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Bovine Dendritic Cells & Their Interaction with *E. coli* O157:H7

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"O Lord God, when Thou givest to thy servants to endeavor any great matter, grant us also to know that it is not the beginning, but the continuing of the same unto the end, until it be thoroughly finished, which yieldeth the true glory."

from Sir Francis Drake's Prayer

May, 1587

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Declaration

I declare that this thesis and the experiments described herein are my own work, except where otherwise indicated. No part of this thesis has been submitted for a degree at this or any other university.

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Abstract

E. coli O157:H7 is the most important serotype of enterohaemorrhagic *E. coli* (EHEC) which is of concern to public health worldwide. As a common cause of haemorrhagic colitis, EHEC infection can progress to life threatening sequelae including haemolytic uraemic syndrome (HUS) in humans. Human infection rates are higher in Scotland than found in the rest of the UK. Cattle are asymptomatic carriers of EHEC and are an important reservoir from which disease outbreaks can spread. The terminal rectum has been indicated as a site of *E. coli* O157:H7 colonisation in the bovine intestinal tract. This is the location of numerous lymphoid follicles which contain dendritic cells (DCs) which are professional antigen presenting cells and important directors of immune responses. DCs are likely to come into contact with EHEC and therefore could be key in this location for enabling EHEC to colonise the bovine host. The first aim of this project was to characterise dendritic cells within the bovine intestinal tract at various anatomical locations, including the terminal rectum, using immunohistochemistry techniques. Following this, work to extract and further phenotype dendritic cells from terminal rectal tissues was undertaken. Finally, a widely-used bovine dendritic cell model was employed to generate dendritic cells from circulating blood monocytes. This model was utilised to investigate the interactions of dendritic cells with EHEC strains compared with responses to bovine enterotoxigenic (ETEC) and bovine commensal *E. coli* strains.

Early work identified that there are potentially numerous DCs within the bovine intestinal tissues and these cells were found in greater numbers at the terminal rectum. Protocols to extract and further characterise these cells were developed but proved inconsistent, with large variation between animals.

Using the monocyte derived dendritic cells (moDCs), differences were observed between immunological responses to challenge with *E. coli* O157:H7 strains and bovine pathogenic or commensal *E. coli* strains. Cytokine production, cell surface molecule expression, cell phenotype and viability as well as intracellular bacterial counts were compared. The data presented here shows that the bovine moDCs respond differently to EHEC strains when compared with commensal or pathogenic *E. coli* in several key areas. This has important implications for the responses of the bovine host to various *E. coli* strains. This work also indicates that dendritic cells could be central to these responses and if studied further still, may hold the key to reducing the colonization and persistence of *E. coli* O157:H7 in cattle, and subsequent human disease outbreaks.

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Abbreviations

A/E	Attaching/effacing
AB_5	A toxin with one A subunit and 5 B subunits
APC	Antigen presenting cell
APS	Ammonium persulphate
β-ME	β-Mercaptoethanol
bp	Base pairs
BSA	Bovine serum albumin
Caco-2	Epithelial colorectal carcinoma cell line-2
CD	Cluster determination
cfu	Colony forming units
cm	Centimetre
CNF	Cell Necrotising Factor
CO_2	Carbon dioxide
DAB	3,3'-Diaminobenzidine
DC	Dendritic Cell
dH_2O	Distilled water
DEFRA	Department for Environment, Food and Rural Affairs
DMEM	Dulbecco's minimal essential medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyriboneucleic acid
dNTPs: dATP, dCTP, dTTP & gGTP	Deoxyribonucleotide triphosphates: deoxyadenosine, deoxycytidine, deoxythymidine, deoxyguanosine
DTT	Dithiothreitol
eae/*eae*	*E. coli* attaching and effacing (intimin)/gene encoding intimin
EBVC	Easter Bush Veterinary Centre, The University of Edinburgh
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohaemorrhagic *E. coli*
Ehx	Enterohaemolysin
ELISA	Enzyme-linked immunosorbent assay
EM	Electron Microscopy
EPEC	Enteropathogenic *E. coli*
Esp	*E. coli* secreted protein
ETEC	Enterotoxigenic *E. coli*
FACS	Fluorescent activated cell sorting
FAE	Follicle associated epithelium
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate conjugated
FliC/*fli*C	Flagellin Protein/gene encoding flagellin

g	Grams/relative centrifugal force(standard coefficient of gravity – approx 9.81ms^{-1})
GALT	Gut-associated lymphoid tissue
Gb_3	Globotriaosylceramide
GIT/GI	Gastrointestinal tract/Gastrointestinal
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H & E	Haematoxylin and eosin
h	Hour
H-antigen (e.g. H7)	Flagellar antigen
HC	Haemorrhagic colitis
HCl	Hydrochloric Acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF	Holstein Friesian
HRP	Horse radish peroxidise
HUS	Haemolytic uraemic syndrome
IAH	Institute of Animal Health
IEC	Intestinal epithelial cell
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	interleukin
ILF	Individual lymphoid follicle
IMDM	Iscove's Modified Dulbecco's Medium
IVOC	*In vitro* organ culture
kg	Kilogram
L	Litres
LB	Luria Bertani
LEE	Locus of enterocyte effacement
LF	Lymphoid follicle
LM	Lamina Muscularis
LN	Lymph Node
LP	Lamina Propria
LPS	lipopolysaccharide
LT	Heat labile enterotoxin of ETEC
M	Molar
mAb	Monoclonal antibody
MALT	Mucosa associated lymphoid tissue
M cells	Membranous or microfold cells
MCI	Microbial and cellular interactions (Group at MRI)
MEM	Minimal essential media
MHC	Major histocompatability complex
min	Minutes
ml	Millilitres

MLN	Mesenteric lymph node
moDC	Monocyte-derived dendritic cell
MOI	Multiplicity of infection
MRI	Moredun Research Institute
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
NGS	Normal goat serum
NMS	Normal mouse serum
O.D.	Optical density
O-antigen	Somatic antigen (e.g. O157)
PAGE	Polyacrylamide gel electrophoesis
PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral blood mononeuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Ph.D.	Doctor of Philosophy
pg	Picograms
PP	Peyer's Patch
PRR	Pattern Recognition Receptors
RAJ	Recto-anal junction
RNA	Ribonucleaic acid
rpm	Revolutions per minute
RPMI	Roslin Park Memorial Institute
RT	Room temperature
SED	Sub-epithelial dome
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
SIRP	Signal regulatory protein
ST	Heat stable toxin of ETEC
STEC	Shiga-toxigenic *E. coli*
Stx	Shiga-like toxin (also verotoxin, see VT)
TAE	Tris-acetate-EDTA
TEMED	N'N'N'-tetremethylethylenediamine
Th1	T helper 1 cell
Th2	T helper 2 cell
Tir	Translocated intimin receptor
TLR	Toll-like receptor
TM	Tunica muscularis
TNFα	Tumour necrosis factor alpha
TR	Terminal rectum
TS	Tela submucosa
TTC	2,3,5 Triphenyl Tetrazolium chloride
TTP	Thrombotic thrombocytopaenic purpura

TTSS	Type three sectretion system
U	Units
UPEC	Uropathogenic *E.coli*
UV	Ultraviolet
VLA	Veterinary Laboratories Agency
v/v	Volume per volume
vol	Volume
V	Volt
VT	Verotoxin
VTEC	Verotoxigenic *E. coli*
VTRI	Veterinary Training and Research Initiative
WT	Wild type (bacterial strain)
w/v	Weight per volume
ZSF	Zinc salts fixative
Δ (delta)	in front of a gene name to denote that the gene has been deleted
µg	Microgram
µl	Microlitre
µm	Micrometer
°C	Degrees centigrade

Chapter 1 General Introduction

1.1 Escherichia coli

Escherichia coli are Gram negative facultative anaerobic bacilli of which numerous strains have been identified not only in diverse animal species but also at varied anatomical locations. As a predominant component of mammalian intestinal microflora, many strains of *E. coli* can be of benefit to the host. However, pathogenic strains can severely affect human and animal health. It is because of this potent health risk that *E. coli* has been the focus of much research over the years.

As a member of the Enterobacteriaceae family, *E. coli* are related to several other bacterial genera which are causative of a wide range of diseases. These strains include *Salmonella, Shigella, Yersinia, Citrobacter* and *Klebsiella.* The *E. coli* species is, however, of particular interest because strains can have very divergent roles. These may range from harmful pathogenic strains, causing serious, sometimes fatal, infections to commensal strains which provide important benefits to the host. Enteric disease, urinary tract infections and septicaemia meningitis can all be caused by pathogenic *E. coli* strains. Non-pathogenic strains, too, can cause pathology and disease if disruptions in the host defences allow access to the body. This can be the case in immunosuppression, or if the host is compromised by some other disease process or in some other way, and this is the case for many predominantly harmless organisms which can be termed opportunistic pathogens. *E. coli* strains that are pathogenic often carry specific arrays of virulence factors; the commensal types tend not to possess these. Pathogenicity factors can be essential in eliciting disease by disrupting host physiological mechanisms. Different strains employ different pathogenicity factors and can show host

and tissue specificity in the species and anatomical environments in which they have their main effects.

Pathogenic *E. coli* strains which predominantly cause gastrointestinal disease are shown in table 1.1. Of these strains, enterohaemorrhagic *E. coli* (EHEC), which is the focus of this study, are most closely related to enteropathogenic *E. coli* (EPEC) strains. Enterotoxigenic *E. coli* (ETEC) are also important intestinal pathogens of animals and man which have been utilised for comparative experiments within this work. The ETEC pathotype is introduced further in section 1.1.2.1.

1.1.1 Commensal *E. coli*

Commensal bacteria are found in the gastrointestinal tract, without causing harm, even though there are around 10^{11} bacteria per gram of intestinal contents. Indeed, commensal micro-organisms play vital roles in host metabolism, host immune system development and prevention of infiltration of pathogenic bacteria (Niedergang & Kweon, 2005). Intestinal commensal *E. coli* strains have been shown to provide nutrients for the host as well as playing a role in protection against colonisation and infection by pathogenic species of bacteria (Dam, 1935; Hudault *et al.*, 2001; Mann & Westblom, 1999; Price, 1988). Non-pathogenic *E. coli* strains have been investigated in human patients as potential pro-biotic organisms. For example the Mutaflor *E. coli* isolate (Nissle 1917), has shown effects on the intestine by reducing the colonisation by pathogenic strains and having some efficacy in the therapeutic regimens of inflammatory bowel disorders IBD and Crohn's Disease (Kamada *et al.*, 2008; Lodinova-Zadnikova *et al.*, 2003; Lodinova-Zadnikova & Sonnenborn, 1997; Trebichavsky *et al.*, 2010).

Pathotype		Clinical Disease Characteristics	Common O serotypes	Toxins
EHEC	Enterohaemorrhagic *E. coli*	Watery diarrhoea +/- blood, progressing to HC and systemic disease	O157:H7, 26, 111, 103, 145	VT, Enterohaemolysin
ETEC	Enterotoxigenic *E. coli*	Watery diarrhoea in naïve adults and weaning diarrhoea in young children	6, 8, 11, 15, 20, 25, 27, 78, 128, 148, 149, 159, 173	Heat labile (LT) and/or Heat stable (ST)
EPEC	Enteropathogenic *E. coli*	Watery diarrhoea in young children and animal species	26, 55, 86, 111, 114, 119, 125, 126, 127, 128, 142, 158	Possible proteins secreted into host cells
EAggEC	Enteroaggregative *E. coli*	Persistent mucoid diarrhoea predominantly in developing countries	3, 15, 44, 86, 111, 125	EAST1, Cytotoxin, PET
EIEC	Enteroinvasive *E. coli*	Watery diarrhoea	28ac, 29, 112ac, 124, 136, 143, 144, 152,159, 164, 167	Possible enterotoxin
DAEC	Diffusely adherent *E. coli*	Watery diarrhoea, often in children	O126:H27	None Described

Table 1.1 Diarrhoeagenic *E. coli* of animals and man. Data compiled from Nataro & Kaper 1998, and Scmidt, 2010. HC: Haemorrhagic Colitis, VT: Verotoxin, EAST1: Enteroagregative heat-stable toxin, PET: plasmid-encoded heat-labile toxin (1994; Nataro & Kaper, 1998; Schmidt, 2010).

1.1.2 Pathogenic *E. coli*

As already mentioned there are numerous pathogenic strains of *E. coli*, which cause disease in a variety of species and diverse body systems. This study focuses on *E. coli* O157:H7, and also utilises ETEC for comparative purposes, therefore, it is these two which will be introduced here. Pathogenic *E. coli* are reviewed by Kaper *et al*. 2004 in more depth than can be included in this study (Kaper *et al.*, 2004).

1.1.2.1 Enterotoxigenic *E. coli* (ETEC)

Enterotoxigenic *E. coli* are responsible for intestinal disease. They are a common cause of human diarrhoea world-wide, and are often the cause of what is commonly known as 'traveller's diarrhoea'. As many as 20 - 60 % of travellers new to areas such as those of the developing world may succumb to ETEC infection. Also a problem in these areas is the numerous cases of infant diarrhoea that occur at the time of weaning. Around 10 – 30% of cases of infant diarrhoea are thought to be caused by ETEC (Elliott *et al.*, 1999; Nataro & Kaper, 1998). Antibiotic resistance is increasing, but as the infective dose required for infection is high and exposed individuals develop mucosal immunity, hygiene has to be the cornerstone of prevention strategies (Nataro & Kaper, 1998). As important as ETEC is as a human pathogen, it was first discovered in piglets and remains a significant cause of death of neonates of this species (Wilson & Francis, 1986). ETEC also cause diarrhoea in cattle and are, in fact, the most common cause of diarrhoeal signs in calves in the first seven days of life (Moxley & Smith, 2010). ETEC are non-invasive but can colonise the GI tract and cause diarrhoea by binding to the host intestinal cells and causing secretion of fluids and electrolytes. ETEC adhere to intestinal epithelia by means of fimbriae, and the fimbrial type directs host

pathogenicity with K99 causing disease in calves, lambs and pigs (Cassels & Wolf, 1995). ETEC also produce toxins: a heat labile enterotoxin (LT) and a heat stable toxin (ST), which affects host cells by activating cellular enzymes (adenylate and guanylate cyclases respectively) and this initiates a sequence of events which induces large volumes of fluid to accumulate within the lumen of the gut (Gill & Richardson, 1980). This leads to profuse watery diarrhoea and ensuing dehydration which in young humans and animals can prove fatal if steps are not taken to maintain fluid intake.

1.1.2.2 Enterohaemorrhagic *E. coli* (EHEC)

Enterohaemorrhagic *E. coli* (EHEC) was first discovered as a cause of human intestinal disease in the 1980s when cases of haemorrhagic colitis (HC) were observed (Riley *et al.*, 1983). The serotype O157:H7 has since been indicated to be involved in outbreaks of disease worldwide and *E. coli* O157:H7 is the most widely documented member of the enterhaemorrhagic group and indeed the term EHEC is often used solely to describe this organism. The first human infection was traced back to fast food restaurants serving hamburgers in USA (Riley *et al.*, 1983), and was thought to have spread during the abattoir or meat preparation process from infected animals at slaughter. Not all cases have been caused by this route and in fact confirmed cases have been food- and water-borne as well as following direct contact with infected animals. However, animals as a reservoir of infection play an integral role in many outbreaks whether directly or indirectly.

The early signs of human EHEC infection include abdominal pain and watery diarrhoea. In some case this can progress to haemorrhagic colitis (HC) with significant blood loss through the intestinal wall (Riley *et al.*, 1983).

Some patients, in particular young children and the elderly may be affected by more serious sequelae such as Haemolytic Uraemic Syndrome (HUS) (Griffin & Tauxe, 1991). HUS can be characterised by microangiopathic haemolytic anaemia, thrombocytopaenia and acute renal dysfunction or failure. The effect of VT acting on the blood vessel endothelial cells in particular within the kidney is the cause of these life-threatening sequelae (Karmali *et al.*, 1983). Neurological symptoms and disorders can also result from EHEC infection with or without a preceding diarrhoeal episode. Thrombotic thrombocytopenic purpura (TTP), which presents as a neurological process is usually observed in adult patients (Pickering *et al.*, 1994).

Symptomatic treatment is at present the only route open to the medical profession, as there is no specialised treatment for human EHEC infection. Fluid therapy and generic treatments for intestinal disorders and damage are commonly used and dialysis when the severity of kidney damage warrants this. No adequate studies have been carried out into the use of antibiotics preventing the progression of disease in EHEC infection, although some reports suggest an effect when used early in treatment. (reviewed in (Nataro & Kaper, 1998)). Successful treatment and control methods for this severe and sometimes fatal condition would undoubtedly be of great benefit for human health. As vaccines can in some cases be used in the face of a disease outbreak as treatment (for example influenza), a full understanding of the immune responses involved and work towards a vaccine strategy in the animal host could also prove beneficial to human treatment regimes.

1.2 *E. coli* O157:H7 Epidemiology

Although *E. coli* O157:H7 is the serotype most commonly found worldwide (Gyles, 2007), other EHEC serotypes have also been found in limited geographical areas. For example, *E. coli* O157:H- (sorbitol-fermenting) isolates were discovered following outbreaks of human disease only in continental Europe until the same organism was recently found to be the cause of outbreaks in Scotland (Karch & Bielaszewska, 2001).

Host factors are likely to play an important role in disease progression following EHEC infection, as a wide range of outcomes have been observed. The more serious sequelae occur with increased regularity in children and elderly patients. Healthy adults may only suffer with watery diarrhoea, and it has even been known for these patients to be asymptomatic (Rios *et al.*, 1999; Silvestro *et al.*, 2004; Wilson *et al.*, 1996). Most human outbreaks of disease have been linked to bovine hosts and consumption by humans of faecally- contaminated goods such as undercooked meat, unpasteurised milk, vegetables, fruit juices or drinking water (Besser *et al.*, 1993; Borczyk *et al.*, 1987; Licence *et al.*, 2001; Morgan *et al.*, 1988; Riley *et al.*, 1983; Swinbanks, 1996; Yarze & Chase, 2000). In the UK, human contact with infected farm animals or environments is by far the most common route of EHEC infections, and this occurs with a greater frequency than food-borne infection (Locking *et al.*, 2001; O'Brien *et al.*, 2001). Wild animals may be important in the spread of EHEC between farms via contact with livestock (discussed further in section 1.4). Although numerous hosts have been found for *E coli* O157:H7; cattle are the reservoir of infection of most concern for human health (Kassenborg *et al.*, 2004; Locking *et al.*, 2001; O'Brien *et al.*, 2001). This is of particular importance in the UK where prevalence of EHEC in cattle is

estimated to be around 4.7% in England and Wales and as high as 8.6% in Scotland (Paiba *et al.*, 2002; Synge & Paiba, 2000) (Fig.1.1.), compared to just 2% found in the herds in Japan, Australia and the USA (Cobbold & Desmarchelier, 2000; Faith *et al.*, 1996; Miyao *et al.*, 1998). The data for incidence of human infection follows the trend of levels of cattle prevalence as Scotland has consistently higher numbers of cases than the rest of the UK (Locking *et al.*, 2006; Lynn *et al.*, 2005). Clearly, the carriage of EHEC by cattle has a significant impact on public health. The number of outbreaks is increasing each year and so there is a real need to research all aspects of *E. coli* O157:H7 in the bovine host.

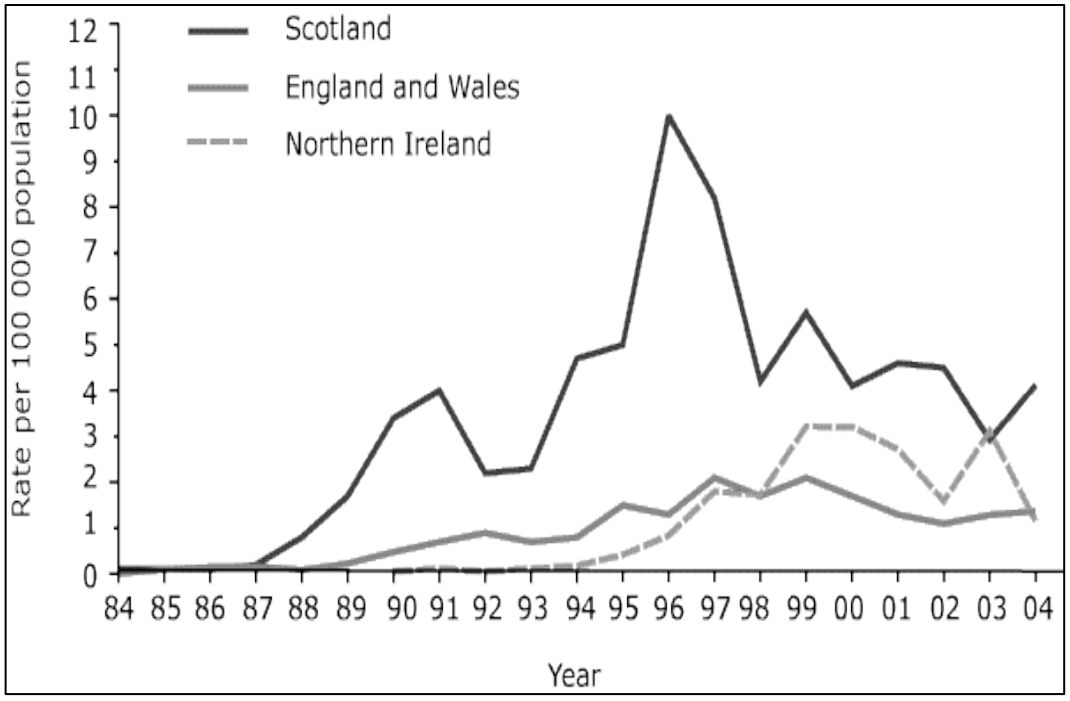


Fig. 1.1 Prevalence of *E. coli* O157:H7 in areas of the UK. (Adapted from Locking et al, 2006: data collated from Health Protection Scotland and Health Protection agencies). Historically cases of *E. coli* O157:H7 have been consistently higher in Scotland than in the rest of the UK.

1.3 Control of human EHEC infections

There is no specific treatment for established EHEC infection in humans, as mentioned earlier, and so prevention of infection is likely to be of greatest benefit to public health. During an outbreak, *E. coli* O157:H7 can spread easily and quickly between patients if stringent hand and food preparation hygiene are not observed. The number of organisms required to cause disease is thought to be small. A couple of studies have addressed this area, one concluded that the infective number of organisms is in the region of 20 - 700 (Tuttle *et al.*, 1999), and another proposes that the number of bacteria required to cause infection is less than 100 (Willshaw *et al.*, 1994). Thus a reduction of EHEC in environmental and animal reservoirs should be the cornerstone of any prevention strategy. Targeting the bovine host to reduce its impact as an important reservoir of infection could in turn reduce the number of human outbreaks. Basic hygiene methods, in particular concentrating on drinking water, feed and pens could go some way to reducing human disease (Dodd *et al.*, 2003; Garber *et al.*, 1999; LeJeune *et al.*, 2001; Smith *et al.*, 2001). Asymptomatic carriage of *E. coli* O157 by humans is difficult to estimate; it has been observed infrequently during outbreaks however it could be a more widespread event because stool samples are rarely collected from people who do not show clinical symptoms of disease.

E. coli O157:H7 has been found in several species of wild animals including animals that graze similar pasture to cattle herds (section 1.4). It is possible that wildlife could be contributing to the spread of this organism between herds or enabling its persistence within a herd. Reducing probable contact between cattle and wildlife is therefore an area to consider for control of this disease. Because EHEC has caused disease in humans following ingestion of contaminated meat, aiming to reduce carriage of the organism prior to

slaughter has been suggested. Research into dietary controls before slaughter, namely choosing a hay diet over grain, to adjust intestinal acidity has undergone some early testing but has not been proven in a large scale study (Diez-Gonzalez *et al.*, 1998; Hovde *et al.*, 1999).

