitner *et al.,* 2000b). In the mammary gland chronically infected by *S. aureus,* an overall increase in the numbers of cells was found, with the major cell population being neutrophils (Riollet *et al.,* 2001; Rivas *et al.,* 2001). The T-lymphocyte percentage increased, with a greater percentage of CD8 cells recruited into the mammary gland in comparison with the CD4 cells (Riollet *et al.,* 2001). In the mammary gland acutely infected with *E. coli* the major cell type in the milk was also neutrophils. However, the predominant T lymphocyte was reported to be CD4 cells (Taylor *et al.,* 1997). Another study observed low percentages of T lymphocytes in acutely infected mammary glands compared with chronically infected mammary glands (Leitner *et al.,* 2000b). The proportion of B lymphocytes in milk from infected bovine mammary glands was low (Leitner *et al.,* 2000b; Riollet *et al.,* 2001).

5.2 Aim and approach

The main aim of the work in this chapter was to characterise the change to lymphocyte populations in mammary gland secretions following two IMM immunisations. In the previous chapter, effects of IMM immunisation had been assessed in tissue samples taken at two time points after the 2nd IMM immunisation; seven days and 12 weeks. This raised the question of the best time to take samples to observe changes induced by the treatment. Using mammary secretions instead of tissues to monitor changes following the IMM immunisations allowed for multiple samples to be collected over an extended time interval.

The potential issue of using naïve animals was also highlighted in Chapter Four. If the relative level of response to immunisation is not known, then interpreting cellular changes becomes difficult. For example, finding no changes in cell counts could be due to there being no induced effects or simply to a poor level of response. The animals that were immunised in this chapter had been immunised in the two previous years with our multi-site regimen and each could be categorised from earlier results as either high- or low-responding animals. Animals were categorised using antigen-specific antibody titres measured in milks collected seven days after parturition: an antigen-specific IgA greater than 2 000 titre units (2.0 kTU) was a high-responding animal and less than 2.0 kTU was a low-responding animal.

The first objective of this work was to collect mammary secretions from pregnant (non-lactating) immunised animals before and after the IMM immunisations. To achieve this, eight high-responding pregnant animals and six low-responding pregnant animals were immunised for a third time using our multi-site immunisation regimen. All four quarters received the IMM immunisations. Mammary secretions were collected at intervals before and after the IMM immunisations, with secretions collected from the left side of the udder only, alternating between the front and back quarters.

The second objective of this work was to analyse cells isolated from mammary secretions by flow cytometry to determine the percentages of lymphocyte sub-populations:

- T-cell sub-populations: CD4, CD8
- Pan B cell: BB2
- Immunoglobulin isotype-specific cells: IgA, IgG, IgM

The third objective was to test the level of antibody response of each animal. To do this, the mammary secretions were analysed for levels of antigen-specific and total IgA antibody. In addition, to assess the effects of multiple sampling of secretions from the left side of the udder, a comparison was made of the levels of antigen-specific IgA antibody in colostrum samples collected from both the left and right quarters of the udder. Milk samples collected seven days *post-partum* were also analysed for antibody levels, to assess how the IgA response compared with that in previous years.

5.3 Materials

5.3.1 *Antibodies*

Primary antibodies used for flow cytometry methods are listed in Table 5.1. All primary antibodies were used at a dilution of 1:400.

Table 5.1 Primary antibodies used for flow cytometry analysis

Clone	Target antigen	Cellular Expression	Isotype	Supplier
ILA-11A	bovine CD4	MHC class II T-cell subset	IgG_{2a}	WSU
CACT80C	bovine CD8	MHC class I T-cell subset	IgM	VMRD
CACT116A	bovine CD25	IL-2 receptor alpha chain	IgG_1	VMRD
BAQ44A	bovine BB2	B cells (non Ig marker)	IgM	VMRD
Rabbit polyclonal*	bovine IgA	IgA-positive cells	-	Bethyl
Rabbit polyclonal*	bovine IgM	IgM-positive cells	-	Bethyl
Sheep polyclonal*	bovine IgG	IgG-positive cells	-	Bethyl
Ci4	neg. control	-	IgG_1	Chemicon
GC270	neg. control	-	IgG_{2a}	Chemicon
GC323	neg. control	-	IgM	Chemicon

* Directly conjugated to fluorescein isothiocyanate
Ig - immunoglobulin

Secondary antibodies used for flow cytometry methods are listed in Table 5.2.

Table 5.2 Secondary antibodies used for flow cytometry analysis

Binding Reactivity	Conjugate	Dilution	Source
Mouse IgG	Fluorescein isothiocyanate	1:50	Jackson
Mouse IgG_1	R-Phycoerythrin	1:1000	Pharmingen
Mouse IgG_{2a}	Fluorescein isothiocyanate	1:400	Pharmingen
Mouse IgM	R-Phycoerythrin	1:400	Jackson

5.4 Methods

5.4.1 *Immunisation procedure*

Fourteen healthy multiparous pregnant cows of mixed Friesian and Jersey breeds were selected from a group that had been previously immunised using our multi-site immunisation regimen (Hodgkinson and Hodgkinson, 2003). The selection was made on the basis of the titres of the specific anti-*C. albicans* IgA in the previous two years, in milk collected seven days *post-partum*. Eight animals that had antibody titres greater than 2.0 kTU were assigned to the High group. Six animals that had antibody titres less than 2.0 kTU were assigned to the Low group. All animals were then re-immunised with *C. albicans* using our multi-site immunisation regimen as outlined in Section 2.2.1. All four mammary gland quarters were immunised. The immunisation schedule is outlined in Table 5.3. The initial immunisation at Week 0 was 14 weeks before the group's average estimated calving date.

Table 5.3 Routes and timing for the multi-site immunisation regimen

Immunisation	Route	Weeks
I	IP/IM	0
II	IP/IM/IMM	4
III	IMM	6
IV	IP/IM	7

IP - intra-peritoneal
IM - intra-muscular
IMM - intra-mammary

5.4.2 *Sample collection and preparation*

Mammary secretions were collected only from the left side of the udder. The collection was alternated between the front and back quarters to minimise any effects sampling may have had on the general mammary secretion parameters. Mammary secretions were collected according to the protocol outlined in Section 2.2.2.3. The schedule for the collection is outlined in Table 5.4. The first sample on Day 0 was collected just prior to the administration of the 1st IMM immunisation. The fifth sample of Day 12 was collected just prior to the administration of the 2nd IMM immunisation.

Table 5.4 Schedule for the collection of mammary secretions from animals before and after the intra-mammary immunisations

Sample number	Days after 1st IMM immunisation	Days after 2nd IMM immunisation
1	0	-
2	2	-
3	5	-
4	7	-
5	12	0
6	14	2
7	15	3
8	16	4
9	19	7
10	21	9
11	35	23
12	Left Colostrum	-
13	Right Colostrum	-
14	Milk (seven days *post-partum*)	-

IMM - intra-mammary

Mammary secretions were processed to isolate the immune cells according to the protocol outlined in Section 2.2.2.3. Cells required for flow cytometry were assayed immediately. An aliquot of each secretion was stored at -20°C for antibody and protein concentration analysis.

Two colostrum samples were collected from each animal as per the protocol outlined in Section 2.2.2.2, one sample from the right quarters and one sample from the left quarters of the udder. These were collected for comparative purposes, to establish any effects that were caused by the removal of multiple samples of mammary secretions from the left side of the udder. Finally, a milk sample was collected seven days after parturition using the protocol outlined in Section 2.2.2.2. Colostrum and milk samples were processed as per the protocol outlined in Section 2.2.2.2.

5.4.3 *Flow cytometry*

Cells from six animals from the High group and six animals from the Low group were analysed at each time point. Cells isolated from mammary secretions were immunostained for flow cytometry using the protocol outlined in Section 2.2.5. The cells were incubated with antibodies specific for the lymphocyte cell-surface antigens listed in Table 5.1. Aliquots of cells were first double-labelled with pairs of primary antibodies with different isotypes, or singly, as shown in Table 5.5. Following this, the cells were incubated with two secondary antibodies that were coupled to either fluorescein isothiocyanate (FITC) or phycoerythrin (PE) fluorescent dyes. The anti-bovine isotype-specific antibodies (IgA, IgM and IgG) were directly conjugated to FITC and did not require a secondary antibody. Measurement was made using a flow cytometer and populations of lymphocytes and neutrophils were gated using SSC and FSC parameters. The percentage of lymphocytes and neutrophils in the total population and the percentage of T and B cells in the lymphocyte population stained by each antibody were calculated using Cellquest™ software (BD).

Table 5.5 Combinations of primary and secondary antibodies used for double staining of cells for flow cytometry analysis

1° MAb - A	1° MAb - B	2° Ab - A	2° Ab - B
CD4 (IgG_{2a})	CD25 (IgG_1)	α-Mu IgG_{2a} - FITC	α-Mu IgG_1 - PE
CD25 (IgG_1)	CD8 (IgM)	α-Mu IgG - FITC	α-Mu IgM - PE
-	BB2 (IgM)	α-Bov IgA - FITC	α-Mu IgM - PE
-	BB2 (IgM)	α-Bov IgM - FITC	α-Mu IgM - PE
-	BB2 (IgM)	α-Bov IgG - FITC	α-Mu IgM - PE

The monoclonal antibody isotype is shown in brackets
FITC - fluorescein isothiocyanate
PE - phycoerythrin
Mu - mouse
Bov - bovine

Non-specific binding of antibodies to cells was determined by the addition of RPMI-medium only or negative isotype control serum in place of the primary antibody. This percentage of non-specific binding was subtracted from the total percentage to give a corrected value for each analysis.

5.4.4 *ELISA*

5.4.4.1 Specific anti-*C. albicans* IgA

Mammary secretions collected from the High and Low groups, plus the colostrum and milk samples, were assayed by ELISA to determine the titre of the specific anti-*C. albicans* IgA, as per the protocol outlined in Section 2.2.6.1. Results were reported as kTU of antibody.

5.4.4.2 Total IgA

Total IgA concentrations were determined by the ELISA protocol, outlined in Section 2.2.6.2, for mammary secretions collected from the High and Low groups on Days 0, 5, 12, 15, 19 and 35, plus milk samples collected seven days *post-partum.* Results were reported as mg/ml IgA.

The titres of specific anti-*C. albicans* IgA and total IgA concentrations measured in the mammary secretions were both adjusted for protein concentration. This allowed for differences in collection procedure; whether or not a saline infusion was required for sample collection. Protein concentrations were determined by the protocol outlined in Section 2.2.7.

5.5 Results

5.5.1 *Characterisation of lymphocytes in mammary secretions following intra-mammary immunisation*

Mammary secretion samples collected before and after IMM immunisations varied considerably in their consistency. There was no visual evidence of the oil adjuvant (FICA) in the samples collected two days after IMM immunisation. Secretions collected prior to IMM immunisation required a saline infusion to produce a sample. Two to four days following IMM immunisation, the gland did not usually require the saline infusion as the gland cistern contained sufficient fluid for sampling. On these days secretions often contained a gelatinous precipitate whereas pre-IMM immunisation samples were relatively clear. Four to five days following IMM immunisation, secretions were clear, similar to that collected prior to IMM immunisation.

Cells were isolated from mammary secretions for analysis by flow cytometry to determine profiles of cells found in the mammary gland cistern following IMM immunisation. Numbers of cells isolated from the 25 ml secretion samples varied amongst animals and from day-to-day, with values ranging from 1×10^6 to 5×10^8 cells. However, total cells could not be isolated from samples containing the gelatinous precipitate described above.

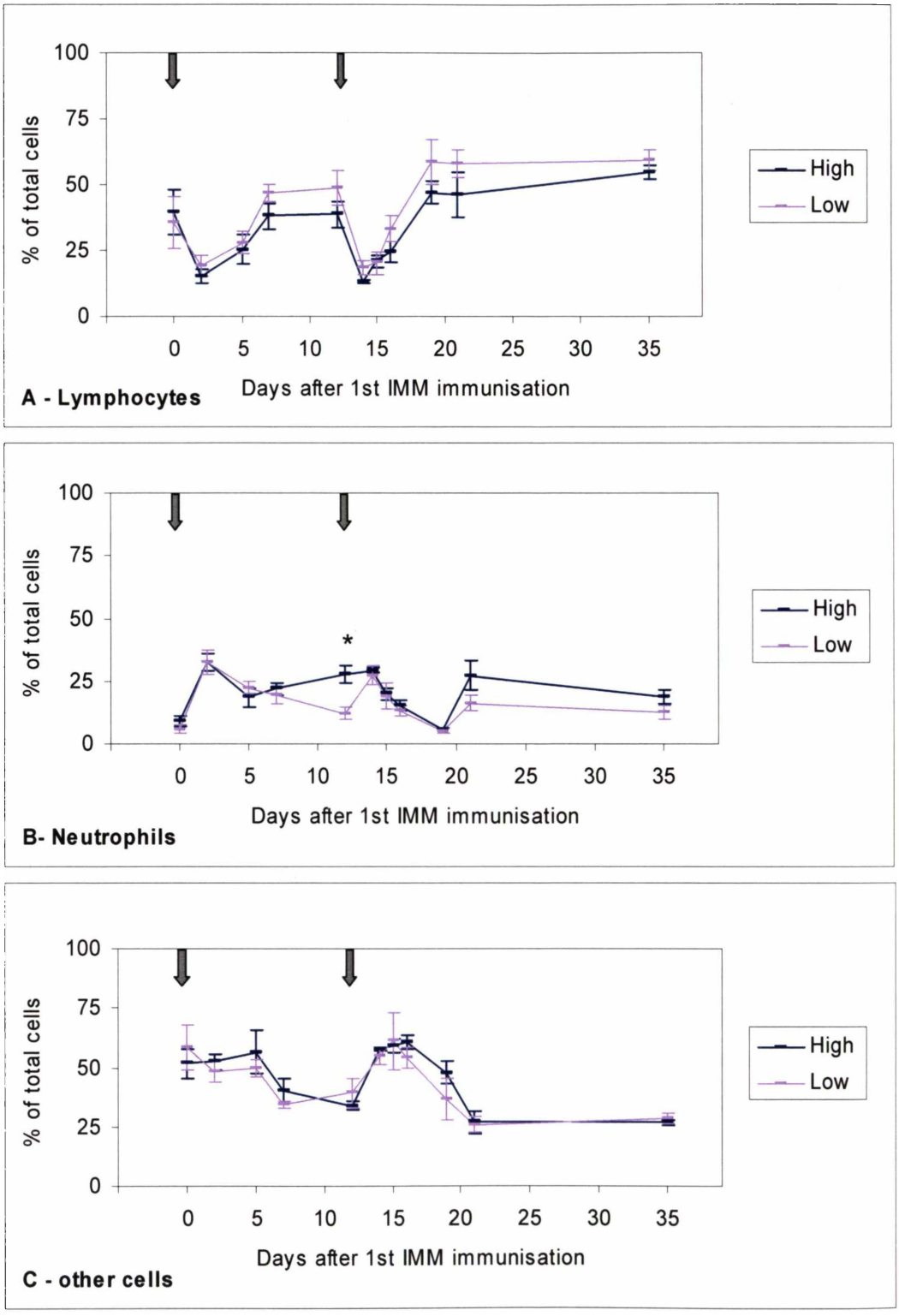
5.5.1.1 Lymphocytes, neutrophils and other cells

Overall comparison of cells isolated from mammary secretions showed that the lymphocyte population (15 – 50%) was greater than the neutrophil population (7 – 33%), with the other cell types accounting for 25 – 60% of the total cells. Few differences were observed between the High and Low groups for cell types across the time points sampled. However, marked response to immunisation was observed with changes occurring to the proportions of different cell types following IMM immunisations. Average percentages of lymphocytes, neutrophils and other cells in the total population of cells isolated from mammary secretions for the High and Low groups (± SEM) are shown in Figure 5.1.

Prior to IMM immunisation, approximately 50% of the total cell population isolated from mammary secretions were lymphocytes. In the samples collected up to five days following the two IMM immunisations, the percentage of lymphocytes

Figure 5.1 Flow cytometry analysis of total cells in mammary secretions for lymphocytes, neutrophils and other cells

Cells were isolated from mammary secretions collected at intervals following intra-mammary immunisation (at times marked with arrows). Graphs show the average percentage of each type of cell for the High and Low groups (± SEM): **A**, Lymphocytes; **B**, Neutrophils; **C**, other cells. Days on which there were significant differences between the groups are marked (* $p < 0.05$).



decreased to 15 – 25% of total cells, a two-fold reduction. Values then recovered to pre-IMM immunisation levels. No significant difference was observed between the percentages of lymphocytes in the high- and low-responding groups.

The percentage of neutrophils in the total population of cells isolated from mammary secretions ranged from 7 to 33%. Average values were similar for the High and Low groups except on Day 12 when neutrophils in the Low group were two-fold lower than in the High group ($p < 0.01$). Following the 1st IMM immunisation, the percentage of neutrophils increased to approximately 20 – 30% of the total cells in the mammary secretions, 3 – 4 times higher than the pre-IMM immunisation levels. The percentage of neutrophils in the total cell population remained at these higher levels until several days after the 2nd IMM immunisation, at which time the percentage of neutrophils declined to pre-IMM immunisation values. On Day 21 (nine days after the 2nd IMM immunisation) there was another rise in the percentage of neutrophils (20 – 30%) which then fell again by Day 35 to approximately pre-IMM immunisation values.

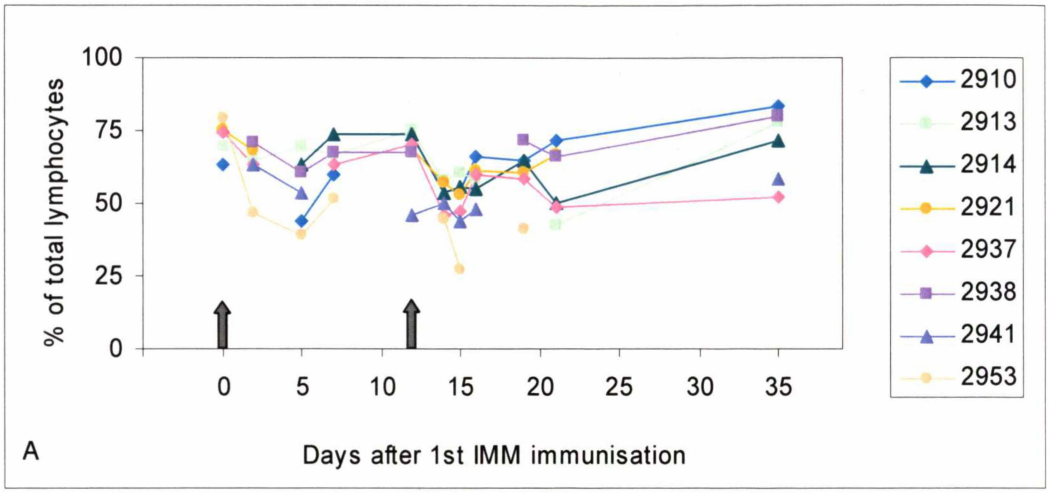
Other cells, not classified, ranged from 30 – 60% of the total population of cells isolated from mammary secretion samples collected over the time course. On Days 0, 2 and 5 (before and up to five days after the 1st IMM immunisation) the percentage of these cells was approximately 60% of total cells isolated from mammary secretions. On Days 7 and 12 the percentage of these cells decreased to approximately 35%. Following the 2nd IMM immunisation, these cells increased again to approximately 60% of total cells. By Day 21, levels of these cells had decreased to 30% and were still at this level on Day 35.

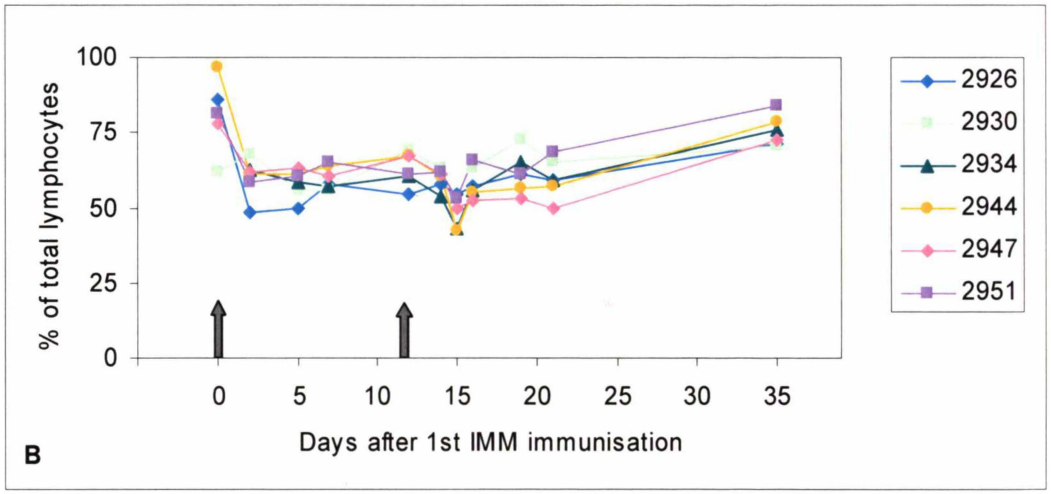
5.5.1.2 CD4- and CD8-positive cells

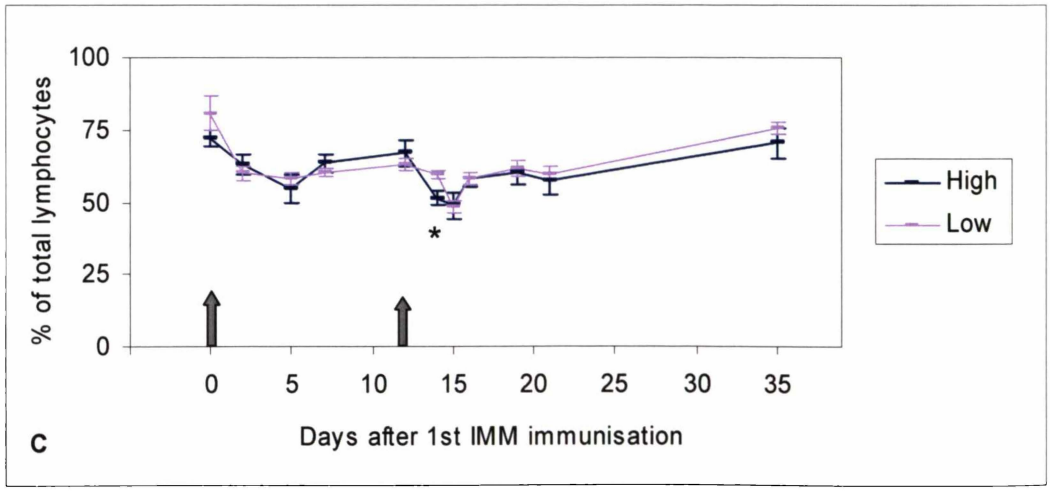
Prior to the IMM immunisations, the predominant lymphocyte population in mammary secretions was the T cell (76% of the total lymphocytes). At the peak of the immunisation response (Day 15, three days after the 2nd IMM immunisation), the percentage of T cells had fallen to approximately 48% of the total lymphocytes. Five weeks after the 1st IMM immunisation (Day 35, 21 days after the 2nd IMM immunisation), the percentage of T cells had returned to pre-immunisation levels (~ 75%). The combined percentage of T lymphocytes that stained positively for CD4 and CD8 are shown in Figure 5.2. Data are shown for individual animal responses over the time course, grouped as either high- or low-responding animals. Group averages (± SEM) are also shown.

Figure 5.2 Flow cytometry analysis of lymphocytes from mammary secretions for combined CD4- and CD8-positive lymphocytes

Cells were isolated from mammary secretions collected at intervals following intra-mammary immunisation (at times marked with arrows). Graphs show the combined percentage of lymphocytes that stained positively for CD4 and CD8: **A**, Individual animals in the High group; **B**, Individual animals in the Low group; **C**, Average response of the High and the Low group (± SEM). Days on which there were significant differences between the groups are marked (* $p < 0.05$).







Comparison of the T-cell responses for the High and Low groups determined that overall there was little difference between the two groups in their response and that only on Day 14 (two days after the 2nd IMM immunisation) was the difference significant ($p < 0.05$). On this day, the Low group had a higher percentage of T cells than the High group (60% and 51%, respectively).

The percentage of lymphocytes that stained positively for CD4 and CD8 T-cell subpopulations is shown in Figure 5.3 and Figure 5.4, respectively. Data are shown for individual animal responses over the time course, grouped as either high- or low-responding animals. Group averages (± SEM) are also shown. CD4 lymphocytes were found to be the predominant T-cell subpopulation in the mammary secretions. Prior to the IMM immunisations, the proportion of CD4-positive cells was 47% compared with 29% CD8-positive cells. On Day 15 (three days after the 2nd IMM immunisation) when the T-lymphocyte percentage was at its lowest point, the value for CD4-positive cells was 36% compared with 12% for CD8-positive cells. This equated to a 23% drop for CD4-positive cells and a 59% drop for CD8-positive cells. There was no significant difference between either the average CD4 or the average CD8 values for the High and Low groups for any of the days that were sampled.

T cells were also examined for expression of the IL-2 receptor (R). The proportion of activated T cells was calculated by dividing the percentage of cells that expressed both the IL-2R and the T-cell marker by the total percentage of cells that expressed that T-cell marker. The values obtained for the High and Low groups are shown in Table 5.6.

The majority of CD4-positive cells in mammary secretions collected over the time course were activated cells (72 - 90%). The proportion of CD4-activated cells increased from approximately 92% of the total CD4-positive cells prior to the IMM immunisation to approximately 98% on the second day following each IMM immunisation and this increase was significantly different ($p < 0.05$). By Day 35, the percentage of activated cells had fallen to approximately 73% of the total CD4-positive cells. There were no significant differences when CD4 values obtained for the High and Low groups over the time course were compared.

Figure 5.3 Flow cytometry analysis of lymphocytes from mammary secretions for CD4-positive lymphocytes

Cells were isolated from mammary secretions collected at intervals following intra-mammary immunisation (at times marked with arrows). Graphs show the percentage of lymphocytes that stained positively for CD4: **A**, Individual animals in the High group; **B**, Individual animals in the Low group; **C**, Average response of the High and the Low group (± SEM).

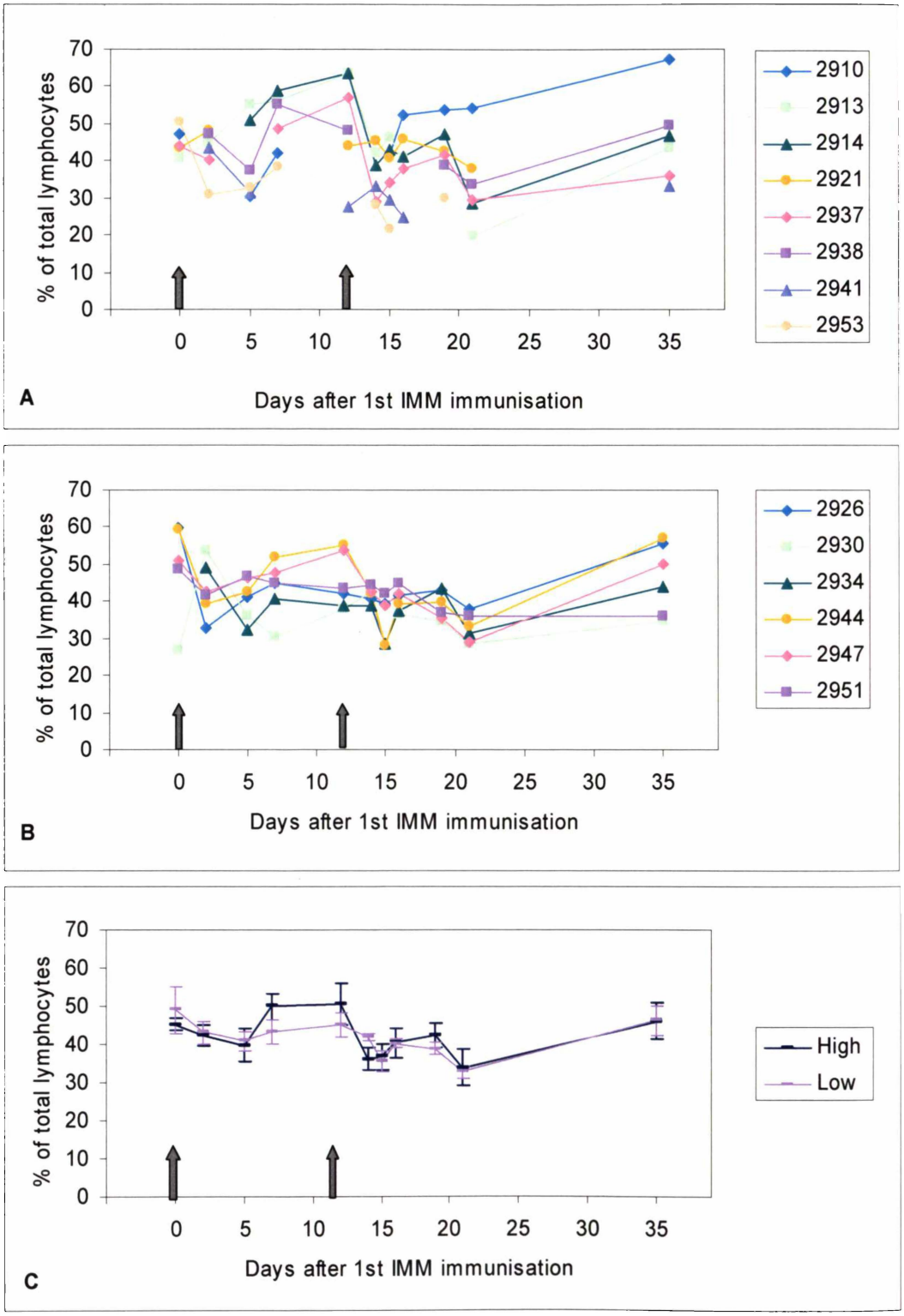


Figure 5.4 Flow cytometry analysis of lymphocytes from mammary secretions for CD8-positive lymphocytes

Cells were isolated from mammary secretions collected at intervals following intra-mammary immunisation (at times marked with arrows). Graphs show the percentage of lymphocytes that stained positively for CD8: **A**, Individual animals in the High group; **B**, Individual animals in the Low group; **C**, Average response of the High and the Low group (± SEM).

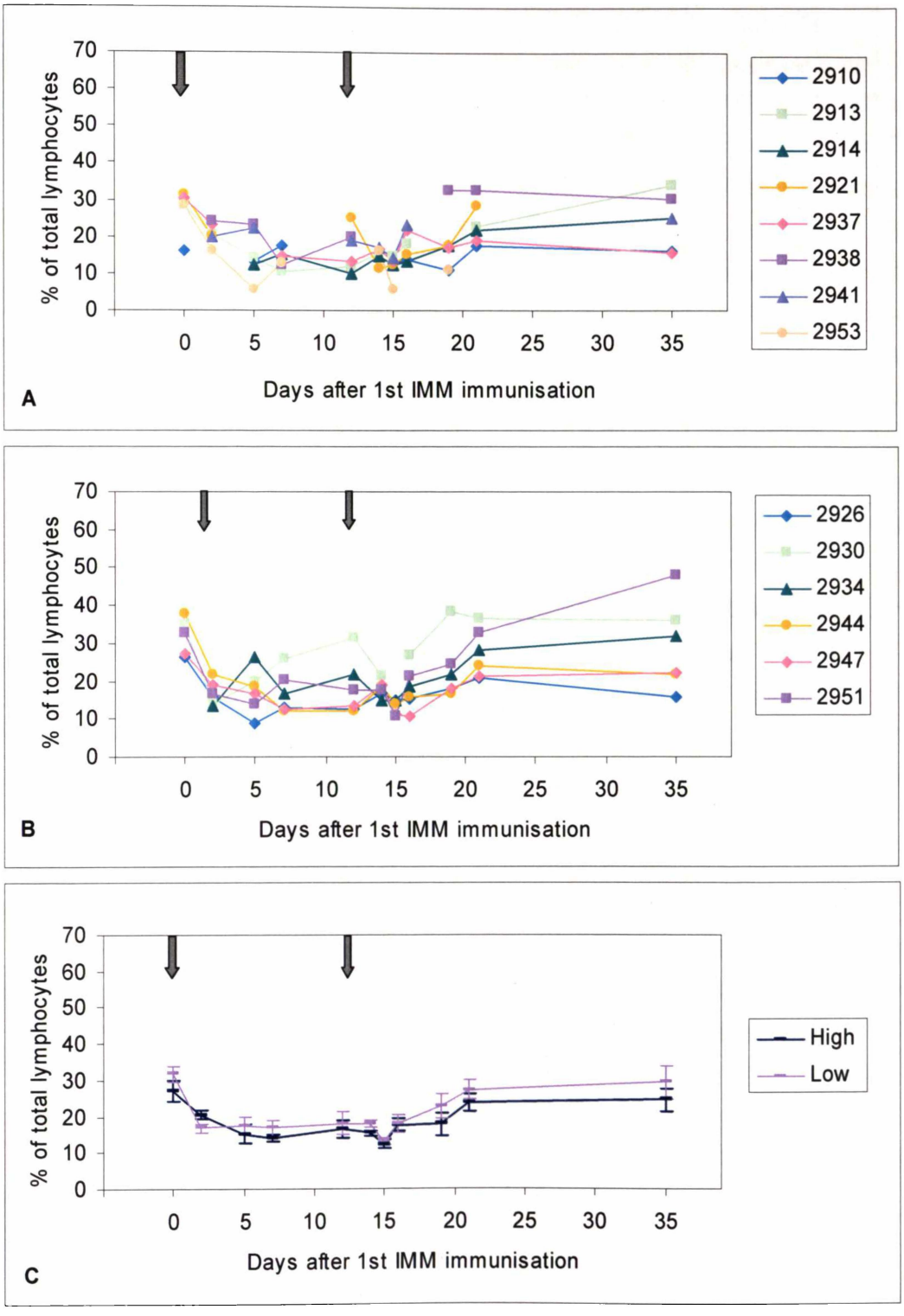


Table 5.6 Percentage of activated T lymphocytes in mammary secretions following intra-mammary immunisation

The proportion of activated T cells was calculated by dividing the percentage of cells that expressed both the IL-2 receptor and the T-cell marker by the total percentage of cells that expressed that T-cell marker.

Days after 1st IMM immunisation	Percentage of activated T cells			
	CD4		*CD8*	
	High Group	*Low group*	*High Group*	*Low group*
0	91.73 ± 1.17	91.62 ± 1.62	34.06 ± 6.49	44.52 ± 11.13
2	97.42 ± 0.50	97.42 ± 0.62	44.75 ± 9.00	55.78 ± 4.91
5	91.04 ± 4.39	96.23 ± 1.52	33.47 ± 7.99	34.18 ± 5.16
12*	90.52 ± 1.48	92.46 ± 1.48	19.62 ± 6.26	21.49 ± 3.52
14	96.54 ± 0.78	97.54 ± 0.79	45.00 ± 7.76	41.13 ± 6.43
15	90.72 ± 1.34	90.65 ± 1.10	25.93 ± 5.57	36.63 ± 3.22
16	94.46 ± 1.05	93.20 ± 1.16	26.45 ± 6.13	23.74 ± 3.80
35	72.29 ± 3.74	74.29 ± 4.75	24.71 ± 5.49	28.64 ± 5.49

IMM- intra-mammary
* Day of 2nd IMM immunisation

Over the time course, the proportion of activated CD8-positive cells ranged from 20 to 55% of the total CD8-positive cells. Similar to CD4-positive cells, the percentage of activated CD8-positive cells increased following each IMM immunisation. The increase in the percentage of activated CD8 cells after the 1st IMM immunisation was not significantly different from pre-IMM immunisation values ($p = 0.4$).

Following the 2nd IMM immunisation, there was a two-fold increase in activated CD8-positive cells from approximately 20% on Day 12 to approximately 40% on Day 14 which was significant ($p < 0.05$). There were no significant differences when activated CD8-positive cell percentages obtained for the High and Low groups over the time course were compared.

5.5.1.3 IgA-positive cells

Data for IgA-positive cells are shown in Figure 5.5 for individual animal responses over the time course, grouped as either high- or low-responding animals. Group averages (± SEM) are also shown.

Prior to the IMM immunisations, IgA-positive cells represented approximately 8% of the total lymphocyte population in mammary secretions. Following the 1st IMM immunisation, the High and Low groups responded in a similar way with the percentage of IgA-positive cells increasing to approximately 12%, two to five days after the IMM immunisation. This increase was significantly different from Day 0 for the High group on Days 2 and 5 ($p < 0.05$), but not for the Low group.

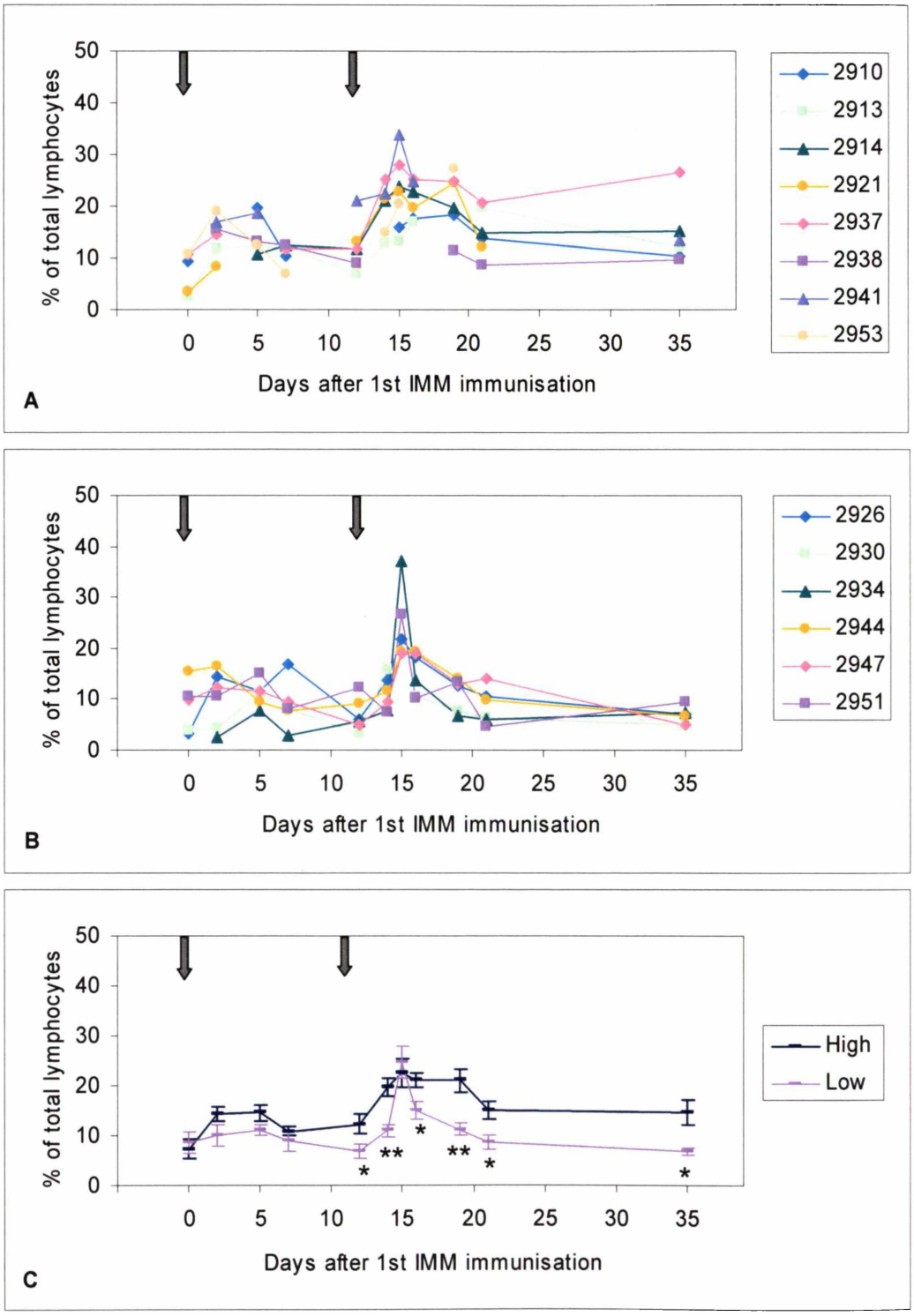
After the 2nd IMM immunisation, the IgA-positive cells in the High group increased to 19% of total lymphocytes by Day 14 compared with the Low group where the IgA-positive cells increased to only 11%. The peak response for both groups was on Day 15 and of the same magnitude (23% IgA-positive cells, a three-fold increase compared with Day 0). Compared with the peak response after the 1st IMM immunisation, the peak response after the 2nd IMM immunisation was significantly higher for the High group ($p < 0.05$) and very significantly higher for the Low group ($p < 0.01$). After Day 15 (three days after the 2nd IMM immunisation) the average percentage for the High group remained elevated until Day 19 (~ 23% IgA-positive cells). For the High group, Days 14, 15 and 19 were very significantly different ($p < 0.01$) and Day 16 was highly significantly different ($p < 0.001$) from the pre-IMM immunisation value for IgA-positive cells. By Day 35, the average percentage of IgA-positive cells for the High group was still higher compared with the pre-IMM immunisation level (14% and 7%, respectively), but this was not significantly different ($p = 0.053$).

