Effect of Body Condition Score at Calving on Adaptive Immune Function During Early Lactation in New Zealand Dairy Cows:

Effect of calving body condition on adaptive immune function

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ABSTRACT

The extent of postpartum negative energy balance in dairy cows is positively associated with body condition score (BCS) at calving. Results of epidemiological studies have indicated that calving BCS is positively correlated with several indicators of immune dysfunction during the post-partum period. Metabolites of negative energy balance (NEB) have been associated with impaired lymphocyte and neutrophil function in vitro. Therefore, cows that calve in greater condition might exhibit a greater degree of periparturient immune dysfunction than thinner cows.

The objective of this thesis was to investigate the effect of BCS at calving on adaptive immune function, using in vivo measures. Two experiments were conducted using dairy cows in the DairyNZ Lye farm herd.

The first experiment determined the level of background exposure to *Candida albicans*. Delayed-type hypersensitivity (DTH) to *C. albicans* is used as an indicator of cell-mediated immune response (CMIR) in cattle. Prior immunisation against *C. albicans* ensures measurable DTH responses from most cows. However, if natural exposure to *C. albicans* is high or variable, background DTH must be measured before immunisation, or an alternate antigen used. To determine background DTH responses in pasture-based cows, responses of ten unimmunised and ten immunised cows were compared. A greater proportion of immunised versus unimmunised cows mounted a positive DTH response (90% vs. 70%), and responses were larger ($P < 0.01$) and more variable ($P < 0.05$) in immunised cows. Results indicate that pasture-based cows in New Zealand have consistently low background exposure to *C. albicans*. Therefore, *C. albicans* is an appropriate antigen for inducing DTH responses as an in vivo measure of CMIR in pasture-based cows.

The second experiment tested the hypothesis that BCS at calving affects immune function. The energy balance of sixty healthy cows with similar mid-lactation BCS was manipulated in late-lactation to achieve three homogenous BCS groups of twenty cows at calving, with calving BCS of 3.4, 4.6 and 5.4 (NZ BCS scale of 1 to 10). Antibody-mediated immune responses (AMIR) and cell-mediated immune responses (CMIR) measured postpartum were compared with responses measured in mid-lactation before BCS manipulation began. Calving BCS had no effect on the magnitude of AMIR ($P = 0.93$) or CMIR ($P = 0.99$), and did not affect the ratio of antigen-specific IgG1 and IgG2 produced during AMIR ($P = 0.48$). These results indicate that, within the range of BCS at calving investigated, and the associated degree of postpartum NEB did not influence ability to mount adaptive immune responses in vivo.

Cows calving within the BCS range examined (between 3.4 and 5.4) did not differ in AMIR or CMIR; therefore, the results support the current recommendations regarding optimal calving BCS for mature cows, and support the minimum BCS allowed by the welfare code.

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List of Abbreviations

AMIR	Antibody-Mediated Immune Response
ANOVA	Analysis of Variance
APP	Acute Phase Protein
AUC	Area Under Curve
BCS	Body Condition Score
BHBA	B-hydroxybutyrate
BWT	Body Weight
CaWC	Dry Matter
CMIR	Cell-Mediate Immune Response
DAMP	Danger- or Damage-Associated Molecule Pattern
dfb	Difference from baseline
DM	Dry Matter
DMI	Dry Matter Intake
DSFT	Double Skin-Fold Thickness
DTH	Delayed-Type Hypersensitivity
ELISA	Enzyme-Linked Immunosorbant Assay
GDP	Gross Domestic Product
HEWL	Hen Egg-White Lysozyme
HSA	Human Serum Albumin
IFN	Interferon
Ig	Immunoglobulin

IL	Interleukin
LSM	Least Squares Mean
MHC	Major Histocompatibility Complex
NA	Not Analysed (Not appropriate)
NEB	Negative Energy Balance
NEFA	Non-esterified Fatty Acid
NET	Neutrophil Extracellular Trap
NK	Natural Killer
NZ	New Zealand
OD	Optical Density
PAMP	Pathogen-Associated Molecule Pattern
PBS	Phosphate-Buffered Saline
PRR	Pattern Recognition Receptor
ROS	Reactive Oxygen Species
SE	Standard Error
SEM	Standard Error Mean
TMR	Total Mixed Ration
TNF	Tumour Necrosis Factor

Chapter 1. General Introduction

1.1 Introduction

Dairy farming occurs in nearly every country in the world and supports the livelihoods of billions of people, ensuring the on-going sustainability of rural communities (The Economic Importance of Dairying, 2013, Schilling et al., 2010). Globally, milk makes up 8.9% of the value of all agricultural products (The Economic Importance of Dairying, 2013).

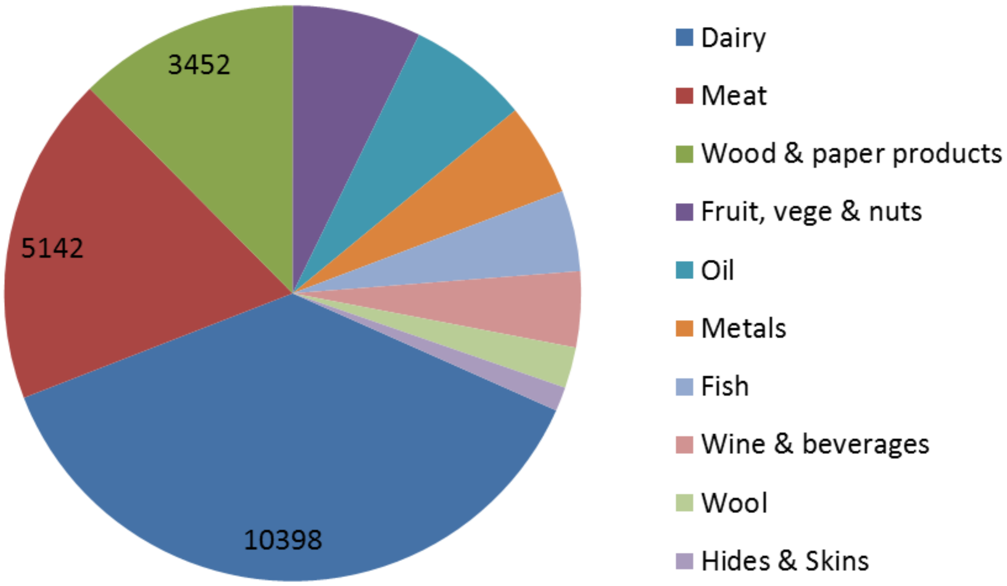

Figure 1. Contribution of individual primary sectors to total value of New Zealand's primary sector exports in 2009, presented in millions of dollars. Data sourced from Schilling et al. (2010).

The industry is especially important in New Zealand, where milk exports account for 26% of agricultural exports (Schilling et al., 2010). Dairy farming contributes 2.8% of New Zealand's GDP, which is over one third of the GDP contribution of the entire primary sector (Figure 1; The Economic Importance of Dairying, 2013, Schilling et al., 2010). The New Zealand dairy sector directly employs 35,000 people and indirectly employs many more in businesses that supply the sector (Schilling et al., 2010). New Zealand produces only 2% of the world's milk but exports 95% of this, thereby contributing one third of the total milk products traded internationally (The Economic Importance of Dairying, 2013, Schilling et al., 2010). Consequently, New Zealand is particularly vulnerable to changes in international demand for milk products and shifts in consumer expectations.

The dairy industry faces complex demands from consumers. As the global population becomes more affluent, demand for milk protein increases. In response, dairy producers strive to increase output. The milk production of the modern North American dairy cow has doubled in the last 50 years (Oltenacu and Broom, 2010) and New Zealand per cow production has increased by at least 50% since the mid-seventies (New Zealand Dairy Statistics 2012/13). Unfortunately, the increased production has been accompanied by greater incidence of disease and arguably decreased welfare for dairy cows (Oltenacu and Broom, 2010). The negative effects of high milk production are an area of risk for the dairy industry because increased affluence also affords consumers the ability to place greater value on animal welfare. Good animal welfare is now synonymous with product quality and sustainability (Oltenacu and Broom, 2010).

In addition to expectations of higher animal welfare, there is increasing public pressure to reduce the reliance on antibiotics in food production animals because of perceived or potential contribution to antibiotic resistance (Oliver et al., 2011, Trevisi et al., 2014). Preventative use of antibiotics is particularly contentious and is associated with increased risk of antimicrobial resistance in North America (Saini et al., 2013). The incidence of mastitis is lower in New Zealand than in overseas dairy industries (Laven and Holmes, 2008) and antibiotic resistance is reported to be very low and declining (McDougall et al., 2013). However, our customers are influenced by the performance of dairy industries in their own country rather than in New Zealand. To maintain our international reputation for producing high quality and sustainable milk products, it is essential that the New Zealand dairy industry responds to changing consumer expectations.

The period of greatest risk to the health of the cow and, therefore, to the industry's animal welfare reputation is during the transition from pregnancy to lactation. During the transition period, dairy cows experience profound metabolic changes and exhibit a transient immune dysfunction (Drackley, 1999, Mallard et al., 1997). Consequently, the incidence of infectious and metabolic disease is high and most antibiotic use in dairy cows is directed toward diseases that occur during early lactation. In many New Zealand dairy herds, long acting intra-mammary antibiotic therapy is routinely administered to every cow at the end of lactation to prevent infection during the early dry period and, therefore, reduce the incidence of clinical mastitis during early lactation (Goff and Horst, 1997, Hogeveen et al., 2011, McDougall et al., 2013). Nevertheless, there is a high incidence of clinical mastitis in the first two weeks of lactation (McDougall et al., 2007); so, there is considerable reliance on antibiotics to manage mastitis in early lactation. Additionally, metritis and endometritis may require treatment with antibiotics and both of these diseases have aetiology originating in the transition period. Because periparturient immune dysfunction contributes to the high incidence of infectious disease in early lactation, selection for increased immune function or management strategies that minimise peripartum immune dysfunction might reduce the use of antibiotics and improve cow welfare in the dairy industry.

Management of body condition score at calving may be one way to address the early lactation immune dysfunction that leads to the high incidence of disease and the reliance on antibiotics. Body condition score, which is a visual assessment of a cow’s energy or fat reserves, is reported to influence cow susceptibility to metabolic disease (Bewley and Schutz, 2008, Roche and Berry, 2006, Roche et al., 2009) and may also influence immune function (Lacetera et al., 2005, Ohtsuka et al., 2008) and, therefore, the risk of infectious disease. However, the relationship between body condition at calving and incidence of clinical infectious disease is inconsistent (Roche et al., 2009). Body condition score is also regarded as an indicator of welfare, in its own right. For example, the New Zealand code of welfare for dairy cattle sets a minimum acceptable body condition score (Code of Welfare: Dairy Cattle 2014), and most welfare assessment protocols for dairy farming include body condition score (De Vries et al., 2011, Matthews et al., 2012). Thin cows are perceived to suffer reduced welfare; however, the evidence in support of this perception is equivocal (Roche et al., 2009). Although thinness has been associated with greater morbidity (Gearhart et al., 1990, Hoedemaker et al., 2009), these studies were not able to determine whether it was a causal relationship or the low BCS was a consequence of illness-induced weight loss (Roche et al., 2009). There is a need for research that directly investigates the relationship between body condition at calving and immune function in early lactation, the period of greatest risk in terms of farm profitability, industry reputation and cow welfare.

1.2 Structure of this thesis

This thesis begins with a review of the literature. Chapter 2 and Chapter 3 provide an outline of the bovine immune system and the metabolic and immune changes reported to occur as cows transition from gestation to lactation. The relationship between body condition and productivity, reproduction and metabolic health is described in Chapter 4, before discussing the evidence for a relationship between BCS and immune function and what might mediate such a relationship in Chapter 5.

Chapter 6 describes a pilot study to establish an in vivo measure of cell-mediated immunity, using cows in the pasture-based herd in which the main experiment would be conducted. Chapter 7 describes the investigation of adaptive immune function in cows with a range of BCS at calving. Salient points arising from these experiments, implications for New Zealand dairy farming and direction for future research are discussed in concluding Chapter 8.

Published manuscripts are included in the Appendices. Raw data is provided on a DVD inside the back cover of this thesis.

Chapter 2. The bovine immune system

Mammals have evolved a sophisticated immune system to detect and remove or neutralise pathogens. The immune system has the ability to distinguish self from non-self, and uses an array of specialised cells and molecules to mount fast and flexible responses against an almost unlimited array of pathogens. The immune system is conceptually divided into two systems illustrated in Figure 2:

- The innate immune system is present from birth and its mechanisms do not evolve with repeated pathogen exposure. It is the “first line of defence”, activated immediately after infection (Borghetti et al., 2009). It responds to a broad range of pathogens and provides most of the machinery and functions needed to fight infection (Mellor and Munn, 2000).
- The adaptive immune system is largely orchestrated by the action of T-lymphocytes and B-lymphocytes, which respond to specific antigens and develop memory following interaction with antigen, allowing faster responses to re-exposure (Mellor and Munn, 2000).

Innate responses are largely non-specific and are generated quickly upon exposure to pathogens. In contrast, adaptive responses are specific but take time to generate. These two systems actually co-operate to prevent infection: innate responses are enhanced by the adaptive immune system (Roth, 2012) and the adaptive system relies on the innate system for activation and initial direction as to the nature of response required (Beutler, 2004, Guzman et al., 2012, Mellor and Munn, 2000). Cell-to-cell communication, mediated via signalling proteins (cytokines) and their cognate surface receptors, allows integration of the two systems and controls the type, intensity and duration of responses. In fact, the interdependence of the two systems means they can hardly be described as separate (Lippolis, 2008).

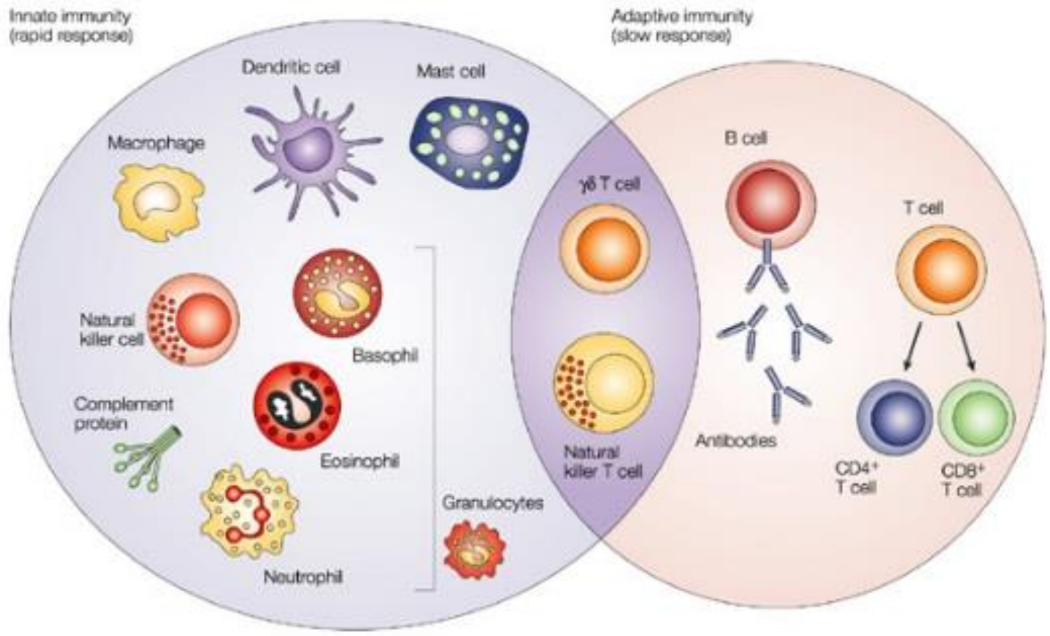

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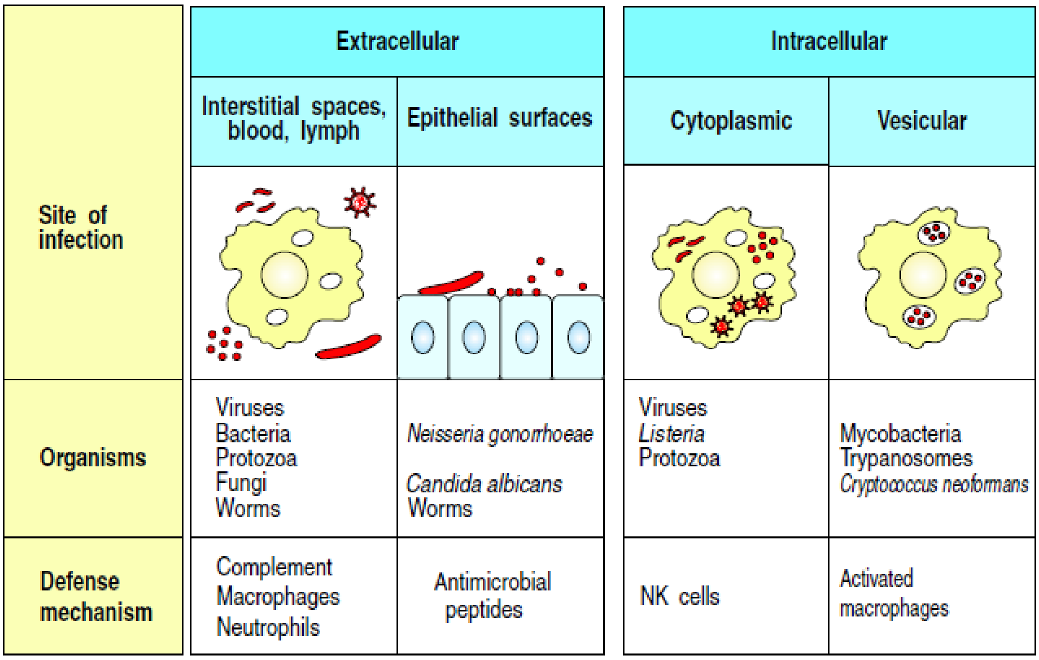
Figure 2. The immune system can be conceptually divided into an innate and an adaptive immune system, although there are some cells that have features of both systems (source: Dranoff, 2004)

2.1 Innate immune system

The innate immune system uses passive barriers and generalised cell-mediated killing mechanisms. It is able to detect common pathogens (e.g. viruses, bacteria, fungi, nematodes) by identifying conserved microbial proteins not typically found in mammalian cells (e.g. lipopolysaccharide, a protein present on the cell walls of all gram-negative bacteria). Such signatures are known as pathogen associated molecular patterns (PAMPs) and are recognised by receptors on the cell known as pattern recognition receptors (PRRs). Additionally, host cells signal to the immune system when damaged or when infected by an intracellular pathogen, such as a virus, by releasing cytokines and altering the expression of surface molecules. The mechanisms employed in an innate response depend on both the type of pathogen and where the pathogen is detected in the body (Figure 3; Parham, 2009). Pathogens in extracellular space activate a different combination of immune cells and defence mechanisms than do intracellular pathogens.



	Extracellular		Intracellular	
	Interstitial spaces, blood, lymph	Epithelial surfaces	Cytoplasmic	Vesicular
Site of infection				
Organisms	Viruses Bacteria Protozoa Fungi Worms	*Neisseria gonorrhoeae* *Candida albicans* Worms	Viruses *Listeria* Protozoa	Mycobacteria Trypanosomes *Cryptococcus neoformans*
Defense mechanism	Complement Macrophages Neutrophils	Antimicrobial peptides	NK cells	Activated macrophages

Figure 3. The innate mechanisms engaged in an immune response are determined by the class of pathogen and where it is detected in the body. NK cells = natural killer cells (source: figure 2.2, pg. 32, Parham, 2009)

2.1.1 Physical and chemical barriers

The first defences against infection are the physical and chemical barriers afforded by the epithelial surfaces of the skin, lungs, mammary glands and the gastrointestinal tract (Lippolis, 2008). Sneezing, urination and the production of tears assist in flushing contaminants from the body, including harmful pathogens. Substances secreted at epithelial barriers in the body, including acids, peptides and digestive enzymes (Lippolis, 2008). For example, lysozyme, which is present in secretory fluids such as tears, destroys the cell wall of gram-negative and gram-positive bacteria (Beutler, 2004). Although not strictly a component of the immune system, the resident commensal bacterial populations in the gut and on skin surfaces also provide protection to the host, by out-competing pathogenic bacteria (Kudsk, 2002). Despite these barriers, pathogens sometimes gain entry through compromised barriers, such as broken skin, or because they have evolved mechanisms specifically to overcome such barriers.

2.1.2 Complement system

The complement system consists of a set of proteolytic enzymes, present in high concentrations in blood and in tissue, from which a cascade of reactions is activated by the presence of extracellular pathogens. Complement proteins work in various combinations to coat microbes for phagocytosis, induce inflammation and attract neutrophils, form a ‘membrane attack complexes’ that can lyse gram-negative bacteria, and inactivate viruses (Ricklin and Lambris, 2007). Coating of microbes (opsonisation) tags them for phagocytosis, which is especially important for bacteria with polysaccharide coatings that innate immune cells can’t otherwise attach to for phagocytosis (e.g. Staphlycoccus spp.; Parham, 2009).

Complement can be activated via three pathways that converge at the cleaving of protein C3 (Figure 4; Parham, 2009, Ricklin and Lambris, 2007). The classical pathway is initiated when complement component C1 interacts with a microbial surface or binds to antibody already bound to pathogen and the lectin pathway is initiated through interactions with carbohydrate structures on microbial surface. The ‘alternative’ pathway is the first pathway activated during infection and can be spontaneously activated. Spontaneous hydrolysis of C3 (known as tick-over), leads to the formation of C3 convertase which further cleaves C3 molecules and leads to complement activation and deposition of C3b molecules on the microbial surface. Complement protein C3b tags the microbe for phagocytosis and is also involved in the initial steps of membrane attack complex formation. Complement protein C3a acts as a chemoattractant to recruit other immune cells to the site, particularly phagocytes.

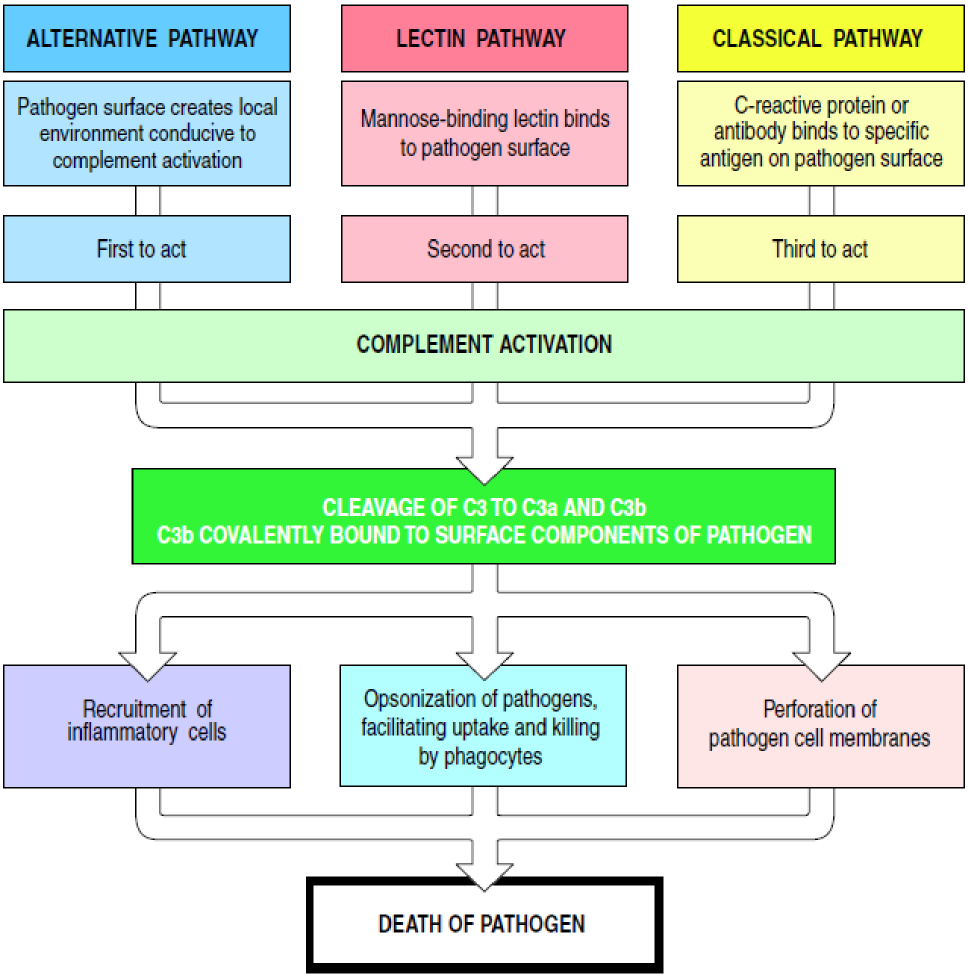


Figure 4. The three pathways of complement activation converge at the cleaving of C3 to C3a and C3b (source: Parham, 2009).

2.1.3 Inflammation and the acute phase response

Inflammatory cytokines secreted by macrophages, and other immune cells, act on the hypothalamus and peripheral tissues to induce fever, which potentiates adaptive immunity and decreases growth and replication of some pathogens. Pro-inflammatory cytokines also stimulate the liver to increase secretion of positive acute phase proteins (APPs) and decrease secretion of negative APPs. For example, increased production of C-reactive protein and mannose-binding lectin enhance the binding of complement to bacterial surfaces and increased haptoglobin binds free plasma haemoglobin, thereby inhibiting iron uptake by microbes. Production of negative APPs such as albumin decreases during an acute phase response, possibly to spare protein for the production of positive APPs (Cray et al., 2009). Combined induction of fever and the acute phase response are aimed at limiting pathogen replication and enhance the innate and adaptive immune responses.

2.1.4 Cellular defences of the innate system

Cell recruitment

During infection, cytokines stimulate cells lining the endothelium to express adhesion molecules that allow white blood cells to roll along and then adhere to the inner surface of the blood vessel where they receive signals to migrate out of the blood vessel and travel along a chemokine gradient to the site of inflammation (a process known as extravasation; Figure 4). Specific combinations of adhesion molecules and chemokines orchestrate recruitment of specific cell types ensuring that appropriate immune cells are recruited for the pathogen type and location.

Phagocytic cells

Phagocytic cells play a critical role in the innate immunity and include neutrophils, monocytes, macrophages and dendritic cells. These cells possess PRRs (e.g. toll-like receptor) which recognise:

- PAMPs: surface-molecule patterns unique to microbes. Most pathogens have several types of PAMPs and, therefore, the innate system can respond to different classes of pathogen with a suitable, but not finely tuned response (Beutler, 2004)
- Damage-associated molecule patterns (also known as danger-associated molecule patterns, DAMPs): molecules normally contained within cells, but released during tissue damage (Seong and Matzinger, 2004).

When PPRs on the surface of phagocytes bind to a PAMP, the phagocyte envelopes the pathogen with its cellular membrane, internalising it in a vesicle called a phagosome. The fate of the pathogen after phagocytosis depends on the class of phagocyte.

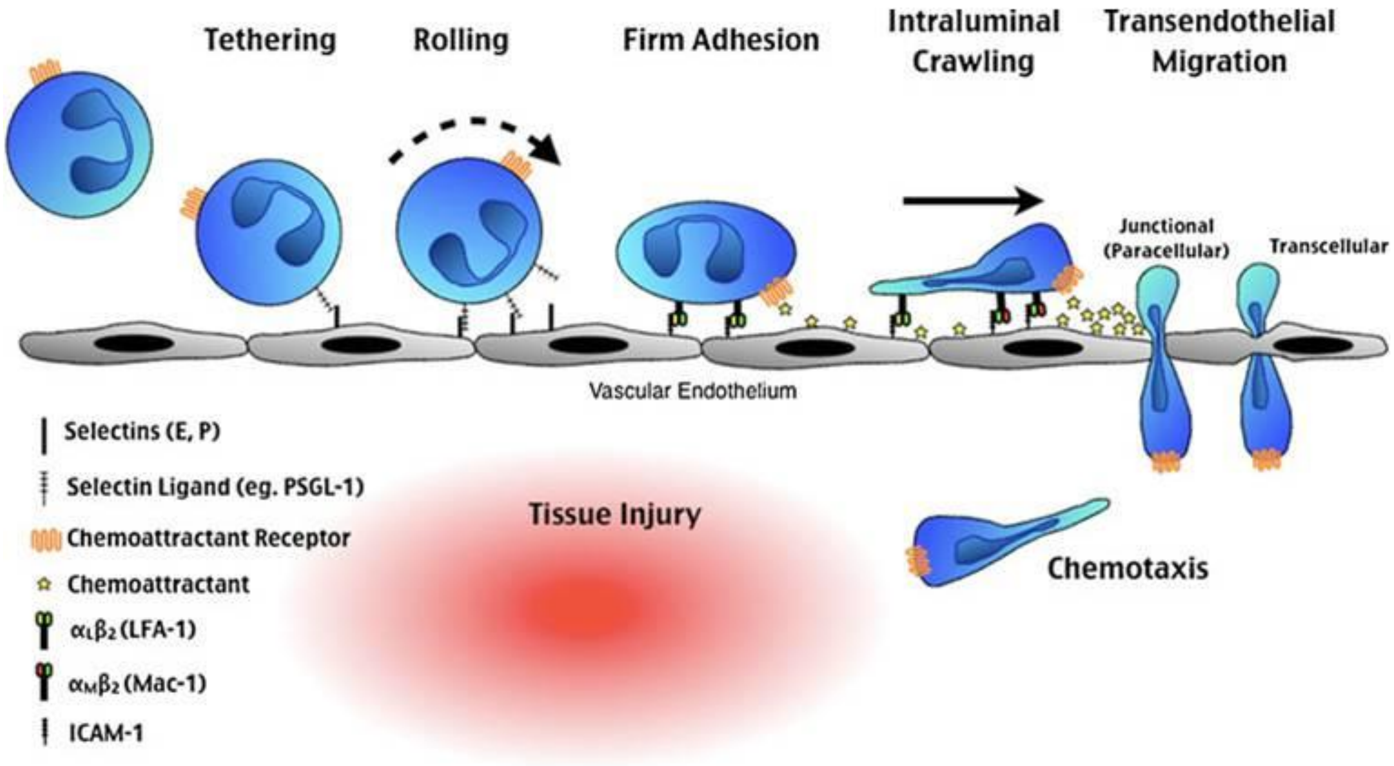


Figure 5. Neutrophils and other white blood cells are recruited to the site of inflammation by obligatory rolling and adhesion, then migration out of the blood vessel and chemotaxis along a chemokine gradient (source: McDonald and Kubes, 2011).