Antimicrobial agents have been investigated with a view to control of EHEC (Elder *et al.*, 2008), however there are disadvantages with this method that could prove detrimental to both human and animal health. Antimicrobial treatment not only alters normal intestinal flora, having downstream effects on host digestion, but also selects for antibiotic-resistant bacterial strains.

Production of a vaccination for *E. coli* O157:H7 is also an interesting area of research and although several ideas have been put forward an effective vaccine is yet to arrive on the market (Ahmed *et al.*, 2006; Dean-Nystrom *et al.*, 2002; Konadu *et al.*, 1999; McNeilly *et al.*, 2008; Potter *et al.*, 2004). Development of an effective vaccine will only be possible with a solid understanding of host immune responses. In particular, the early interactions with host immune cells are an important area of investigation for the assessment of whether it is possible to harness or alter these interactions to reduce carriage and spread of O157:H7 or the pathogenic sequelae of colonisation. It is important to understand how *E. coli* O157:H7 can colonise and persist in the bovine host without causing overt clinical disease.

Mouse models have been used to assess the efficacy of bacteriophages to prohibit *E. coli* O157:H7 and while this has been successful, these methods have yet to be proven in the bovine host (Sheng *et al.*, 2006). Probiotic bacteria, most commonly *Lactobacillus acidophilus*, are suggested to reduce

numbers of pathogenic strains of bacteria within the bovine intestine. This *Lactobacillus* strain is widely obtainable in the United States as a feed additive (Elam *et al.*, 2003). Probiotic *E. coli* strains have also been assessed for their potential to reduce EHEC carriage in ruminants with some success (Hakkinen & Schneitz, 1996; Schamberger *et al.*, 2004; Schamberger & Diez-Gonzalez, 2004; Tkalcic *et al.*, 2003; Zhao *et al.*, 1998).

1.4 Animal hosts

Several animal hosts may also be sub-clinical carriers of *E. coli* O157:H7 and it has been discovered in several species to date. Ruminants are the most common carrier of EHEC, while cattle are the predominant source of human disease, goats (Bielaszewska *et al.*, 1997) and sheep (Ogden *et al.*, 2002) have also been implicated in outbreaks. EHEC has, in addition, been detected in other domestic species including pigs, dogs, cats and chickens (Beutin *et al.*, 1993; Doyle & Schoeni, 1987; Griffin & Tauxe, 1991; Nakazawa & Akiba, 1999) Wildlife species which often share the same grazing grounds as cattle too have been discovered as reservoirs of infection, and has been demonstrated for both deer and rabbits (Garcia & Fox, 2003; Garcia-Sanchez *et al.*, 2007; Pritchard *et al.*, 2001; Renter *et al.*, 2001). However cattle are deemed to be the principal reservoir hosts, and have on many occasions been confirmed as the source of organisms responsible for disease outbreaks (Besser *et al.*, 1993; Chapman, 2000; Riley *et al.*, 1983).

1.5 EHEC in cattle

The carriage of *E. coli* O157:H7 by cattle is of obvious importance to human outbreaks of disease; however cattle are largely asymptomatic carriers. Only very young calves have been shown to develop clinical symptoms of diarrhoea associated with the presence of *E. coli* O157:H7 (Kang *et al.*, 2004).

Other human EHEC serotypes however, such as O5, O26 and O118, have caused diarrhoeal symptoms in weaned cattle associated with the presence of massive attaching and effacing (A/E) lesions along the large intestine (Moxley & Smith, 2010; Stordeur *et al.*, 2000). The haemorrhagic symptoms seen in man are not replicated in the bovine situation except for in very young calves and similar to the human case, because there are no specific treatments, it is only possible to provide symptomatic care (Pearson *et al.*, 1999; Sandhu & Gyles, 2002; Stevens *et al.*, 2002; Wieler *et al.*, 1996). The above strains that cause bovine diarrhoea have their effect along the length of the bovine large intestine. *E. coli* O157:H7 however has been found to primarily locate to the recto-anal junction (RAJ) (Lim *et al.*, 2007; Low *et al.*, 2005; Naylor *et al.*, 2003). It was thought that O157:H7 could colonise multiple intestinal sites in the bovine host and early research suggested a general predilection for large intestinal areas (Brown *et al.*, 1997; Cray, Jr. & Moon, 1995; Dean-Nystrom *et al.*, 1998; Laven *et al.*, 2003). Later it was noted that large intestinal colonisation was found up to 16 days post infection, yet faecal shedding of the organism was still occurring at 34 days (Grauke *et al.*, 2002). More recently it has been shown that this extended shedding could be due to colonisation of the terminal rectum where the O157:H7 organisms were found to localise to the terminal intestine up to 5 cm proximal to the RAJ following experimental infection (Naylor *et al.*, 2003). Naturally colonised cattle have since been shown to demonstrate this colonisation pattern also (Lim *et al.*, 2007; Low *et al.*, 2005).

Bovine 'super-shedders' have been identified and it is suggested that they are largely responsible for the dissemination of *E. coli* O157:H7 in cattle herds and therefore are an important risk factor for human health (Chase-Topping

et al., 2007; Matthews *et al.*, 2006a; Matthews *et al.*, 2006b). Persistent rectal colonisation has been proposed to be an element of such 'super-shedders' (Cobbold *et al.*, 2007) The organism enters the environment coating the faecal matter as it passes through the RAJ and as such it allows infections to persist within herds.

The terminal rectal localisation of *E. coli* O157:H7 may indicate an important area for research into a targeted approach to reducing the carriage of this organism by cattle.

The last 5cm of bovine intestines are densely populated with lymphoid follicles (LF) which contain large numbers of immune cells (Parsons *et al.*, 1989). This population of immune cells is likely to come into contact with the *E. coli* O157:H7 bacteria (via several methods discussed further in section 1.10.1 and Fig. 1.5), and as such could be an important area of study with the aim to target control of this organism within the cattle herds. Although the terminal rectum is fairly accessible in terms of treatment, for example by topical application of an antimicrobial, this may however not be a practical solution. This method has been investigated under experimental conditions and has shown some ability to reduce shedding of the organism; however this is yet to be implemented or investigated in naturally-infected cattle (Naylor *et al.*, 2007).

The carriage and shedding into the environment of *E. coli* O157:H7 by cattle is an obvious risk factor for human infection. Cases of infection occur following contact with infected animals or their environments (Chapman, 2000) or following ingestion of contaminated food or water (Besser *et al.*,

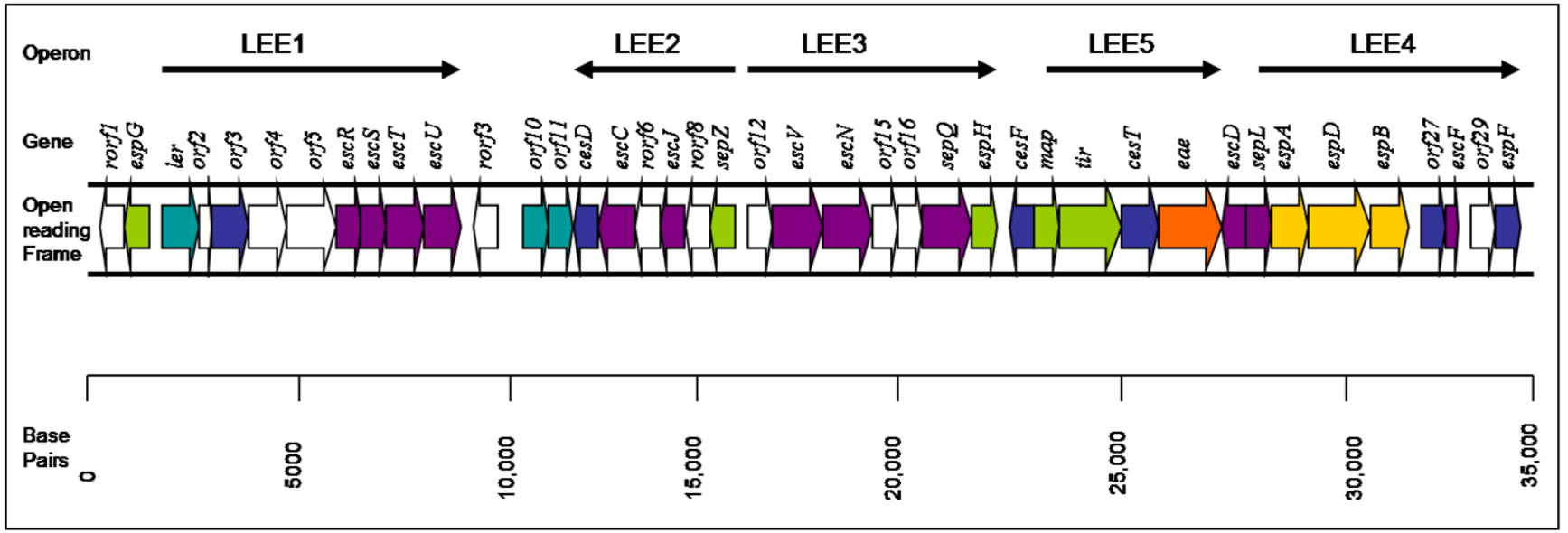
1993; Riley *et al.*, 1983; Yarze & Chase, 2000). Reducing the contamination of environments and the food chain by cattle is likely to have its effect by reducing the outbreaks of human illness and so is an important area of investigation.

1.6 Colonisation and virulence factors of *E. coli* O157:H7

The mechanisms of pathogenicity employed by EHEC are numerous and multifaceted. The ways in which EHEC has developed to evade host defences and cause disease are multitudinous. Key virulence factors of *E. coli* O157:H7 are Verotoxin (VT), a type three secretion system (TTSS), and flagella, although other factors also contribute.

1.6.1 Attaching and Effacing lesions (A/E lesions)

Attaching and effacing lesions (A/E lesions) are a histopathological finding in EHEC infections. Intestinal epithelium has its brush border microvilli destroyed and the bacteria are then found intimately attached to the epithelial cells. A pedestal-like structure can be seen and is formed by reorganisation of the host cell cytoskeleton (Frankel *et al.*, 1998). The genes which encode the A/E phenomenon are found within the Locus of Enterocyte Effacement (LEE). The LEE is likely to have arisen in *E. coli* through horizontal transfer from another bacterial species. This has been postulated due to its lower content of C and G than the rest of the *E. coli* chromosome. The A/E phenotype is determined by this LEE, which is a 35.5kbp pathogenicity island (McDaniel *et al.*, 1995). This A/E phenotype of EHEC is determined by LEE genes which are arranged as five operons (LEE1 to 5) (Fig.1.2). The LEE of EHEC O157:H7 necessitates other factors encoded in a



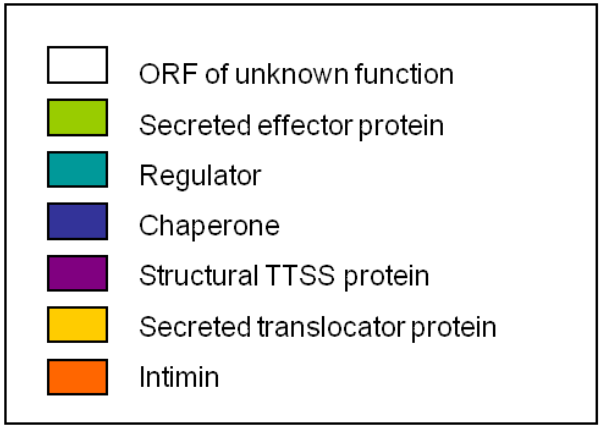


Fig.1.2. Organisation of the genes of the *E. coli* O157:H7 LEE

Adapted from Castillo *et al.* 2005 and Garmendia *et al.* 2005 (Castillo *et al.*, 2005; Garmendia *et al.*, 2005)

separate part of the genome to form the A/E lesions (Elliott *et al.*, 1999; McDaniel & Kaper, 1997). Several cytoskeletal proteins are directed to the EHEC attachment site including structural and effector proteins (Cantarelli *et al.*, 2002; Goosney *et al.*, 2001; Ismaili *et al.*, 1995). A translocated intimin receptor (Tir) encoded by EHEC requires *E. coli* secreted protein F_u ($EspF_u$) a proline rich actin assembly effector protein (Campellone *et al.*, 2004). $EspF_u$ or Tir-cytoskeleton coupling protein (TccP) is a novel, non-LEE encoded EHEC effector protein that has been shown to be similar to the host adaptor protein Nck (non-catalytic region of tyrosine kinase). EHEC-encoded Tir requires this TccP to mediate the EHEC neuronal Wiskott-Aldrich Syndrome protein (N-WASP) signalling (Campellone *et al.*, 2004; Garmendia *et al.*, 2004). TccP has an effect on the recruitment of α-actinin, actin-related protein 3 (Arp3), N-WASP and actin to the bacterial adhesion site. This condensation of F actin produces the effacement and intimate attachment and results in the characteristic pedestal formation.

1.6.2 TTSS

An essential part of the A/E lesion is the formation of a functional Type Three Secretion System (TTSS) (Jarvis *et al.*, 1995; Jerse *et al.*, 1990) (Fig.1.3). The TTSS is encoded by the *E. coli* secretory genes (*esc* genes) found at LEE1, 2 and 3 (Elliott *et al.*, 1998; Kaper, 1998; Perna *et al.*, 1998). Following contact of the bacterium with the host cell the TTSS can allow proteins to be directly inserted into the cytoplasm of the host cells. These proteins travel through the TTSS across both the bacterial and host cell membranes (Hueck, 1998). The complex through which secreted effector proteins reach the host cell cytoplasm is formed by bacterial secreted proteins EspA, B and D (*E. coli* secreted proteins A, B & D) which are coded for by LEE4. EspA is the main

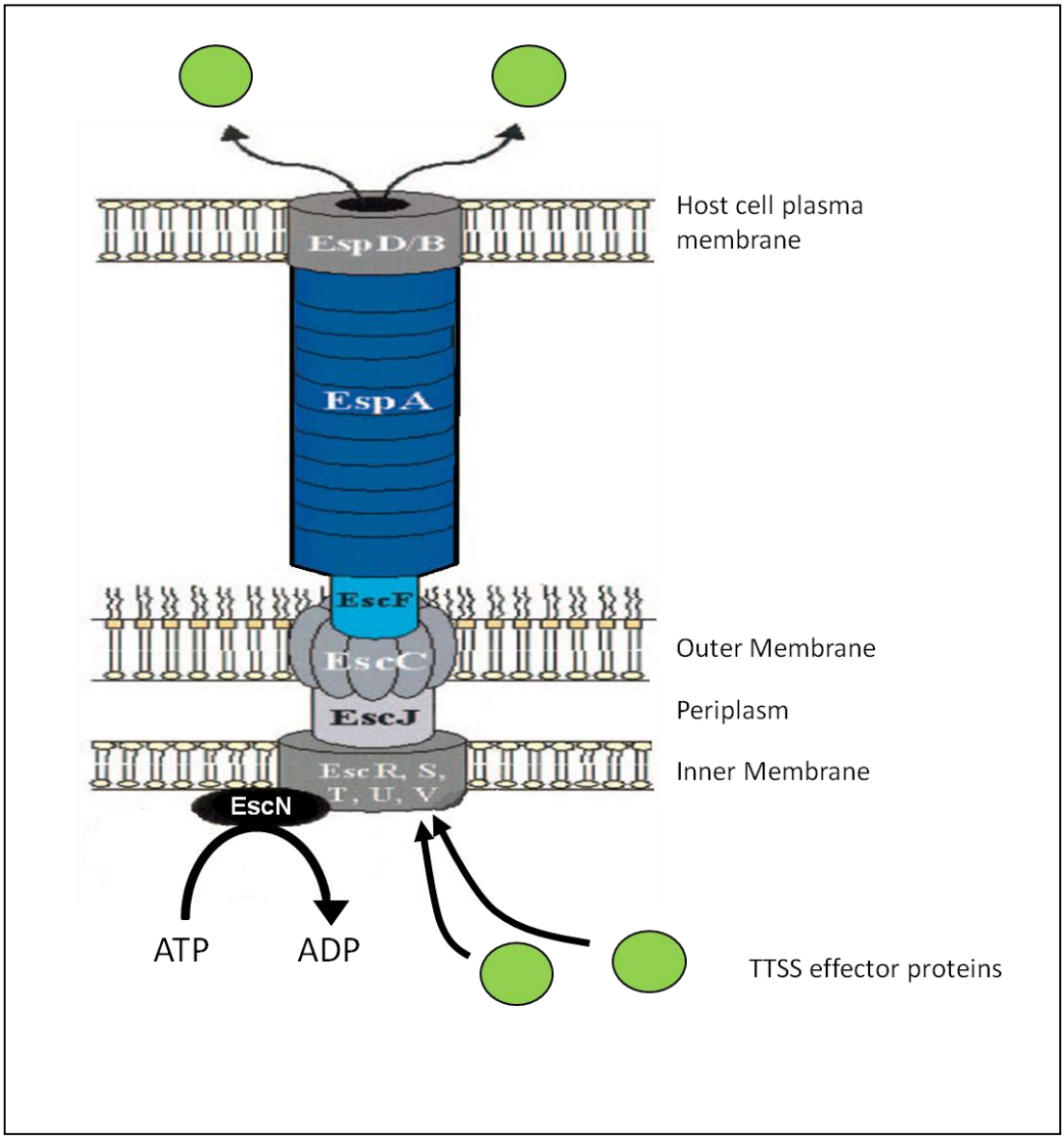


Fig. 1.3 Diagrammatic representation of the structure of the Type Three Secretion System (TTSS) of *E. coli* O157:H7. The tube-like arrangement of the TTSS facilitates its function to secrete effector molecules directly into the host cell cytoplasm. The long EspA structure protrudes from the bacterial outer membrane connected to the EspF needle complex. The effector molecules travel through this assembly eventually emerging through an EspB and EspD pore in the host cell plasma membrane. This apparatus is anchored to the bacteria via the basal body of EscR, S, T, U, & V proteins within the inner membrane, the transmembrane EscJ, and the outer membrane EscC ring. EscN provides energy for the TTSS by hydrolysis of ATP. Adapted from (Garmendia & Frankel, 2005)

structural protein and forms the needle of the TTSS. The proteins which form the pore are EspB and EspD (Delahay *et al.*, 1999; Garmendia *et al.*, 2005; Ogino *et al.*, 2006). Operon LEE5 encodes *eae* and *tir* genes. *Eae* encodes for intimin, an outer membrane protein whose receptor Tir (translocated intimin receptor) is inserted into the host cell and becomes part of the host cell membrane.

The role of the TTSS may well be more wide reaching than initially thought. Bioinformatic analysis of the *E. coli* O157:H7 genome has yielded several additional potential effector proteins and further studies confirmed that at least 39 of these are injected into the host cell (Tobe *et al.*, 2006) although functions are yet to be ascribed to them all.

To conclude, the TTSS has effects that are wide ranging and still to be fully defined. It plays a role in affecting the host cells to the likely benefit of the bacterium and knowledge of the methods by which it does this are likely to evolve as further work is carried out into the nature of the effector proteins.

The LEE also contains other smaller operons and ORFs (open reading frames) which encode for other proteins, many of which have roles in changing the host cell cytoskeletal structure. EspF (Crane *et al.*, 2001) and Map (mitochondrial associated protein) (Kenny & Jepson, 2000) are likely to interfere with transepithelial resistance and mitochondrial membrane potentials (McNamara & Donnenberg, 1998; McNamara & Basbaum, 2001). Map, EspH (Tu *et al.*, 2003) and EspB modulate the host cell skeleton and initiates filopodia. EspF also plays a significant role in the initiation of cell death (Crane *et al.*, 2001; Viswanathan *et al.*, 2004). EspG has been shown to

disrupt the microtubule network (Tomson *et al.*, 2005). A chaperone protein for Tir, CesT is also encoded (Abe *et al.*, 1999). There are several LEE-encoded effector proteins that have not as yet been assigned a function for example EspZ. There are other TTSS effector proteins that are non-LEE encoded, NleA, NleB, NleC, NleD, NleE, NleF and NleH (Deng *et al.*, 2004; Gruenheid *et al.*, 2004; Kanack *et al.*, 2005). Of these, NleA may inhibit host cell protein export (Kim *et al.*, 2007) and it is proposed that NleD is somehow involved in the colonisation of calves in the experimental environment. The exact role which it plays has not been established (Dziva *et al.*, 2004).

LEE1 encodes Ler, which regulates the expression of LEE-encoded operons. This regulator allows the TTSS to be expressed at the most useful time during the interaction between the bacterium and the host cells. Ler upregulates virulence factors (both LEE-encoded and non-LEE-encoded) (Friedberg *et al.*, 1999; Mellies *et al.*, 1999; Sanchez-Sanmartin *et al.*, 2001; Sperandio *et al.*, 2000). Quorum sensing may be a factor in the initiation of the transcription of the LEE-encoded genes (Sperandio *et al.*, 1999).

1.6.3 Tir & Intimin

Intimin is an adhesin which is central in the process of forming A/E lesions (Deibel *et al.*, 2001; Kenny *et al.*, 1997; Kenny & Finlay, 1997). A second method in which intimin is proposed to have a role in adherence is interaction with nucleolin, a host protein. This method is Tir independent (Sinclair & O'Brien, 2002). Intimin is a 94 - 97 kDa outer membrane protein which the bacterial cells use to become intimately attached to the host cells. The TTSS injects the Tir, intimin's primary receptor, into the host cell which enables this attachment to take place. Binding of intimin to Tir causes

Phosphorylation of Tir's C-terminal 170 amino acids which precedes actin polymerisation. Following intimin/Tir binding, the actin polymerisation which occurs forms the pedestal part of the A/E lesion (Cantarelli *et al.*, 2002; Goosney *et al.*, 2001). This intimate adherence allows bacterial microcolonies to form on host cells. Underneath the microcolonies on the cell surface aggregation of filamentous actin (F-actin) occurs which gives rise to effacement of the microvilli and formation of the pedestal like structures. Tir, as well as acting as a receptor for intimin also initiates this pedestal formation.

At present there has been found to be 14 different intimin types, which have been named using Greek letters α (alpha) to ξ (xi) (Adu-Bobie *et al.*, 1998; Agin *et al.*, 1998; Agin & Wolf, 1997; Jores *et al.*, 2003; Oswald *et al.*, 2000; Tarr & Whittam, 2002; Zhang *et al.*, 2002). *In vitro* organ culture (IVOC) experiments have shown that substitution of the intimin type will change the tropism of *E. coli* (Fitzhenry *et al.*, 2002; Hartland *et al.*, 1999; Phillips *et al.*, 2000). This may indicate a receptor independent of Tir such as the intimin γ attraction for nucleolin in *E. coli* O157:H7 (Sinclair & O'Brien, 2002). This suggests the possibility that these subtypes of intimin play a role in tissue and host specificity. A role for intimin-Tir binding has been found in the colonisation of ruminants. A/E lesions have been observed on both bovine and ovine intestinal tissues infected with EHEC, even though these hosts generally remain sub-clinical (Cornick *et al.*, 2002; Dean-Nystrom *et al.*, 1998; Naylor *et al.*, 2005a; Naylor *et al.*, 2005b; Woodward *et al.*, 2003).

1.6.4 Verotoxin (VT)

A key virulence feature of many bacteria is the ability to produce toxins. EHEC produces a toxin called verotoxin (VT), named for its potent ability to rapidly kill Vero cells. This toxin is also termed Shiga-toxin (Stx) as it is closely related to the toxin produced by *Shigella dysenteriae*. For these reasons EHEC are also known as Shiga-toxigenic *E. coli* (STEC) or Vero-toxigenic *E. coli* (VTEC). EHEC has acquired the VT genes via integrative bacteriophages (Konowalchuk *et al.*, 1977; Scotland *et al.*, 1983). Translocation of VT across the intestinal interface is responsible for serious human illness from intestinal, renal and CNS complications and can ultimately be fatal (Frankel *et al.*, 1998). VT mediates disease processes in several ways. Locally, diarrhoea follows from the disruption of epithelial integrity, and this can progress to haemorrhagic diarrhoea due to damaged mucosal blood vessels. Systemically, if the toxin becomes disseminated several body systems may be affected with the most serious affects observed on the kidneys, the circulatory vasculature and the neurological tissues. These effects can progress the clinical condition to include HUS, TTP or neurological deficits (O'Brien & Holmes, 1987).