In contrast, the average percentage of IgA-positive cells for the Low group declined on Day 16 to 15% of the total lymphocytes and had returned to the pre-IMM immunisation level (8%) by Day 21. For the Low group, only Day 15 and Day 16 were significantly different ($p < 0.01$ and $p < 0.05$, respectively) from the pre-IMM immunisation value for IgA-positive cells.

Analysis comparing the average IgA-positive cell responses for the High and the Low groups over the time course determined that overall the response was higher for the High group. Statistical analysis showed that this difference was only significant on Days 12, 16, 21 and 35 ($p < 0.05$) and very significantly different on Days 14 and 19 ($p < 0.01$). There was no significant difference between the two groups for their peak response on Day 15.

Figure 5.5 Flow cytometry analysis of lymphocytes from mammary secretions for IgA-positive lymphocytes

Cells were isolated from mammary secretions collected at intervals following intra-mammary immunisation (at times marked with arrows). Graphs show the percentage of lymphocytes that stained positively for IgA: **A**, Individual animals in the High group; **B**, Individual animals in the Low group; **C**, Average response of the High and Low group (± SEM). Days on which there were significant differences between the groups are marked (* $p < 0.05$; ** $p < 0.01$).



5.5.1.4 IgM-positive cells

Similar to IgA-positive cells, a biphasic response was observed for IgM-positive cells in mammary secretions following the two IMM immunisations. Data for IgM-positive cells are shown in Figure 5.6 for individual animal responses over the time course, grouped as either high- or low-responding animals. Group averages (± SEM) are also shown.

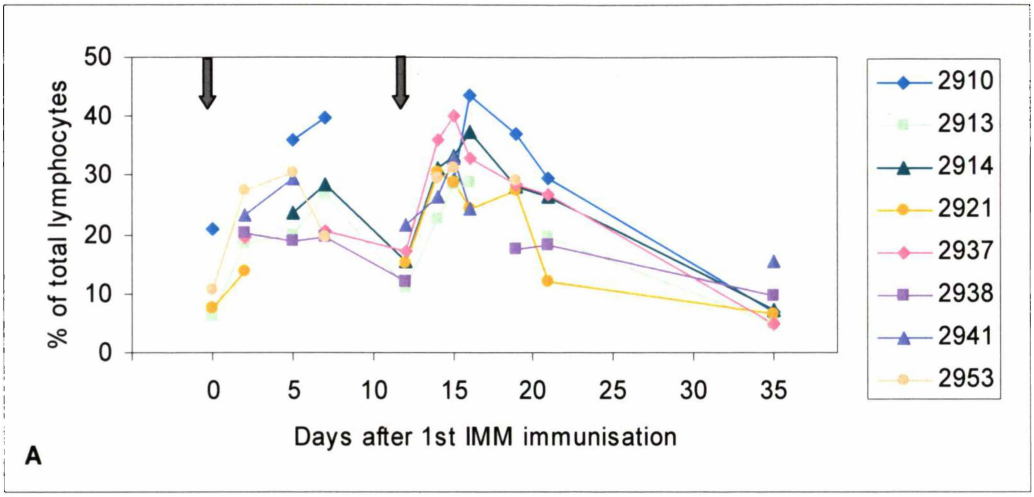
Prior to the 1st IMM immunisation, IgM-positive cells represented approximately 16% of the total lymphocyte population. There was a large variation for this baseline value for the Low group, ranging from 8 to 40%, a five-fold difference. After the 1st IMM immunisation, there was an increase for both groups in IgM-positive cells to an average of approximately 24% of lymphocytes, which then returned to baseline levels. This increase was significantly different from Day 0 for the High group on Days 2, 5 and 7 ($p < 0.05$). In contrast, there was no significant difference from pre-immunisation values for the Low group in this phase of the response.

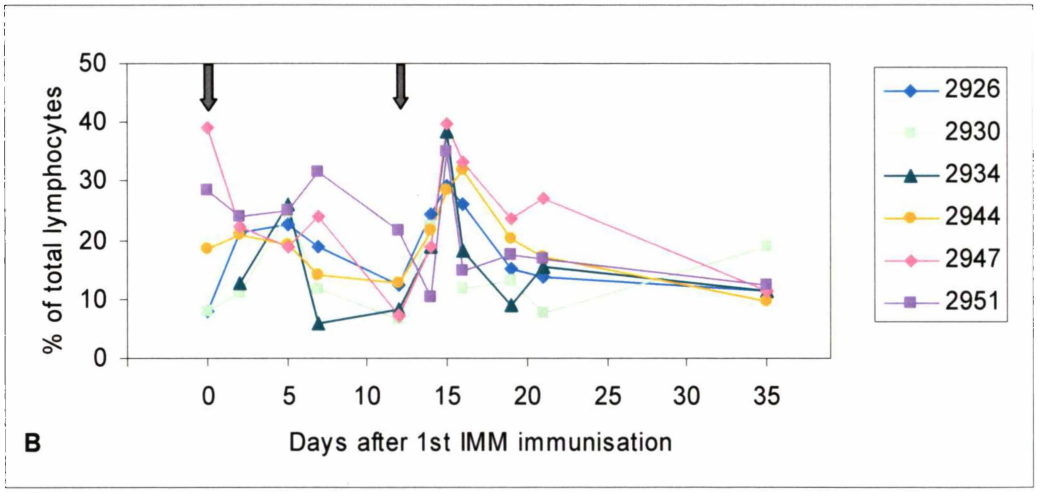
Similar to the results for the IgA-positive cells, the IgM-positive cells for the High and Low groups appeared to have a different pattern of response following the 2nd IMM immunisation. Two days after the 2nd IMM immunisation (Day 14), the average percentage of IgM-positive cells for the animals in the High group had increased to 29% whereas the Low group IgM-positive cells only increased to 19%. On the third day following the 2nd IMM immunisation (Day 15), both groups reached their peak response (~ 33% IgM-positive cells). Compared with the 1st IMM immunisation, the peak response after the 2nd IMM immunisation was significantly higher for the Low group ($p < 0.01$) but not for the High group ($p = 0.09$). After Day 15, the average percentage of IgM-positive cells for the High group remained elevated at this level until Day 19. By Day 35 the average percentage of IgM-positive cells for the High group was still higher compared with the pre-IMM immunisation level (21% and 11%, respectively) and this difference was significant ($p< 0.05$). In contrast, the average percentage of IgM-positive cells for the Low group declined on Day 16 to 23% and had returned to pre-IMM immunisation levels (16%) by Day 19.

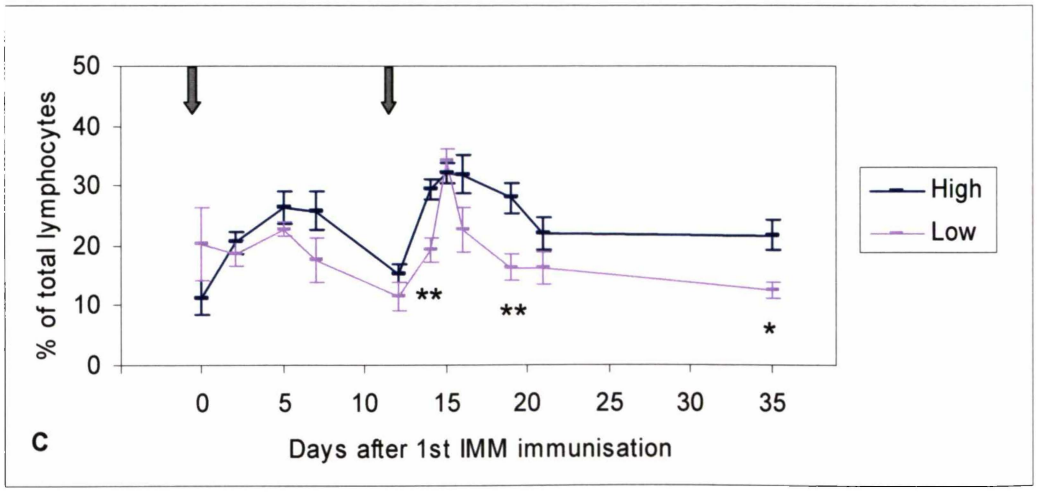
Comparison of the average IgM-positive cell responses for the High and the Low groups over the time course showed a significant difference between the groups only on Days 14 and 19 ($p < 0.01$), and 35 ($p < 0.05$).

Figure 5.6 Flow cytometry analysis of lymphocytes from mammary secretions for IgM-positive lymphocytes

Cells were isolated from mammary secretions collected at intervals following intra-mammary immunisation (at times marked with arrows). Graphs show the percentage of lymphocytes that stained positively for IgM: **A**, Individual animals in the High group; **B**, Individual animals in the Low group; **C**, Average response of the High and the Low group (± SEM). Days on which there were significant differences between the groups are marked (* $p < 0.05$; ** $p < 0.01$).







5.5.1.5 IgG-positive cells

Values for IgG-positive cells in mammary secretions were much lower than those found for IgA and IgM. IgG-positive cells represented approximately 2% of the total lymphocyte population prior to IMM immunisation. After the 1st IMM immunisation, IgG-positive cells increased to around 4% then returned to baseline levels. A peak level of 6% of the total lymphocytes was attained three days after the 2nd IMM immunisation (Day 15). The percentage of IgG-positive cells then declined, and by Day 35 it was at pre-immunisation values (2%).

Data for IgG-positive cells are shown in Figure 5.7 for individual animal responses over the time course, grouped as either high- or low-responding animals. Group averages (± SEM) are also shown. There was no significant difference for IgG-positive cells when values for the High and Low groups were compared, for any of the days that were sampled.

Although the values of IgG-positive cells were low, Days 15 and 19 were significantly different from pre-IMM immunisation levels ($p < 0.05$) for the High group. There was no significant difference from pre-IMM immunisation levels for the Low group for any of the days following the IMM immunisations.

5.5.1.6 BB2-positive cells

BB2 antibody has been used in this study as a general B cell marker. This antibody is thought to recognise a cell-surface antigen, other than immunoglobulin, that is common to all mature B cells (Pallares *et al.,* 1999). B cells isolated from mammary secretions that stained positively for BB2 represented approximately 3% of the total lymphocytes prior to IMM immunisation. Animals in the High group showed a large variation in lymphocytes staining positive for BB2 whereas animals in the Low group showed a more uniform pattern of response.

Data for BB2-positive cells are shown in Figure 5.8 for individual animal responses over the time course, grouped as either high- or low-responding animals. Group averages (± SEM) are also shown.

Figure 5.7 Flow cytometry analysis of lymphocytes from mammary secretions for IgG-positive lymphocytes

Cells were isolated from mammary secretions collected at intervals following intra-mammary immunisation (at times marked with arrows). Graphs show the percentage of lymphocytes that stained positively for IgG: **A**, Individual animals in the High group; **B**, Individual animals in the Low group; **C**, Average response of the High and the Low group (± SEM).

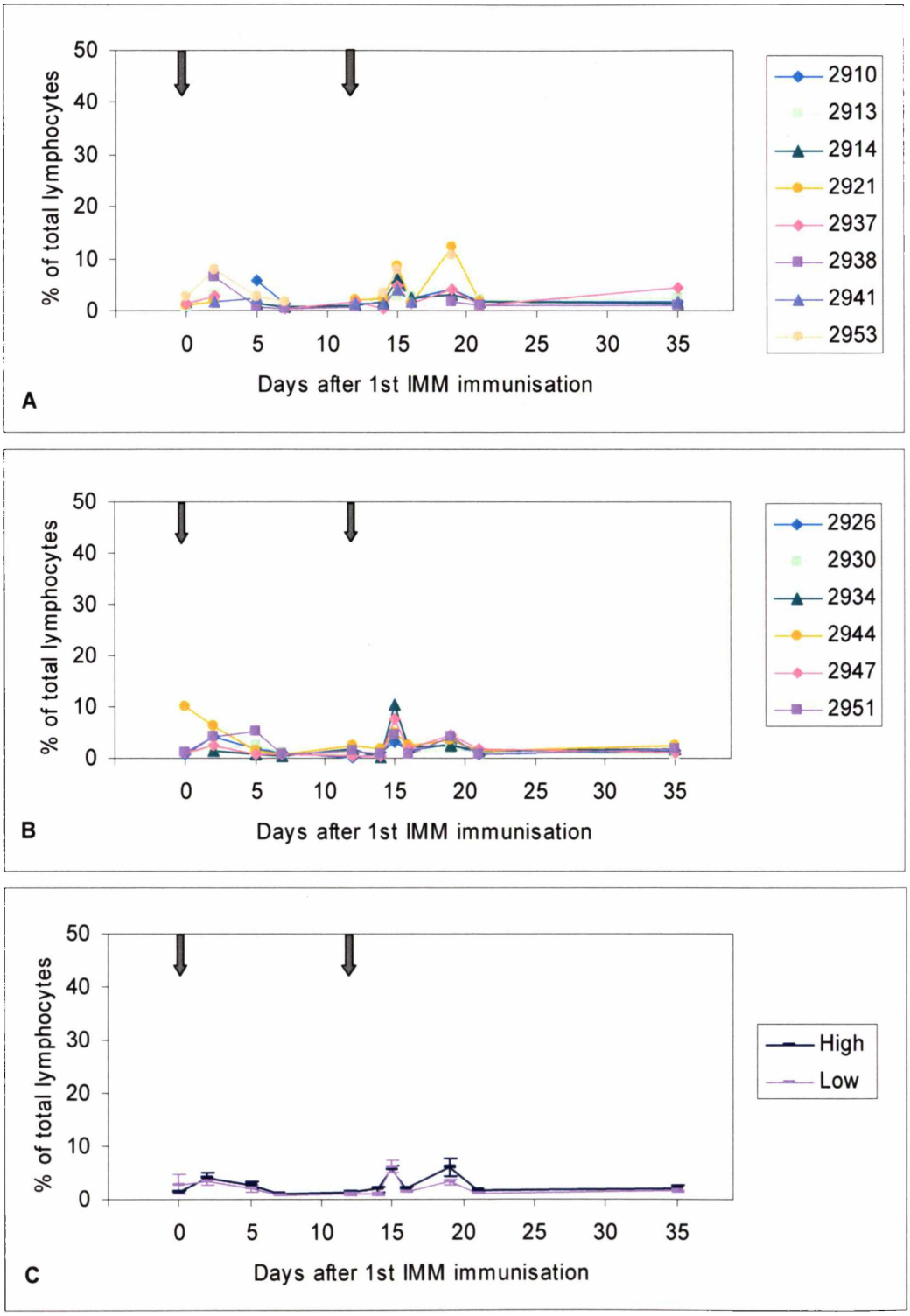
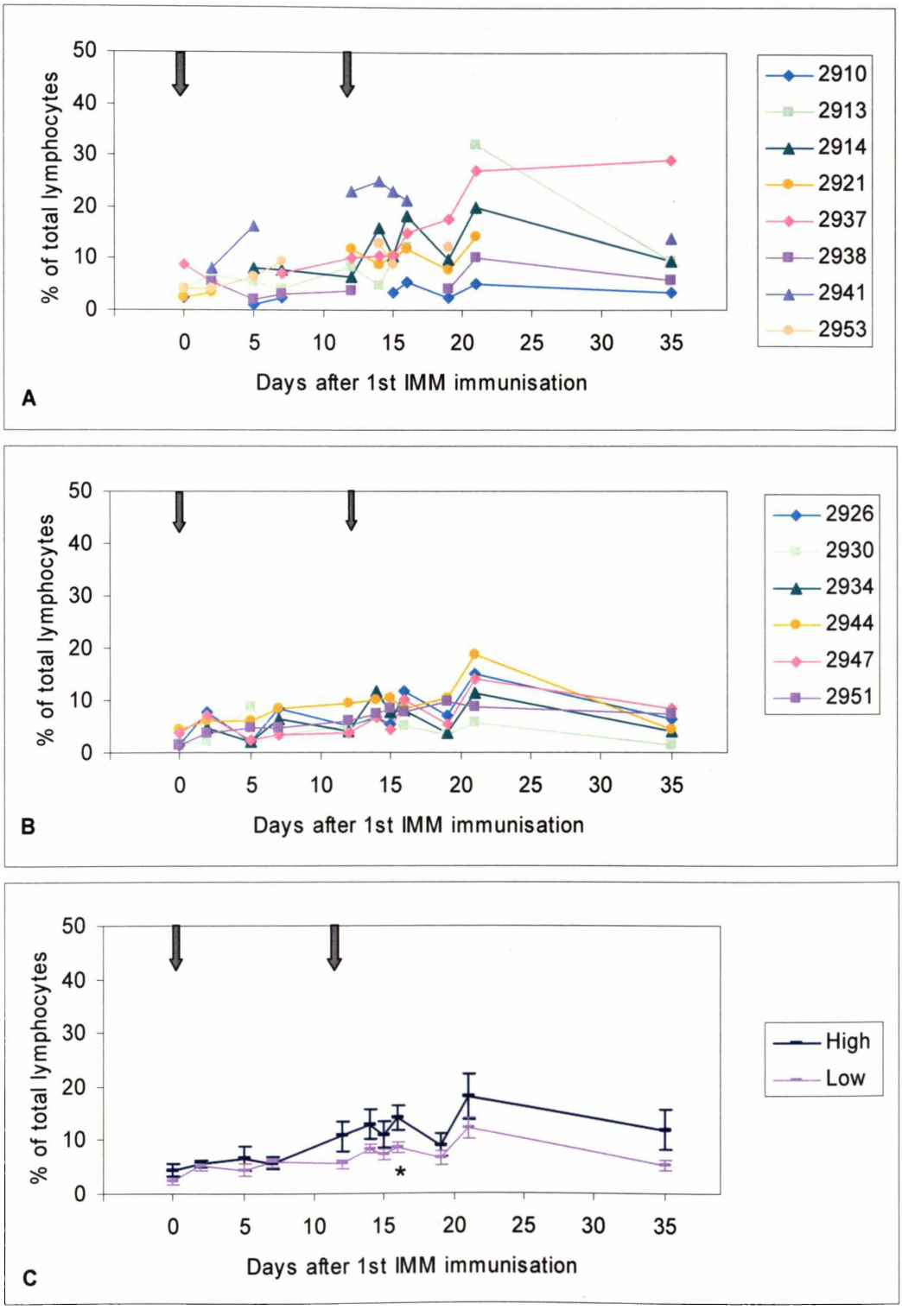


Figure 5.8 Flow cytometry analysis of lymphocytes from mammary secretions for BB2-positive lymphocytes

Cells were isolated from mammary secretions collected at intervals following intra-mammary immunisation (at times marked with arrows). Graphs show the percentage of lymphocytes that stained positively for BB2: **A**, Individual animals in the High group; **B**, Individual animals in the Low group; **C**, Average response of the High and the Low group (± SEM). Day on which there were significant differences between the groups are marked (* $p < 0.05$).



There was only a slight increase in the proportion of the BB2-positive cells in the total lymphocyte population following the 1st IMM immunisation. Following the 2nd IMM immunisation the percentage of BB2-positive cells increased from 3% of the total lymphocytes to an average peak of 18% for the High group and 12% for the Low group on Day 21 (nine days after the 2nd IMM immunisation). However, there were large variations in the values for the High group ranging from 5 to 32%, a six-fold difference, but only a three-fold variation (6 – 18%) in values for the Low group on this day.

By Day 35, the average percentage of BB2-positive cells for the Low group had returned to approximately pre-IMM immunisation values (5%). The average value of BB2-positive cells on Day 35 for the High group was still elevated at 12% of total lymphocytes compared with the pre-IMM immunisation value of 4% but again there was a large spread in values amongst the individual animals (3 – 29%), similar to Day 21.

Comparison of the average BB2-positive cell responses of the High versus the Low group over the time course determined that there was a significant difference on only one of the days sampled, Day 16 ($p < 0.05$).

Cells stained for the immunoglobulin isotypes IgA or IgM were double-stained with BB2 antibody to determine the proportion of cells that were positive for both cell-surface markers. There was no statistical difference between the High and Low groups in the relative proportions of cells that stained positively for both BB2 and IgA and both BB2 and IgM (data not shown). Therefore, the data are presented as a combined average of the High and Low groups. The percentages of total lymphocytes that stained positively for isotype IgA or IgM plus BB2, in comparison with the percentage of cells that stained positively for the immunoglobulin isotype alone are shown in Figure 5.9.

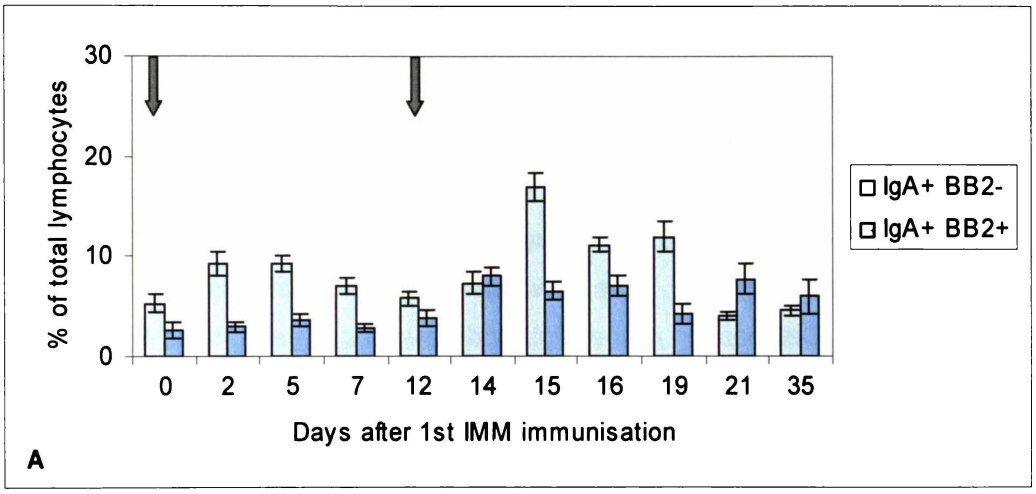
For all the samples collected over the time course, a larger percentage of the cells were positively stained with the immunoglobulin isotype antibody alone than were doubled-stained with the immunoglobulin isotype plus BB2 antibody.

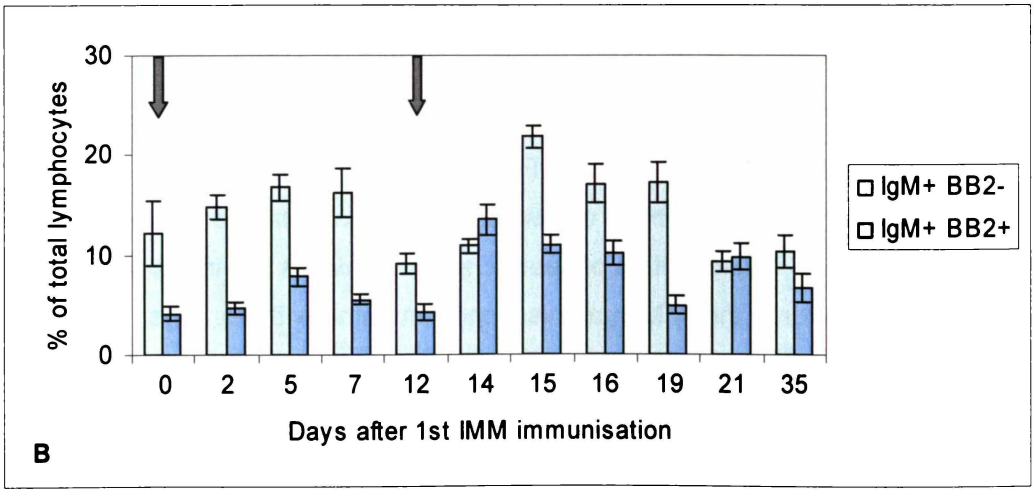
For IgA-positive cells, the percentage of cells that stained positively for IgA alone ranged from 4.0 to 17.0% and the percentage of cells that double-stained positively with both IgA and BB2 ranged from 2.8 to 8.0%. For IgM-positive cells, the percentage of cells that stained positively for IgM alone ranged from 9.0 to 21.8%

and the percentage of cells that double-stained positively for both IgM and BB2 ranged from 4.2 to 13.5%. The peak of cells positively doubled-stained was on Day 14 (two days following the 2nd IMM immunisation) for both IgA and IgM. In contrast, the peak of cells that stained positively only with the immunoglobulin isotype antibody was three days following the 2nd IMM immunisation, on Day 15.

Figure 5.9 Flow cytometry analysis of lymphocytes from mammary secretions double stained for immunoglobulin isotypes IgA and IgM, and BB2

Cells were isolated from mammary secretions collected at intervals following intra-mammary immunisation (at times marked with arrows). Cells were double-stained with immunoglobulin isotype-specific antibody and BB2 antibody. The data are for the combined average of the High and Low groups. The graphs show the average percentage of total lymphocytes (± SEM) that stained positively with antibody for cell-surface markers: **A**, IgA+BB2-; IgA+BB2+; **B**, IgM+BB2-; IgM+BB2+





5.5.2 *Analysis of antibody levels in mammary secretions following intra-mammary immunisation*

Mammary secretion samples collected from the mammary gland before and after the IMM immunisations were analysed by ELISA for specific anti-*C. albicans* IgA and total IgA antibody. Animal responses for the High and Low groups were compared. A ratio of the protein concentration to the level of antibody for individual mammary secretions was calculated. This adjusted for any dilution of the mammary secretion sample that may have occurred when saline infusion was used during the sample collection process.

5.5.2.1 Total protein

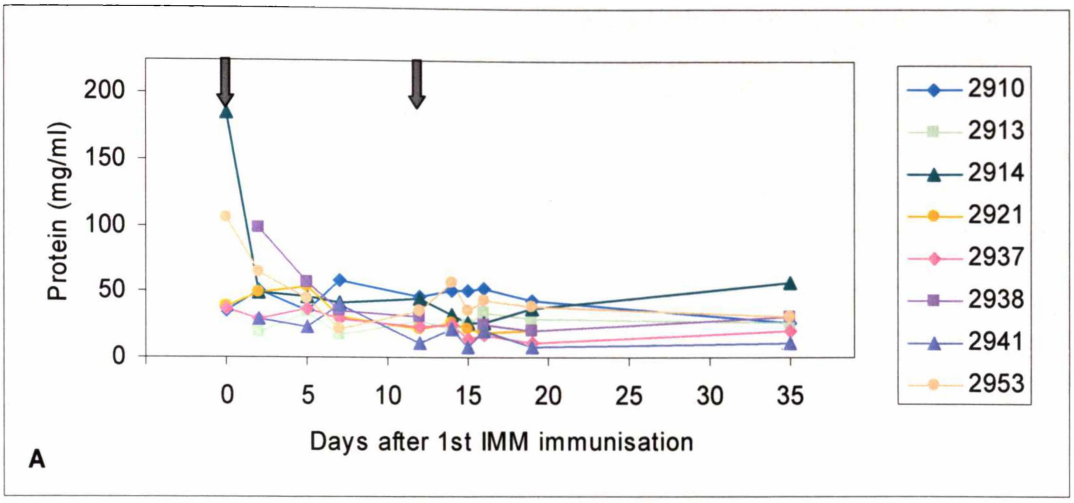
Data for total protein concentration in mammary secretions collected over the time course are shown in Figure 5.10 for individual animals, grouped as either high- or low-responding animals. Group averages (± SEM) are also shown. The initial protein concentrations, in samples collected prior to the 1st IMM immunisation, tended to be the highest of all times points measured but considerable animal-to-animal variation was observed, with values ranging from 15 – 185 mg/ml of protein. After the 1st IMM immunisation, concentrations decreased and plateaued at a level of 25 – 30 mg/ml of protein. The between-animal variability also decreased after the first sample. Comparison of the High and Low groups determined that there was no significant difference between the groups for protein concentrations on any of the days sampled.

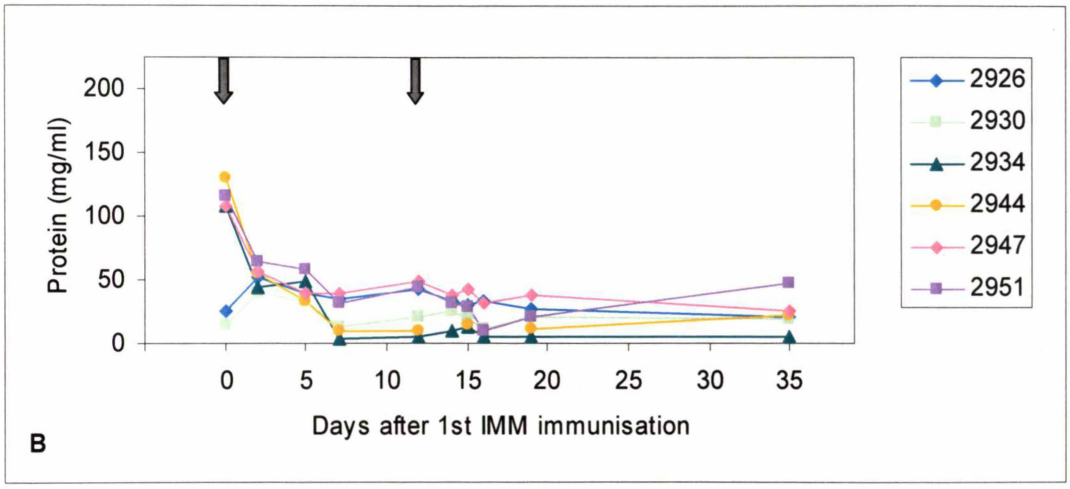
5.5.2.2 Specific anti-*C. albicans* IgA

Data for the ratio of specific anti-*C. albicans* IgA titres to protein concentration in mammary secretions are shown in Figure 5.11 for individual animals over the time course, grouped as either high- or low-responding animals. Group averages (± SEM) are also shown. Overall, titres of specific anti-*C. albicans* IgA in mammary secretions were higher prior to the IMM immunisations compared with the days following the two IMM immunisations. After the 1st IMM immunisation, titres fell then plateaued prior to falling again after the 2nd IMM immunisation. The titres then rose steadily and by Day 35 had returned to pre-IMM immunisation levels. The patterns of response for the High and Low groups were very similar but the antigen-specific IgA titre values in mammary gland secretions for the High group were considerably higher than for the Low group (3.5 – 13.0 times). This difference between the groups in antigen-specific

Figure 5.10 Analysis of the protein concentration in mammary secretions

Graphs show the protein concentration in mammary secretions collected at intervals following intra-mammary immunisation (at times marked with arrows): **A**, Individual animals in the High group; **B**, Individual animals in the Low group; **C**, Average response of the High and the Low group (± SEM).





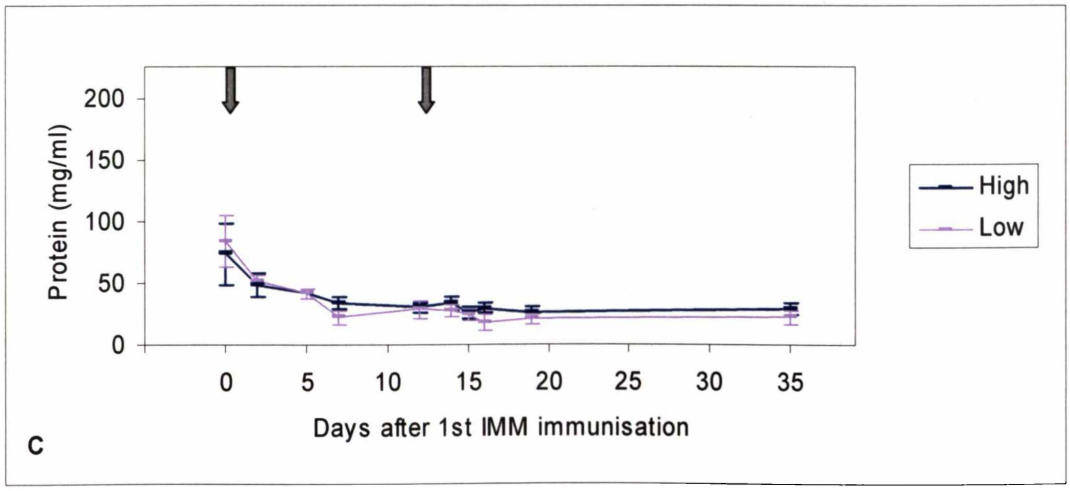
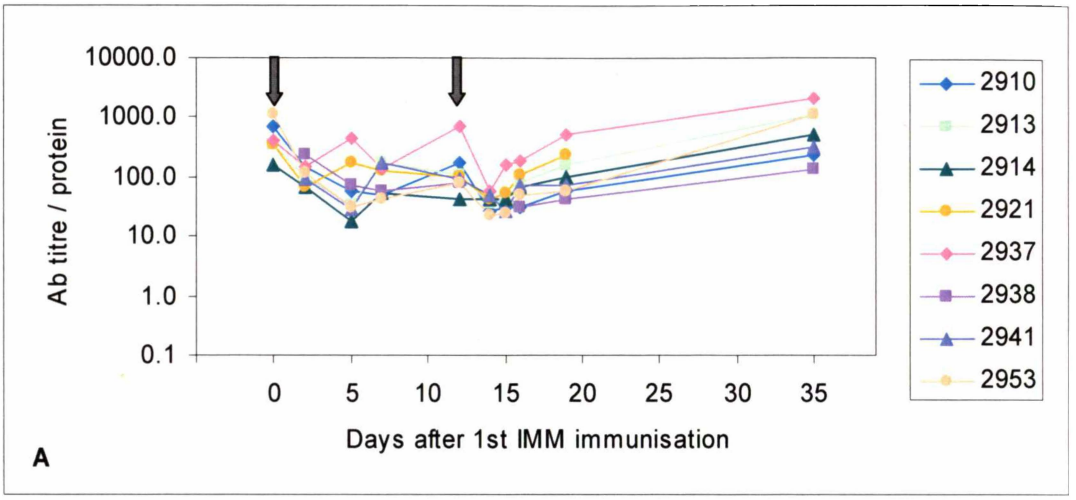
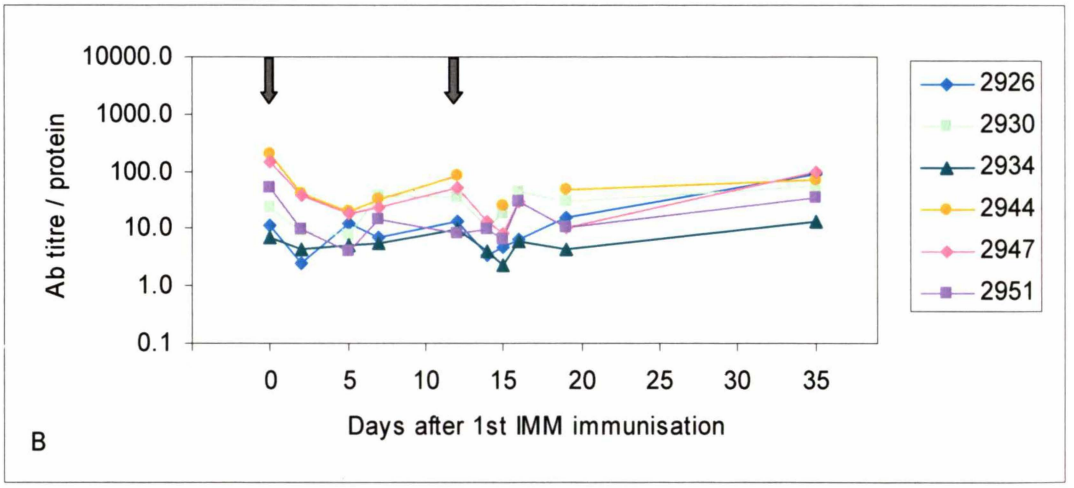
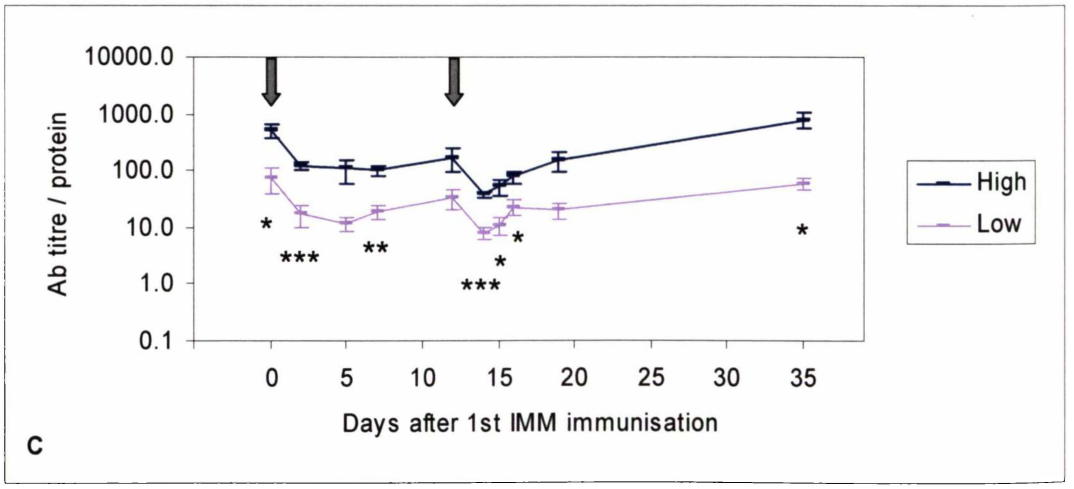


Figure 5.11 Analysis of the level of specific anti-*C. albicans* IgA in mammary secretions

Graphs show the ratio of the titre of specific anti-*C. albicans* IgA and protein concentration in mammary secretions collected at intervals following intra-mammary immunisation (at times marked with arrows): **A**, Individual animals in the High group; **B**, Individual animals in the Low group; **C**, Average response of the High and the Low group (± SEM). Days on which there were significant differences between the groups are marked (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).







IgA titre values was significant on all days sampled (p values ranging from < 0.05 to < 0.001), except Days 5, 12 and 19. In the High group, pre-IMM immunisation and Day 35 values were significantly higher than the other days sampled ($p < 0.05$). In contrast, the Low group showed no significant difference in titre values compared with pre-IMM immunisation values on any of the days over the time course.