Neutrophils

Polymorphonuclear neutrophils are recruited to the site of infection in large numbers within hours of pathogen recognition. Neutrophils are short lived and only survive in circulation for 1-2 days, before undergoing spontaneous apoptosis. Neutrophils once recruited to the site of infection are only capable of one phagocytic event; they cannot replenish their granule contents so die by undergoing apoptosis and are ingested by macrophages or accumulate as pus. The mechanisms by which neutrophils eliminate microbes include phagocytosis-induced respiratory burst and degranulation, and the formation of neutrophil extracellular traps (NETs; Kaplan and Radic, 2012):

- **Phagocytosis induced respiratory burst.** After engulfing pathogens, neutrophils fuse granules containing microbicidal molecules with the phagocytic vesicle, which generates reactive oxygen species (ROS) in the vesicle to kill the pathogen.
- **Neutrophil extracellular traps.** NETs are constructed of chromatin from the nucleus and microbicidal molecules from granules. They are able to entrap pathogens that neutrophils are unable to phagocytose; for example, *Staphylococcus aureus* is too hydrophilic for neutrophils to attach to and engulf, but is a potent stimulator of NET formation (Kaplan and Radic, 2012). NETs trap and kill (or contain) bacteria, and also enhance innate inflammatory responses.

The mechanisms neutrophils use to clear infections also damage host tissues, particularly the release of reactive oxygen species (ROS); therefore, they are short lived and undergo spontaneous apoptosis to limit collateral damage to the host.

Monocytes, macrophages and dendritic cells

Monocytes circulate in blood and upon entering tissue mature into long-lived macrophages and dendritic cells. Macrophages and dendritic cells can phagocytose and kill pathogens using microbicidal agents, but, play an even more important role as antigen presenting cells, presenting antigens to cells of the adaptive immune system and secreting cytokines that recruit other monocytes and neutrophils to the site of infection (Beutler, 2004). Dendritic cells reside in tissues that are exposed to the external environment. When they engulf a pathogen, they mature and migrate into the lymph nodes, where they present fragments of the pathogen to naïve lymphocytes. Macrophages also reside in tissue, but act more locally, presenting antigen to lymphocytes and secreting cytokines that recruit other innate immune cells and direct the type of immune response induced. Antigen-presenting cells also modulate the adaptive immune system by controlling nutrient supply, for example, activated macrophages and dendritic cells increase the availability of cysteine to nearby T-lymphocytes (Fox et al., 2005). Macrophages and dendritic cells are usually the first cells to respond to an infection; so, they play an important role in determining the nature of the subsequent response.

Eosinophils, basophils, and mast cells

Eosinophils, basophils, and mast cells are specialised for destroying large extracellular parasites, such as Helminth worms. Eosinophils circulate in blood and lymph and are also resident in the lining of the gastrointestinal and reproductive tracts. Basophils circulate in blood while mast cells are resident in connective tissue and mucosal surfaces. The three cell types often work together; mast cells are activated first, which in turn recruit eosinophils and basophils to the site of inflammation. When activated, these cells degranulate, releasing microbicidal substances into their environment. The granules of mast cells and basophils contain histamine and heparin, which increase vascular permeability and promote blood flow to the infected tissue. Eosinophils, mast cells and basophils have IgE antibody bound to their cell surface, and are activated when IgE on their surface is cross-linked by simultaneously binding their cognate antigen. This means that they are restricted to responses against multicellular pathogens, although they can also be also activated by tissue damage. These cells produce cytokines that influence the inflammatory response but also respond to cytokines produced by cells of the adaptive immune system (Beutler, 2004). Basophils may be particularly important to the establishment of type-2 antibody-mediated responses because of their ability to produce IL-4 in the initial stages of an immune response which promotes type-2 responses (Parham, 2009).

Natural killer cells

Natural killer (NK) cells are specialised cells with the capacity to identify and kill infected cells and tumour cells. NK cells destroy cells by inducing apoptosis, either by delivering enzymes into the cell via perforin proteins or by binding to the Fas protein on the cell surface (Sompayrac, 2012). Natural killer cells have features of the innate and the adaptive immune system: the NK cell response is modified by repeat exposure to a pathogen (Sun et al., 2010), but they have a limited diversity of antigen receptors relative to T-lymphocytes (Sompayrac, 2012). Like neutrophils, NK cells are ‘on-call’ from the blood system. Natural killer cells proliferate rapidly at the site of infection and can be activated by binding directly to pathogens or in response to interferon cytokines secreted by virus infected cells. Interleukin-12 induces NK cells to secrete IFN-γ, which primes macrophages to produce more TNF-α and IL-12, which induces NK cells to secrete yet more IFN-γ, creating a positive feedback loop. These cells kill infected and tumour host cells that express an unusually high ratio of foreign or unusual molecules: MHC I on their surface (Sompayrac, 2012), and foreign cells, which do not express MHC-I molecules.

2.2 Cell-to-cell signalling by cytokines

Cytokines are small proteins, secreted by cells which influence the behaviour of other immune cells. There are many different cytokines, some of which are outlined in Table 1. They bind to specific receptors on other immune cells, influencing their growth, movement and function. A subset of cytokines, called chemokines, facilitates the extravasation of immune cells to sites of infection (Beutler, 2004). Immune cells are the predominant source of cytokines: however, non-immune cells, such as epithelial and adipose cells, also contribute to the cytokine milieu during infection or tissue damage (Lippolis, 2008).

Table 1. Cell source and actions of prominent cytokines in the bovine immune system.

Cytokine	**Cell source**	**Target and action**
IL-2	Macrophages, T-lymphocytes, Natural killer (NK) cells	T- and B-lymphocyte proliferation, NK cell activation & proliferation
IL-8	Macrophages	Attracts neutrophils
Pro-inflammatory cytokines		
TNF-α	Macrophages, Dendritic cells, T-lymphocytes	Activates vascular epithelium facilitating entry of complement & immune cells into tissue Neutrophil and NK cell activation, Hypothalamus – fever, Liver - acute-phase response
IFN-γ	T-lymphocytes, Natural killer cells	Up regulates type-1 cytokine secretion by T-lymphocytes Switches antibody isotype to IgG2 isotype, Macrophage, neutrophils and natural killer cell activation
IL-12	Macrophages, Dendritic cells	Polarises T-lymphocyte cytokine secretion toward type-1 profile NK cell activation
IL-6	Macrophages	Hypothalamus – fever, Liver - acute-phase response, Promotes type-2 bias by promoting IL-4 secretion and making CD4+ T-lymphocytes unresponsive to IFN-γ
Anti-inflammatory cytokines		
IL-10	Macrophages, T cells	Inhibits IL-12 production, Inhibits Type-1 cytokine production, Deactivates natural killer cells
IL-4	T cells, Mast cells	Up regulates Type-2 cytokine secretion by T-lymphocytes, Switches antibody isotype to IgG1 and IgE, Inhibits IFN-γ activation of macrophages, Down regulates IFN-γ production

The first cytokines released during infection are usually pro-inflammatory cytokines (e.g., TNF-α and IL-12), which mediate recruitment of immune cells to site of infection by inducing expression of adhesion molecules and chemokines on the endothelium and also induce maturation of dendritic cells (Lippolis, 2008). Later in infection, anti-inflammatory cytokines (e.g., IL-10 and IL-4) start to dominate and dampen the inflammatory response, thereby preventing excessive damage to the host (Lippolis, 2008). Antigen presentation and cytokine production by cells of the innate immune system provides an important link between the innate and adaptive immune system (Beutler, 2004).

2.3 Adaptive immune system

The adaptive immune response is much slower to develop than the innate response. However, the adaptive response is tailored specifically to individual pathogens and each time the animal is exposed to the same pathogen, the response becomes faster and stronger. Adaptive responses can be broadly divided into two types:

- Cell-mediated immune responses (CMIR), which are commonly generated in response to intracellular pathogens and are mediated by T-lymphocytes.
- Antibody-mediated immune responses (AMIR), which are commonly generated in response to extracellular pathogens and are mediated by B-lymphocytes.

T- and B-lymphocytes recognise specific antigens. The fundamental difference between T- and B-lymphocytes, apart from their effector mechanisms, is that receptors on T-lymphocytes only bind antigens presented to them by other host cells, whereas B-lymphocyte receptors bind antigens directly in the extracellular environment. The T- or B-lymphocyte receptors on each individual cell can only recognise a single specific antigen. Therefore during development, the genes encoding these receptors are re-arranged to create millions of different receptors. Receptors that are specific for self-antigens are destroyed during maturation. Although each lymphocyte is specific for a single antigen, there can be multiple lymphocytes which recognise the same antigen. When infected by a new pathogen, each cell with a receptor that recognises the pathogen proliferates to produce a clone of thousands of identical cells recognising the same antigen.

2.3.1 Immunological Memory

The adaptive immune system acquires memory of pathogens, which facilitate much more effective responses to re-invasion. When infection occurs, naïve lymphocytes with receptors for an antigen on the pathogen rapidly differentiate and proliferate into a small clone of memory cells and a large clone of effector cells. Effector cells control the infection, and then undergo apoptosis when the infection has cleared. In contrast, memory cells remain in circulation, and can immediately differentiate into effector cells on subsequent exposure, allowing a more rapid and effective response to re-invasion.

2.3.2 T-lymphocytes

Naïve T-lymphocytes are activated by dendritic cells that have migrated into lymph nodes after engulfing pathogen. T-lymphocytes can differentiate into two sub-populations, as determined by cytokines released by the dendritic cell during activation. Each sub-population has distinct functions and is distinguishable by cell surface receptors. Receptors on T-lymphocytes are composed of either α and β chains or γ and δ chains. T-lymphocytes expressing αβ receptors are the most populous and have the largest receptor diversity. In contrast, γδ T-lymphocytes straddle the interface of innate and adaptive immune function (Dranoff, 2004) and function as regulatory T-lymphocytes in bovines (Guzman et al., 2012).

αβ T-lymphocytes

T-lymphocytes with αβ T-lymphocyte receptors recognise antigen fragments presented in association with major histocompatibility complex (MHC) molecules on the surface of other cells (Beutler, 2004, Lippolis, 2008). There are two types of MHC molecule, class I and class II which present different types of antigen and have different expression patterns in the body:

- Intracellular antigens are presented by MHC-I molecules. All cells, except red blood cells and platelets, express MHC-I, which display fragments of proteins that are being synthesised or degraded within the cell. Foreign protein fragments presented by MHC-I on infected cells invite destruction by the immune system.

- Extracellular pathogens are presented by MHC-II molecules. Only specialised antigen presenting cells (i.e. macrophages, dendritic cells, and B-lymphocytes) normally express MHC-II. The type of antigen presenting cell influences the type of immune response that develops.

There are two subpopulations of αβ T-lymphocytes, defined by co-receptors that bind to either MHC-I or MHC-II. T-lymphocytes with the co-receptor CD4 are restricted to antigen presented in complex with MHC-II (Figure 6) and are, therefore, specialised to controlling extracellular pathogens. T-lymphocytes with the co-receptor CD8 are restricted to antigen presented in complex with MHC-I and are, therefore, specialised in control of intracellular pathogens. It should be noted however, that CD4+ T-lymphocytes also contribute to control of intracellular pathogens when presented antigen by antigen presenting cells harbouring microbes in intracellular vesicles.

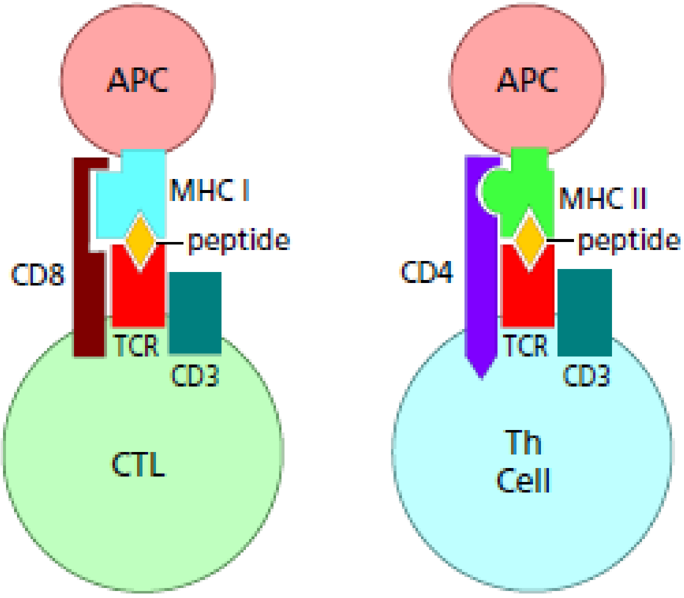


Figure 6. Activation of naïve αβ T-lymphocytes. **Cytotoxic T-lymphocytes (CTL) express co-receptor CD8, which restricts them to antigen presented in MHC-I molecules. T helper (Th) lymphocytes express co-receptor CD4, which restricts them to antigen presented in MHC-II molecules. (source: Sompayrac, 2012)**

CD4+ T helper lymphocytes

T-lymphocytes that express αβ receptors and CD4 co-receptors are known as T-helper lymphocytes because they help other immune cells to eliminate pathogens, rather than killing directly. Naive CD4+ T-lymphocytes are primarily activated by dendritic cells but once differentiated, primed T-lymphocytes can be activated by any antigen presenting cell. Cytokines secreted by the dendritic cell during activation of naïve T-lymphocytes influence the cytokines expressed by that T-lymphocyte once mature (Lippolis, 2008). There are several discrete subpopulations of CD4+ T-lymphocytes described in mice, including T_H1, T_H2, T_H17, and Trig & T_{FH}, each with a signature cytokine expression profile. In bovines, the same discrete subpopulations are not present; but rather the cytokine expression profiles of bovine CD4+ T-lymphocytes exist on a spectrum and secrete a range of cytokines (Brown et al., 1998). However, polarised responses to different types of antigens do occur in cattle and CD4+ cells in this species can be described in terms of polarised rather than discrete subpopulations. In mice, each subpopulation secretes a different combination of cytokines to direct immune responses, as illustrated in Figure 7 (Lippolis, 2008).

T_H1 – drives cell-mediated immunity

T_H1 lymphocytes secrete IFN-γ to activate adjacent macrophages and enhance killing of phagocytosed pathogens by macrophages. Interferon-γ inhibits IL-4 secretion by other T-lymphocytes. They also stimulate and enhance the cytotoxic activity of CD8+ T-lymphocytes. The IFN-γ mediated response is associated with inflammation. T_H1 cells are the principal regulators of type-1 (cell-mediated) responses aimed at eliminating intracellular pathogens.

T_H2 – drives antibody-mediated immunity

T_H2 cells secrete IL-4 to stimulate naïve B-lymphocyte proliferation and differentiation to antibody secreting cells and memory cells. Interleukin-4 also inhibits IFN-γ secretion by other T-lymphocytes and macrophages. T_H2 lymphocytes are the principal regulators of type-2 (antibody-mediated) responses aimed at eliminating extracellular pathogens.

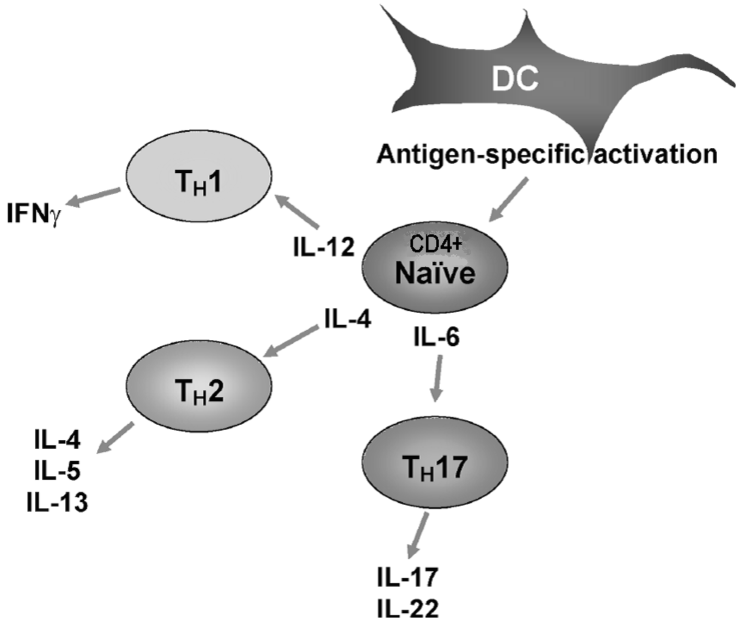


Figure 7. Dendritic cells secrete cytokines during activation of naïve T-lymphocytes, which influence the cytokine expression profile of the resulting mature T-lymphocytes. In this illustration, activation of naïve T-helper lymphocytes in the presence of IL-12 produces mature T-helper (Th) cells that tend to secrete more IFN-γ than other cytokines. IFN = interferon; IL = interleukin. (Modified from: Lippolis, 2008)

CD8+ Cytotoxic T-lymphocytes

T-lymphocytes that express the CD8 co-presentation molecule are known as CD8+ or cytotoxic T-lymphocytes. After activation, CD8+ T-lymphocytes migrate to the site of infection and destroy cells displaying their cognate antigen in complex with MHC-I. Killing of infected cells is achieved by secretion of perforin, which makes a hole in the cell membrane, through which secretion of another protein induces apoptosis of the cell. Their function is similar to that of NK cells, in that they are specialised in destroying virus infected cells, before the virus is able to replicate and leave the cell. However, CD8+ T-lymphocytes are able to identify infected cells that still have normal MHC-I expression, which NK cells cannot. Like CD4+ T-lymphocytes, CD8+ T-lymphocytes secrete cytokines to regulate the immune response; cells that secrete IFN-γ stimulate macrophages and NK cells, whereas cells that secrete IL-4 and IL-10 inhibit macrophage and NK cell activity. CD8+ T-lymphocytes contribute to escalation, and then regulation of responses against intracellular infections.

Regulatory γδ T-lymphocytes

T-lymphocytes with γδ T-lymphocyte receptors play an important role in T-lymphocyte regulation in the bovine (Coussens et al., 2012, Hoek et al., 2009); they contribute to both the establishment and resolution of the immune response (Bailey et al., 2013). In contrast to most other mammals, a large proportion of circulating T-lymphocytes in cattle are γδ T-lymphocytes, especially in calves, and the γδ receptor repertoire is much more diverse (Bailey et al., 2013, Guzman et al., 2012). They are not restricted by MHC-I or MHC-II; therefore, naïve γδ T-lymphocytes do not rely on antigen presenting cells for activation; in fact, they are able to present antigen in MHC-II complex to CD4+ T-lymphocytes (Guzman et al., 2012). They appear to be involved in responses against intracellular pathogens, such as Johne's (*Mycobacterium paratuberculosis*); (Plattner et al., 2014). They can secrete pro-inflammatory cytokines (TNF-α and IFN-γ) and anti-inflammatory cytokines (IL-10); their rapid response at the start of infection supports development of the adaptive immune response, but, as infection progresses, the γδ T-lymphocyte population takes on a regulatory role (Coussens et al., 2012, Plattner et al., 2014).

2.3.3 B-lymphocytes

The B-cell receptor on B-lymphocytes is composed of a transmembrane immunoglobulin molecule. The basis of antibody-mediated immune responses is antibodies secreted by plasma B-lymphocytes. The help of CD4+ T-lymphocytes is required for antibody production against most pathogens and this help occurs in the secondary lymphoid organs where IL-4-expressing CD4+ T-lymphocytes accumulate. When a naïve B-lymphocyte's receptor binds to its cognate antigen on the surface of a pathogen, it engulfs the pathogen and presents antigen fragments in complex with MHC-II on its surface. This activates T-lymphocytes with a receptor for the same antigen fragment to secrete cytokines that trigger B-lymphocyte proliferation and differentiation into plasma cells, capable of producing copious amounts of antibody specific for the original antigen. Antibodies are selectively transported from blood to the extracellular fluid, where they opsonise extracellular pathogens for removal or killing by phagocytes, activate the complement cascade, and coat toxins and viruses to neutralise them.

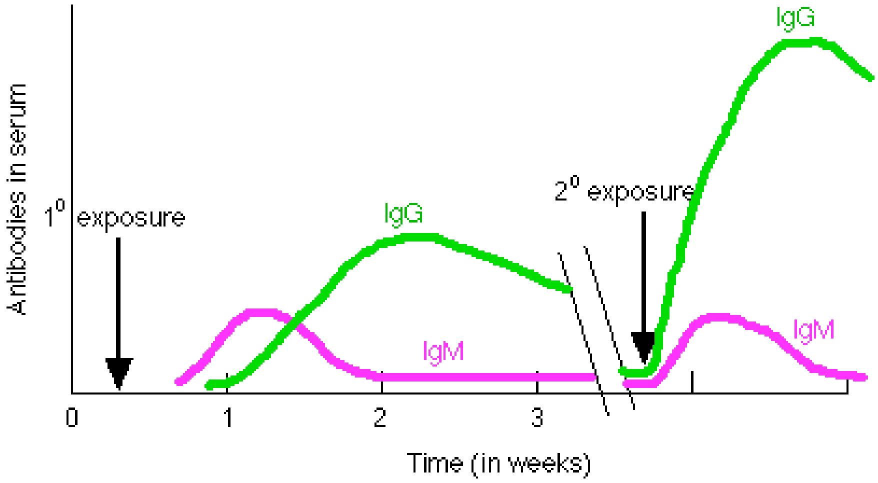


Figure 8. The first antibody response to antigen exposure (the primary response) is small; however, the memory lymphocytes generated during initial antigen exposure remain in circulation for a long time and allow a much faster and more specific secondary antibody response to subsequent antigen exposure.

Antibody isotypes and switching

As an antibody-mediated immune response progresses, the affinity of the secreted antibody for its cognate antigen improves as a consequence of somatic hyper mutation leading to affinity maturation of the antibody population. Clonal expansion of the naïve B-lymphocyte occurs in two phases. In the initial phase, the plasma cells produced secrete mostly IgM, which tags the pathogen and activates complement. Later in the response, the cytokine milieu signals the plasma cells to switch to secreting antibody isotypes more suited to the target (Figure 8; Heriazon et al., 2011). This switch occurs during primary and secondary antibody responses, except that during secondary responses, memory B-lymphocytes proliferate into plasma cells that are already isotype switched; so, large amounts of the appropriate isotype are quickly produced. Isotype switching produces isotypes with different effector functions and is critical to the establishment of type-1 and type-2 responses (Begley et al., 2009a).

The isotypes differ in their ‘constant’ region, the shape of which determines how flexible the antibody structure is and what effector functions they can engage:

- **IgG** is the most abundant isotype in body fluids, present in lymph, serum and milk, and is essential for combating systemic disease. It is smaller and more flexible than IgM, which affords it better access to antigens in small spaces. There are two main subtypes of IgG, with IgG1 usually more abundant than IgG2, especially in colostrum as a consequence of IgG1 being selectively transported from circulation into the mammary gland during colostrogenesis (Butler et al., 1971). IgG1 and IgG2 both contribute to responses against extracellular and intracellular pathogens (Heriazon et al., 2011); however IgG1 is more efficient at fixing complement (McGuire et al., 1979). In contrast, bovine IgG2 does not require complement fixation for potent opsonin activity and is considered the main opsonin supporting neutrophil phagocytosis in the mammary gland (Atalla et al., 2010, Guidry, 1993). IgG2 is considered a type-1 because it supports inflammatory mechanisms; whereas, IgG1 production is considered more a type-2 reaction because type-2 cytokines induce IgG1 production (Estes and Brown, 2002).

- **IgA** is also produced by plasma cells in lymph tissue near mucosal surfaces, such as in the gut and respiratory tract, and in exocrine glands secretions, such as tears (Butler et al., 1971), where it binds extracellular pathogens and toxins (Parham, 2009).
- **IgE** binds with very high affinity to eosinophils, mast cells and basophils and is essential to activating their degranulation during parasitic infections.

2.4 Response type

Antibody-mediated and cell-mediated immune responses are not mutually exclusive; during any adaptive response, both responses will be present. The combination of innate and adaptive functions engaged in an immune response tends to polarise reactions towards one of two types of response depending on the pathogen encountered. Each responses being defined by the innate mechanisms at play, cytokine environment established and antibody isotypes produced:

Type-1

Cytokines produced by T_H1 lymphocytes promotes type-1 or CMIR against intracellular pathogens. Type-1 responses are characterised by intense phagocytic activity by cytotoxic CD8 cells, NK cells and macrophages, mediated by production of pro-inflammatory cytokines (IFN-y, TNF-a, IL-12), and enhanced B-lymphocyte production of antibodies, including IgG2 in cattle which act as opsonins (Estes and Brown, 2002, Estes et al., 1994, Goldszmid and Trinchieri, 2012, Heriazon et al., 2011)

Type-2

Cytokines produced by T_H2 lymphocytes promote type-2 or AMIR against extracellular pathogens. Type-2 responses are characterised by the production of antibody, mediated by the production of anti-inflammatory cytokines (IL-4, IL-6, IL-10), which enhance B-lymphocyte production of IgG1 and suppress macrophage IFN-γ secretion and deactivate neutrophils (Dosogne et al., 1999, Estes and Brown, 2002, Estes et al., 1995, Goldszmid and Trinchieri, 2012).

Cytokines involved in each type of reaction tend to inhibit or modulate the action of cytokines produced in the other type of reaction. Therefore adaptive immune responses may be polarised towards CMIR (type 1) or AMIR (type2), depending on the antigen. In cattle, polarisation of the response can be assessed by looking at the ratio of IgG1:IgG2 and the ratio of IL-4:IFN-γ produced during a response (Heriazon et al., 2011, Hine et al., 2011). A balanced ability to mount both type-1 and type-2 responses is important for control of intra- and extra-cellular pathogens. Responses that are initially type-1 in nature are followed by a shift to type-2 mechanisms, which resolve the response by promoting AMIR and suppressing CMIR (Spellberg and Edwards, 2001).

2.5 Costs of immunity

Maintaining a responsive immune system does not come without cost. A constant supply of protein and energy is needed to maintain the cell populations and physical barriers of the immune system (Beisel, 1977, Calder, 1995, 2006, Healy et al., 2002, Kim et al., 2007, Lochmiller and Deerenberg, 2000, Newsholme et al., 1999, Scrimshaw and SanGiovanni, 1997). For example, about 1 billion replacement neutrophils are generated per kilogram of body weight (BWT) per day (Dancey et al., 1976). To mount an active immune response further increases protein and energy requirements (Fox et al., 2005, Kominsky et al., 2010, MacIver et al., 2008, Matarese and La Cava, 2004), for synthesis of antibodies, cytokines, complement and acute phase proteins (Li et al., 2007), and for cell structures and enzymes of proliferating cells (Beisel, 1977). Initiating an immune response is reported to increase energy requirements by 25-60% of basal metabolic requirements (Demas, 2004, Straub et al., 2010).

Each immune response also incurs costs in terms of collateral damage. An inflammatory response always incurs some tissue damage to the host (Goldszmid and Trinchieri, 2012). During inflammation, ROS and RNS are produced by immune cells to kill pathogens, but the oxidants are not selective and affect host cells as well. Additionally, an excessive or unresolved response can lead to systemic inflammation, changes in metabolism, and impaired response to other infections (Goldszmid and Trinchieri, 2012). Finally, the adaptive immune system sometimes responds inappropriately to harmless antigens, as in allergies, and can direct effector mechanisms toward host cells, as in auto-immune diseases.

However, although immune responses have a high cost in terms of collateral damage and partitioning of nutrients away from production, production losses due to occurrence of disease in animals with less than optimal immune responsiveness may outweigh those losses (Colditz, 2008).

2.6 Summary of the immune system

The immune system comprises an innate system, which immediately mounts an immune response to pathogens upon initial exposure and is largely non-specific in its nature, and an adaptive system, which takes time to develop, but produces a response that is highly specific to the invading pathogen and results in immunological memory. The two systems synergise via intricate cell-to-cell communication, using cytokines, adhesion molecules and cell receptors; this integration enhances the innate response and is essential to the development of the adaptive response. Adaptive immune responses can be broadly classified as type-1 (CMIR) and type-2 (AMIR), which are largely directed at intracellular and extracellular pathogens, respectively. Competent immune function depends on a fine balance between effector and regulatory mechanisms to ensure efficient pathogen clearance, while limiting collateral damage; any imbalance can lead to either chronic infection or chronic inflammation (Hoek et al., 2009).