There are two sub-groups of VT, named VT1 and VT2; these can be expressed individually or together by *E. coli* O157:H7 strains (Scotland *et al.*, 1983; Strockbine *et al.*, 1986). VT1 is highly conserved, but VT2 has multiple variants, these two groups vary in toxicity and affinity for tissues (Jacewicz *et al.*, 1999). The structure of VT is relevant to its function. There are two subunits termed A and B forming a structure comprising a single central A subunit surrounded by 5 B subunits. These B subunits bind to a receptor found on eukaryotic cells,

called globotriaosylceramide (Gb3 or CD77) (Lingwood, 1996) and the toxin is then internalised into the cell cytosol (Sandvig & van, 2002). During this process the A subunit is cleaved to facilitate its action. Furin, a protease, cleaves the A subunit to release A1 peptide which is inhibitory to cellular protein synthesis by affecting the 28S subunit of 60S rRNA. Affected cells undergo apoptosis due to this inhibitory effect on vital protein synthesis as well as other proposed mechanisms such as VT inhibition of cellular anti-apoptotic factors and triggering of caspase and mitochondrial apoptosis pathways (Erwert *et al.*, 2003; Tetaud *et al.*, 2003).

VT action on host cells is clearly a key determinant of pathogenicity and the progression of disease in human EHEC infection. Epithelia are the first cells to come into contact with EHEC following ingestion and although human epithelia lack the VT receptor CD77; these interactions are an important factor in the local effects of EHEC and VT (Holgersson *et al.*, 1991; Kasai *et al.*, 1985). VT in the sub-epithelial tissues targets endothelial cells of the vasculature, causing haemorrhagic conditions. The epithelia are induced to produce several pro-inflammatory cytokines by VT; these include IL-1, TNFα, IL-6 and IL-8. These can cause inflammatory damage to the intestinal tissues as well as providing signals to stimulate infiltration of phagocytic cells (Harel *et al.*, 1993; Tesh *et al.*, 1994; Thorpe *et al.*, 1999; Yamasaki *et al.*, 1999). Of these cells $CD77^{+}$ phagocytes may take up VT and facilitate spread of the toxin around the body, and this factor is important for the development of systemic complications (Hurley *et al.*, 2001; te Loo *et al.*, 2000).

Although VT has been detected in the faecal matter of cattle colonised with EHEC, these animals remain clinically normal (Ball *et al.*, 1994; Hyatt *et al.*, 2001). However, anti-VT antibodies have been detected in the serum and colostrum of colonised cattle, suggesting that the bovine immune system does indeed recognise VT (Johnson *et al.*, 1996; Pirro *et al.*, 1995).

These disparate consequences to VT in human and bovine hosts could be due to a number of factors. VT may be important in cattle for modulating the mucosal immune response and reducing local tissue damage as VT has been shown *in vitro* to affect immune cells by reducing proliferation of PBMCs and limiting cytokine production by intestinal lymphocytes (Menge *et al.*, 2004; Menge *et al.*, 1999). This could be an important factor for the species differences because if the pro-inflammatory effects of the immune cell populations, including dendritic cells (DCs), are reduced *in vivo*, then a consequent reduction in tissue damage and limiting of systemic features is likely to ensue, as well as potentially permitting persistent colonisation with EHEC (Westerholt *et al.*, 2003). There could however be other elements to these disparate responses relating to CD77 expression. In cattle CD77 is not expressed by vascular endothelial cells (Pruimboom-Brees *et al.*, 2000), and the CD77 expressing crypt cells of the intestinal tissue have been shown to internalise VT into lysosomes which results in functional abrogation (Hoey *et al.*, 2003).

1.6.5 Flagella

Flagella are an important virulence factor as they confer motility on bacteria enabling them to effectively select preferred microenvironments (Monday *et al.*,

2003). *E. coli* flagella have been implicated in several modes of virulence including roles in adhesion, microcolony formation, and persistence (Giron *et al.*, 2002; La Ragione *et al.*, 2000; Mahajan *et al.*, 2009). H7 flagellin has been implicated in mucus binding in the intestine, which could point to a role in early localisation of bacteria to the intestinal surface (Erdem *et al.*, 2007). H7 has also been demonstrated to produce specific serum IgA responses in *E. coli* O157:H7 challenged cattle (McNeilly *et al.*, 2008). The structure and functions of the EHEC flagella share some aspects with the TTSS. The assembly of the flagella filaments is similar to the mechanisms involved in TTSS injection of effector proteins into host cells, with exported flagellin molecules travelling down the centre of the structure to be assembled at its distal end (Galan *et al.*, 1992; Macnab, 2003). Polymerised flagellin monomers form chains to make up the long filamentous structure of the flagella. These filaments are anchored to the bacterium via a membrane embedded rotary motor and hook (Macnab, 2003).

Flagella are recognised by the host innate immune system via toll-like receptor (TLR) -5 (Hayashi *et al.*, 2001). This is one of ten related pattern recognition receptors (PRR) which are discussed further in section 1.9.1. Binding of the highly conserved D1 region of the flagellin monomer to the TLR5 receptor on the host cell surface initiates signalling which prompts various defences for example mucin, antimicrobial peptides, nitric oxide and IL-8 (Eaves-Pyles *et al.*, 2001; McNamara & Basbaum, 2001; Ogushi *et al.*, 2004; Zhou *et al.*, 2003).

1.7 Pathogenic Bacteria and the Intestinal Environment

There are numerous ways in which hosts have developed to prevent colonisation and disease with pathogens. Both structural and functional adaptations aim to circumvent the pathogenicity factors of common disease-causing microorganisms.

1.7.1 Intestinal microflora

As mentioned previously, there can be approximately 10^{11} commensal bacteria per gram of intestinal contents within the colon, which can consist of up to 500 various bacterial species (Eckburg *et al.*, 2005; Evaldson *et al.*, 1982). The bacteria and their hosts have a symbiotic relationship whereby hosts provide favourable environments in terms of nutrients and temperature and the microorganisms in turn provide some protection from cellular insult, regulation of lipid storage, stimulation of development of the mucosal tissues and inhibition of colonisation by pathogenic bacteria (Backhed *et al.*, 2004; Rakoff-Nahoum *et al.*, 2004; Stappenbeck *et al.*, 2002). The normal intestinal flora is important for development of the gut associated lymphoid tissue (GALT) as well as epithelial function (Steege *et al.*, 1997), and this feature is highlighted in the intestines of animals which are reared in germ-free environments. These animals have a much thinner gut wall with smaller areas of GALT, compared to conventionally reared animals.

Although under normal circumstances commensal bacteria are harmless, the study of some human intestinal diseases suggests that the way in which the host perceives bacteria, either as pathogen or commensal, can depend upon host

"sensing" and responding to microbial stimuli as much as factors associated with the bacterial strain. For instance, inappropriate inflammatory responses in Crohn's disease and Inflammatory Bowel Disease can cause significant damage to the normal structure and function of the gastrointestinal system; and these have been proposed to be instigated in part by commensal bacterial flagellin (Lodes *et al.*, 2004; Sansonetti, 2004). Moreover, for protection of the gut and optimal intestinal function it is important that inflammatory responses are generated against pathogenic bacteria without responses directed at the commensal types which may share many of the same features.

1.7.2 Intestinal physiology and anatomy

A common feature of many of the diarrhoeagenic *E. coli* including O157:H7 strains is that they are able to colonise the intestinal tract despite numerous physiological and structural host factors designed to make the intestine a sub-optimal environment for such accomplishments. These factors include peristalsis, intestinal mucus and the presence of commensal microflora among others (Nataro & Kaper, 1998).

1.7.3 The Epithelial Layer

Epithelial cells are found covering the full length of the intestine and are therefore the first line of contact with the host for all ingesta including pathogenic and commensal organisms. The epithelium is a barrier, the thickness of a single cell, between the external and internal environments and is faced with a massive antigenic milieu on a day-to-day basis. The mechanisms that have evolved to cope with this load of antigen are highly complex. To

differentiate between commensal and pathogenic organisms, as well as food and self-antigens, requires the immune system to be highly sophisticated and well-regulated. Some disease states indicate the complex role of the mucosal system as proposed in the situation in Crohn's disease patients. One theory suggests that the pathogenesis of this disease is triggered by inappropriate mucosal responses to intestinal commensal bacteria (Blaser *et al.*, 1984; Walmsley *et al.*, 1996). There are several cell types within the epithelial layer, and regular replacement of these cells is driven by the rapid turnover of the crypt cells from which all intestinal epithelia are derived. The cells which originate in the crypts gradually migrate upwards to an apical position, mature to form enterocytes and goblet cells within 3-5 days. The rapid turnover of these cells provides defence in itself as the intestinal lining is constantly being shed into the lumen, inhibiting adherence and colonisation by pathogens. Tight junctions between the epithelial cells maintain integrity of the layer and function to maintain epithelial cell polarity and regulate any paracellular movement of molecules. This paracellular permeability is affected by host as well as extrinsic factors (McKay & Baird, 1999).

1.7.4 The Mucus Layer

The intestine is covered with a layer of mucus which in itself makes it difficult for pathogens to contact the epithelial layer, but it also contains anti-microbial peptides and secretory IgA which are further protective of this continually challenged environment. The mucus layer is produced mainly by goblet cells and is highly viscous and elastic (Rhodes, 1990). The intestinal mucus is a glycoprotein secretion of these goblet cells of high molecular weight (Lamont,

1992) Mucus is resistant to enzyme digestion and in this feature is thought to inhibit its breakdown by host enzymes but also to protect the host epithelia from enzymes produced by microbes. Mucus further protects epithelial cells from pathogenic bacteria by trapping organisms in its matrix as well as masking cellular binding sites with mucin and secretory IgA (LaMont, 1992);

1.7.5 Secretory IgA

Secretory IgA is the main mucosal antibody (Lamm, 1997). Plasma cells produce dimeric IgA within the lamina propria (LP) and this is then secreted into the lumen of the intestine via the basolateral epithelial Ig receptors. Secretory IgA is transported across the epithelial layer captured within vesicles attached to a polymeric immunoglobulin receptor (pIgR) and released at the luminal surface following proteolysis of pIgR (Macpherson *et al.*, 2001). The main role for secretory IgA involves inhibition of microbial attachment to host cells as well as neutralisation of bacterial products (Brandtzaeg *et al.*, 1999; Brandtzaeg, 2003; Silvey *et al.*, 2001).

1.8. The Structure of the Intestinal Immune System

The intestinal immune system is complex in structure and function. Numerous immune cells are found within the tissues of the intestine. The function of these cells is primarily to perform and respond to continual sampling of the antigenic load of the luminal contents. A diagrammatic representation of the mucosal immune system is shown in Fig. 1.4. The intestinal immune system can be broadly divided into inductive sites including mesenteric lymph nodes (MLN)

and the lymphoid follicles as well as effector sites such as the epithelium and lamina propria (LP).

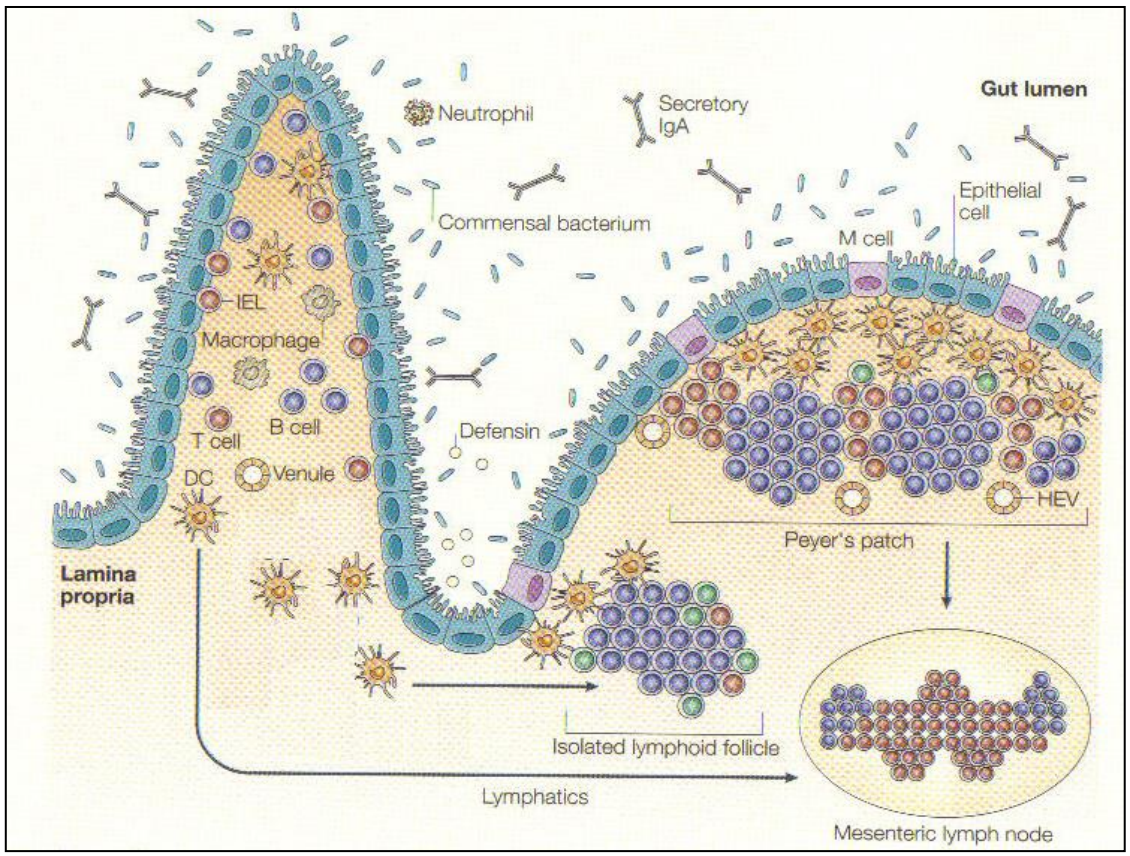


Fig. 1.4 The structure of the Intestinal Immune System (modified from Eberl G, Nature Reviews Immunology Vol 5: 413 – 420, 2005) Individual DCs are present throughout the lamina propria and many within discrete lymphoid tissues of the intestine as indicated here by the Peyer's patch. Numerous other cell types are involved in the immune system of the intestine including macrophages, T and B lymphocytes and M cells (Eberl, 2005).

1.8.1 Gut Associated Lymphoid Tissue (GALT)

As well as individual immune cells scattered throughout the intestine tissues, individual intraepithelial lymphocytes have been observed within the epithelial layer close to the lumen contents. The function of such cells is unclear, however it is possible that they respond to features of the microenvironment and secrete cellular signals or are cytotoxic to pathogens (Cerf-Bensussan & Guy-Grand, 1991). The intestinal tissues also have associated with them numerous aggregations of immune cells. These immune cell clusters are called lymphoid follicles and these small collections of immune cells may be found as individual lymphoid follicles (ILF) in the intestinal tissues or grouped together in some numbers to form patches of GALT. These patches are observable by eye as thickenings of the gut wall. The precise locations of GALT tend to be species specific, albeit with some individual animal variation however there are some areas which are consistently present for example the tonsillar tissues and the PP of the small intestine; reviewed in (Liebler-Tenorio & Pabst, 2006).

Close to the Recto Anal Junction (RAJ) in several species, including cattle, there are dense patches of lymphoid follicles (LF). These follicles contain numerous immune cells. The structure of the follicles consist of follicular B cell areas and parafollicular T cells zones, overlaid by a sub-epithelial dome highly populated by DCs, as can been seen in Fig.1.4. The epithelium immediately covering the lymphoid follicles contains a greater number of specialised epithelial cells called M cells (see section 1.8.2) and is termed follicle associated epithelium (FAE) (Mahajan *et al.*, 2005; Owen *et al.*, 1986). The main function of the GALT is

antigen sampling of the intestinal contents and to facilitate generation of the appropriate local immune responses at the gut surface.

1.8.2 M cells

Specialised epithelial cells called M cells (microvillous or microfold cells) are found in the FAE of LF, PP, isolated lymphoid follicles (ILF) and appendix. They were discovered in 1965 in the rabbit appendix but were not named until 1974 when electron microscopy (EM) was used to view the microfolds on the surface of these cells (Owen, 1975). M cells function in transportation from the intestinal lumen to the subepithelial dome region. Microorganisms as well as various macromolecules and soluble molecules are all transported. The subjacent cells within the subepithelial dome then have access to these luminal antigens and can initiate immune responses (Gebert *et al.*, 1999).

M cells have shorter, more irregular microvilli when compared to enterocytes. The M cells have only a thin glycocaylx layer, which has been suggested to permit them greater access to the intestinal luminal antigens. Enterocyte brush borders have alkaline phosphatase and sucrose-isomaltase, but these are not found on M cells and therefore have been used as negative markers (Gebert *et al.*, 1996). One of the most striking features of the M cell is an invagination of its membrane at the basolateral surface (Neutra *et al.*, 1996). It is in this pocket that immune cells are often found including DCs and B and T lymphocytes, which are the cell types required to generate immune responses. This close association of M cells and DCs is thought to facilitate antigen transfer from one cell type to another.

1.8.3 Lamina propria (LP)

The LP is a highly vascular layer of loose connective tissue which underlies the epithelium providing support. There are numerous cell types within the LP to facilitate its effector functions. These cell types have varied roles in structure, such as smooth muscle cells and fibroblasts, as well as function; for example, the cells of the immune system. Numerous plasma cells in the LP function to produce secretory IgA (section 1.7.5). Immune responses are generated in the LP by interaction of DCs with T and B lymphocytes, including production of cytokines and other signals directed towards eliminating pathogens and maintaining tolerance to self. The populations of these cells between species is heterogeneous (Haverson & Riffault, 2006).

1.9. The Intestinal Immune Response

The immune response generated within the mucosal tissues of the intestine may be broadly categorised into the innate and adaptive (or acquired) immune responses. Both innate and adaptive arms of the immune response involve activity of various leukocytes to protect the host.

1.9.1 The Innate Immune Response

The innate immune response is the first line of defence against foreign material. It is a more immediate response and has evolved to recognise conserved features of pathogens, termed pathogen associated molecular patterns (PAMP). Recognition of a PAMP induces cellular responses and secretion of soluble factors, such as complement components and cytokines, all directed towards

elimination of the organism. Cell types which are involved in the recognition and elimination of foreign material include macrophages, natural killer cells, basophils, eosinophils, neutrophils and DCs. An important discrimination between self and non-self molecules must be made to prevent inappropriate damaging inflammatory responses towards harmless molecules. The innate immune system has evolved to effectively meet this challenge (Janeway, Jr. & Medzhitov, 2002). Central to this discriminatory role are the TLR (Beutler, 2003) which recognise PAMPs. TLR are transmembrane proteins with a high degree of evolutionary conservation. To date, at least ten TLR have been discovered in mammals including cattle (Werling *et al.*, 2006). Of these several are of importance to the recognition of bacterial factors which will be involved in responses to *E. coli* O157:H7 *in vivo*. TLR2 recognises bacterial membrane components including lipoproteins (Cario, 2005; Medzhitov & Janeway, Jr., 1997b). TLR4 recognises the lipid A portion of bacterial lipopolysaccharide (LPS) in conjunction with LPS binding protein and CD14 (Cario, 2005; Chow *et al.*, 1999). Flagella and flagellated bacteria have been shown to be a specific ligand for TLR5 (Hayashi *et al.*, 2001), and TLR9 is involved in recognition of bacterial CpG motifs (Hemmi *et al.*, 2000). Binding of the specific ligands to the TLR initiates signalling cascades via activation of MyD88 which promotes the action of NF-κB and MAP kinases on transcription of pro-inflammatory genes (Takeda & Akira, 2004). The cell types which express these TLRs are often the cell types which will first encounter foreign material and are in anatomical positions to provide effective clearance; these include epithelial cells, DCs, macrophages and neutrophils (Akira *et al.*, 2001; Werling *et al.*, 2006).

1.9.2 The Adaptive Immune Response

The adaptive immune response takes longer to become activated but is highly specific to the pathogen involved. (Abbas, 2000) The innate immune system acts in the first instance to prevent spread of infection, however while this is occurring, antigenic information about the causative organism is transported from the local site to secondary lymphoid organs often via antigen-presenting cell types such as DCs. It is in this location that the host lymphocytes are stimulated to generate effector T cells which are able to specifically recognise the pathogen. This response is characterised by clonal selection and differentiation and includes stimulation of B cells to produce specific antibodies (Medzhitov & Janeway, Jr., 1997a). These specific cells and antibodies are often able to clear the pathogen and during this process memory T and B cells are also generated for host protection against future challenge. The two types of immune response while seemingly distinct from each other, nonetheless are integrated to provide a comprehensive host defence system against innumerable pathogens (Hoebe *et al.*, 2004). It is important to the host that all responses generated are appropriate to the level of threat and therefore immune recognition is a delicate balance. As an example of aberrant immune responses, autoimmune disorders exist whereby inappropriate response to self tissues is poorly regulated such as arthritis or asthma (Hoebe *et al.*, 2006; Hoebe *et al.*, 2004; Janeway, 1989). DCs are central cell types linking both the innate and adaptive arms of the immune response

1.10 Dendritic cells

Dendritic cells are one of the most important cell types involved in initiating intestinal immune responses or generating tolerance to encountered molecules. DCs are potent stimulators of both primary and secondary immune responses to infections (Bell *et al.*, 1999). These professional antigen presenting cells (APCs) ingest and process antigens, to present to T cells which promote the immune responses. Proliferation of the T cell follows, along with cytokine release and alteration of expression of surface co-stimulatory molecules to direct immune responses.

Characterisation of DCs is complex; phenotypically they elaborate long dendritic processes, and functionally they are highly active cells, but they are multifarious and take up many positions both anatomically and functionally (Haverson & Riffault, 2006). Traditionally, identification of DCs has been based upon investigation of cellular morphology (Steinman & Cohn, 1973). The irregular cellular structure coupled with constitutive expression of MHC II is now often used to identify DCs. Expression of other cell surface molecules tends to vary between tissues and species and state of maturity. Identification of DCs and cell surface molecule expression is discussed further in Chapter Two. Surprisingly, the early DC development from haematopoietic precursors is one of the least understood areas of DC biology. Predominantly, research has been carried out with mice and human cells (Wu & Dakic, 2004). Traditionally DCs were categorised as developing from myeloid precursors, then further work suggested that DCs can develop from lymphoid precursors (Manz *et al.*, 2001a; Manz *et al.*, 2001b). These lineages were thought to remain separate during

development. More recent views are that the lineages are not so distinct and that DCs may demonstrate differentiation and plasticity within and between DC subsets (Grohmann *et al.*, 2003) (A. MacDonald, BSI Summer School Lecture, 2006). Similarly, bovine DCs are widely accepted to be heterogeneous with a poorly characterised lineage (Mirkovitch *et al.*, 2006).

DCs are found in various tissues throughout the body; however this study is concerned with intestinal dendritic cells and methods by which they may interact with *E. coli* O157:H7. Intestinal DCs are found in discrete lymphoid tissue of the intestine such as Peyer's patches and lymphoid follicles (as described in section 1.8.1) and also individually throughout the lamina propria (Stagg *et al.*, 2004). Subsets of intestinal dendritic cells have been identified in porcine and ovine intestinal tissues but no such study has been carried out for the bovine intestinal DC populations.