5.5.2.3 Total IgA

Total IgA concentrations in mammary secretions were also adjusted for protein concentrations in the samples to allow for differences due to collection procedures, *i.e.* whether or not a saline infusion was required to obtain the mammary secretion sample. Data for the ratio of total IgA concentration to protein concentration are shown in Figure 5.12 for individual animal responses over the time course, grouped as either high- or low-responding animals. Group averages (± SEM) are also shown.

Similar to results for titres of antigen-specific IgA, levels of total IgA tended to be higher in the secretion sample collected prior to IMM immunisation compared with secretion samples collected after IMM immunisation. The results showed high between-animal variability, ranging from 7 – 50, with an average of 24.6 ± 4.8 for the High group and 16.7 ± 4.2 for the Low group. There was no significant difference between the groups for Day 0 total IgA values ($p = 0.126$). By Day 5 after the 1st IMM immunisation, the ratio of total IgA to protein had fallen to 11.7 ± 1.6 and 10.6 ± 1.4 for the High and Low groups, respectively. The level of total IgA remained constant over most of the time course, and then rose between Day 19 and 35 to 52.4 ± 6.6 for the High group and 30.5 ± 3.7 for the Low group. Day 35 was the only time-point where there was a significant difference between the two groups for the total IgA to protein ratio ($p < 0.05$).

Comparison of the titre of specific anti-*C. albicans* IgA and the total IgA concentration (both adjusted for protein concentration) is shown in Figure 5.13. For the High group a relationship between the titres of antigen-specific antibody and the total IgA concentrations ($r^2 = 0.7513$) was observed. For the Low group no such relationship was found (r^2 value = 0.1641).

Figure 5.12 Analysis of the total IgA concentration in mammary secretions

Graphs show the ratio of the total IgA concentration and protein concentration in mammary secretions collected at intervals following intra-mammary immunisation (at times marked with arrows): **A**, Individual animals in the High group; **B**, Individual animals in the Low group; **C**, Average response of the High and the Low group (± SEM). Days on which there were significant differences between the groups are marked (* $p < 0.05$).

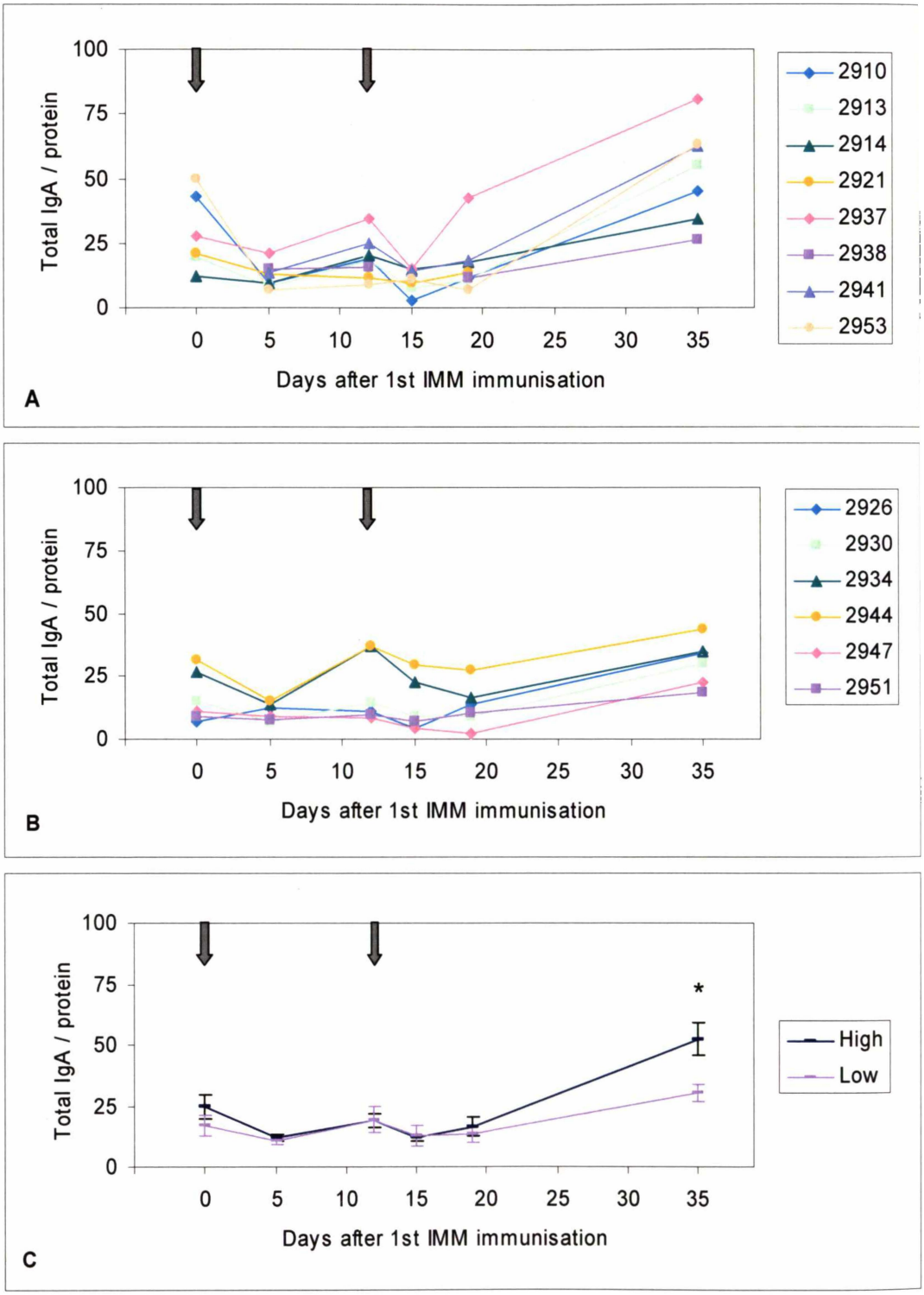
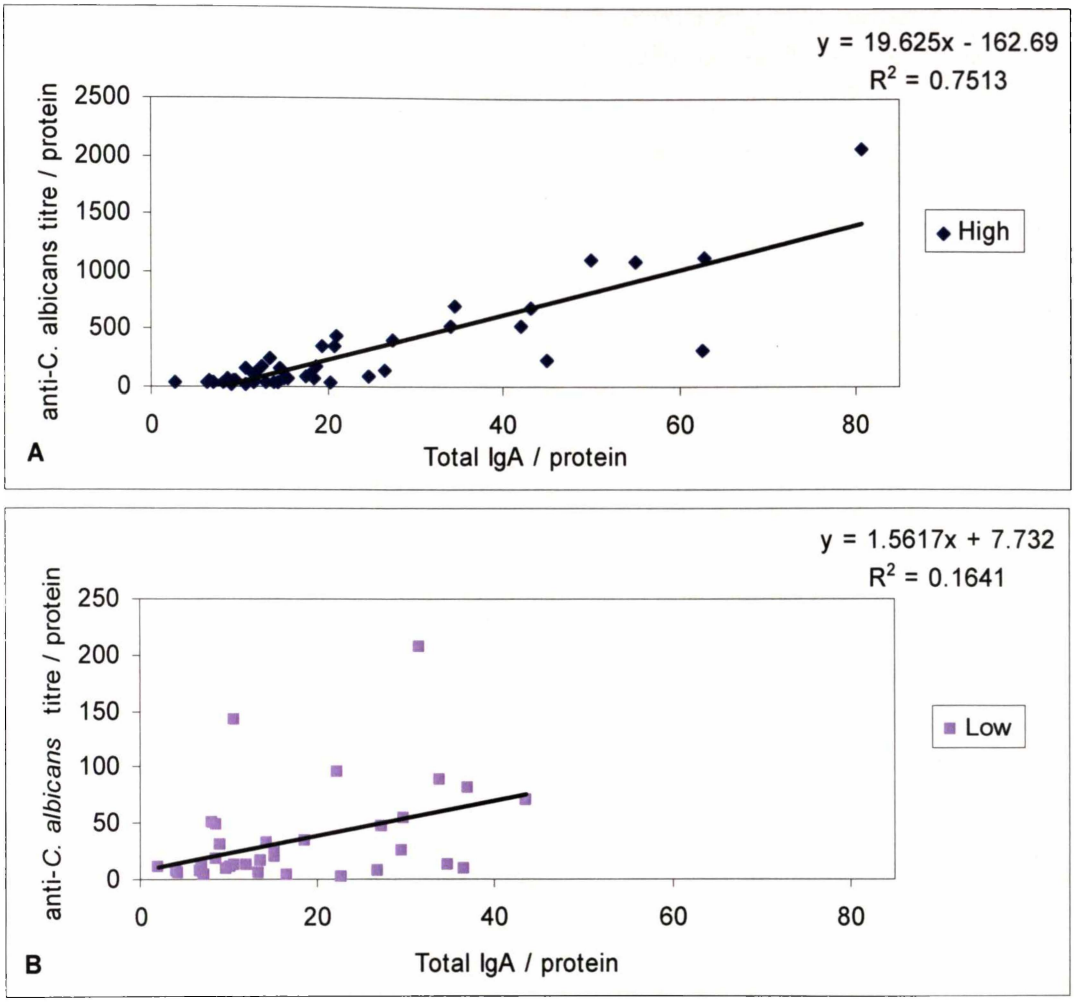


Figure 5.13 Comparison of concentrations of total IgA and titres of specific anti-*C. albicans* IgA in mammary secretions

Graphs show the correlation of total IgA concentrations and titres of specific anti-*C. albicans* IgA (both adjusted for protein) in individual animal mammary secretions collected over the time course: **A**, High group; **B**, Low group

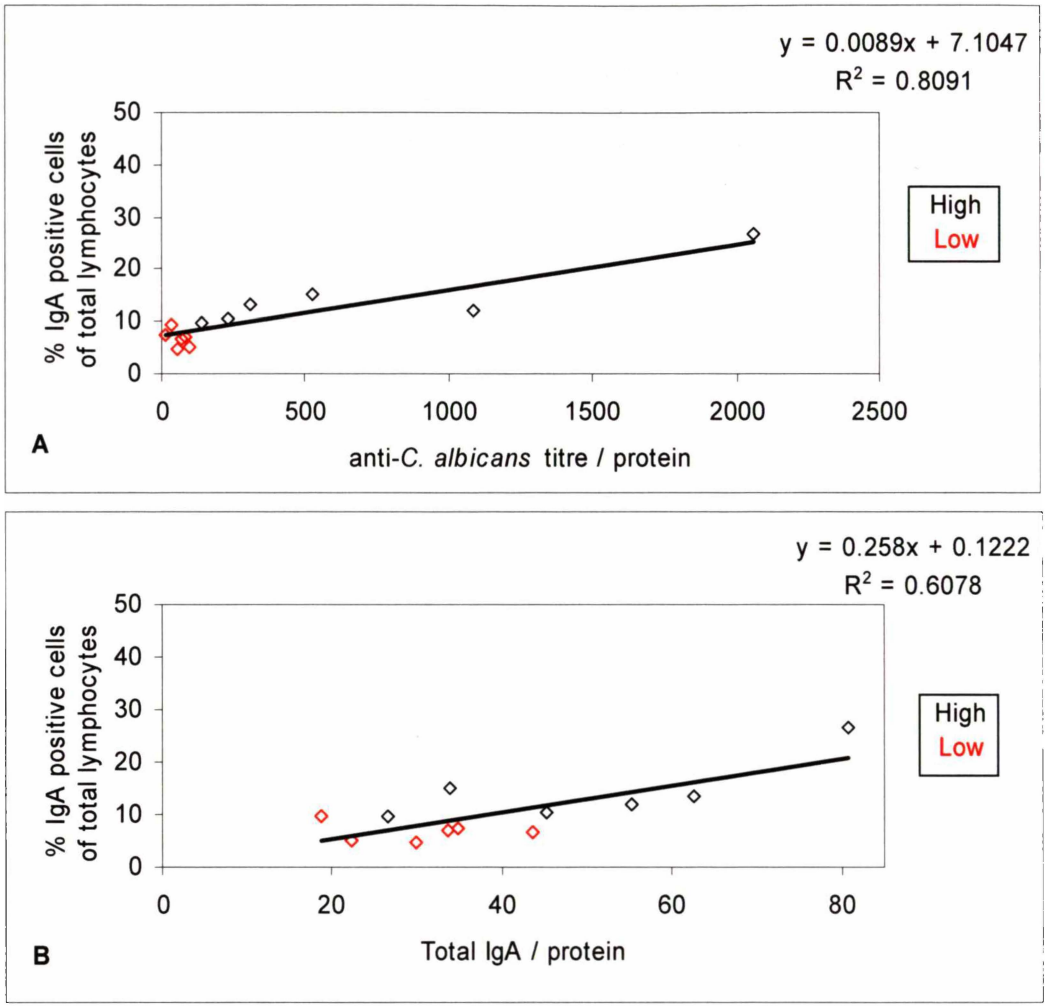


A comparison between the percentage of IgA-positive cells (of the total lymphocytes) and the titres of specific anti-*C.albicans* IgA (adjusted for protein concentration) in mammary secretions collected from all individual animals at the end of the time course (Day 35) is shown in Figure 5.14 A. A highly positive correlation was observed (r^2 value = 0.8091).

Comparison of the percentage of IgA-positive cells (of the total lymphocytes) and the total IgA concentrations (adjusted for protein concentration) in mammary secretions collected from individual animals on Day 35, also showed a positive correlation (r^2 = 0.6078). Data are shown in Figure 5.14 B.

Figure 5.14 Comparison between the percentage of IgA-positive cells and IgA antibody levels

Graphs show the correlation of the percentage of IgA-positive cells (of the total lymphocytes) and IgA antibody (adjusted for protein concentration) in mammary secretions collected on Day 35 following the 1st intra-mammary immunisation: **A**, IgA-positive cells and titres of specific anti-*C. albicans* IgA; **B**, IgA-positive cells and total IgA concentrations



5.5.3 *Analysis of antibody levels in colostrum and milk*

Colostrum samples collected from the left and right quarters of the udder were analysed to determine whether multiple sampling of mammary secretions from the left side had influenced the response to immunisation. Titre results for specific anti-*C. albicans* IgA (adjusted for protein) are shown in Table 5.7. Comparison of the data for the left and right sides revealed that there was no significant difference between antibody responses for the two sides of the udder ($p = 0.345$ and 0.657 for the High and Low groups, respectively).

Table 5.7 Comparison of antigen-specific IgA titres in colostrum samples from the left and right sides of the udder

The ratio of the average titre of specific anti-*C. albicans* IgA and protein concentration (± SEM) in colostrum samples collected from the left and right sides of the udder of the High and Low groups.

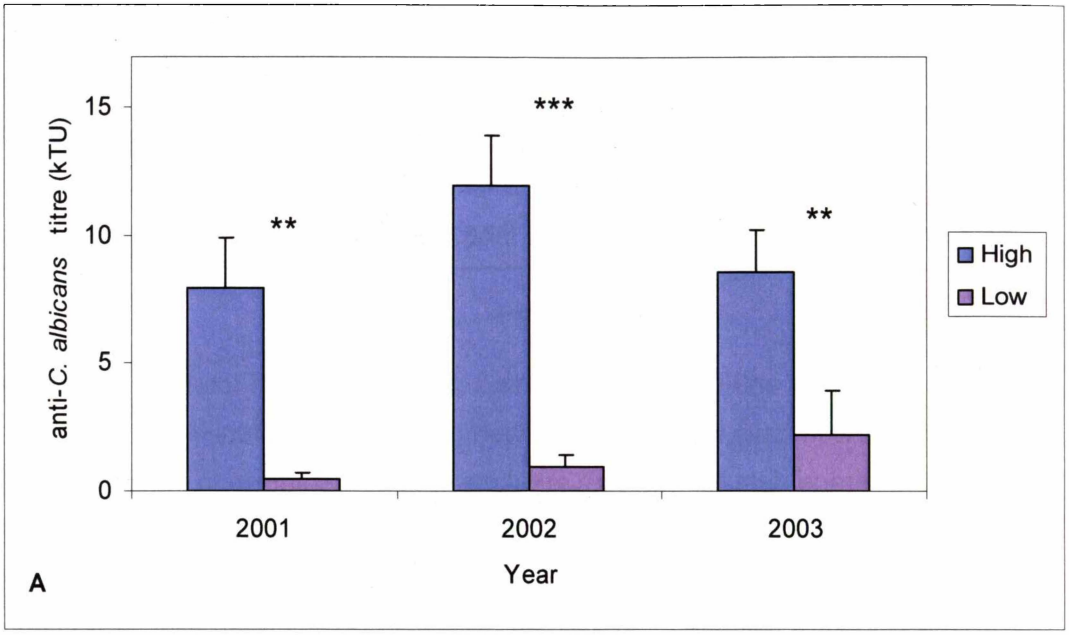
Side sampled	Ratio of antigen-specific antibody titre and protein concentration	
	High group	*Low group*
Left	182.77 ± 44.62	38.81 ± 21.07
Right	214.66 ± 48.91	34.88 ± 16.07

Titres of specific anti-*C. albicans* IgA and concentrations of total IgA in milk samples collected seven days after parturition were compared with results of the two previous years. Average data for the High and Low groups are shown in Figure 5.15. For the titres of specific anti-*C. albicans* IgA there was a significant difference between the High and Low groups in all three years ($p < 0.01$ in 2001, $p < 0.001$ in 2002, $p < 0.01$ in 2003). There was also a significant difference between the High and Low groups in all three years for concentrations of total IgA ($p < 0.01$ in 2001, $p < 0.001$ in 2002, $p < 0.05$ in 2003).

Comparison between titres of specific anti-*C. albicans* IgA (adjusted for protein concentration) in mammary secretions collected on different days are shown in Figure 5.16. There was no correlation between the antibody titres for Day 0 and Day 35 mammary secretion samples ($r^2 = 0.2821$). There was a weak correlation between the antibody titres for Day 35 mammary secretions and milks collected seven days *post-partum* ($r^2 = 0.5606$).

Figure 5.15 Comparison of antibody levels over three years of immunisation, in milks collected seven days *post-partum*

Graphs show data for average responses of the High and Low group (± SEM) for: **A**, Titres of specific anti-*C. albicans* IgA; **B**, Total IgA concentration. Years in which there were significant differences between the groups are marked (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)



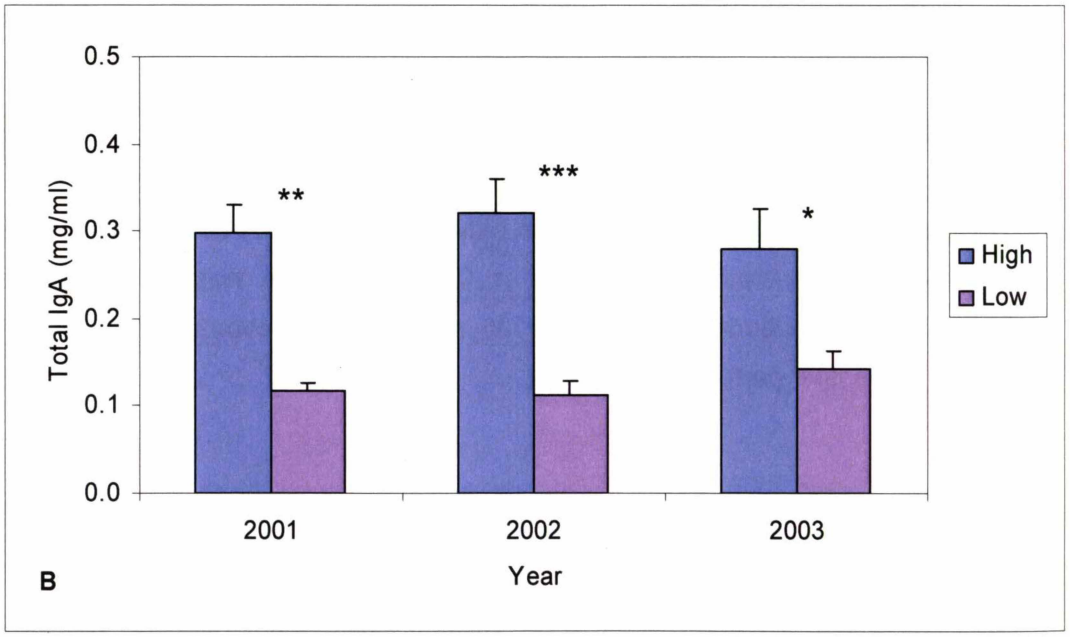
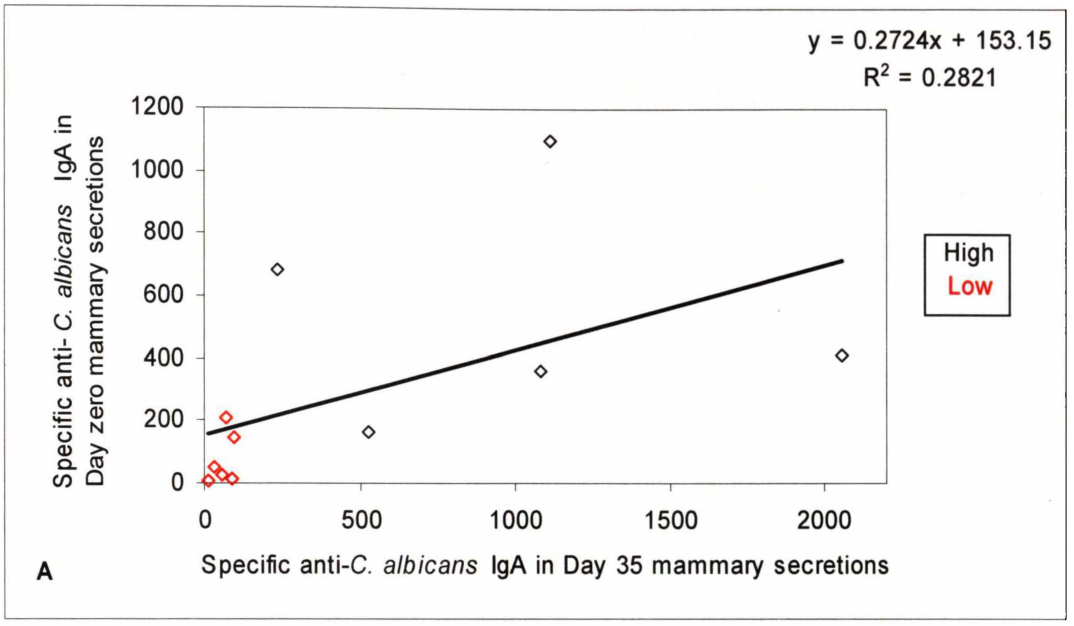
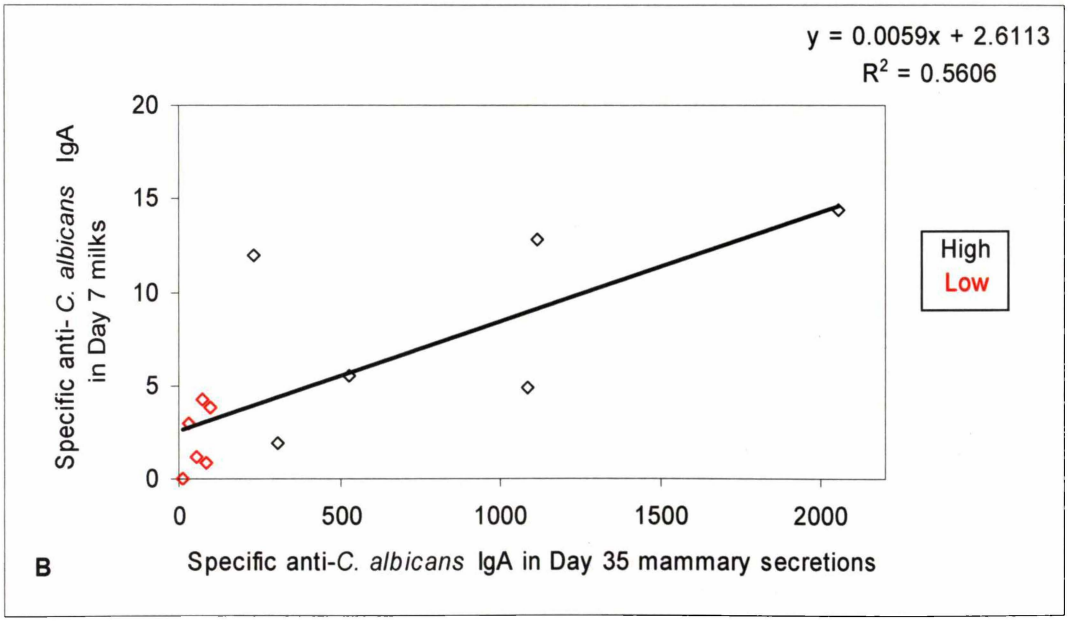


Figure 5.16 Comparison of titres of specific anti-*C. albicans* IgA in mammary secretions collected at different time points

Graphs show the correlation for: **A**, mammary secretions collected on Day 0 and Day 35 following the 1st intra-mammary immunisation; **B**, mammary secretions collected on Day 35 following the 1st intra-mammary immunisation and milk collected seven days *post-partum*





5.6 Discussion

In this chapter the cellular responses in the mammary gland to intra-mammary immunisation have been compared in animals that have been classified as high- and low-responding animals. The animals had been previously immunised and were assigned to their group based on their titre of specific anti-*C. albicans* IgA in the two preceding years, in milks collected seven days after parturition. The two groups were re-immunised using our immunisation regimen and the lymphocyte populations and the level of antibody were assessed in mammary secretions collected at intervals before and after the two IMM immunisations. In the current study, the response of the High and Low group to the multi-site immunisation was found to be similar to the two previous years in respect to antigen-specific IgA, *i.e.* animals in the High group produced significantly increased antigen-specific IgA titres compared with animals in the Low group.

The overall objective of the immunisation regimen is the production of IgA so the lymphocyte population of greatest interest in mammary gland secretions following immunisation was the IgA-positive cell type. The pattern of response shown by the IgA-positive cells appeared to be a classical immune response to a priming antigen dose followed by a booster dose. Data from the experiment showed that the percentage of IgA-positive cells increased following both IMM immunisations in a biphasic pattern and the increase was greater following the 2nd IMM immunisation. A distinct difference was observed in the pattern of response between the High and Low groups following the 2nd IMM immunisation, with respect to IgA-positive cells. The data suggests that the High group had a faster response to the IMM immunisation booster and sustained the response for a longer interval when compared with the response of the Low group. It is of interest to note that the response of both the High and Low groups peaked on the same day following the 2nd IMM immunisation (Day 15) and the peak percentage of IgA-positive cells was the same. However, overall it can be seen that animals that produced a high level of antibody in response to immunisation had a greater response to the immunisation with respect to the percentage of IgA-positive cells in mammary secretions compared with animals that produced a low level of antibody in response to immunisation.

The percentage of lymphocytes that were IgA-positive in Day 35 secretions correlated well with the antigen-specific antibody concentration for the same day. Previous studies have indicated that the IgA antibody is produced locally by cells in the mammary gland (Hodgkinson and Hodgkinson, 2003). Therefore, the observed

correlation implies that cells isolated from mammary secretions reflect the cellular environment of the mammary gland. This has been demonstrated by other researchers working with goat mammary glands. They found that mRNA extracted from cells isolated from milk and cells isolated from mammary gland tissue samples gave comparable results for gene analyses (Boutinaud *et al.,* 2002). The correlation between the percentage of IgA-positive cells and titres of antigen-specific IgA, in this study, suggests that the higher titres in the mammary secretions are due to an increase in the number of cells producing IgA rather than individual cells producing more IgA. This relationship also confirms the trend observed in Chapter Four, that higher numbers of IgA-positive cells correlate with higher antigen-specific IgA levels in secretions. However, the observation does not exclude the possibility that IgA-positive cells may demonstrate a range of IgA productivity.

The finding that there was no significant difference between the High and Low groups for the BB2-positive cell population would suggest that this population of lymphocytes was not involved in the cellular response to IMM immunisation. The observation that there was no significant increase in the percentage of the BB2-positive cell population prior to the increase in IgA-positive cells would suggest that the IgA-positive cells are trafficking to the mammary gland as differentiated plasmablasts rather than as B cells that are then differentiated in the mammary gland. The reason for the increase in the BB2 lymphocyte population observed on Day 21, nine days after the 2nd IMM immunisation and the similar increase in the percentage of neutrophils on this day could not be deduced from the data.

The very low percentage of IgG-positive cells compared with the percentage of IgA-positive cells is in contrast to the findings in Chapter Four where numbers of IgG- and IgA-positive cells in the tissue of the immunised mammary gland were comparable. One explanation is that the antibody used (sheep anti-bovine IgG heavy-chain specific) did not detect all of the IgG-positive cells using the flow cytometry cellular staining protocol, although this was the same antibody that had been used to successfully detect IgG-positive cells in tissues using the immunohistochemical staining protocol.

It should be noted that the flow cytometry method used for measuring lymphocyte populations would not have detected the presence of plasma cells in mammary secretions, only plasmablasts. This is because the method detects cell surface immunoglobulin, not intracellular antibody. Mature plasma cells down-regulate the B-

cell receptor and membrane immunoglobulin is low or absent on plasma cells (Calame *et al.,* 2003b).

Prior to IMM immunisations, the predominant lymphocyte population in mammary secretions was the T cell (~ 75% of total lymphocytes), in agreement with other studies for the dry (non-lactating) period of the cow (Park *et al.,* 1992; Yang *et al.,* 1997). The proportion of CD4-positive cells was higher than CD8-positive cells (49% and 29% of total lymphocytes, respectively), which is also in agreement with other studies (Inchaisri *et al.,* 2000; Yang *et al.,* 1997). As the overall percentage of T cells decreased following the IMM immunisation, the relative proportions of CD4- and CD8-positive cells changed. Prior to the IMM immunisation, CD8-positive cells comprised of 40% of the T-cell population. When the T-cell percentage was at its lowest point (Day 15, three days after the 2nd IMM immunisation) the CD8-positive cells made up only 25% of the T-cell population. This decrease in CD8-positive cells was the opposite result to that found in bovine mammary glands chronically infected with *S. aureus,* where the proportion of CD8 cells increased relative to CD4 cells (Riollet *et al.,* 2001). However, the response was similar to the pattern observed in mammary glands acutely infected with *E. coli* where CD4 cells increased relative to CD8 cells (Taylor *et al.,* 1997).

Overall, these findings would suggest that the type of immune response induced by our immunisation regimen is somewhat different from the immune response induced in a cow with chronic or acute mastitis. The relative decrease in the percentage of T lymphocytes with an increase in the percentage of CD4-positive cells compared with CD8-positive cells, along with an increase in plasmablasts, could indicate that our immunisation regimen induces a T-cell-dependent antibody response involving CD4 T cells, in contrast to a cell-mediated response involving CD8 T cells.

The T lymphocytes examined for the expression of the IL-2R, to determine if these cells were in an activated state, found that the greater majority of the CD4 cells in the mammary secretions were activated cells. In other studies where T cells expressing the IL-2R have been examined, it was found that only 10% of lymphocytes were activated in bovine milk and this proportion did not change six weeks post-involution in the dry period (Inchaisri *et al.,* 2000). No data have been reported for IL-2R expression on T cells in mammary secretions in late pregnancy. It is also of interest that there was a greater percentage of activated CD4 cells in the mammary gland prior to the 1st IMM immunisation, compared with three weeks after the 2nd IMM immunisation. These animals had been immunised the two preceding years, with the

same antigen. The finding of a high percentage of activated CD4 cells in the mammary gland prior to the 1st IMM immunisation may suggest that the priming IP/IM immunisation administered four weeks previously was already inducing an effect in the mammary gland.

The finding that there was no significant difference between the percentage of activated cells (CD4 or CD8) for the High and Low groups suggests that the T cells were part of an overall general immune response to the IMM immunisations. There was a significant increase seen in the percentage of activated CD4- and CD8-positive cells following the 2nd IMM immunisation. This may suggest that the memory T cells, upon re-exposure to antigen, proliferated with a second clonal expansion phase which was greater than the primary response, in a manner similar to that reported by others (Rogers *et al.,* 2000; Schluns and Lefrancois, 2003).

Analysis of the proportion of total lymphocytes in the entire population of cells isolated from the mammary secretion samples determined that the percentage of lymphocytes decreased after each IMM immunisation. There are several explanations for this reduction. This change may have been due to an influx of neutrophils, characteristic of an infected bovine mammary gland, leading to a relative decrease in the lymphocyte population. Rivas *et al* (2001) reported that prior to infection of the mammary gland, bovine milk contained approximately 7% neutrophils which then increased to 30 – 70% after acute infection. Another study found 3 – 22% neutrophils in uninfected bovine milk and 55 – 96% in chronically infected bovine milk (Riollet *et al.,* 2001). The numbers for uninfected cows are similar to those observed in this study in the mammary secretions prior to IMM immunisation (7%), although the percentage of neutrophils (30%) observed following IMM immunisation is at the lower end of the range found in infected animals (Riollet *et al.,* 2001; Rivas *et al.,* 2001). Other cells, including macrophages and dendritic cells, may also have increased in numbers after IMM immunisation, as has been reported to occur in the infected bovine mammary gland (Concha, 1986; Rivas *et al.,* 2001), and thereby decreased the proportion of lymphocytes in mammary secretions. Another explanation for the observed decreases in the proportion lymphocytes in this current study, may be that following IMM immunisation the mammary gland retained more lymphocytes in the tissues rather than releasing them into the gland cistern. In this study, secretion samples were collected to represent the cellular environment of the mammary gland tissues, but this may only be indicative. Future studies are required where corresponding tissue samples are collected to accurately validate the results.

It should be noted that the percentages of cells were not converted to numbers of cells, in this study. Although total cell counts were performed, these were counts of the cells isolated from the secretion sample. This did not always equate to the total number of cells in the sample. Samples that were collected two – five days after each IMM immunisation contained a gelatinous precipitate that made it difficult to extract all the cells from the samples. Therefore, although it would have been interesting to compare numbers of cells rather than percentages of the total cell population, this was not possible.

The level of antibody found in mammary secretions collected over the time course of the experiment also suggest that an immune response had already been activated in the mammary gland prior to the 1st IMM immunisation. The titres of specific anti-*C. albicans* IgA were elevated in Day 0 mammary secretions: the values of 509.6 ± 135.5 and 73.4 ± 33.5 kTU (adjusted for protein concentration) for the High and Low group, respectively were high compared with the value of 1.10 ± 0.45 kTU measured in secretions samples that were have obtained from untreated animals (n = 5) at a similar stage of pregnancy (unpublished data). The total IgA also appeared to be elevated in the secretion samples collected prior to IMM immunisation compared with that in later samples. However, the overall average of 20.29 ± 3.21 mg/ml was comparable to the total IgA in mammary secretions (24.31 ± 3.40 mg/ml) collected from untreated pregnant animals (n = 5; unpublished data). Others have reported levels of 0.54 mg/ml total IgA in the mammary secretions of dry cows, although these animals were not pregnant (Inchaisri *et al.,* 2000). It is interesting to consider the source of the antigen-specific antibody-producing cells. These cells may have been long-lived plasma cells recruited by the previous years' immunisation and resident in the mammary gland. Studies in mice have shown that plasma cells can live for at least a year (Slifka *et al.,* 1998).

There are several possible explanations for the finding that both antigen-specific and total IgA antibody levels in the secretions decreased after the 1st IMM immunisation and then increased again. Whether or not saline was used during the sample collection procedure could account for the observed decrease, however, antibody levels were adjusted for the protein concentration in the samples. Another possibility is that the observed fall in the antibody levels may have been due to the introduction of antigen to the mammary gland in the IMM immunisation. This antigen could have bound to antigen-specific antibody already present in the mammary gland and thus interfered with the measurement of this antibody by the antigen-specific ELISA method, resulting in lower levels of antibody being reported. But examination of the

data show that the decrease of antibody titre from Day 0 to Day 2 in individual animals ranged from approximately 3 – 1000 kTU, yet all the animals received the same antigen dose. If the introduced antigen was interfering in the ELISA then the level of decrease should have been constant. On the other hand, the possibility that different animals processed the antigen differently cannot be ruled out. A fall in total IgA was also observed which could not have been due to introduced antigen interfering in the ELISA method, and this may indicate that antigen binding to antibody was not the cause for the observation of a decrease in antibody levels. However, if the antigen-specific antibody was binding antigen then being removed by macrophages, this could account for the fall in the antibody levels observed. Another explanation could be that the IMM immunisations induced a change in the antibody production by the plasma cells, either by reducing the numbers of antibody-producing cells or inducing a change in the rate of protein synthesis by the plasma cells.

As described earlier, there was a significant difference ($p < 0.01$) between the High and Low groups in the titres of specific anti-*C. albicans* IgA in mammary secretions. However, there was a trend for the titres of antigen-specific IgA of the Low group to be higher than in previous years. This was also the case for the total IgA concentrations. Although the average concentration for total IgA for the two groups was still significantly different, the significance level had changed from less than 0.01 to less than 0.05, with a higher concentration of total IgA in the Low group compared with that in earlier years. The positive correlation between the antigen-specific IgA and total IgA in the samples for the High group and not the Low group would suggest that only the High group produced sufficient quantity of specific anti-*C. albicans* IgA to appreciably increase the concentration of total IgA.

The lack of significant difference between the titres of specific anti-*C. albicans* IgA in the colostrum samples collected from the left and right glands of the animals indicated that removing secretions samples and their cells from the mammary gland over the time course of the study had no effect on the final antibody response. This would indicate that the immunogen is dispersed rapidly and in support of this was the observation that there was no sign of the oil adjuvant in samples collected two days after the IMM immunisations. It is interesting that removal of cells contained in the secretions sample did not influence the final antibody response. This may suggest that the cells being released into the cistern of the gland are only a small proportion of the total number in the tissues of the mammary gland.

In summary, there were significant differences in the cellular responses to the IMM immunisations observed for the High and Low groups. The High group responded more rapidly to the 2nd IMM immunisation and sustained the response for longer when compared with the Low group. Overall the High group had an increased percentage of IgA-positive cells and this correlated with a greater antibody response. Therefore, this would imply that for animals to produce higher levels of antibody they require higher numbers of IgA-producing cells.

Chapter 6

Expression of key immunomodulatory genes in the bovine mammary gland

6.1 Introduction

The work in Chapter Six characterises the expression of various key immunomodulatory genes in the bovine mammary gland. Knowledge of gene expression profiles in the mammary gland may help elucidate the immune mechanisms involved in the recruitment of B cells to the bovine mammary gland, their differentiation and production of IgA for secretion into milk. Profiles of the expression of cytokine genes may provide insight into the type of immune response that is induced by our IgA immunisation procedure.

Many of the studies investigating the immune mechanisms in the healthy and infected mammary gland of the dairy cow have focused on the identification of the cells in milk, classifying the cell types by markers expressed on their cell surface. More recently, studies have investigated the mammary gland cells in more detail by characterising the immunoregulatory genes that are being expressed by these cells (Alluwaimi and Cullor, 2002; Riollet *et al.,* 2000b; Riollet *et al.,* 2001; Taylor *et al.,* 1997). Cytokine genes are of key interest because of their known functions. Of particular interest are those genes for the cytokines linked to the inflammatory process (TNF-α and IL-1) and the cytokines associated with the type Th1 (IL-2, IL-12, IFN-γ and TNF-β) and Th2 (IL-4, IL-5, IL-6, IL-10 and IL-13) immune responses (Kelso, 1995).