Chapter 3. The transition period and immune suppression

3.1 What is the transition period?

The '*transition period*', defined as three weeks before calving to three weeks after calving, is a significant metabolic challenge for the dairy cow, due to dramatic changes in nutrient demands and sinks (Bell, 1995, Drackley, 1999, Goff, 2006, Moyes et al., 2010, Roche et al., 2013a, Roche et al., 2009). Failure to transition successfully is believed to affect individual cow's productivity and disease susceptibility (Drackley, 1999, Roche et al., 2013a). Consistent with this view, Godden et al. (2003) reported that 25% of cows culled in dairy herds, in Minnesota, left the herd in the first 60 DIM. The onset of most metabolic diseases occurs within the first two weeks postpartum, and the incidence of infectious disease is highest during this period (Goff and Horst, 1997). Disease occurrence during the transition period impacts productivity beyond the period of clinical symptoms (Bar et al., 2007, Fetrow et al., 1991, Wilson et al., 2008) and may also have a negative effect on a cow's subsequent reproductive success (Roche et al., 2009, Staples et al., 1990, Wilson et al., 2008). Therefore, successful navigation of the transition from gestation to lactation is of high importance to dairy farming profitability (Drackley, 1999, Grummer, 1995).

A fully functioning immune system is essential for protection from infection in the mammary gland during lactation and for remodelling the uterus after parturition. A competent immune system also supports immunity in the immunologically-naïve new-born calf through the passive transfer of maternal antibodies via colostrum. And yet, during the transition from pregnancy to lactation, dairy cows exhibit a profound immune dysfunction, which contributes to higher incidence of mastitis and the clinical manifestation of many diseases during the early post-partum period.

3.2 Peripartum immune dysfunction

Hormonal and metabolic changes during the transition period lead to impaired immune function which negatively impacts on the ability of the immune system to fight infections. Dairy cows exhibit a transient type-2 shift in immune function during gestation (Hine et al., 2011, Maeda et al., 2013) and the transition to lactation that is common to mammals (Goff, 2006, Kehrli et al., 2006, Mallard et al., 1997, Mellor and Munn, 2000, Schwarm et al., 2013, Shafer-Weaver et al., 1999, Trowsdale and Betz, 2006). Suppression of type-1 cellular immune responses during pregnancy is thought to prevent rejection of the conceptus, which expresses non-maternal antigens (Kehrli Jr and Harp, 2001, Maeda et al., 2013, Mellor and Munn, 2000, Trowsdale and Betz, 2006) and to minimise unwanted immune responses against self- and fetal-antigens exposed as a result of tissue damage during parturition (Kehrli Jr and Harp, 2001). Up-regulation of type-2 functions may also support production of IgG1 for transport to colostrum, which is especially important for ruminants because there is no placental transfer of maternal antibodies and the ability of the neonate to absorb antibody through the gut wall declines rapidly after birth (Quigley and Drewry, 1998, Shafer-Weaver et al., 1999). Although suppression of type-1 immunity is typical for gestating mammals and helps prevent harmful immune responses, the changes that occur in dairy cows during the periparturient period are more profound and extend further beyond parturition (Mallard et al., 1998, Theodorou et al., 2007). Changes are apparent in both the innate and adaptive arms of immunity, normally from about 3 weeks before parturition to 3 weeks after parturition with most severe immune dysfunction being observed at parturition, though the timing depends on the immune function measured (Mallard et al., 1998). This immune dysfunction contributes to the high incidence of clinical infectious disease observed during the periparturient period (Burvenich et al., 2007, Drackley, 1999, Mallard et al., 1998, Smith et al., 1985).

3.3 Lactation contributes to immune suppression

Many of the changes in immune function postpartum are attributable to the presence of the mammary gland and lactation (Kimura et al., 1999, Kimura et al., 2002, Nonnecke et al., 2003). A series of experiments by Kimura, Nonnecke and Goff et al. (Goff et al., 2002, Kimura et al., 1998, 1999, 2002, Nonnecke et al., 2003) studied neutrophil and lymphocyte function in cows that were mastectomised, compared with cows that were intact, during the transition period. In mastectomised cows, most immune functions returned to normal approximately one week after parturition. In contrast, immune dysfunction of intact cows was not resolved until several weeks after parturition. The ability of neutrophils to kill ingested bacteria (as indicated by myeloperoxidase activity) decreased before parturition in both intact and mastectomised cows, but was quickly recovered in mastectomised cows; in comparison, intact cows continued to have depressed neutrophil function for at least three weeks (Figure 9; Kimura et al., 1999). Mastectomised cows did not exhibit the same changes in peripheral blood immune cell populations, even before parturition (Figure 10; Kimura et al., 2002), and their lymphocyte functions (proliferation, IFN-γ secretion, and T-lymphocyte dependent IgM secretion) were not suppressed to the same extent (Nonnecke et al., 2003). These studies demonstrated that, although a degree of immune suppression was evident as a result of parturition, the demands of lactation may be responsible for the delayed recovery of normal immune function postpartum (Goff, 2006, Kimura et al., 1999).

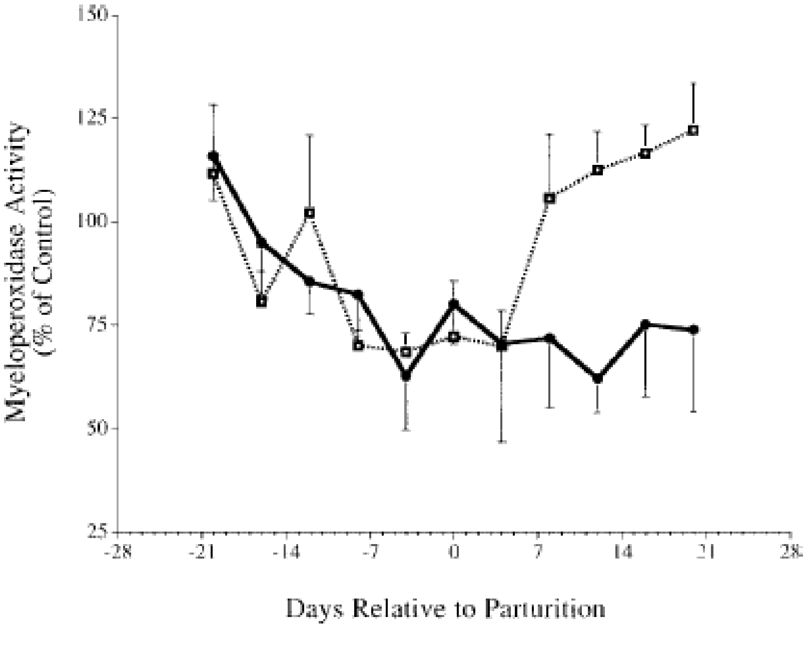


Figure 9. Ability of neutrophils to kill ingested bacteria, indicated by Myeloperoxidase activity, in mastectomised cows (n = 10, ◘) and intact cows (n = 8,solid line ●), during the periparturient period (mean ± SEM; sourced from Kimura et al., 1999).

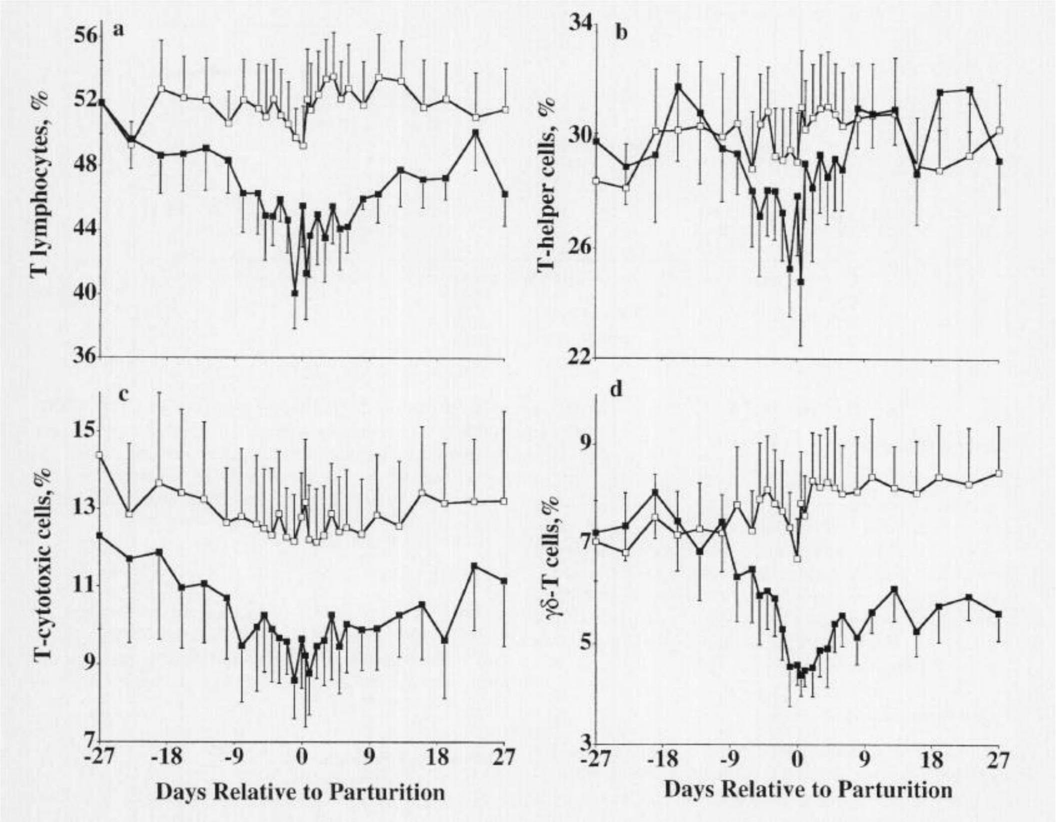


Figure 10. Percentage of total T-lymphocytes (a), T-helper lymphocytes (b), T-cytotoxic cells (c), and gamma-delta T-lymphocytes (d) in peripheral whole blood from intact (n = 8, solid line ●) and mastectomised cows (n = 10, ◘) during the periparturient period (mean ± SEM; sourced from Kimura et al., 2002)

3.3.1 Metabolic stress

Lactation, in dairy cows, contributes to extended immune dysfunction for several weeks postpartum probably because it induces metabolic stress. Metabolic stress occurs when an imbalance between nutrient demand and nutrient intake forces some metabolic processes to be compromised or down-regulated. During the transition from pregnancy to lactation, a dramatic increase in nutrient requirements (for milk production) causes metabolic stress because nutrient intake does not increase as quickly (Roche et al., 2009). Additionally, changes in the concentration of circulating hormones, and tissue sensitivity to hormones, skew the partitioning of nutrients to the mammary gland, further exacerbating metabolic stress in the rest of the cow. Cows that exhibit greater metabolic stress during transition, indicated by more negative energy balance postpartum, have increased systemic inflammation and oxidative stress, and poorer neutrophil function than cows with less negative postpartum energy balance (Graugnard et al., 2012).

3.4 Changes to the innate immune system around calving

3.4.1 Monocytes

Changes in the monocyte population are thought to contribute to the increased inflammatory state observed in periparturient dairy cows. The proportion of monocytes in blood is increased during the periparturient period (Kimura et al., 2002, Park et al., 1992, Shafer-Weaver et al., 1999, Shafer-Weaver and Sordillo, 1997). Additionally, monocytes isolated from blood and the supramammary lymph nodes of periparturient cows secrete more TNF-α when stimulated with lipopolysaccharide, than those from mid-lactation cows (Sordillo et al., 1995). Monocyte secretion of TNF-α in response to antigen exposure usually induces inflammatory, cell-mediated immune responses by activating and recruiting neutrophils. On this basis it is thought that monocytes exacerbate inflammation in periparturient cows.

3.4.2 Neutrophils

There are changes in both number and effector functions of neutrophils during the peripartum period (depicted in Figure 11). Blood neutrophil count increases from

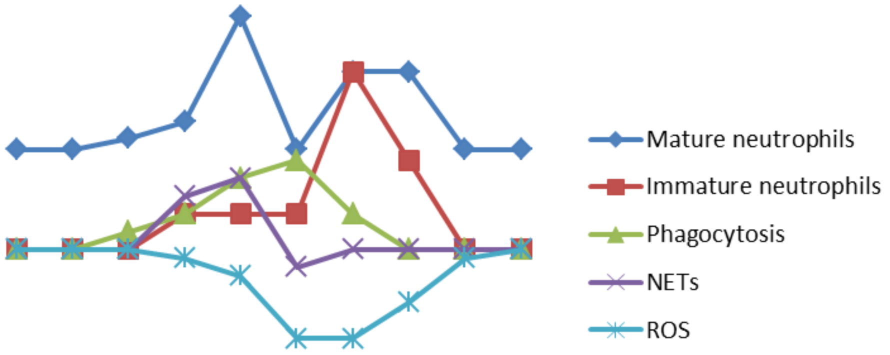


Figure 11. A stylised representation of the changes that occur in the peripheral blood neutrophil population of dairy cows during the periparturient period. The normal number of mature neutrophils is 25 times the normal number of immature neutrophils. NETs = neutrophil extracellular traps; ROS = reactive oxygen species.

about five weeks pre-calving, then declines to normal levels in the first week of lactation (Detilleux et al., 1995, Gilbert et al., 1993). In the second week of lactation, large numbers of immature neutrophils enter circulation (Detilleux et al., 1995). Neutrophil expression of NETs increases pre-partum and then decreases (Revelo and Waldron, 2010), in line with the maturity of the neutrophil population; immature neutrophils do not produce NETs (Lukášová et al., 2013). Phagocytic ability is generally increased around parturition, although not for all pathogens (Detilleux et al., 1995, Dosogne et al., 1999, Revelo and Waldron, 2010, Saad et al., 1989). In contrast, neutrophil capacity to generate intracellular and extracellular ROS is reduced in the first three weeks postpartum (Detilleux et al., 1995, Dosogne et al., 1999, Revelo and Waldron, 2010) and does not reflect population changes. Reduced expression of adhesion molecules and chemo-attractant receptors also renders neutrophils less able to leave circulation and enter tissue, delaying recruitment to the site of infection (Heyneman et al., 1990, Hill, 1981, Kehrli et al., 1999, Kehrli et al., 2006, Seo et al., 2013). Consequently, despite increased TNF-α secretion by periparturient macrophages, the ability of neutrophils to migrate to the site of infection and eliminate pathogens is impaired in early lactation (Detilleux et al., 1995, Kehrli et al., 2006).

Increased impairment of neutrophil function during the periparturient period is associated with increased incidence of retained foetal membranes and uterine infections (Cai et al., 1994, Gunnink, 1984) and with increased incidence and severity of mastitis (Heyneman et al., 1990, Hill, 1981, Kremer et al., 1993, Mehrzad et al., 2005, Shuster et al., 1996, Zecconi et al., 1994). Delayed neutrophil recruitment and impaired oxygen-dependent killing contributes to an increase in the incidence and severity of infection in periparturient cows and as a consequence compromises innate immune system function and subsequent initiation of an appropriate adaptive response.

3.5 Changes to the adaptive immune system around calving

Adaptive immune function is altered around calving, with changes apparent in both the B- and T-lymphocyte populations during the periparturient period. Peripheral blood lymphocyte and milk lymphocyte blastogenesis and proliferation in response to stimulation with mitogens decrease in the first week following parturition (Kehrli Jr et al., 1989, Nonnecke et al., 2003, Saad et al., 1989, Wells et al., 1977). Blood lymphocyte responsiveness recovers within two weeks of parturition, whereas milk lymphocyte responsiveness remains low for several weeks (Saad et al., 1989). Changes are more evident and broader in scope in the T-lymphocyte population whose effector function is biased away from type-1 inflammatory responses.

3.5.1 T-lymphocytes

There are major changes in the T-lymphocyte population during the periparturient period which result in suppression of type-1 responses in favour of type-2 responses. Numbers of T-lymphocytes in blood (irrespective of type) are reduced for about two weeks before and after parturition, with nadir at parturition (Kehrli et al., 2006, Kimura et al., 2002, Shafer-Weaver et al., 1999, Shafer-Weaver and Sordillo, 1997). Similarly, the percentage of T-lymphocytes in milk during the postpartum period is a quarter of the percentage present in mid-lactation (Park et al., 1992). The ratio of CD4:CD8 is lower in blood and milk and a higher proportion of the CD8+ T-lymphocytes are activated and of the suppressor rather than cytotoxic phenotype (Kehrli et al., 2006, Shafer-Weaver and Sordillo, 1997). The percentage of γδ T-lymphocytes decreases postpartum, although the magnitude of the decrease is not always significant (Kimura et al., 2002, Shafer-Weaver et al., 1999), so it is likely that the differences in cytokine expression of peripartum T-lymphocytes is the influence of suppressive CD8+ T-lymphocytes rather than γδ T-lymphocytes (Shafer-Weaver et al., 1999). Suppressor CD8+ T-lymphocytes secrete IL-4, which upregulates the expression of type-2 cytokines and downregulates the expression of type-1 cytokines by CD4+ T-lymphocytes (Brown et al., 1998, Shafer-Weaver et al., 1999, Shafer-Weaver and Sordillo, 1997). Consequently, the cytokine expression profile of CD4+ and CD8+ T-lymphocytes is type-2 biased, secreting less IL-2 and IFN-γ and more IL-4 and IL-10 during the periparturient period as compared with mid lactation (Shafer-Weaver et al., 1999, Shafer-Weaver and Sordillo, 1997). Interferon-γ is the central cytokine in development of CMIR (Young and Hardy, 1995); therefore, periparturient T-lymphocytes are less able to mount strong cell-mediated responses when required.

3.5.2 B lymphocytes

During the peripartum period, B-lymphocyte responses are suppressed, despite a supportive type-2 cytokine milieu secreted by T-lymphocytes. The capacity for antibody-mediated immune responses decreases for a short period around parturition but returns to normal within the first two weeks of lactation. The percentage of B-lymphocytes in blood tends to decline at calving (Kimura et al., 2002, Mallard et al., 1998, Nagahata et al., 1992, Shafer-Weaver et al., 1999), whereas the population of B-lymphocytes in milk is increased (Park et al., 1992). In vitro B-lymphocyte proliferation and antibody secretion decline several weeks pre-calving and are lowest immediately after calving, then recover within the first two weeks of lactation (Detilleux et al., 1995, Ishikawa, 1987, Nagahata et al., 1992, Nonnecke et al., 2003, Sasaki et al., 1976, Stabel et al., 1991). In the two weeks pre-partum, IgG1 is selectively transported from the blood circulation and is present in colostrum at a much higher ratio of IgG1:IgG2 than that in blood (Sasaki et al., 1976). Unlike IgG1, serum IgG2 concentration increases in the week before parturition (Detilleux et al. (1995) and remains elevated during early lactation (Mallard et al., 1983). It is well documented that IgG1 is selectively transferred to colostrum and that IgG2 only enters the mammary gland in significant quantities during infection (Mallard et al., 1983, Sasaki et al., 1976). Overall, the changes observed in the B-lymphocyte population reflect a generalised suppression and a type-2 shift in immune response bias.

3.6 Infectious disease

Due to periparturient immune dysfunction, the incidence of clinical infectious disease is highest during early lactation. The periparturient udder is particularly susceptible to new infections and many subclinical intramammary infections progress to clinical mastitis during early lactation (Goff and Horst, 1997, Kehrli et al., 2006, Oliver and Sordillo, 1988, Smith et al., 1985). At least 25-50% of clinical mastitis occurs in the first month after calving (Loker et al., 2012, McDougall et al., 2007, Miltenburg et al., 1996) and, the severity of new intramammary infections is greater in the periparturient period than at other stages of lactation. For example, *Escherichia coliform* infections are less likely to self-cure without antibiotic during the periparturient period (Hill, 1981, Shuster et al., 1996, Vangroenweghe et al., 2005). Additionally, many other infectious diseases that the immune system had previously been able to control become clinically apparent during the first two weeks of lactation (Kehrli et al., 2006). For example, clinical manifestation of Johne’s (*Mycobacterium paratuberculosis)* usually occurs during the periparturient period (Stabel and Goff, 2004, Stabel et al., 2003) and immunity to gastrointestinal nematodes is also suppressed (Barger and Gibbs, 1981, Houdijk, 2008, Kloosterman et al., 1985). The increased incidence and severity of infection indicates a reduced ability to resist new infection and to continue to contain latent subclinical infections during the periparturient period.

3.6.1 Infectious disease is associated with immune dysfunction

Although periparturient immune dysfunction has been reported in all dairy systems (Detilleux et al., 1995), not all cows exhibit periparturient immune dysfunction. Mallard et al. (1997) and Wagter et al. (2000) reported that one third of cows did not exhibit depressed AMIR postpartum; these cows had lower incidence of disease, especially mastitis, higher response to J5 *E. coli* vaccination, and lower somatic cell count, than cows with depressed AMIR (Mallard et al., 1997, Wagter et al., 2000), indicating that AMIR may contribute to defence of the mammary gland.

The adaptive CMIR is also critical to periparturient health. A competent type-1 response is required to control Johne’s, *Theileria* spp and the small colony variant form of *Staphylococcus aureus* (Atalla et al., 2010, Grönlund et al., 2006, Katzer et al., 2007, McKeever et al., 1994, Plattner and Hostetter, 2011). For example, progression from subclinical to clinical *M. paratuberculosis* is associated with a switch from inflammatory and cytotoxic type-1 response to a type-2 response (Coussens et al., 2012, Plattner and Hostetter, 2011). The bias towards type-2 responses post-calving may contribute to increased clinical manifestation of diseases best controlled by type-1 responses in early lactation.

3.7 Summary of immune dysfunction in the transition period

During the periparturient period, both innate and adaptive immune defences become dysfunctional, resulting in inefficient pathogen clearance. The overriding feature of bovine peripartum immunity is suppression of inflammatory responses and bias of T-lymphocytes toward a type-2 cytokine profile immediately after calving (Kehrli et al., 2006, Shafer-Weaver et al., 1999). The type-2 bias may prevent unwanted immune responses toward self- and fetal-antigens (Kehrli Jr and Harp, 2001) and support production of maternal antibody for transport into colostrum (Shafer-Weaver et al., 1999). Type-2 bias may also limit the impact of excessive pro-inflammatory cytokine secretion by monocytes and macrophages postpartum (Shafer-Weaver et al., 1999). However, in the absence of enhanced by type-1 cytokine production, the innate immune system operates at a reduced capacity, leading to increased incidence and severity of clinical infections.

Chapter 4. Body Condition Score

4.1 Assessment of energy reserves.

4.1.1 Anatomy considered in body condition score assessment

Body condition score (BCS) is a subjective assessment of an animal's energy reserves, based on a visual and/or tactile assessment of subcutaneous fat deposits (Bewley and Schutz, 2008, Roche et al., 2009). The anatomy considered most important include the thoracic and vertebral region of the spinal column (chine, loin and rump), the ribs, the spinous processes (loin), the tuber sacrale (hip or hook bones), the tuber ischii (pin bones), the anterior coccygeal vertebrae (tail head) and the thigh region (Figure 12., Earle, 1976, Macdonald and Roche, 2004, Wildman et al., 1982).

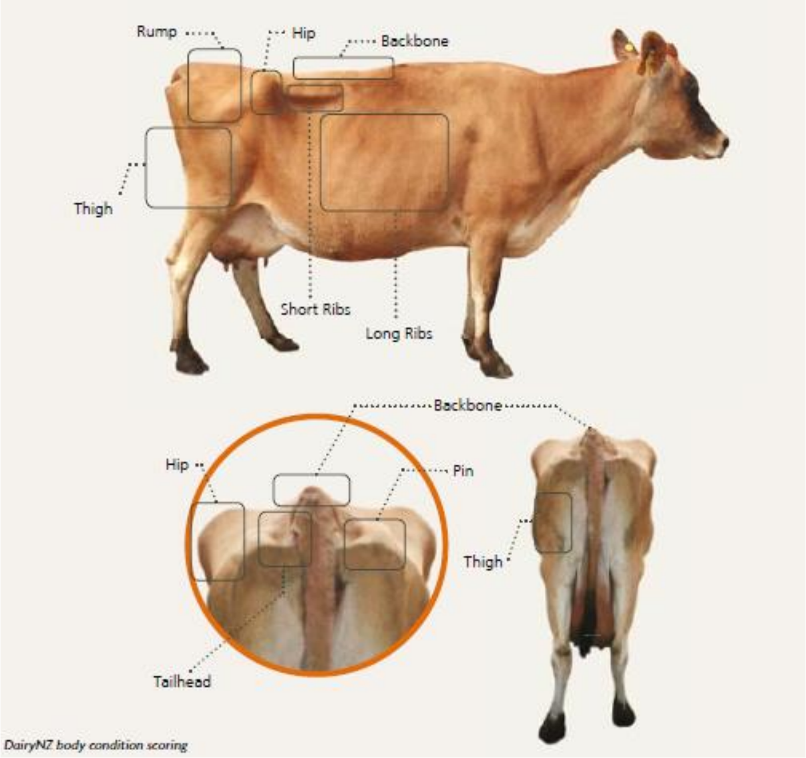


Figure 12. The eight points of anatomy considered when assessing body condition score using the New Zealand system (source: DairyNZ body condition scoring: The reference guide for New Zealand dairy farmers, 2012). This cow has a BCS of 4.0 on a scale of 1 to 10.

4.1.2 International Body condition score systems

Multiple BCS systems have evolved around the world; however, common to all scales is that low values represent emaciation and high values represent obesity (Bewley and Schutz, 2008, Roche et al., 2009). The systems' vary in their reliance on visual versus tactile assessment, and there are subtle differences in the emphasis placed on the different body parts considered during assessment (Bewley and Schutz, 2008, Roche et al., 2009) . Assessments can be converted between scales, allowing comparison of international results (Bewley et al., 2010, Roche et al., 2004). All BCS in this thesis will be presented using the New Zealand scale of 1 to 10 (Macdonald and Roche, 2011), on which, scores below 3.0 reflect emaciation and those above 6.0 are considered obese. Body condition scores originally reported using different BCS scales are converted to the New Zealand scale using the equations of Roche et al. (2004).

4.1.3 Accuracy of body condition score systems

The systems of BCS were developed because of recognition that changes in body weight (BWT) didn't adequately reflect changes in stores of fat and protein (Bewley and Schutz, 2008, Roche et al., 2009). Cows of a specific BWT may be tall and thin or short and fat, and within cow, BWT is highly variable due to gut fill and changing pregnancy status (Bewley and Schutz, 2008). Andrew et al. (1994) and Gibb et al. (1992) reported that energy stores varied by as much as 40% in cows of similar BW, highlighting the futility and inaccuracy of relying on BWT alone as an index of cow condition.

In comparison, dissection studies have reported that BCS is correlated with total body fat (Waltner et al., 1994, Wright and Russel, 1984), internal fat and dissected fat (Gregory et al., 1998, Otto et al., 1991), ultrasound subcutaneous fat thickness (Bewley and Schutz, 2008, Domecq et al., 1995, Hussein et al., 2013), and the muscle to bone ratio (Gregory et al., 1998); therefore, BCS is regarded as a valid measure/estimate of total energy reserves (Bewley and Schutz, 2008, Roche et al., 2009). Although the subjective nature of BCS remains a challenge (Roche et al., 2009), there is high repeatability and inter-observer agreement when assessors have been trained. However, accuracy of scoring is lower in cows with BCS below 3.0 or above 7.0, or when assessors have limited experience (Ferguson et al., 1994, Roche et al., 2009). Body condition score is widely accepted as the primary measure of energy reserves in the dairy industry, upon which recommendations and management decisions are based (Alawneh et al., 2012, Buckley et al., 2003, Oltenacu and Broom, 2010, Roche et al., 2009).

4.2 Inter-lactation profile of Body Condition changes

All mammals are able to lose condition to support milk production in early lactation (Dall and Boyd, 2004). Elasticity of body energy reserves is an important feature of the mammalian reproductive strategy, because it buffers the offspring against unreliable food supplies (Dall and Boyd, 2004, Friggens et al., 2013, Roche et al., 2009). However, the dairy cow is extreme in this regard, losing large amounts of BCS for 50-100 days post-calving and only re-gaining BCS slowly, if at all, in mid-late lactation (Figure 13; Roche et al., 2007a). Genetically driven changes increase lipolysis and partitioning of nutrients to the mammary gland, at the onset of lactation (Chilliard et al., 2000, Friggens et al., 2013, Lucy, 2003). As lactation progresses, the partitioning of nutrients eventually swings back towards increasing body reserves and pregnancy, rather than millk production (Friggens et al., 2013, Yan et al., 2006). The inter-lactation profile of BCS change is affected by three things primarily: genetics, nutrition, and BCS at calving.