1.10.1 DC Antigen Capture in the Intestine

The role of DCs in antigen uptake and presentation is more widely understood. Immature DCs migrate from the bone marrow to seed peripheral tissues including the intestine. These peripheral DCs encounter antigen which stimulates them to mature and migrate. In the intestine, the DCs may sample antigen by a number of methods, both directly and indirectly. DCs can extend dendrites into the intestinal lumen between the cells of the epithelial layer to directly capture antigen. By expressing epithelial tight junction molecules the DCs do not interrupt the epithelial integrity (Rescigno *et al.*, 2001b). Physical damage to the epithelial cell layer also allows DCs to access and uptake luminal

antigen. Indirectly, DCs may receive antigen via epithelial cells; either by uptake and transport by the epithelial cell, or following DC phagocytosis of apoptotic cells. M cells also provide DCs directly with antigenic material (Milling *et al.*, 2005; Mohamadzadeh *et al.*, 1993; Stagg *et al.*, 2003). DCs phagocytise antigenic molecules obtained via these multiple routes and process the antigens for presentation to T cells. This processing occurs during maturation and migration of the DCs. The DCs employ three main methods of internalising antigens. 1) Phagocytosis of particles following binding of Fc or complement receptors mediates actin polymerisation and then internalisation (Carroll, 1998). 2) Macropinocytosis is a method by which DCs can sample large amounts of fluid from the surrounding microenvironment. Any antigens present are quickly concentrated and processed for presentation on the DC surface. The initial events also require actin polymerisation, but direct antigen contact is not required, and in fact this process appears to occur constitutively in human DCs (Sallusto *et al.*, 1995). 3) The third method of uptake is initiated by binding of PAMPs to PRR such as TLR (section 1.9.1.) and is termed receptor-mediated endocytosis. Other PRR exist such as CD205, DC-SIGN, and the mannose receptor (MR) (Engering *et al.*, 2002; Sallusto *et al.*, 1995; Swiggard *et al.*, 1995). Following binding to the receptor, uptake occurs into invaginations of the host cell membrane called clathrin-coated pits, which then contain the antigen and receptor. These pits then separate from the cell membrane and the resulting intracellular vesicles may bind with lysosomes to facilitate inactivation of the antigen (Steinman & Swanson, 1995).

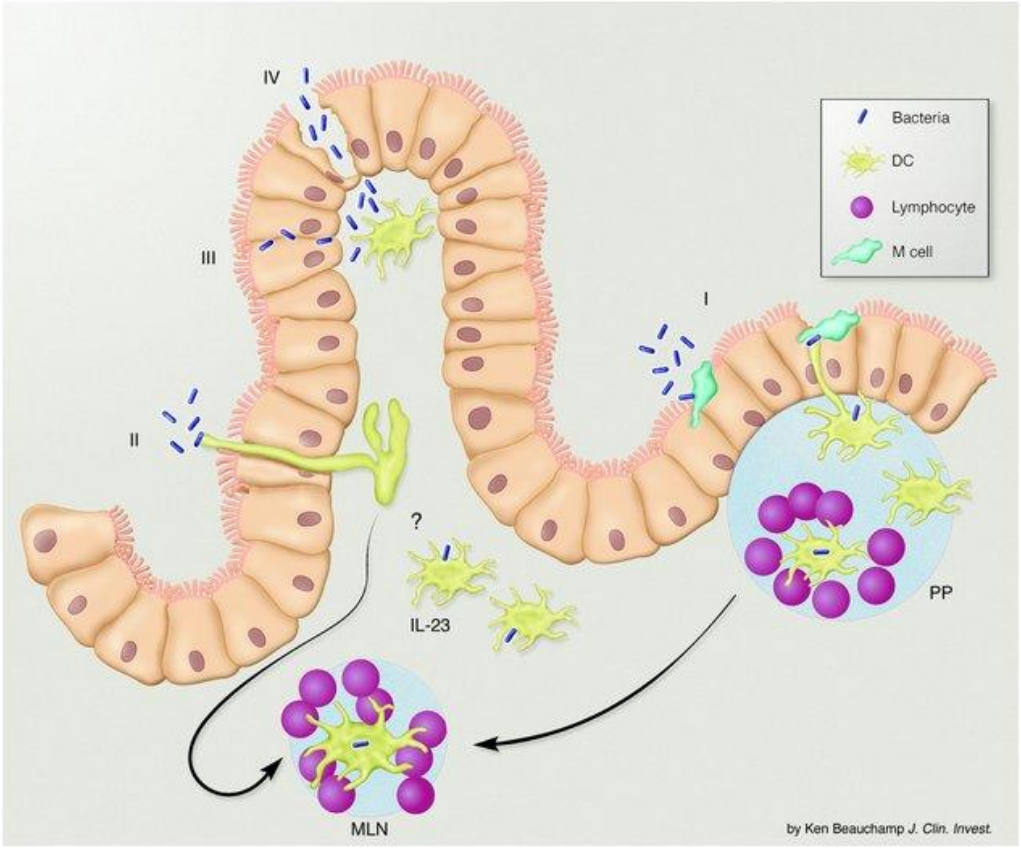


Fig. 1.5 Intestinal DCs: antigen sampling methods. Imange adapted from Uhlig, 2003, Journal of Clinical Investigation. Four methods which DCs use to capture antigen are demonstrated here (Uhlig & Powrie, 2003).

I: Via M cells

II: DC extending dendrites between epithelial cells to directly contact antigens in the luminal contents

III: Uptake of translocated bacteria which arrive in the LP via transcellular routes or via transport by epithelial cells

IV: Uptake of bacteria which reach the lamina propria following disruption of the epithelial layer.

Following uptake of antigen the DCs migrate to draining lymph nodes (MLN - mesenteric lymph node) to initiate immune responses via lymphocyte interactions.

1.10.2. Migration & maturation of DCs

Maturation of DCs, characterised by upregulation of MHC II expression, cytokine secretion and expression of high levels of cell surface co-stimulatory molecules, occurs soon after encountering antigen. The levels of cellular expression of MHC II, CD40, CD80 and CD86 are all upregulated in response to antigen uptake (Cella *et al.*, 1996). The DCs will then migrate to lymphoid tissue in order to drive the appropriate T cell response (Niedergang & Kweon, 2005; Stagg *et al.*, 2004). The changes in cell surface expression and the production of cytokines during DC migration and maturation facilitate T cell interactions (Cella *et al.*, 1996).

1.10.3. Antigen presentation

The ability of APCs to stimulate naïve T cells is unique to DCs. Memory T cells are targeted by other APC types. This means the DCs are in a unique position to direct inflammatory and immune responses against, or tolerance to, encountered molecules. The interaction of DCs and T cells involves two main signals. Firstly, interaction of the MHC II/antigen complex with the T cell receptor and secondly, ligation of DC CD80 or CD86 with T cell CD28, or interaction of CD40 with CD154. Whether an immune response is generated or not, and indeed what type of immune response, (for example Th1 or Th2), is driven through a number of mechanisms by DCs. For instance, cytokine production by DCs determines qualitatively any immune response by stimulation of different arms of immune responses. Production of IL-12, IL-18 and IL-23 by DCs is more likely to drive a Th1 response, whereas a Th2 response is more likely to result from IL-4 or IL-10 secretion (Stagg *et al.*, 2003).

Furthermore CD80 ligation with CD28 has been shown to bias towards a Th1 response, with the Th2 bias resulting from CD86-CD28 interaction (Sansom *et al.*, 2003).

1.11 DCs *in situ*

DCs have been found in many tissues of the body subsequent to *in situ* studies. These studies have successfully identified tissue- and species-specific subsets of DCs. In ruminant species the ovine pulmonary and intestinal DCs have been investigated using immunohistochemistry (IHC) techniques (Akesson *et al.*, 2008; McNeilly *et al.*, 2006). In cattle, the tissue resident DC populations have not been extensively examined in this way. The *in situ* IHC techniques are useful to indicate tissue distribution of cells and cellular expression of molecules of interest. However, they cannot fully characterise the complex cellular expression of the DC subsets or permit functional studies of these cells, thus, further techniques are required.

1.12 *Ex vivo* DCs

Several limitations to working with *ex vivo* DCs are evident. Firstly, there are relatively few DCs within the peripheral blood. DCs from tissues are also relatively difficult to collect. A pure DC population from tissues may contain contaminating cell types and involve surgical or necropsy techniques. Furthermore, the resulting cells may have to undergo separation, digest and culture techniques which have potential to affect the phenotype of the cell population present. In addition to this a lack of DC specific molecules in most species and a relative scarcity of ruminant reagents in particular have

necessitated development of other methods of studying *ex vivo* DCs. Because these cells are migratory in nature and demonstrate plasticity in their phenotype, the same cell is likely to display different properties depending at which point in its development it is collected. However, the majority of the knowledge of bovine DC phenotype and function comes from work on afferent lymph dendritic cells (ALDCs). These are DCs which have captured antigen in the peripheral tissues and are travelling back to the local lymph node to present antigen. Cannulation of lymph ducts in both cattle and sheep has been described (Epardaud *et al.*, 2004; Hope *et al.*, 2006). These techniques have provided a means of collecting circulating DCs over extended periods. Information on DCs returning from various tissues including, for example, the skin of cattle (Hope *et al.*, 2006), mucosa of the ovine head (Epardaud *et al.*, 2004) and rat intestinal tissues (Milling *et al.*, 2006) have been provided. These techniques however are not suited to all species and tissues due to the complexities of the anatomical features and surgery involved.

1.13 *In-vitro* culture of DC

To facilitate investigation of the properties of DCs it is necessary to carry out *in vitro* experiments. One widely used method of generating numerous DCs for *in vitro* studies is to stimulate differentiation of blood monocytes using cytokines and culture techniques. This method was first demonstrated for human cells by Sallusto *et al* in 1994 (Sallusto & Lanzavecchia, 1994). These techniques have been utilised for DC studies with numerous species. In cattle, the *in vitro* culture of $CD14^+$ blood monocytes with granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4) produces cells of an immature DC

phenotype after 3 days (Hope *et al.*, 2000a; Werling *et al.*, 2002) (see also Chapter 4) and has been employed in the investigation of various bovine diseases. Similar culture methods with bone marrow precursors have also been described in cattle (Hope *et al.*, 2000b). The surface phenotype of GM-CSF- and IL-4-stimulated cells is comparable but not identical to *in vivo* cells, and cultures must also contain cells at various stages of differentiation. Despite these limitations, culture techniques are still a useful model for studying DC interactions with various infectious agents.

1.14 Bovine DCs

To date relatively little work has been carried out on bovine intestinal DCs in comparison with rodent and human DC research; hence it is a rapidly-growing, exciting and relevant area of investigation. As mentioned earlier, studies of the distribution and presence of subsets of DCs within intestinal tissues have been published for pigs (Bimczok *et al.*, 2005) and sheep (Akesson *et al.*, 2008), but no comparable study is available for bovine cells. It is important to recognise species differences in phenotype and function of cells. While investigating the relationship between DCs and EHEC in cattle, we must be aware that the human EHEC interactions are likely to be different to that which occurs in the ruminant host. Not all of the investigative techniques may be appropriate for all species and there may be ways in which bovine DCs differ from that which has been discovered so far. Most work has discovered DC subsets that differ in cell surface molecule expression; however these subsets are not the same in all the veterinary species (Haverson & Riffault, 2006).

1.15 Bovine DCs and interaction with *E. coli* O157:H7

During infection EHEC is likely to encounter intestinal DCs. The terminal rectum of cattle, which is this organism's predilection site for colonisation, is also an area which contains numerous lymphoid follicles. Lymphoid follicles, like other GALT, contain numerous immune cells, and although the presence of DCs within bovine terminal rectal tissue has not been confirmed prior to this study, it is postulated that these are a predominant cell type underlying the epithelium. Furthermore, bovine primary epithelial cells express CCL-20, a DC chemo-attractant chemokine, in response to challenge with *E. coli* O157 *in vitro* (Dr. Mahajan, personal communication), and secondly, DCs can directly sample the intestinal lumen and collect antigens from food, organisms and apoptotic epithelial cells (Rescigno *et al.*, 2001b; Rescigno *et al.*, 2001a). DCs (as well as other cells) express TLR and these PRR recognise PAMPs. EHEC possesses several PAMPs that may be recognised by TLRs. Among these, flagella (the H7 antigen) which is recognised by TLR5, has a significant role in host recognition (Hayashi *et al.*, 2001; Tsujimoto *et al.*, 2005). Other PAMPs such as LPS (recognised via TLR4), lipoproteins (TLR2), or CpG (TLR9) may also be of importance.

E. coli O157:H7 could in some ways be classified as a 'commensal' bacteria in cattle because it does not appear to induce significant clinical changes in this species. However, A/E lesions have been shown to be formed on cattle epithelial cells, on intestinal explants and during calf challenges (Sandhu & Gyles, 2002). These lesions are a characteristic more commonly associated with disease (Dean-Nystrom *et al.*, 1998; Phillips *et al.*, 2000). Coinciding with A/E lesion

formation is the secretion of IL-8, a chemokine that is key in the promotion of inflammation (Moxley, 2004). IL-8 acts as a neutrophil chemoattractant, however there is no conclusive evidence that neutrophil infiltration is observed as a feature of *E. coli* O157:H7 colonisation in cattle. It is therefore possible that this bacterium alters and controls mucosal defences to promote its colonisation, potentially to include signals affecting DC function. Although phagocytic cells and antigen-presenting cells play central roles in directing host defences, the interplay between these cells and *E. coli* O157:H7 in cattle has received relatively little attention. Dendritic cells are of major significance in directing defences at mucosal surfaces but have as yet not been studied in this context.

In Summary, *E. coli* O157:H7 is a significant cause of human disease. Cattle have been shown to be an important reservoir for this pathogen and are often the cause of outbreaks of disease within the human population. Cattle carry *E. coli* O157:H7 asymptomatically within the intestine, in particular at the terminal rectum. Due to the tissue distribution of lymphoid follicles there are likely to be numerous DCs within the terminal rectum. DCs are key directors of immune responses and will interact with intestinal bacteria in the bovine host. The hypothesis of this study is that these terminal rectal DCs are important for the colonisation and persistence of cattle with *E. coli* O157:H7.

Aims

1. To characterise the dendritic cell population in the bovine intestine at various sites to include the terminal rectum.
2. Evaluate extraction of *ex vivo* DCs using several methods to generate a protocol for extraction, and if successful, to characterise the cells and compare phenotype and function with the monocyte-derived dendritic cell (moDC) system.
3. To characterise the response of moDCs to various *E. coli* strains and components, looking at cytokine responses, cell surface molecule expression and cell viability after challenge. Intracellular bacterial survival will also be investigated.

Chapter 2 Investigation of cell surface molecule expression by bovine intestinal dendritic cells *in situ*.

2.1 Introduction

The intestine constantly comes into contact with a wide variety of foreign material. Pathogens must be differentiated from commensal organisms and harmless food antigens by the host, and an appropriate response has to be initiated. It is important to prevent an excessive inflammatory response to harmless food or self-antigens and it is equally vital not to neglect to respond to potentially harmful stimuli.

There are several sites in the intestine at which immune responses can be generated and distinct anatomical locations in which numerous immune cells can be found. Individual lymphoid follicles can be found within the intestinal wall at numerous sites; however there are patches where abundant follicles are found in the same location in thick aggregations. These aggregations are observable by eye and are generally referred to as mucosa-associated lymphoid tissue (MALT) or gut associated lymphoid tissue (GALT). MALT is found in all species and the locations are species specific, although there are parallels between species. In cattle, MALT occurs most notably as the Peyer's patches of the ileum, within the proximal colon and at the terminal rectum. Reviewed in (Liebler-Tenorio & Pabst, 2006)

These aggregates of lymphoid follicles (LF) which make up the MALT at the bovine terminal rectum occur for only a short distance up to 3cm proximal to the recto-anal junction (RAJ). Individual LFs can be observed becoming more sparse proximal to this (Mahajan *et al.*, 2005). The lymphoid follicles are so-named because they consist of organised groups of lymphocytes: a large number of B cells are found in the germinal centres within a network of DCs and T cells are found in the inter-follicular areas. It is within these follicles that immune responses are generated. The follicles are covered by a single

layer of specialised epithelium which is termed the follicle associated epithelium (FAE). The FAE is a thinner layer with less prominent microvilli than that found in the villous regions and contains a higher number of specialised M cells. M cells, along with DCs, are responsible for antigen sampling from the intestinal lumen. M cells transfer captured antigen to DCs which can be found inside a specialised invagination of the M cell basal membrane. The area directly below the FAE is sometimes termed the sub-epithelial dome (SED) and can contain large numbers of DCs, as well as other immune cells, at a prime location for collecting antigens from the intestinal contents.

It is known that FAE secretes chemokines, including the DC chemoattractant CCL20 (Cook *et al.*, 2000; Iwasaki & Kelsall, 2000). DCs are recruited to the SED in response to this chemokine in mice and humans *in vitro* (Iwasaki & Kelsall, 2000; Thorley *et al.*, 2005). It is known that bovine primary rectal epithelial cells also express CCL20 (Dr. Mahajan, personal communication) and so it seems likely that there could be numerous DCs within the follicles of the bovine terminal rectum although this has not been investigated prior to this study. DCs at this site then will come into contact with *E. coli* O157:H7 due to their antigen uptake abilities.

Intestinal DCs obtain antigens by several methods and subsequently generate immune responses. They are able to obtain antigens via specialist M cells which have been shown to occur in higher numbers in the FAE. M cells have been identified within the FAE of the bovine ileum and the terminal rectum (Momotani *et al.*, 1988) (Dr. Mahajan, thesis, 2005) DCs have been shown to directly sample the gut lumen by expressing tight junction proteins to extend dendrites directly into contact with the gut contents (Chieppa *et al.*,

2006; Collan, 1972; Rescigno *et al.*, 2001b; Rescigno *et al.*, 2001a). Apoptotic epithelial cells can be efficiently phagocytosed by DCs and in this way even self-antigen can be taken up and processed. It has been shown in rats that a population of SIRPα^- DCs draining the intestine constitutively phagocytoses and transports apoptotic epithelial cells to T cell rich lymph node areas and it is proposed that these cells are involved in the maintenance of peripheral self-tolerance (Huang *et al.*, 2000). Epithelial cells in mice have been shown to have a rapid turn-over, dependent on intestinal location, of one to four days (CREAMER *et al.*, 1961) and so are continually being replaced. This rapid epithelial shedding is known to be a feature in all species. Although many of these cells are shed into the intestinal lumen, DCs can obtain food and micro-organism antigens from these apoptotic epithelial cells. DCs which have captured antigen travel in the lymph to the local draining lymph node. It is here that DCs can present antigen to naive T cells and in this way generate a response, whether it be tolerance, Th1 or Th2 type.

DCs in peripheral tissues are central to directing immune responses. They are the only cell type so far discovered capable of presenting antigen to naive T cells and thus directing immune responses. The presence of these highly inductive follicles at the site of preferential colonisation of EHEC implies a central role for DCs in the asymptomatic persistence of EHEC in cattle.

The study of DCs in various species and tissues has been an area of much interest in recent years, however the DC populations in the bovine intestine have yet to be characterised.

Ovine dendritic cells have been studied by immunohistochemistry at different anatomical locations within the lung and differential expression of

cell surface markers CD205, CD1b and SIRPα have been found (McNeilly *et al.*, 2006). Ovine intestinal dendritic cells have been characterised phenotypically using immunohistochemistry and immunofluorescence techniques. A wider range of markers were assessed including MHC II, CD11c, CD205, CD1b and CD209 and populations of immune cells were identified. The majority of DCs in peripheral tissues were found to co-express CD11c, CD205 and MHC II and very often were located in the lamina propria and SED, at a prime location for sampling intestinal antigens (Akesson *et al.*, 2008).

Porcine DC populations in the small intestine have been studied in a similar way. Subsets of DCs were discovered in the jejunum and ileum by using SIRPα and CD11b co-staining with MHC II (Bimczok *et al.*, 2005). Immunofluorescence in mice has also yielded information about DC subsets in intestinal tissues and in particular the Peyer's patches. CD11b, CD11c, CD8α and CD205 have been used to localise subsets to anatomical locations within the Peyer's patches. Some functional properties, involving the role of chemokines in the migration of DCs, have been attributed to these subsets (Iwasaki & Kelsall, 2000).

Molecules specific to DCs in humans, in particular Dendritic cell lysosome-associated membrane protein (DC-LAMP or CD208) (de Saint-Vis *et al.*, 1998) and Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN; also known as CD209) (Geijtenbeek *et al.*, 2000), have helped in the identification of these cells in this host. In earlier murine DC research, CD11c had been used as a specific DC cell surface molecule. However, it is clear now that this is not the case. In fact, in veterinary species studied to date, there has been no individual cell surface molecule identified

that is specific for dendritic cells. The isolation and identification of DCs therefore relies on a series of identifying features that must be assessed in conjunction with one another including morphology as well as expression of various molecules. DCs are generally defined as large irregular cells which express MHC II with heterogeneous expression of additional molecules including CD205, CD1b, SIRPα and CD11c among others. These molecules associated with DCs tend to have different expression patterns and functions which can be used to partition DC populations into phenotypic and functional subsets. Characterisation of these subsets in different anatomical compartments and species is an active and dynamic area of research.

MHC II molecules are involved in presenting antigen to T cells in conjunction with co-stimulatory molecules. Any captured antigen is phagocytosed into MHC II lined endosomes and this promotes up-regulation of MHC II and movement of the MHC II-antigen complex to the cell surface where it can be presented (Shin *et al.*, 2006).

CD205 is a member of the C-type multilectins and is thought to act as an endocytic receptor for antigen uptake (Gliddon *et al.*, 2004b). In sheep CD205 has been shown to be expressed by the majority of afferent lymph dendritic cells (ALDCs) draining tissue such as the skin, intestine and mucosal surfaces of the cranium, and has also been shown to be expressed by ovine dendritic cells within lung and intestinal tissues *in situ* (Akesson *et al.*, 2008; McNeilly *et al.*, 2006; Ryan *et al.*, 2000; Watkins *et al.*, 2005). Bovine ALDC draining the skin also express CD205 (Gliddon *et al.*, 2004b). CD205 is of further interest in dendritic cell research: a recent study has indicated that $CD205^{+}$ dendritic cells can be targeted and manipulated in cancer models *in vitro* to increase antigen presenting capacity (Birkholz *et al.*, 2010).

SIRPα, a member of the signal regulatory protein family, is involved in the interactions of DCs with $CD4^+$ T cells. In rat intestinal lymph there have been found to be two DC subsets can be defined by expression or lack of SIRPα (Liu *et al.*, 1998). SIRPα has also been used to identify subsets of DCs within porcine intestinal tissues using immunohistochemistry techniques. These subsets can also be defined by their expression of SIRPα as well as CD11b (Bimczok *et al.*, 2005). SIRPα, when bound to its ligand CD47, produces a negative effect on phagocytosis of host cells by APCs (Yamao *et al.*, 2002). Mononuclear phagocytic cells in cattle show SIRPα expression, specifically monocytes, neutrophils, eosinophils, myeloblasts and macrophages (Ellis *et al.*, 1988). SIRPα expression in bovine cells has been identified in tissues of the lymph nodes, spleen and skin (Brooke *et al.*, 1998).

CD1b is a cell surface glycoprotein which is also involved in the presentation to T cells; however the molecules which it is thought to present are lipid or glycolipid in nature (Porcelli *et al.*, 1998). A small sub-set of ALDCs draining the skin of cattle express this molecule. CD1b has been used, in conjunction with CD14, to identify sub-sets of ALDCs in Sheep (Ryan *et al.*, 2000). CD1b was found on few cells within ovine intestinal and lung tissues using immunohistochemistry techniques (Akesson *et al.*, 2008; McNeilly *et al.*, 2006).

CD40 is a membrane protein expressed by B cells and antigen presenting cells and functions as a co-stimulatory molecule. It is a part of the TNF receptor family and binding by its ligand can induce proliferation or activation of APCs (Banchereau *et al.*, 1994).

CD11c is an integrin expressed by DCs involved with cell adhesions. Studies of ovine lymph DCs have found ubiquitous CD11c expression of these cells (Gupta *et al.*, 1995) while within ovine intestinal cells CD11c has been localised to dendritic cells within the Peyer's patches (Gupta *et al.*, 1993). Expression by murine DCs of CD11c is often used as a specific marker for DCs in this species, although, may not be as specific as widely thought. CD11c was originally generated against murine macrophages (Shortman & Liu, 2002). CD11c has also been identified on bovine ALDCs draining the skin (McKeever *et al.*, 1991).