Cytokines act as intercellular messengers and have a crucial role in the signalling for inflammatory and immune processes. Central to the adaptive immune response is the production of IL-2. This cytokine stimulates T cells to express cytokines and drives clonal expansion and differentiation of activated T and B cells. Activated T cells are a major producer of IL-2 (Smith, 1988). In the cow, IL-2 mRNA has been detected in cells from normal and mastitic mammary glands (Alluwaimi and Cullor, 2002; Sordillo *et al.,* 1991b) although others have been unable to measure mRNA transcripts of this gene in milk cells (Riollet *et al.,* 2000b; Riollet *et al.,* 2001; Taylor *et al.,* 1997). Usage of IL-2 as adjuvant in the bovine mammary gland has been shown to increase specific immune response to keyhole limpet haemocyanin compared with Freund's incomplete adjuvant (Pighetti and Sordillo, 1995). In addition, infusion of IL-2 has been shown to induce recruitment of plasma cells into the involuting bovine mammary gland (Nickerson *et al.,* 1992).

IL-4 has a key role in the induction of activated B cells to proliferate and secrete IgG_1 (Kuhn *et al.,* 1991; Turaga *et al.,* 1993). IL-4 is produced mainly by T lymphocytes,

both CD4 and CD8 cells (Paul, 1991). In bovine cell culture studies, IL-4 has been shown to increase IgG_1 and IgE in preference to IgG_2 (Estes *et al.,* 1995). Studies that have investigated the transcription levels of IL-4 in the bovine mammary gland have failed to detect any levels of mRNA for this cytokine in cells isolated from milk collected from healthy or infected glands (Riollet *et al.,* 2000b; Riollet *et al.,* 2001; Taylor *et al.,* 1997).

IL-6 was first described as a cytokine that enhances immunoglobulin secretion by B cells (Kishimoto, 1989). It is secreted by many different cell types, including T and B cells and macrophages (Hirano *et al.,* 1989). The expression of IL-6 has been determined in the healthy bovine mammary gland at different physiological stages. IL-6 protein levels were higher in early colostrum compared with milk (Hagiwara *et al.,* 2000) but no significant IL-6 mRNA was detected in milk cells from the middle and late stages of lactation (Alluwaimi and Cullor, 2002). Other studies have found IL-6 mRNA expression in milk cells from mastitic animals to be greater than those in healthy animals, implicating IL-6 in the pathogenesis of mastitis (Taylor *et al.,* 1997). Studies have shown that glands infected with *E. coli* have increased transcription levels of IL-6 by 14 h (Shuster *et al.,* 1997) and earlier in endotoxin-infused mammary glands (Shuster *et al.,* 1993). However, in *S. aureus* infections transcription of IL-6 was less evident (Alluwaimi *et al.,* 2003).

IL-8, originally termed a cytokine, has been re-classified as the chemokine CXCL8 (Graves and Jiang, 1995). It is a well-known neutrophil-chemoattractant produced by stimulated T cells, macrophages and endothelial cells (Bickel, 1993). Using *in vitro* studies, it has also been demonstrated to be chemotactic to both human T and B cells (Bacon *et al.,* 1989; Schratzberger *et al.,* 1997). In the bovine mammary gland the primary role for IL-8 is to attract neutrophils into the infected gland. Bacterial endotoxin lipopolysaccharide (LPS) has been shown to induce the production of large quantities of IL-8 in bovine mammary epithelial cell lines (Boudjellab *et al.,* 1998). In studies investigating the effects of mastitis inducing pathogens in the bovine mammary gland, *E. coli* was shown to induce increased protein levels of IL-8 in milk, peaking at 16 hours (Bannerman *et al.,* 2004; Riollet *et al.,* 2000a). However, *S. aureus* challenge did not show an effect. Transcriptional levels of IL-8 mRNA are detectable in the healthy gland with no differences observed between mid- and late-lactational stages (Alluwaimi and Cullor, 2002).

IL-10 is described as an anti-inflammatory cytokine involved in the process of suppressing cell-mediated immunity while promoting humoral responses (Burdin *et*

al., 1997; Spits and de Waal Malefyt, 1992). It is produced by T cells and macrophages, and by B cells that are activated by CD40 ligand. In conjunction with IL-6, IL-10 acts to increase antibody production in B cells (Burdin *et al.,* 1997). In the bovine mammary gland, levels of IL-10 mRNA were low or negligible when determined in milk cells from healthy or mastitic cows (Riollet *et al.,* 2000b; Taylor *et al.,* 1997). In studies where the IL-10 protein was measured, a significant transient increase in the cytokine was observed in mammary gland quarters infected with *E. coli* but not with *S. aureus* (Bannerman *et al.,* 2004).

IL-12 is an important regulatory molecule that enhances the differentiation of Th1 type cells (Trinchieri, 1997). Produced by macrophages, IL-12 induces T cells to produce IFN-γ (Trinchieri, 1995). In the healthy bovine mammary gland, IL-12 mRNA has been shown to be elevated in late-lactation compared with mid-lactation (Alluwaimi and Cullor, 2002). A comparison of milk from healthy and mastitic cows showed transcriptional expression of IL-12 only in the infected cows (Taylor *et al.,* 1997), and an increase in mRNA and protein levels following *S. aureus* (Bannerman *et al.,* 2004; Riollet *et al.,* 2000b) and *E. coli* challenge (Bannerman *et al.,* 2004).

IFN-γ, which is produced predominantly by CD4 (Th1) and CD8 cells, is a major positive regulator of cell-mediated immunity (Farrar and Schreiber, 1993). In the bovine mammary gland, IFN-γ is an important mediator in the activation of recruited neutrophils and enhancement of their phagocytic activity (Wedlock *et al.,* 2000). In cell culture studies using bovine cells, IFN-γ has been shown to induce IgG_{2a} production by B cells rather than IgG_1 (Estes *et al.,* 1994). Similar to IL-6, IFN-γ protein was elevated in colostrum compared with early, mid- and late-lactation milk (Hagiwara *et al.,* 2000), although IFN-γ mRNA was present in cells from mature milk at all stages of lactation (Alluwaimi and Cullor, 2002; Hagiwara *et al.,* 2000). During infection with mastitis-inducing pathogens, IFN-γ protein production increased to high levels and remained elevated over an extended time (Bannerman *et al.,* 2004).

TNF-α is a potent pro-inflammatory cytokine produced by many different cells including epithelial cells, macrophages, monocytes and T cells. In conjunction with IL-1β, TNF-α mediates the inflammatory response at both the local and systemic levels (Vassalli, 1992). TNF-α initiates the migration of neutrophils to the site of infection by inducing VCAM-1 expression on endothelial cells (Van Kampen and Mallard, 2001). Infusion of recombinant TNF-α into a healthy bovine mammary gland results in an increase in the influx of neutrophils (Watanabe *et al.,* 2000). In addition,

TNF-α stimulation of phagocytic killing by bovine neutrophils has been demonstrated using *in vitro* studies (Rainard *et al.,* 2000). The protein concentration of TNF-α is higher in colostrum compared with that of later milk (Hagiwara *et al.,* 2000) and transcriptional levels of TNF-α are significantly elevated at middle and late lactation compared with levels for the cytokines IFN-γ, GM-CSF, IL-6 and IL-2 (Alluwaimi and Cullor, 2002). Conflicting results have been reported regarding expression of TNF-α mRNA in mastitic glands. Taylor *et al.* (1997) was not able to detect TNF-α mRNA expression in cells isolated from the milk of normal or mastitic cows. Alluwaimi *et al.* (2003) found elevated transcription levels of TNF-α in the *S. aureus* infected bovine mammary gland whereas Bannerman *et al.* (2004) detected TNF-α mRNA expression after *E. coli* intra-mammary infection and not *S. aureus* infection.

B-lymphocyte stimulator (BLyS), also termed B-cell activating factor, belonging to the TNF family (BAFF) and TNF- and ApoL-related leukocyte-expressed ligand (TALL-1), is a recently discovered member of the TNF super-family (Moore *et al.,* 1999; Schneider *et al.,* 1999). Three receptors for BLyS expressed by B cells have been identified: B-cell maturation antigen (BCMA) (Thompson *et al.,* 2000); BAFF-receptor (BAFF-R) (Thompson *et al.,* 2001) and transmembrane-activator and cyclophilin ligand interactor (TACI) (Xia *et al.,* 2000; Yu *et al.,* 2000). BLyS has been shown to be required for the prolonged survival of mature B cells and plasma cells (Avery *et al.,* 2003; MacKay and Browning, 2002; Schneider *et al.,* 2001). Expression of this cytokine has been detected in the spleen, lymph nodes, peripheral blood mononuclear cells, macrophages and dendritic cells (Moore *et al.,* 1999; Schneider *et al.,* 1999) and more recently it has been reported to be expressed by neutrophils (Scapini *et al.,* 2003). Macrophages and dendritic cells have been shown to directly regulate T-cell independent B-cell proliferation and the effect is mediated by BLyS (Craxton *et al.,* 2003). Expression of BLyS by macrophages and dendritic cells has been reported to be stimulated by IFN-γ and IL-10 (Nardelli *et al.,* 2001). There are no reports in the literature about the role of BLyS in the ruminant.

With regard to the production of IgA by B cells, cytokines act at multiple stages of B-cell development, differentiation and secretion of antibody. TGF-β plays a major role in the differentiation of B cells and has been shown to be essential in the class-switching process to IgA (Kim and Kagnoff, 1990; Lebman and Edmiston, 1999). The T-cell derived cytokines, IL-2, IL-4, IL-5 and IL-10 (either alone or in combination) have all been demonstrated to be involved in the increased production of IgA in mouse studies (Husband *et al.,* 1999). Transport of IgA into mammary

secretions is mediated by the polyimmunoglobulin receptor (pIgR). Following sequencing of the bovine pIgR gene (Kulseth *et al.,* 1995; Verbeet *et al.,* 1995), pIgR has been shown to be expressed in the mammary gland of the lactating cow (Verbeet *et al.,* 1995). Recently, expression of pIgR mRNA has been reported in the ovine mammary gland, with the highest expression during established lactation (Rincheval-Arnold *et al.,* 2002).

6.2 Aim and approach

The aim of the work in this chapter was to characterise the expression of various key immunomodulatory genes in the bovine mammary gland. To achieve this, mammary tissues that had been characterised for immune cell distribution and adhesion molecule expression in earlier chapters of this thesis were investigated. The tissues included:

- alveolar samples from the udders of healthy untreated cows at four different physiological stages of the mammary gland (Chapter Three) – late pregnancy, colostral phase, lactation phase and involution phase
- alveolar samples from the immunised and non-immunised (control) mammary glands of cows at two time points following the multi-site immunisation regimen (Chapter Four) – seven days following the 2nd IMM immunisation and five weeks *post-partum*, in established lactation

In addition, cells isolated from the mammary secretions and characterised for lymphocyte populations (Chapter Five), were also investigated. These samples were collected from immunised animals before, and at multiple times after, the IMM immunisations. These animals had been immunised in two previous years using our multi-site immunisation regimen and had been characterised as high- or low-responding animals based on their titre of antigen-specific antibody measured in milks collected seven days *post-partum*.

The objective was to determine transcription levels of genes for various cytokines in mRNA extracted from tissue and cell samples described above, using real-time PCR. The cytokines chosen were: IL-2; IL-6; IL-8; IL-10; IFN-γ; TNF-α; TGF-β; and BLyS. These cytokines were selected because they may be key immunomodulatory molecules in the immune response elicited by our IgA immunisation protocol, as determined by a literature search.

To measure the level of induction of IgA in tissues and cells of the immunised mammary gland, the transcription levels of IgA were also determined. Additionally, the gene expression of the receptor involved in the transcytosis of IgA across the mammary gland epithelium, pIgR, was quantified in the mammary tissue samples to determine whether this molecule is a limiting factor for IgA levels in bovine mammary secretions.

6.3 Materials

6.3.1 *Primers*

Oligonucleotide primers, used in PCR for detection of mRNA from the expression of genes in mammary tissues, are listed in Table 6.1 below. Primer sets were designed using Vector NTI bioinformatics software (Informax, Invitrogen) or obtained from a published bovine gene database (Coussens and Nobis, 2002).

Table 6.1 Oligonucleotide primers used for PCR

Target	Primer sequence (5' – 3')	Product size (bp)	Accession Number
IL-2*	F:GTGAAGTCATTGCTGCTGGA	202	AF348423
	R:GGTTCAGGTTTTTGCTTGGA		
IL-6	F:GACACCACCCCAGGCAGACTACTT	551	NM_173923
	R:GGAATGCCCAGGAACTACCACAAT		
IL-8	F:TGCTCTCTGCAGCTCTGTGTGAA	259	AF232704
	R:TCTTGCTTCTCAGCTCTCTTCACAA		
IL-10	F:GATGCGAGCACCCTGTCT	538	NM_174086
	R:CCGTTTACGTCATGGAGTCTA		
IFN-γ	F:AGCCAAATTGTCTCCTTCTACTTC	261	NM_174088
	R:CTGACTTCTCTTCCGCTTTCTG		
TNF-α	F:CCCCAGGGCTCCAGAAGTT	579	AF348421
	R:GGCGATGATCCCAAAGTAGACC		
TGF-β	F:GGGTGGCCGGGGAAAGTG	496	M36271
	R:CCGTGAATGGTGGCGAGGTC		
BLyS	F:CTCGAGCAGAAGCTTCAGGGCTCCGAA	458	BE753440
	R:CATATGGCCCTGCAGGAGGCAGAA		
pIgR	F:CATATGGTGTCCATCAAGTGCTACTA	307	L04797
	R:CTCGAGAAGGGGTTTGTTCTGGAT		
IgA**	F:GCGTGACTCCTGTGGCTGCTA	471	AF109167
	R:AGGCGGTCGATGGTCTTCTGG		
β-actin*	F:CAGAAGGACTCGTACGTGGG	200	AF191490
	R:TTGGCCTTAGGGTTCAGGG		

* Primer set obtained from a published bovine gene database (Coussens and Nobis, 2002)
** Heavy chain region
F - Forward primer
R - Reverse primer

6.4 Methods

6.4.1 *Sample collection and preparation*

Three sets of samples were analysed for gene expression. These were mammary gland tissues and mammary gland cells (isolated from mammary secretion samples) that had been previously studied in Chapters Three, Four and Five.

The first set of samples was collected from the alveolar areas of the udders of four untreated cows at each of four different physiological stages of the mammary gland - late pregnancy, colostral phase, lactation phase and involution (see Section 3.4.1).

The second set of samples was collected from eight cows that had been immunised using our multi-site immunisation protocol. Only one side of each cow's udder received the IMM immunisation while the other side was not immunised and acted as a control. Alveolar tissue was collected from immunised and control mammary glands, at two time points following the immunisation schedule (see Section 4.4.2). Four pregnant cows were sampled seven days following the 2^{nd} intra-mammary immunisation and four cows were sampled five weeks *post-partum*, in established lactation.

The third set of samples consisted of cells isolated from mammary secretions. Cows that had been previously immunised with our multi-site immunisation protocol were classified as high- and low-responding animals, based on their antibody response, as described in Section 5.4.1. These cows were re-immunised and mammary secretions collected over the time course of the two IMM immunisations (see Section 5.4.2). Based on the cellular data described in Chapter Five, only selected days were chosen for mRNA analysis: Days 2, 12, 14, 15, 19, and 35 following the 1^{st} IMM immunisation. Due to technical difficulties, no samples were available for Day 0. Samples from four high-responding cows and four low-responding cows were analysed for each selected day.

Tissues samples were processed and frozen as per the protocol outlined in Section 2.2.2.1. Mammary secretions were processed to extract the cells according to the protocol outlined in Section 2.2.2.3 and an aliquot of cells (10^7) was frozen at -80°C.

6.4.2 *Quantitative real-time PCR analysis*

Extraction and purification of mRNA from the tissue and cell samples was performed using the protocol outlined in Section 2.2.4.1. First-strand synthesis of cDNA from the purified mRNA was performed using the protocol outlined in Section 2.2.4.2. In order to determine the efficiency of the real-time PCR reaction, PCR products of cDNA were prepared for each gene by amplifying a mammary gland cDNA sample by PCR as described in Section 2.2.4.3. Annealing temperatures for PCR had been previously customised for each gene and are listed in Table 6.2. The products were isolated on agarose gel as described in Section 2.2.4.4, purified as described in Section 2.2.4.5 and five 10-fold serial dilutions prepared.

To determine the mRNA levels of target genes in unknown samples, real-time PCR was performed using the protocol outlined in Section 2.2.4.6 with the primers listed in Table 6.1 and annealing temperatures, customised for each gene, listed in Table 6.2. Five ten-fold serial dilutions of the PCR products for each gene were also analysed by real-time PCR to determine the efficiency of the real-time PCR reaction and to calculate the relative quantity of mRNA in unknown samples, as described in Section 2.2.4.6. The mRNA levels of the target genes were reported relative to the mRNA levels of β-actin. This adjusted for any variation in the quantity of total mRNA used for each reaction.

Table 6.2 The annealing temperature for the PCR and real-time PCR reaction, customised for target genes

Gene	Annealing temperature for PCR (°C)	Annealing temperature for real-time PCR (°C)
IL-2	62	65
IL-6	60	64
IL-8	55	59
IL-10	60	64
IFN-γ	51	64
TNF-α	64	64
TGF-β	60	64
BLyS	60	64
pIgR	62	65
IgA*	65	64
β-actin	60	60

* Heavy chain region

6.5 Results

The efficiencies of the real-time PCR reaction for the target genes, calculated by the Lightcycler software (Roche) as described in Section 2.2.4.6, are listed in Table 6.3.

Table 6.3 The efficiencies of the real-time PCR reaction for target genes

Efficiencies were calculated by the Lightcycler software (Roche) using the slope of the line found by plotting the log concentration of PCR products against their cycle number at the crossing point, for each of the five 10-fold serial dilutions of PCR product.

Gene	Efficiency of the real-time PCR reaction
IL-2	1.910
IL-6	1.977
IL-8	1.807
IL-10	1.839
IFN-γ	1.853
TNF-α	1.951
TGF-β	1.919
BLyS	1.837
pIgR	1.905
IgA*	1.814
β-actin	1.811

* Heavy chain region

6.5.1 *Gene expression profiles in the untreated mammary gland*

The levels of gene expression (relative to β-actin) in mammary alveolar tissue samples collected from untreated animals at each of four different physiological stages of the mammary gland (pregnant, colostral, lactation and involution) are listed in Table 6.4 for various key immunomodulatory genes. For many of the genes, there was a wide range in mRNA expression for animals within the groups. This variation is reflected in the large SEM values relative to the average value for the group. Many of the genes had low or undetectable levels of mRNA in the mammary alveolar tissues of the untreated animals.

Table 6.4 Expression level of genes (relative to β-actin) in the untreated mammary gland

Mammary alveolar tissues were collected from four untreated animals at each of four different physiological stages of the mammary gland - pregnant, colostral, lactation and involution. (Group average ± SEM, n = 4).

	Pregnant	Colostrum	Lactation	Involution
Cytokine				
IL-2	0.20 ± 0.20	1.03 ± 0.75	0.62 ± 0.39	0.55 ± 0.55
IL-6	0.22 ± 0.08	0.23 ± 0.21	0.82 ± 0.38	0.40 ± 0.20
IL-8	5.20 ± 1.72	19.8 ± 16.3	2.85 ± 2.12	9.08 ± 3.18
IL-10	0.01 ± 0.00	0.01 ± 0.01	0.02 ± 0.01	1.65 ± 1.63
IFN-γ	19.5 ± 7.47	0.01 ± 0.01	6.53 ± 3.76	9.79 ± 2.61
TNF-α	0.05 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
TGF-β	0.90 ± 0.20	0.81 ± 0.50	1.60 ± 0.26	0.49 ± 0.35
BLyS	0.13 ± 0.12	1.04 ± 0.90	0.02 ± 0.01	0.32 ± 0.30
Other				
pIgR	0.03 ± 0.02	0.00 ± 0.00	0.43 ± 0.34	0.04 ± 0.03
IgA*	4940 ± 1220	1660 ± 1040	8070 ± 3690	6440 ± 2340

* Heavy chain region

All samples of alveolar mammary tissue analysed had low or undetectable levels of IL-2 mRNA. The average expression of IL-2 was lowest in pregnancy compared with other physiological stages and highest in colostral phase tissues, however, there was no significant difference between the physiological stages.

Similarly, alveolar mammary tissues had low or undetectable levels of IL-6 mRNA. The average expression of IL-6 was lowest in the pregnant and colostral phase, compared with four-fold higher levels in the lactation phase and two-fold higher levels in the involution phase. However, there was no significant difference between the physiological stages.

The levels of expression of IL-8 in alveolar mammary tissues were variable amongst animals at each stage, ranging from undetectable to 68.56. The colostral group average of IL-8 mRNA was highest compared with two-fold, four-fold and seven-fold lower levels in the involution, pregnant and lactation groups, respectively. However,

these differences were not statistically significant due to the large variation amongst individual animals within each physiological stage

Levels of IL-10 mRNA were very low in all mammary tissues sampled except for one animal in the involution group. This animal had a level of 6.55 compared with an average of 0.01 ± 0.003 for the remainder of the animals.

The average level of mRNA for IFN-γ was highest in the pregnant stage and lowest in the colostral stage. The expression level for the colostrum group was significantly different from both the pregnant ($p < 0.05$) and involution groups ($p < 0.01$).

TNF-α gene expression was undetectable in the mammary alveolar tissues of all the animals tested except one and the level for this animal was very low (0.18).

Levels of TGF-β mRNA were similar for all physiological stages of the mammary gland. All but two of the animals had detectable levels of mRNA. Compared with pregnant and colostral stages, the TGF-β mRNA levels for the lactation phase were two-fold higher and for the involution phase two-fold lower, however, there was no significant difference between any of the physiological stages.

There were low levels of expression for BLyS in alveolar mammary tissues for all physiological stages of the mammary gland with only one or two animals per group having detectable levels. There was no significant difference in level of BLyS expression between any of the physiological stages.

The average level of pIgR mRNA was higher in the lactation stage compared with the other physiological stages of the mammary gland but only two of the four animals had detectable levels. Overall the pIgR mRNA levels were very low and there was no significant difference between any of the groups.

The level of IgA mRNA in the alveolar mammary tissue samples in the pregnant, lactation and involution groups were similar. In the colostral group the average IgA mRNA level was approximately 2.5 - 4.0 times lower compared with the other groups, although this difference was not significant. Of all the various genes, IgA had the highest expression level relative to β-actin expression.

6.5.2 *Gene expression profiles in the immunised mammary gland*

The levels of gene expression (relative to β-actin) in mammary alveolar tissues collected from the immunised and control (non-immunised) sides of the udder of cows treated with our multi-site immunisation regimen are shown in Figure 6.1 for IL-2, IL-6, IL-8, and IL-10 and Figure 6.2 for IFN-γ, TNF-α, TGF-β and BLyS. The level of gene expression was determined in tissues collected at two time points following immunisation: seven days after the 2nd intra-mammary immunisation in pregnant cows and five weeks *post-partum* in lactating cows.

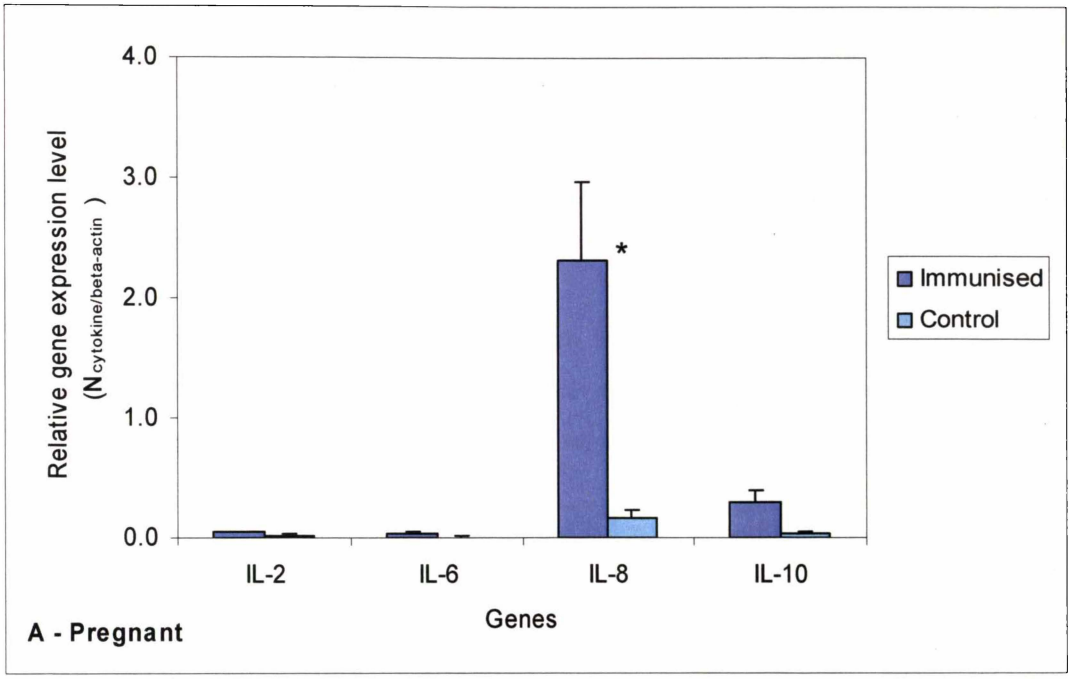
IL-2 mRNA levels were low in both the immunised and control mammary alveolar tissue samples collected from the pregnant (0.03 ± 0.01) and lactating animals (0.07 ± 0.06). There was no significant difference in IL-2 expression between the immunised and control mammary glands sampled at the two different times points. There was also no significant difference for the level of IL-2 expression between the two time points in either immunised or control glands. The pattern of expression of IL-6 was very similar to that of IL-2.

For IL-8, in the mammary alveolar tissues collected from the pregnant cows seven days after the 2nd IMM immunisation, there was a 15-fold increased level of mRNA in the immunised glands (2.31 ± 0.66) compared with the control glands (0.16 ± 0.08), and this difference was statistically significant ($p < 0.05$). A similar difference was not observed in mammary alveolar tissues collected from the lactation group five weeks *post-partum*. In the lactation group, the average IL-8 mRNA levels in the alveolar tissues of the immunised and control mammary glands were similar to the levels in the control mammary glands of the pregnant group.

The level of expression for IL-10 mRNA was low, ranging from undetectable to 0.58. In the pregnant group, although the average level of IL-10 expression was seven-fold higher in the mammary alveolar tissues of the immunised glands compared with the control glands, there was no significant difference between the two sets of samples ($p = 0.06$). In the lactation group, there was no detectable expression of IL-10 in the immunised mammary glands compared with an average level of 0.16 ± 0.09 in the control mammary glands, but this difference was not significant.

Figure 6.1 Comparison of the expression levels of the immunised mammary gland versus control gland for the cytokines IL-2, IL-6, IL-8 and IL-10

Alveolar tissue samples were collected from the immunised and control (non-immunised) sides of the udder. Graphs show the expression levels of target genes (relative to β-actin) in: **A**, samples from pregnant animals seven days after the 2nd intra-mammary immunisation; **B**, samples from lactating animals five weeks *post-partum*. (Group average ± SEM, n = 4). Significant differences between the groups are marked (* $p < 0.05$)



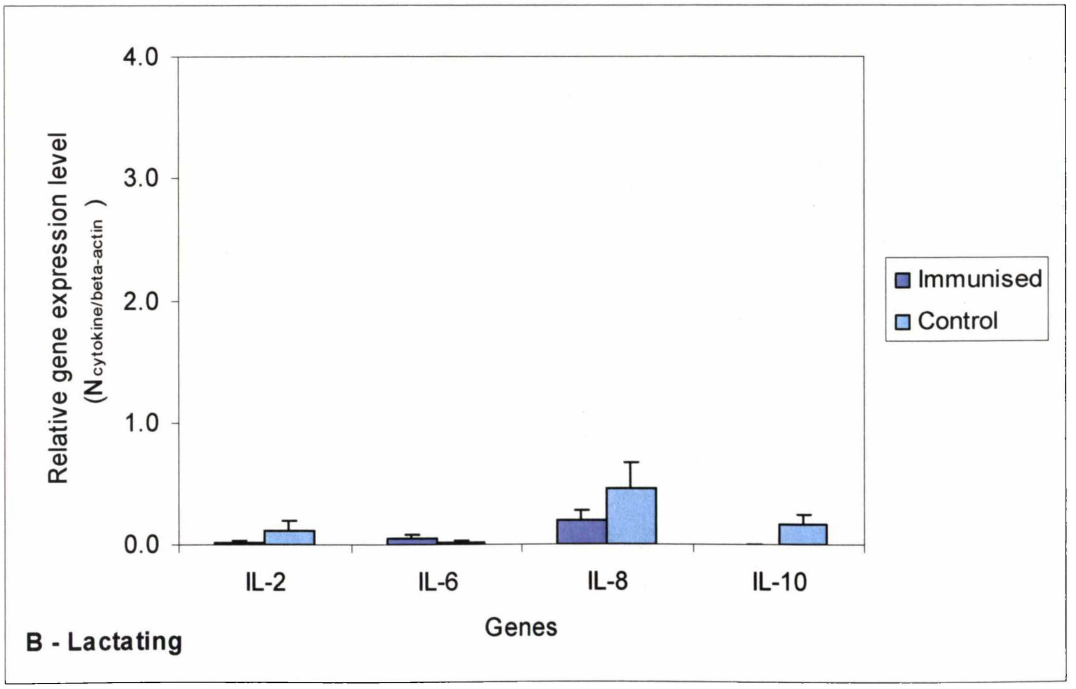
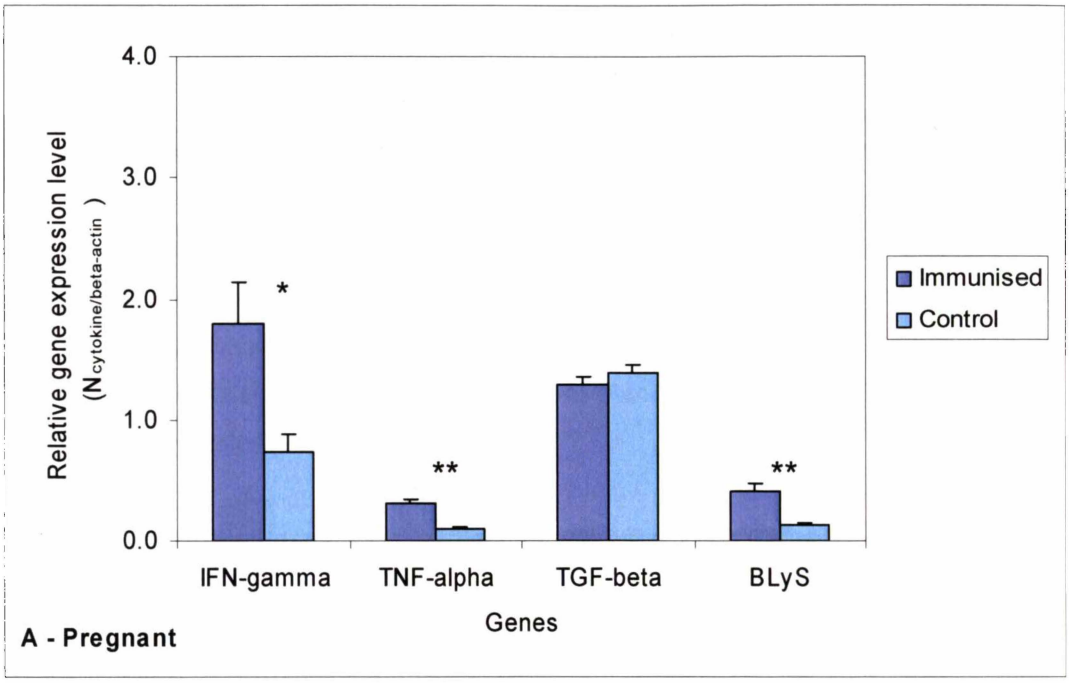
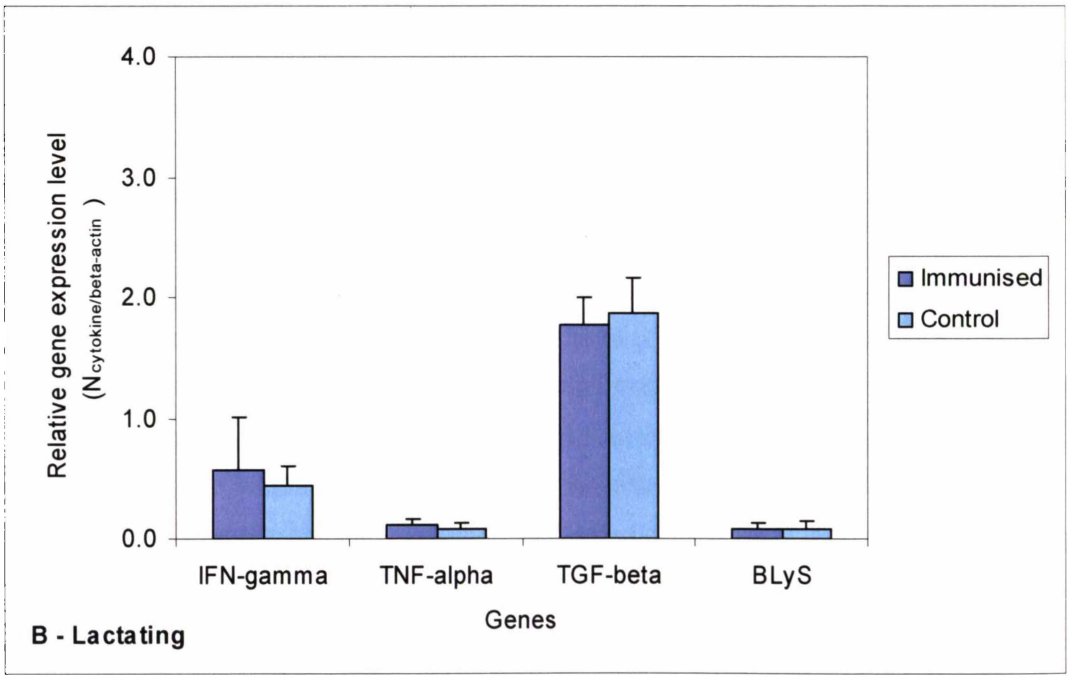


Figure 6.2 Comparison of the expression levels of the immunised mammary gland versus control gland for cytokines IFN-γ, TNF-α, TGF-β and BLyS

Alveolar tissue samples were collected from the immunised and control (non-immunised) sides of the udder. Graphs show the expression levels of target genes (relative to β-actin) in: **A**, samples from pregnant animals seven days after the 2nd intra-mammary immunisation; **B**, samples from lactating animals five weeks *post-partum*. (Group average ± SEM, n = 4). Significant differences between the groups are marked (* $p < 0.05$, ** $p < 0.01$)





In the pregnant group, the average level of expression for IFN-γ was increased two-fold in the alveolar tissues of the immunised mammary glands (1.79 ± 0.34) compared with the control mammary glands (0.74 ± 0.15), and this difference was statistically significant ($p < 0.05$). In the lactation group, the average IFN-γ mRNA levels in the alveolar tissues of the immunised and control mammary gland were similar to the levels in the control mammary gland of the pregnant group.

Although the expression for TNF-α was low, there was a significant difference between the alveolar mammary tissues from the immunised and control glands when the samples for the pregnant group were compared ($p < 0.01$). The level of TNF-α mRNA in the lactating group, both the immunised and control mammary glands, was similar to the value for the control tissue samples of the pregnant group.

There was no significant difference between the TGF-β mRNA for the immunised and control alveolar mammary gland tissue samples for both the pregnant and lactation groups. The level of TGF-β mRNA was similar at the two time points.

In the pregnant group, there was a three-fold increased level of BLyS mRNA in the alveolar tissue samples of the immunised mammary glands (0.40 ± 0.07) compared with the controls (0.13 ± 0.02) and this difference was statistically significant ($p < 0.01$). The average level of BLyS expression in the alveolar tissue of both immunised and control samples of the lactation group was similar to the control samples of the pregnant group.

Overall the levels of pIgR mRNA were low in the alveolar mammary tissues of the pregnant group and while the expression was three-fold higher in immunised tissues (0.11 ± 0.04) compared with the control tissues (0.04 ± 0.01), this difference was not significant ($p = 0.1$). Levels of pIgR mRNA in the immunised and control tissues for the lactation group were very similar (0.43 ± 0.12 and 0.51 ± 0.13, respectively) and significantly higher compared with those levels in the pregnant group ($p < 0.05$).

The level of IgA mRNA in the alveolar mammary tissues of the pregnant immunised mammary glands was three-fold higher compared with the control mammary glands (771.1 ± 108.4 and 262.2 ± 85.6; $p < 0.01$). In the lactation group the level of IgA mRNA in the immunised (998.7 ± 108.4) and control tissues (958.4 ± 85.6) was similar to the immunised tissues in the pregnant group. The transcription levels of IgA and pIgR correlated poorly ($r^2 = 0.39$).

6.5.3 *Gene expression profiles in cells isolated from mammary secretions following intra-mammary immunisation*

The levels of gene expression (relative to β-actin) for cells isolated from bovine mammary secretions on Days 2, 12, 14, 15, 19 and 35 following the 1st IMM immunisation are shown in Figure 6.3 for IL-8, IL-10 and IFN-γ and in Figure 6.4 for TNF-α, TGF-β and IgA. The level of gene expression in samples was determined for both high- and low-responding animals, however, no significant difference was found between the two groups for their level of expression. Therefore, data for the same day are pooled for the two groups.

When interpreting the data for the mammary secretion cells, it needs to be remembered that the proportions of the different cell populations within the mammary secretion samples were not constant. Lymphocyte percentages fell following each of the IMM immunisations and in addition the percentages of neutrophils also changed over the time course, in relation to other cell types (see Figure 5.1). In the approach taken here, the source of mRNA from day-to-day may be from a varied population of cells. Cytokine mRNA levels may have been a contribution from one type of cell or from several types of cells. In addition, due to technical difficulties, no sample of cells was retained for mRNA analysis for Day 0 and therefore, there was no baseline value obtained for comparison with post-IMM immunisation values.

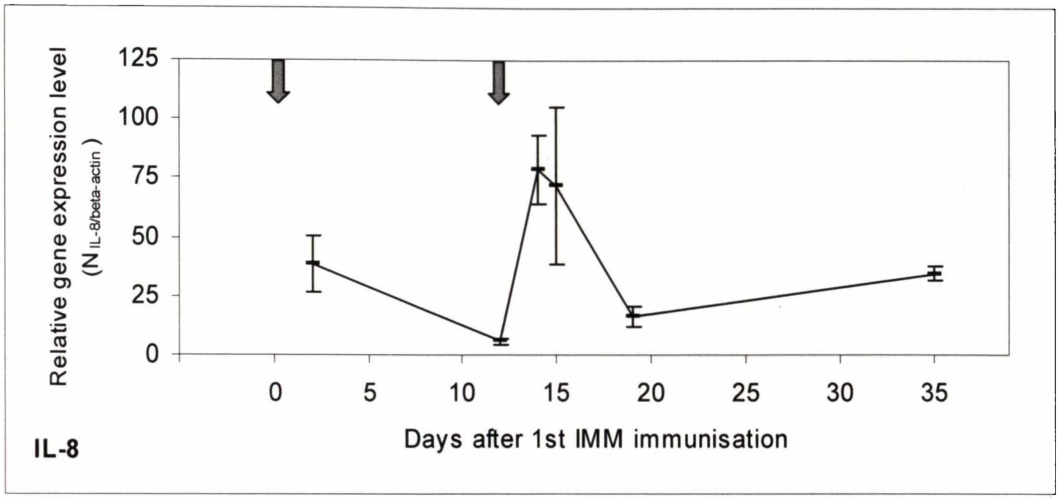
There were very low levels of mRNA expression for IL-2 and IL-6, in cells isolated from mammary secretions, for all days analysed (data not shown), with approximately half of the samples having levels being below the level of detection.