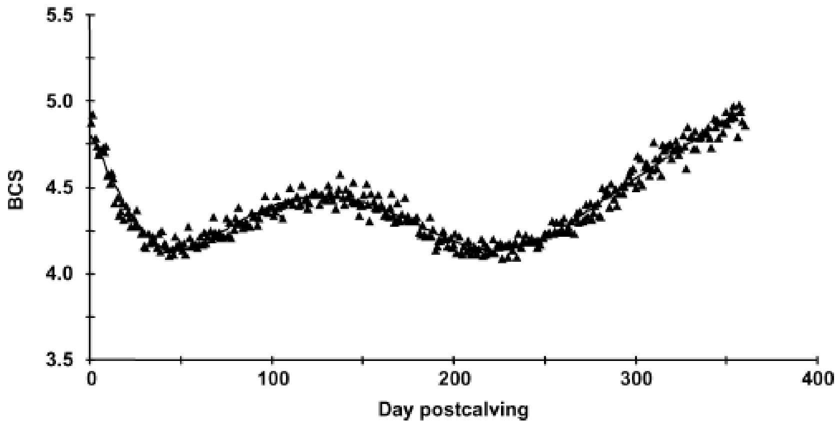


Figure 13. The inter-calving BCS profile of grazed dairy cows (source: Roche et al., 2007a).

4.2.1 Genetic effects on the inter-calving BCS profile

Intense selection for increased milk production has exaggerated the homeorhetic response to lactation, increasing the partitioning of nutrients from body reserves toward milk (Friggens et al., 2013) and increasing the degree and duration of negative energy balance post-partum (Oltenacu and Broom, 2010). Breeding has increased early lactation milk production but this has not been accompanied by a comparable increase in dry matter intake (Goff, 2006, Roche et al., 2009, Van Arendonk et al., 1991) with peak dry matter intake lagging behind peak milk yield by several weeks (Berry et al., 2006, Oltenacu and Broom, 2010, Roche et al., 2009). Dairy strains within breed differ in their propensity to lose BCS, depending on the intensity of selection for high milk production (Friggens et al., 2013, Lucy et al., 2009, Macdonald et al., 2008, McCarthy et al., 2007, Piccand et al., 2013, Pyman and Macmillan, 2010, Roche et al., 2006, Yan et al., 2006). For example, North American Holstein-Friesian cows partition more energy to lactation and lose more BCS in early lactation, and additional energy intake does little to ameliorate BCS loss, compared with New Zealand Holstein-Friesian cows in the same herd (Lucy et al., 2009, Macdonald et al., 2008). Genetic drive for production is such that offering a more energy dense diet in early lactation often results in higher milk production without improvement in post-partum energy balance (Roche et al., 2006, Roche et al., 2009).

4.2.2 Nutrition and system effects on inter-calving BCS profile

In all systems, cows lose BCS in early lactation, before ideally returning to the desired BCS before the next calving (Berry et al., 2006, Roche et al., 2007a, Roche et al., 2009). In systems where cows are fed large amounts of energy dense supplements, cows lose less BCS postpartum and the rate of BCS gain post-nadir is greater than in grazing cows receiving little supplementation (Figure 14; Roche et al., 2007a, Roche et al., 2009). In fact, cows in such systems may require feed restriction to ensure they are not over-conditioned at calving (Wildman et al., 1982). In contrast, the BCS of cows in pasture-based systems in NZ tends to plateau and even decline for a period during summer, when pasture is of lower quality (Roche et al., 2007a). As a result, dairy cows end lactation thinner than they would in systems offering large amounts of concentrate feeds (Roche et al., 2007a) and either calve at less than optimal BCS or require extra feeding to regain an ideal BCS prior to calving. Although the BCS toward the end of lactation and into the dry period differs depending on the feed system, the loss of BCS in early lactation is common to dairy cows in all systems.

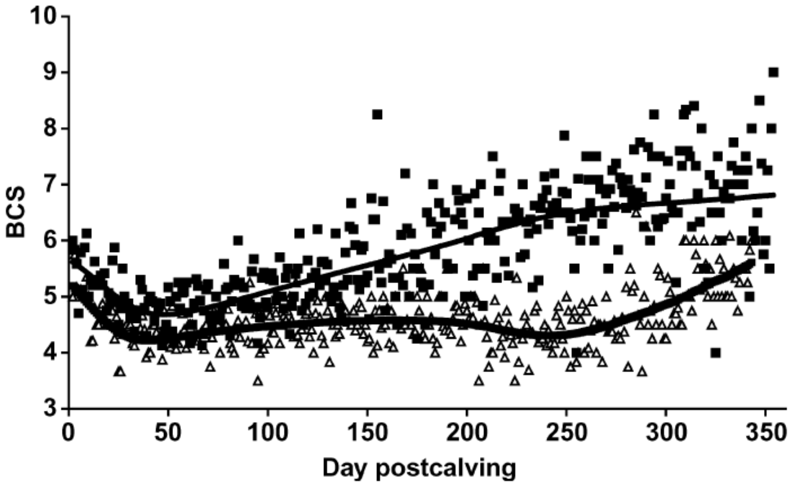


Figure 14. The inter-calving BCS profile of grazing cows (∆, heavy line) is different to the inter-calving BCS profile of TMR-fed cows (■, light line), although cows lose BCS for the first 50 days of lactation in both systems. Body condition score was assessed on the New Zealand 1 to 10 scale (source: Roche et al., 2007a).

4.2.3 Calving BCS and the inter-calving BCS profile.

There are moderate correlations between BCS at different stages of lactation (Berry et al., 2007a, Roche et al., 2007c). Cows that have low BCS at calving will usually have lowest BCS in the herd throughout lactation, and high BCS cows will usually be highest BCS throughout lactation (Berry et al., 2007a, Roche et al., 2007c). As illustrated in Figure 15, there is a positive relationship between calving BCS and rate of BCS loss postpartum, such that cows that calve at high BCS lose more condition and experience a more severe negative energy balance (NEB) than cows that are low BCS at calving (Roche et al., 2007a, Ruegg and Milton, 1995). Together these results suggest that body condition score at nadir and mid-lactation, and BCS loss postpartum are largely determined by BCS at calving, because feeding and management have little influence over BCS change (Roche et al., 2009).

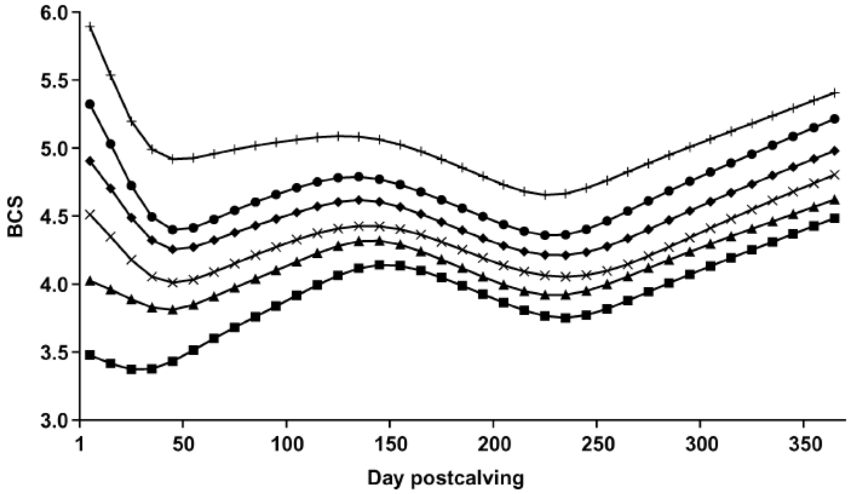


Figure 15. Inter-calving BCS profiles for cows calving at a BCS of ≤3.5 (■), 4.0 (▲), 4.5 (X), 5.0 (♦), 5.5 (●), or ≥6.0 (+). Body condition score was assessed on the New Zealand 1 to 10 scale (source: Roche et al., 2007a).

4.3 Associations between BCS and milk production and reproductive performance

There is general agreement in the literature that BCS at calving and loss of BCS, or NEB, during early lactation have a significant effect on milk production and reproduction (Bewley and Schutz, 2008, Roche et al., 2009). However, because BCS at calving has a strong influence on the energy balance of a cow in early lactation and because early lactation NEB is not substantially affected by feed or management in an otherwise healthy cow during very early lactation, BCS at calving is the most important BCS target on the lactation calendar (Roche et al., 2009).

4.3.1 Milk production

Higher BCS at calving and greater BCS loss postpartum are associated with higher lactation milk yield (Bewley and Schutz, 2008, Roche et al., 2009). The relationship between calving BCS and milk yield is curvilinear; milk yield increases with BCS but the increase in milk relative to BCS diminishes up to BCS 6.5, beyond which, milk yield declines (Berry et al., 2007a, Roche et al., 2007b). There is a similar non-linear relationship between BCS lost postpartum and milk yield in the subsequent lactation, with increasing milk yield up to 1.5 BCS units loss and decreased yield when BCS loss exceeds this value (Berry et al., 2007a, Roche et al., 2007b).

4.3.2 Reproduction

Reproductive performance generally improves with increasing calving BCS (Bewley and Schutz, 2008, López-Gatius et al., 2003, Roche et al., 2009). Higher BCS at nadir and smaller BCS loss between calving and nadir are associated with shorter postpartum anoestrus intervals and increased pregnancy rates (Beam and Butler, 1999, Markusfeld et al., 1997, Roche et al., 2009, Roche et al., 2007c). Greater BCS at calving contributes to better reproductive performance by association with nadir BCS; however, as with milk production, excessive postpartum BCS loss in cows with very high calving BCS may diminish the positive effect of high BCS (Roche et al., 2009) .

4.4 Calving BCS and health

Associations between BCS and various measures of health have been studied extensively and are less clear than relationships between BCS and milk production or reproduction (Roche et al., 2009). Low BCS is associated with greater morbidity (Gearhart et al., 1990, Hoedemaker et al., 2009); however, this relationship may reflect the effect of disease on BCS rather than the effect of BCS on disease incidence. A positive relationship between calving BCS and metabolic health is regularly observed but the relationship between calving BCS and resistance to infectious disease is ambiguous. Some studies have reported relationships between BCS or energy balance and immune function. Leptin, oxidative stress, and NEB are reported to modify the type-1/type-2 bias of immune function; however, these factors have primarily been studied in vitro, and independently of each other, and therefore it is unclear how such factors translate to disease resistance in the cow during the transition period.

4.4.1 Metabolic disease

Excessive loss of BCS combined with very high milk production, often occurring in cows with higher calving BCS, contributes to the development of several metabolic disorders, including ketosis, fatty liver, milk fever and displaced abomasum (Bewley and Schutz, 2008, Goff and Horst, 1997, Roche et al., 2009, Rukkwamsuk et al., 1999).

Ketosis and fatty liver: Most studies report increased risk of ketosis and fatty liver in cows with high BCS at calving (Bewley and Schutz, 2008, Roche et al., 2009). High rates of gluconeogenesis, such as occurs in early lactation, deplete hepatic oxaloacetate; consequently, oxaloacetate is limiting for oxidisation of NEFA and a greater proportion of NEFA are used for ketogenesis or accumulated as triglycerides in hepatocytes (Goff and Horst, 1997, Roche et al., 2009, Rukkwamsuk et al., 1999). Excessive accumulation of triglycerides (fatty liver) decreases gluconeogenic capacity, which exacerbates the NEB and induces further lipid mobilisation (Rukkwamsuk et al., 1999, Wathes et al., 2009). Depressed gluconeogenesis causes ketosis, which is characterised by lethargy, reduced dry matter intake (DMI) and decreased milk production. Ketosis is diagnosed by elevated ketone levels in blood and urine (Goff and Horst, 1997). Cows that are greater than BCS 6.0 at calving are twice as likely to develop ketosis when compared to cows with a BCS of 5.5 at calving (Gillund et al., 2001).

Milk fever: Risk of hypocalcaemia, or milk fever, is increased in cows with high BCS at calving due to their high milk production and greater NEB (Roche and Berry, 2006). Milk fever occurs when secretion of milk calcium far exceeds dietary intake of calcium and plasma calcium concentrations are inadequate for muscle and nerve function (Goff and Horst, 1997, Roche et al., 2009). Cows that calve below BCS 3.5 also have a higher risk of developing milk fever than moderately conditioned cows, although the reason for this in unclear (Roche and Berry, 2006, Roche et al., 2009).

Generally, the risk of metabolic disorders is higher in cows that calve at higher BCS because they produce more milk from nutrients released from body stores rather than from DMI.

4.4.2 Incidence of infectious disease.

The relationship between BCS and the incidence of infectious disease is inconsistent. Generally disease incidence is too low to accurately identify differences in susceptibility between different BCS groups, with the exception of intramammary and uterine infections. Most studies have reported no relationship between calving BCS and incidence of clinical mastitis (Breen et al., 2009, Gearhart et al., 1990, Heuer et al., 1999), whereas, Berry et al. (2007b) reported a weak positive relationship between calving BCS and incidence of intramammary infection in mature cows, and a negative relationship in young cows. Similarly, the relationship between BCS and uterine infection is unclear. High rates of fat mobilisation before or after calving are reported to increase the incidence of metritis (Bewley and Schutz, 2008, Kaneene et al., 1997, Moyes et al., 2009a, Ospina et al., 2010, Roche et al., 2009). However, low BCS at calving is usually associated with greater incidence of uterine infection or inflammation, especially in young cows (Bewley and Schutz, 2008, Hoedemaker et al., 2009, Markusfeld et al., 1997, Roche et al., 2009), although Huzzey et al. (2009) reported no association between pre-partum BCS and metritis. The tendency for low BCS to be associated with a greater risk of mammary and uterine infections in young cows, but not in older cows, indicates that NEB, which is more severe in older cows, may influence the relationship between BCS and immune function.

Much of the research examining these relationships is drawn from epidemiological studies, and as such it is difficult to determine whether the relationship is causative or associative. For example, the low BCS of a cow that presents with a uterine infection post-calving may be a consequence of the infection, rather than the cause of increased risk of infection (Roche et al., 2009). Some associations between BCS and incidence of infectious disease may be mediated by the effects of metabolic disease rather than by altered immune function. For example, cows with high calving BCS are at greater risk of milk fever and the consequent reduction in muscle function may reduce effectiveness of the teat sphincter and impair the expulsion of placenta, increasing the risk of mastitis and metritis, respectively (Goff and Horst, 1997).

Another challenge to determining the nature of relationships as identified from epidemiological studies is that many studies do not record BCS at calving, or before calving, so the ‘calving’ BCS may in fact refer to BCS measured several days to several weeks after calving (e.g. Heuer et al., 1999, Ruegg and Milton, 1995). Approximately 1/3 of the total BCS that a cow loses during early lactation is lost in the first 10 days post-partum (Roche et al., 2007a), and this BCS loss is exacerbated by infection (Ruegg and Milton, 1995). Therefore, any BCS recorded more than 1-2 days post calving cannot be accurately used to determine the relationship between calving BCS and incidence of disease.

Equally, many reports are focussed on the relationship between over-conditioning at calving and subsequent health, rather than on under-conditioning. In the New Zealand dairy industry and in grazed dairy systems generally, low BCS at calving is more common than high BCS at calving, and average BCS tends to be lower throughout lactation, than in total mixed ration (TMR) systems (Roche et al., 2007a, Washburn et al., 2002).

4.4.3 Recommended body condition scores are based on relationships between BCS, milk production, fertility and metabolic disease.

The relationships between BCS and milk production, reproductive performance and risk of metabolic disease, have led to the development of BCS targets that aim to optimise milk yield, fertility and health. For the New Zealand dairy industry, the recommendation is for mature cows to calve at 5.0, and young cows to calve at 5.5 BCS. Although calving BCS is positively associated with increased milk yield, the association between higher calving BCS and greater risk of metabolic disease means that there is little benefit in moving calving BCS of mature cows from 5.5 to 6.5.

Young cows have lower milk yield and don’t experience such severe NEB postpartum; therefore they are less susceptible to metabolic disease. Furthermore, young cows tend to be less dominant in the herd and so are less likely to regain adequate BCS before mating and before subsequent calving (Bewley and Schutz, 2008, Ruegg and Milton, 1995). In addition, the interaction between calving BCS, parity and animal health measures suggest additional BCS at calving in younger cows is beneficial. Additional recommendations are to avoid cows losing more than 1.0 BCS after calving and for cows to be BCS 4.0 or greater by the start of mating. However, as described, famers have little influence over postpartum BCS loss; therefore, the most important target is calving BCS.

It is apparent from the relationship between reproduction and BCS that when BCS or energy status is low, non-essential functions are compromised in favour of milk production in early lactation. Yet there is limited research examining the relationship between low BCS at calving and immune function, and the recommendations for farmers do not incorporate immune competence, apart from considering the effects of BCS on the incidence of mammary and uterine infections. The incidence of other diseases may be relatively low but collectively their impact on productivity and welfare is substantial and warrants direct investigation of the relationship between BCS at various stages of the lactation cycle and immune function.

Chapter 5. Interface between BCS and immune function

5.1 Functional trade-offs

An animal that has large energy reserves can normally afford a more competent immune system (Houston et al., 2007, Lochmiller and Deerenberg, 2000). However, when energy intake is insufficient, an animal must divert nutrients from non-essential functions, such as growth or immunity, to those functions essential to survival, such as brain function (Demas, 2004, Houston et al., 2007, Lochmiller and Deerenberg, 2000). Once significant investment has been made in reproduction, the survival of offspring generally takes priority over immune function (French et al., 2007). This is reflected in the capacity for mammals to mobilise adipose and muscle tissue to support pregnancy and lactation (Dall and Boyd, 2004, Roche et al., 2009). During lactation, the ability of the mammary gland to take up glucose independent of insulin means that milk production is afforded higher priority for nutrient resources than reproductive tissues.

Low body fat reserves are associated with altered immunity in many species (Demas, 2004, French et al., 2011). When energy reserves/supply are limited, type-1 responses are inhibited and type-2 responses are favoured, most likely because a type-1 cellular response consumes more energy and protein than a type-2 antibody response, although both may be impaired by nutritional deficiency (Demas, 2004, Fekete and Kellems, 2007, Gilot-Fromont et al., 2012, Lochmiller and Deerenberg, 2000). For example, wild deer living in a low resource environment and, therefore were in poor body condition, had less circulating neutrophils and lower serum APP, higher circulating antibodies, higher circulating lymphocytes and monocytes, and lower CD4+/CD8+ and IFN-γ:IL-4 ratios, than deer living in a high resource environment (Gilot-Fromont et al., 2012). Similarly, low BCS cows (BCS < 3.75) in mid-lactation have reduced numbers of T cells and reduced cytokine expression, particularly IFN-γ, resulting in a lower IFN-γ:IL-4 ratio when compared to higher BCS cows (Ohtsuka et al., 2009). Cows of moderate body condition (BCS 4.0-5.0) in mid-lactation have lower circulating TNF-α and lower TNF-α production in response to lipopolysaccharide than cows with BCS greater than 6.5 (O'Boyle et al., 2006). These results support the concept of a type-2 biased immune system in cows with lower BCS.

5.2 Leptin

The bias toward type-1 or type-2 immune responses, in relation to degree of adiposity, is possibly mediated by leptin, a hormone secreted primarily by adipocytes and which increases in direct proportion to adiposity (Borghetti et al., 2009, French et al., 2011, Lago et al., 2008, Meyers et al., 2005). The concentration of leptin signals energy status, facilitating functional trade-offs during periods of limited resource, for example, suppressing immune responsiveness to conserve energy for vital organs (Abrams and Miller, 2011, Block et al., 2001, French et al., 2007, French et al., 2011, Ingvartsen and Boisclair, 2001, Matarese and La Cava, 2004). Most immune cells possess leptin receptors, including macrophages and T- and B-lymphocytes (Fenandez-Riejos10). Administration of leptin to rodents with limited energy availability restores adaptive immune responses (Demas, 2004, Lord et al., 1998), indicating that suppression of immune function is facultative rather than obligatory (French et al., 2007). Leptin suppresses expression of type-2 cytokines and, therefore, decreases antibody responsiveness (Lago et al., 2008, Meyers et al., 2005). Leptin also induces ROS secretion from neutrophils and expression of inflammatory cytokines by macrophages and dendritic cells (Borghetti et al., 2009, Lago et al., 2008, Meyers et al., 2005). Naïve T-lymphocytes that are activated in the resulting inflammatory cytokine milieu are polarised toward a type-1 cytokine expression pattern (Lago et al., 2008, Meyers et al., 2005). The sum effect of high leptin levels is to support a type-1 immune response bias in animals with ample energy reserves and to inhibit type-1 in favour of type-2 responses in animals of limited energy reserves (Borghetti et al., 2009, Lago et al., 2008, Meyers et al., 2005).

5.3 Oxidative stress

Oxidative stress and the consequent inflammatory state generated may contribute to greater immune dysfunction in high BCS cows. Immune cells are sensitive to oxidative stress because the lipids in their cell membranes are vulnerable to peroxidation and because immune cells produce high quantities of ROS (Esposito et al., 2014, Spears and Weiss, 2008). Elevated leptin levels increase neutrophil production of ROS (Lago et al., 2008), and postpartum, the metabolic demand of high milk production and rapid lipolysis accelerate ROS production in all tissues (Esposito et al., 2014, Morris et al., 2009) Additionally, elevated NEFA concentrations change the activity of serum albumin from antioxidant to pro-inflammatory (Contreras and Sordillo, 2011). Consequently, oxidative stress occurs because antioxidant pools are depleted by excessive ROS production. Cows with higher BCS pre-calving and greater BCS loss post-partum have lower antioxidant potential and higher levels of reactive oxygen metabolites and markers of peroxidative damage to cell membranes, indicative of oxidative stress (Bernabucci et al., 2005). Oxidative stress and, consequently, systemic inflammation increase with adiposity (O'Boyle et al., 2006), and would possibly exacerbate the type-1 bias observed in high BCS cows.

5.4 Negative energy balance

High BCS promotes type-1 immune bias through the effects of leptin and oxidative stress; however, the relationship between BCS and immune function is complicated by NEB during the post-partum period. The onset of lactation and NEB causes a rapid decrease in serum leptin, with the decline greatest in cows with higher calving BCS (Block et al., 2001, Kokkonen et al., 2005, Meikle et al., 2004). During the postpartum period, serum leptin levels in high BCS cows are almost as low as those observed in low BCS cows (Kokkonen et al., 2005). Since leptin levels are similar between high and low BCS cows during the post-partum period, both high and low BCS cows might have inhibited type-1 responses in favour of type-2 responses.

Additionally, as a consequence of NEB, postpartum serum NEFA and ketone body concentrations are usually higher in cows with high BCS. Higher concentrations of serum NEFA and ketone bodies are associated with a higher incidence of retained placenta, metritis, and mastitis (Holtenius et al., 2004, Kaneene et al., 1997, Moyes et al., 2009a, Ospina et al., 2010). Neutrophils incubated with NEFA and/or ketones, or isolated from ketotic cows, have reduced ROS production and NET formation, impaired phagocytosis and chemotaxis, and reduced viability (Grinberg et al., 2008, Scalia et al., 2006), although intracellular killing is not consistently affected (Grinberg et al., 2008, Suriyasathaporn et al., 2000). Higher concentration of serum NEFA, but not ketones, has been associated with decreased levels of circulating natural antibodies (van Knegsel et al., 2012). Incubation or exposure to elevated NEFA concentrations is reported to impair several lymphocyte functions in vitro, including proliferation (Lacetera et al., 2004, Ster et al., 2012), antibody production (Lacetera et al., 2004), and production of pro-inflammatory cytokines (Lacetera et al., 2004, Ster et al., 2012). Franklin et al. (1991) and Sato et al. (1995) reported impaired lymphocyte proliferation in the presence of elevated ketone concentrations; whereas, Ster et al. (2012) found no effect of ketone concentration on in vitro lymphocyte proliferation, and Ster et al. (2012) and Nonnecke et al. (1992) reported no effect on in vitro production of inflammatory cytokines. In any case, both NEFA and ketone body concentrations are more elevated postpartum in cows that calve with higher BCS; therefore, cows that calve in high BCS probably have more severe impairment of lymphocyte function postpartum than cows with lower calving BCS.

5.5 In vivo evidence for the relationship between BCS and immune function at calving

It is well documented that greater NEB contributes to immune dysfunction in dairy cows during the transition period. However, it is not entirely clear how the interplay between calving BCS and NEB affects immune response bias, particularly in low BCS cows. Most research has been directed at high BCS cows, rather than low BCS, because excessive BCS at calving is more prevalent in many dairy systems. However, in systems that rely heavily on seasonal grass growth, such as in NZ, low to moderate BCS at calving is typical (Roche et al., 2007a). Additionally, the energy allowance offered to dairy cows before calving is lower in NZ, than other countries; limited pasture availability means that cows in systems reliant on seasonal grass growth are usually partially underfed in the last few weeks pre-calving (Holmes et al., 2002, Stafford and Gregory, 2008). The level of energy intake pre-calving is reported to affect adaptation to lactation, with reduced postpartum NEB, fewer metabolic diseases, reduced systemic inflammation, lower oxidative stress, and better neutrophil function in cows that were fed a lower energy diet pre-partum (Graugnard et al., 2012). Consequently, the relationships between calving BCS and immune function reported previously may not translate to the NZ system.

There has been limited in vivo research investigating the relationship between BCS and immune function; however, what research has been done indicates that cows that calve in high BCS exhibit greater impairment of immune function post-partum, than in lower BCS cows. Lacetera et al (2005) reported that the degree of impairment of both type-1 and type-2 lymphocyte functions was proportional to BCS loss, being greatest in cows with higher BCS at calving. Lymphocytes isolated from cows with BCS below 4.0 pre-calving had higher IFN-γ responses, before and after calving, and higher IgM responses after calving, than lymphocytes collected from cows of BCS > 6.5 and BCS 4.5-6.0. Ohtsuka et al. (2008) reported that cows which lost the most body weight postpartum, and presumably had higher calving BCS, had a type-2 biased immune cell population, with lower CD4:CD8 and IFN- γ:IL-4 ratios. These results contradict what is observed outside the periparturient period and indicate that the NEB may counteract the type-1 immune response bias associated with high BCS cows during other stages of the inter-calving profile. Cows with the lowest energy balance postpartum likely have the lowest leptin levels, because leptin indicates energy status in terms of both reserves and demand on reserves, and this may contribute to greater type-2 bias in cows experiencing greater NEB postpartum.

5.6 Summary and hypotheses

Management of cows during the transition between pregnant and lactating states is one of the key determinants of cow welfare and the profitability of a dairy enterprise (Drackley, 1999, Mulligan and Doherty, 2008). Despite this, there is still much that isn’t understood about the transition period. In particular, the role of calving BCS on the post-partum health of dairy cows is poorly understood. The New Zealand dairy industry has established a target calving BCS of 5.0 for mature cows and 5.5 for first and second calvers (DairyNZ body condition scoring: The reference guide for New Zealand dairy farmers, 2012). This target is optimal for production, fertility and metabolic health (Roche et al., 2009, Roche et al., 2013b); however, the relationship between calving BCS and infectious disease is not well understood. There is evidence from epidemiological and in vitro studies that the degree of periparturient immune dysfunction is influenced by the extent of the NEB in early lactation (Lacetera et al., 2005, Lacetera et al., 2004, Ohtsuka et al., 2008). Since farmers have limited ability to manipulate energy balance in early lactation (Roche et al., 2006, Roche et al., 2009), management of BCS at calving would be the most accessible way to manage periparturient immune dysfunction, if that link exists.

This study will investigate the effect of BCS at calving on AMIR and CMIR of dairy cows in early lactation. Antigen-specific antibody produced in response to immunisation with type-2 antigens will be used as the measure of AMIR and delayed-type hypersensitivity (DTH) against *Candida albicans* will be used as the measure of CMIR. These in vivo measures have been used extensively in Canada and Ireland; however, the CMIR measure relies on low background exposure to *C. albicans*. Therefore, the first objective is to investigate the level of background exposure to *C. albicans*, indicated by magnitude of DTH responses, in a pasture-based herd.

To address the main objective, the energy levels of healthy cows will be manipulated to achieve specific BCS at calving. Post-partum AMIR and CMIR will be compared with responses measured during the previous lactation, when all cows had similar BCS. In addition to measuring DTH and antigen-specific IgG, the ratio of antigen-specific IgG1:IgG2 in response to immunisation with type-2 antigen will be measured as an indicator of type-1/type-2 bias. The hypotheses for this study are:

1. Cows in a New Zealand pasture-based herd have small DTH responses relative to cows immunised with *C. albicans.*
2. The degree of immune suppression is positively correlated with BCS at calving.
3. Degree of type-2 bias is positively correlated with BCS at calving.

Chapter 6. Experiment One: Induction of delayed-type hypersensitivity reactions to *Candida albicans* in pasture-based dairy cows

6.1 Introduction

Selection for production traits with little or no emphasis on health traits has led to an increase in the incidence of disease in dairy herds (Oltenacu and Broom, 2010). Development of methods to assess immune response phenotypes in pasture-based NZ dairy cattle will allow selection pressure to be placed on health traits, and when used in combination with selection for important production traits, improve productivity, health & welfare in dairy herds. Cell-mediated immunity is crucial to protection against viruses and other intracellular parasites (Baldridge and Ward, 1997). Delayed-type hypersensitivity (DTH) reactions are localised cell-mediated immune responses (CMIR), mediated primarily by antigen-specific CD4+ T-lymphocytes which act to recruit macrophages to the reaction site. DTH reactions generally occur in response to intracellular pathogens (Baldridge and Ward, 1997, Kobayashi et al., 2001) and are marked by a hard swelling at the site 24-72 hours later post antigen exposure (Rosenstreich, 1993). A DTH response only occurs if the animal has been exposed to the antigen previously and has memory T-lymphocytes specific for the antigen (Rosenstreich, 1993).