CD14 is commonly a marker of macrophages and monocytes and functions as a pattern recognition receptor (PRR). Its primary ligand is bacterial LPS and it works alongside TLR4 during binding. CD14 has been found on ALDCs in sheep at low levels (Ryan *et al.*, 2000) and also on porcine and bovine BMDCs (Carrasco *et al.*, 2001; Hope *et al.*, 2000b). CD14 may recognise other pathogen associated molecular patterns (PAMPS) as well as endogenous particles and apoptotic cells (Anas *et al.*, 2010; Kitchens, 2000; Tapping & Tobias, 2000).

In a similar study to the one described in this chapter, which made use of ovine intestinal tissues, a large proportion of cells displaying dendritic morphology within the rectal mucosa were MHC II $^{+}$ and co-expressed CD11c and CD205. Only sparse staining of CD1b was observed (Akesson *et al.*, 2008). Studies into similar molecules within tissues of the ovine respiratory tract showed staining profiles that were related to anatomical location. MHC II staining was widespread within the tissues including airways and lung parenchyma. CD1b was found expressed by a small number of DCs in all anatomical locations studied. SIRPα was found on a

population of DCs within the airways but was absent from tissues of the lung. Likewise the $CD205^+$ DCs were more abundant within the airways and less often observed in the lungs (McNeilly *et al.*, 2006).

Although the FAE and follicular tissues have been characterised in the cow, and a great deal of work has been carried out on ALDC returning from peripheral tissues, distinct populations of DCs within the tissues of the bovine intestine have not been studied *in situ.* The aim of this work was to identify and characterise DC populations within the bovine intestinal tract.

The aims of this chapter were:

1. To identify DC populations within the tissues of the bovine intestine using immunohistochemistry for immune cell molecules.
2. To investigate any differences between the cell populations identified at various sites within the large intestine to include the terminal rectum.

2.2 Materials and Methods

2.2.1 Post-Mortem Cattle Tissue

Post-Mortem tissue was obtained from six male Holstein-Friesian calves which were approximately six months old. Four different intestinal sites were sampled: proximal colon, distal colon, proximal rectum and terminal rectum (Fig. 2.1). Sections of approximately 1 - 1.5 cm^2 were collected using a sterile scalpel blade and rat-toothed forceps.

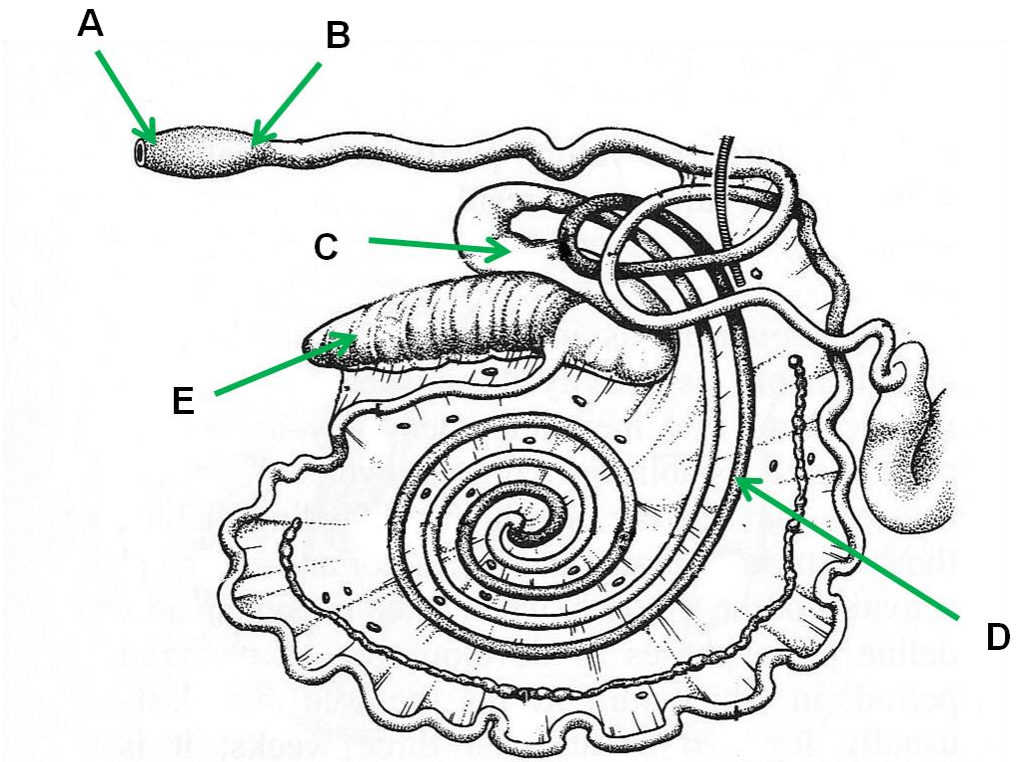


Fig. 2.1 Representation of the bovine gastrointestinal tract indicating the locations of post-mortem tissue sampling used in this study. A: Terminal Rectum; B: Proximal Rectum; C: Proximal Colon; D: Distal Colon. The terminal rectum was sampled no more than 5 cm proximal to the recto-anal junction (RAJ). The proximal rectum was sampled approximately 15-20 cm from the RAJ. The proximal colon was sampled from the sigmoid flexure close to the caecum (E). Distal colon sampling was carried out at an area where the centrifugal turns of the spiral colon straighten out towards the distal loop and transverse colon (Image adapted from Dyce *et al* 2nd ed. 1996).

2.2.2 Preservation and Fixation of Tissues

Two preservation methods were used for the tissue: snap-freezing in liquid nitrogen, fixing in formal saline and fixing in zinc salts fixative (ZSF, Appendix A.4.5.2). Three methods were chosen to give a wide range of tissues for IHC while maintaining tissue morphology. The efficacy of some antibodies was affected by fixation technique but the frozen tissues were difficult to section and resulted in poorer morphology. Tissues were placed in ZSF for 6-8 hours and then trimmed to approx 5-10 mm^2 and placed in fresh ZSF for a further 24 – 72 h. The formalin and ZSF tissue were mounted in paraffin wax blocks. These tissue sections were placed in plastic cassettes and placed in 70% ethanol for 30 min before mounting in paraffin wax using an automated procedure (80% ethanol, 45 min; 95% ethanol, 75 min; 99% ethanol, 3×75 min; iso-propanol, 2×90 min; isopropanol/xylene [1:1 v:v], 90 min; xylene, 2×90 min; medium temperature melting [56–58°C] paraffin-wax, 2×105 min) (Gonzalez *et al.*, 2001). The frozen sections were embedded in O.C.T. compound (Tissue-Tek, Sakura-Finitek, The Netherlands) before snap-freezing in liquid nitrogen. These samples were stored at -80°C until required for analysis. Sections 5 μm thick on treated glass slides (Superfrost plus®™, Menzel-glaser, Germany) were used for IHC. All the immunohistochemistry results presented in this thesis were carried out on the frozen sections or the Zinc salt fixed sections.

2.2.3 Single labelling Immunohistochemistry protocol

Both the single and double staining methods were adapted from published work in ovine tissues (McNeilly *et al.*, 2006).

Frozen sections were removed from -80°C and air dried under a fume hood for 10 min (forced air). These slides were then fixed in -20°C chilled methanol for 10 min. Further air drying in Bio *MAT*^{2} Class II Microbiological safety cabinet for 10 min was followed by two washes with PBS. Paraffin embedded tissue sections were re-hydrated using a Thermo-Shandon Varistain 24-4™ automatic stainer which placed the slides in graduated alcohols to take them to water. At this stage the method proceeded similarly for the paraffin embedded and the frozen tissues. All slides were then placed in PBS containing 0.5% Tween 80 (PBST80) to wash for 5 min. The slides were then incubated in PBST80 0.3% H_2O_2 v/v in a glass staining dish or coplin jar for 20 min at room temperature. The slides were washed twice, 5 min for each, in PBST80. The slides were then loaded into a Sequenza™ immunostaining centre (Shandon; Runcorn, UK) rack using PBS to rinse each slide as it was loaded. Each slide was then washed once with PBS, where a wash corresponds to one fill of the reservoir chamber for each slide. The slides were incubated in 25% v/v Normal Goat Serum (NGS; Vector) and PBST80 for one hour at room temperature. The slides were incubated overnight at 4°C in primary antibody diluted to appropriate concentration in 10% NGS/PBST80. The negative control used was incubation with mouse/rat IgG at 2 μg/ml in 10% NGS/PBST80, to correspond with the species of the primary antibody used (see Appendix A.1 for antibodies used and concentrations). After overnight incubation in primary antibody the slides were washed twice with PBS before incubation in Dako goat anti-mouse labelled polymer-HRP (included in EnVision kit) for 30 min at room temperature. Two further washes in PBS were carried out before the slides were incubated in Dako DAB substrate. After a final wash with distilled water the slides were removed from the Sequenza™ immunostaining centre

and placed into distilled water before haematoxylin counterstaining. The counterstaining was carried out using a Thermo-Shandon Varistain 24-4™ automatic stainer. The programme places slides automatically in haemotoxylin for 1 min, followed by 2 separate water rinses of 30 sec each. The slides are then placed in Scott's tap water for 30 sec before a further rinse. The slides were then protected by a cover slip using mounting medium (DPX, BDH Laboratory Supplies Poole, UK). Slides were left to set for at least 1 h before visualisation on an Olympus BX50 microscope. Images were captured using an Olympus DP70 digital camera and Cell F imaging software.

Numbers of positively stained cells in each section (one section for each animal at each anatomical location) were assessed subjectively and described using the key shown in Table 2.1. This is a published method employed by Dagleish *et al*..

0	no significant immunostaining
1	perceptible staining
2	mild staining
3	moderate staining
4	severe staining
5	most severe staining

Table 2.1 Immunohistochemistry: positive staining scoring system used for sections of bovine intestinal tissues. This method is an objective way of assessing the staining. The scoring refers in the main to numbers of positive cells per section and also takes into account the strength of staining. This method is used in order to produce a numerical output which can then be subjected to statistical analyses (Dagleish *et al.*, 2010).

Statistical analyses were carried out to determine if there were any differences between the staining patterns observed at the four anatomical sites. A Kruskal-Wallis one way analysis of variance test was used within the Minitab (v13) programme for Microsoft Windows because the data sets did not conform to normal distribution. A p value of less than 0.05 was considered significant.

2.2.4 Dual immunohistochemistry fluorescence staining protocol

Slides were removed from -80°C and treated as for the initial part of the method above. They were air dried under a fume hood for 10 min using forced air. They were fixed in chilled methanol at -20°C for 10 min and air dried in a laminar flow tissue culture hood for 10 minutes. Two rinses in PBS were followed by a 5 min wash in PBS + 0.5% Tween 80 (PBST80). The slides were then incubated in PBST80 + 0.3% H_2O_2 in a coplin jar for 20 min and washed with 2 x 5 min rinses in PBST80. All slides were then loaded into the Sequenza rack using PBS and washed once using PBS. Incubation in 25% normal goat serum (NGS; Vector)/PBST80 was carried out for 1 h. Following this blocking step the slides were incubated overnight at 4°C in either primary antibody in 10% NGS/PBST80 or mouse IgG (2 μg/ml). Two rinses in PBS were then followed by 30 min incubation in Dako goat anti-mouse polymer at ambient temperature. Two washes in PBS were followed by an incubation for 10 min in Alexa Fluor-568 tyramide substrate (Molecular probes) in the dark. The tyramide substrate was made up according to the manufacturer's instructions immediately before applying to the sections. All subsequent incubation stages were now carried out in the dark and all fluorescent antibodies were briefly pulsed in the centrifuge to remove residues. Two washes in PBS were followed by a further serum blocking

step. The slides were incubated in 10% normal mouse serum (NMS, Sigma)/PBST80 for 1 h at RT. The slides were then incubated for 1 h at RT in either SW73.2 mAb, to detect MHC II, or rat IgG (2 μg/ml) diluted in 10% NMS/PBST80. A further two washes in PBS were followed by an incubation for 30 min at ambient temperature with rabbit anti-rat FITC (Vector, FI-4001) diluted 1:500 in NMS/PBST80. Again, 2 washes in PBS were carried out before an incubation for 30 min at ambient temperature with donkey anti-rabbit LC Alexa Fluor-488 (1:1000 i.e. 2 μg/ml). Two final washes in PBS were carried out before the slides were mounted in Mowiol (Calbiochem). The slides were left for at least 1 h for the Mowiol to set and the slides were stored, protected from light, at 4°C prior to visualisation. The slides were visualised on a Zeiss Axiovert 200 and images were captured using Axiocam MRm and Axiovision Rel 4.7.2. multidimensional acquisition software.

2.2.5 Cell counts of positively stained cells and data analysis

Counts of positively stained cells were made using ImageJ software. ImageJ is a public domain Java image processing program inspired by NIH Image (Rasband W.S. & Bright D. S., 1995). Downloadable distributions are available on the internet at http://rsbweb.nih.gov/ij/download.html. 400 to 600 positively stained cells were counted and the number of these cells that were single- and double-stained were noted. The percentage of co-expressing cells in each anatomical location could then be calculated. Data was analysed using a Kruskal-Wallis test for populations with unequal variance within in the Minitab software

2.3 Results

In this study the bovine intestine was subjected to immunohistochemical staining for cell surface molecules. Four intestinal sites were selected for investigation: the distal colon, the proximal colon, the proximal rectum and terminal rectum (See Fig. 2.1). Post-mortem samples were collected from six male Holstein Friesian calves and immunohistochemistry was carried out as described in section 2.2. Representative formal saline fixed samples were H&E stained and no significant histopathological changes were observed (Fig. 2.2).

2.3.1 Single Immunohistochemical Staining

The presence of MHC II, CD205, SIRPα, CD1b, CD40, CD11c and CD14 in the tissue sections was analysed by immunohistochemistry. MHC II, CD205, CD40 and CD14 staining was carried out with zinc salt fixed tissues. SIRPα, CD1b and CD11c staining was carried out with frozen tissue sections. The numbers of positively stained cells were objectively analysed and categorised as shown in Table 2.1. This allowed statistical analysis to be carried out to determine any differences in staining at the four intestinal sites. The results of the immunohistochemistry for the six animals studied are shown in Table 2.2 and Fig. 2.4. All animals were examined for each molecule of interest at four intestinal locations where numbers of sections allowed. A Kruskal-Wallis test was used to assess differences in positive staining at the terminal rectum, proximal rectum, proximal colon and the distal colon. All control slides were clear of staining (Fig. 2.2).

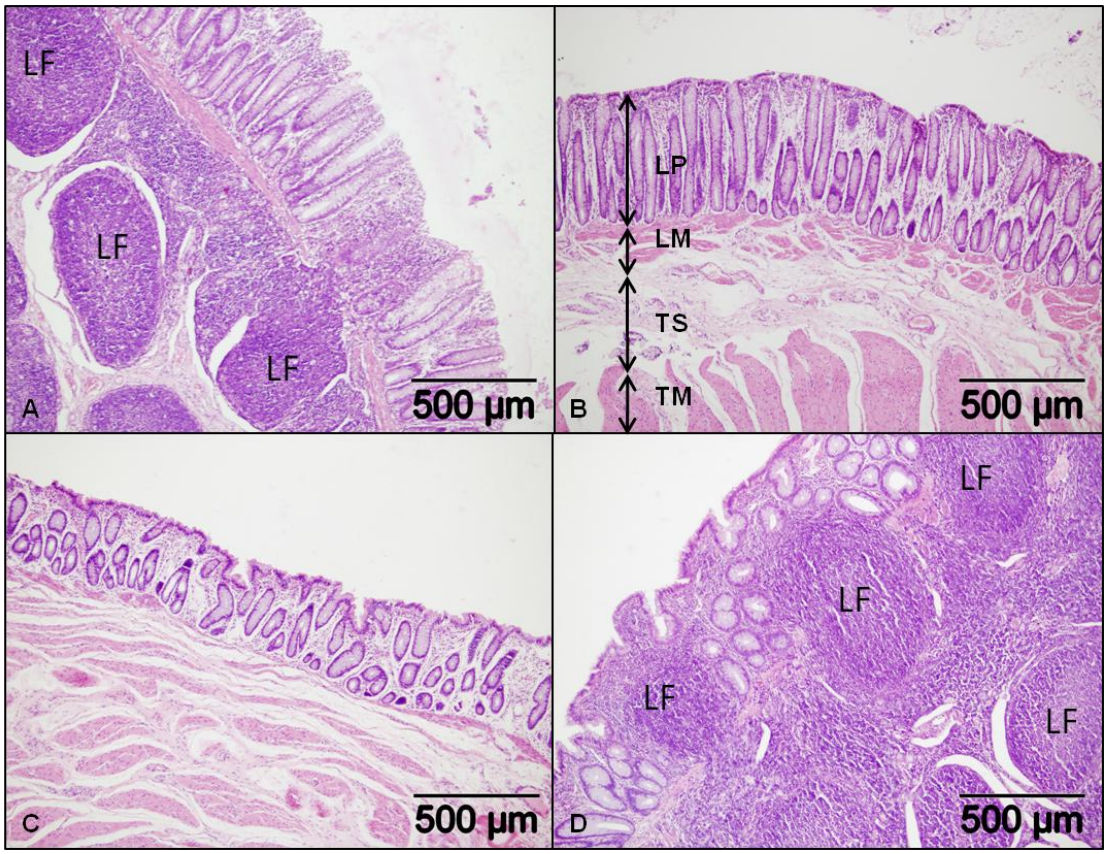


Fig. 2.2 Representative Haematoxylin and Eosin stained sections of formal saline fixed calf intestine at sites utilised for immunohistochemistry analysis. (A) Proximal Colon, (B) Distal Colon, (C) Proximal Rectum, (D) Distal Rectum. LF: Lymphoid Follicle, LP: Lamina Propria, LM: Lamina Muscularis, TS: Tela Submucosa, TM: Tunica Muscularis. These sections are representative of the normal intestinal morphology. Panels (A) and (D) make clear the abundance of lymphoid tissues within the proximal colon and the terminal rectum, and the relative lack in corresponding tissues of the distal colon and proximal rectum. Panel (B) is annotated to show the anatomical layers, which are similar for all sections. Lymphoid follicles can be seen within the tela submucosa, often extending through the lamina muscularis into the lamina propria, and directly up to the epithelium.

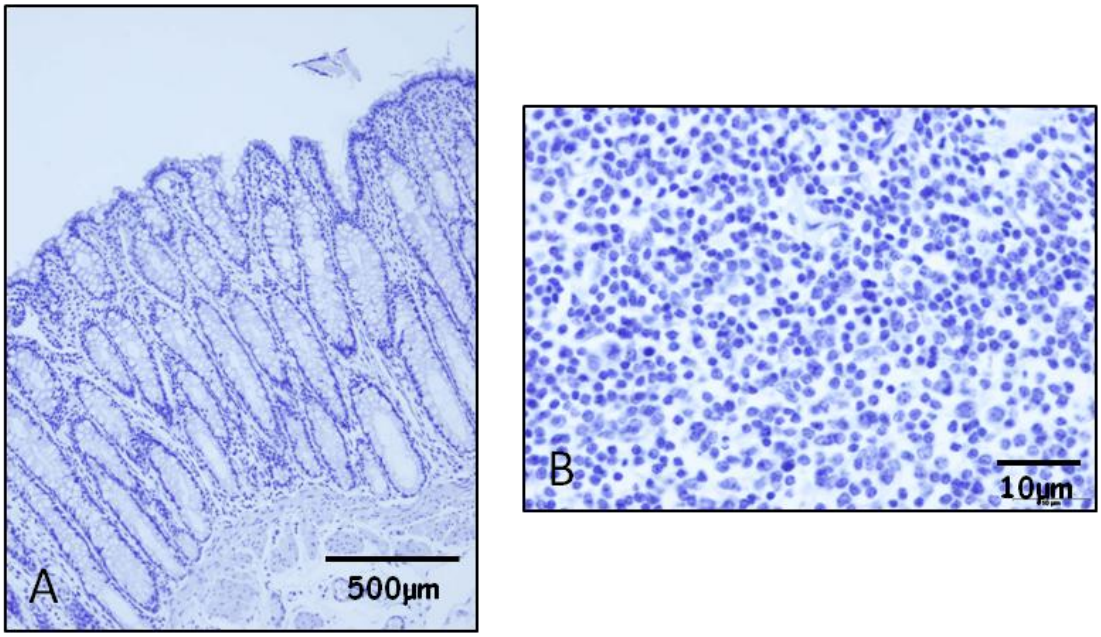


Fig 2.3 Representative isotype control sections of calf intestine. (A) Rat IgG isotype control, distal colon epithelium. (B) Mouse IgG isotype control, proximal colon showing a lymphoid follicle. All control slides were clear of staining.

	MHC II				CD1b				CD205				SIRPα				CD40			
	DiC	PC	PR	TR	DiC	PC	PR	TR	DiC	PC	PR	TR	DiC	PC	PR	TR	DiC	PC	PR	TR
Calf 1	3	5	3	5	n.d.	n.d.	1	3	3	5	3	5	1	3	3	2	4	5	5	5
Calf 2	2	4	3	5	1	1	1	3	4	4	4	5	3	3	3	4	4	4	4	5
Calf 3	2	4	2	5	1	1	1	3	3	4	3	5	3	1	1	2	5	4	4	4
Calf 4	3	5	5	4	1	3	1	3	3	5	4	5	2	4	5	3	3	5	5	5
Calf 5	3	5	4	5	1	2	1	4	4	5	2	5	1	1	1	3	3	5	3	5
Calf 6	4	5	3	5	2	1	1	n.d.	3	4	4	5	3	3	3	4	4	4	4	5

	CD14				CD11c				IgG				IgA			
	DiC	PC	PR	TR	DiC	PC	PR	TR	DiC	PC	PR	TR	DiC	PC	PR	TR
Calf 1	2	5	3	5	n.d.	n.d.	2	1	1	1	1	1	0	0	0	1
Calf 2	2	1	3	5	2	n.d.	2	1	0	0	1	1	0	0	0	1
Calf 3	n.d.	1	2	5	2	2	2	3	1	1	0	1	0	0	1	1
Calf 4	1	5	5	2	3	n.d.	2	2	0	1	0	0	0	1	1	1
Calf 5	1	5	n.d.	5	1	0	1	2	1	0	0	0	1	1	1	0
Calf 6	3	1	2	5	1	1	1	n.d.	1	0	1	1	1	0	0	1

Table 2.2 Individual animal data for immunohistochemistry of intesitinal sections. Intensity of staining scored 0 – 5 where 0 = no significant immunolabelling and 5 = most severe. n.d. = not determined, due to lack of tissues or tissues where the morphology was too poor to assess staining accurately. Severity of staining correlates to numbers of positive cells as well as intensity of stain. DiC: Distal Colon, PC: Proximal Colon, PR: Proximal Rectum, TR: Terminal Rectum. Positive staining was observed for all the molecules of interest. There was some variation noted between the six animals studied. Significant differences between intestinal sites were observed for MHC II, CD1b and CD205 ($p<0.05$). For each of these molecules a greater number of positive cells was observed at the terminal rectum (CD1b), or the terminal rectum and proximal colon (MHC II & CD205) compared with the remaining anatomical locations.