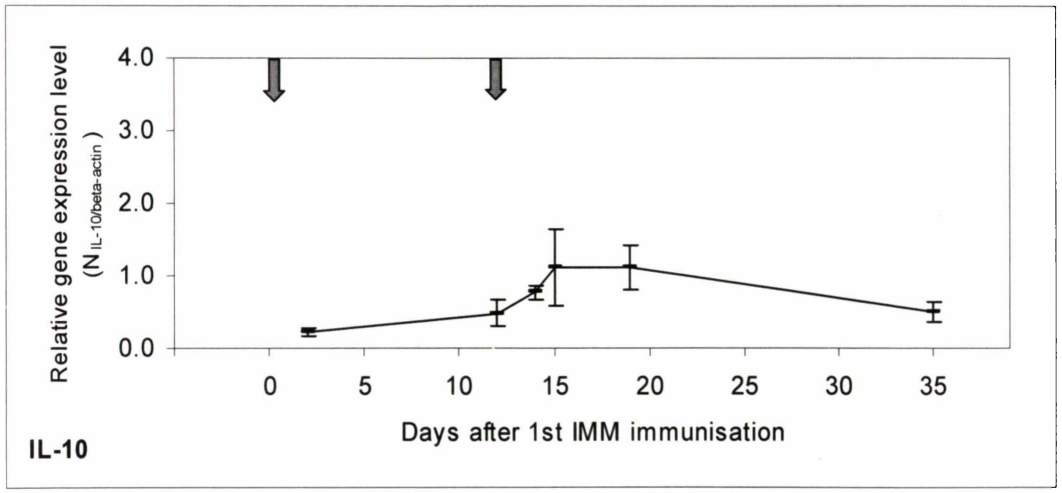
The expression of IL-8 in cells isolated from mammary secretions appeared to be higher in the days shortly after IMM immunisation (Day 2, 14 and 15) compared with the other days analysed. Levels of IL-8 mRNA were 14-fold higher on Day 14 (two days after the 2nd IMM immunisation) compared with Day 12 (the day of the 2nd IMM immunisation) and this difference was highly significant ($p < 0.001$).

IL-10 levels of mRNA in cells isolated from mammary secretions were generally low over the time course. On Day 15 and 19 (three and seven days after the 2nd IMM immunisation, respectively) some individual animals had increased levels of IL-10 mRNA. However, the average values for all animals at these time points were not significantly different from other days analysed.

Figure 6.3 Relative gene expression of the cytokines IL-8, IL10 and IFN-γ in the mammary secretion cells

Cells were isolated from mammary secretions collected at intervals following intra-mammary immunisation (at times marked with arrows). Graphs show the level of gene expression (relative to β-actin) in the cells. (Combined average of the high- and low-responding animals ± SEM, n = 8)





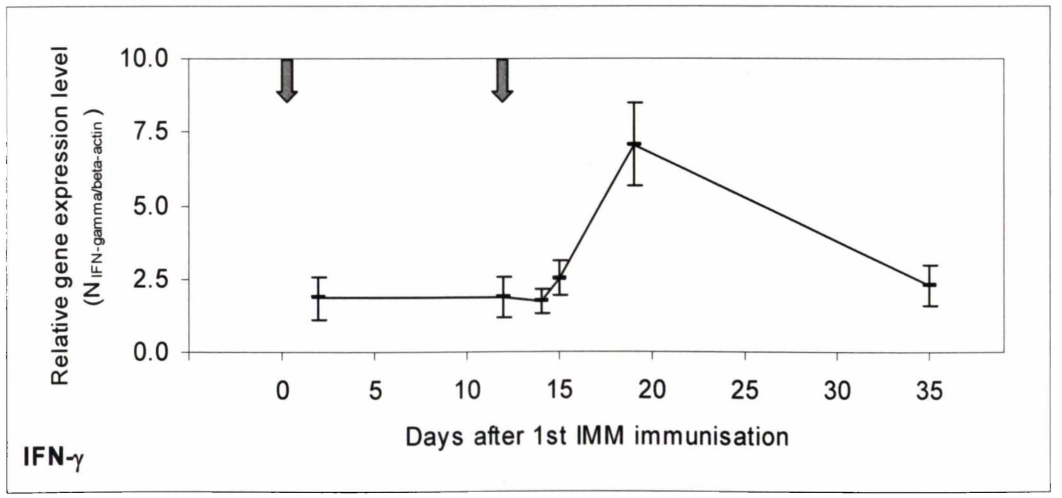
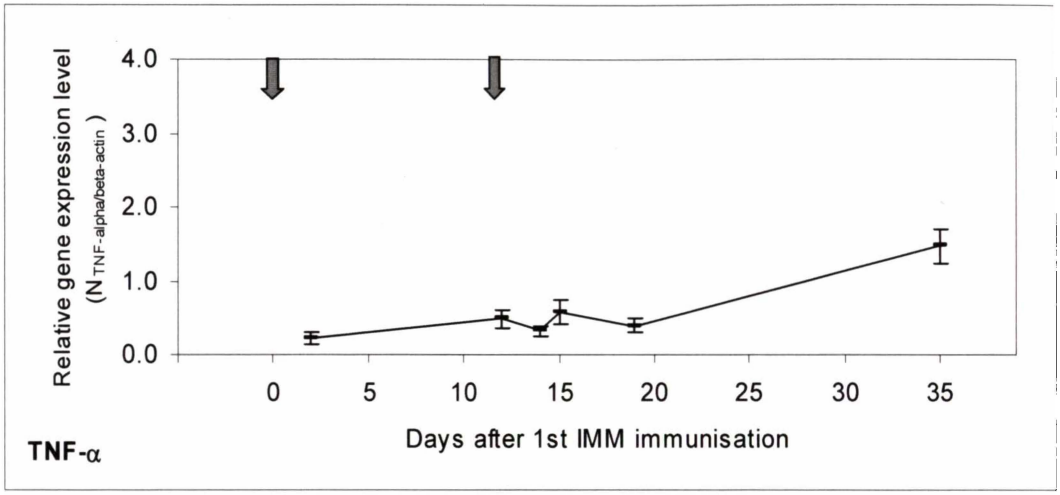
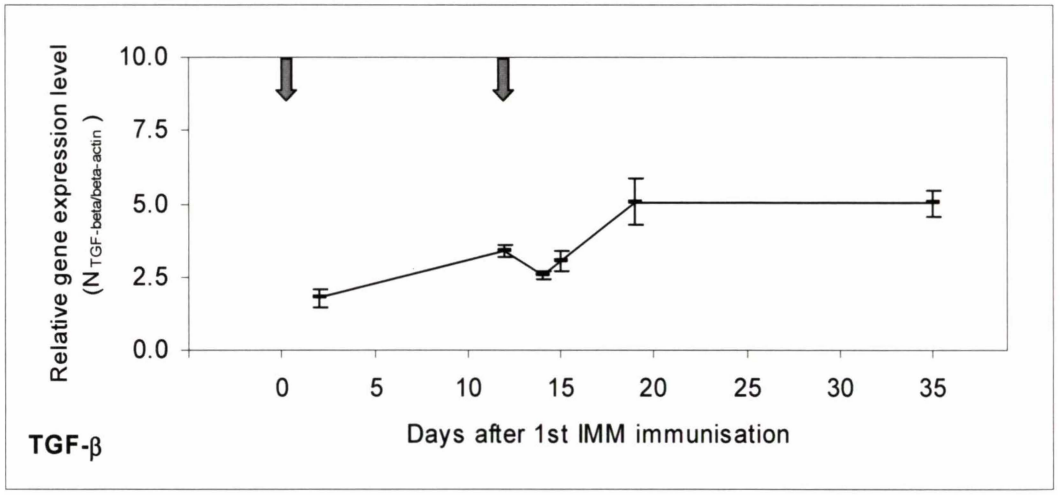
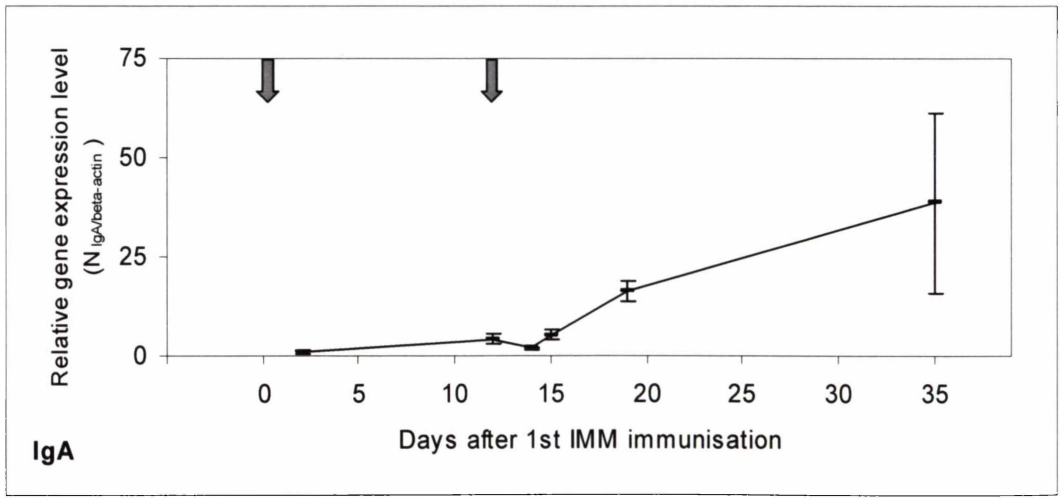


Figure 6.4 Relative gene expression of the cytokines TNF-a, TGF-β and IgA in the mammary secretion cells

Cells were isolated from mammary secretions collected at intervals following intra-mammary immunisation (at times marked with arrows). Graphs show the level of gene expression (relative to β-actin) in the cells. (Combined average of the high- and low-responding animals ± SEM, n = 8)







All days analysed had low levels of IFN-γ in cells isolated from mammary secretions except Day 19 (seven days after the 2nd IMM immunisation). On this day, levels of IFN-γ mRNA were four-fold higher compared with others days and this difference was statistically significant ($p < 0.01$).

The level of TNF-α mRNA in the cells isolated from mammary secretions for days analysed over the time course did not vary until Day 35 (three weeks after the 2nd IMM immunisation). On this day, TNF-α mRNA levels were three-fold higher compared with other days analysed and this difference was statistically significant ($p < 0.01$).

Although the levels of TGF-β mRNA were readily detected in cells isolated from mammary secretions the values did not appear to change with IMM immunisation although there was a trend for levels to increase over the time course of the experiment.

Levels of BLyS mRNA in the cells isolated from mammary secretions were very low (data not shown).

Cells isolated from mammary secretions were not analysed for pIgR mRNA because the proportion of total cells in mammary secretions that express pIgR i.e. epithelial cells, were considered to be low based on the literature (Jensen and Eberhart, 1981; Lee *et al.*, 1980). However, identification of epithelial cells in cells isolated from mammary secretions was not undertaken in this study.

The IgA mRNA level in the cells isolated from mammary secretions was at a constant level until Day 19 (seven days after the 2nd IMM immunisation). At this time point, the levels increased 2.0 – 4.0 times compared with the previous days analysed ($p < 0.01$). By Day 35 the average level of IgA mRNA was higher than on Day 19 but there was considerable animal variation for this time point. A comparison of the percentages of IgA-positive cells as measured by flow cytometry (Chapter Five, Section 5.5.1.3) and IgA mRNA levels showed no correlation (data not shown).

6.6 Discussion

The regulation of humoral immune responses is complex, involving the interaction between different cell types and soluble stimulatory/inhibitory factors such as cytokines and chemokines. Data from mouse studies using cloned cells have shown specific cytokines to be associated with either a Type 1 or Type 2 immune response (Mosmann *et al.,* 1986). However, there is some debate about the delineation of cells into strict subsets *in vivo* (Kelso, 1995). In addition, work undertaken in cattle has found that the classical roles of many cytokines in the laboratory mouse may not be extrapolated to the bovine species (Estes and Brown, 2002). The objective of this chapter was to establish cytokine expression profiles in mammary gland samples that may give a better understanding of the immune response being elicited by our immunisation procedure.

In the healthy untreated animals, analysis of the cytokine profiles of the mammary gland alveolar tissues showed that there was a wide range of mRNA expression for individual animals. For many of the cytokines, the variation amongst individual cows within each of the four physiological stages of the mammary gland was as large as the variation between the four different stages.

In accordance with other studies (Alluwaimi and Cullor, 2002; Leutenegger *et al.,* 2000), levels of IL-2 mRNA were generally low. Similarly the cytokines associated with the Th2 response, IL-6 and IL-10, were also found to be low in this study. This was also in agreement with other studies where these cytokines were reported to be low in healthy cows (Alluwaimi and Cullor, 2002; Riollet *et al.,* 2000b; Taylor *et al.,* 1997). Conversely, mRNA levels of IFNγ were significantly different between physiological stages with levels higher in pregnant, lactation and involution phases of the mammary gland compared with the colostrum phase. This observation was different to reports in the literature where IFN-γ measured as protein in milk was observed to be elevated in colostrum compared with early, middle and late lactation (Hagiwara *et al.,* 2000). However, this disparity in results may reflect that IFN-γ measured as protein in milk in the Hagiwara *et al.* study (2000) is not equivalent to IFN-γ mRNA levels in tissues, as measured in this study. Aside from the fact that there may be differences in comparing mRNA and protein, there may also be a different repertoire of cells contributing to the protein levels of this cytokine in milk versus cells contributing to the mRNA levels in the mammary tissues.

Mammary gland levels of IL-8 mRNA in the untreated animals appeared to be elevated in the colostrum phase. However, because of very large variations amongst individual animals there was no significant difference in values between the physiological stages of the mammary gland. IL-8 is a chemoattractant for neutrophils and elevated levels of this cytokine may reflect a response to bacterial infection, although all animals in this study were supposedly free of mastitis infection. In contrast, levels of TNF-α mRNA were undetectable at all physiological stages of the mammary gland suggesting that they were indeed healthy uninfected mammary glands. The undetectable levels of TNF-α mRNA in this study were different to the observations described by Alluwaimi and Cullor (2000) where the levels of TNF-α mRNA in milk cells were significantly elevated in mid- and late-lactation milk compared with other cytokines measured.

In the mammary gland tissues of untreated animals, the mRNA levels of IgA relative to β-actin mRNA were the highest of all of the genes tested. Interestingly, the mRNA levels of IgA were lowest in colostral mammary tissues and highest in lactation mammary tissues. This is the opposite of that observed for protein levels of IgA, which are highest in colostrum mammary secretions compared with other physiological stages of the mammary gland. However, this may simply reflect the fact that IgA is concentrated in colostrum over many weeks *pre-partum*. Why expression of the gene should be elevated in lactation when levels of the IgA protein are low in milk (0.08 g/l in milk compared with 0.37 g/l in colostrum) (Butler, 1983), could not be ascertained from the data in this study but may possibly indicate that IgA mRNA is not being translated into protein or the translated protein is not being transported into secretions. This finding warrants further investigation.

The transport of IgA into mammary secretions is controlled by pIgR (Brandtzaeg, 1985; Kaetzel *et al.,* 1991). Overall the levels of pIgR mRNA detected in the untreated bovine mammary gland was in accordance with that found in studies using sheep (Rincheval-Arnold *et al.,* 2002) and mice (Van der Feltz *et al.,* 2001). An interesting finding was that mRNA levels of pIgR did not correlate with IgA mRNA levels. This may suggest that pIgR is a limiting factor for the secretion of IgA into bovine mammary secretions, similar to that suggested by others for ovine mammary secretions (Scicchitano *et al.,* 1986; Sheldrake *et al.,* 1984).

Overall, the data suggest that in the healthy untreated animals, cytokine expression in the mammary gland is generally low, consistent with a non-inflammatory, non-

infected physiological state although a wide variation for individual animal expression levels within groups at the four different physiological stages of the mammary gland was observed.

Cytokine profile analysis of immunised and control (non-immunised) mammary tissue, collected at two different time points after the 2nd IMM immunisation, also found low expression levels for many of the cytokines tested, similar to the untreated animals. This was the case for both IL-2 and IL-6, and the low levels were similar when immunised and control alveolar tissues were compared. There was a trend towards increased mRNA expression of IL10 mRNA in the immunised mammary tissues seven days after the 2nd IMM immunisation, in comparison to the control tissues and this was also the pattern for IFN-γ expression. All of these cytokines are expressed by subpopulations of activated T cells, for example in a Th1 type immune response CD4 cells express IL-2 and IFN-γ, whereas in a Th2 type response, CD4 cells express IL-6 and IL-10. However, overall data for these cytokines did not yield any conclusive information about the relative activity of these cells types except to suggest that they were not actively expressing cytokines at the time points sampled or the levels of cytokines required for biological activity are below the level of detection by this method of analysis.

Transcription levels of the pro-inflammatory cytokine TNF-α were increased in the immunised mammary tissues compared with the control tissues, sampled seven days after the 2nd IMM immunisation. Immunised animals sampled during lactation had reduced mRNA levels of this cytokine compared with the immunised samples collected from the pregnant animals. Taken together, this would suggest that the IMM immunisation had induced an inflammatory response in the mammary gland. However, the generally low levels of TNF-α would suggest that a prolonged inflammatory response was not invoked.

The observation that immunised mammary tissues, sampled seven days after the 2nd IMM immunisation, showed markedly increased levels of expression of IL-8 (15-fold) in comparison to control tissues suggests that IMM immunisation may induce an influx of cells via stimulation of this cytokine in mammary tissues. This is based on the knowledge that IL-8 has been shown to be a potent inducer of neutrophil infiltration into tissues (Bickel, 1993), and *in vitro* studies have shown that it may also be chemotactic for T and B cells (Bacon *et al.,* 1989; Schratzberger *et al.,* 1997). Transcriptional levels of IL-8 from tissues collected five weeks *post-partum* from the

immunised and control mammary glands of the lactating group were similar and reduced compared with pregnant immunised tissues, suggesting that the IMM immunisation stimulation of IL-8 production was not sustained over weeks.

The cytokine shown to be involved in IgA class-switching in mice, TGF-β, (Kim and Kagnoff, 1990; Lebman and Edmiston, 1999), showed no discernible difference in mRNA levels between the immunised and control mammary tissues of both time points following IMM immunisation. However, transcriptional levels for this cytokine were readily detectable in most tissue samples analysed. Of course, it must be remembered that TGF-β has a myriad of functions. One of the most pronounced properties of TGF-β is that it is anti-proliferative for immune cells, including B cells (Lebman and Edmiston, 1999). Given its suppressive nature, it may be important for this cytokine to be highly regulated and changes in expression of TGF-β may not have been observed in this study due to the timing of sampling.

The TNF family member, BLyS has been attributed with the function of maintaining the survival of both mature B cells and plasma cells (Avery *et al.,* 2003; MacKay and Browning, 2002). Expression levels of BLyS were three-fold higher in the immunised mammary tissues than control tissues, suggesting that the higher numbers of plasma cells detected in immunised tissues (as described in Chapter Four) may be, at least in part, attributable to the action of this cytokine.

On the whole, levels of IgA mRNA were found to be increased when immunised mammary tissues were compared with control tissues, collected seven days after the 2nd IMM immunisation, suggesting that the immunisation had induced increased levels of IgA mRNA. In contrast, levels of pIgR mRNA in the immunised animal were low, with levels higher in mammary tissues collected five weeks *post-partum* compared with tissue collected seven days after the 2nd IMM immunisation, similar to the finding in the untreated animals and in accordance with the findings of other studies on sheep (Rincheval-Arnold *et al.,* 2002) and mice (Van der Feltz *et al.,* 2001). Taken together these results would indicate that the up-regulation of IgA production does not induce a corresponding increase in pIgR transcriptional levels and may suggest that pIgR is limiting IgA secretion in the ruminant mammary gland.

Cells that were isolated from mammary secretions over the IMM immunisation time course had low IL-2 mRNA levels, similar to the other two sample sets analysed. T cells are a major producer of IL-2 (Smith, 1988). Therefore, a more significant level

of IL-2 might have been expected in the mammary secretion cells because T cells comprised approximately 10 – 25% of the total cells in these samples and greater than 90% of these were activated cells (see section 5.5.1.2). However, IL-2 acts in a paracrine/autocrine fashion (Janeway *et al.,* 2001b) and thus, *in vivo*, a low level may be all that is required for effective biological activity. Cell culture studies have demonstrated that IL-2 can be a potent immunomodulator of bovine immune cells (Shafer-Weaver and Sordillo, 1996; Sordillo *et al.,* 1991a; Wedlock *et al.,* 2000).

The finding that cells isolated from mammary secretions showed an increase in IFN-γ at the same time point corresponding to that when IFN-γ mRNA levels in immunised mammary tissues were also elevated suggests that IFN-γ expression is elevated by IMM immunisation. IFN-γ is produced by T cells and the observation that there was no increase in IFN-γ mRNA levels from Day 2 until Day 19 (seven days after the 2nd IMM immunisation) may reflect the reduction in the percentage of total lymphocytes observed in mammary secretions following IMM immunisation. The expression profile for IL-10 over the time course was less well-defined. Although there appeared to be a trend for levels of IL-10 mRNA to increase following the 2nd IMM immunisation, there was a variation in response between individual animals. In another bovine study, measurement of the IL-10 protein by ELISA showed the levels increased for a short time after *E. coli* mammary gland infection (Bannerman *et al.,* 2004). Human recombinant IL-10 has been shown to be suppressive on bovine T-cell proliferation and IFN-γ production (Brown *et al.,* 1994). This did not appear to be the case in this study; levels of IFN-γ mRNA were at their peak on Day 19 when IL-10 levels were also at their peak. This may indicate that the level of IL-10 was not sufficient to suppress the T-cell production of IFN-γ or that the cells producing IL-10 were not in the proximity of the T cells producing IFN-γ

In the cells isolated from the mammary secretions over the time course, mRNA levels of the pro-inflammatory cytokine TNF-α were generally low, similar to those levels found in immunised mammary tissues, although these levels were increased by Day 35, (three weeks after the 2nd IMM immunisation). However, in general the TNF-α mRNA levels in immunised animals were detectable, in contrast to the undetectable levels in the untreated animals. In other studies, transcriptional levels of TNF-α in milk cells from mastitic animals have found a variety of results, ranging from no detection to elevated levels, compared with healthy animals (Alluwaimi *et al.,* 2003; Taylor *et al.,* 1997). In cows with naturally occurring mastitis, TNF-α mRNA levels were undetectable in milk cells (Taylor *et al.,* 1997) in contrast to cows experimentally

infected where levels of TNF-α in milk cells were increased at 24 h and then decreased by 36 h post-infection (Alluwaimi *et al.,* 2003). This would suggest that either cells expressing TNF-α mRNA levels occur transiently in milk or cells express TNF-α only in the acute stages of the infection. This could also explain why an increase in TNF-α was not observed in mammary secretions because in this study the first samples were collected 48 h post IMM immunisation. The reason for an increase in TNF-α mRNA in Day 35 samples cannot be explained with the data to hand.

In the cells isolated from mammary secretions, the marked increase in IL-8 mRNA levels following the 2nd IMM immunisation were mirrored by the infiltration of neutrophils as measured by flow cytometry (Chapter Five). In addition these data were in agreement with IL-8 mRNA levels observed in immunised mammary tissues, where levels were elevated in samples collected seven days after the 2nd IMM immunisation compared with control samples. Taken together, this would indicate that the IMM immunisation induced an innate immune response in the mammary gland with increased levels of IL-8 inducing an influx on neutrophils to the gland. However, an innate response is generally characterised as a rapid response yet IL-8 expression levels were still elevated in mammary secretion cells at Day 35, three weeks post-IMM immunisation. This may suggest that IL-8 is playing a different role at this time point that cannot be ascertained from the data in these studies.

In mammary secretion cells the general observation was that TGF-β mRNA levels tended to be lower in the early stages of the time course following IMM immunisation compared with the later stages. This late stage increase in TGF-β may explain why no difference was observed in TGFβ mRNA levels between the immunised and control tissues samples collected from animals seven days after the IMM immunisation. In addition, expression levels of this cytokine were similar for immunised animals both seven days after IMM immunisation and five weeks *post-partum* suggesting that this is the normal level for mRNA TGF-β and that immediately following IMM immunisation the expression of this cytokine in cells isolated from mammary secretions was reduced. Down-regulation of this cytokine during the early immune response would help promote cell proliferation as TGF-β has been demonstrated to be anti-proliferative for both T and B cells (Lebman and Edmiston, 1999; Stoeck *et al.,* 1989). In this manner TGF-β plays a important role in the mediation of immune regulation (Wahl *et al.,* 2004).

Interestingly, levels of BLyS mRNA were lower in samples of mammary secretion cells compared with samples of mammary tissues. This may reflect the differences in cell types that were in the different samples, although neutrophils (present in the secretion cell population) have been shown to express BLyS (Scapini *et al.,* 2004).

The IgA mRNA levels in cells isolated from the mammary secretions, which increased over the time period sampled, did not correlate with the percentages of IgA-positive cells as measured by flow cytometry. This may have been due to the fact that flow cytometry did not measure plasma cells but only plasmablasts. Contribution to IgA mRNA levels would have been from both populations of these cells.

Overall data for the immunised animals suggest that IMM immunisations induce a complex immune response. Low levels of TNF-α would suggest that a prolonged inflammatory response is not invoked although a transient inflammatory response cannot be ruled out. A mammary secretion sample was not collected until two days after each IMM immunisation, by which time a spike in TNF-α may have occurred undetected. The observed increases in IL-8, along with rises in IFN-γ, may indicate that the IMM immunisations induced innate and cell-mediated types of immune responses, in addition to a humoral response. This was supported by the data that there were fluctuations in both neutrophils and lymphocyte cell populations in the total cells isolated from mammary secretions after the IMM immunisations. No conclusion could be drawn regarding the relative activity of Th1 and Th2 type CD4 cells from the cytokine gene expression profiles. Many of the cytokine genes were expressed at low levels and changes occurred within a relatively short time following the IMM immunisations, and this was reflected in the comparison of the data for the immunised tissue trial and the time course trial.

Surprisingly, there was no difference in the mRNA cytokine expression profile in the cells isolated from mammary secretions when high- and low-responding animals were compared. There are several potential explanations for this observation. The results may suggest that the cells released into the cistern and collected in mammary secretions did not truly reflect the mammary gland cellular environment. Alternatively, these results may indicate that mRNA transcription levels in cells do not translate into protein bioactivity in the mammary gland. Another possibility is that the cytokine profile reflected not only the IgA induction but also a more generalised inflammatory response to the immunisation challenge which one would expect to be similar in both the high- and low-responding animals. However, the observation that

there was no difference in cytokine expression levels in the mammary gland of these animals may simply mean that the factors that determine a high- and low-responding animal occur prior to IMM immunisation and are induced by the priming of the animal by intra-peritoneal and intra-muscular immunisations.

Chapter 7

General Discussion

7.1 General discussion

The overall aim of this thesis was to investigate the molecular and cellular mechanisms that are involved in the local induction of IgA secretion in the bovine mammary gland. In our earlier studies, it has been shown that each of the intra-peritoneal (IP), intra-muscular (IM) and intra-mammary (IMM) routes for immunisation are required for maximal IgA responses (Hodgkinson and Hodgkinson, 2003). All the combinations of IP, IM, and IMM routes were trialled but the highest titre of IgA antibody in colostrum and milk in the greatest number of animals was found when a combination of all three routes was used. In particular, earlier studies have shown that IMM immunisation is critical for IgA production in mammary secretions signifying that this immunisation is essential for inducing pathways involved in the process. If one side of the udder is immunised and not the other, then IgA is produced only in the milk from the side that has been treated. This suggests that IgA produced by our regimen is localised to the mammary gland and that the IMM immunisation activates molecular and cellular processes in the treated gland to induce this response. For local IgA secretion, it is essential to have immune cells in the mammary gland. At the start of the work for this thesis, the key questions were: what cells are required *in situ*; where do they come from; how do they get into the mammary gland; and how do they interact to induce the IgA production in mammary secretions? It was these key 'knowledge gaps' pertaining to the cow, identified from the proposed mechanism of action of our multi-site immunisation regimen (section 1.5.3.3), that provided the focus for the investigations detailed in this thesis.

The concept of a common mucosal immune response suggests that antigenic challenge at one mucosal surface can be reflected in immune effector responses at distant mucosal sites (McDermott and Bienenstock, 1979; McDermott *et al.,* 1980). Our multi-site immunisation regimen is based on this principle. In the proposed mechanism of IgA production (Section 1.5.3.3), the cells that are generated by the administration of antigen at the IP and IM sites migrate into the blood and circulate the body. These cells home to the mammary gland after the gland has been exposed to the same antigenic challenge via the IMM immunisation. Homing of effector cells has been shown in mice to be coordinated by the interaction of adhesion molecules and chemokines expressed on the vascular endothelium of the local tissues and their receptors displayed on the cell surface of immune cells (Butcher *et al.,* 1999; Picker, 1994; Radi *et al.,* 2001).

From the work in this thesis, it can be seen that following the IMM immunisations there was an increase in the numbers of T and differentiated B cells in the alveolar tissue of the mammary gland. In this study, the characterisation of adhesion molecules expressed by the bovine mammary gland found differences when the results were compared with studies on mice. MAdCAM-1 and VCAM-1 did not appear to be involved in the recruitment of lymphocytes to the mammary gland in the cow, unlike in the mouse. A study on mice observed a correlation between T cell immigration to the mammary gland and MAdCAM-1 levels on mammary vascular endothelium (Tanneau *et al.,* 1999). In another study on mice, there was evidence of a link between VCAM-1 and IgA plasmablast recruitment to the mammary gland (Finke and Acha-Orbea, 2001).

The observed differences in adhesion molecule expression between the mouse and cow may account for the variation in the immunoglobulin repertoire secreted in the milk of the two species. In the cow, the predominant immunoglobulin class is IgG, in contrast to the mouse, where the major immunoglobulin class is IgA (Brambell, 1970). However, in the work in this thesis, when IgA production was induced in the bovine mammary gland there was no evidence of changes to MAdCAM-1 or VCAM-1 expression in immunised mammary tissues compared with non-immunised (control) mammary tissues. In support of the non-detection of MAdCAM-1 in immunised mammary alveolar tissues by immunohistochemical methods, very low levels of MAdCAM-1 mRNA were found in these same tissues compared with bovine Peyer's patch tissues.

Overall the results of the investigations suggest that the adhesion molecules involved in lymphocyte homing to the bovine mammary gland differ from those described in the literature for the mouse. Contrary to the situation in the mouse, MAdCAM-1 and VCAM-1 may not be involved in the recruitment of lymphocytes to the bovine mammary gland. In the cow, other factors such as chemokines may be responsible for this process.

Recently it has been proposed that CCL28 is a key regulator of IgA plasma-cell accumulation in the mammary gland and this chemokine has been shown to be up-regulated in the mouse mammary gland during lactation (Wilson and Butcher, 2004). In the Wilson and Butcher (2004) study, IgA plasma cells isolated from mammary tissues expressed CCR10, the receptor for CCL28, while *in vivo* treatment with anti-CCL28 blocked IgA plasma-cell accumulation. In future studies, it would be of interest to measure mRNA levels of this chemokine and its receptor in mammary

tissues and cells (isolated from mammary secretions) collected from cows immunised using our multi-site immunisation regimen.

Although a clear mechanism for recruitment of lymphocytes to the bovine mammary gland has not resulted from these studies, a marked increase in the numbers of lymphocytes in tissues following IMM immunisation was observed. This suggests that there is a mechanism in operation to recruit cells to the mammary gland in response to IMM immunisation. Whether all of the lymphocytes were recruited to the mammary gland or just a small percentage of the cells which then proliferated *in situ* could not be elucidated from these studies. In other studies, it has been shown that memory T cells (both Th1 and Th2 type CD4 cells) respond rapidly to re-exposure to antigen that originally induced them, by proliferating and secreting cytokines (Rogers *et al.,* 2000). Therefore, it is possible that memory T cells recruited to the mammary gland, proliferated when re-exposed to antigen. Following IMM immunisation, an increase was observed in the proportion of CD4 cells compared with CD8 cells, however, cytokine gene expression analysis was not able to differentiate whether these CD4 cells were Th1 or Th2 type cells. Overall, it can be seen that IMM immunisation had a marked stimulatory effect on the alveolar tissues of immunised mammary glands compared with the non-immunised (control) glands, as shown by a significant accumulation of lymphocytes.

In response to foreign antigen exposure, the first line of defence is usually an innate immune response. This is typified by an influx of neutrophils into tissues in response to pro-inflammatory cytokines and the expression of the chemokine IL-8 by endothelial cells (Bickel, 1993). In the studies in this thesis, the same pattern of response, an increase in neutrophils and a rise in IL-8 mRNA expression, was observed following the IMM immunisations. A key component of the host innate immune response is the up-regulation of pro-inflammatory cytokines. Measurement of mRNA expression for the pro-inflammatory cytokine TNF-α determined that levels were not highly elevated following IMM immunisation. However, as samples were not collected until two days after the immunisation, a rise and subsequent fall in TNF-α prior to the first sampling cannot be discounted. In other studies where the bovine mammary gland was experimentally infused with mastitis-inducing organisms, TNF-α protein levels in milk were reported to be elevated 1 – 5 h later, with levels reduced to normal by 24h (Paape *et al.,* 2002b) and levels of TNF-α mRNA in milk cells were reported to be increased at 24 h and decreased by 36 h (Alluwaimi *et al.,* 2003). In the studies in this thesis, there were physical signs of inflammation, including

oedema in the mammary gland and generalised non-specific changes in the consistency of mammary secretions, for several days following the IMM immunisations, suggesting that levels of the pro-inflammatory cytokine TNF-α may have been transiently elevated following IMM immunisation.

A significant increase in both T cells and differentiated B cells was found in the alveolar tissues that were collected seven days after IMM immunisation. In another study in this thesis, analysis of mammary secretion cells before and after IMM immunisation illustrated the rate of lymphocytes infiltrating into and/or proliferating in the mammary gland in response to the immunisations. The increased levels of mRNA for the cytokines, IL-10 and IFN-γ following the IMM immunisation, suggest that these cells were activated and interacting with one another. For example, the cytokine IL-10 has been shown to induce B cells to increase antibody production (Burdin *et al.,* 1997) and may be inducing this effect here in the immunised mammary gland. On the other hand and in contrast to IL-10, transcriptional levels of IL-6 were very low in mammary secretion cells following IMM immunisation, although IL-6 has been demonstrated in other studies to be essential for IgA production (Morse *et al.,* 1997). IL-6 knockout mice have greatly reduced numbers of intestinal plasma cells and poorly developed IgA responses to mucosal immunisation (Ramsay *et al.,* 1994a).

Some of the 'classical' cytokines of the adaptive immune response were found to be raised following IMM immunisation, *e.g.* IFN-γ, and IL-10. Activation of this cytokine repertoire suggests that both cell-mediated and humoral responses may be induced by the IMM immunisation. The concept that expression patterns of cytokines may be linked to a particular immune response is based on *in vitro* cell culture studies using cloned mouse cells (Mosmann *et al.,* 1986; Mosmann *et al.,* 1997; Mosmann and Sad, 1996). However, the paradigm may not extrapolate to *in vivo* conditions or to other species and in particular cows (Estes and Brown, 2002). Also, it should be noted that in the studies in this thesis, the cytokine mRNA levels were measured in samples that contained a mixture of cells; therefore, it is difficult to distinguish which cells are responsible for individual changes in cytokine transcription. Many of the cytokines are produced by several different cell types. For example IL-10 is produced by T cells, macrophages and activated B cells (Burdin *et al.,* 1997; Spits and de Waal Malefyt, 1992). The other factor that must be considered is that the same cytokine produced by different cells can have different actions. For example, TGF-β produced by T cells plays a major role in the class-switching process to IgA

(Kim and Kagnoff, 1990; Lebman and Edmiston, 1999) while TGF-β produced by epithelial cells has been shown to have an inhibitory effect on lymphocyte proliferation (Ebert, 1999). Overall a cautious approach is needed when interpreting cytokine gene expression profiles.

Of primary interest in these studies were the B cells. This cell type is essential for the production of IgA antibody in mammary secretions. The main questions are: where do the B cells come from, and at what stage in the development of a response do they enter the gland? The IMM immunisation is administered four weeks after the primary immunisations at the IP/IM sites, in conjunction with a booster dose at the IP/IM sites. Therefore, memory T and B cells generated in response to the primary immunisation will respond to the subsequent antigen booster. From the characterisation studies of cells isolated from mammary secretions, it was shown that there were increased percentages of plasmablasts in the mammary gland as early as two days after IMM immunisation (Chapter Five). Many of these B cells consisted of the IgA isotype and this, in conjunction with priming at mucosal sites and the concept of the common mucosal immune system, would suggest that these cells were of mucosal origin.

There were low numbers of BB2 cells observed in mammary secretions with no apparent difference between the High and Low group for this cell type. In addition, from the immunohistochemical studies it can be said categorically that seven days after IMM immunisation there are increased numbers of plasma cells in the tissues of the immunised mammary gland (Chapter Four). Taken together, these data would suggest that the B cells enter the gland as plasmablasts that then differentiate into antibody-secreting plasma cells presumably under the influence of Th2 type CD4 cells, antigen-presenting cells and associated secretory cytokines. Proliferation of plasmablasts would, therefore, probably occur *in situ*. It is thought that differentiation of plasmablasts into plasma cells is preceded by a burst of proliferation, with cessation of cell division being a prerequisite of differentiation (Calame *et al.,* 2003a; Chen-Kiang, 2003). The finding, in the studies in this thesis, of antibody-containing cells with double nuclei in mammary gland tissues may be suggestive that these cells do proliferate in the mammary gland. On the other hand, the cells had the histological appearance of antibody-secreting cells (plasma cells) rather than immature plasmablasts, having their cytoplasm heavily stained by antibody specific for IgA or IgM. This raises the debate about whether or not plasma cells are able to proliferate. In the literature, plasma cells are definitively described as terminally differentiated cells (Chen-Kiang, 2003; Cyster, 2003; O'Connor *et al.,* 2003).

The final step in the process of producing IgA in mammary secretions, as described in Section 1.5.3.3, is the transfer of IgA antibody across the alveoli epithelial cells and into the lumen. The transport of IgA into secretions is controlled by the poly-immunoglobulin receptor (pIgR) with one pIgR molecule transporting one molecule of IgA (Brandtzaeg, 1985; Kaetzel *et al.,* 1991) . The pIgR molecule is not recycled. In the studies in this thesis, transcriptional levels of pIgR were found to be generally low in mammary tissues from untreated animals, although there was higher expression during the lactation stage. This pattern of expression of pIgR mRNA is in accordance with the findings of others in sheep (Rincheval-Arnold *et al.,* 2002) and mice (Van der Feltz *et al.,* 2001). Therefore, this may suggest that the low levels of IgA characteristic of bovine mammary secretions are due to a deficiency in the transport of this immunoglobulin by pIgR, as suggested by others for sheep (Scicchitano *et al.,* 1986; Sheldrake *et al.,* 1984). There was no significant difference in the expression of this gene between immunised and control alveolar tissues, from both pregnant and lactating animals, although levels of mRNA pIgR were higher in the lactation samples, similar to the untreated animals. In tissues samples, transcriptional levels of pIgR correlated poorly with transcriptional levels of IgA, again suggesting a deficiency in the IgA transport system.