Delayed-type hypersensitivity is an accepted in vivo measure of CMIR in many species, including humans, rodents and pigs (Hessing et al., 1995, Martin et al., 2008, Neuvonen and Salo, 1984). To assess CMIR in cattle, DTH responses to the intracellular yeast *Candida albicans* have been used (Heriazon et al., 2011). Importantly, the use of *C. albicans* does not interfere with the diagnosis of bovine infectious diseases, such as Tuberculosis (Heriazon et al., 2009a). *C. albicans* is a common commensal of many mammals and it has been reported that dairy cows produce a mild DTH response to *C. albicans* without priming suggesting previous natural exposure (Heriazon et al., 2009a). To minimise the effects of individual variability in background exposure most DTH testing protocols involve priming the test animals by immunising with *C. albicans* prior to DTH testing to generate an expanded pool of antigen-specific T-lymphocytes. Immunised animals respond more consistently and display larger responses than cows that have not been immunised (Heriazon et al., 2009a).

Measurement of DTH responses to *C. albicans* has been used successfully in Canadian and Irish dairy herds to assess CMIR (Begley et al., 2009b, Heriazon et al., 2009a, Mallard, 2007) but has not been investigated in NZ dairy herds. Cows assessed in both the Canadian and Irish dairy herds spend a large proportion of their time in housed environments. This is in contrast to NZ dairy herds which are predominantly pasture-based and not housed for extended periods of time. There has been limited research about the prevalence of *C. albicans* in dairy-farm environments because it is not an important cause of disease (Heriazon et al., 2009a, Richard et al., 1980); however, it is plausible that housed cows are exposed to different pathogens and have different commensal micro-flora to their pasture-based counterparts. For example, cows that are permanently housed have a higher prevalence of digital dermatitis than cows with access to pasture (Holzhauer et al., 2012, Laven and Holmes, 2008), and the prevalence of mastitis-causing pathogens is different in pasture-based versus housed cows (Laven and Holmes, 2008).

Differences in pathogen and commensal micro-flora exposure between farming systems may also extend to exposure to *C. albicans.* Yeast are isolated from a lower proportion of milk samples submitted for bacteriological culture in New Zealand (0.3%; Petrovski et al., 2011), than in other countries (1.3% to 25%; Casia dos Santos and Marin, 2005, Hayashi et al., 2013, Moretti et al., 1998), indicating that exposure to *C.albicans* might be lower in New Zealand herds. If natural exposure to *C. albicans* is not consistently low in pasture-based cows then background exposure would need to be determined prior to priming with antigen and subsequent DTH testing, or an alternative antigen used to elicit the DTH response. The primary objective of this experiment was to determine the magnitude of background DTH responses to *C. albicans* in New Zealand pasture-based dairy cows and ascertain whether *C. albicans* is a suitable antigen to elicit measurable DTH responses for assessment of immune responsiveness on farm.

6.2 Methods

The experiment was conducted at the DairyNZ Lye Farm, Hamilton (37°76'S 175°37'E, 45 m above sea level), during November and December 2010. All procedures were approved by the Ruakura Animal Ethics Committee, Hamilton, New Zealand, in accordance with the New Zealand Animal Welfare Act 1999.

6.2.1 Animals and Immunisation.

Twenty healthy, mid-lactation dairy cows of mixed age and breed were selected from the Lye Farm research herd (DairyNZ Ltd, Hamilton, New Zealand). The Lye Farm herd is milked twice per day and grazes a ryegrass and white clover pasture, with pasture silage and maize silage offered when pasture is limited. The cows were run together in their normal herd, and no supplementary feed was offered, for the duration of this experiment. Average milk production at the time of experiment was 22.3kg per cow per day. The cows were run together in their normal herd for the duration of the experiment. Cows were grouped on body condition score (BCS) and then cows within each BCS group were randomly assigned to one of two experimental groups; immunised (n=10), control (n=10). For the immunised group, the mean age was 4.1 ± 1.6 yr (mean ± SD) and mean BCS was 3.8 ± 0.4 (1-10 BCS scale, where 1 is emaciated and 10 obese; Roche et al., 2004). For the unimmunised group, the mean age was 4.6 ± 1.9 yr and the mean BCS was 3.8 ± 0.3.

The immune testing protocol involved immunisation on d0 and d14, followed by DTH testing on d21, as described by Heriazon et al. (2009b). Cows in the immunised group received an intramuscular (IM) injection of 0.5 mL of phosphate-buffered saline (PBS, pH7.4) containing 0.5 mg of type-1 antigen *C. albicans* (CaWC, My15 crude whole cell; Greer Laboratories Inc, Lenoir, NC, USA) and 0.5 mg of adjuvant (QuilA, 848051, Brenntag Biosector A/S, Frederikssund, Denmark) on D0 and D14. The injection also contained 0.5 mg of type-2 antigen; either hen egg-white lysozyme (HEWL) or human serum albumin (HSA). Immunisation with HEWL (n=5) or HSA (n=5) did not significantly influence DTH responses (P>0.05), therefore DTH responses from these cows were combined (immunised group, n=10) for analysis. Cows in the control group received an IM injection of 0.5mL PBS on day 0 (d0) and d14 (n=5), or received no injection (n=5). Immunisation with saline or receiving no immunisation did not significantly influence DTH responses (P>0.05), therefore DTH responses from these cows were combined (unimmunised group, n=10) for analysis.

6.2.2 Delayed-type Hypersensitivity.

The DTH skin test was performed on all cows on d 21, in the caudal skin folds on either side of the tail, as described previously (Heriazon et al., 2009a, Hine et al., 2011). Briefly, 0.1 ml PBS alone (control) or containing either 0.1mg of CaWC (test) were injected intradermally into the skin fold on opposite sides of the tail. The site of each injection was marked with correction fluid (twink) to allow subsequent measurements in the same site. Double skin fold thickness (DSFT) measures (3 repetitions), were taken at both test and control sites, prior to injection (0 h) and 7, 24 and 47 h post injection, using a spring loaded calliper (Harpenden, Mentone Educational Centre, Victoria, Australia). Photos in Figure 16 depict several stages of the DTH test.

Hine et al (2011) defined a positive DTH response to CaWC as an increase in DSFT of >1mm at the test site relative to the control site. A similar criterion was used to define positive DTH responses in the current study. The number of cows that exhibited positive responses was calculated for each experimental group. The responses of all cows were included in statistical analyses, regardless of whether they met the threshold for a positive response.

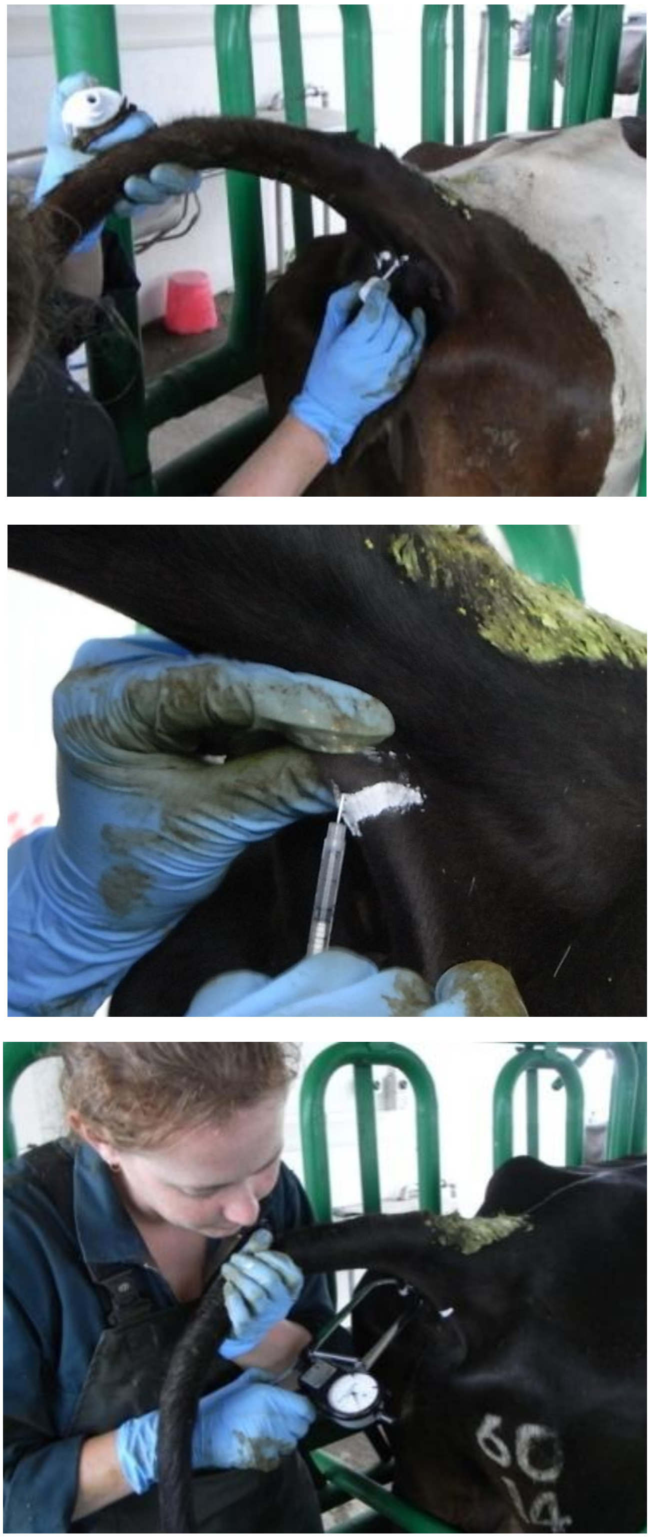


Figure 16. Measurement of delayed-type hypersensitivity. From top to bottom: 1) Twink lines are drawn on both caudal folds to facilitate consistent placement of spring loaded callipers. 2) An insulin needle was used to inject 0.1ml of test or control formula within the dermal layer of opposite caudal folds. 3) Spring loaded callipers were used to measure the thickness of the caudal fold immediately before the injection and at 7, 24 and 47 hours post-injection.

6.2.3 Statistical Analyses.

For analysis, responses to DTH testing were expressed as the change in log of (DSFT at test site/DSFT at control site) from 0 h, as described by Hine et al. (2011):

$$\text{Response} = \log (T_h/C_h) - \log (T_0/C_0)$$

Where T_h = mean test DSFT at time h; T_0 = mean test DSFT at 0 h; C_h = mean control DSFT at time h; C_0 = mean control DSFT at 0 h; and time h was 7, 24 or 48 h. This response variable was used for all statistical tests. Responses were analysed in SAS® software (version 9.3, SAS Institute, Cary, NC, USA), using mixed models approach to repeated measures with time, treatment, and their interaction included as fixed effects. Tukey's test was used for pairwise comparisons. F-tests were used to determine whether the variability of DTH responses differed between immunised cows and unimmunised cows, and between 24 and 47 h for immunised cows. Pearson product-moment correlation coefficients were computed between 0 h DSFT of the test fold and maximum relative DSFT, and between 24 and 47 h for immunised cows. Significance is declared if $P < 0.05$.

6.3 Results

All cows had increased DSFT at the test site relative to the control site following the CaWC injection at 0 h. Seven of the ten unimmunised cows and nine of the ten immunised cows produced a positive DTH reaction. No positive reactions at control injection sites were observed. The responses observed at the test sites were limited to swelling of the caudal fold, with no apparent changes to the skin surface of any injection sites.

Mean maximum relative DSFT increases were 4.3 mm (±1.4; SE) and 1.4 mm (±0.5) for immunised and unimmunised cows, respectively. Responses were larger in immunized cows at all time-points relative to unimmunised cows (Figure 18). The increase in relative DSFT from 0 h to 7 h was 5-fold greater in immunised cows than in unimmunised cows. The maximum response was more variable in immunised relative to unimmunised cows ($p<0.01$).

The average DTH response for both groups peaked at 24 h but was not significantly greater than responses observed at 47 h (immunised: $P = 0.649$; unimmunised: $P = 0.989$). There was no difference in variability of 24 and 47 h responses in immunised cows ($P = 0.788$). Responses at 24 and 47 h were highly correlated in immunised cows ($r^2 = 0.79$, $P < 0.01$). There was no correlation between maximum relative DSFT increase and 0 h test DSFT in the immunised group ($r^2 = 0.08$, $P > 0.05$).

The time-dependent DTH responses for individual animals in each group depicted in Figure 19 illustrate the increased variability of response magnitude in immunised cows. In the immunised group, the maximum relative DSFT increase was at 24 h for six cows, 47 h for three cows, and 7 h for one cow. In the unimmunised group, the maximum relative DSFT increase was at 24 h for four cows, 47 h for five cows, and 7 h for one cow (Figure 19). Examples of DTH responses in immunised cows are illustrated in Figure 17.

Figure 17. Four examples of delayed-type hypersensitivity response, 24 hours after the injection. In all photos, the test fold is on the left and the control fold is on the right. The top right image shows an unusually strong DTH response with hard swelling spread up the caudal fold from the injection site.

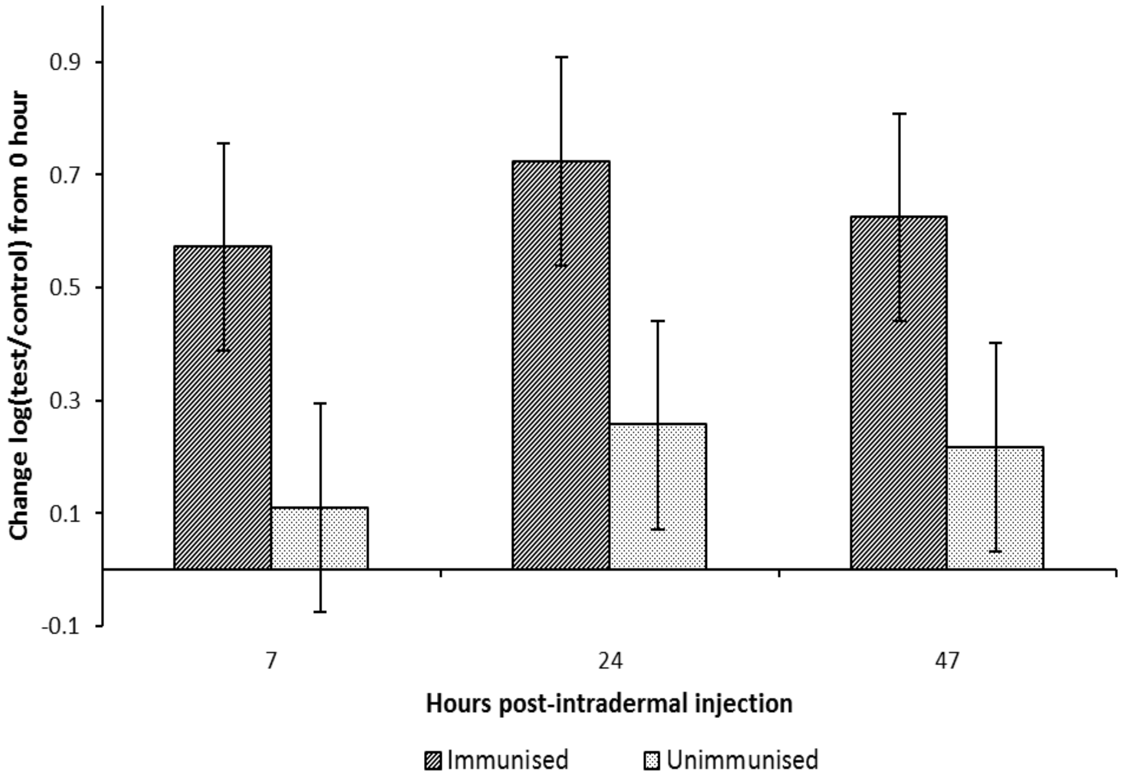


Figure 18. Delayed-type hypersensitivity (DTH) response to *Candida albicans* whole-cell, in dairy cows previously sensitised via immunisation (n=10), and in unimmunised cows (n=10). Responses are expressed as change in the log of (double skin fold thickness at the test site/doubles skin fold thickness at the control site) from 0 h, measured at 7, 24 and 47 h post-injection. Error bars indicate 95% confidence intervals. Responses in immunised cows were significantly larger than responses in unimmunised cows at every time point (P < 0.05).

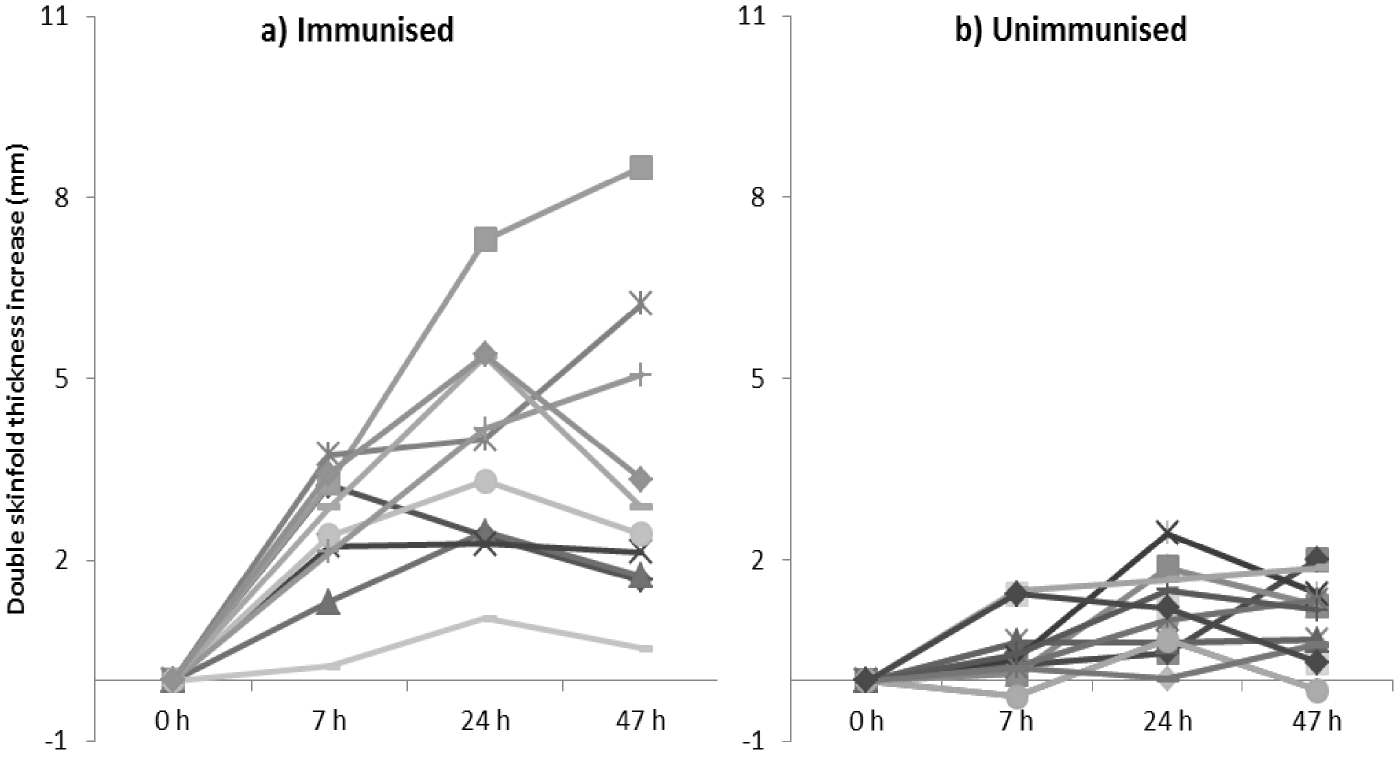


Figure 19. Delayed-type hypersensitivity responses to *Candida albicans* of individual dairy cows that were previously immunised with *C. albicans* (a) or unimmunised (b). Responses are expressed as relative increase in double skin fold thickness from 0 h relative to the control site, at 0, 7, 24 and 47 h post-injection.

6.4 Discussion

Delayed-type hypersensitivity reactions to *C. albicans* have been used extensively in North American dairy cattle to assess cell mediated immune responsiveness (Heriazon et al., 2011, Heriazon et al., 2009a, Heriazon et al., 2009b, Hine et al., 2011). Studies have shown that these largely housed dairy cows produce mild DTH responses to *C. albicans* as a consequence of natural exposure. Nevertheless, previous experiments have included priming the immune system by immunising with *C. albicans* prior to DTH testing to minimise the effects of individual variation in natural exposure and to stimulate greater DTH responses. Natural exposure levels of cows in New Zealand's pasture-based dairy herds may be different to their largely housed counterparts. Therefore, the objectives of this experiment were to investigate both background and pre-primed DTH responses of pasture-based dairy cows and to determine if DTH testing using *C. albicans* can be used successfully as an indicator of CMIR in pasture-based dairy cows.

6.4.1 Background DTH responses to C.albicans

Results of the current study demonstrate that the pasture-based NZ dairy cows in the herd tested had low level background DTH responses to *C. albicans* suggesting low level natural exposure to *C. albicans.* Although several unimmunised cows produced mild DTH responses, responses measured in immunised cows were much larger. Furthermore, responses were five-fold larger in immunised cows relative to unimmunised cows at 7 h post-injection. This contrasts with the results of Heriazon et al. (2009a), who reported that the magnitude of responses at 6 h were similar in immunised and unimmunized cows. Heriazon et al. (2009b) reported that cows immunised twice exhibited skin lesions such as pustules, crusting and dermatitis at the *C. albicans* injection site. In the current experiment, all DTH responses were limited to swelling of the caudal fold, with no apparent changes to the skin surface in immunised cows. These results indicate that the natural exposure to *C. albicans* was lower in the pasture-based cows tested here relative to housed cows and therefore, it is likely not necessary to determine background DTH prior to priming with *C. albicans*. The number of cows used to determine background DTH in this experiment was small (n=10), and the results pertain to a single farm. Testing of background DTH to *C. albicans* in other pasture-based herds, in varied geographical locations and operating under various management systems will be required to confirm that natural exposure to *C. albicans* is typically low in pasture-based herds in New Zealand.

6.4.2 Pre-primed DTH responses in immunised cows

Results demonstrated that immunisation prior to conducting DTH testing successfully enhanced DTH responses. A greater proportion of positive DTH responses were observed in immunised cows and responses were three-fold larger at 24 and 47 h than in unimmunised cows. Testing of unimmunised cows showed that background sensitivity to *C. albicans* was consistently very low therefore the high variability observed in DTH responses from immunised cows is likely due to individual differences in immune responsiveness, rather than from differences in previous natural exposure to *C. albicans.* These results confirm that *C. albicans* can be successfully used to elicit measurable DTH responses in pasture-based cows to rank animals based on their cell-mediated immune responsiveness.

6.4.3 Kinetics of pre-primed DTH

In the current study, peak DTH response times varied between cows indicating that measurement at both 24 and 48 h may be better than either time-point alone. Delayed-type hypersensitivity reactions are characterised by a hard swelling at the site 24-72 hours post antigen exposure (Rosenstreich, 1993). While DTH responses at 7 h were significantly higher in immunised versus control cows, responses observed at 7 h are not expected to be indicative of true DTH responses but rather earlier phase immune reactions (Black, 1999, Hernández et al., 2005). Begley et al. (2009b) identified 48 h post dermal injection as the optimal time to assess DTH response. However, other researchers have measured DTH at both 24 and 48 h and found no significant difference between the magnitude of DTH responses at each time-point (Heriazon et al., 2011, Heriazon et al., 2009a, Heriazon et al., 2009b, Hernandez et al., 2003). Heriazon et al. (2009b) reported that cell infiltration to the DTH site following injection with *C. albicans* was similar at 24 and 48 h and concluded that the response observed at both time points represented a classic DTH reaction. Hernandez et al. (2003) suggested that using the average of 24 and 48 h or selecting the time-point with greatest variability between cows is appropriate when using DTH responses to classify cows. In this experiment, 24 h responses were highly correlated with 47 h responses, however, for individual cows the time of peak response occurred equally at 24 and 47 h and ranking of cows based on the magnitude of the DTH response was variable between 24 and 47 h. Additionally, the variability was the same at 24 and 47 h. While measuring at 24 h would capture the peak response for most of the cows in the current experiment, our results suggest that DSFT should be measured at both time points when using DTH response to classify cows, as suggested by Hernandez et al. (2003).

6.5 Conclusion

Background exposure to *C. albicans* was minimal in the pasture-based New Zealand herd tested and DTH reactions to *C. albicans* can be successfully used as a measure of cell-mediated immune responsiveness. Cows produced only mild DTH responses to CaWC, when not pre-immunised with *C. albicans*. Furthermore immunised cows had a greater proportion of positive DTH responses, and produced significantly larger and more variable DTH responses than non-immunised cows.

Chapter 7. Experiment Two - Adaptive immune responses in early lactation were not affected by body condition score at calving.

7.1 Introduction

Dairy cows exhibit transient immune dysfunction during the period of transition between pregnancy and lactation (Goff, 2006, Schwarm et al., 2013, Wagter et al., 2000), This is believed to contribute to a higher incidence and severity of infectious disease during the first few weeks of lactation (Drackley, 1999, Mallard et al., 1998, Vangroenweghe et al., 2005). In support of this hypothesis, 25 to 56% of clinical mastitis occurs in the three weeks after calving (Kehrli Jr, 2012, McDougall et al., 2007) and gastrointestinal worm burdens and egg counts are elevated in periparturient cows (Houdijk, 2008). These results indicate a reduced ability to resist new infection or contain latent subclinical infections during the transition period.

Negative energy balance (NEB) may contribute to this impaired immune function. Homeorhetic adaptations support a rapid increase in milk production, but the increase in voluntary food intake is not sufficient to meet energy and protein requirements until several weeks after calving (Bauman and Currie, 1980, Oltenacu and Broom, 2010, Roche et al., 2009). Consequently, dairy cows are in negative energy and protein balance during early lactation (Drackley, 1999, Gearhart et al., 1990, Goff and Horst, 1997) and mobilize fat and protein from body tissues to support milk production (Morris et al., 2009, Roche et al., 2009). Fat mobilization elevates serum levels of NEFA and ketones, such as BHBA (Roche et al., 2009, Suriyasathaporn et al., 2000), and levels of these metabolites have been associated with increased incidence of retained placenta, metritis and mastitis (Kaneene et al., 1997, Moyes et al., 2009b, Ospina et al., 2010), and impaired lymphocyte and neutrophil function (Grinberg et al., 2008, Ster et al., 2012, Suriyasathaporn et al., 2000). These results suggest that a greater level of immune dysfunction occurs in cows that experience a greater NEB postpartum, resulting in reduced ability to resist disease. The extent of postpartum NEB is positively correlated with BCS at calving (Broster and Broster, 1998, Roche et al., 2007a, Roche et al., 2009); therefore, cows that calve at higher BCS might exhibit a greater degree of immune dysfunction than cows that calve at lower BCS. In support of this hypothesis, high BCS at calving has been associated with increased incidence of mastitis (Berry et al., 2007b, Morrow, 1976) and Lacetera et al. (2005) reported greater impairment of both IFN-γ and IgM secretion in cows that calved at higher BCS and lost more BCS post-calving. However, in contrast, low BCS has been associated with greater incidence of uterine infection (Hoedemaker et al., 2009, Markusfeld et al., 1997, Roche et al., 2009). Therefore, associations between calving BCS and cow health are not consistent (Breen et al., 2009, Gearhart et al., 1990, Heuer et al., 1999). Much of the research has been epidemiological, making it impossible to determine whether the relationship is causative or associative, and there has been limited in vivo research concerning the relationship between calving BCS and postpartum immune function. Additionally, limited research has been conducted in pasture-fed cows; this is important because, compared with cows in other systems, pasture-fed cows tend to calve at lower BCS (Roche et al., 2007a) and are exposed to different environmental pathogens (Laven & Holmes, 2008).

The objectives of this study were to compare in vivo measures of adaptive immunity in pasture-fed cows postpartum, and to investigate the effect of differences in NEB associated with differing BCS at calving on adaptive immune function in early lactation. It was hypothesized that, as a result of more profound negative energy balance, cows that have higher BCS at calving would exhibit greater immune suppression during early lactation compared with cows calving at lower BCS. The immune system becomes type-2 biased during the periparturient period (Shafer-Weaver et al., 1999, Shafer-Weaver and Sordillo, 1997), and that type-2 shift is reported to be increased in cows with greater postpartum loss of BWT Ohtsuka et al. (2008). Since postpartum BWT loss is associated with BCS at calving, I hypothesised that cows with a higher calving BCS will exhibit a stronger type-2 bias than cows with lower calving BCS. To test these hypotheses, the energy balance of healthy cows at similar mid-lactation BCS was manipulated in late-lactation, to generate groups of cows with differing BCS at calving (Roche et al., 2013c). Antibody-mediated immune responses (AMIR) and cell-mediated immune responses (CMIR) in early lactation were compared with responses measured in mid-lactation before BCS manipulation began. Previous studies have reported that cows with enhanced immune responsiveness as assessed with these immune testing protocols have decreased incidence of infectious disease, including mastitis, increased levels of antibody in colostrum, and respond more strongly to commercial vaccines (De La Paz, 2008, Mallard et al., 1997, Wagter et al., 2000).