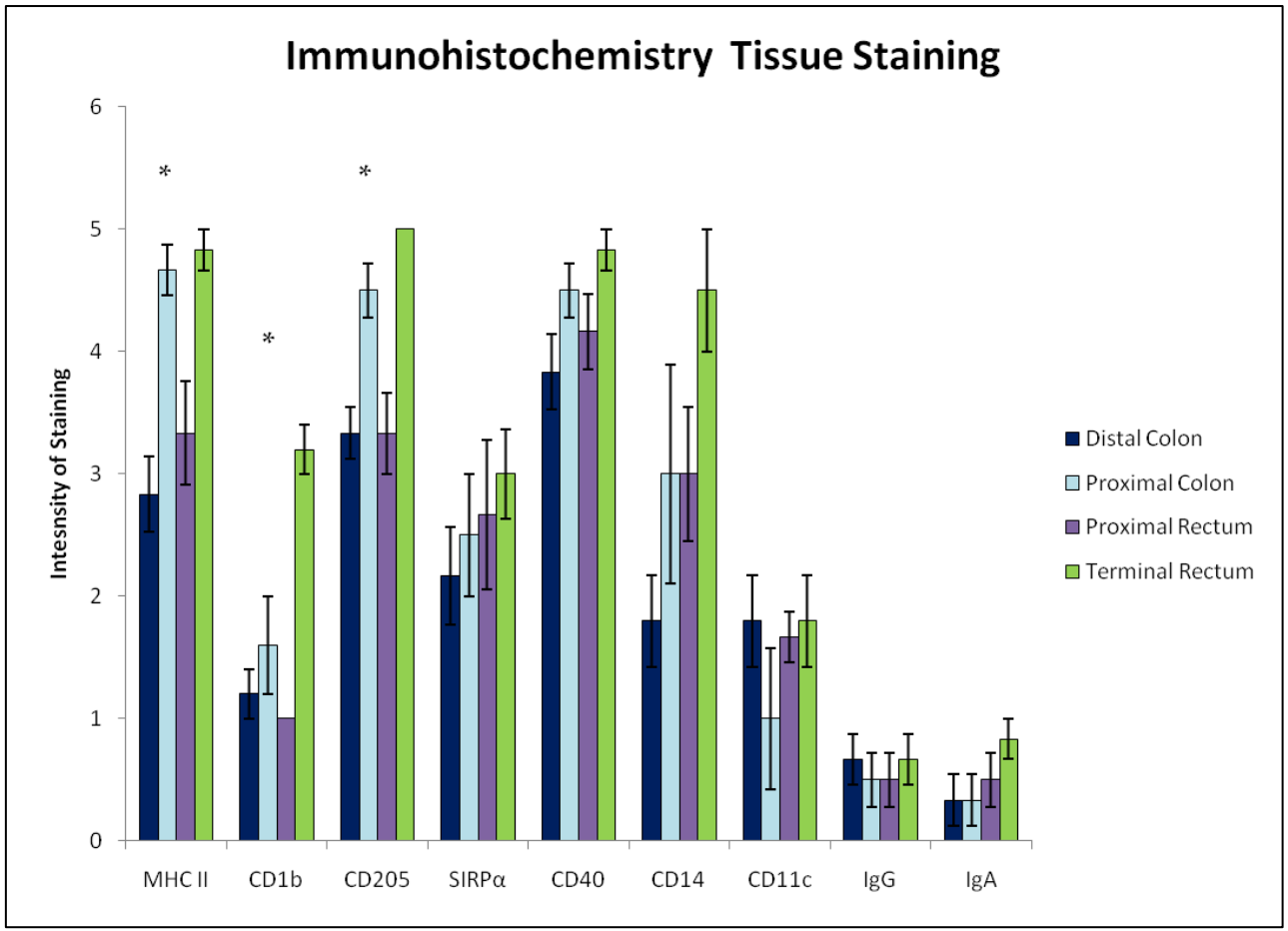


Fig. 2.4 Histogram showing anatomical distribution of molecules detected by immunohistochemistry in the bovine intestine. Histogram shows the pooled data presented in table 2.2. Significant differences in staining between intestinal sites were observed for MHC II, CD1b and CD205 ($p<0.05$ *). Data is pooled from six animals where morphology of tissues allowed (Table 2.2 details the tissues from which results could not be obtained). Error bars represent the standard error of the mean.

2.3.1.1 MHC II Expression

Labelling of MHC II was observed throughout the intestinal tract. Representative results of the immunohistochemistry are shown in Fig. 2.5. Many positive cells were observed in the lymphoid follicles at both the proximal colon and the terminal rectum, to the extent that very little counter stain was visible in these areas. Small groups and single MHC II^+ cells were observed in epithelial areas and within the lamina propria. Higher numbers were present just below the epithelial layer at all sites, often at the tips of the villi. Individual MHC II^+ cells were observed less frequently in the lamina propria than in villous areas along the whole large intestine. In general fewer positive cells were observed at the proximal rectum (Fig 2.5 E & F) and distal colon (Figure 2.5 C & D) compared with the proximal colon (Fig 2.5 A & B) and terminal rectum (Fig. 2.5 G & H).This finding was statistically significant ($p<0.05$). Most of the positive cells within the follicles exhibited pleomorphism when observed at higher magnification (OM x 400), with an irregular shape and the elaboration of dendritic type structures (Fig. 2.5 H). A much smaller population of large, strongly staining, rounded cells, likely to be B cells, was also present (Fig. 2.5 F). In the epithelium and lamina propria the positive cells again appeared to fall into these two main populations in roughly similar proportions.

2.3.1.2 CD205 expression

The results of CD205 staining can be observed in Fig. 2.6. Widespread staining was observed for CD205. Positive cells were observed frequently within the epithelial layer, along the basement membrane and bordering the intestinal lumen (Fig. 2.6 D, F & H). Positive cells were noted individually

and in small groups throughout the lamina propria (Fig. 2.6 B, C & E). Follicles were positively stained in their entirety (Fig. 2.6 A & G). More staining was observed at the terminal rectum and the proximal colon due to strong and widespread staining of the follicles in these areas ($p<0.05$). Cell phenotypes observed included a population of large irregular cells which were likely to be DCs, other cells with a flattened morphology and a sizeable population of rounded cells which were likely to represent T and B cell populations.

2.3.1.3 SIRPα expression

Positive staining for SIRPα was observed at all intestinal locations in all the animals studied. Representative results are shown in Fig 2.7. Staining for SIRPα was widespread, with large numbers of positive cells within the lamina propria and particularly sub-epithelially (Fig. 2.7 C & F). Occasionally individual SIRPα^+ cells were observed within the epithelial layer between two epithelial cells making up the luminal edge of the tissue (Fig. 2.7 D). Aggregations of positive cells were observed around the edges of the follicles, almost forming a ring of strongly positive staining surrounding the follicle (Fig. 2.7 F, G & H).Very few strongly positive cells were noted within the follicular areas themselves, although a very weak stain was observed over the cells in the very centre of some follicles. The majority of the positive cells were large with an irregular morphology. A small number of the positive cells were more rounded in appearance (Fig. 2.7 D, G & H). There was a trend towards a larger number of positive cells within the tissues of the terminal rectum than at other sites however any differences were not significant ($p=0.637$).

2.3.1.4 CD1b Expression

Positive staining for CD1b was observed in all animals tested and at all sites. The results are shown in Fig. 2.8. There were many fewer CD1b-positive cells than MHC II-positive cells, although CD1b-positive cells stained more strongly. There were significantly more CD1b-positive cells recorded at the terminal rectum than at other sites ($p<0.05$). There were few positively stained cells noted in the epithelial layers and none within the centre of the follicles. Positive cells could be found individually throughout the lamina propria at all sites. Darkly staining cells were observed individually and in small groups, in moderate numbers surrounding the edges of lymphoid follicles (Fig. 2.8 D). All of the positively stained cells were large with an irregular appearance and dendrites were often identifiable (Figure 2.8 E).

2.3.1.5 CD40 Expression

The results of CD40 staining are shown in Fig. 2.9. Positive staining for CD40 was observed in all animals and at all of the intestinal sites. Staining for CD40 was fairly widespread and intense, being scored as severe in most sections. A large number of the positive cells were grouped together under the epithelium near the basement membrane. Individual positive cells were scattered through the lamina propria (Fig. 2.9 C, D & E). No cells were observed between the epithelial cells contacting the lumen (Fig. 2.9 B, D & F). The lymphoid follicles were strongly positively stained throughout making it difficult to identify any negative cells in this location (Fig. 2.9 A & G). The positive cells within the follicles were largely irregular in shape (Fig. 2.9 H). $CD40^+$ cells with a flattened, elongated morphology were observed directly

below the epithelium. There was no significant difference between the anatomical sites for levels of positive staining observed (p= 0.083).

2.3.1.6 CD11c Expression

The results of CD11c staining are shown in Fig. 2.10. CD11c staining was generally weak although a small proportion of larger, more strongly-stained cells were present. There were no significant differences in CD11c expression at different anatomical locations (p=0.607), however a smaller sample size for some of the locations may have contributed to this. CD11c staining was carried out on frozen tissues and in some sections the morphology was not preserved sufficiently to accurately assess the staining pattern or cell morphology. Positive cells were observed at all sites and in all animals. However there was section to section variation, which could be due to the weaker nature of the positive staining with these reagents or variation in cryosectioning techniques. Positive cells were generally found within the lamina propria as large individual, irregularly shaped cells (Fig. 2.10 E) and sometimes in small aggregations (Fig. 2.10 B & D). Few positive cells were noted within the follicles.

2.3.1.7 CD14 Expression

The results of CD14 staining are shown in Fig. 2.11. Mild $CD14^+$ staining was observed in all animals and at all of the intestinal sites. The positive staining was generally weaker than that observed for the other molecules. Individual cells were observed throughout the epithelium and lamina propria. The $CD14^+$ cells tended to have a more flattened or elongated morphology (Fig. 2.11 D). However, infrequent, more strongly staining cells with a round morphology were also observed (Fig. 2.11 F). Positive staining was observed

in follicular areas and surrounding the follicles but was not pronounced. No significant difference was found between the staining patterns observed at the four intestinal sites ($p= 0.082$). A variation in staining patterns between the animals studied was observed and could be caused by several factors. It could be that heterogeneity of CD14 expression exists within the cattle population and this technique reflects these animal differences; or it could also be due to limitations of the technique. The generally weak nature of CD14 staining could indicate that the combination of tissue, fixation technique and reagents employed here may be close to the threshold of detection for CD14, and therefore in some sections positive staining is not perceptible.

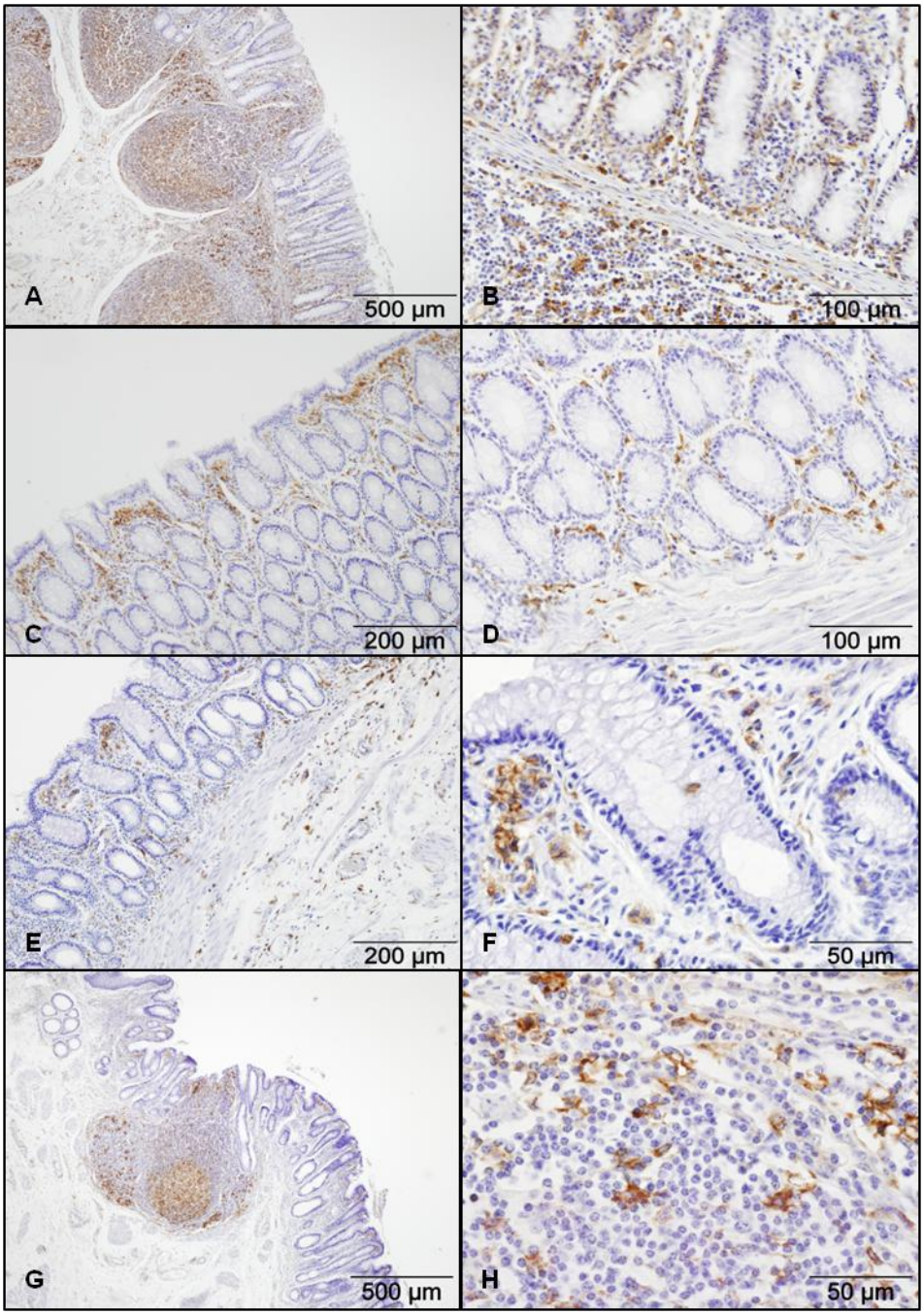


Fig. 2.5 Representative MHC II staining of bovine intestinal tissues. (A) & (B) Proximal Colon, (C) & (D) Distal Colon, (E) & (F) Proximal Rectum, (G) & (H) Terminal Rectum. Widespread staining of all tissues was observed. (A) Lymphoid follicles were stained strongly (B) positive cells were observed between the epithelial cells, contacting the intestinal lumen. (C) aggregations of positive cells were observed at the apical villi. (D) pleomorphic cells near the lamina muscularis. (E) Fewer positive cells were noted within the tissues of the proximal rectum. Aggregations of cells noted at the apical villi. Individual positive cells were scattered throughout the tela submucosa. (F) Pleomorphic cells at the apical villi and a positive cell within the epithelium. (G) Numerous positive cells within a lymphoid follicle of the terminal rectum. (H) Positively stained cells with a dendritic morphology within the lymphoid follicle.

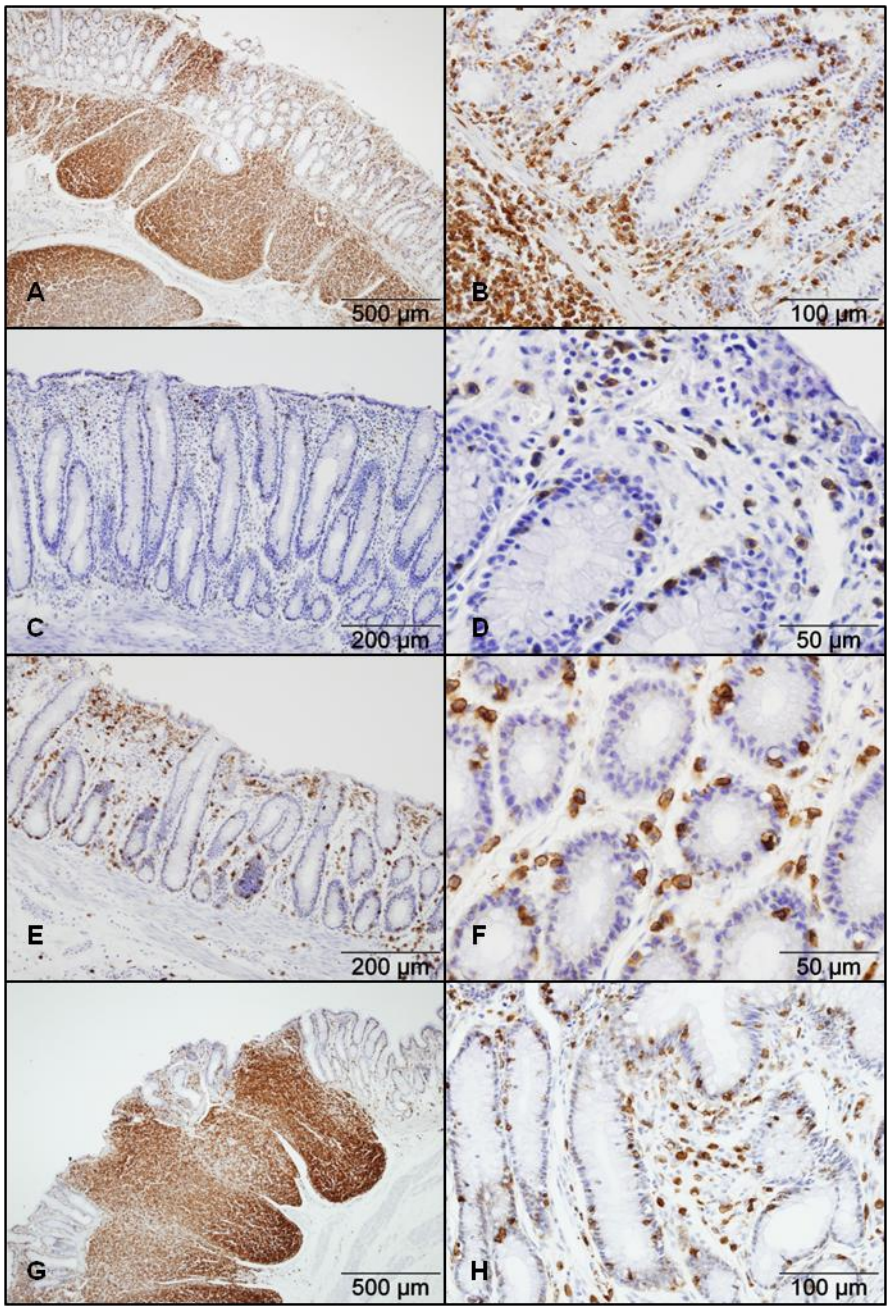


Fig. 2.6 Representative CD205 staining of bovine intestinal tissues. (A) & (B) Proximal Colon, (C) & (D) Distal Colon, (E) & (F) Proximal Rectum, (G) & (H) Terminal Rectum. Widespread staining of all tissues was observed. (A) Intense staining of the lymphoid follicles of the proximal colon (B) Many positive cells were observed between the epithelial cells, contacting the intestinal lumen; a lymphoid follicle observed below the lamina muscularis (C) Fewer positive cells are noted within the tissues of the distal colon, mainly scattered through the lamina propria. (D) Mainly rounded or elongated cells were found near the apical villi. (E) Aggregations of cells noted at the apical villi. Individual positive cells were scattered throughout the tela submucosa. (F) Pleomorphic cells within the lamina propria, several positively stained cells were found within the epithelial layer (G) Abundant positive cells within the lymphoid follicles of the terminal rectum. (H) Numerous positively stained cells within the epithelial layer. Many large round cells, other morphologies noted, including some elongated cells.

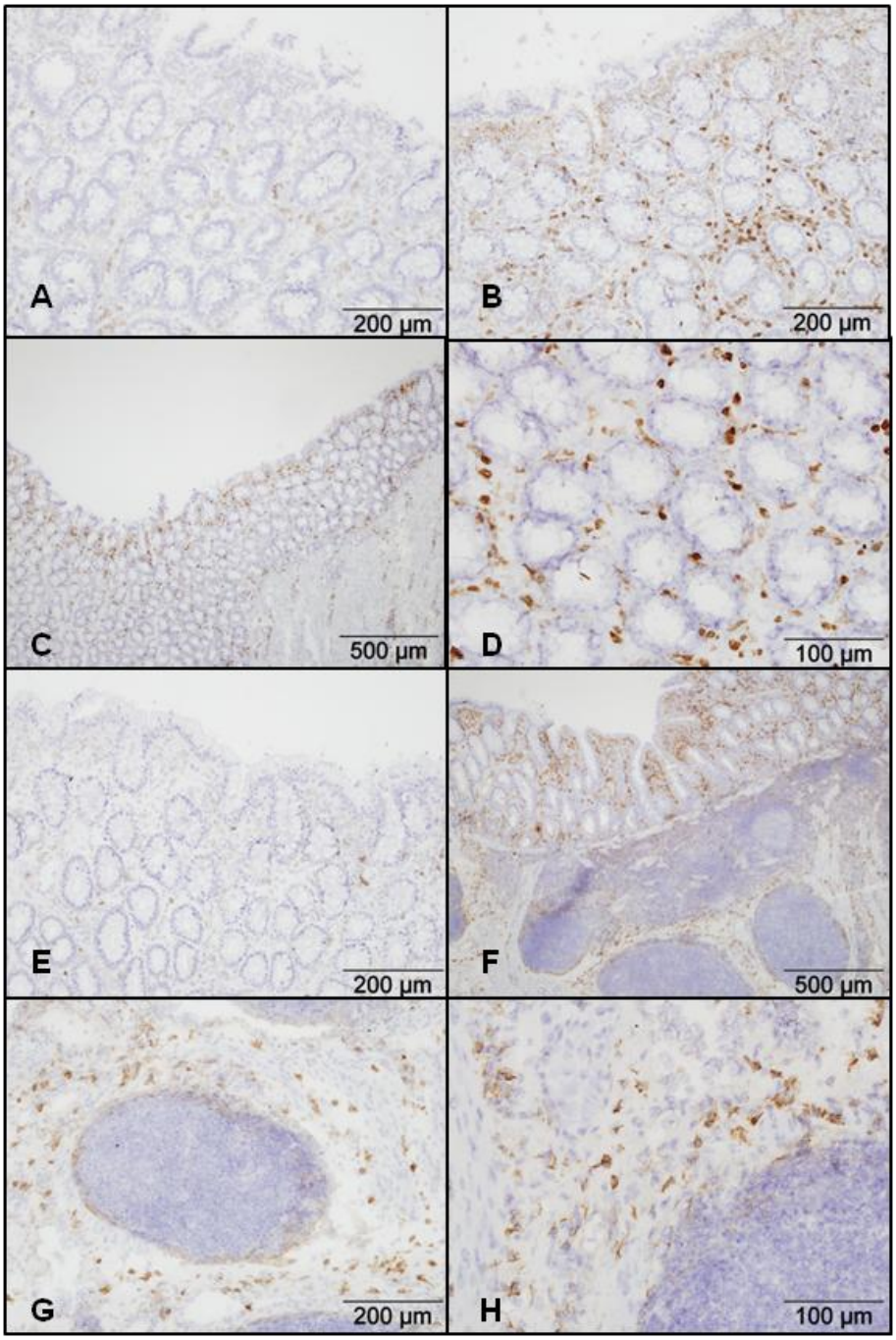


Fig. 2.7 Representative SIRPα staining of bovine intestinal tissues. (A) & (B) Proximal Colon, (C) & (D) Distal Colon, (E) Proximal Rectum, (F) (G) & (H) Terminal Rectum. Moderate staining of all tissues was observed. (A) & (B) Staining of the lamina propria of the proximal colon showing some variation in the strength of positive staining (C) Staining of the distal colon shows that positive cells occur in aggregates at the apical villi and scattered through the lamina propria and tela submucosa. (D) Rounded and elongated cells were observed as well as cells with a more dendritic morphology. (E) Few positive cells within the proximal rectum. (F) Numerous strongly staining cells within the lamina propria of the terminal rectum, Positive cells were observed throughout the villi in groups as well as scattered throughout the tela submucosa. Positively stained cells surround the lymphoid follicles and are generally excluded from the centre. (G) Abundant positive cells within the tela submucosa surrounding a follicle at the terminal rectum. (H) Cells surrounding the follicles demonstrated pleomorphism.