The use of animals that had been previously immunised with our multi-site regimen allowed for a valuable comparison between animals that responded well to the immunisation and animals that responded poorly. From the work in Chapter Five, the main finding was a significant difference between the high- and low-responding animals in the pattern of cellular response following IMM immunisation. Interestingly, a difference between the groups for their cytokine expression levels in mammary secretion cells could not be detected. This could be explained by the fact that there was no significant difference between the T-cells populations for these two groups, if this cell type contributed a significant proportion of the mRNA detected in cell samples. This again highlights the difficulty in interpreting mRNA cytokine data for samples that have a mixture of cell types.

Another valuable outcome from using high- and low-responding animals is that it reinforced the finding that animals respond in a consistent manner to the multi-site immunisation regimen each year they are immunised. This was the third consecutive year that these animals had been immunised. The results for IgA levels in milk confirmed that a high-responding cow appears to always produce a high titre antibody response and conversely a low-responding cow appears to always produce a low titre antibody response. These data imply that there are different genetic

characteristics in these animals that are influencing the outcome of their immune response. This has widespread ramifications and may lead to the discovery of genetic factors that control or regulate immune responses to vaccination.

There are several other factors that need to be considered when drawing conclusions from the data produced in this thesis. Relatively small numbers of animals were used for experimentation. Since the experimental animal was a dairy cow, there were significant cost implications for trial design. Small experimental groups mean that individual animal variation has a large influence on results. For example, the levels of cytokine mRNA in the mammary gland tissues of untreated animals at four different physiological stages of the mammary gland often had larger variation within the group than compared with the different stages. This meant that the only conclusion that could be drawn is that the cytokine expression is highly variable. Small numbers of animals were also an issue in the experiment in Chapter Four where naïve animals were immunised. At least one of the animals did not respond with significant levels of IgA as determined by the level of antigen-specific IgA in mammary secretions. On the other hand, animals in this experiment were immunised on one side of the udder only, therefore, the other side acted as an individual control for each animal. Clearly with the dairy cow being such an expensive animal, this places constraints on experimental design but it is contended that within the boundaries of the resources available, the experimental design was appropriate for addressing the key questions in this thesis.

The timing of collection was another issue when data were being interpreted. For example, in Chapter Four, tissues samples were collected from the mammary gland seven days after the 2nd IMM immunisation. The timing meant that although the expression of MAdCAM-1 was not detected in these tissues samples, it cannot be discounted that expression of the adhesion molecule may have occurred earlier. If cell migration occurred within a short time period of the IMM treatment, it is possible that expression of the adhesion molecules on the vascular endothelium may have occurred undetected in the interval prior to tissue samples being collected. However, data from the experiment where cells in mammary secretion were analysed showed that increased levels of T and B cells were sustained for more than seven days after the IMM immunisation. This would suggest that the infiltration of lymphocytes to the mammary gland was still occurring when the tissues samples were collected from the immunised mammary glands. On the other hand, it is possible that the observed increased level of cells may have been due to proliferation *in situ* of a small number of cells that had been recruited immediately following IMM immunisation.

Another example of timing influencing the interpretation of results was in the experiment where mammary secretions were collected from the mammary gland after the IMM immunisations. In this case it was decided that it would be prudent not to sample the gland until two days after the IMM immunisation in order to reduce the chance of removing immunogen along with the secretions. The timing meant that early events in the immune response, for example a transient inflammatory response, would not be detected using this approach, Secretion samples taken within the first 24 h following IMM immunisation would provide valuable information.

Another issue relates to the use of mammary secretion cells to reflect the cellular environment of the mammary gland. Many studies investigating the health of the dairy cow udder have used cells isolated from the milk. While colostrum and milk samples from healthy animals have low numbers of cells (Concha, 1986), mastitis is characterised by a high somatic cell count in milk (Jensen and Eberhart, 1981). One study suggested that at the *pre-partum* stage of mammary development (the dry period) the gland is more susceptible to infection, therefore, there are increased numbers of immune cells in *pre-partum* mammary secretions to counteract bacterial infection (Jensen and Eberhart, 1981). The mechanism of transport of cells into the lumen of the healthy cow is not completely understood although several modes of transport in the infected mammary gland have been suggested (Nickerson and Pankey, 1985). In the experiment in Chapter Five, cells from mammary secretion were utilised to monitor the immune response of the mammary gland to the IMM immunisation. In the pre-IMM immunisation secretion samples, which were collected during the dry-period from *pre-partum* animals, there were high numbers of lymphocytes. The percentages of lymphocytes decreased following each of the two IMM immunisations then recovered to their previous levels. The reason for this was not determined from the data but may be due to a greater number of neutrophils being transported into mammary secretions using the same pathways. Another explanation may be that the mammary gland retained lymphocytes in the tissues following IMM immunisation in a manner similar to that process in infected lymph nodes (Janeway *et al.,* 2001a). Using mammary secretion cells allowed for multiple non-invasive sampling over an extended time period. However, the validity of the results from these studies needs to be verified using tissue sampling.

In summary, the data presented in this thesis have increased the understanding of the molecular and cellular mechanisms underlying our multi-site immunisation procedure. The main focus of this work was investigating the effects of the IMM immunisations in the mammary gland. Many of the key elements of the response

identified in the proposed mechanism in Section 1.5.3.3 have now been characterised for the cow.

The work in this thesis has shown that the immune response invoked by the IMM immunisations is not a simple response but is complex, and in many ways follows the 'classical' immune response to foreign pathogenic invasion. After each of the IMM immunisations there is firstly an inflammatory response as characterised by the physical changes to the mammary gland and its secretions. This is quickly followed by a rise in IL-8 and an influx of neutrophils, distinctive of an innate immune response. The actual mechanism for recruiting the circulating lymphocytes into the bovine mammary gland is not as yet determined. In mammary secretions prior to the IMM immunisation, the T-cell population is a combination of helper T cells (CD4) and cytotoxic T cells (CD8), in a ratio of approximately 1.5:1 respectively. As the cellular response progresses, there is a change in this ratio to 3:1 indicating a greater proportion of CD4-positive cells compared with CD8-positive cells in mammary secretions. These changes may indicate that CD4 (MHC class II) type cells are recruited in preference to CD8 (MHC class I) type cells and/or the CD4 cells are preferentially proliferating in response to antigen exposure. Whether the CD4 cells are Th1 or Th2 type could not be determined from the data to hand. The known fact that the multi-site immunisation regimen activates a humoral response may indicate that a proportion of the CD4 cells are of the Th2 type.

As the cellular response of the mammary gland progresses, the percentage of B lymphocytes increases from 30 to 50% of the total lymphocyte population. These B cells consist of a combination of IgA-, IgM- and IgG-positive cells. Following the IMM immunisations, the IgA-positive cells increase in a biphasic pattern with the response being greater after the 2nd IMM immunisation. In contrast, the magnitude of response for the IgM-positive cells is similar for both IMM immunisations. High-responding animals react more rapidly to the 2nd IMM immunisation and sustain the response for longer when compared with the Low-responding animals. The High-responding animals also produce a greater percentage of IgA-positive cells and this correlates with a greater antibody response. Overall the data indicate that the B cells enter the gland as plasmablasts that then differentiate into antibody-secreting plasma cells, presumably under the influence of the Th2 CD4 cells and antigen-presenting cells and their secreted cytokines. By seven days after the 2nd IMM immunisation, there are significantly increased numbers of IgA, IgM and IgG plasma cells in immunised mammary gland alveolar tissues in comparison with non-immunised tissues. By Day 35, antigen-specific and total IgA antibody levels in mammary secretions are

significantly higher in High-responding animals compared with the Low-responding animals.

Many of the underlying molecular and cellular mechanisms for the induction of IgA secretion in the bovine mammary gland have been established by the work undertaken in this thesis. Our multi-site immunisation regimen induces a complex immune response and there is still considerable work required to fully elucidate the underlying mechanisms. Future avenues for investigation are outlined in Section 7.3 below.

7.2 Future work

There are several areas of investigation that could be explored to further increase the understanding of the immune response induced by our multi-site immunisation regimen. As with many scientific investigations, further questions are raised by the research. This work is no exception.

The mechanism by which circulating lymphocytes are recruited into the mammary gland from the blood was not established, although the role of several adhesion molecules was investigated and found to be unlikely candidates. A further study would be to investigate the function of chemokines in lymphocyte homing to the bovine mammary gland, in particular the chemokine CCL28 and its receptor CCR10. A recently published paper has identified these two molecules as key components in the recruitment of IgA antibody-producing cells to the mouse mammary gland (Wilson and Butcher, 2004). Mammary gland tissue samples and the mammary secretion cells that were collected from immunised animals could be used to measure transcription levels of the CCL28 and CCR10 genes. Likewise, mammary secretion samples that were already collected and stored could be used for measurement of the CCL28 protein levels and for migration studies, using purified bovine lymphocytes and transmembrane wells.

Data about the timing of the immune response in the mammary gland following IMM immunisation has been generated from the study characterising cells in mammary secretions. A follow-up study collecting tissue samples from immunised mammary glands and control glands at key time points is required to verify the molecular and cellular data. The key time points to sample may be two and seven days following each the two IMM immunisations. These tissues would be used for immunohistochemical studies and mRNA analysis, using both real-time PCR and microarrays. This study would use high- and low-responding animals. A third subset of animals would also be of interest to include, a group of animals that do not respond at all to our immunisation, *i.e.* have undetectable IgA antibody levels in milk following the multi-site immunisation regimen.

Studies in this thesis used one tissue sample per animal. RT-PCR studies of genes that control milk production in the mammary gland have highlighted the variation in gene expression in alveolar tissue collected from different regions of the gland (Molenaar *et al.*, 1992). It would be valuable to collect several tissues samples, at

one time point, from one or two animals for comparative studies of tissue sampling. This could be incorporated into the study described above.

Another beneficial study would involve a cell culture study. The correlation between the number of IgA-positive cells and specific antibody titre indicates that numbers of plasma cells are important for high-titre antibody levels in mammary secretions. In addition the data from this work would suggest that the plasmablasts migrate to the mammary gland and then proliferate prior to undergoing terminal differentiation. The study would involve extracting IgA plasmablasts from blood, after the IP/IM immunisation, and investigate *in vitro* factors that induce their proliferation. Some potential candidates may include IL-6 and IL-10. Potentially these factors could be incorporated into the intra-mammary immunogen in slow-release formulations to discharge at the appropriate time to induce cell proliferation.

Other cell culture studies that may be interesting would be to characterise by real-time PCR the cytokines being expressed in single cell preparations, isolated from mammary secretions following IMM immunisation. This would provide more definitive information about the cytokine dynamics of the induced immune response in the mammary gland.

Identification of genetic markers that differentiate the animals that respond to the multi-site immunisation regimen with high-titre antibodies levels in milk and animals that respond with low-titre antibody levels is another study that would yield very important data that would aid selection of animals to produce a herd of high-responding animals.

References

Abitorabi M. A., Mackay C. R., Jerome E. H., Osorio O., Butcher E. C. and Erle D. J. (1996) Differential expression of homing molecules on recirculating lymphocytes from sheep gut, peripheral, and lung lymph. *Journal of Immunology* **156**, 3111-7.

Akira S., Takeda K. and Kaisho T. (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. *Nature Immunology* **2**, 675-80.

Alluwaimi A. M. and Cullor J. S. (2002) Cytokines gene expression patterns of bovine milk during middle and late stages of lactation. *Journal of veterinary medicine. B, Infectious diseases and veterinary public health.* **49**, 105-10.

Alluwaimi A. M., Leutenegger C. M., Farver T. B., Rossitto P. V., Smith W. L. and Cullor J. S. (2003) The cytokine markers in Staphylococcus aureus mastitis of bovine mammary gland. *Journal of veterinary medicine. B, Infectious diseases and veterinary public health.* **50**, 105-11.

Altmann K. and Mukkur T. K. (1983) Passive immunisation of neonatal lambs against infection with enteropathogenic Escherichia coli via colostrum of ewes immunised with crude and purified K99 pili. *Research in Veterinary Science* **35**, 234-9.

Amichay D., Gazzinelli R. T., Karupiah G., Moench T. R., Sher A. and Farber J. M. (1996) Genes for chemokines MuMig and Crg-2 are induced in protozoan and viral infections in response to IFN-gamma with patterns of tissue expression that suggest nonredundant roles in vivo. *Journal of Immunology* **157**, 4511-20.

Andrew D. P., Berlin C., Honda S., Yoshino T., Hamann A., Holzmann B., Kilshaw P. J. and Butcher E. C. (1994) Distinct but overlapping epitopes are involved in alpha 4 beta 7-mediated adhesion to vascular cell adhesion molecule-1, mucosal addressin-1, fibronectin, and lymphocyte aggregation. *Journal of Immunology* **153**, 3847-61.

Apodaca G., Katz L. A. and Mostov K. E. (1994) Receptor-mediated transcytosis of IgA in MDCK cells is via apical recycling endosomes. *Journal of Cell Biology* **125**, 67-86.

Asai K. I., Komine Y., Kozutsumi T., Yamaguchi T., Komine K. I. and Kumagai K. (2000) Predominant subpopulations of T lymphocytes in the mammary gland secretions during lactation and intraepithelial T lymphocytes in the intestine of dairy cows. *Veterinary Immunology and Immunopathology* **73**, 233-240.

Avery D. T., Kalled S. L., Ellyard J. I., Ambrose C., Bixler S. A., Thien M., Brink R., MacKay F., Hodgkin- Philip D. and Tangye Stuart G. (2003) BAFF selectively enhances the survival of plasmablasts generated from human memory B cells. *Journal of Clinical Investigation* **112**, 286-297.

Bacon K. B., Westwick J. and Camp R. D. (1989) Potent and specific inhibition of IL-8-, IL-1 alpha- and IL-1 beta-induced in vitro human lymphocyte migration by calcium channel antagonists. *Biochemical and biophysical research communications* **165**, 349-54.

Banchereau J. and Steinman R. M. (1998) Dendritic cells and the control of immunity. *Nature* **392**, 245-52.

Bannerman D. D., Paape M. J., Lee J. W., Zhao X., Hope J. C. and Rainard P. (2004) Escherichia coli and Staphylococcus aureus elicit differential innate immune responses following intramammary infection. *Clinical and diagnostic laboratory immunology* **11**, 463-72.

Bao S., Beagley K. W., Allanson M. and Husband A. J. (1998) Exogenous IL-6 promotes enhanced intestinal antibody responses in vivo. *Immunology and Cell Biology* **76**, 560-562.

Barclay A. N., Brady R. L., Davis S. J. and Lange G. (1993) CD4 and the immunoglobulin superfamily. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences.* **342**, 7-12.

Bargatze R. F., Jutila M. A. and Butcher E. C. (1995) Distinct roles of L-selectin and integrins alpha 4 beta 7 and LFA-1 in lymphocyte homing to Peyer's patch-HEV in situ: the multistep model confirmed and refined. *Immunity* **3**, 99-108.

Beale D. (1987) The structure of bovine secretory component. *Veterinary Immunology and Immunopathology* **17**, 37-49.

Berek C., Jarvis J. M. and Milstein C. (1987) Activation of memory and virgin B cell clones in hyperimmune animals. *European Journal of Immunology* **17**, 1121-9.

Berlin C., Bargatze R. F., Campbell J. J., von Andrian U. H., Szabo M. C., Hasslen S. R., Nelson R. D., Berg E. L., Erlandsen S. L. and Butcher E. C. (1995) Alpha 4 integrins mediate lymphocyte attachment and rolling under physiologic flow. *Cell* **80**, 413-22.

Berlin C., Berg E. L., Briskin M. J., Andrew D. P., Kilshaw P. J., Holzmann B., Weissman I. L., Hamann A. and Butcher E. C. (1993) Alpha 4 beta 7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. *Cell* **74**, 185-95.

Bernasconi N. L., Traggiai E. and Lanzavecchia A. (2002) Maintenance of serological memory by polyclonal activation of human memory B cells. *Science* **298**, 2199-202.

Bickel M. (1993) The role of interleukin-8 in inflammation and mechanisms of regulation. *Journal of Periodontology* **64**, 456-60.

Bienenstock J. and Clancy R. (1994) Broncial mucosal lymphiod tissue. In *Handbook of Mucosal Immunology* (Edited by Ogra P. L., Mestecky J., Lamm M. E., Strober W., McGhee J. R. and Bienenstock J.), p. 529-538. Academic Press, San Diego.

Bleul C. C., Fuhlbrigge R. C., Casasnovas J. M., Aiuti A. and Springer T. A. (1996) A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). *Journal of Experimental Medicine* **184**, 1101-9.

Boenisch T. (2001) *Handbook Immunochemical Staining Methods*. DakoCytomation, Carpinteria, CA.

Boesman-Finkelstein M., Walton N. E. and Finkelstein R. A. (1989) Bovine lactogenic immunity against cholera toxin-related enterotoxins and Vibrio cholerae outer membranes. *Infection and Immunity* **57**, 1227-34.

Boudjellab N., Chan-Tang H. S., Li X. and Zhao X. (1998) Interleukin 8 response by bovine mammary epithelial cells to lipopolysaccharide stimulation. *American Journal of Veterinary Research* **59**, 1563-7.

Boutinaud M., Rulquin H., Keisler D. H., Djiane J. and Jammes H. (2002) Use of somatic cells from goat milk for dynamic studies of gene expression in the mammary gland. *Journal of Animal Science* **80**, 1258-1269.

Bowersock T. L., Hogenesch H., Torregrosa S., Borie D., Wang B., Park H. and Park K. (1998) Induction Of Pulmonary Immunity In Cattle By Oral Administration Of Ovalbumin In Alginate Microspheres. *Immunology Letters* **60**, 37-43.

Bowman E. P., Campbell J. J., Soler D., Dong Z., Manlongat N., Picarella D., Hardy R. R. and Butcher E. C. (2000) Developmental switches in chemokine response profiles during B cell differentiation and maturation. *Journal of Experimental Medicine* **191**, 1303-18.

Bowman E. P., Kuklin N. A., Youngman K. R., Lazarus N. H., Kunkel E. J., Pan J. L., Greenberg H. B. and Butcher E. C. (2002) The intestinal chemokine thymus-expressed chemokine (CCL25) attracts IgA antibody-secreting cells. *Journal of Experimental Medicine* **195**, 269-275.

Brambell F. W. R. (1970) The transfer of passive immunity from mother to young. In *Frontiers of Biology* (Edited by Neuberger A. and Tatum E. L.), Vol. 18. North-Holland Publishing Co., Amsterdam.

Brandon M. R. and Lascelles A. K. (1971) Relative efficiency of absorption of IgG 1 , IgG 2 , IgA and IgM in the newborn calf. *Australian Journal of Experimental Biology and Medical Science* **49**, 629-33.

Brandon M. R., Watson D. L. and Lascelles A. K. (1971) The mechanism of transfer of immunoglobulin into mammary secretion of cows. *Australian Journal of Experimental Biology and Medical Science* **49**, 613-23.

Brandtzaeg P. (1983) The secretory immune system of lactating human mammary glands compared with other exocrine organs. *Annals of New York Academy of Sciences* **409**, 353-82.

Brandtzaeg P. (1985) Role of J chain and secretory component in receptor-mediated glandular and hepatic transport of immunoglobulins in man. *Scandinavian Journal of Immunology* **22**, 111-46.

Brandtzaeg P., Baekkevold E. S., Farstad I. N., Jahnsen F. L., Johansen F. E., Nilsen E. M. and Yamanaka T. (1999a) Regional specialization in the mucosal immune

system: what happens in the microcompartments? *Immunology Today* **20**, 141-51.

Brandtzaeg P., Farstad I. N., Johansen F. E., Morton H. C., Norderhaug I. N. and Yamanaka T. (1999b) The B-cell system of human mucosae and exocrine glands. *Immunological Reviews* **171**, 45-87.

Briskin M., Winsor-Hines D., Shyjan A., Cochran N., Bloom S., Wilson J., McEvoy L. M., Butcher E. C., Kassam N., Mackay C. R., Newman W. and Ringler D. J. (1997) Human mucosal addressin cell adhesion molecule-1 is preferentially expressed in intestinal tract and associated lymphoid tissue. *American Journal of Pathology* **151**, 97-110.

Briskin M. J., McEvoy L. M. and Butcher E. C. (1993) MAdCAM-1 has homology to immunoglobulin and mucin-like adhesion receptors and to IgA1. *Nature* **363**, 461-4.

Brock J. H., Arzabe F. R., Ortega F. and Pineiro A. (1977) The effect of limited proteolysis by trypsin and chymotrypsin on bovine colostral IgG1. *Immunology* **32**, 215-9.

Brock S. C., McGraw P. A., Wright P. F. and Crowe J. E. (2002) The human polymeric immunoglobulin receptor facilitates invasion of epithelial cells by Streptococcus pneumoniae in a strain-specific and cell type-specific manner. *Infection and Immunity* **70**, 5091-5095.

Brown W. C., Woods V. M., Chitko-McKown C. G., Hash S. M. and Rice-Ficht A. C. (1994) Interleukin-10 is expressed by bovine type 1 helper, type 2 helper, and unrestricted parasite-specific T-cell clones and inhibits proliferation of all three subsets in an accessory-cell-dependent manner. *Infection and Immunity* **62**, 4697-708.

Brown W. R., Newcomb R. W. and Ishizaka K. (1970) Proteolytic degradation of exocrine and serum immunoglobulins. *Journal of Clinical Investigation* **49**, 1374-80.

Brunser O., Espinoza J., Figueroa G., Araya M., Spencer E., Hilpert H., Link-Amster H. and Brussow H. (1992) Field trial of an infant formula containing anti-rotavirus and anti-Escherichia coli milk antibodies from hyperimmunized cows. *Journal of Paediatric Gastroenterology and Nutrition* **15**, 63-72.

Burdin N., Rousset F. and Banchereau J. (1997) B-cell-derived IL-10: production and function. *Methods* **11**, 98-111.

Burdin N., Van Kooten C., Galibert L., Abrams J. S., Wijdenes J., Banchereau J. and Rousset F. (1995) Endogenous IL-6 and IL-10 contribute to the differentiation of CD40-activated human B lymphocytes. *Journal of Immunology* **154**, 2533-44.

Burton J. L. and Erskine R. J. (2003) Immunity and mastitis. Some new ideas for an old disease. *The Veterinary Clinics of North America. Food animal practice* **19**, 1-45.

Butcher E. C. (1999) Lymphocyte homing and intestinal immunity. In *Mucosal Immunology* (Edited by Ogra P. L., Mestecky J., Lamm M. E., Strober W., Bienenstock J. and McGhee J. R.), p. 507-522. Academic Press, San Diego.

Butcher E. C., Williams M., Youngman K., Rott L. and Briskin M. (1999) Lymphocyte trafficking and regional immunity. *Advances in Immunology* **72**, 209-53.

Butler J. E. (1969) Bovine immunoglobulins: A review. *Journal of Dairy Science* **52**, 1895-1909.

Butler J. E. (1974) Immunoglobulins of the mammary secretions. In *Lactation: A comprehensive Treatise* (Edited by Larson B. L. and Smith V. R.), Vol. 3, p. 217-255. Academic Press, New York.

Butler J. E. (1981) A concept of humoral immunity among ruminants and an approach to its investigation. *Advances in Experimental Medicine and Biology.* **137**, 3-55.

Butler J. E. (1983) Bovine immunoglobulins: an augmented review. *Veterinary Immunology and Immunopathology* **4**, 43-152.

Butler J. E. (1986) Biochemistry and biology of ruminant immunoglobulins. *Progress in Veterinary Microbiology and Immunology* **2**, 1-53.

Butler J. E. (1994) Passive immunity and immunoglobulin diversity. In *Indigenous antimicrobial agents of milk: recent developments*, p. 14-50. International Dairy Federation, Brussels.

Butler J. E., Heyermann H., Borca M., Bielecka M. and Frenyo L. V. (1987) The isotypic, allotypic and idiotypic heterogeneity of bovine IgG2. *Veterinary Immunology and Immunopathology* **17**, 1-16.

Butler J. E. and Kennedy N. (1978) The differential enzyme susceptibility of bovine immunoglobulin G1 and immunoglobulin G2 to pepsin and papain. *Biochimica et Biophysica Acta* **535**, 125-37.

Calame K., L., Lin Kuo I. and Tunyaplin C. (2003a) Regulatory mechanisms that determine the development and function of plasma cells. *Annual review of immunology* **21**, 205-30.

Calame K. L. (2001) Plasma cells: finding new light at the end of B cell development. *Nature Immunology* **2**, 1103-1108.

Calame K. L., Lin K. I. and Tunyaplin C. (2003b) Regulatory mechanisms that determine the development and function of plasma cells. *Annual Review of Immunology* **21**, 205-230.

Campbell J. J., Hedrick J., Zlotnik A., Siani M. A., Thompson D. A. and Butcher E. C. (1998) Chemokines and the arrest of lymphocytes rolling under flow conditions. *Science* **279**, 381-4.

Cassese G., Arce S., Hauser Anja E., Lehnert K., Moewes B., Mostarac M., Muehlinghaus G., Szyska M., Radbruch A. and Manz Rudolf A. (2003) Plasma cell survival is mediated by synergistic effects of cytokines and adhesion-dependent signals. *Journal of Immunology* **171**, 1684-1690.

Casswall T. H., Sarker S. A., Albert M. J., Fuchs G. J., Bergstrom M., Bjorck L. and Hammarstrom L. (1998) Treatment Of Helicobacter Pylori Infection In Infants In Rural Bangladesh With Oral Immunoglobulins From Hyperimmune Bovine Colostrum. *Alimentary Pharmacology & Therapeutics* **12**, 563-568.

Casswall T. H., Sarker S. A., Faruque S. M., Weintraub A., Albert M. J., Fuchs G. J., Alam N. H., Dahlstrom A. K., Link H., Brussow H. and Hammarstrom L. (2000) Treatment of enterotoxigenic and enteropathogenic Escherichia coli-induced diarrhoea in children with bovine immunoglobulin milk concentrate from hyperimmunized cows: a double-blind, placebo-controlled, clinical trial. *Scandinavian Journal of Gastroenterology* **35**, 711-718.

Castrucci G., Frigeri F., Ferrari M., Cilli V., Caleffi F., Aldrovandi V. and Nigrelli A. (1984) The efficacy of colostrum from cows vaccinated with rotavirus in protecting calves to experimentally induced rotavirus infection. *Comparative Immunology, Microbiology and Infectious Diseases* **7**, 11-8.

Cepek K. L., Parker C. M., Madara J. L. and Brenner M. B. (1993) Integrin alpha E beta 7 mediates adhesion of T lymphocytes to epithelial cells. *Journal of Immunology* **150**, 3459-70.

Cerf-Bensussan N., Jarry A., Gneragbe T., Brousse N., Lisowska-Grospierre B., Griscelli C. and Guy-Grand D. (1988) Monoclonal antibodies specific for intestinal lymphocytes. *Monographs Allergy* **24**, 167-72.

Chang C. C., Winter A. J. and Norcross N. L. (1981) Immune response in the bovine mammary gland after intestinal, local, and systemic immunization. *Infection and Immunity* **31**, 650-9.

Chen-Kiang S. (2003) Cell-cycle control of plasma cell differentiation and tumorigenesis. *Immunological Reviews* **194**, 39-47.

Clark B. R. and Engvall E. (1980) Enzyme-linked immunosorbent assay (ELISA): Theoretical and practical aspects. In *Enzyme-immunoassay* (Edited by Maggio E. T.), p. 167-180. CRC Press, Boca Raton, Florida.

Classon B. J., Brown M. H., Garnett D., Somoza C., Barclay A. N., Willis A. C. and Williams A. F. (1992) The hinge region of the CD8 alpha chain: structure, antigenicity, and utility in expression of immunoglobulin superfamily domains. *International Immunology* **4**, 215-25.

Collins R. A. and Oldham G. (1995) Effect of recombinant bovine IL-1 and IL-2 on B cell proliferation and differentiation. *Veterinary Immunology and Immunopathology* **44**, 141-150.

Concha C. (1986) Cell types and their immunological functions in bovine mammary tissues and secretions--a review of the literature. *Nordic Veterinary Medicine* **38**, 257-72.

Conley M. E. and Delacroix D. L. (1987) Intravascular and mucosal immunoglobulin A: two separate but related systems of immune defense? *Annals of Internal Medicine* **106**, 892-9.

Coussens P. M. and Nobis W. (2002) Bioinformatics and high throughput approach to create genomic resources for the study of bovine immunobiology. *Veterinary Immunology and Immunopathology* **86**, 229-44.

Crago S. S., Kulhavy R., Prince S. J. and Mestecky J. (1978) Secretory component of epithelial cells is a surface receptor for polymeric immunoglobulins. *Journal of Experimental Medicine* **147**, 1832-7.

Craxton A., Magaletti D., Ryan Elizabeth J. and Clark Edward A. (2003) Macrophage- and dendritic cell--dependent regulation of human B-cell proliferation requires the TNF family ligand BAFF. *Blood* **101**, 4464-71.

Cripps A. W., Husband A. J. and Lascelles A. K. (1974) The origin of immunoglobulins in intestinal secretion of sheep. *Australian Journal of Experimental Biology and Medical Science* **52**, 711-6.

Cripps A. W. and Lascelles A. K. (1976) The origin of immunoglobulins in salivary secretion of sheep. *Australian Journal of Experimental Biology and Medical Science* **54**, 191-5.

Croft M. and Swain S. L. (1991) B cell response to T helper cell subsets. II. Both the stage of T cell differentiation and the cytokines secreted determine the extent and nature of helper activity. *Journal of Immunology* **147**, 3679-89.

Crottet P. and Corthesy B. (1998) Secretory Component Delays the Conversion Of Secretory Iga Into Antigen-Binding Competent F(Ab')(2) - a Possible Implication For Mucosal Defense. *Journal of Immunology* **161**, 5445-5453.

Crouch C. F. (1985) Vaccination against enteric rota and coronaviruses in cattle and pigs: enhancement of lactogenic immunity. *Vaccine* **3**, 284-91.

Crouch C. F., Oliver S. and Francis M. J. (2001) Serological, colostral and milk responses of cows vaccinated with a single dose of a combined vaccine against rotavirus, coronavirus and Escherichia coli F5 (K99). *Veterinary Record* **149**, 105-108.

Crouch C. F., Oliver S., Hearle D. C., Buckley A., Chapman A. J. and Francis M. J. (2000) Lactogenic immunity following vaccination of cattle with bovine coronavirus. *Vaccine* **19**, 189-96.

Csencsits K. L., Jutila M. A. and Pascual D. W. (2002) Mucosal addressin expression and binding-interactions with naive lymphocytes vary among the cranial, oral, and nasal-associated lymphoid tissues. *European Journal of Immunology* **32**, 3029-3039.

Csencsits K. L. and Pascual D. W. (2002) Absence of L-selectin delays mucosal B cell responses in nonintestinal effector tissues. *Journal of Immunology* **169**, 5649-5659.

Csencsits K. L., Walters N. and Pascual D. W. (2001) Cutting edge: Dichotomy of homing receptor dependence by mucosal effector B cells: alpha(E) versus L-selectin. *Journal of Immunology* **167**, 2441-2445.

Cyster J. G. (1999) Chemokines and cell migration in secondary lymphoid organs. *Science* **286**, 2098-102.

Cyster J. G. (2003) Homing of antibody secreting cells. *Immunological Reviews* **194**, 48-60.

Cyster J. G., Ansel K. M., Reif K., Ekland E. H., Hyman P. L., Tang H. L., Luther S. A. and Ngo V. N. (2000) Follicular stromal cells and lymphocyte homing to follicles. *Immunological Reviews* **176**, 181-93.

Czinn S. J. and Lamm M. E. (1986) Selective chemotaxis of subsets of B lymphocytes from gut-associated lymphoid tissue and its implications for the recruitment of mucosal plasma cells. *Journal of Immunology* **136**, 3607-11.

Dallas S. D. and Rolfe R. D. (1998) Binding Of Clostridium Difficile Toxin a to Human Milk Secretory Component. *Journal of Medical Microbiology* **47**, 879-888.

Davidson G. P., Whyte P. B., Daniels E., Franklin K., Nunan H., McCloud P. I., Moore A. G. and Moore D. J. (1989) Passive immunisation of children with bovine colostrum containing antibodies to human rotavirus. *Lancet* **2**, 709-12.

de Groot N., Van Kuik-Romeijn P., Lee S. H. and De Boer H. A. (2000) Increased immunoglobulin A levels in milk by over-expressing the murine polymeric immunoglobulin receptor gene in the mammary gland epithelial cells of transgenic mice. *Immunology* **101**, 218-224.

De Haan A., Geerligs H. J., Huchshorn J. P., Van Scharrenburg G. J. M., Palache A. M. and Wilschut J. (1995) Mucosal immunoadjuvant activity of liposomes: Induction of systemic IgG and secretory IgA responses in mice by intranasal immunization with an influenza subunit vaccine and coadministered liposomes. *Vaccine* **13**, 155-162.

de Oliveira I. R., de Araujo A. N., Bao S. N. and Giugliano L. G. (2001) Binding of lactoferrin and free secretory component to enterotoxigenic Escherichia coli. *FEMS Microbiology Letters* **203**, 29-33.

Defrance T., Vanbervliet B., Briere F., Durand I., Rousset F. and Banchereau J. (1992) Interleukin 10 and transforming growth factor beta cooperate to induce anti-CD40-activated naive human B cells to secrete immunoglobulin A. *Journal of Experimental Medicine* **175**, 671-82.

Della Corte E. and Parkhouse R. M. (1973) Biosynthesis of immunoglobulin A (IgA) and immunoglobulin M (IgM). Requirement for J chain and a disulphide-exchanging enzyme for polymerization. *The Biochemical Journal.* **136**, 597-606.

Dickinson E. C., Gorga J. C., Garrett M., Tuncer R., Boyle P., Watkins S. C., Alber S. M., Parizhskaya M., Trucco M., Rowe M. I. and Ford H. R. (1998) Immunoglobulin A supplementation abrogates bacterial translocation and preserves the architecture of the intestinal epithelium. *Surgery* **124**, 284-90.

Dixon F. J., Weigle W. O. and Vazquez J. J. (1961) Metabolism and mammary secretion of serum proteins in the cow. *Laboratory investigation; a journal of technical methods and pathology* **10**, 216-37.

Dosogne H., Vangroenweghe F., Mehrzad J., Massart-Leen A. M. and Burvenich C. (2003) Differential leukocyte count method for bovine low somatic cell count milk. *Journal of Dairy Science* **86**, 828-34.

Doymaz M. Z., Sordillo L. M., Oliver S. P. and Guidry A. J. (1988) Effects of Staphylococcus aureus mastitis on bovine mammary gland plasma cell populations and immunoglobin concentrations in milk. *Veterinary Immunology and Immunopathology* **20**, 87-93.

Duhamel G. E., Bernoco D., Davis W. C. and Osburn B. I. (1987) Distribution of T and B lymphocytes in mammary dry secretions, colostrum and blood of adult dairy cattle. *Veterinary Immunology and Immunopathology* **14**, 101-22.

Dunn-Walters D. K., Isaacson P. G. and Spencer J. (1997) Sequence analysis of human IgVH genes indicates that ileal lamina propria plasma cells are derived from Peyer's patches. *European Journal of Immunology* **27**, 463-467.

Ebert E. C. (1999) Inhibitory effects of transforming growth factor-beta (TGF-beta) on certain functions of intraepithelial lymphocytes. *Clinical & Experimental Immunology* **115**, 415-420.

Ebina T., Ohta M., Kanamaru Y., Yamamoto Osumi Y. and Baba K. (1992) Passive immunizations of suckling mice and infants with bovine colostrum containing antibodies to human rotavirus. *Journal of Medical Virology* **38**, 117-123.

Ebina T., Sato A., Umezu K., Ishida N., Ohyama S., Ohizumi A., Aikawa K., Katagiri S., Katsushima N., Imai A. and et al. (1983) Prevention of rotavirus infection by cow colostrum antibody against human rotaviruses. *Lancet* **2**, 1029-30.

Eskeland T. and Brandtzaeg P. (1974) Does J chain mediate the combination of 19S IgM and dimeric IgA with the secretory component rather than being necessary for their polymerization? *Immunochemistry* **11**, 161-3.

Estes D. M. and Brown W. C. (2002) Type 1 and type 2 responses in regulation of Ig isotype expression in cattle. *Veterinary Immunology and Immunopathology* **90**, 1-10.

Estes D. M., Closser N. M. and Allen G. K. (1994) IFN-gamma stimulates IgG2 production from bovine B cells costimulated with anti-mu and mitogen. *Cellular Immunology* **154**, 287-95.

Estes D. M., Hirano A., Heussler V. T., Dobbelaere D. A. and Brown W. C. (1995) Expression and biological activities of bovine interleukin 4: effects of recombinant bovine interleukin 4 on T cell proliferation and B cell differentiation and proliferation in vitro. *Cellular Immunology* **163**, 268-79.

Fagarasan S., Kinoshita K., Muramatsu M., Ikuta K. and Honjo T. (2001) In situ class switching and differentiation to IgA-producing cells in the gut lamina propria. *Nature* **413**, 639-643.

Fahey K. J., Snodgrass D. R., Campbell I., Dawson A. M. and Burrells C. (1981) IgG1 antibody in milk protects lambs against rotavirus diarrhoea. *Veterinary Immunology and Immunopathology* **2**, 27-33.

Fallgreen-Gebauer E., Gebauer W., Bastian A., Kratzin H. D., Eiffert H., Zimmermann B., Karas M. and Hilschmann N. (1993) The covalent linkage of secretory component to IgA. Structure of sIgA. *Biological Chemistry Hoppe-Seyler* **374**, 1023-8.

Farrar M. A. and Schreiber R. D. (1993) The molecular cell biology of interferon-gamma and its receptor. *Annual Review of Immunology* **11**, 571-611.

Farstad I. N., Halstensen T. S., Fausa O. and Brandtzaeg P. (1994) Heterogeneity of M-cell-associated B and T cells in human Peyer's patches. *Immunology* **83**, 457-64.

Fearon D. T. and Locksley R. M. (1996) The instructive role of innate immunity in the acquired immune response. *Science* **272**, 50-3.

Fernandez F. M., Conner M. E., Hodgins D. C., Parwani A. V., Nielsen P. R., Crawford S. E., Estes M. K. and Saif L. J. (1998) Passive immunity to bovine rotavirus in newborn calves fed colostrum supplements from cows immunized with recombinant SA11 rotavirus core-like particle (CLP) or virus-like particle (VLP) vaccines. *Vaccine* **16**, 507-16.

Fernandez F. M., Conner M. E., Parwani A. V., Todhunter D., Smith K. L., Crawford S. E., Estes M. K. and Saif L. J. (1996) Isotype-specific antibody responses to rotavirus and virus proteins in cows inoculated with subunit vaccines composed of recombinant SA11 rotavirus core-like particles (CLP) or virus-like particles (VLP). *Vaccine* **14**, 1303-1312.

Finke D. and Acha-Orbea H. (2001) Differential migration of in vivo primed B and T lymphocytes to lymphoid and non-lymphoid organs. *European Journal of Immunology* **31**, 2603-11.

Fitzpatrick J. L., Cripps P. J., Hill A. W., Bland P. W. and Stokes C. R. (1992) MHC class II expression in the bovine mammary gland. *Veterinary Immunology and Immunopathology* **32**, 13-23.

Forster R., Schubel A., Breitfeld D., Kremmer E., Renner-Muller I., Wolf E. and Lipp M. (1999) CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* **99**, 23-33.

Freedman D. J., Tacket C. O., Delehanty A., Maneval D. R., Nataro J. and Crabb J. H. (1998) Milk immunoglobulin with specific activity against purified colonization factor antigens can protect against oral challenge with enterotoxigenic Escherichia coli. *Journal of Infectious Diseases* **177**, 662-7.