7.2 Method

The experiment was conducted at the DairyNZ Lye Farm, Hamilton, New Zealand (37°76’S 175°37’E, 45 m above sea level) from January until September 2011. All procedures had prior approval of the Ruakura Animal Ethics Committee, Hamilton, New Zealand, and are in accordance with the New Zealand Animal Welfare Act 1999.

7.2.1 Animals and BCS treatment

Sixty mid-lactation dairy cows of mixed age and breed between body condition score 3.75 and 4.25 (1-10 BCS scale, where 1 is emaciated and 10 obese; Roche et al., 2004) were enrolled in the study. All cows had no previous history of disease, including mastitis, were confirmed pregnant, and passed a veterinary clinical examination when enrolled in the experiment on 5th January 2011 Cows were allocated randomly to treatment groups (High, Medium and Low, 20 cows per group) on 1st February 2011, ensuring treatments were balanced for BCS at the time of enrolment, expected calving date, production, age, breed, somatic cell count and live weight. Age at enrolment was 4.0 ± 1.4 yr (mean ± SD). Mean expected calving date was 9 July ± 9 d. Fourteen cows in each group were Holstein-Friesian, with the balance being 75% Holstein-Friesian/25% Jersey cross.

Between mid-lactation and calving, feeding levels were manipulated with the intention of generating three homogenous BCS groups before the end of lactation: 3.0, 4.0 and 5.0, for the Low, Medium and High groups, respectively. Following drying off, cows were offered sufficient pasture and supplement to gain 0.5 BCS unit towards target calving BCS of 3.5, 4.5 and 5.5 at calving for the Low, Medium and High groups, respectively. Final mean BCS at calving were 3.4, 4.6

and 5.4 for the Low, Medium and High BCS groups, respectively. The mean BCS of each group over time is presented in Figure 20.

Body condition score was determined by two experienced assessors twice per week (Monday and Thursday) following the morning milking or at an equivalent time during the non-lactating period. All BCS assessors were trained and recalibrated at the start of the experiment following the procedures set out by (Macdonald and Roche, 2004). The inter-observer variance component for BCS estimates was 0.05 (SD = 0.2).

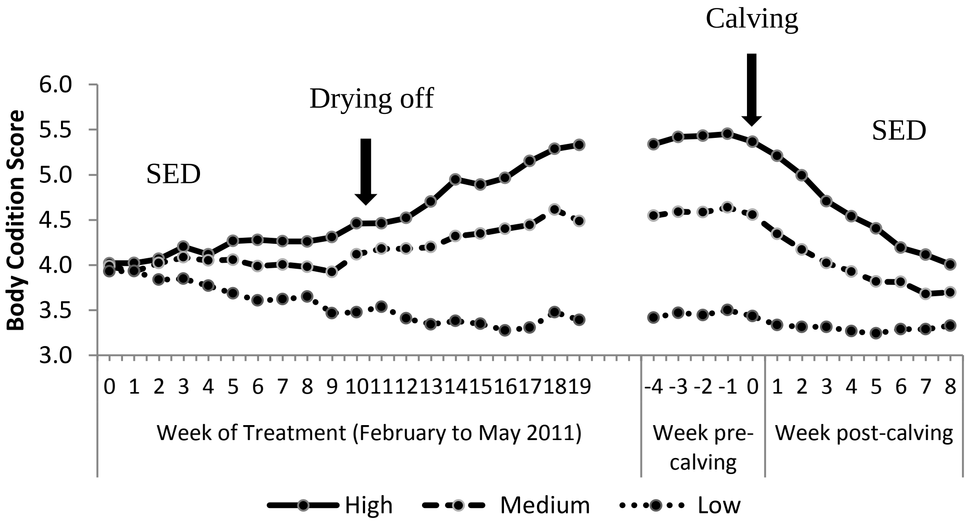


Figure 20. Mean body condition score (1 to 10 scale) of each group from selection in mid-lactation until 6 weeks post-calving. Data sourced from Roche et al. (2013c).

During late lactation, daily feed allowances were adjusted to account for the average BCS state of the group and the proposed trajectory of BCS change. Cows in the High, Medium, and Low treatments had estimated daily DMI of fresh pasture of 12.3 (±3.38), 11.2 (±2.26), and 6.8 (±1.69) kg DM, respectively. Pasture and maize silage, and rolled maize grain, were provided to supplement DMI when sufficient pasture was not available. Allocated feed supplements were weighed, and estimations of wastage included in the final calculation of DMI from these sources. Cows in the Low group received 1.9 (±1.03) kg dry matter (DM) of pasture silage daily, those in the Medium group received 2.0 (±2.08) kg DM of pasture silage, 0.5 (±0.90) kg DM of maize silage, and 1.0 (±1.03) kg DM of rolled maize grain daily, and those in the High group received 2.4 (±2.04) kg DM of pasture silage, 1.6 (±2.86) kg DM of maize silage, and 2.6 (±1.27) kg DM of rolled maize grain daily. When an individual cow's BCS was not tracking towards the treatment target, she was moved to a group with a feeding regime that facilitated the desired change. Groups grazed the same paddock with electric fences separating them. Mean pasture DMI was calculated as the product of the difference between the pre- and post-grazing pasture mass and area grazed on three d/wk, as outlined by Roche et al. (2005).

General management of the cows, including mineral and trace element supplementation, was in accordance with research farm practice. From 30th May until calving, cows were supplemented with 10 g elemental Mg/cow per day via the water reticulation system (Dosatron Water Dispensers, Bell-Booth Ltd., Palmerston North, New Zealand)and received oral Mg supplement weekly as Mg pidolate (100 ml More-Mag, Dominion Chemicals (1988) Ltd., Auckland, New Zealand). Following calving, cows received oral Mg salts, sufficient to provide 20 g elemental Mg/cow per day.

On each day during calving, cows that had calved between 1000h the previous day and 1000h that day were brought to the farm dairy at 1300h (except where cows had observed calving difficulty (n = 2) or milk fever (n = 2), which were attended to immediately). Cows were milked twice a day. Cows entered a 'colostrum herd' for their first 10 milkings, before being transferred to the 'milking herd'. From entry to the milking herd onward, the cows were managed as a single group and offered a generous feed allowance, with mean intakes of 10.9 and 6.0 kgDM/cow/day, of pasture and pasture silage, respectively.

Fifty-six cows completed the experiment: 20, 18, and 18, in the High, Medium, and Low groups, respectively. Results of the four cows that did not complete the sampling schedule were excluded from the analysis. One cow did not receive an immunisation on d0, one died of misadventure in autumn, another aborted her pregnancy, and the fourth developed aspiration pneumonia after calving and was euthanized.

7.2.2 Immune testing protocol

Immune responses were measured during mid-lactation, to obtain baseline values before cows were allocated to their BCS treatment group, and immediately postpartum (d 0 was the day of calving). Cows were immunized intramuscularly, on d 0 and d 14, with type-1 (*Candida albicans*) and type-2 (hen egg white lysozyme, HEWL, or human serum albumin, HSA) antigens known to induce polarised CMIR and AMIR, respectively (Begley et al., 2009b, Cartwright et al., 2012, Holland et al., 2001). The effect of calving BCS on AMIR was assessed using a cross-over design, with the type-2 antigen order crossed over within each level of BCS (Table 2) to permit assessment of both primary and secondary AMIR post-calving. Half of the cows in each BCS group were immunized with HEWL in mid-lactation and HSA postpartum (HEWL-HSA group); the remainder were immunized with HSA in mid-lactation and HEWL postpartum (HSA-HEWL group). The vaccine contained 0.5 mg of HEWL (L6876, Sigma Aldrich, Auckland, NZ) or 0.5 mg of HSA (A3782, Sigma Aldrich, Auckland, NZ), 0.5 mg *C. albicans* (CaWC, Greer Laboratories Inc, Lenoir, NC, USA) and 0.5 mg adjuvant (QuilA, Brenntag Biosector A/S, Frederikssund, Denmark) in 1mL sterile phosphate-buffered saline pH 7.4 (PBS).

Table 2. Antibody-mediated immune function was assessed using a cross-over arrangement, with the type-2 antigen order crossed over within each body condition score treatment. Cows in the HEWL-HSA antigen treatment were immunized with HEWL in mid-lactation and HSA post-partum; cows in the HSA-HEWL treatment were immunized with HSA in mid-lactation and HEWL post-partum. The number of cows that completed the experiment in each group is presented in the table.

	Low BCS	Med BCS	High BCS
HEWL-HSA	10	11	9
HSA-HEWL	10	8	8

Delayed-type hypersensitivity (DTH) reactions induced by the type 1 antigen were assessed, as an indicator of CMIR (Heriazon et al., 2011, Heriazon et al., 2009b, Kobayashi et al., 2001). DTH reactions were induced on d 21 during baseline testing and d 8 during post-partum testing. Blood samples were collected by caudal venepuncture on d 0, 14, and 21 of each immune test. Serum was prepared after coagulation, by centrifugation (1500 x *g* for 10 min at 4°C) and stored at -20°C for later analysis of immunoglobulin content.

7.2.3 Evaluation of antibody-mediated immune response

Serum anti-HEWL specific IgG was measured using a previously described ELISA method (Begley et al., 2009a, Cartwright et al., 2011). Flat-bottomed 96-well polystyrene plates (Nunc-Immuno^{TM} Plate, Maxisorp^{TM} surface, Thermofisher) were coated with HEWL dissolved in carbonate-bicarbonate buffer (1.44 mg/mL, pH 9.6, C3041, Sigma-aldrich) and incubated at 4°C for 24 h. Plates were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween20 (wash buffer), using a Thermoscientific Wellwash Versa automatic plate-washer. Plates were then blocked with 200 µL per well of PBS (pH 7.4) containing 5 % Tween20 and 1 % gelatine (G7041, Sigma Aldrich) and incubated for 1 h at room temperature. Plates were again washed three times with wash buffer. Serum samples and controls diluted 1/400 and 1/1600 in wash buffer were added in quadruplicate (100µL per well), using a quadrant system (Wright. 1987) and incubated at room temperature for 2 h. Negative and positive controls were a pooled sample of d 0 sera and a pooled sample of d 21 sera, respectively. Plates were washed five times, then 100 µL of alkaline phosphatase-conjugated sheep anti-bovine IgG (heavy chain specific, A10118AP, Bethyl Laboratories Inc, Montgomery, TX) diluted in Tris buffered saline (pH 7.4, T5912, Sigma Aldrich) containing 0.05% Tween20 was added to each well. Plates were incubated at room temperature for 1 h. Plates were washed a final five times then, the substrate p-Nitrophenyl phosphate disodium (p-NPP) in Tris buffered saline (pNPP substrate system, N2770, Sigma Aldrich) was added at 100 µL per well. Plates were incubated in the dark at room temperature and the optical density (OD) measured once OD of positive controls reached approximately 1.0. Optical density was measured with an ELISA plate reader (Thermoscientific Multiscan FO) at a wavelength of 405 nm.

Antibody-mediated response to HSA was measured using a previously described ELISA method (Cartwright et al., 2012). Briefly, the ELISA protocol is as described above for HEWL, except that plates were coated with 0.8mg/mL of HSA dissolved in carbonate-bicarbonate buffer, samples and controls were added at dilutions of 1/40 and 1/160.

The ratio of IgG1 to IgG2-related antibodies produced in immune responses can provide an indication of type-1 or type-2 IR bias (Estes and Brown, 2002, Hine et al., 2011). Type-2 antigens (e.g. IL-4) promote production of IgG1 and type-1 cytokines (e.g. IFN-γ) promote production of IgG2, therefore, IgG1 and IgG2 are considered to be type-2 and type-1 antibodies, respectively (Estes and Brown, 2002). A balanced ability to mount both type-1 and type-2 responses is important for control of intra- and extra-cellular pathogens (Estes and Brown, 2002, Hine et al., 2011, Spellberg and Edwards, 2001). To assess type-1 or type-2 response bias, IgG1 and IgG2 antibody activity against HEWL and HSA were also determined in each serum sample by ELISA. The ELISA protocols used to determine antigen-specific IgG1 and IgG2 to HEWL and HSA were similar to the respective protocols described above for IgGT with the following modifications:

For anti-HEWL and anti-HSA IgG1, conjugated sheep anti-bovine IgG1 (A10116AP, Bethyl Laboratories Inc, Montgomery, TX) was added. For anti-HEWL and anti-HSA IgG1, conjugated sheep anti-bovine IgG2 (A10117AP, Bethyl Laboratories Inc, Montgomery, TX) was added.

Any replicate OD greater than 20% above or below the median OD for the sample-dilution was excluded from analysis, as described by Stone et al (1991); 0.4% of replicate ODs were removed for HEWL and 1.3% for HSA, with at least 3 replicates analysed for all samples and controls at each dilution. CVs were calculated for the raw OD of the positive controls on each plate to determine whether plate results were accepted or rejected. Plates with a CV greater than 15% for positive control samples were reanalysed. CVs were calculated from the raw ODs for each sample, at both dilutions, to determine whether sample results were accepted or rejected. Samples with CV greater than 15% for either dilution were reanalysed on a subsequent plate. Inter-assay CVs using standardised data (as described below) were 2.2% and 2.0% for anti-HEWL and anti-HSA IgG, respectively. Intra-assay CVs, using standardised data, ranged from 3.6% to 4.6%.

To compare samples run across all plates, mean optical density values were corrected based on the mean OD value of the positive control sample run on the same plate, as a previously described (Hine et al., 2011, Mallard et al., 1997). For each plate, the mean OD of the blank wells were subtracted from the mean OD of all sample and control replicates And replicates were standardized using the positive control at the same dilution on the plate as described above. Briefly, the mean OD of the positive control on each plate was corrected to an OD of 1.0, and all mean test values on the same plate were corrected by the same factor (the inverse of the mean positive control). Corrected means for each test serum dilution were then added together to give an additive OD value for each test sample, and these values were used for statistical analysis.

7.2.4 Evaluation of cell-mediated immune response

Cutaneous DTH to *C. albicans* was measured, following previously described protocols (Hernández et al., 2005, Hine et al., 2011, McGowan et al., 2014). Intradermal injections of 0.1 mg CaWC in 0.1ml PBS (test) and 0.1 ml PBS (control) were each administered to an opposing caudal skin-fold of the tail. All intradermal injections were administered with a 29 g needle/insulin syringe (Terumo, Shoof International, Cambridge, New Zealand). The site of each injection was marked with correction fluid (twink) to allow subsequent measurements at the same site. The double skin-fold thickness (DSFT) at each injection site was measured (3 repetitions) using a Harpenden spring-loaded dial-calliper (Mentone Educational Centre, Victoria, Australia), immediately before injection and at both 24 and 48 hours after injection. Pilot studies of DTH testing with CaWC in grazing cows had demonstrated that the time to maximal response varied between individual cows, with similar numbers having maximal responses at both time points (McGowan et al., 2014).

Relative increases in DSFT were calculated at both 24 and 48 h post-injection and expressed as the change in log of DSFT at test site / DSFT at control site, from 0 h, as described by Hine et al. (2011):

$$\text{Response} = \log (T_h/C_h) - \log (T_0/C_0)$$

Where T_h = mean test DSFT at time h; T_0 = mean test DSFT at 0 h; C_h = mean control DSFT at time h; C_0 = mean control DSFT at 0 h; and time h was 24 or 48 h.

7.2.5 Colostrum antibody

A pooled colostrum sample was collected from each cow before her first milking and stored at -20°C for later analysis. Colostrum samples were analysed in duplicate for their immunoglobulin content (mg/ml) by colorimetric ELISA methods using commercial kits (Bethyl Laboratories, Inc., Montgomery, TX). These assays had been demonstrated by the manufacturer to be highly specific for each respective immunoglobulin isotype in bovine samples by immunoelectrophoresis and ELISA methods. Dilution rates for samples varied between 1:20000 and 1:40000 for IgA, 1:40000 for IgM, 1:600000 and 1:800000 for IgG1, and 1:10000 to 1:30000 for IgG2. Inter-assay CV's were 21%, 19%, 15% and 25% for IgG1, IgG2, IgM and IgA, respectively.

7.2.6 Statistical Analyses

Statistical analyses were undertaken to assess AMIR and CMIR. Specific antibody responses were examined based on the OD value at each time point and the area under the curve between time points. The primary response was determined using the concentration of IgG on d 14 and the secondary response was evaluated as the concentration of IgG on d 21. Delayed-type hypersensitivity (CMIR) was evaluated at 24 and 48 h post intradermal injection. An increase in DSFT of >1 mm from 0 h was considered a positive DTH response and an increase in OD of >0.1 induced by immunisation was considered a positive AMIR response, as per Hine et al (2011).

Observations were $\log_{10}$-transformed to ensure normalisation of the residuals. The transformed data satisfied the assumptions of ANOVA and were used to calculate *P*-values. However, the corresponding least squares means and standard errors of the non-transformed data are presented in the results for clarity.

Results from mid-lactation were analysed separately from those at calving. For mid-lactation, responses (raw and differences from baseline; dfb) were analysed using mixed models approach to repeated measures (Proc Mixed, SAS 9.3) with time, antigen, and their interactions included as fixed effect and cow as random effect. Area under the curve (AUC) based on dfb were analysed using two-way analysis of variance (ANOVA) for the effect of antigen. For post-calving, responses (raw and dfb) were analysed using mixed models approach to repeated measures (Proc Mixed, SAS 9.3) with time, antigen, BCS, and their interactions included as fixed effect and cow as random effect. AUC based on difference from baseline were analysed using two-way ANOVA for the effects of antigen, BCS, and their interaction. The individual response in mid-lactation was accounted for when assessing responses post-calving by including AUC from mid-lactation as a covariate. To estimate the stage of lactation effect (mid-lactation to calving), a repeated measures analysis of AUC for IgG and DTH, and of raw data for IgG1:IgG2 was conducted (Proc Mixed, SAS 9.3), with antigen, BCS and stage of lactation included as fixed effects and cow as random effect. Tukey's test was used for pairwise comparisons. Results are presented as least-squares means and standard errors. Significance is declared if $P < 0.05$).

One outlier, in the Med BCS group, was removed from the analysis of IgG1:IgG2 ratio because the magnitude of her IgG1:1gG2 ratio was 4-5 times greater than the other cows on d0 and d14 and consequently changed the response patterns and outcomes for IgG1:IgG2 ratio to an unacceptable degree. The IgGT, IgG1, IgG2 and DTH responses for this cow were within the normal range and inclusion or exclusion of this cow did not affect the relationship between calving BCS and those immune measures, therefore she was not excluded from other analyses.

7.3 Results

7.3.1 Mid-lactation immune responses

Antigen-specific IgG responses to immunisation with HEWL or HSA in mid-lactation are presented in Figure 21. There was a significant interaction between antigen type and time ($P < 0.05$). Primary immunisation with HEWL did not significantly increase IgG antibody concentrations at d14 ($P = 0.20$); whereas, primary immunisation with HSA increased IgG concentrations at d14 ($P < 0.001$). Secondary immunisation with either HEWL or HSA increased IgG concentrations at d21 relative to d14 ($P < 0.001$) in 100% of cows. The AUC of IgG was not affected by antigen type ($P = 1$).

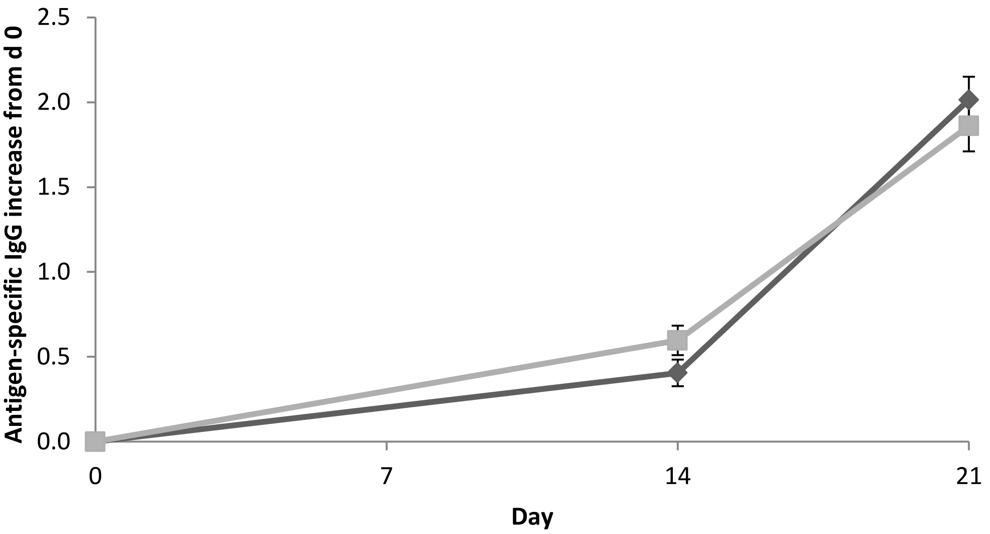


Figure 21. Least-squares means and SE of antigen-specific IgG antibody response of moderately producing pasture-fed cows in mid-lactation, to immunisation with hen eggwhite lysozyme ◆ or human serum albumin ■ , on d 0 and 14. Responses expressed as change in the log of optical density (OD) value from d0, measured at d 14 and 21.

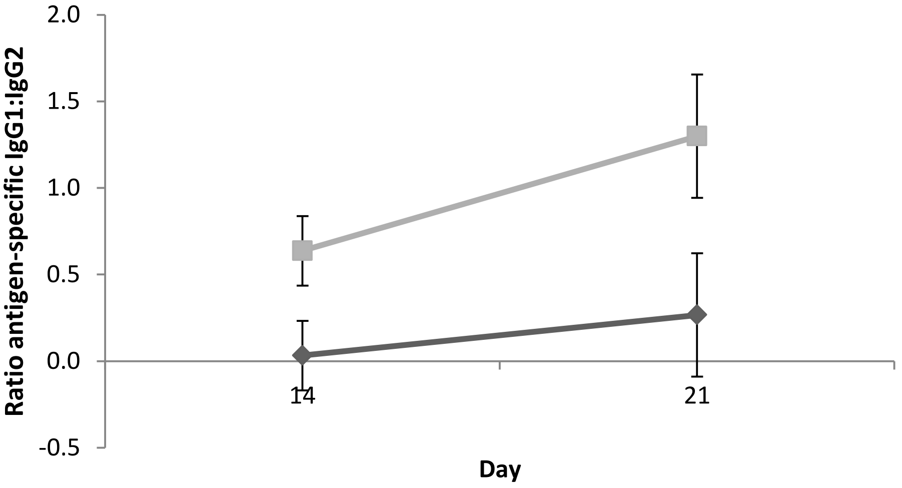


Figure 22. Least-squares means and SE of antigen-specific IgG1:IgG2 ratio of antibody responses, in moderately producing pasture-fed cows in mid-lactation, to immunisation with hen eggwhite lysozyme ◆ or human serum albumin ■ , on d 0 and 14. Data presented are IgG1:IgG2 ratio, relative to d 0.

Overall, the IgG1:IgG2 ratio increased from d14 to d21 following immunisation with type-2 antigen (0.45 ± 0.21, $P < 0.05$, Figure 22); however, the difference between IgG1:IgG2 on d14 and d21 was not significant for HEWL (0.24 ± 0.86, $P = 0.86$) or HSA (0.66 ± 0.31, $P = 0.16$).

Delayed-type hypersensitivity responses to *C. albicans* in mid-lactation are presented in Figure 23. Positive DTH responses were evident in 100% of cows. Responses at 24 and 48 hours were larger than at 0 hours ($P < 0.001$), but did not differ between 24 and 48 hours ($P = 0.90$). The type-2 antigen included in the immunisation protocol (HEWL or HSA) did not affect the subsequent DTH response to *C. albicans* ($P = 0.66$).

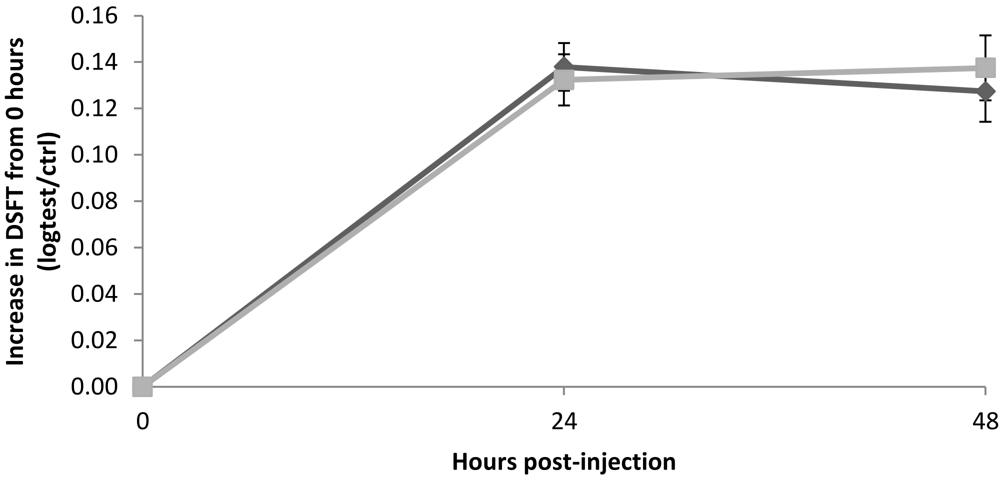


Figure 23. Least-squares means and SE of delayed-type hypersensitivity responses of moderately producing pasture-fed cows in mid-lactation, to *C. albicans* whole cell co-immunized with either hen eggwhite lysozyme —◆— or human serum albumin —■— , on d 0 and 14. Responses expressed as change in the log of test site / control site from 0 h, measured at 24 and 48 h post–injection.

7.3.2 Effect of calving on immune responses

Antibody-mediated immune responses were smaller at calving than in mid-lactation; whereas, cell-mediated immune responses were larger. Positive antigen-specific IgG responses were evident in 90% and 96% of cows immunised with HEWL and HSA, respectively. Mean AUC of antigen-specific IgG, IgG1 and IgG2 responses were lower at calving than in mid-lactation ($P < 0.01$, Table 3). There was a significant increase in IgG1:IgG2 ratio from d14 to d21 post-calving (0.99 ± 0.16, $P < 0.001$) and that increase was larger than the increase recorded in mid-lactation (Figure 24). Positive DTH responses were evident in 100% of cows. The magnitude of DTH responses was bigger at calving than in mid-lactation ($P < 0.001$, Table 3).

Table 3. Difference in magnitude of adaptive immune responses measured at calving compared with responses measured in mid-lactation, in moderate producing pasture-fed dairy cows. Results presented are the difference in the response area under curve, for calving compared with mid-lactation.

	Difference LSM	Std Error	Adjusted *P*-value	% change
IgG	-2.7	0.85	<0.01	- 31
IgG1	-2.4	0.80	<0.01	- 28
IgG2	-3.8	1.14	<0.01	- 51
DTH	1.9	0.39	<0.001	+ 38

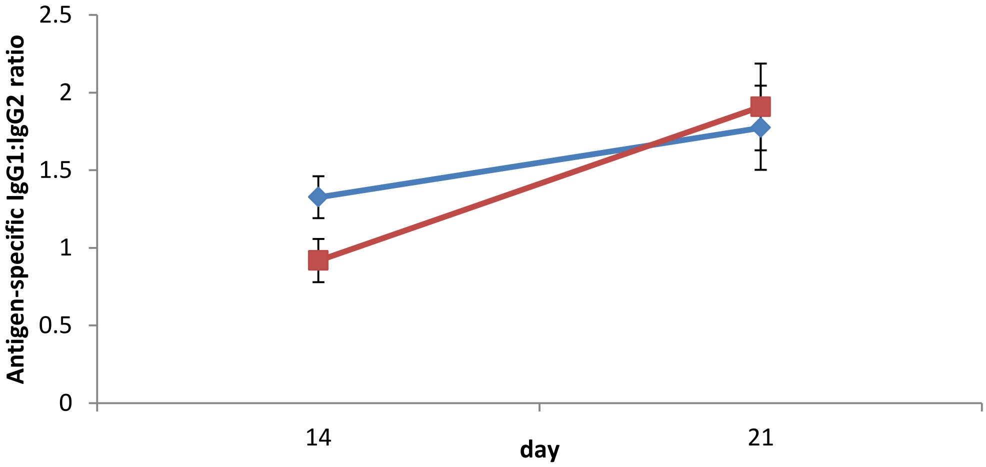


Figure 24. Least-squares means and SE of antigen-specific IgG1:IgG2 ratio of antibody responses to type-2 antigen, in pasture-fed dairy cows in mid-lactation ◆ and at calving ■.

Cows that produced large adaptive immune responses in mid-lactation generally produced large responses postpartum. There was a significant effect of mid-lactation AUC for DTH, IgG, and IgG1 response on the AUC of equivalent measurements postpartum ($P < 0.01$, $P < 0.05$, and $P < 0.01$, respectively; Table 4). However, there was no effect of mid-lactation values on AUC for IgG2 on IgG2 at calving ($P = 0.16$; Table 4).

Table 4. Probabilities for the effects of mid-lactation response magnitude, type-2 antigen, and BCS on immune responses at calving in mixed aged dairy cows.