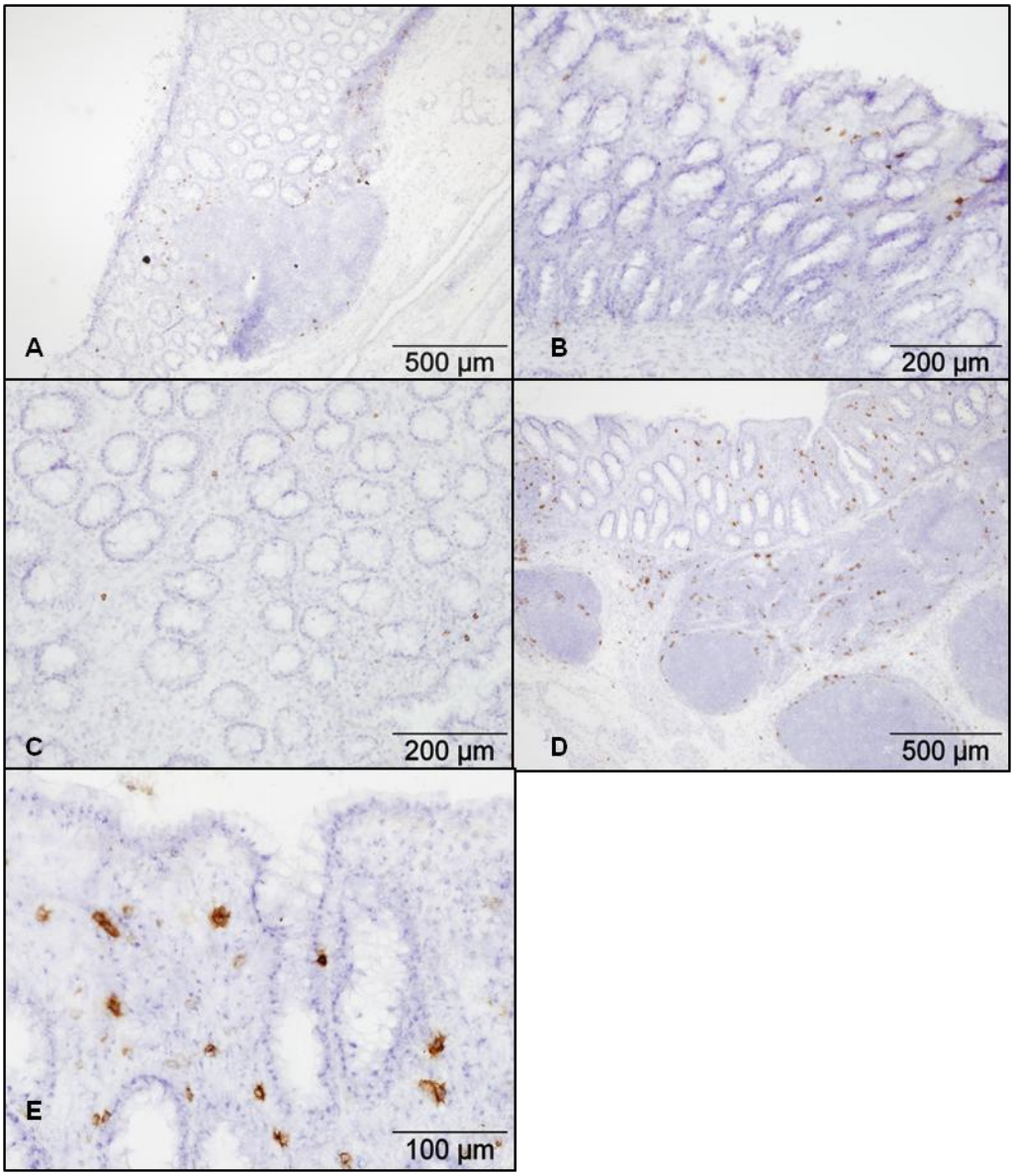


Fig. 2.8 Representative CD1b staining of bovine intestinal tissues. (A) Proximal Colon, (B) Distal Colon, (C) Proximal Rectum, (D) & (E) Terminal Rectum. Mild staining of tissues was observed. (A) Staining of the proximal colon showing few strongly stained cells in the lamina propria and at the periphery of a lymphoid follicle. No positive staining was observed in the centres of the follicles (B) Sparse, strongly staining cells at the apical villi (C) Staining of the proximal rectum shows that a few positive cells occur scattered through the lamina propria (D) Positive cells within tissues of the terminal rectum surrounding the lymphoid follicles, and absent from the follicle centres. Strongly staining cells with dendritic morphology can be seen within the lamina propria, and also scattered between the lymphoid follicles within the tela submucosa. (E) Strongly staining dendritic – type cells within the lamina propria of the terminal rectum.

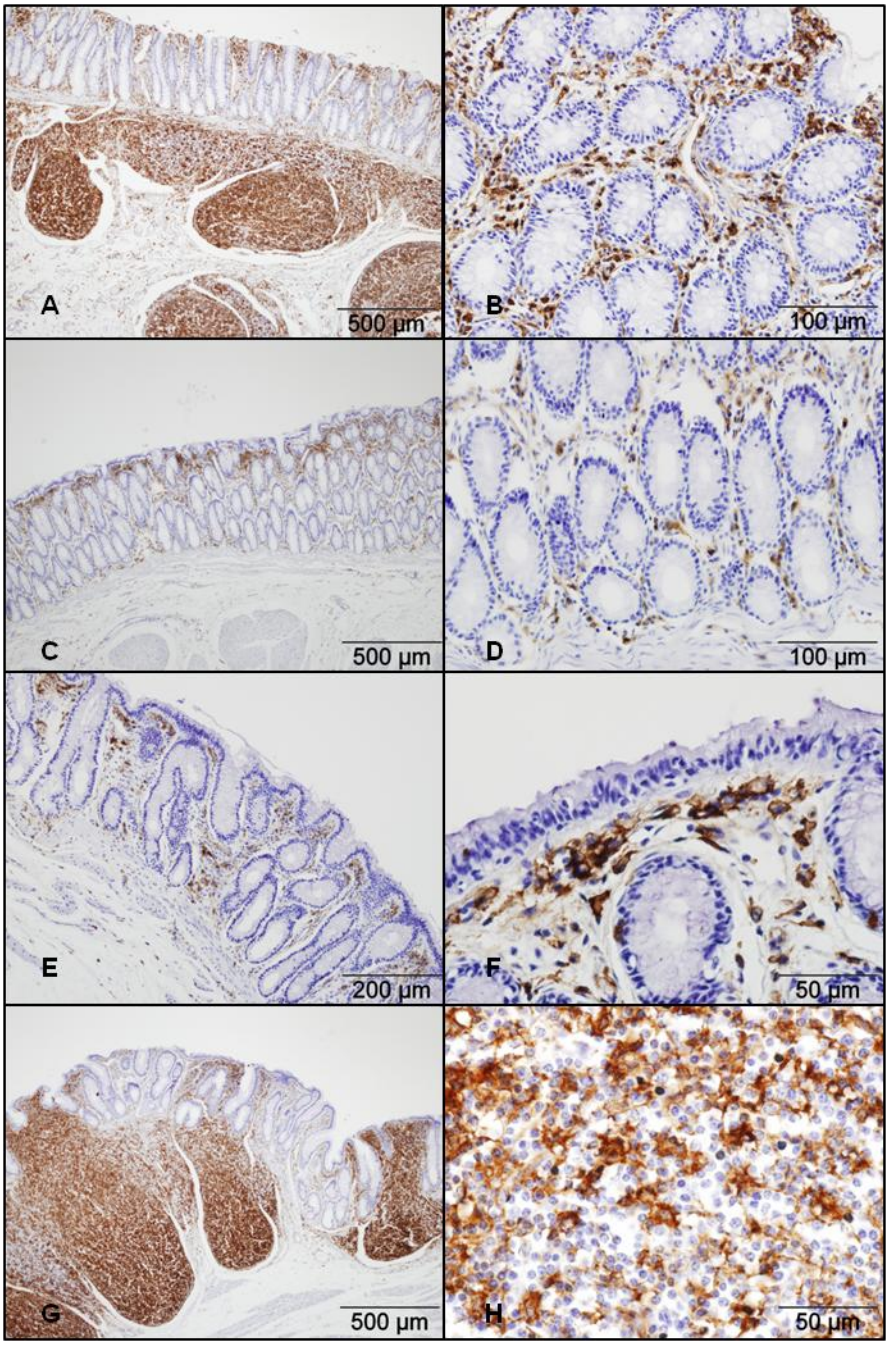


Fig. 2.9 Representative CD40 staining of bovine intestinal tissues. (A) & (B) Proximal Colon, (C) & (D) Distal Colon, (E) & (F) Proximal Rectum, (G) & (H) Terminal Rectum. Widespread staining of all tissues was observed. (A) Strong staining of lymphoid follicles and apical villous areas (B) Numerous positive cells throughout the lamina propria, very few positive cells observed within the epithelial layer. (C) Aggregations of positive cells at the apical villi. (D) pleomorphic cells within the lamina propria, no positive cells observed within or penetrating the epithelium. (E) Fewer positive cells are noted within the tissues of the proximal rectum. Small groups of positive cells within the lamina propria and individual positive cells scattered throughout the tela submucosa. (F) Pleomorphic cells at the apical villi and a positive cell within the epithelium. (G) Profuse staining of lymphoid follicles of the terminal rectum. (H) Large strongly stained cells with a dendritic morphology within the lymphoid follicle.

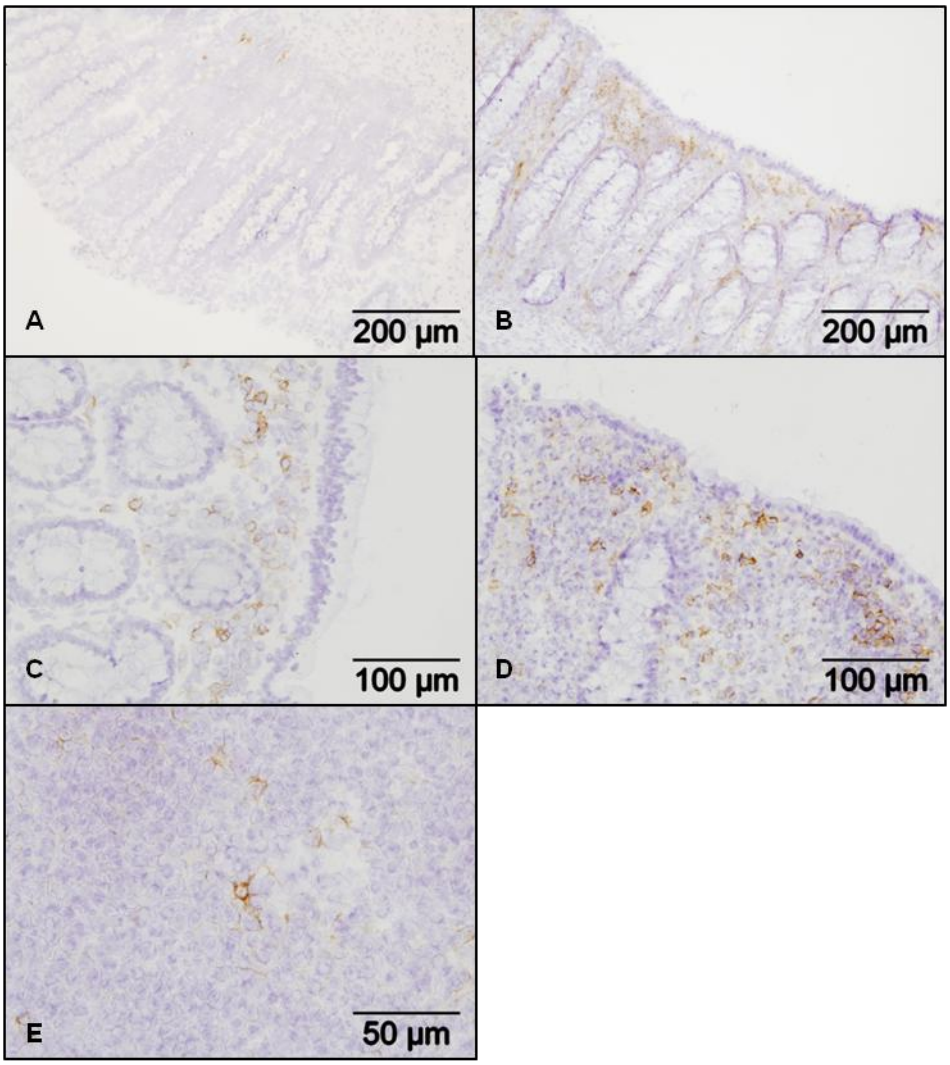


Fig. 2.10 Representative CD11c staining of bovine intestinal tissues. (A) Proximal Colon, (B) Distal Colon, (C) Proximal Rectum, (D) & (E) Terminal Rectum. Weaker staining of tissues, with a varied distribution was observed. (A) Staining of the proximal colon showing very few weakly stained cells in the lamina propria near the base of the intestinal glands (B) Groups of moderately stained cells at the apical villi (C) Staining of the proximal rectum shows weaker staining pleomorphic cells at the apical villi (D) Positive cells within tissues of the terminal rectum were slightly stronger staining, here pleomorphic cells are observed at the apical villi. (E) Positively stained dendritic – type cell within a follicle at the terminal rectum.

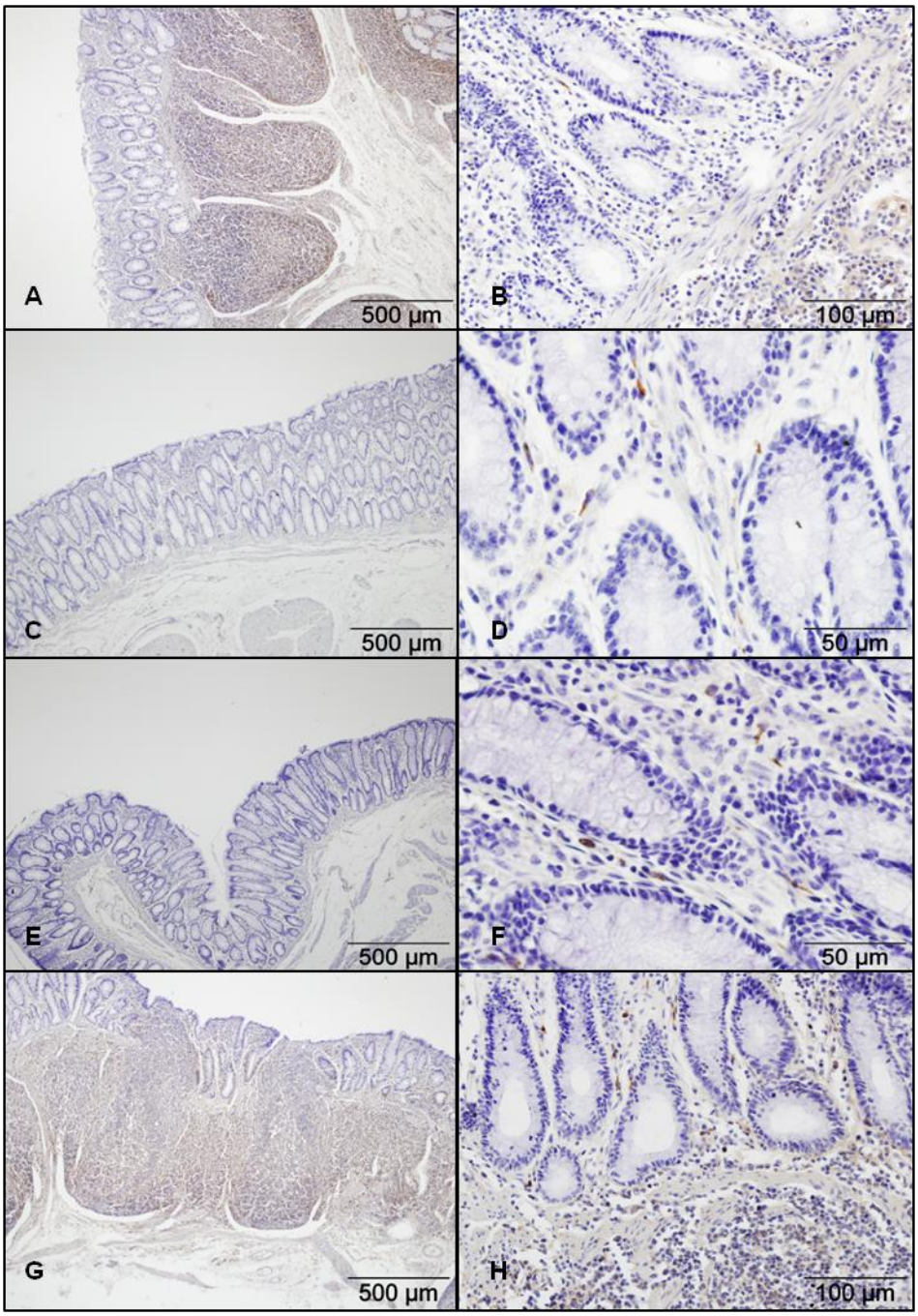


Fig. 2.11 Representative CD14 staining of bovine intestinal tissues. (A) & (B) Proximal Colon, (C) & (D) Distal Colon, (E) Proximal Rectum, (F) (G) & (H) Terminal Rectum. Moderate staining of all tissues was observed. (A) Diffuse, weak staining of the follicles of the proximal colon (B) Positively stained elongated cell in the lamina propria and diffuse, weak stain of the follicle tissue below the lamina muscularis. (C) Staining of the distal colon shows that positive cells occur in aggregates at the apical villi and scattered through the lamina propria and tela submucosa. (D) Elongated cells observed within the lamina propria of the distal colon. (E) Little positive staining within the tissues of the proximal rectum (F) Sparse positive cells within the lamina propria were elongated or rounded in shape. (G) Weak staining of the lymphoid follicles of the terminal rectum. (H) Moderately stained cells were generally of an elongated or rounded phenotype within the lamina propria. A weak stain can be seen on cells within the lymphoid tissue below the lamina muscularis.

2.3.2 Double immunohistochemical staining

The results described in section 2.3.1.1 and Fig. 2.5 identified widespread populations of MHC II$^+$ cells associated with the epithelium and lymphoid tissues of the bovine intestine. Further staining revealed other cell surface molecules in similar anatomical locations to the MHC II$^+$ cells (sections 2.3.1.2 – 2.3.1.7 and Fig. 2.6 – 2.11). A selection of these molecules was chosen for further investigation by double immunofluorescence, co-staining with MHC II as described in section 2.2.4. The molecules of interest selected were SIRPα, CD205 and CD1b. The same six animals and the same intestinal sites were used as for the single staining protocols. Approximately 500 (range 400-600) MHC II$^+$ cells per tissue section were counted and the number of these cells that were dual stained was recorded. The areas selected for analysis were the epithelium and lamina propria because the large numbers of positive cells within the follicles would make it difficult to identify individual cells. This study was directed towards identifying DC populations within the tissues and, as such, these cells would be large irregular cells which express MHC II in addition to other cell molecules. Calculation of the percentage of cells which are dual stained from the total MHC II$^+$ population can potentially indicate prevalence of DC subsets. However, not all SIRPα^+, CD1b$^+$ or CD205$^+$ cells were MHC II$^+$ and it was appropriate to also consider these cells. It was important to take into account that this method of statistical analysis does not indicate actual numbers of dual stained cells per section, only the proportions of cells single or double stained for various molecules and how these proportions may relate to anatomic locations. The relative numbers of positively stained cells are indicated by the earlier single cell staining data.

Firstly, this numerical system enabled the calculation of the percentage of the total MHC II$^+$ cell population (FITC stained – green fluorescence) which co-expressed each molecule (yellow fluorescence). Calculation of the percentage of CD205$^+$, SIRPa$^+$ and CD1b$^+$ cells (Tyramide stained – red fluorescence) which were also MHC II$^+$ (yellow fluorescence) was also performed. Representative images obtained for the double staining are shown in Fig. 2.12. All controls slides were clear (data not shown). The percentage of the total number of MHC II $^+$ cells which also expressed each of the molecules of interest is shown in Table. 2.3. SIRPα was expressed on a small percentage of MHC II $^+$ cells (<5%) at each site along the intestine. CD205 was widely expressed by the MHC II$^+$ population in the intestine with variations that were site specific ($p<0.05$). The largest percentage of CD205$^+$ cells which were also MHC II$^+$ were observed within the tissues of the colon (73%). The percentages at the proximal rectum and proximal colon were similar at 43-45% and the smallest percentage of co-localisation was observed at the terminal rectum (35%).

There was great anatomical variation in the percentage of MHC II$^+$ cells which expressed CD1b. The smallest percentages were found at the proximal rectum (8%) and the terminal rectum (12%). The colon tissues contained higher percentages of MHC II$^+$ cells which also expressed CD1b; 28% at the proximal colon and 58% within the distal colon.

Next, the counts of positive cells recorded for each section were used to calculate the percentages of each of the individual cell surface molecules (CD1b, CD205 and SIRPα) which also expressed MHC II. This data showing the percentage co-localisation of MHC II with each molecule is shown in

Table. 2.4. This provided different information about the positively stained cells in the intestinal tissues. Co-localisation of SIRPα and MHC II was observed in all the animals and at all the intestinal sites at low levels; 15 - 20% of SIRPα^+ cells were also MHC II$^+$ at all sites except the colon. At the colon, less than 10% of SIRPα^+ cells were co-localised. These findings indicate that 80 – 90% of SIRPα^+ cells were not found to co-express MHC II when using this method in these tissues. The majority of CD205$^+$ cells also expressed MHC II. Greater than 50% of CD205$^+$ cells also expressed MHC II at all intestinal sites except the terminal rectum. The percentage of CD205$^+$ cells co-localising within the tissues of the terminal rectum were lower at 33%. It was found that 50 – 70% of CD1b$^+$ cells also expressed MHC II and this percentage was found throughout the intestine.