Geijtenbeek T. B., Torensma R., van Vliet S. J., van Duijnhoven G. C., Adema G. J., van Kooyk Y. and Figdor C. G. (2000) Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell* **100**, 575-85.

Germain R. N. (1994) MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. *Cell* **76**, 287-99.

Girard J. P. and Springer T. A. (1995) High endothelial venules (HEVs): specialized endothelium for lymphocyte migration. *Immunology Today* **16**, 449-57.

Goodrich M. E. and McGee D. W. (1999) Effect of intestinal epithelial cell cytokines on mucosal B-cell IgA secretion: Enhancing effect of epithelial-derived IL-6 but not TGF-beta on IgA+ B cells. *Immunology Letters* **67**, 11-14.

Gorin A. B., Stewart P. and Gould J. (1979) Concentrations of immunoglobulin classes in subcompartments of the sheep lung. *Research in Veterinary Science* **26**, 126-8.

Graves D. T. and Jiang Y. (1995) Chemokines, a family of chemotactic cytokines. *Critical Reviews in Oral Biology and Medicine* **6**, 109-18.

Grayson J. M., Harrington L. E., Lanier J. G., Wherry E. J. and Ahmed R. (2002) Differential sensitivity of naive and memory CD8+ T cells to apoptosis in vivo. *Journal of Immunology* **169**, 3760-70.

Greenberg P. D. and Cello J. P. (1996) Treatment of severe diarrhea caused by Cryptosporidium parvum with oral bovine immunoglobulin concentrate in patients

with AIDS. *Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology* **13**, 348-54.
Grewal I. S. and Flavell R. A. (1996) The role of CD40 ligand in costimulation and T-cell activation. *Immunological Reviews* **153**, 85-106.
Groves M. L. and Gordon W. G. (1967) Isolation of a new glycoprotein-a and a gamma-G-globulin from individual cow milks. *Biochemistry* **6**, 2388-94.
Gunzer M., Schafer A., Borgmann S., Grabbe S., Zanker K. S., Brocker E. B., Kampgen E. and Friedl P. (2000) Antigen presentation in extracellular matrix: interactions of T cells with dendritic cells are dynamic, short lived, and sequential. *Immunity* **13**, 323-32.
Gutierrez M., Forster F. I., McConnell S. A., Cassidy J. P., Pollock J. M. and Bryson D. G. (1999) The detection of CD2+, CD4+, CD8+, and WC1+ T lymphocytes, B cells and macrophages in fixed and paraffin embedded bovine tissue using a range of antigen recovery and signal amplification techniques. *Veterinary Immunology and Immunopathology* **71**, 321-34.
Hagiwara K., Kataoka S., Yamanaka H., Kirisawa R. and Iwai H. (2000) Detection of cytokines in bovine colostrum. *Veterinary Immunology and Immunopathology* **76**, 183-90.
Hajishengallis G., Nikolova E. and Russell M. W. (1992) Inhibition of Streptococcus mutans adherence to saliva-coated hydroxyapatite by human secretory immunoglobulin A (S-IgA) antibodies to cell surface protein antigen I/II: reversal by IgA1 protease cleavage. *Infection and Immunity* **60**, 5057-64.
Hamada H., Hiroi T., Nishiyama Y., Takahashi H., Masunaga Y., Hachimura S., Kaminogawa S., Takahashi-Iwanaga H., Iwanaga T., Kiyono H., Yamamoto H. and Ishikawa H. (2002) Identification of multiple isolated lymphoid follicles on the antimesenteric wall of the mouse small intestine. *Journal of Immunology* **168**, 57-64.
Han S., Hathcock K., Zheng B., Kepler T. B., Hodes R. and Kelsoe G. (1995) Cellular interaction in germinal centers. Roles of CD40 ligand and B7-2 in established germinal centers. *Journal of Immunology* **155**, 556-67.
Hanson L. A. and Johansson B. G. (1970). In *Milk proteins: Chemistry and molecular biology* (Edited by McKenzie H. A.), Vol. 1, p. 45-123. Academic Press, New York.
Hargreaves D. C., Hyman P. L., Lu T. T., Ngo V. N., Bidgol A., Suzuki G., Zou Y. R., Littman D. R. and Cyster J. G. (2001) A coordinated change in chemokine responsiveness guides plasma cell movements. *Journal of Experimental Medicine* **194**, 45-56.
Harp J. A., Stabel J. R., Pesch B. A. and Goff J. P. (2004) Expression of adhesion molecules on milk and blood lymphocytes from periparturient dairy cattle with Johne's disease. *Veterinary Immunology and Immunopathology* **98**, 69-76.
Harriman G. R., Kunimoto D. Y., Elliott J. F., Paetkau V. and Strober W. (1988) The role of IL-5 in IgA B cell differentiation. *Journal of Immunology* **140**, 3033-9.
Hathaway L. J. and Kraehenbuhl J. P. (2000) The role of M cells in mucosal immunity. *Cellular & Molecular Life Sciences* **57**, 323-332.
Hauser A. E., Debes G. F., Arce S., Cassese G., Hamann A., Radbruch A. and Manz R. A. (2002) Chemotactic responsiveness toward ligands for CXCR3 and CXCR4 is regulated on plasma blasts during the time course of a memory immune response. *Journal of Immunology* **169**, 1277-82.
Hein W. R. and Mackay C. R. (1991) Prominence of gamma delta T cells in the ruminant immune system. *Immunology Today* **12**, 30-4.
Heyneman R., Burvenich C. and Vercauteren R. (1990) Interaction between the respiratory burst activity of neutrophil leukocytes and experimentally induced Escherichia coli mastitis in cows. *Journal of Dairy Science* **73**, 985-94.
Higuchi R., Fockler C., Dollinger G. and Watson R. (1993) Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology (N Y)* **11**, 1026-30.
Hilpert H., Brussow H., Mietens C., Sidoti J., Lerner L. and Werchau H. (1987) Use of bovine milk concentrate containing antibody to rotavirus to treat rotavirus gastroenteritis in infants. *Journal of Infectious Diseases* **156**, 158-66.

Hirano T., Taga T., Yamasaki K., Matsuda T., Tang B., Muraguchi A., Horii Y., Suematsu S., Hirata Y., Yawata H. and et al. (1989) A multifunctional cytokine (IL-6/BSF-2) and its receptor. *International Archives of Allergy and Applied Immunology* **88**, 29-33.

Hodgkinson A. J. and Hodgkinson S. C. (2003) Processes for production of immunoglobulin A in milk. In *Official Gazette of the United States Patent and Trademark Office Patents*, Vol. 1274, p. No Pagination.

Hodgkinson A. J., McQuoid M. R. and Hodgkinson S. C. (1995) Antibody class-specific immune responses in ovine milk measured by ELISA. *Proceedings of the New Zealand Society of Animal Production* **55**, 218-220.

Holmes C. W., Wilson G. F., Mackenzie D. D. S., Flux D. S., Brookes I. M. and Davey A. W. F. (1984a) Lactation: Mammary gland development and maintenance of lactation. In *Milk production from pasture*, p. 176-195. Butterworths of New Zealand, Wellington.

Holmes C. W., Wilson G. F., Mackenzie D. D. S., Flux D. S., Brookes I. M. and Davey A. W. F. (1984b) Lactation: Mammary gland structure and milk secretion. In *Milk production from pasture*, p. 162-175. Butterworths of New Zealand, Wellington.

Holmes W. E., Lee J., Kuang W. J., Rice G. C. and Wood W. I. (1991) Structure and functional expression of a human interleukin-8 receptor. *Science* **253**, 1278-80.

Holt P. G., Schon-Hegrad M. A. and McMenamin P. G. (1990) Dendritic cells in the respiratory tract. *International Reviews of Immunology* **6**, 139-49.

Honjo T., Kinoshita K. and Muramatsu M. (2002) Molecular mechanism of class switch recombination: linkage with somatic hypermutation. *Annual Review of Immunology* **20**, 165-96.

Horsfall D. J., Heddle R. J., Gormly A. A. and Rowley D. (1978) In vitro degradation of mouse, rabbit and dog antibodies to Vibrio cholerae by succus entericus. *Australian Journal of Experimental Biology and Medical Science* **56**, 681-93.

Hosseini S., Inserra P., Araghi-Niknam M. and Watson R. R. (2001) Colostrum and milk in the treatment of disease. *Advances In Nutritional Research* **10**, 201-212.

Hsu S. M., Raine L. and Fanger H. (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *The Journal of Histochemistry and Cytochemistry* **29**, 577-80.

Husband A. J., Beagley K. W. and McGhee J. R. (1999) Mucosal cytokines. In *Mucosal Immunology* (Edited by Ogra P. L., Mestecky J., Lamm M. E., Strober W., Bienenstock J. and McGhee J. R.), p. 541-557. Academic Press, San Diego.

Husband A. J. and Gowans J. L. (1978) The origin and antigen-dependent distribution of IgA-containing cells in the intestine. *Journal of Experimental Medicine* **148**, 1146-60.

Iijima H., Takahashi I. and Kiyono H. (2001) Mucosal immune network in the gut for the control of infectious diseases. *Reviews in Medical Virology* **11**, 117-133.

Inchaisri C., Waller K. P. and Johannisson A. (2000) Studies on the modulation of leucocyte subpopulations and immunoglobulins following intramammary infusion of beta 1,3-glucan into the bovine udder during the dry period. *Zentralblatt fur Veterinarmedizin - Reihe B* **47**, 373-386.

Iwasaki A. and Kelsall B. L. (1999) Freshly isolated Peyer's patch, but not spleen, dendritic cells produce interleukin 10 and induce the differentiation of T helper type 2 cells. *Journal of Experimental Medicine* **190**, 229-239.

Iwasaki A. and Kelsall B. L. (2000) Localization of distinct Peyer's patch dendritic cell subsets and their recruitment by chemokines macrophage inflammatory protein (MIP)-3alpha, MIP-3beta, and secondary lymphoid organ chemokine. *Journal of Experimental Medicine* **191**, 1381-94.

Janeway C. A., Travers P., Walport M. and Schlomchik M. J. (2001a) Adaptive immunity to infection. In *Immunobiology: The immune system in health and disease.* (Edited by Gibbs S.), p. 381-423. Garland Publishing, New York.

Janeway C. A., Travers P., Walport M. and Schlomchik M. J. (2001b) Cell-mediated Immunity. In *Immunobiology: The immune system in health and disease.* (Edited by Gibbs S.), p. 295-340. Garland Publishing, New York.

Janeway C. A., Travers P., Walport M. and Schlomchik M. J. (2001c) The generation of lymphocyte antigen receptors. In *Immunobiology: The immune system in Health and Disease.* (Edited by Gibbs S.), p. 123-154. Garland Publishing, New York.

Janeway C. A., Travers P., Walport M. and Schlomchik M. J. (2001d) The humoral immune response. In *Immunobiology: The immune system in health and disease.* (Edited by Gibbs S.), p. 341-380. Garland Publishing, New York.

Jensen D. L. and Eberhart R. J. (1981) Total and differential cell counts in secretions of the nonlactating bovine mammary gland. *American Journal of Veterinary Research* **42**, 743-7.

Jertborn M., Ahren C., Holmgren J. and Svennerholm A. M. (1998) Safety and Immunogenicity Of an Oral Inactivated Enterotoxigenic Escherichia Coli Vaccine. *Vaccine* **16**, 255-260.

Jertborn M., Svennerholm A. M. and Holmgren J. (1986) Saliva, breast milk, and serum antibody responses as indirect measures of intestinal immunity after oral cholera vaccination or natural disease. *Journal of Clinical Microbiology* **24**, 203-9.

Johansen F. E., Braathen R. and Brandtzaeg P. (2001) The J chain is essential for polymeric Ig receptor-mediated epithelial transport of IgA. *Journal of Immunology* **167**, 5185-5192.

Jutila M. A., Berg E. L., Kishimoto T. K., Picker L. J., Bargatze R. F., Bishop D. K., Orosz C. G., Wu N. W. and Butcher E. C. (1989a) Inflammation-induced endothelial cell adhesion to lymphocytes, neutrophils, and monocytes. Role of homing receptors and other adhesion molecules. *Transplantation* **48**, 727-31.

Jutila M. A., Rott L., Berg E. L. and Butcher E. C. (1989b) Function and regulation of the neutrophil MEL-14 antigen in vivo: comparison with LFA-1 and MAC-1. *Journal of Immunology* **143**, 3318-24.

Kaetzel C. S., Robinson J. K., Chintalacharuvu K. R., Vaerman J. P. and Lamm M. E. (1991) The polymeric immunoglobulin receptor (secretory component) mediates transport of immune complexes across epithelial cells: a local defense function for IgA. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 8796-800.

Kansas G. S. (1996) Selectins and their ligands: current concepts and controversies. *Blood* **88**, 3259-87.

Kantele A., Kantele J. M., Savilahti E., Westerholm M., Arvilommi H., Lazarovits A., Butcher E. C. and Makela P. H. (1997) Homing potentials of circulating lymphocytes in humans depend on the site of activation: Oral, but not parenteral, typhoid vaccination induces circulating antibody-secreting cells that all bear homing receptors directing them to the gut. *Journal of Immunology* **158**, 574-579.

Kato S., Hokari R., Matsuzaki K., Iwai A., Kawaguchi A., Nagao S., Miyahara T., Itoh K., Ishii H. and Miura S. (2000) Amelioration of murine experimental colitis by inhibition of mucosal addressin cell adhesion molecule-1. *Journal of Pharmacology and Experimental Therapeutics* **295**, 183-189.

Kato T. and Owen R. L. (1999) Structure and function of intestinal mucosal epithelium. In *Mucosal Immunology* (Edited by Ogra P. L., Mestecky J., Lamm M. E., Strober W., Bienenstock J. and McGhee J. R.), p. 115-132. Academic Press, San Diego.

Kawabata K., Ujikawa M., Egawa T., Kawamoto H., Tachibana K., Iizasa H., Katsura Y., Kishimoto T. and Nagasawa T. (1999) A cell-autonomous requirement for CXCR4 in long-term lymphoid and myeloid reconstitution. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 5663-7.

Kehrli M. E., Jr. and Harp J. A. (2001) Immunity in the mammary gland. *The Veterinary Clinics of North America. Food animal practice* **17**, 495-516.

Kehrli M. E., Jr., Nonnecke B. J. and Roth J. A. (1989) Alterations in bovine lymphocyte function during the periparturient period. *American Journal of Veterinary Research* **50**, 215-20.

Kelly C. P., Pothoulakis C., Vavva F., Castagliuolo I., Bostwick E. F., C O. K. J., Keates S. and Lamont J. T. (1996) Anti-Clostridium difficile bovine immunoglobulin concentrate inhibits cytotoxicity and enterotoxicity of C. difficile toxins. *Antimicrobial Agents and Chemotherapy* **40**, 373-379.

Kelly K. A. and Rank R. G. (1997) Identification Of Homing Receptors That Mediate the Recruitment Of Cd4 T Cells to the Genital Tract Following Intravaginal Infection With Chlamydia Trachomatis. *Infection and Immunity* **65**, 5198-5208.

Kelso A. (1995) Th1 and Th2 subsets: paradigms lost? *Immunology Today* **16**, 374-9.

Kemler R., Mossmann H., Strohmaier U., Kickhofen B. and Hammer D. K. (1975) In vitro studies on the selective binding of IgG from different species to tissue sections of the bovine mammary gland. *European Journal of Immunology* **5**, 603-8.

Kilian M., Reinholdt J., Mortensen S. B. and Sorensen C. H. (1983) Perturbation of mucosal immune defence mechanisms by bacterial IgA proteases. *Bulletin of European Physiopathology and Respiratory* **19**, 99-104.

Kim P. H. and Kagnoff M. F. (1990) Transforming growth factor beta 1 increases IgA isotype switching at the clonal level. *Journal of Immunology* **145**, 3773-8.

Kinoshita K. and Honjo T. (2001) Linking class-switch recombination with somatic hypermutation. *National Reviews of Molecular and Cell Biology* **2**, 493-503.

Kishimoto T. (1989) The biology of interleukin-6. *Blood* **74**, 1-10.

Knight K. L., Suter M. and Becker R. S. (1988) Genetic engineering of bovine Ig. Construction and characterization of hapten-binding bovine/murine chimeric IgE, IgA, IgG1, IgG2, and IgG3 molecules. *Journal of Immunology* **140**, 3654-9.

Korhonen H., Marnila P. and Gill H. S. (2000) Bovine milk antibodies for health. *British Journal of Nutrition* **84**, S135-S146.

Korhonen H., Syvaoja E. L., Ahola Luttila H., Sivela S., Kopola S., Husu J. and Kosunen T. (1994) Helicobacter pylori-specific antibodies and bactericidal activity in serum, colostrum and milk of immunized and non-immunized cows. In *Indigenous antimicrobial agents of milk: recent developments*, p. 131-136. International Dairy Federation, Brussels.

Koshland M. E. (1975) Structure and function of the J chain. *Advances in Immunology* **20**, 41-69.

Kraehenbuhl J. P. and Neutra M. R. (1992) Molecular and cellular basis of immune protection of mucosal surfaces. *Physiological Reviews* **72**, 853-79.

Kruschwitz M., Fritzsche G., Schwarting R., Micklem K., Mason D. Y., Falini B. and Stein H. (1991) Ber-ACT8: new monoclonal antibody to the mucosa lymphocyte antigen. *Journal of Clinical Pathology* **44**, 636-45.

Kruse V. (1970) Absorption of immunoglobulin from colostrum in newborn calves. *Animal Production* **12**, 627-638.

Kuhn R., Rajewsky K. and Muller W. (1991) Generation and analysis of interleukin-4 deficient mice. *Science* **254**, 707-10.

Kulseth M. A., Krajci P., Myklebost O. and Rogne S. (1995) Cloning and characterization of two forms of bovine polymeric immunoglobulin receptor cDNA. *DNA and Cell Biology* **14**, 251-256.

Kunkel E. J., Campbell J. J., Haraldsen G., Pan J., Boisvert J., Roberts A. I., Ebert E. C., Vierra M. A., Goodman S. B., Genovese M. C., Wardlaw A. J., Greenberg H. B., Parker C. M., Butcher E. C., Andrew D. P. and Agace W. W. (2000) Lymphocyte CC chemokine receptor 9 and epithelial thymus-expressed chemokine (TECK) expression distinguish the small intestinal immune compartment: Epithelial expression of tissue-specific chemokines as an organizing principle in regional immunity. *Journal of Experimental Medicine* **192**, 761-8.

Kunkel E. J., Kim C. H., Lazarus N. H., Vierra M. A., Soler D., Bowman E. P. and Butcher E. C. (2003) CCR10 expression is a common feature of circulating and mucosal epithelial tissue IgA Ab-secreting cells. *Journal of Clinical Investigation* **111**, 1001-1010.

Kupfer A., Swain S. L., Janeway C. A., Jr. and Singer S. J. (1986) The specific direct interaction of helper T cells and antigen-presenting B cells. *Proceedings of the Nationall Academy of Science U S A* **83**, 6080-3.

Labib R. S., Calvanico N. J. and Tomasi T. B. (1976) Bovine secretory component. Isolation, molecular size and shape, composition, and NH2-terminal amino acid sequence. *Journal of Biology and Chemistry* **251**, 1969-74.

Lamm M. E. (1997) Interactions of antigens and anitbodies at mucosal surfaces. *Annual Review of Microbiology* **51**, 311-340.

Lascelles A. K. (1963) A review of the literature on some aspects of immune milk. *Dairy Science Abstracts* **25**, 359-364.

Lascelles A. K., Beh K. J. and Husband A. J. (1981) Origin of antibody in mammary secretion with particular reference to the IgA system. *Advances in Experimental Medicine and Biology.* **137**, 493-511.

Lascelles A. K., Beh K. J., Kerlin R. L., Watson D. L. and Mukkur T. K. (1986) The generation of antibody of different isotype specificity in relation to mucosal defence in ruminants. In *Immunology of the sheep* (Edited by Morns and Miyaska), p. 410-435. Roche, Basle.

Lazarus N. H., Kunkel E. J., Johnston B., Wilson E., Youngman K. R. and Butcher E. C. (2003) A common mucosal chemokine (mucosae-associated epithelial chemokine/CCL28) selectively attracts IgA plasmablasts. *Journal of Immunology* **170**, 3799-3805.

Lebman D. A. and Edmiston J. S. (1999) The role of TGF-beta in growth, differentiation, and maturation of B lymphocytes. *Microbes and Infection* **1**, 1297-1304.

Lebman D. A., Lee F. D. and Coffman R. L. (1990) Mechanism for transforming growth factor beta and IL-2 enhancement of IgA expression in lipopolysaccharide-stimulated B cell cultures. *Journal of Immunology* **144**, 952-9.

Lee C. S. and Lascelles A. K. (1969a) Distribution of lymphatic vessels in mammary glands of ewes. *American Journal of Anatomy* **126**, 489-95.

Lee C. S. and Lascelles A. K. (1969b) The histological changes in involuting mammary glands of ewes in relation to the local allergic response. *Australian Journal of Experimental Biology and Medical Science* **47**, 613-23.

Lee C. S. and Lascelles A. K. (1970) Antibody-producing cells in antigenically stimulated mammary glands and in the gastro-intestinal tract of sheep. *Australian Journal of Experimental Biology and Medical Science* **48**, 525-35.

Lee C. S., Meeusen E. and Brandon M. R. (1989) Subpopulations of lymphocytes in the mammary gland of sheep. *Immunology* **66**, 388-93.

Lee C. S., Meeusen E. and Brandon M. R. (1992) Local immunity in the mammary gland. *Veterinary Immunology and Immunopathology* **32**, 1-II.

Lee C. S., Wooding F. B. and Kemp P. (1980) Identification, properties, and differential counts of cell populations using electron microscopy of dry cows secretions, colostrum and milk from normal cows. *Journal of Dairy Research* **47**, 39-50.

Leitner G., Chaffer M., Krifucks O., Glickman A., Ezra E. and Saran A. (2000a) Milk leucocyte populations in heifers free of udder infection. *Zentralblatt fur Veterinarmedizin - Reihe B* **47**, 133-138.

Leitner G., Shoshani E., Krifucks O., Chaffer M. and Saran A. (2000b) Milk leucocyte population patterns in bovine udder infection of different aetiology. *Zentralblatt fur Veterinarmedizin - Reihe B* **47**, 581-589.

Letesson J. J., Van den Broecke A., Marbaix-Cleuter Y., Delcommenne M., Mager A., Mammerickx M., Burny A. and Depelchin A. (1991) FACS analysis of bovine leukemia virus (BLV)-infected cell lines with monoclonal antibodies (mAbs) to B cells and to monocytes/macrophages. *Veterinary Immunology and Immunopathology* **27**, 207-13.

Leung E., Berg R. W., Langley R., Greene J., Raymond L. A., Augustus M., Ni J., Carter K. C., Spurr N., Choo K. H. and Krissansen G. W. (1997) Genomic organization, chromosomal mapping, and analysis of the 5' promoter region of the human MAdCAM-1 gene. *Immunogenetics* **46**, 111-9.

Leung E., Greene J., Ni J., Raymond L. G., Lehnert K., Langley R. and Krissansen G. W. (1996) Cloning of the mucosal addressin MAdCAM-1 from human brain: identification of novel alternatively spliced transcripts. *Immunology and Cell Biology* **74**, 490-6.

Leutenegger C. M., Alluwaimi A. M., Smith W. L., Perani L. and Cullor J. S. (2000) Quantitation of bovine cytokine mRNA in milk cells of healthy cattle by real-time TaqMan polymerase chain reaction. *Veterinary Immunology and Immunopathology* **77**, 275-87.

Liebler-Tenorio E. M., Riedel-Caspari G. and Pohlenz J. F. (2002) Uptake of colostral leukocytes in the intestinal tract of newborn calves. *Veterinary Immunology and Immunopathology* **85**, 33-40.

Lindh E. (1975) Increased risistance of immunoglobulin A dimers to proteolytic degradation after binding of secretory component. *Journal of Immunology* **114**, 284-6.

Loimaranta V., Laine M., Soderling E., Vasara E., Rokka S., Marnila P., Korhonen H., Tossavainen O. and Tenovuo J. (1999) Effects of bovine immune and non-immune whey preparations on the composition and pH response of human dental plaque. *European Journal of Oral Science* **107**, 244-50.

Loimaranta V., Tenovuo J., Virtanen S., Marnila P., Syvaoja E. L., Tupasela T. and Korhonen H. (1997) Generation Of Bovine Immune Colostrum Against Streptococcus Mutans and Streptococcus Sobrinus and Its Effect On Glucose Uptake and Extracellular Polysaccharide Formation By Mutans Streptococci. *Vaccine* **15**, 1261-1268.

Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J. (1951) Protein measurement with the Folin Phenol reagent. *Journal of Biological Chemistry* **193**, 265-275.

Luther S. A., Bidgol A., Hargreaves D. C., Schmidt A., Xu Y., Paniyadi J., Matloubian M. and Cyster J. G. (2002) Differing activities of homeostatic chemokines CCL19, CCL21, and CXCL12 in lymphocyte and dendritic cell recruitment and lymphoid neogenesis. *Journal of Immunology* **169**, 424-33.

Ma Q., Jones D. and Springer T. A. (1999) The chemokine receptor CXCR4 is required for the retention of B lineage and granulocytic precursors within the bone marrow microenvironment. *Immunity* **10**, 463-71.

Mackay C. R., Andrew D. P., Briskin M., Ringler D. J. and Butcher E. C. (1996) Phenotype, and migration properties of three major subsets of tissue homing T cells in sheep. *European Journal of Immunology* **26**, 2433-9.

Mackay C. R., Marston W. and Dudler L. (1992) Altered patterns of T cell migration through lymph nodes and skin following antigen challenge. *European Journal of Immunology* **22**, 2205-10.

MacKay F. and Browning J. L. (2002) BAFF: A fundamental survival factor for B cells. *Nature Reviews Immunology* **2**, 465-475.

MacLennan I. C. and Gray D. (1986) Antigen-driven selection of virgin and memory B cells. *Immunological Reviews* **91**, 61-85.

Macpherson A. J., Hunziker L., McCoy K. and Lamarre A. (2001) IgA responses in the intestinal mucosa against pathogenic and non-pathogenic microorganisms. *Microbes and Infection* **3**, 1021-1035.

Magnusson K. E. and Stjernstrom I. (1982) Mucosal barrier mechanisms. Interplay between secretory IgA (SIgA), IgG and mucins on the surface properties and association of salmonellae with intestine and granulocytes. *Immunology* **45**, 239-48.

Mallard B. A., Dekkers J. C., Ireland M. J., Leslie K. E., Sharif S., Vankampen C. L., Wagter L. and Wilkie B. N. (1998) Alteration in immune responsiveness during the peripartum period and its ramification on dairy cow and calf health. *Journal of Dairy Science* **81**, 585-95.

Manz R. A., Arce S., Cassese G., Hauser A. E., Hiepe F. and Radbruch A. (2002) Humoral immunity and long-lived plasma cells. *Current Opinion in Immunology* **14**, 517-21.

Manz R. A. and Radbruch A. (2002) Plasma cells for a lifetime? *European Journal of Immunology* **32**, 923-7.

Maric I., Holt P. G., Perdue M. H. and Bienenstock J. (1996) Class II MHC antigen (Ia)-bearing dendritic cells in the epithelium of the rat intestine. *Journal of Immunology* **156**, 1408-14.

Marshall L. J., Perks B., Ferkol T. and Shute J. K. (2001) IL-8 released constitutively by primary bronchial epithelial cells in culture forms an inactive complex with secretory component. *Journal of Immunology* **167**, 2816-2823.

Mazanec M. B., Kaetzel C. S., Lamm M. E., Fletcher D. and Nedrud J. G. (1992) Intracellular neutralization of virus by immunoglobulin A antibodies. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 6901-5.

McDermott M. R. and Bienenstock J. (1979) Evidence for a common mucosal immunologic system. I. Migration of B immunoblasts into intestinal, respiratory, and genital tissues. *Journal of Immunology* **122**, 1892-8.

McDermott M. R., Clark D. A. and Bienenstock J. (1980) Evidence for a common mucosal immunologic system. II. Influence of the estrous cycle on B immunoblast migration into genital and intestinal tissues. *Journal of Immunology* **124**, 2536-9.

McGhee J. R., Czerkinsky C. and Mestecky J. (1999) Mucosal vaccines: an overview. In *Mucosal Immunology* (Edited by Ogra P. L., Mestecky J., Lamm M. E., Strober W., Bienenstock J. and McGhee J. R.), p. 741-757. Acedemic Press, San Diego.

McIntyre T. M. and Strober W. (1999) Gut-associated lymphoid tissue: regulation of IgA B-cell development. In *Mucosal immunology* (Edited by Ogra P. L., Mestecky J., Lamm M. E., Strober W., Bienenstock J. and McGhee J. R.), p. 319-356. Academic Press, San Diego.

Meeusen E., Lee C. S. and Brandon M. (1991) Differential migration of T and B cells during an acute inflammatory response. *European Journal of Immunology* **21**, 2269-72.

Mepham T. B. (1987) *Physiology of lactation*. Open University Press, Milton Keynes, UK.

Mestecky J. (1987) The common mucosal immune system and current strategies for induction of immune responses in external secretions. *Journal of Clinical Immunology* **7**, 265-76.

Mestecky J. and McGhee J. R. (1987) Immunoglobulin A (IgA): molecular and cellular interactions involved in IgA biosynthesis and immune response. *Advances in Immunology* **40**, 153-245.

Mestecky J., Moldoveanu Z., Michalek S. M., Morrow C. D., Compans R. W., Schafer D. P. and Russell M. W. (1997) Current Options For Vaccine Delivery Systems By Mucosal Routes. *Journal of Controlled Release* **48**, 243-257.

Mietens C., Keinhorst H., Hilpert H., Gerber H., Amster H. and Pahud J. J. (1979) Treatment of infantile E. coli gastroenteritis with specific bovine anti-E. coli milk immunoglobulins. *European Journal of Pediatrics* **132**, 239-52.

Mikulowska-Mennis A., Xu B. H., Berberian J. M. and Michie S. A. (2001) Lymphocyte migration to is mediated by vascular inflamed lacrimal glands cell adhesion molecule-1/alpha(4)beta(1) integrin, peripheral node addressin/L-selectin, and lymphocyte function-associated antigen-1 adhesion pathways. *American Journal of Pathology* **159**, 671-681.

Miller C. J., McChesney M. and Moore P. F. (1992) Langerhans cells, macrophages and lymphocyte subsets in the cervix and vagina of rhesus macaques. *Laboratory investigation; a journal of technical methods and pathology* **67**, 628-34.

Minami Y., Kono T., Miyazaki T. and Taniguchi T. (1993) The IL-2 receptor complex: its structure, function, and target genes. *Annual Review of Immunology* **11**, 245-68.

Molenaar A. J., Davis S. R. and Wilkins R. J. (1992) Expression of alpha-lactalbumin, alpha-S1-casein, and lactoferrin genes is heterogeneous in sheep and cattle mammary tissue. *Journal of Histochemistry & Cytochemistry* **40**, 611-8.

Monfardini E., Paape M. J., Wang Y., Capuco A. V., Husheem M., Wood L. and Burvenich C. (2002) Evaluation of L-selectin expression and assessment of protein tyrosine phosphorylation in bovine polymorphonuclear neutrophil leukocytes around parturition. *Veterinary Research* **33**, 271-81.

Moore P. A., Belvedere O., Orr A., Pieri K., LaFleur D. W., Feng P., Soppet D., Charters M., Gentz R., Parmelee D., Li Y. L., Galperina O., Giri J., Roschke V., Nardelli B., Carrell J., Sosnovtseva S., Greenfield W., Ruben S. M., Olsen H. S., Fikes J. and Hilbert D. M. (1999) BLyS: Member of the tumor necrosis factor family and B lymphocyte stimulator. *Science* **285**, 260-263.

Morgan K. L., Bourne F. J., Newby T. J. and Bradley P. A. (1981) Humoral factors in the secretory immune system of ruminants. In *The Ruminant Immune System* (Edited by Butler J. E.), p. 391-411. Plenum Press, NY and London.

Morrison L. A., Lukacher A. E., Braciale V. L., Fan D. P. and Braciale T. J. (1986) Differences in antigen presentation to MHC class I-and class II-restricted influenza virus-specific cytolytic T lymphocyte clones. *Journal of Experimental Medicine* **163**, 903-21.

Morse L., Chen D., Franklin D., Xiong Y. and Chen-Kiang S. (1997) Induction of cell cycle arrest and B cell terminal differentiation by CDK inhibitor p18(INK4c) and IL-6. *Immunity* **6**, 47-56.

Mosmann T. R. (1992) T lymphocyte subsets, cytokines, and effector functions. *Annals of New York Academy of Sciences* **664**, 89-92.

Mosmann T. R., Cherwinski H., Bond M. W., Giedlin M. A. and Coffman R. L. (1986) Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *Journal of Immunology* **136**, 2348-57.

Mosmann T. R., Li L., Hengartner H., Kagi D., Fu W. and Sad S. (1997) Differentiation and functions of T cell subsets. *Ciba Foundation Symposium* **204**, 148-58.

Mosmann T. R. and Sad S. (1996) The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunology Today* **17**, 138-46.

Motegi Y., Kita H., Kato M. and Morikawa A. (2000) Role of secretory IgA, secretory component, and eosinophils in mucosal inflammation. *International Archives of Allergy & Immunology* **122**, 25-27.

Mukhopadhyay A., Ni J., Zhai Y., Yu G. L. and Aggarwal B. B. (1999) Identification and characterization of a novel cytokine, THANK, a TNF homologue that activates apoptosis, nuclear factor-kappaB, and c-Jun NH2-terminal kinase. *The Journal of Biological Chemistry* **274**, 15978-81.

Mukwedeya D. T., Takamatsu H., Denyer M. S. and Parkhouse R. M. (1996) Analysis of bovine B cell reactive monoclonal antibodies. *Veterinary Immunology and Immunopathology* **52**, 285-94.

Murphy P. M., Baggiolini M., Charo I. F., Hebert C. A., Horuk R., Matsushima K., Miller L. H., Oppenheim J. J. and Power C. A. (2000) International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacological Reviews* **52**, 145-76.

Musil L. S. and Baenziger J. U. (1987) Cleavage of membrane secretory component to soluble secretory component occurs on the cell surface of rat hepatocyte monolayers. *Journal of Cell Biology* **104**, 1725-33.

Mutwiri G., Bowersock T., Kidane A., Sanchez M., Gerdts V., Babiuk L. A. and Griebel P. (2002) Induction of mucosal immune responses following enteric immunization with antigen delivered in alginate microspheres. *Veterinary Immunology and Immunopathology* **87**, 269-276.

Nansen P. (1972) Selective immunoglobulin deficiency in cattle and susceptibility to infection. *Acta Pathologica et Microbiologica Scandinavica [B] Microbiology and Immunology* **80**, 49-54.

Nardelli B., Belvedere O., Roschke V., Moore P. A., Olsen H. S., Migone T. S., Sosnovtseva S., Carrell J. A., Feng P., Giri J. G. and Hilbert D. M. (2001) Synthesis and release of B-lymphocyte stimulator from myeloid cells. *Blood* **97**, 198-204.

Nashar T. O., Stokes C. R. and Cripps P. J. (1991) Immune responses to intramammary infusion with soluble (ovalbumin) and particulate (S. uberis) antigens in the preparturient bovine udder. *Research in Veterinary Science* **50**, 145-151.

Nashar T. O., Webb H. M., Eaglestone S., Williams N. A. and Hirst T. R. (1996) Potent immunogenicity of the B subunits of Escherichia coli heat-labile enterotoxin: Receptor binding is essential and induces differential modulation of lymphocyte subsets. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 226-230.

Nashar T. O., Williams N. A. and Hirst T. R. (1998) Importance Of Receptor Binding In the Immunogenicity, Adjuvanticity and Therapeutic Properties Of Cholera Toxin and Escherichia Coli Heat-Labile Enterotoxin. *Medical Microbiology and Immunology* **187**, 3-10.

Neuberger M. S. and Milstein C. (1995) Somatic hypermutation. *Current Opinion in Immunology* **7**, 248-54.

Neumann B., Engelhardt B., Wagner H. and Holzmann B. (1997) Induction of acute inflammatory lung injury by staphylococcal enterotoxin B. *Journal of Immunology* **158**, 1862-1871.

Neutra M., Weltzin R., Winner L., Mack J., Michetti P., Morrison L., Fields B. N., Mekalanos J. J. and Kraehenbuhl J. P. (1991) Identification and use of protective monoclonal IgA antibodies against viral and bacterial pathogens. *Advances in Experimental Medicine and Biology*. **310**, 179-82.

Neutra M. R., Frey A. and Kraehenbuhl J. P. (1996a) Epithelial M cells: gateways for mucosal infection and immunization. *Cell* **86**, 345-8.
Neutra M. R. and Kraehenbuhl J. P. (1992) Transepithelial transport and mucosal defence I: the role of M cells. *Trends in Cell Biology* **2**, 134-8.
Neutra M. R., Mantis N. J. and Kraehenbuhl J. P. (2001) Collaboration of epithelial cells with organized mucosal lymphoid tissues. *Nature Immunology* **2**, 1004-9.
Neutra M. R., Pringault E. and Kraehenbuhl J. P. (1996b) Antigen sampling across epithelial barriers and induction of mucosal immune responses. *Annual Review of Immunology* **14**, 275-300.
Newby T. J. and Bourne J. (1977) The nature of the local immune system of the bovine mammary gland. *Journal of Immunology* **118**, 461-5.
Nickerson S. C. (1987) Resistance mechanisms of the bovine udder: new implications for mastitis control at the teat end. *Journal of the American Veterinary Medical Association* **191**, 1484-8.
Nickerson S. C. (1989) Immunological aspects of mammary involution. *Journal of Dairy Science* **72**, 1665-78.
Nickerson S. C., Owens W. E., Boddie R. L. and Boddie N. T. (1992) The effect of chronic immunostimulation of the nonlactating bovine mammary gland with interleukin-2, pokeweed mitogen, and lipopolysaccharide. *Journal of Dairy Science* **75**, 3339-3351.
Nickerson S. C. and Pankey J. W. (1985) Electron microscopic study of leucocytic infiltration of the mammary teat duct during infection with Staphylococcus aureus. *Research in Veterinary Science* **38**, 167-73.
Nickerson S. C., Pankey J. W. and Boddie N. T. (1984) Distribution, location, and ultrastructure of plasma cells in the uninfected, lactating bovine mammary gland. *Journal of Dairy Research* **51**, 209-17.
Nord J., Ma P., DiJohn D., Tzipori S. and Tacket C. O. (1990) Treatment with bovine hyperimmune colostrum of cryptosporidial diarrhea in AIDS patients. *Aids* **4**, 581-4.
O'Connor B. P., Cascalho M. and Noelle R. J. (2002) Short-lived and long-lived bone marrow plasma cells are derived from a novel precursor population. *Journal of Experimental Medicine* **195**, 737-45.
O'Connor B. P., Gleeson Michael W., Noelle Randolph J. and Erickson- Loren D. (2003) The rise and fall of long-lived humoral immunity: Terminal differentiation of plasma cells in health and disease. *Immunological Reviews* **194**, 61-76.
Okada T., Ngo V. N., Ekland E. H., Forster R., Lipp M., Littman D. R. and Cyster J. G. (2002) Chemokine requirements for B cell entry to lymph nodes and Peyer's patches. *Journal of Experimental Medicine* **196**, 65-75.
Okato S., Magari S., Yamamoto Y., Sakanaka M. and Takahashi H. (1989) An immuno-electron microscopic study on interactions among dendritic cells, macrophages and lymphocytes in the human palatine tonsil. *Archives of Histology and Cytology* **52**, 231-40.
Okazaki I. M., Kinoshita K., Muramatsu M., Yoshikawa K. and Honjo T. (2002) The AID enzyme induces class switch recombination in fibroblasts. *Nature* **416**, 340-5.
Onorato I. M., Modlin J. F., McBean A. M., Thoms M. L., Losonsky G. A. and Bernier R. H. (1991) Mucosal immunity induced by enhance-potency inactivated and oral polio vaccines. *Journal of Infectious Diseases* **163**, 1-6.
Opdebeeck J. P. and Norcross N. L. (1984) Comparative effect of selected adjuvants on the response in the bovine mammary gland to staphylococcal and streptococcal antigens. *Veterinary Immunology and Immunopathology* **6**, 341-51.
Osborn L., Hession C., Tizard R., Vassallo C., Luhowskyj S., Chi-Rosso G. and Lobb R. (1989) Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. *Cell* **59**, 1203-11.
Outteridge P. M. and Lee C. S. (1981) Cellular immunity in the mammary gland with particular reference to T, B lymphocytes and macrophages. *Advances in Experimental Medicine and Biology.* **137**, 513-34.
Outteridge P. M. and Lee C. S. (1988) The defence mechanisms of the mammary gland of domestic ruminants. *Progress in Veterinary Microbiology and Immunology* **4**, 165-96.