Antigen-specific response	variable	mid-lactation AUC	type-2 Antigen	BCS	Antigen X BCS	Time (day or hour)	BCS X time	Antigen X time
IgG	raw dfb	< 0.01	0.37	0.93	0.78	< 0.0001	0.67	0.36
	AUC dfb	0.0175	0.097	0.9333	0.6086	NA	NA	NA
IgG1	raw dfb	< 0.001	0.16	0.84	0.74	< 0.0001	0.68	0.99
	AUC dfb	< 0.005	< 0.05	0.94	0.66	NA	NA	NA
IgG2	raw dfb	0.21	< 0.05	0.79	0.34	< 0.0001	0.13	< 0.0001
	AUC dfb	0.16	0.18	0.81	0.41	NA	NA	NA
IgG1:IgG2	raw dfb	0.94	0.12	0.49	0.49	0.12	0.36	0.95
	AUC dfb	NA	NA	NA	NA	NA	NA	NA
DTH	raw dfb	< 0.005	0.18	0.95	0.07	< 0.0001	0.54	0.36
	AUC dfb	< 0.005	0.13	0.99	< 0.05	NA	NA	NA

AUC = area under curve; dfb = difference from baseline (day 0 or hour 0); type-2 Antigen = HEWL/HSA; BCS = body condition score at calving.

7.3.3 Effect of BCS on immune responses at calving

Postpartum antigen-specific IgG responses to immunisation with a type-2 antigen are presented in Figure 25. There were no significant effects of BCS at calving on circulating concentrations of antigen-specific IgG (*P* = 0.93; Figure 25), IgG1 (*P* = 0.95; Figure 26), or IgG2 (*P* = 0.81; Figure 27), and no interactions between BCS and day, or BCS and antigen for IgG, IgG1 or IgG2 concentration (Table 4). There was also no significant effect of BCS on the IgG1:IgG2 ratio (*P* = 0.48; Figure 28), and no interaction between BCS and day or BCS and antigen for IgG1:IgG2 ratio (Table 4).

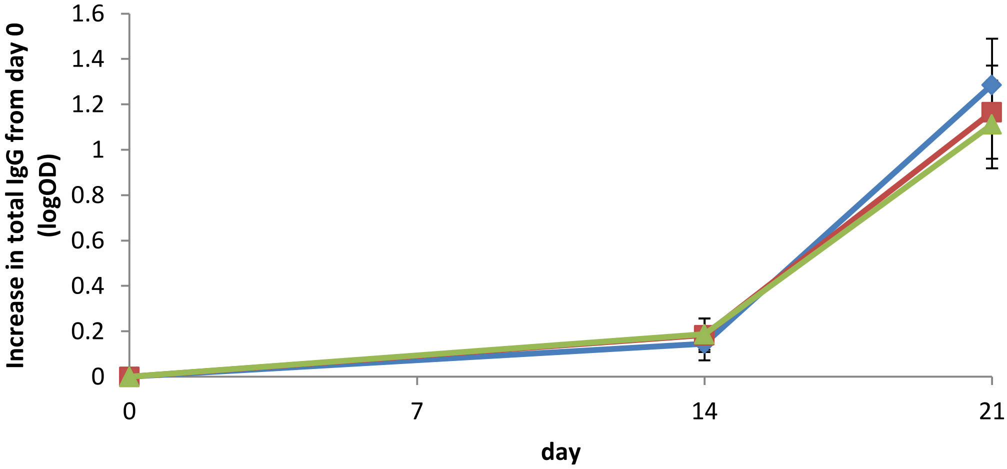


Figure 25. Least-squares means and SE of antigen-specific IgG antibody response to immunisation with type-2 antigen on d 0 and 14, in dairy cows with body condition score 3.4 , 4.6 and 5.4 at calving (1-10 BCS scale, where 1 is emaciated and 10 obese; Roche et al., 2004). Responses expressed as change in the log of optical density (OD) value from d0, measured at d 14 and 21.

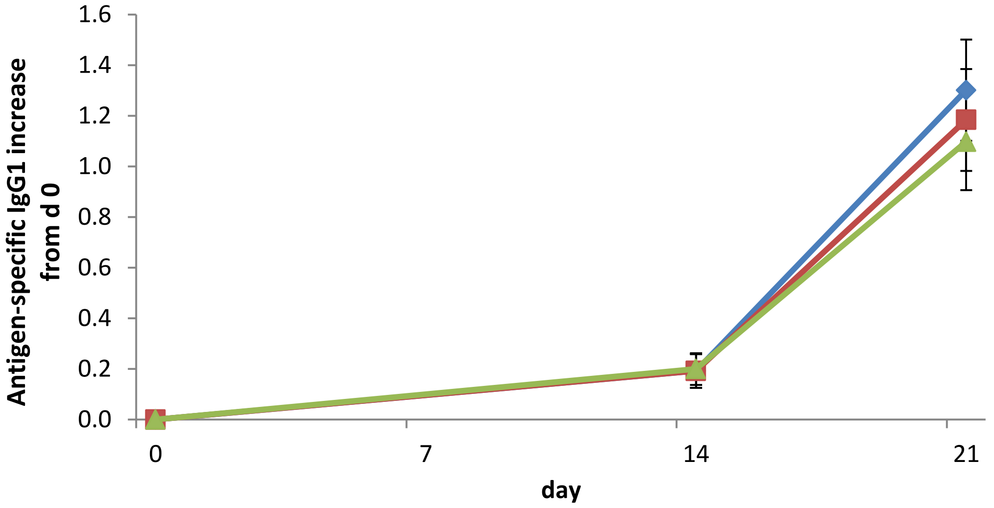


Figure 26. Least-squares means and SE of antigen-specific IgG1 antibody response to immunisation with type-2 antigen on d 0 and 14, in dairy cows with body condition score 3.4 ◆ , 4.6 ■ and 5.4 ▲ at calving 1-10 BCS scale, where 1 is emaciated and 10 obese; Roche et al., 2004). Responses expressed as change in the log of optical density (OD) value from d0, measured at d 14 and 21.

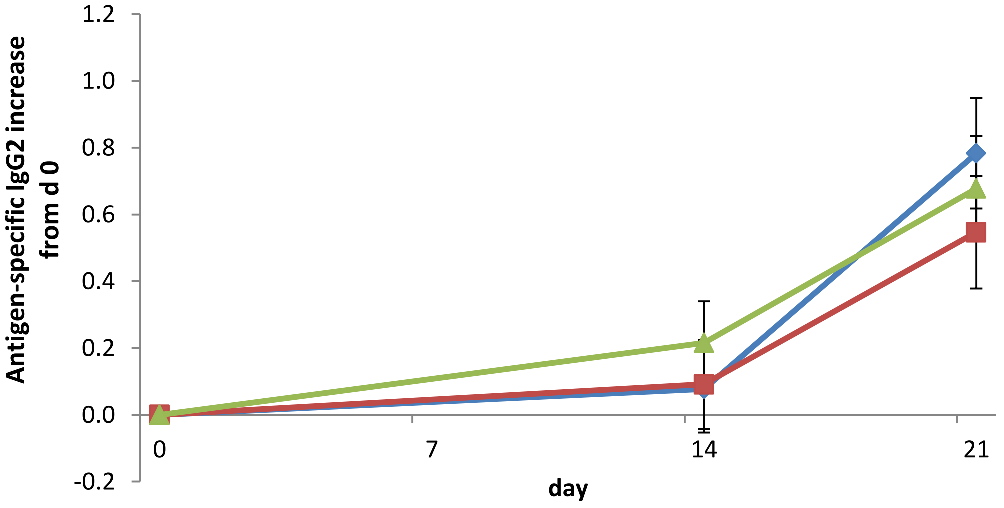


Figure 27. Least-squares means and SE of antigen-specific IgG1 antibody response to immunisation with type-2 antigen on d 0 and 14, in dairy cows with body condition score 3.4 ◆ , 4.6 ■ and 5.4 ▲ at calving 1-10 BCS scale, where 1 is emaciated and 10 obese; Roche et al., 2004). Responses expressed as change in the log of optical density (OD) value from d0, measured at d 14 and 21.

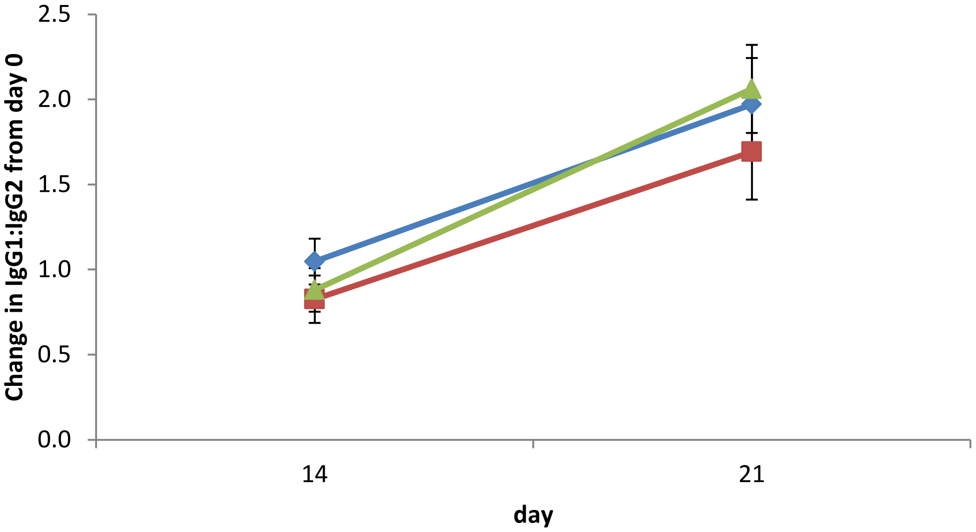


Figure 28. Least-squares means and SE of antigen-specific IgG1:IgG2 ratio of antibody response to immunisation with type-2 antigen on d 0 and 14, in dairy cows with body condition score 3.4 ◆ , 4.6 ■ and 5.4 ▲ at calving 1-10 BCS scale, where 1 is emaciated and 10 obese; Roche et al., 2004). Responses expressed as change in the log of optical density (OD) value from d0, measured at d 14 and 21.

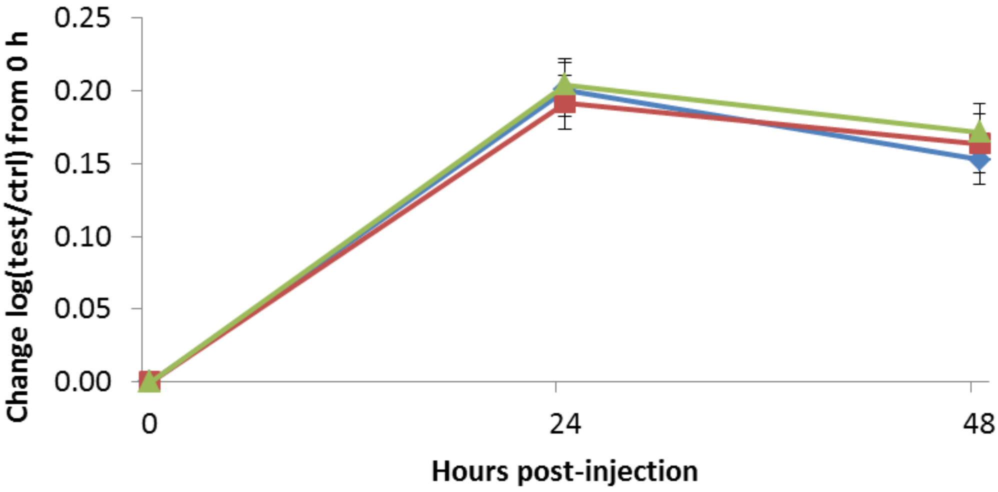


Figure 29. Least-squares means and SE of delayed-type hypersensitivity responses to *C. albicans* whole cell, in dairy cows with body condition score 3.4 ◆ , 4.6 ■ and 5.4 ▲ at calving (1-10 BCS scale, where 1 is emaciated and 10 obese; Roche et al., 2004). Responses expressed as change in the log of test site / control site from 0 h, measured at 24 and 48 h post–injection.

Postpartum DTH responses to *C.albicans* are presented in Figure 29. Body condition score at calving did not affect the AUC of DTH responses (P = 0.99).

However, there was an interaction between antigen type (co-administered type-2 antigen) and BCS on the DTH response ($P < 0.05$; Table 4), with the AUC of DTH responses for cows co-immunized with HSA at calving higher than for cows immunized with HEWL at calving for high and medium BCS cows, but not low BCS cows. There were no other interactions between BCS, antigen or time on DTH response.

Concentrations of total IgA, IgM, IgG1, and IgG2, and the ratio IgG1:IgG2 in colostrum are presented in Table 5. Cows in the Low BCS group had lower IgM concentration in colostrum ($P < 0.05$), whereas concentrations of IgA, IgG1 and IgG2 were similar for all BCS treatments ($P = 0.23$, $P = 0.85$ and $P = 0.69$, respectively).

Table 5. Mean immunoglobulin concentrations (mg/ml) in colostrum samples taken at the first milking for cows in the High, Medium and Low treatments. Shared letters indicate no significant difference.

	High	Medium	Low	sed	P
n	20	18	18		
IgA	7.9	9.7	7.3	1.37	0.23
IgM	8.5 ab	10.6 a	7.3 b	1.30	<0.05
IgG1	43.0	43.1	45.8	5.53	0.85
IgG2	3.3	2.9	3.0	0.52	0.69

7.4 Discussion

The objectives of this study were to determine the effect of BCS at calving on post-partum immune function in pasture-fed cows, measured by assessing CMIR to type-1 antigen (*C. albicans*) and AMIR to type-2 antigens (HEWL and HSA). To our knowledge, this is the first in vivo investigation of postpartum adaptive immune responses in pasture-fed dairy cows with imposed differences in BCS at calving. The energy balance of healthy cows was manipulated in mid- and late gestation to generate groups differing in BCS at calving. We hypothesized that both CMIR and AMIR would be suppressed postpartum, and that cows with higher BCS at calving would have a greater NEB in early lactation, and as a result, would have reduced AMIR and CMIR and increased type-2 immune response bias relative to cows with lower BCS at calving..

7.4.1 Immune function at calving

The presented results support the paradigm of immune dysfunction in the peripartum cow but do not support the hypothesis of a suppression of both CMIR and AMIR post-partum. Antibody-mediated responses were markedly impaired postpartum, as evidenced by a 31% reduction in the mean AUC of the IgG response to an antigen challenge. In contrast, the mean magnitudes of the DTH responses were 38% greater during the postpartum period than in mid-lactation. Suppressed AMIR is consistent with previous research, in which it was reported that peripartum dairy cows have lower B-lymphocyte proliferation and antibody production in vitro compared with cows at other stages of lactation (Lacetera et al., 2005, Nagahata et al., 1992, Nonnecke et al., 2003). Unlike AMIR, which is consistently reported to be impaired postpartum, previous reports of CMIR in peripartum dairy cows have been mixed. Interferon-γ is the central cytokine involved in development of DTH (Nonnecke et al., 2003, Young and Hardy, 1995). Lacetera et al. (2005) reported that mean in vitro IFN-γ secretion did not decrease postpartum; whereas, Ishikawa et al. (1994) and Nonnecke et al. (2003) reported reduced ability of stimulated T-lymphocytes to secrete IFN-γ in the early postpartum period The increased magnitude of postpartum DTH reported here may be largely attributed to additional priming induced by the immunisation on day of calving. Nevertheless, increased DTH magnitude indicates that lymphocyte secretion of IFN-γ and recruitment of macrophages and neutrophils is not impaired postpartum. Our results provide further evidence of impaired AMIR in peripartum cows, consistent with previous studies, but indicate, at least for the cows in this study, that the changes in CMIR are less severe than implicated in previous in vitro studies.

In addition to the reduced AMIR magnitude, the results indicate a type-2 shift in immune responses, meaning postpartum AMIR was biased toward anti-inflammatory mechanisms. The increase in IgG1:IgG2 ratio from d14 to d21 was larger post-calving, relative to mid-lactation AMIR responses. The ratio

IgG1:IgG2 indicates the relative balance of type-1 and type-2 mechanisms in an immune response, with higher values indicative of a response that is more biased toward type-2 immune response mechanisms (Estes and Brown, 2002, Hine et al., 2011). Type-2 responses promote a higher rate of IgG1 production, relative to IgG2 production; therefore, an increase in IgG1:IgG2 ratio indicates that post-calving AMIR had a stronger type-2 bias than AMIR in mid-lactation. This type-2 shift is consistent with Shafer-Weaver et al. (1999) and Shafer-Weaver and Sordillo (1997), who reported a type-2 shift peripartum, as evidenced by a higher ratio of IL4:IFN-γ cytokine secretion in T-lymphocytes isolated from peripartum cows than in T-lymphocytes isolated at other times. Most infections, with the exception of large eukaryotes, are controlled by type-1 mechanisms before a shift to type-2 mechanisms resolves the response (Spellberg and Edwards, 2001), therefore, the apparent type-2 bias postpartum may lead to inadequate cellular defences against infection.

7.4.2 Effect of BCS on measures of immune function

Body condition score at calving did not influence adaptive immune responsiveness or the balance between type-1 and type-2 immune response in healthy cows and within the range of BCS investigated. Evidence for this includes the lack of effect of BCS on DTH responses and on the production of antigen-specific IgG2 antibodies (reflecting type-1 immune responses) and on the production of antigen-specific IgG1 concentrations (reflecting type-2 responses). The ratio of IgG1:IgG2, for which higher values indicate response biased toward type-2 mechanisms (Estes and Brown, 2002, Hine et al., 2011), also did not differ between BCS groups. These results are not consistent with those of Lacetera et al. (2005), who reported peripartum impairment of both type-1 and type-2 lymphocyte functions increased as calving BCS increased. The BCS range included in this experiment was narrower than that studied by Lacetera et al. (2005; approximately BCS 3.0 to 7.5 on the 10 point scale: Roche et al., 2004). However, the range of NEFA concentrations reported by Lacetera et al. (2005) were similar to those reported in the cows investigated here (Roche et al., 2013c). The range of NEFA concentrations reported by Lacetera et al. (2005) for the first week of lactation was approximately 0.55, 0.7 and 1.0 mmol/L for BCS 3.0, 5.5 and 7.5, respectively. Roche et al. (2013c) reported mean NEFA concentrations for the cows investigated here, of 0.48, 0.71 and 0.93 mmol/L for BCS 3.4, 4.6 and 5.4, respectively, for the first 6 weeks of lactation.

Ohtsuka et al. (2008) reported that type-2 bias increased with greater postpartum loss of BWT. In the current experiment, cows that calved at higher BCS lost more BWT postpartum and had higher blood NEFA than cows with lower calving BCS (Roche et al., 2013c), but there was no significant difference in the IgG1:IgG2 ratio between the groups. Ohtsuka et al. (2008) and Lacetera et al. (2005) both measured the function of isolated lymphocytes in vitro. This may not reflect function in vivo, where other immune cells and proteins contribute to the immune response (Sander et al., 2011). Although the BCS range investigated here was narrower than previous investigations, it reflected the range of calving BCS evident in New Zealand dairy herds. Our results indicate that, for cows calving within the BCS range investigated, BCS at calving and the associated degree of postpartum NEB did not influence ability to mount an effective adaptive immune response in vivo.

Calving BCS did not influence colostrum quality. Although the concentration of IgM in colostrum was lower in the Low BCS group, the difference was small, and concentration of IgG1, IgG2 and IgA did not differ between BCS groups. IgG1 is selectively transported to the mammary gland during colostrogenesis and is the most important antibody in colostrum, being present in much greater concentration than other isotypes and more protective against rotavirus (Franklin et al., 2005, Mallard et al., 1998, Sasaki et al., 1976). Therefore, from the perspective of passive transfer of immunity to the calf, BCS at calving did not affect colostrum quality.

7.4.3 The role of negative energy balance in immune dysfunction

These and other results indicate that the severity of postpartum NEB is not the primary contributor to peripartum immune dysfunction. The BCS treatment imposed in this experiment generated three groups with very different degrees of postpartum NEB but the groups did not differ in the magnitude of AMIR or CMIR, or in their bias towards type 1 or type 2 responses. The difference and range of serum NEFA concentrations in early lactation was comparable in this study (Roche et al., 2013c) to those reported in previous investigations of periparturient immune function (Goff et al., 2002, Lacetera et al., 2005, Ster et al., 2012). Increased adipose mobilisation and elevated serum NEFA and BHBA concentrations have been associated with retained placenta, metritis and mastitis (Holtenius et al., 2004, Kaneene et al., 1997, Ospina et al., 2010), and with impaired lymphocyte and neutrophil function (Grinberg et al., 2008, Lacetera et al., 2004, Ster et al., 2012). However, the association between measures of NEB and immune dysfunction has been inconsistent. Nonnecke et al. (1992) reported that in vitro secretion by lymphocytes was not affected by physiologically relevant levels of glucose, ketones and acetate. Similarly, elevated NEFA and BHBA in later lactation, as a result of dietary-induced NEB, is reported to have inconsistent or negligible effect on immune function (Ingvartsen and Moyes, 2013, Moyes et al., 2009a), and has even been reported to stimulate phagocytosis and production of both type-1 and type-2 cytokines (Ingvartsen and Moyes, 2013). Recent work, using whole-blood assays to measure neutrophil function and number during the transition period, reported increased rather than decreased capacity for phagocytosis postpartum, with no clear relationship between phagocytic power and serum NEFA and BHBA concentrations (Sander et al., 2011). This finding, which differs from reports using isolated neutrophils, was attributed to the presence of opsonising factors in whole blood that are not present in a purified preparation (Sander et al., 2011). Previous investigations of the relationship between BCS or NEB on lymphocyte function have mostly been in vitro using purified cell preparations. The differences between the results reported here and those of Lacetera et al. (2005) indicate that the function of isolated lymphocytes in vitro may not reflect function in vivo, where other immune cells and proteins contribute to the immune response (Sander et al., 2011). Our results indicate that, for cows calving within the range of BCS investigated, ability to mount an effective adaptive immune response in vivo was not influenced by the degree of postpartum NEB.

7.4.4 The role of hypocalcaemia in immune dysfunction

Low serum concentration of calcium has previously been associated with impaired lymphocyte function, possibly because calcium plays critical role in activation and function of immune cells (Kimura et al., 2006); the results of this experiment do not contradict nor confirm this association. The serum concentration of calcium, did not differ between BCS groups on the day of calving or during the first 6 weeks of lactation (Roche et al., 2013c). Serum calcium in the first week of lactation was within the normal range for healthy cows (Roche et al., 2013c), and higher than reported for mastectomised and intact cows on the day of calving (Goff et al., 2002, Kimura et al., 2006). Although there was no apparent relationship between BCS and either serum calcium concentration in early lactation or adaptive immune responsiveness during this time, possible links between serum calcium and peripartum immune dysfunction cannot be excluded.

7.4.5 The role of negative protein balance in immune dysfunction

Dietary protein deficiency has been reported to suppress adaptive immunity (Dai and McMurray, 1998), because protein is required for the proliferation of lymphocytes and secretion of cytokines and immunoglobulins. Cows in the Low BCS group had lower protein stores and were in a greater negative protein balance during early lactation (reported in Roche et al 2013); however, the view of the authors was that, although milk production was reduced, other functions, such as the cows' ability to mount an acute phase response, were not compromised. The results reported here support the premise of Roche et al. (2013c), that the negative protein balance observed in the low BCS cows peripartum was not sufficient to impair immune function to a greater degree than was observed in the other BCS groups.

7.5 Conclusion

Under the conditions of this experiment, and the range of BCS tested, BCS at calving did not significantly affect the ability of cows to mount an adaptive immune response. Neither elevated NEB in high BCS cows nor low protein balance in low BCS cows postpartum appeared to affect cows' ability to mount AMIR and CMIR. Periparturient immune dysfunction was evident, with reduced AMIR and increased type-2 bias of AMIR postpartum relative to mid-lactation.

Chapter 8. General discussion

In this thesis, I set out to directly investigate the relationship between calving BCS and adaptive immune function in early lactation, using in vivo measures that have been associated with incidence of clinical diseases, such as mastitis and metritis. Here I discuss several key points arising from this study, comment on the implications and suggest where future research should focus.

Cows in a New Zealand pasture-based herd had low background sensitivity to Candida albicans.

I established the use of two in vivo measures of adaptive immune function in New Zealand pasture-based dairy cows. In the first experiment (Chapter 6), I reported low background DTH responses to *C. albicans*, in a New Zealand pasture-based herd. Although this test had been used extensively in North American and Irish dairy herds, the only reported investigation of background DTH responses was in a housed system (Heriazon et al., 2009a), which may have a different level of exposure to *C. albicans* than a pasture-based system. Having confirmed low background DTH in the Lye farm herd, I could confidently use DTH reactions to *C. albicans* as a measure of CMIR in the second experiment.

For the second experiment (Chapter 7), I used change in antigen-specific antibody concentrations after immunisation with type-2 antigens (HEWL and HSA) as a measure of AMIR, in addition to using DTH as a measure of CMIR. These tests provide a low impact assessment of immune function, without causing a clinical disease that would require antibiotic or other therapy to resolve. Previous in vivo measures have largely been limited to infectious challenges, such as intramammary infusion of mastitis bacteria to induce clinical mastitis; these challenges can have a large impact on the productivity and welfare of cows enrolled in those experiments. This is the first study that I am aware of to use low impact, in vivo measures of adaptive immune function and imposed calving BCS to investigate the relationship between calving BCS and immune function in pasture-based dairy cows.

Although adaptive responses were assessed here, these responses depend on innate function for activation so they may also act as de-facto measures of innate immune function. High responses on these tests have been associated with lower incidence of several diseases usually thought to be primarily controlled by the innate immune system (Thompson-Crispi et al., 2013). Low CMIR has been associated with lower expression of IL-8 (Nino-Soto et al., 2008), which contributes to increased RFM and increased metritis in low CMIR cows (Thompson-Crispi et al., 2012). Cows with low AMIR responses have also been reported to have increased incidence of metritis (Thompson-Crispi et al., 2012). It would be interesting to investigate if, when adaptive immune function is impaired, whether that impairment is mediated by innate functions, such as reduced macrophage secretion of IL-8, or whether it can be wholly attributed to altered lymphocyte function.

Body condition score at calving did not affect adaptive immune responses.

There is a perception that thin cows are less healthy than fatter cows and, on this basis, are of welfare concern. The research undertaken in the compilation of this thesis indicates no relationship between BCS at calving and adaptive immune function. It could be argued that a wider BCS range would need to be investigated to completely rule out a relationship between calving BCS and early lactation adaptive immune function. However, the BCS range investigated here represented very thin to moderately conditioned cows and reflected the range of calving BCS evident in New Zealand dairy herds. At the very least, the research results support the lower allowable BCS of 3.0 that has been mandated in the New Zealand Code of Welfare for Dairy Cattle (Code of Welfare: Dairy Cattle 2014).

The extent of early lactation NEB, and associated serum NEFA, was not related to adaptive immune responses.

Despite the welfare concerns for low BCS cows, high BCS cows lose more BCS in early lactation and this greater NEB may pre-dispose them to a greater risk of disease. It had previously been reported that impairment of in vitro IgM and IFN-γ responses is proportional to BCS loss (Lacetera et al., 2005) and incubation of lymphocytes in high concentrations of NEFA and ketones reduces antibody responses (Lacetera et al., 2004, Wentink et al., 1997) and production of pro-inflammatory cytokines (Franklin et al., 1991, Lacetera et al., 2004, Sato et al., 1995, Ster et al., 2012, Suriyasathaporn et al., 1999, Suriyasathaporn et al., 2000). However, in this study, despite greater loss of BCS and higher serum NEFA concentrations in early lactation (Roche et al., 2013c), cows with high calving

BCS did not exhibit greater immune dysfunction than cows with lower calving BCS. Therefore other factors may be responsible for the increased immune dysfunction in over-conditioned dairy cows reported in previous studies.

On the basis of this research, there is no cause for changing the recommended calving BCS for mature cows or the minimum BCS allowed by the welfare code. However, the cows in this experiment were very well managed and were well fed pre- and post-calving. The level of energy intake pre-calving is reported to affect postpartum NEB, systemic inflammation and neutrophil function (Graugnard et al., 2012). Reduced DMI in the weeks preceding calving increases the risk of metritis and retained fetal membranes (Huzzey et al., 2007, LeBlanc, 2008) and low CMIR has also been associated with increased RFM and increased metritis (Thompson-Crispi et al., 2012). Therefore, an interesting follow up experiment to the current work would be to investigate the interaction between calving BCS and feeding level pre- and post-calving on adaptive immune function.

Periparturient immune dysfunction was apparent in antibody-mediated responses but not cell-mediated immune responses.

In Chapter 7, I was able to evaluate the effect of calving on the immune system's ability to respond to a prescribed antigen by comparing the CMIR and AMIR responses in mid-lactation with those immediately post-calving. My results reflect a dysfunctional immune system consistent with previous research. Although cell-mediated immune responses did not appear to be impaired at calving; in fact, the magnitude of DTH responses was increased by 38%, this increase can possibly be attributed to the additional priming provided by immunisation at calving. In contrast, AMIR responses to type-2 antigens were reduced by 31%. The majority of mastitis pathogens are extracellular; therefore, lower AMIR in early lactation likely contributes to increased incidence of clinical mastitis, as reported in Canadian herds by Wagter et al. (2000) and Thompson-Crispi et al. (2013). The reduction in AMIR is also consistent with the relaxation of immunity to nematodes reported in many species during late gestation and early lactation (Barger and Gibbs, 1981, Houdijk, 2008, Kloosterman et al., 1985), although, there was no effect of BCS on immunity like that reported in sheep, which have lower worm burdens if they have greater adipose reserves in late gestation (Valderrábano et al., 2006, Valderrábano and Uriarte, 2003). The difference between my results and those reported in sheep may be due to the equalising effect of NEB, which is more extensive in dairy cows than in sheep: while high BCS cows have greater reserves, NEB induces a reduction in leptin such that the perceived availability of energy would be similar between low and high BCS cows in early lactation (Kokkonen et al., 2005).