Paape M., Mehrzad J., Zhao X., Detilleux J. and Burvenich C. (2002a) Defense of the bovine mammary gland by polymorphonuclear neutrophil leukocytes. *Journal of Mammary Gland Biology and Neoplasia* **7**, 109-21.

Paape M. J., Rautiainen P. M., Lilius E. M., Malstrom C. E. and Elsasser T. H. (2002b) Development of anti-bovine TNF-alpha mAb and ELISA for quantitating TNF-alpha in milk after intramammary injection of endotoxin. *Journal of Dairy Science* **85**, 765-773.

Pallares F. J., Seva J., Bernabe A., Gomez M. A. and Navarro J. A. (1999) Characterization and distribution of B cells in the lymphoid organs of goats. *Anatomia, Histologia, Embryologia* **28**, 171-176.

Pan J., Kunkel E. J., Gosslar U., Lazarus N., Langdon P., Broadwell K., Vierra M. A., Genovese M. C., Butcher E. C. and Soler D. (2000) A novel chemokine ligand for CCR10 and CCR3 expressed by epithelial cells in mucosal tissues. *Journal of Immunology* **165**, 2943-9.

Park Y. H., Fox L. K., Hamilton M. J. and Davis W. C. (1992) Bovine mononuclear leukocyte subpopulations in peripheral blood and mammary gland secretions during lactation. *Journal of Dairy Science* **75**, 998-1006.

Paul W. E. (1991) Interleukin-4: a prototypic immunoregulatory lymphokine. *Blood* **77**, 1859-70.

Phalipon A., Cardona A., Kraehenbuhl J. P., Edelman L., Sansonetti P. J. and Corthesy B. (2002) Secretory component: A new role in secretory IgA-mediated immune exclusion in vivo. *Immunity* **17**, 107-115.

Picker L. J. (1994) Control of lymphocyte homing. *Current Opinion in Immunology* **6**, 394-406.

Picker L. J., Martin R. J., Trumble A., Newman L. S., Collins P. A., Bergstresser P. R. and Leung D. Y. (1994) Differential expression of lymphocyte homing receptors by human memory/effector T cells in pulmonary versus cutaneous immune effector sites. *European Journal of Immunology* **24**, 1269-77.

Picker L. J., Treer J. R., Ferguson-Darnell B., Collins P. A., Buck D. and Terstappen L. W. (1993) Control of lymphocyte recirculation in man. I. Differential regulation of the peripheral lymph node homing receptor L-selection on T cells during the virgin to memory cell transition. *Journal of Immunology* **150**, 1105-21.

Pighetti G. M. and Sordillo L. M. (1994) Regulation of mammary gland macrophage tumour necrosis factor-alpha production with interferon-gamma. *Research in Veterinary Science* **56**, 252-5.

Pighetti G. M. and Sordillo L. M. (1995) Enhanced Antigen-Specific Responses in Bovine Mammary Glands Following Administration of Interleukin-2. *Journal of Dairy Science* **78**, 528-537.

Pighetti G. M. and Sordillo L. M. (1996) Specific immune responses of dairy cattle after primary inoculation with recombinant bovine interferon-gamma as an adjuvant when vaccinating against mastitis. *American Journal of Veterinary Research* **57**, 819-824.

Piskurich J. F., Blanchard M. H., Youngman K. R., France J. A. and Kaetzel C. S. (1995) Molecular cloning of the mouse polymeric Ig receptor: Functional regions of the molecular are conserved among five mammalian species. *Journal of Immunology* **154**, 1735-1747.

Plaut A. G. (1983) The IgA1 proteases of pathogenic bacteria. *Annual Review of Microbiology* **37**, 603-22.

Pollard K., Lunny D., Holgate C. S., Jackson P. and Bird C. C. (1987) Fixation, processing, and immunochemical reagent effects on preservation of T-lymphocyte surface membrane antigens in paraffin-embedded tissue. *The Journal of Histochemistry and Cytochemistry* **35**, 1329-38.

Porter P. and Noakes D. E. (1970) Immunoglobulin IgA in bovine serum and external secretions. *Biochimica et Biophysica Acta* **214**, 107-16.

Poupon V. and Cerf-Bensussan N. (1999) Adhesion molecules on mucosal lymphocytes. In *Mucosal Immunology* (Edited by McGhee J. R.), p. 523-540. Academic Press, San Diego.

Premier R. R. and Meeusen E. N. (1998) Lymphocyte surface marker and cytokine expression in peripheral and mucosal lymph nodes. *Immunology* **94**, 363-7.

Qin S., Rottman J. B., Myers P., Kassam N., Weinblatt M., Loetscher M., Koch A. E., Moser B. and Mackay C. R. (1998) The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. *Journal of Clinical Investigation* **101**, 746-54.

Quiding- Jarbrink M., Nordstrom I., Granstrom G., Kilander A., Jertborn M., Butcher E. C., Lazarovits A. I., Holmgren J. and Czerkinsky C. (1997) Differential Expression Of Tissue-Specific Adhesion Molecules On Human Circulating Antibody-Forming Cells After Systemic, Enteric, and Nasal Immunizations - a Molecular Basis For the Compartmentalization Of Effector B Cell Responses. *Journal of Clinical Investigation* **99**, 1281-1286.

Radi Z. A., Kehrli M. E., Jr. and Ackermann M. R. (2001) Cell adhesion molecules, leukocyte trafficking, and strategies to reduce leukocyte infiltration. *Journal of Veterinary Internal Medicine* **15**, 516-29.

Rainard P., Riollet C., Poutrel B. and Paape M. J. (2000) Phagocytosis and killing of Staphylococcus aureus by bovine neutrophils after priming by tumor necrosis factor-alpha and the des-arginine derivative of C5a. *American Journal of Veterinary Research* **61**, 951-9.

Ramsay A. J., Husband A. J., Ramshaw I. A., Bao S., Matthaei K. I., Koehler G. and Kopf M. (1994a) The role of interleukin-6 in mucosal IgA antibody responses in vivo. *Science* **264**, 561-3.

Ramsay A. J., Leong K. H., Boyle D., Ruby J. and Ramshaw I. A. (1994b) Enhancement of mucosal IgA responses by interleukins 5 and 6 encoded in recombinant vaccine vectors. *Reproduction, Fertility, and Development* **6**, 389-92.

Rasmussen R. (2001) Quantification on the Lightcycler. In *Rapid cycle Real-time PCR, Methods and Applications* (Edited by Meuer S., Wittwer C. and Nakagawara K.), p. 21 - 34. Springer Press, Heidelberg.

Rebelatto M. C., Mead C. and HogenEsch H. (2000) Lymphocyte populations and adhesion molecule expression in bovine tonsils. *Veterinary Immunology and Immunopathology* **73**, 15-29.

Reif K., Ekland E. H., Ohl L., Nakano H., Lipp M., Forster R. and Cyster J. G. (2002) Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position. *Nature* **416**, 94-9.

Renegar K. B., Jackson G. D. F. and Mestecky J. (1998) In Vitro Comparison Of the Biologic Activities Of Monoclonal Monomeric Iga, Polymeric Iga, and Secretory Iga. *Journal of Immunology* **160**, 1219-1223.

Rescigno M., Urbano M., Valzasina B., Francolini M., Rotta G., Bonasio R., Granucci F., Kraehenbuhl J. P. and Ricciardi-Castagnoli P. (2001) Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nature Immunology* **2**, 361-7.

Riedel Caspari G. (1993) The influence of colostral leukocytes on the course of an experimental Escherichia coli infection and serum antibodies in neonatal calves. *Veterinary Immunology and Immunopathology* **35**, 275-288.

Rincheval-Arnold A., Belair L. and Djiane J. (2002) Developmental expression of pIgR gene in sheep mammary gland and hormonal regulation. *Journal of Dairy Research* **69**, 13-26.

Riollet C., Rainard P. and Poutrel B. (2000a) Differential induction of complement fragment C5a and inflammatory cytokines during intramammary infections with Escherichia coli and Staphylococcus aureus. *Clinical and diagnostic laboratory immunology* **7**, 161-7.

Riollet C., Rainard P. and Poutrel B. (2000b) Kinetics of cells and cytokines during immune-mediated inflammation in the mammary gland of cows systemically immunized with Staphylococcus aureus alpha-toxin. *Inflammation Research* **49**, 486-96.

Riollet C., Rainard P. and Poutrel B. (2001) Cell subpopulations and cytokine expression in cow milk in response to chronic Staphylococcus aureus infection. *Journal of Dairy Science* **84**, 1077-84.

Rivas A. L., Quimby F. W., Blue J. and Coksaygan O. (2001) Longitudinal evaluation of bovine mammary gland health status by somatic cell counting, flow cytometry, and cytology. *Journal of Veterinary Diagnostic Investigation* **13**, 399-407.

Rogers P. R., Dubey C. and Swain S. L. (2000) Qualitative changes accompany memory T cell generation: faster, more effective responses at lower doses of antigen. *Journal of Immunology* **164**, 2338-46.

Rosato R., Jammes H., Belair L., Puissant C., Kraehenbuhl J. P. and Djiane J. (1995) Polymeric-Ig receptor gene expression in rabbit mammary gland during pregnancy and lactation: Evolution and hormonal regulation. *Molecular and Cellular Endocrinology* **110**, 81-87.

Rossi D. and Zlotnik A. (2000) The biology of chemokines and their receptors. *Annual Review of Immunology* **18**, 217-42.

Rudzik R., Clancy R. L., Perey D. Y., Day R. P. and Bienenstock J. (1975) Repopulation with IgA-containing cells of bronchial and intestinal lamina propria after transfer of homologous Peyer's patch and bronchial lymphocytes. *Journal of Immunology* **114**, 1599-604.

Ruggeri F. M., Johansen K., Basile G., Kraehenbuhl J. P. and Svensson L. (1998) Antirotavirus Immunoglobulin a Neutralizes Virus In Vitro After Transcytosis Through Epithelial Cells and Protects Infant Mice From Diarrhea. *Journal of Virology* **72**, 2708-2714.

Ruiz L. P., Jr. (1994) Antibodies from milk for the prevention and treatment of diarrheal disease. In *Indigenous antimicrobial agents of milk: recent developments*, p. 108-121. International Dairy Federation, Brussels.

Russell M. W., Kilian M. and Lamm M. E. (1999) Biological activities of IgA. In *Mucosal immunology* (Edited by Ogra P. L., Mestecky J., Lamm M. E., Strober W., Bienenstock J. and McGhee J. R.), p. 225-240. Academic Press, San Diego.

Saif L. J., Redman D. R., Smith K. L. and Theil K. W. (1983) Passive immunity to bovine rotavirus in newborn calves fed colostrum supplements from immunized or nonimmunized cows. *Infection and Immunity* **41**, 1118-31.

Saif L. J., Smith K. L., Landmeier B. J., Bohl E. H., Theil K. W. and Todhunter D. A. (1984) Immune response of pregnant cows to bovine rotavirus immunization. *American Journal of Veterinary Research* **45**, 49-58.

Sallusto F., Mackay C. R. and Lanzavecchia A. (2000) The role of chemokine receptors in primary, effector, and memory immune responses. *Annual Review of Immunology* **18**, 593-620.

Sampaio S. O., Li X., Takeuchi M., Mei C., Francke U., Butcher E. C. and Briskin M. J. (1995) Organization, Regulatory Sequences, and Alternatively Spliced Transcripts of the Mucosal Addressin Cell Adhesion Molecule-1 (MAdCAM-1) Gene. *Journal of Immunology* **155**, 2477-2486.

Sanz-Rodriguez F., Hidalgo A. and Teixido J. (2001) Chemokine stromal cell-derived factor-1alpha modulates VLA-4 integrin-mediated multiple myeloma cell adhesion to CS-1/fibronectin and VCAM-1. *Blood* **97**, 346-51.

Sarker S. A., Casswall T. H., Mahalanabis D., Alam N. H., Albert M. J., Brussow H., Fuchs G. J. and Hammerstrom L. (1998) Successful treatment of rotavirus diarrhea in children with immunoglobulin from immunized bovine colostrum. *The Pediatric Infectious Disease Journal* **17**, 1149-54.

Scapini P., Carletto A., Nardelli B., Calzetti F., Roschke V., Merigo F., Tamassia N., Pieropan S., Biasi D., Sbarbati A., Sozzani S., Bambara L. and Cassatella M. A. (2004) Proinflammatory mediators elicit the secretion of the intracellular B-lymphocyte stimulator (BLyS) pool that is stored in activated neutrophils: implications for inflammatory diseases. *Blood.*

Scapini P., Nardelli B., Nadali G., Calzetti F., Pizzolo G., Montecucco C. and Cassatella M. A. (2003) G-CSF-stimulated neutrophils are a prominent source of functional BLyS. *Journal of Experimental Medicine* **197**, 297-302.

Schaller J. P., Saif L. J., Cordle C. T., Candler E., Jr., Winship T. R. and Smith K. L. (1992) Prevention of human rotavirus-induced diarrhea in gnotobiotic piglets using bovine antibody. *Journal of Infectious Diseases* **165**, 623-30.

Schiemann B., Gommerman J. L., Vora K., Cachero T. G., Shulga-Morskaya S., Dobles M., Frew E. and Scott M. L. (2001) An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. *Science* **293**, 2111-4.

Schiffer S. G., Day E., Latanision S. M., Tizard R. and Osborn L. (1995) An alternately spliced mRNA encoding functional domains of murine MAdCAM-1. *Biochemical and Biophysical Research Communications* **216**, 170-176.

Schluns K. S. and Lefrancois L. (2003) Cytokine control of memory T-cell development and survival. *Nature Reviews Immunology* **3**, 269-79.

Schneider P., MacKay F., Steiner V., Hofmann K., Bodmer J. L., Holler N., Ambrose C., Lawton P., Bixler S., Acha-Orbea H., Valmori D., Romero P., Werner-Favre C., Zubler R. H., Browning J. L. and Tschopp J. (1999) BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. *Journal of Experimental Medicine* **189**, 1747-1756.

Schneider P., Takatsuka H., Wilson A., Mackay F., Tardivel A., Lens S., Cachero T. G., Finke D., Beermann F. and Tschopp J. (2001) Maturation of marginal zone and follicular B cells requires B cell activating factor of the tumor necrosis factor family and is independent of B cell maturation antigen. *Journal of Experimental Medicine* **194**, 1691-7.

Schratzberger P., Dunzendorfer S., Reinisch N., Kahler C. M. and Wiedermann C. J. (1997) Interleukin-8-induced human peripheral blood B-lymphocyte chemotaxis in vitro. *Immunology Letters* **58**, 167-70.

Schroten H., Stapper C., Plogmann R., Kohler H., Hacker J. and Hanisch F. G. (1998) Fab-Independent Antiadhesion Effects Of Secretory Immunoglobulin a On S-Fimbriated Escherichia Coli Are Mediated By Sialyloligosaccharides. *Infection and Immunity* **66**, 3971-3973.

Schwartz R. N. (1984) The role of gene products of the major histocompatability complex in T cell activation and cellular interactions. In *Fundamental Immunology* (Edited by Paul W. E.), p. 385-392. Raven press, New York.

Scicchitano R., Husband A. J. and Clancy R. L. (1984) Contribution of intraperitoneal immunization to the local immune response in the respiratory tract of sheep. *Immunology* **53**, 375-84.

Scicchitano R., Sheldrake R. F. and Husband A. J. (1986) Origin of immunoglobulins in respiratory tract secretion and saliva of sheep. *Immunology* **58**, 315-21.

Shafer-Weaver K. A., Pighetti G. M. and Sordillo L. M. (1996) Diminished mammary gland lymphocyte functions parallel shifts in trafficking patterns during the postpartum period. *Proceedings of the Society for Experimental Biology and Medicine* **212**, 271-80.

Shafer-Weaver K. A. and Sordillo L. M. (1996) Enhancing bactericidal activity of bovine lymphoid cells during the periparturient period. *Journal of Dairy Science* **79**, 1347-52.

Sheldrake R. F. and Husband A. J. (1985) Intestinal uptake of intact maternal lymphocytes by neonatal rats and lambs. *Research in Veterinary Science* **39**, 10-5.

Sheldrake R. F., Husband A. J. and Watson D. L. (1985a) Specific antibody-containing cells in the mammary gland of non-lactating sheep after intraperitoneal and intramammary immunisation. *Research in Veterinary Science* **38**, 312-6.

Sheldrake R. F., Husband A. J. and Watson D. L. (1988) Origin of antibody-containing cells in the ovine mammary gland following intraperitoneal and intramammary immunisation. *Research in Veterinary Science* **45**, 156-9.

Sheldrake R. F., Husband A. J., Watson D. L. and Cripps A. W. (1984) Selective transport of serum-derived IgA into mucosal secretions. *Journal of Immunology* **132**, 363-8.

Sheldrake R. F., Husband A. J., Watson D. L. and Cripps A. W. (1985b) The effect of intraperitoneal and intramammary immunization of sheep on the numbers of antibody-containing cells in the mammary gland, and antibody titres in blood serum and mammary secretions. *Immunology* **56**, 605-14.

Shu H. B., Hu W. H. and Johnson H. (1999) TALL-1 is a novel member of the TNF family that is down-regulated by mitogens. *Journal of Leukocyte Biology* **65**, 680-3.

Shuster D. E., Kehrli M. E., Jr., Rainard P. and Paape M. (1997) Complement fragment C5a and inflammatory cytokines in neutrophil recruitment during intramammary infection with Escherichia coli. *Infection and Immunity* **65**, 3286-92.

Shuster D. E., Kehrli M. E., Jr. and Stevens M. G. (1993) Cytokine production during endotoxin-induced mastitis in lactating dairy cows. *American Journal of Veterinary Research* **54**, 80-5.

Shuster J. (1971) Pepsin hydrolysis of IgA--delineation of two populations of molecules. *Immunochemistry* **8**, 405-11.

Shyjan A. M., Bertagnolli M., Kenney C. J. and Briskin M. J. (1996) Human mucosal addressin cell adhesion molecule-1 (MAdCAM-1) demonstrates structural and functional similarities to the alpha-4-beta-7-integrin binding domains of murine MAdCAM-1, but extreme divergence of mucin-like sequences. *Journal of Immunology* **156**, 2851-2857.

Sjolander A., Drane D., Maraskovsky E., Scheerlinck J. P., Suhrbier A., Tennent J. and Pearse M. (2001) Immune responses to ISCOM (R) formulations in animal and primate models. *Vaccine* **19**, 2661-2665.

Slifka M. K., Antia R., Whitmire J. K. and Ahmed R. (1998) Humoral immunity due to long-lived plasma cells. *Immunity* **8**, 363-72.

Smith D. J., King W. F., Barnes L. A., Trantolo D., Wise D. L. and Taubman M. A. (2001) Facilitated intranasal induction of mucosal and systemic immunity to mutans streptococcal glucosyltransferase peptide vaccines. *Infection and Immunity* **69**, 4767-4773.

Smith K. A. (1988) Interleukin-2: inception, impact, and implications. *Science* **240**, 1169-1176.

Smits E., Burvenich C., Guidry A. J. and Massart Leen A. (2000) Adhesion receptor CD11b/CD18 contributes to neutrophil diapedesis across the bovine blood-milk barrier. *Veterinary Immunology and Immunopathology* **73**, 255-265.

Smits E., Burvenich C., Guidry A. J. and Roets E. (1998) In vitro expression of adhesion receptors and diapedesis by polymorphonuclear neutrophils during experimentally induced Streptococcus uberis mastitis. *Infection and Immunity* **66**, 2529-34.

Snapper C. M., Kehry M. R., Castle B. E. and Mond J. J. (1995) Multivalent, but not divalent, antigen receptor cross-linkers synergize with CD40 ligand for induction of Ig synthesis and class switching in normal murine B cells. A redefinition of the TI-2 vs T cell-dependent antigen dichotomy. *Journal of Immunology* **154**, 1177-87.

Snapper C. M., Peschel C. and Paul W. E. (1988) IFN-gamma stimulates IgG2a secretion by murine B cells stimulated with bacterial lipopolysaccharide. *Journal of Immunology* **140**, 2121-7.

Snodgrass D. R., Fahey K. J., Wells P. W., Campbell I. and Whitelaw A. (1980) Passive immunity in calf rotavirus infections: maternal vaccination increases and prolongs immunoglobulin G1 antibody secretion in milk. *Infection and Immunity* **28**, 344-9.

Snodgrass D. R., Nagy L. K., Sherwood D. and Campbell I. (1982) Passive immunity in calf diarrhea: vaccination with K99 antigen of enterotoxigenic Escherichia coli and rotavirus. *Infection and Immunity* **37**, 586-91.

Sordillo L. M., Campos M. and Babiuk L. A. (1991a) Antibacterial activity of bovine mammary gland lymphocytes following treatment with interleukin-2. *Journal of Dairy Science* **74**, 3370-5.

Sordillo L. M. and Nickerson S. C. (1988a) Morphologic changes in the bovine mammary gland during involution and lactogenesis. *American Journal of Veterinary Research* **49**, 1112-20.

Sordillo L. M. and Nickerson S. C. (1988b) Quantification and immunoglobulin classification of plasma cells in nonlactating bovine mammary tissue. *Journal of Dairy Science* **71**, 84-91.

Sordillo L. M., Redmond M. J., Campos M., Warren L. and Babiuk L. A. (1991b) Cytokine activity in bovine mammary gland secretions during the periparturient period. *Canandian Journal of Veterinary Research* **55**, 298-301.

Sordillo L. M., Shafer-Weaver K. and DeRosa D. (1997) Immunobiology of the mammary gland. *Journal of Dairy Science* **80**, 1851-65.

Sordillo L. M. and Streicher K. L. (2002) Mammary gland immunity and mastitis susceptibility. *Journal of Mammary Gland Biology and Neoplasia* **7**, 135-46.

Spits H. and de Waal Malefyt R. (1992) Functional characterization of human IL-10. *International archives of allergy and immunology* **99**, 8-15.

Staats H. F. and McGhee J. R. (1996) Application of Mucosal Vaccination. In *Mucosal Vaccines* (Edited by Kiyono H., Ogra P. L. and McGhee J. R.), p. 17-39. Academic Press, San Diego.

Stoeck M., Ruegg C., Miescher S., Carrel S., Cox D., Fliedner V. v., Alkan S. and Von Fliedner V. (1989) Comparison of the immunosuppressive properties of milk growth factor and transforming growth factors beta1 and beta2. *Journal of Immunology* **143**, 3258-3265.

Streeter P. R., Berg E. L., Rouse B. T., Bargatze R. F. and Butcher E. C. (1988a) A tissue-specific endothelial cell molecule involved in lymphocyte homing. *Nature* **331**, 41-6.

Streeter P. R., Rouse B. T. and Butcher E. C. (1988b) Immunohistologic and functional characterization of a vascular addressin involved in lymphocyte homing into peripheral lymph nodes. *Journal of Cell Biology* **107**, 1853-62.

Sullivan D. A., Underdown B. J. and Wira C. R. (1983) Steroid hormone regulation of free secretory component in the rat uterus. *Immunology* **49**, 379-86.

Sullivan D. A. and Wira C. R. (1983) Hormonal regulation of immunoglobulins in the rat uterus: uterine response to a single estradiol treatment. *Endocrinology* **112**, 260-8.

Szabo M. C., Butcher E. C. and McEvoy L. M. (1997) Specialization of mucosal follicular dendritic cells revealed by mucosal addressin-cell adhesion molecule-1 display. *Journal of Immunology* **158**, 5584-8.

Tacket C. O., Binion S. B., Bostwick E., Losonsky G., Roy M. J. and Edelman R. (1992) Efficacy of bovine milk immunoglobulin concentrate in preventing illness after Shigella flexneri challenge. *The American Journal of Tropical Medicine and Hygiene* **47**, 276-83.

Tacket C. O., Losonsky G., Link H., Hoang Y., Guesry P., Hilpert H. and Levine M. M. (1988) Protection by milk immunoglobulin concentrate against oral challenge with enterotoxigenic Escherichia coli. *The New England Journal of Medicine* **318**, 1240-3.

Tanneau G. M., Oyant L. H. S., Chevaleyre C. C. and Salmon H. P. (1999) Differential recruitment of T- and IgA B-lymphocytes in the developing mammary gland in relation to homing receptors and vascular addressins. *Journal of Histochemistry & Cytochemistry* **47**, 1581-1592.

Tatarczuch L., Philip C., Bischof R. and Lee C. S. (2000) Leucocyte phenotypes in involuting and fully involuted mammary glandular tissues and secretions of sheep. *Journal of Anatomy* **196 (Pt 3)**, 313-26.

Taylor B. C., Dellinger J. D., Cullor J. S. and Stott J. L. (1994) Bovine milk lymphocytes display the phenotype of memory T cells and are predominantly CD8+. *Cellular Immunology* **156**, 245-53.

Taylor B. C., Keefe R. G., Dellinger J. D., Nakamura Y., Cullor J. S. and Stott J. L. (1997) T cell populations and cytokine expression in milk derived from normal and bacteria-infected bovine mammary glands. *Cellular Immunology* **182**, 68-76.

Thompson J. S., Bixler S. A., Qian F., Vora K., Scott M. L., Cachero T. G., Hession C., Schneider P., Sizing I. D., Mullen C., Strauch K., Zafari M., Benjamin C. D., Tschopp J., Browning J. L. and Ambrose C. (2001) BAFF-R, a newly identified TNF receptor that specifically interacts with BAFF. *Science* **293**, 2108-11.

Thompson J. S., Schneider P., Kalled S. L., Wang L., Lefevre E. A., Cachero T. G., MacKay F., Bixler S. A., Zafari M., Liu Z. Y., Woodcock S. A., Qian F., Batten M., Madry C., Richard Y., Benjamin C. D., Browning J. L., Tsapis A., Tschopp J. and Ambrose C. (2000) BAFF binds to the tumor necrosis factor receptor-like molecule B cell maturation antigen and is important for maintaining the peripheral B cell population. *Journal of Experimental Medicine* **192**, 129-35.

Tidswell M., Pachynski R., Wu S. W., Qiu S. Q., Dunham E., Cochran N., Briskin M. J., Kilshaw P. J., Lazarovits A. I., Andrew D. P., Butcher E. C., Yednock T. A. and Erle D. J. (1997) Structure-function analysis of the integrin beta 7 subunit: identification of domains involved in adhesion to MAdCAM-1. *Journal of Immunology* **159**, 1497-505.

Toellner K. M., Gulbranson-Judge A., Taylor D. R., Sze D. M. and MacLennan I. C. (1996) Immunoglobulin switch transcript production in vivo related to the site and time of antigen-specific B cell activation. *Journal of Experimental Medicine* **183**, 2303-12.

Tollemar J., Gross N., Dolgiras N., Jarstrand C., Ringden O. and Hammarstrom L. (1999) Fungal prophylaxis by reduction of fungal colonization by oral administration of bovine anti-Candida antibodies in bone marrow transplant recipients. *Bone Marrow Transplant* **23**, 283-90.

Tomoda T., Morita H., Kurashige T. and Maassab H. F. (1995) Prevention of influenza by the intranasal administration of cold-recombinant, live-attenuated influenza virus vaccine: Importance of interferon-gamma production and local IgA response. *Vaccine* **13**, 185-190.

Trinchieri G. (1995) Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annual Review of Immunology* **13**, 251-76.

Trinchieri G. (1997) Cytokines acting on or secreted by macrophages during intracellular infection (IL-10, IL-12, IFN-gamma). *Current Opinion in Immunology* **9**, 17-23.

Turaga P. S., Berton M. T. and Teale J. M. (1993) Frequency of B cells expressing germ-line gamma 1 transcripts upon IL-4 induction. *Journal of Immunology* **151**, 1383-90.

Turner C. A., Jr., Mack D. H. and Davis M. M. (1994) Blimp-1, a novel zinc finger-containing protein that can drive the maturation of B lymphocytes into immunoglobulin-secreting cells. *Cell* **77**, 297-306.

Tzipori S., Roberton D., Cooper D. A. and White L. (1987) Chronic cryptosporidial diarrhoea and hyperimmune cow colostrum. *Lancet* **2**, 344-5.

Underhill D. M., Bassetti M., Rudensky A. and Aderem A. (1999) Dynamic interactions of macrophages with T cells during antigen presentation. *Journal of Experimental Medicine* **190**, 1909-14.

Van der Feltz M. J. M., De Groot N., Bayley J. P., Lee S. H., Verbeet M. P. and De Boer H. A. (2001) Lymphocyte homing and Ig secretion in the murine mammary gland. *Scandinavian Journal of Immunology* **54**, 292-300.

Van Kampen C. and Mallard B. A. (2001) Regulation of bovine intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) on cultured aortic endothelial cells. *Veterinary Immunology and Immunopathology* **79**, 129-38.

Van Kampen C., Mallard B. A. and Wilkie B. N. (1999) Adhesion molecules and lymphocyte subsets in milk and blood of periparturient Holstein cows. *Veterinary Immunology and Immunopathology* **69**, 23-32.

Vassalli P. (1992) The pathophysiology of tumor necrosis factors. *Annual Review of Immunology* **10**, 411-52.

Verbeet M. P., Vermeer H., Warmerdam G. C. M., De Boer H. A. and Lee S. H. (1995) Cloning and characterization of the bovine polymeric immunoglobulin receptor-encoding cDNA. *Gene* **164**, 329-333.

Viney J. L., Jones S., Chiu H. H., Lagrimas B., Renz M. E., Presta L. G., Jackson D., Hillan K. J., Lew S. and Fong S. (1996) Mucosal addressin cell adhesion molecule-1 - A structural and functional analysis demarcates the integrin binding motif. *Journal of Immunology* **157**, 2488-2497.

Vudhichamnong K., Walker D. M. and Ryley H. C. (1982) The effect of secretory immunoglobulin A on the in-vitro adherence of the yeast Candida albicans to human oral epithelial cells. *Archives of Oral Biology* **27**, 617-21.

Wahl S. M., Swisher J., McCartney-Francis N. and Chen W. (2004) TGF-beta: the perpetrator of immune suppression by regulatory T cells and suicidal T cells. *Journal of Leukocyte Biology* **76**, 15-24.

Wang S., Zhu G., Chapoval A. I., Dong H., Tamada K., Ni J. and Chen L. (2000a) Costimulation of T cells by B7-H2, a B7-like molecule that binds ICOS. *Blood* **96**, 2808-13.

Wang W., Soto H., Oldham E. R., Buchanan M. E., Homey B., Catron D., Jenkins N., Copeland N. G., Gilbert D. J., Nguyen N., Abrams J., Kershenovich D., Smith K., McClanahan T., Vicari A. P. and Zlotnik A. (2000b) Identification of a novel

chemokine (CCL28), which binds CCR10 (GPR2). *Journal of Biology and Chemistry* **275**, 22313-23.
Watanabe A., Yagi Y., Shiono H. and Yokomizo Y. (2000) Effect of intramammary infusion of tumour necrosis factor-alpha on milk protein composition and induction of acute-phase protein in the lactating cow. *Zentralblatt fur Veterinarmedizin - Reihe B* **47**, 653-662.
Watson D. L. (1980) Immunological functions of the mammary gland and its secretion--comparative review. *Australian Journal of Biological Sciences* **33**, 403-22.
Watson D. L. (1981) Immunologically-specific resistance to infection with particular reference to staphylococcal mastitis. *Advances in Experimental Medicine and Biology.* **137**, 579-90.
Watson D. L. (1984) Evaluation of attenuated, live staphylococcal mastitis vaccine in lactating heifers. *Journal of Dairy Science* **67**, 2608-13.
Watson D. L. and Kennedy J. W. (1981) Immunisation against experimental staphylococcal mastitis in sheep - effect of challenge with a heterologous strain of Staphylococcus aureus. *Australian Veterinary Journal* **57**, 309-13.
Wedlock D. N., Doolin E. E., Parlane N. A., Lacy-Hulbert S. J., Woolford M. W. and Buddle B. M. (2000) Effects of yeast expressed recombinant interleukin-2 and interferon-gamma on physiological changes in bovine mammary glands and on bactericidal activity of neutrophils. *Journal of Dairy Research* **67**, 189-97.
Wehrli N., Legler D. F., Finke D., Toellner K. M., Loetscher P., Baggiolini M., MacLennan I. C. and Acha-Orbea H. (2001) Changing responsiveness to chemokines allows medullary plasmablasts to leave lymph nodes. *European Journal of Immunology* **31**, 609-16.
Weiner C., Pan Q., Hurtig M., Boren T., Bostwick E. and Hammarstrom L. (1999) Passive immunity against human pathogens using bovine antibodies. *Clinical and Experimental Immunology* **116**, 193-205.
Weisz-Carrington P., Roux M. E. and Lamm M. E. (1977) Plasma cells and epithelial immunoglobulins in the mouse mammary gland during pregnancy and lactation. *Journal of Immunology* **119**, 1306-7.
Weisz-Carrington P., Roux M. E., McWilliams M., JM P. H.-Q. and Lamm M. E. (1979) Organ and isotype distribution of plasma cells producing specific antibody after oral immunization: evidence for a generalized secretory immune system. *Journal of Immunology* **123**, 1705-8.
Weisz-Carrington P., Roux M. E., McWilliams M., Phillips-Quagliata J. M. and Lamm M. E. (1978) Hormonal induction of the secretory immune system in the mammary gland. *Proceedings of the National Academy of Sciences of the United States of America* **75**, 2928-32.
Weltzin R., Lucia-Jandris P., Michetti P., Fields B. N., Kraehenbuhl J. P. and Neutra M. R. (1989) Binding and transepithelial transport of immunoglobulins by intestinal M cells: demonstration using monoclonal IgA antibodies against enteric viral proteins. *Journal of Cell Biology* **108**, 1673-85.
Wilhelm J. and Pingoud A. (2003) Real-time polymerase chain reaction. *Chembiochem* **4**, 1120-8.
Wilkening S. and Bader A. (2004) Quantitative real-time polymerase chain reaction: methodical analysis and mathematical model. *Journal of Biomolecular Techniques* **15**, 107-11.
Wilson E. and Butcher E. C. (2004) CCL28 Controls Immunoglobulin (Ig)A Plasma Cell Accumulation in the Lactating Mammary Gland and IgA Antibody Transfer to the Neonate. *Journal of Experimental Medicine* **200**, 805-9.
Wilson R. A., Linn J. A. and Eberhart R. J. (1986) A study of bovine T-cell subsets in the blood and mammary gland secretions during the dry period. *Veterinary Immunology and Immunopathology* **13**, 151-64.
Winner L., 3rd, Mack J., Weltzin R., Mekalanos J. J., Kraehenbuhl J. P. and Neutra M. R. (1991) New model for analysis of mucosal immunity: intestinal secretion of specific monoclonal immunoglobulin A from hybridoma tumors protects against Vibrio cholerae infection. *Infection and Immunity* **59**, 977-82.
Xia X. Z., Treanor J., Senaldi G., Khare S. D., Boone T., Kelley M., Theill L. E., Colombero A., Solovyev I., Lee F., McCabe S., Elliott R., Miner K., Hawkins N.,

Guo J., Stolina M., Yu G., Wang J., Delaney J., Meng S. Y., Boyle W. J. and Hsu H. (2000) TACI is a TRAF-interacting receptor for TALL-1, a tumor necrosis factor family member involved in B cell regulation. *Journal of Experimental Medicine* **192**, 137-43.

Yajima T., Nishimura H., Ishimitsu R., Watase T., Busch D. H., Pamer E. G., Kuwano H. and Yoshikai Y. (2002) Overexpression of IL-15 in vivo increases antigen-driven memory CD8+ T cells following a microbe exposure. *Journal of Immunology* **168**, 1198-203.

Yamaguchi T., Hiratsuka M., Asai K., Kai K. and Kumagai K. (1999) Differential distribution of T lymphocyte subpopulations in the bovine mammary gland during lactation. *Journal of Dairy Science* **82**, 1459-64.

Yan D., Zhou H. R., Brooks K. H. and Pestka J. J. (1997) Potential role for IL-5 and IL-6 in enhanced IgA secretion by Peyer's patch cells isolated from mice acutely exposed to vomitoxin. *Toxicology* **122**, 145-158.

Yang T. J., Ayoub I. A. and Rewinski M. J. (1997) Lactation stage-dependent changes of lymphocyte subpopulations in mammary secretions: inversion of CD4+/CD8+ T cell ratios at parturition. *American Journal of Reproductive Immunology* **37**, 378-83.

Yang T. J., Mather J. F. and Rabinovsky E. D. (1988) Changes in subpopulations of lymphocytes in peripheral blood, and supramammary and prescapular lymph nodes of cows with mastitis and normal cows. *Veterinary Immunology and Immunopathology* **18**, 279-85.

Yoshimura T., Matsushima K., Tanaka S., Robinson E. A., Appella E., Oppenheim J. J. and Leonard E. J. (1987) Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 9233-7.

Yu G., Boone T., Delaney J., Hawkins N., Kelley M., Ramakrishnan M., McCabe S., Qiu W. R., Kornuc M., Xia X. Z., Guo J., Stolina M., Boyle W. J., Sarosi I., Hsu H., Senaldi G. and Theill L. E. (2000) APRIL and TALL-I and receptors BCMA and TACI: system for regulating humoral immunity. *Nature Immunology* **1**, 252-6.

Yurchak A. M., Butler J. E. and Tomasi T. B., Jr. (1971) Fluorescent localization of immunoglobulins in the tissues of the cow. *Journal of Dairy Science* **54**, 1324-5.

Zabel B. A., Agace W. W., Campbell J. J., Heath H. M., Parent D., Roberts A. I., Ebert E. C., Kassam N., Qin S., Zovko M., LaRosa G. J., Yang L. L., Soler D., Butcher E. C., Ponath P. D., Parker C. M. and Andrew D. P. (1999) Human G protein-coupled receptor GPR-9-6/CC chemokine receptor 9 is selectively expressed on intestinal homing T lymphocytes, mucosal lymphocytes, and thymocytes and is required for thymus-expressed chemokine-mediated chemotaxis. *Journal of Experimental Medicine* **190**, 1241-56.

Zhang J. R., Mostov K. E., Lamm M. E., Nanno M., Shimida S., Ohwaki M. and Tuomanen E. (2000) The polymeric immunoglobulin receptor translocates pneumococci across human nasopharyngeal epithelial cells. *Cell* **102**, 827-837.

Zinkernagel R. M. and Doherty P. C. (1979) MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction-specificity, function, and responsiveness. *Advances in Immunology* **27**, 51-177.