Maintenance of CMIR, while AMIR is decreased, is opposite to what is expected during NEB. The physiological state of a transition cow has been likened to that of humans and rodents undergoing severe protein-energy malnutrition (Goff, 2008); however, in protein-energy malnutrition, CMIR is the most impaired response and AMIR is either maintained or elevated (Abrams and Miller, 2011). Decreased leptin, as occurs during negative energy balance or when adipose reserves are limited, usually leads to a decrease in CMIR. However, leptin acts on naïve T-lymphocytes rather than memory T-lymphocytes (Lord et al., 1998). Therefore, in experiment two, reduced leptin in early lactation may have reduced AMIR because the antigen was novel, whereas CMIR was unaffected because the cows had already been exposed to *C. albicans* in mid-lactation and already had memory T-lymphocytes for *C. albicans*. Consequently, the DTH response may have been able to proceed without the requirement for leptin to support activation of naïve T-lymphocytes. Future research could investigate the role of leptin in secondary AMIR and CMIR responses.

Concluding remarks

This study offers convincing evidence that BCS at calving, and the associated degree of NEB does not affect adaptive immune function in early lactation. Cows calving within the BCS range examined (between 3.5 and 5.5) did not differ in AMIR or CMIR; therefore, the results support the current recommendations regarding optimal calving BCS for mature cows, and support the minimum BCS standards allowed by the welfare code. An investigation of the interaction between calving BCS and feeding level during the transition period would confirm whether or not the lack of BCS effect reported here is influenced by feeding level.

Chapter 9. References

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Appendix 1: Manuscript published in the Proceedings of the New Zealand Society of Animal Production, Napier, June 2014

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Induction of delayed-type hypersensitivity reactions to Candida albicans in pasture-based dairy cows

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Abstract

Delayed-type hypersensitivity (DTH) reactions to Candida albicans are regularly used as an in-vivo indicator of cell-mediated immune function in cattle. Prior immunisation against C. albicans ensures a large clone of antigen-specific memory T cells, resulting in larger DTH responses and positive responses from a greater proportion of cows. Previous experimental results indicate that natural exposure to C. albicans is minimal in cows housed indoors and does not interfere with the interpretation of DTH responses to C. albicans in pre-immunised cows. However, housed cows may have different natural exposure to C. albicans than cows grazing fresh pasture outdoors. To determine if C. albicans is an appropriate antigen for inducing measurable DTH responses in pasture-based cows, DTH responses to C. albicans were assessed in ten cows pre- immunised with C. albicans and ten unimmunised control cows. A greater proportion of immunised versus unimmunised cows mounted a positive DTH response (90% vs. 70%), and responses were larger ($P<0.01$) and more variable ($P<0.05$) in immunised cows. Maximum relative double skinfold thickness increased 4.3 mm (±0.7; SEM) and 1.4mm (±0.2) in immunised and unimmunised cows, respectively. The results of this experiment suggest that pasture-based dairy cows in New Zealand have a low background exposure to C. albicans and pre-immunisation with C. albicans is required when using DTH responses as an indicator of cell-mediated immune function in pasture-based cows.

Keywords: Candida albicans; delayed-type hypersensitivity (DTH); dairy cow; pasture-based; housed; cell-mediated immune response (CMIR)

Introduction

Selection for production traits with little or no emphasis on health traits has led to an increase in the incidence of disease in dairy herds (Oltenacu & Broom, 2010). Development of methods to assess immune response phenotypes in pasture-based NZ dairy cattle will allow selection pressure to be placed on health traits, and when used in combination with selection for important production traits, improve productivity, health & welfare in dairy herds. Cell-mediated immunity is crucial to protection against viruses and other intracellular parasites (Baldridge & Ward, 1997). Delayed-type hypersensitivity (DTH) reactions are localised cell-mediated immune responses (CMIR), mediated primarily by antigen-specific CD4+ T-cells which act to recruit macrophages to the reaction site. DTH reactions generally occur in response to intracellular pathogens (Baldridge & Ward, 1997, Kobayashi et al. 2001) and are marked by a hard swelling at the site 24-72 hours after antigen exposure (Rosenstreich, 1993). A DTH response only occurs if the animal has been exposed to the antigen previously and has memory T-cells specific for the antigen (Rosenstreich, 1993).

Delayed-type hypersensitivity is an accepted in-vivo measure of CMIR in many species, including humans, rodents and pigs (Hessing et al. 1995, Martin et al. 2008, Neuvonen & Salo, 1984). To assess CMIR in cattle, DTH responses to the intracellular yeast Candida albicans have been used (Heriazon et al. 2011). Importantly, the use of C. albicans does not interfere with the diagnosis of bovine infectious diseases, such as tuberculosis (Heriazon et al. 2009a). C. albicans is a common commensal of many mammals and it has been reported that dairy cows produce a mild DTH response to C. albicans without priming suggesting previous natural exposure (Heriazon et al. 2009a). To minimise the effects of individual variability in background exposure, most DTH testing protocols involve priming the test animals by immunising with C. albicans prior to DTH testing to generate an expanded pool of antigen-specific T cells. Immunised animals respond more consistently and display larger responses than cows that have not been immunised (Heriazon et al. 2009a).

Measurement of DTH responses to C. albicans has been used successfully in Canadian and Irish dairy herds to assess CMIR (Begley et al. 2009, Heriazon et al. 2009a, Mallard, 2007) but has not been investigated in NZ dairy herds. Cows assessed in both the Canadian and Irish dairy herds spend a large proportion of their time in housed environments. This is in contrast to NZ dairy herds which are predominantly pasture-based and not housed for extended periods of time. There has been limited research about the prevalence of C. albicans in dairy-farm environments because it is not an important cause of disease (Heriazon et al., 2009, Richard et al., 1980); however, it is plausible that housed cows are exposed to different pathogens and have different commensal micro-flora to their pasture-based counterparts. For example, cows that are permanently housed have a higher prevalence of digital dermatitis than cows with access to pasture (Holzhauer et al. 2012, Laven & Holmes, 2008), and the prevalence of mastitis-causing pathogens is different in pasture-based versus housed cows (Laven & Holmes, 2008).

Differences in pathogen and commensal micro-flora exposure among farming systems may also extend to exposure to C. albicans. Yeast are isolated from a lower proportion of milk samples submitted for bacteriological culture in New Zealand (0.3%; Petrovski et al., 2011), than in other countries (1.3% to 25%; Casia dos Santos & Marin, 2005, Hayashi et al., 2013, Moretti et al., 1998), indicating that exposure to C.albicans might be lower in New Zealand herds. If natural exposure to C. albicans is not consistently low in pasture-based cows then background exposure would need to be determined prior to priming with antigen and subsequent DTH testing, or an alternative antigen used to elicit the DTH response. The primary objective of this experiment was to determine the magnitude of background DTH responses to C. albicans in New Zealand pasture-based dairy cows and ascertain whether C. albicans is a suitable antigen to elicit measurable DTH responses for assessment of immune responsiveness on farm.

Materials and Methods

The experiment was conducted at the DairyNZ Lye Farm, Hamilton (37°76'S 175°37'E, 45 m above sea level), during November and December 2010. All procedures were approved by the Ruakura Animal Ethics Committee, Hamilton, New Zealand, in accordance with the New Zealand Animal Welfare Act 1999.

Animals and immunisation

Twenty healthy, mid-lactation dairy cows of mixed age and breed were selected from the Lye Farm research herd (DairyNZ Ltd, Hamilton, New Zealand). The Lye Farm herd is milked twice per day and grazes a ryegrass and white clover pasture, with pasture silage and maize silage offered when pasture is limited. . The cows were run together in their normal herd, and no supplementary feed was offered, for the duration of this experiment. Average milk production at the time of experiment was 22.3kg per cow per day. Cows were grouped on body condition score (BCS) and then cows within each BCS group were randomly assigned to one of two experimental groups; immunised (n=10) or control (n=10). For the immunised group, the mean age was 4.1 ± 1.6 yr (mean ± SD) and mean BCS was 3.8 ± 0.4 (mean ± SD; 1-10 BCS scale, where 1 is emaciated and 10 obese; Roche et al. 2004). For the unimmunised group, the mean age was 4.6 ± 1.9 yr and the mean BCS was 3.8 ± 0.3.

The immune testing protocol involved immunisation on d0 and d14, followed by DTH testing on d21, as described by Heriazon et al. (2009b). Cows in the immunised group received an intramuscular (IM) injection of 0.5 mL of phosphate-buffered saline (PBS, pH7.4) containing 0.5 mg of type 1 antigen C. albicans (CaWC, My15 crude whole cell; Greer Laboratories Inc, Lenoir, NC, USA) and 0.5 mg of adjuvant (QuilA, 848051, Brenntag Biosector A/S, Frederikssund, Denmark) on D0 and D14. The injection also contained 0.5 mg of type 2 antigen; either hen egg-white lysozyme (HEWL) or human serum albumin (HSA). Immunisation with HEWL (n=5) or HSA (n=5) did not significantly influence DTH responses (P>0.05), therefore DTH responses from these cows were combined (immunised group, n=10) for analysis. Cows in the control group received an IM injection of 0.5mL PBS on day 0 (d0) and d14 (n=5), or received no injection (n=5). Immunisation with saline or receiving no immunisation did not significantly influence DTH responses (P>0.05), therefore DTH responses from these cows were combined (unimmunised group, n=10) for analysis.

Delayed-type hypersensitivity

The DTH skin test was performed on all cows on d 21, in the caudal skin folds on either side of the tail, as described previously (Heriazon et al. 2009a, Hine et al. 2011). Briefly, 0.1 ml PBS alone (control) or containing 0.1 mg of CaWC (test) were injected intradermally into the skin fold on opposite sides of the tail. Double-skin-fold thickness (DSFT) measures (3 repetitions), were taken at both test and control sites, prior to injection (0 h) and 7, 24 and 47 h after injection, using a spring loaded calliper (Harpenden, Mentone Educational Centre, Victoria, Australia).

Hine et al, (2011) defined a positive DTH response to CaWC as an increase in DSFT of >1mm at the test site relative to the control site. A similar criterion was used to define positive DTH responses in the current study. The number of cows that exhibited a positive response was calculated for each experimental group. The responses of all cows were included in statistical analyses, regardless of whether they met the threshold for a positive response.

Statistical analyses

For analysis, responses to DTH testing were expressed as the change in log of (DSFT at test site/DSFT at control site) from 0 h, as described by Hine et al. (2011):

$$\text{Response} = \log(T_h/C_h) - \log(T_0/C_0)$$

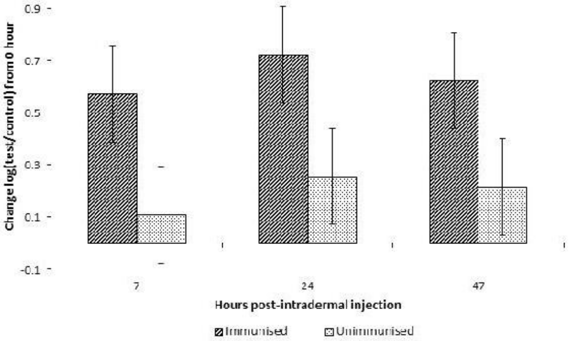
Where T_h = mean test DSFT at time h; T_0 = mean test DSFT at 0 h; C_h = mean control DSFT at time h; C_0 = mean control DSFT at 0 h; and time h was 7, 24 or 48 h. This response variable was used for all statistical tests. Responses were analysed in SAS® software (version 9.3, SAS Institute, Cary, NC, USA), using mixed models approach to repeated measures with time, treatment, and their interaction included as fixed effects. Tukey's test was used for pairwise comparisons. F-tests were used to determine whether the variability of DTH responses differed between immunised cows and unimmunised cows, and between

24 and 47 h for immunised cows. Pearson product-moment correlation coefficients were computed between 0 h DSFT of the test fold and maximum relative DSFT, and between 24 and 47 h for immunised cows. Significance is declared if P<0.05.

Results

All cows had increased DSFT at the test site relative to the control site following the CaWC injection at 0 h. Seven of the ten unimmunised cows and nine of the ten immunised cows produced a positive DTH reaction. No positive reactions at control injection sites were observed. The responses observed at the test sites were limited to swelling of the caudal fold, with no apparent changes to the skin surface of any injection sites.

Figure 1 Delayed-type hypersensitivity (DTH) response to Candida albicans whole-cell, in dairy cows previously sensitised via immunisation (, n=10), and in unimmunised cows (, n=10). Responses are expressed as change in the log of (double skin fold thickness at the test site/doubles skin fold thickness at the control site) from 0 h, measured at 7, 24 and 47 h post-injection. Error bars indicate 95% confidence intervals. Responses in immunised cows were significantly larger than responses in unimmunised cows at every time point (P<0.05).



Mean maximum relative DSFT increases were 4.3 mm (±0.7; SEM) and 1.4 mm (±0.2) for immunised and unimmunised cows, respectively. Responses were larger in immunized cows at all time-points relative to unimmunised cows (Fig. 1). The increase in relative DSFT from 0 h to 7 h was five-fold greater in immunised cows than in unimmunised cows. The maximum response was more variable in immunised relative to unimmunised cows (P<0.01).

The average DTH response for both groups peaked at 24 h but was not significantly greater than responses observed at 47 h (immunised: P=0.649; unimmunised: P=0.989). There was no difference in variability of 24 and 47 h responses in immunised cows (P=0.788). Responses at 24 and 47 h were highly correlated in immunised cows (r^2=0.79, P<0.01). There was no correlation between maximum relative DSFT increase and 0 h test DSFT in the immunised group (r^2=0.08, P>0.05).

The time-dependent DTH responses for individual animals in each group depicted in Fig. 2 illustrate the increased variability of response magnitude in immunised cows. In the immunised group, the maximum relative DSFT increase was at 24 h for six cows, 47 h for three cows, and 7 h for one cow. In the unimmunised group, the maximum relative DSFT increase was at 24 h for four cows, 47 h for five cows, and 7 h for one cow (Fig.2).

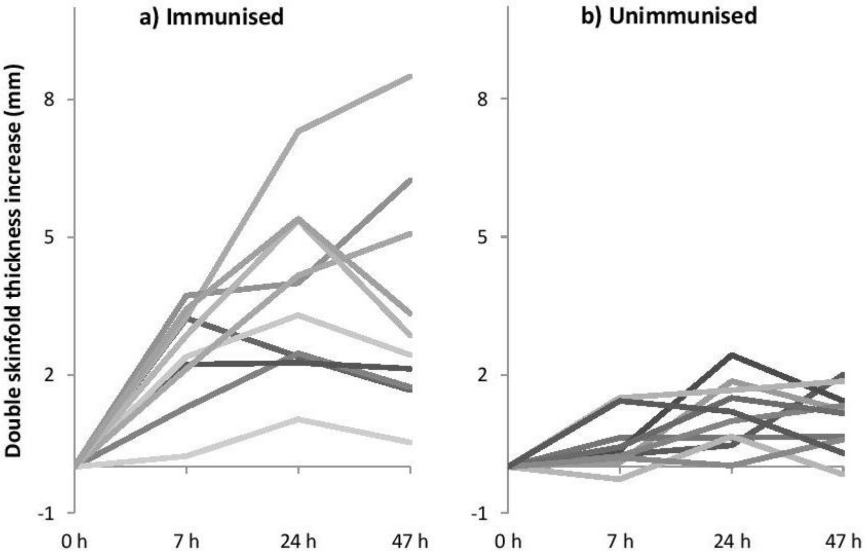
Discussion

Delayed-type hypersensitivity reactions to C. albicans have been used extensively in North-American dairy cattle to assess cell-mediated immune responsiveness (Heriazon et al. 2011, Heriazon et al. 2009a, Heriazon et al. 2009b, Hine et al. 2011). Studies have shown that these largely housed dairy cows produce mild DTH responses to C. albicans as a consequence of natural exposure. Nevertheless, previous experiments have included priming the immune system by immunising with C. albicans prior to DTH testing to minimise the effects of individual variation in natural exposure and to stimulate greater DTH responses. Natural C.albicans exposure of cows in New Zealand's pasture-based dairy herds may be different to their largely housed counterparts. Therefore, the objectives of this experiment were to investigate both background and pre-primed DTH responses of pasture-based dairy cows and to determine if DTH testing using C. albicans can be used successfully as an indicator of CMIR in pasture-based cows.

Background DTH responses to C.albicans

Results of the current study demonstrate that the pasture-based NZ dairy cows in the herd tested had low-level background DTH responses to C. albicans indicating low-level natural exposure to C. albicans. Although several unimmunised cows produced mild DTH responses, responses measured in immunised cows were much larger. Furthermore, responses were five-fold larger in immunised cows relative to unimmunised cows at 7 h post-injection. This contrasts with the results of Heriazon et al. (2009a), who reported that the magnitude of responses at 6 h were similar in immunised and unimmunized cows. Heriazon et al. (2009b) reported that cows immunised twice exhibited skin lesions such as pustules, crusting and dermatitis at the C. albicans injection site. In the current experiment, all DTH responses were limited to swelling of the caudal fold, with no apparent changes

Figure 2. Delayed-type hypersensitivity responses to Candida albicans of individual dairy cows that were previously immunised with C.albicans (a) or unimmunised (b). Responses are expressed as relative increase in double skin fold thickness from 0 h relative to the control site, at 0, 7, 24 and 47 h post-injection.



to the skin surface in immunised cows. These results indicate that the natural exposure to C. albicans was lower in the pasture-based cows tested here relative to housed cows and, therefore, it is likely not necessary to determine background DTH prior to priming with C.albicans. The number of cows used to determine background DTH in this experiment was small (n=10), and the results pertain to a single farm. Testing of background DTH to C.albicans in other pasture-based herds, in varied geographical locations and operating under various management systems will be required to confirm that natural exposure to C.albicans is typically low in pasture-based herds in New Zealand.

Pre-primed DTH responses in immunised cows

Results demonstrated that immunisation prior to conducting DTH testing successfully enhanced DTH responses. A greater proportion of immunised cows produced positive DTH responses, and their responses were three-fold larger at 24 and 47 h than in unimmunised cows. Testing of unimmunised cows showed that background sensitivity to C. albicans was consistently very low, therefore, the high variability observed in DTH responses from immunised cows is likely due to individual differences in immune responsiveness, rather than from differences in previous natural exposure to C. albicans. These results confirm that C. albicans can be successfully used to elicit measurable DTH responses in pasture-based cows to rank animals based on their cell-mediated immune responsiveness.

Kinetics of pre-primed DTHs

In the current study, peak DTH response times varied among cows indicating that measurement at both 24 and 48 h may be better than either time-point alone. Delayed-type hypersensitivity reactions are characterised by a hard swelling at the site 24-72 hours post antigen exposure (Rosenstreich, 1993). While DTH responses at 7 h were significantly higher in immunised versus control cows, responses observed at 7 h are not expected to be indicative of true DTH responses but rather indicative of earlier-phase immune reactions (Black 1999; Hernández et al. 2005). Begley et al. (2009) identified 48 h after antigen exposure as the optimal time to assess DTH response. However, other researchers have measured DTH at both 24 and 48 h and found no significant difference between the magnitude of DTH responses at each time-point (Heriazon et al. 2011, Heriazon et al. 2009a, Heriazon et al. 2009b, Hernandez et al. 2003). Heriazon et al. (2009b) reported that cell infiltration to the DTH site following injection with C.albicans was similar at 24 and 48 h and concluded that the response observed at both time points represented a classic DTH reaction. Hernandez et al. (2003) suggested that using the average of 24 and 48 h or selecting the time-point with greatest variability between cows is appropriate when using DTH responses to classify cows. In this experiment, 24-h responses were highly correlated with 47-h responses, however, for individual cows the time of peak response occurred equally at 24 and 47 h and ranking of cows based on the magnitude of the

DTH response was variable between 24 and 47 h. Additionally, the variability was the same at 24 and 47 h. While measuring at 24 h would capture the peak response for most of the cows in the current experiment, our results suggest that DSFT should be measured at both time points when using DTH response to classify cows, as suggested by Hernandez et al. (2003).

Conclusion

Background exposure to C. albicans was minimal in the pasture-based New Zealand herd tested and DTH reactions to C. albicans can be successfully used as a measure of cell-mediated immune responsiveness. Cows produced only mild DTH responses to CaWC, when not pre-immunised with C. albicans. Furthermore immunised cows had a greater proportion of positive DTH responses, and produced significantly larger and more variable DTH responses than non-immunised cows.

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Title

Condition score at calving did not affect adaptive immune responses in early lactation.

Short title

Body condition score and immune function

Author names

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Abstract

The greater the body condition score (BCS) at calving, the more severe the postpartum negative energy balance (NEB). Metabolites reflective of the energy balance status of the cow, such as non-esterified fatty acids and ketone bodies, have been associated with impaired lymphocyte and neutrophil function in vitro. Therefore, we hypothesised that cows that calve at higher BCS may exhibit a greater degree of immune dysfunction than cows that calve at lower BCS. To test this hypothesis, the energy balance of healthy cows was manipulated during mid-lactation to generate groups with differing BCS at calving and antibody-mediated and cell-mediated immune responses were assessed. Calving BCS had no effect on the magnitude of antibody-mediated ($P = 0.93$) or cell-mediated ($P = 0.99$) responses. Our results indicate that BCS at calving and the associated degree of postpartum NEB does not influence the cow's ability to mount an effective adaptive immune response in vivo over the range of BCS tested.

Keywords

Transition; dairy; negative energy balance; *Candida albicans*; HEWL; HSA; DTH; BCS

Introduction

Dairy cows exhibit transient immune dysfunction during the period of transition between pregnancy and lactation, which contributes to a higher incidence and severity of infectious disease during the first few weeks of lactation (Mallard *et al.* 1998). For example, 50% of clinical mastitis occurs in the two weeks after calving (McDougall *et al.* 2007). In early lactation, voluntary food intake is insufficient to meet the energy and protein requirements of milk production so dairy cows mobilise fat and protein from body tissues, which elevates serum levels of non-esterified fatty acids (NEFA) and ketone bodies (Roche *et al.* 2009, Suriyasathaporn *et al.* 2000). These metabolites have been associated with impaired lymphocyte and neutrophil function in vitro (Ster *et al.* 2012, Suriyasathaporn *et al.* 2000) and with increased incidence of metritis and mastitis (Ospina *et al.* 2010). Based on these findings, it is hypothesised that cows that experience a greater negative energy balance (NEB) postpartum may have increased immune dysfunction.

Body condition score (BCS) at calving is positively correlated with postpartum NEB (Roche *et al.* 2009). Cows with a greater BCS at calving experience more severe NEB in early lactation and may exhibit a greater degree of immune dysfunction than low BCS cows. Reported associations between BCS at calving and incidence of infectious disease in early lactation have been inconsistent (Gearhart *et al.* 1990, Roche *et al.* 2009) and much of the research has been epidemiological and unable to distinguish causation or association. The objective of this study was to determine the effect of calving BCS on adaptive immune responses of cows during early lactation.

Methods

Sixty pasture-fed Friesian and Friesian-Jersey dairy cows between body condition 3.75 and 4.25 (1-10 BCS scale, where 1 is emaciated and 10 is obese; Roche *et al.* 2009) were allocated randomly to BCS treatment groups. Feeding levels were manipulated so that mean BCS at calving was 3.4, 4.6 and 5.4 for the low, medium and high BCS groups, respectively. For a detailed description of the experimental set up, see Roche *et al.* (2013).

Immune responses were measured before allocation to BCS treatment group, coinciding with mid-lactation (baseline), and immediately postpartum (day 0 (d 0) was the day of calving). On both occasions, cows were immunised on d 0 and 14, with *Candida albicans* and *a* type-2 antigens (HEWL or HSA), known to induce polarised cell-mediated immune responses (CMIR) and antibody-mediated immune responses (AMIR), respectively (Cartwright *et al.* 2012). To allow assessment of both primary and secondary AMIR postpartum, a cross-over design was used. Half the animals in each BCS treatment group recieved HSA at baseline testing and HEWL at postpartum testing and the other half, HEWL at baseline testing and HSA at postpartum testing. Blood samples were collected by caudal venepuncture on d 0, 14, and 21 and serum prepared.

As an indicator of AMIR, antigen-specific IgG for HEWL and HSA were measured using previously described ELISA methods (Cartwright *et al.* 2012) and expressed as change from d0.

As an indicator of CMIR, delayed-type hypersensitivity (DTH) reactions to *C.albicans* were assessed on d 21 of baseline testing and on d 8 of post-partum testing using a previously described skin-testing protocol (Hine *et al.* 2011, McGowan *et al.* 2014). Responses to DTH testing were expressed as change (DSFT at test site/DSFT at control site) from 0 h.

Specific antibody and DTH responses were examined based on value at each time point and area under the curve (AUC) between time points. Data were $\log_{10}$-transformed and responses were analysed using a mixed models approach to repeated measures (Proc Mixed, SAS 9.3) with time, antigen, BCS, and their interactions as fixed effects. Response AUC was analysed using two-way ANOVA for the effects of antigen, BCS, and their interaction, including AUC from baseline as a covariate. Tukey's test was used for pairwise comparisons. Results are presented as least-squares means and standard errors (SE). Significance is declared if $P<0.05$.

Results and discussion

The objectives of this study were to investigate the hypothesis that differences in NEB associated with differing BCS at calving influence adaptive immune function in early lactation cows. To our knowledge, this is the first in vivo investigation of postpartum adaptive immune responses in pasture-fed dairy cows, with imposed differences in calving BCS.

Within the range of BCS investigated, body condition score at calving did not affect the ability of pasture-fed cows to mount adaptive immune responses in-vivo. Differing levels of postpartum NEB were observed as a consequence of differing calving BCS, and the serum NEFA concentrations observed in these cows (Roche *et al.* 2013) were similar to those reported in periparturient cows with impaired lymphocyte secretion of both IgM and IFN-γ in vitro (Lacetera *et al.* 2005). Despite this, the effect of BCS on circulating antigen specific IgG antibody production (Fig. 1) and DTH responses (Fig. 2) was not significant (*P*= 0.93 and 0.99, respectively). Our results indicate that, for cows calving within the range of BCS investigated, their ability to mount an effective adaptive immune response in vivo was not influenced by BCS at calving and the associated degree of postpartum NEB, supporting the view that periparturient immune dysfunction is not a direct consequence of the level of postpartum NEB.

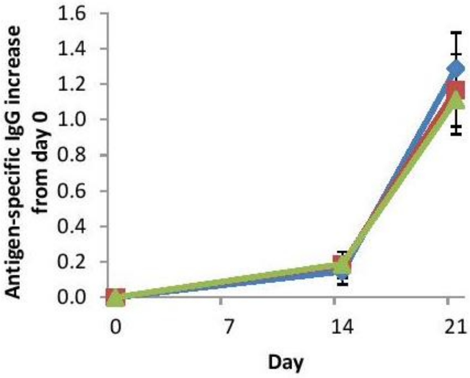

Figure 1. Least-squares means and SE of antigen-specific IgG antibody response to immunisation with type-2 antigen on d 0 and 14, in dairy cows with body condition score 3.4 ◆, 4.6 ■ and 5.4 ▲ at calving.

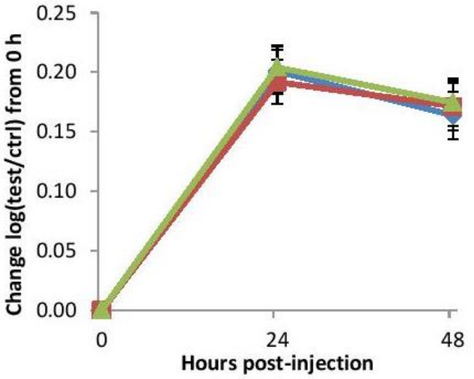

Figure 2. Least-squares means and SE of delayed-type hypersensitivity responses to *C.albicans* (whole cell), in dairy cows with body condition score 3.4 ◆, 4.6 ■ and 5.4 ▲ at calving.

Elevated serum NEFA and β-hydroxybutyrate (BHBA) concentrations have been associated with impaired bovine lymphocyte function in vitro (Lacetera *et al.* 2004, Ster *et al.* 2012). However, reported associations between measures of NEB and immune dysfunction have been inconsistent. Nonnecke *et al.* (1992) reported that high concentrations of ketones and acetate did not affect in vitro IgM secretion when glucose concentration was physiologically relevant. Similarly, dietary-induced NEB in later lactation had an inconsistent effect on immune function (Ingvartsen and Moyes 2013).

Whole-blood assays to measure neutrophil function during the transition period show increased rather than decreased capacity for phagocytosis postpartum, and no clear relationship with serum NEFA and BHBA concentrations (Sander *et al.* 2011). These researchers attributed their unexpected results to the presence of opsonising factors in whole blood that are not present in purified preparations. Previous investigations of lymphocyte function have been largely based on in vitro testing using purified cell preparations. In the current study, the effects of BCS at calving on immune function were assessed in vivo, where the influence of other immune cells and proteins contribute to the observed response improving the biological relevance of the findings.

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