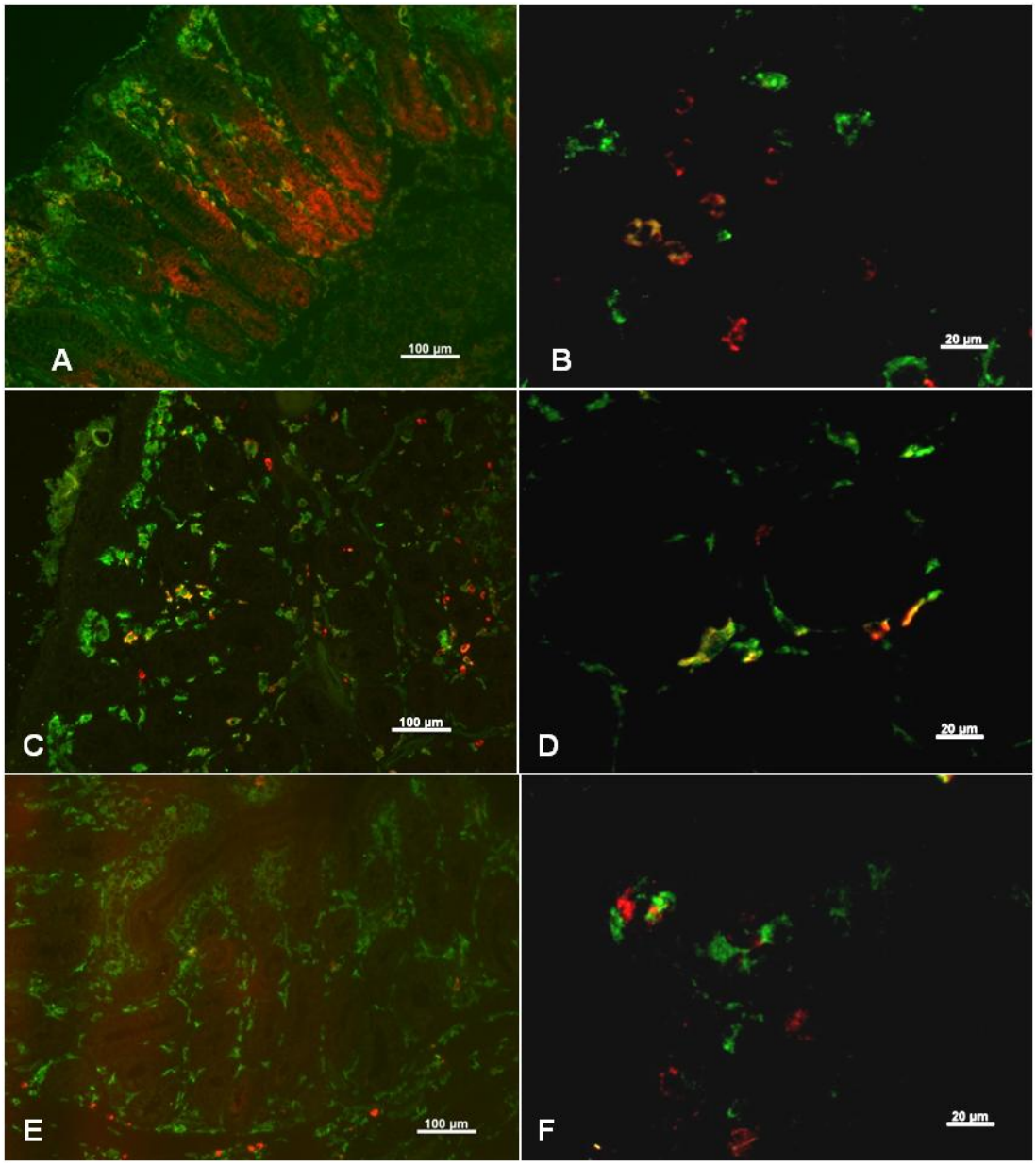


Fig. 2.12 Representative fluorescent stained sections of calf intestine. Green fluorescence in all images corresponds to MHC II expression. Red fluorescence: (A) & (B) CD205; (C) & (D) SIRPα; (E) & (F) CD1b. Yellow fluorescence indicates co-localisation of molecules. All sections are from rectal tissues. (A) Staining for MHC II and CD205 at the bovine terminal rectum. Numerous MHC II$^+$ cells (green) within the lamina propria, Many CD205$^+$ cells also expressed MHC II (yellow). Few CD205$^+$ single stained cells. (B) Higher power image of proximal rectum tissue showing a few CD205$^+$ cells (red), some large MHC II$^+$ cells (green) and two double stained cells (yellow). (B) Tissue of the proximal rectum stained for MHC II (green) and SIRPα (red). Many MHC II$^+$ cells present, several SIRPα^+ cells and occasional larger double stained cells near the apical villi. (D) Co-localisation of MHC II and SIRPα (yellow) in a large cell with dendritic morphology. (E) Tissues of the proximal rectum stained for MHC II (green) and CD1b (red) showing numerous MHC II cells in the lamina propria region. Fewer cells were CD1b$^+$ and co-localised cells (yellow) were sparse. (F) Large cell with dendritic morphology stained positively for MHC II, with one cell which seemed to express both molecules although at different cellular locations.

Cell surface molecule†	% of total MHC II cell population co-expressing cell surface molecules*			
	Proximal colon	Distal Colon	Proximal Rectum	Terminal Rectum
SIRPα	5.04 ± 1.91	3.45 ± 1.31	3.84 ± 0.77	4.67 ± 1.19
CD205	43.21 ± 9.41	73.23 ± 5.69	45.40 ± 5.14	35.00 ± 5.09
CD1b	28.54 ± 8.08	58.33 ± 8.24	8.43 ± 2.53	12.78 ± 6.39

Table 2.3 The percentage of MHC II^+ cells which co-express DC cell surface molecules: (†) A double immunohistochemical staining method was used for DC molecules and MHC II in the proximal colon, distal colon, proximal rectum and terminal rectum of 6 calves. (*) Values are the mean percentages of cells expressing MHC II which also express SIRPα, CD205 and CD1b ± SEM. Significant differences ($p<0.05$) for anatomical location were observed for CD205 and CD1b. No significant difference for anatomical location was observed for MHC II^+ cells co-expressing SIRPα, these values were similar at the intestinal sites investigated.

Cell surface molecule†	% co-expression of MHC II*			
	Proximal colon	Distal Colon	Proximal Rectum	Terminal Rectum
SIRPα	16.46 ± 4.16	7.3 ± 1.73	18.81 ± 4.27	18.46 ± 5.17
CD205	61.26 ± 6.10	84.11 ± 10.49	51.32 ± 6.16	33.82 ± 8.13
CD1b	47.16 ± 10.39	69.65 ± 4.48	51.88 ± 4.58	54.47 ± 8.08

Table 2.4 Co-expression of DC cellular molecules with MHC II; the percentage of cells expressing the DC molecule of interest which co-express MHC II: (†) A double immunohistochemical staining method was used for DC molecules and MHC II in the proximal colon, distal colon, proximal rectum and terminal rectum of 6 calves. (*) Values are the mean percentages of cells positively stained for SIRPα, CD205 and CD1b which also express MHC II ± SEM. Significant differences ($p<0.05$) were observed at anatomical locations for CD205. There were no significant differences between intestinal sites observed for CD1b or SIRPα positive cells which also co-expressed MHC II.

2.4 Discussion

For this chapter, immunohistochemical techniques were used to characterise dendritic cells in bovine intestinal tissue and to investigate any differences in the population of cells within selected locations within the tissues of the large intestine, including the terminal rectum.

Widespread staining of MHC II within the tissues of the terminal rectum suggests there are numerous immune cells in this area. The additional cell surface molecules investigated, the co-localisation of selected molecules with MHC II, the locations of the positively stained cells and their morphology make it quite clear that there are numerous DCs within all bovine large intestinal tissue including the terminal rectum which is the principal site of *E. coli* O157:H7 colonisation.

There were significantly more positively stained cells found associated with the lymphoid follicle areas within the proximal colon and terminal rectum than found within the tissues of the proximal rectum and the distal colon. This was evident by visual inspection and confirmed by statistical analysis of data derived from the stained sections.

There was widespread staining of MHC II, CD205 and CD40 within the follicles. The results of the single colour IHC indicates that there is a population of cells within bovine intestinal tissues that are likely to be MHC II^+, $CD205^+$ and $CD40^+$, suggested by the profuse staining for these three epitopes within the tissues of the lymphoid follicles. This does not fully define these follicular cell populations, however, and multiple-colour IHC could more accurately identify the molecules which are co-expressed by these cells. Additionally, if the cells could be extracted from this area,

multiple colour FACS analysis would assist further characterisation. The possibility of characterising *ex vivo* DCs in this way is investigated in more depth in Chapter 3.

The staining pattern observed for SIRPα and CD1b, where few positive cells were observed within the follicles but were clustered around the follicular margins and scattered within the lamina propria, suggests these populations of cells take up similar anatomical locations. Although there were many fewer CD1b$^+$ cells than SIRPα^+, it is possible that some of these cell are either co-expressing or are in close proximity. The morphology of these cells is strongly suggestive of DCs. Further techniques such as three colour immunofluorescence would be required to confirm any co-localisation of these epitopes with MHC II. Within ALDCs draining the skin of cattle, a subset of SIRPα^+ cells have been shown to co-express CD1b (Howard *et al.*, 1999). The SIRPα staining pattern, (strongly stained cells with dendritic morphology surrounding the lymphoid follicles) was interesting and is similar to findings in bovine mediastinal lymph node and spleen, where SIRPα^+ cells were identified but were excluded from the B cell germinal centres of these tissues (Brooke *et al.*, 1998).

CD11c staining, although weak, was observed in anatomical positions where MHC II$^+$ cells were located and dendritic morphology was observed, suggesting a small subpopulation of bovine DCs could be CD11c$^+$. Double staining with MHC II could verify this. CD11c has been found to be expressed by SIRPα^+ bovine DCs in blood and the thymic medulla (Miyazawa *et al.*, 2006).

Macrophage populations, represented by CD14 staining, indicated two populations of cells within the intestinal tissues. Weak staining of the follicle tissues suggests that cell types in this location weakly express CD14. More strongly staining cells within the lamina propria were either very elongated, or more rounded in appearance, neither of which are a classic dendritic morphology. Although some DCs may be weakly expressing CD14 within the follicles, the majority of the CD14 cells are likely to represent macrophages.

Double staining allowed the calculation of the percentages of MHC II^+ cells which co-expressed DC molecules at each intestinal location.

When comparing the results of the double staining with the study by McNeilly *et al* (2006), which examined tissues of the respiratory tract, many similarities can be drawn. The percentage of MHC II^+ cells co-expressing SIRPα was similar for intestinal and airway tissues at 5% or less. This suggests that a significant population of SIRPα^- DCs are likely to be present in the tissues. It has been shown that SIRPα^- DCs from both sheep mucosae and rat intestine contain apoptotic bodies and are likely to be tolerogenic in function, and are therefore an important DC sub-population (Epardaud *et al.*, 2004; Huang *et al.*, 2000). CD205 expression by MHC II^+ cells in the intestine was also similar to that found in the airway tissues: around 60% for the airways and between 43% and 73% for the intestinal tissues. No cells co-expressing SIRPα were found within the lung tissue and less than 10% of MHC II^+ cells were also $CD205^+$ in this area. It is possible that this may reflect the reduced antigen challenge within the lung tissues compared with the airways and intestine. There were marked differences in the percentages of MHC II^+ cells co-expressing CD1b depending on anatomical location. Within

all the respiratory locations the percentage was less than 10%, which was similar to the values calculated for the proximal and terminal rectum (8% and 12% respectively). This study found a much greater percentage of $CD1b^+$ cells co-expressing MHC II in the tissues of the proximal and distal colon (28% and 58% respectively). The sheep pulmonary study (McNeilly *et al.*, 2006) found that 96% - 100% of SIRPα^+ and $CD205^+$ cells in the airway also expressed MHC II but that around 87% of $CD205^+$ cells in the lung parenchyma did not co-express MHC II. Although this study also shows that $CD205^+$ cells co-expressing MHC II is significantly varied with anatomical site such as found by McNeilly *et al.*, in contrast to the airway findings this study suggests that 16 – 64% of intestinal $CD205^+$ cells were MHC II^-. These cells are likely to be B and T lymphocytes. Furthermore, upward of 80% of SIRPα^+ cells in this study appeared not to express MHC II. SIRPα is expressed by bovine DCs, monocytes, macrophages and granulocytes (neutrophils and eosinophils) (Ellis *et al.*, 1988; McKeever *et al.*, 1991). Of these cells only the granulocytes do not express MHC II in resting states, however both neutrophils and eosinophils can be stimulated to express MHC II (Celestin *et al.*, 2001; Hansch & Wagner, 2003). A study by Stamm *et al.* (Stamm *et al.*, 2008) also found a population of SIRPα^+, MHC II^- cells within the lamina propria of the bovine colon and the authors concluded that these cells were intestinal macrophages, despite the fact that macrophages would also be expected to show some level of MHC II expression. It is unclear whether the SIRPα data here represents an actual finding or is a result of the more intense SIRPα staining compared with the moderate MHC II staining when using the fluorescent antibodies. The imaging software was occasionally hampered by the varied levels of fluorescence when producing a multidimensional image. It is also possible that the dual staining protocol

blocked some of the MHC II sites due to the SIRPα, CD1b or CD205 mAbs being incubated with the tissues before MHC II. A further possibility is that the SIRPα^+ cells were expressing MHC II at a low level which was below the level of detection by these methods, and these cells could represent a population of immature peripheral DCs.

50 to 70% of CD1b$^+$ cells co-expressed MHC II in the bovine intestine, which is only marginally lower than the results for ovine respiratory tract where 60 – 90% of CD1b$^+$ cells co-expressed MHC II.

In summary, this study found similar populations of DCs in the bovine intestine to those found within the ovine respiratory system with widespread MHC II$^+$ cells throughout the tissues. DC populations expressing CD205 were widespread with smaller sub-populations of SIRPα^+ and CD1b$^+$ cells. However this study identified a population of cells that was SIRPα^+, MHC II$^-$ which was not observed by McNeilly *et al.* in ovine tissues. CD205$^+$, MHC II$^-$ cells were observed in ovine lung parenchyma (87%) but very infrequently within the upper airways, thus showing an anatomical variation. In this work too CD205$^+$, MHC II$^-$ cells appeared to be significantly linked to the anatomical location sampled ($p<0.05$).

Within the intestinal tissues 40-60% of CD1b$^+$ cells did not express MHC II. As MHC II expression is characteristic of DCs, it is probable that these cells are not DCs and as discussed in McNeilly *et al.* (2006), it is possible that they represent cortical thymocytes.

In contrast to the McNeilly study, where SIRPα staining of the ovine DCs was relatively weak, SIRPα staining was strong in the bovine cells. This

could represent a genuine increase in SIRPα expression by the bovine cells compared to ovine, or could be due to differences in protocols or reagents, and may have affected the dual staining results as discussed above. The monoclonal antibody used by McNeilly was ILA-24 whereas this study used CC149. Both antibodies have been investigated by Brooke *et al* (1998), and they were each found to recognise a different epitope of SIRPα which could account for the differences in staining observed here. In addition, although both mAbs functioned to inhibit binding of T cells to SIRPα, CC149 did this with higher efficacy than ILA-24 (Brooke *et al.*, 1998).

The lower numbers of SIRPα^+ cells in the intestinal tissue possibly suggests that a SIRPα^- population is more widespread here than SIRPα^+. This is similar to the situation found in sheep where SIRPα^- cells were more commonly found in lymph draining the mucosal surfaces of the head compared to the skin (Epardaud *et al.*, 2004).

The work on ALDCs in ruminants has identified differences in the DC populations draining different anatomical locations. The original bovine studies were carried out on lymph draining the skin, and this is where the majority of the information on bovine DCs, their function and phenotype has originated (Gliddon *et al.*, 2004a; Gliddon & Howard, 2002; Hope *et al.*, 2001; Howard *et al.*, 1996; Howard *et al.*, 1997; Stephens *et al.*, 2003). More recent work has been carried out on lymph draining the ovine head and has shown that SIRPα^- cells may be more common within mucosal tissue than the skin. This could be reflected in this study, as SIRPα^+ cells were only a small population within the DCs of the intestinal mucosa.

There are differences between the cellular expression of SIRPα and CD1b in the tissues of the intestine found in this study when compared to ALDC expression levels. A greater proportion of cells within bovine lymph express both SIRPα and CD1b. This could be due to anatomical variation as the majority of ALDC work employs DCs migrating from the skin. The results shown here could indicate genuine differences in the populations within intestinal tissues compared to the skin. These findings could also relate to state of maturity of the cells involved. DCs within peripheral tissues, such as the intestine or the skin or the lungs, are widely accepted to be immature, with maturation occurring upon uptake of antigen and during migration to the draining lymph node (Banchereau *et al.*, 2000). Therefore, afferent lymph cells have begun their migration, are likely to be carrying antigen, will have undergone some initial maturation characterised by changes in cell surface molecule expression and may not accurately represent the DC populations of the peripheral tissues which they have left. Finally, work carried out on ALDCs has for the most part utilised flow cytometry, a technique which may have inherent differences in sensitivity to the IHC techniques used here. This could account for some of the observed differences in cellular expression.

Positively stained MHC II cells appeared occasionally to be within or between adjacent epithelial cells, in direct contact with the intestinal lumen. This finding may indicate that the sampling method used by DCs, in which they extend dendrites into the intestinal lumen to collect antigens, does occur in cattle as has been reported in murine hosts (Chieppa *et al.*, 2006; Collan, 1972; Rescigno *et al.*, 2001b). A higher number of $CD205^{+}$ cells were found to be in contact with the intestinal lumen than any of the other molecules. Since not all of these cells were co-localised for MHC II it is possible that they may

not be DCs. However, these cells could be immature DCs expressing MHC II at levels below the threshold for detection by this technique. However, a proportion of the cells within the epithelial layer were MHC II positive and are likely to represent DCs in a location ideal for sampling antigens directly from the intestinal lumen.

This study was performed on tissues from six month old calves and thus may not be representative of adult animals. It is also possible that there could be age, breed, management or age related changes. A similar study observed no age-related differences in the distribution and expression of DC markers in ovine intestinal tissues (Akesson *et al.*, 2008), and so it possible that this situation is mirrored in other ruminant species. However, there have been differences noted in immune cell locations within the ileal immune follicles when comparing newborn or neonatal calves with animals of 2 months old (Lwin *et al.*, 2009). Investigations into dendritic cell subsets within Peyer's patches of rats did reveal age-related differences in DCs; differences reached a steady state at approximately 11wks post partum, corresponding with the time of adult maturity in rats. Differences were observed at times of major dietary change such as weaning (Zhou *et al.*, 2009). The calves in this experiment were weaned and fed a solid diet. Their intestinal maturity would more closely resemble that of an adult than a neonatal animal on a purely milk diet, so it is reasonable to assume that the results obtained here reflect the situation in adult animals. A similar study on fully mature bovine intestinal tissues would be necessary to confirm this.

Several antibodies were trialled with the calf tissues to attempt to visualise B cell populations in order to determine their anatomical locations and to

confirm that these are distinct from those of DCs. Antibodies for IgG, IgA, IgM and CD79 were used on ZSF and frozen tissues. CD79, IgG and IgA antibodies showed non-specific or no staining, or patterns of staining that varied greatly between sections. Staining for IgM was successful and stained up the mucosal edges of epithelial cells, cells within the lamina propria and the centre of follicles but not the interfollicular areas. Individual positively stained cells that were observed were large and round and strongly stained (data not shown). These results made it difficult to definitively identify B cell populations; however the morphologies of the positively stained cells and the co-localisation observed using the antibody panel in sections 2.3 - 2.9 make it likely that DCs have been positively identified in this work.

In conclusion, this work shows that the bovine large intestine contains many immune cells. DCs are found in all tissues of the large intestine, in particular within the lymphoid follicles of the terminal rectum. These DCs are defined primarily by their morphology and expression of MHC II. Their cellular morphology displays numerous cytoplasmic processes and large eccentric nuclei. Expression of various other cell molecules including SIRPα, CD1b, CD205 & CD11c can help to identify DC subsets. SIRPα^+ DCs were a smaller subset found at all intestinal sites; no significant differences between the anatomical sites were detected. CD205$^+$ DCs were more widespread and showed a distribution significantly linked to anatomical location. CD1b was expressed by a moderate subset of intestinal DCs, and some site specific differences were noted. The work presented here also highlights the importance of recognising the limitations of any technique when interpreting any resulting data. Further techniques such as three colour IHC or flow cytometry of *ex vivo* cells would provide additional characterisation

information for the DC populations within the intestine to support the discoveries presented here.

Chapter 3 Development of a protocol to isolate dendritic cells from bovine terminal rectal tissue

3.1 Introduction

The results from Chapter 2 give a clear indication that there are potentially numerous DCs within the tissues of the terminal rectum of cattle. To assess any possible interactions between *E. coli* O157:H7 and DCs, it would be useful to extract these local DCs for further investigation *in vitro*.

Dendritic cells from diverse anatomical locations and which encounter different micro-organisms are likely to respond uniquely to their surrounding environment. These different responses are likely only to be able to be studied accurately *in vitro* with cells extracted from their specific anatomical location.

Many studies have been carried out on cells generated from blood, but it is argued that these may not behave as specifically as *ex-vivo* tissue cells. The role cytokines play in producing DCs *in vitro* has been reviewed and the extent to which this may reflect the *in vivo* situation. It has been postulated that the GM-CSF stimulated cells represent not the normal state DCs but are more likely to correspond to DCs present during inflammatory processes (Conti & Gessani, 2008).

One aim of this study was to look at the possibility of extracting DCs from the bovine terminal rectum as the site of localisation of *E. coli* O157:H7. It was presumed that viable DCs could be extracted in sufficient numbers to perform downstream assays including their phenotypic characterisation as well as assessing their interactions with *E. coli* O157:H7 and its components.

DCs have been generated *in vitro* from bone marrow in mouse (Inaba *et al.*, 1992), rat (Talmor *et al.*, 1998), human (Reid *et al.*, 1992) and bovine systems

(Hope *et al.*, 2000b). Stem cells from cord blood have also been used in this way (Zheng *et al.*, 2000).

A common method to generate DCs *in vitro* has emerged with the use of blood monocytes as precursor cells in humans (Sallusto & Lanzavecchia, 1994), rats (Richters *et al.*, 2002), mice (Schreurs *et al.*, 1999), pigs (Carrasco *et al.*, 2001; Paillot *et al.*, 2001) as well as cattle (Hope *et al.*, 2000a; Werling *et al.*, 2002). These methods involve extended culture of cells in the presence of cytokines.

DCs from the tissues of interest have also been studied directly, including spleen, liver, lymph node, lungs and skin, using various techniques. DCs from the thymus and spleen of rats have been collected for study by enriching for dendritic cells using metrizamide and plastic adherence techniques subsequent to collagenase tissue digestion (Josien *et al.*, 1997). Cells are more readily released from lymph nodes than intestinal or other organ tissues and lend themselves well to producing a single cell suspension.

However, all these methods described involve many laboratory practices including extensive culturing, wash steps, separation techniques and other manipulations, all of which may give rise to cells which do not closely resemble the *in vivo* counterparts which are the cells of interest.

One method for the study of *ex vivo* DCs is the afferent lymphatic cannulation model, whereby lymph DCs returning from the tissue of interest to the draining lymph node are collected. This has been achieved by removal of local lymph nodes and cannulation of lymph ducts in various species.

These techniques lend themselves necessarily to anatomical sites which are accessible using surgical techniques.

DC populations in rodents have been collected and studied following lymph duct cannulation. Rat intestinal lymph DCs are shown to belong to several sub-groups with varied cell surface molecule expression and functional activity (Yrlid & MacPherson, 2003). Two main subsets have been identified: SIRPα^-, CD4$^-$ and SIRPα^+, CD4$^+$. These two subsets have been characterized in terms of their phenotype, anatomical distribution, T cell stimulatory functions and *in vitro* viability. The SIRPα^+ cells have been shown to have superior immunostimulatory capabilities *in vitro* compared with the SIRPα^- cells as well as superior viability in culture conditions. This has been demonstrated using mixed leukocyte reactions (MLR) and naïve CD8$^+$ T cell proliferation assays (Liu *et al.*, 1998; Voisine *et al.*, 2002). The SIRPα^- cell subset constitutively endocytose apoptotic enterocytes and migrate to T cell areas indicating a role for these cells in self-tolerance in the intestine (Huang *et al.*, 2000). SIRPα^- cells have been localised to the T cell areas of the mesenteric lymph node while the SIRPα^+ were not observed in this location, further supporting a role for the tolerogenic nature of SIRPα^- and a stimulatory role for the SIRPα^+ cells (Liu *et al.*, 1998).

Porcine DCs have been studied by both cannulation and immunohistochemistry methods. Four main populations of cells were found using IHC on porcine intestinal tissue however, only two of these were then found in the lymph draining this tissue. The sub-sets found in the lymph corresponded to DCs found in the lamina propria and not other anatomical locations within the intestine. This could suggest that either migration from tissues occurs mainly from the lamina propria or that DCs alter in their

expression of cell surface molecules in response to migration (Bimczok *et al.*, 2005).

The removal of the bovine pre-scapular lymph node and cannulation of the resulting pseudo afferent lymph duct is a well documented technique and has provided valuable information about DCs phenotype and function in this species (Hope *et al.*, 2006). Again, these cells are not found to be homogeneous, and two main sub-groups have been identified based on cell surface molecule expression, although there is an indication that there could be yet further sub-groups within this definition that are yet to be identified (Howard *et al.*, 1999). As for rodent afferent lymph DC subsets, bovine cell subsets defined on the basis of phenotypic differences were found to be functionally divergent with different capacity to interact with T cells. The two broad groups of cells in afferent lymph could be identified by their expression of SIRPα. The SIRPα^+ cells were found to induce proliferation of both CD4 and CD8 T cells at a greater level than the SIRPα^- cells. SIRPα^- and SIRPα^+ subsets were able to present soluble protein antigen (OVA) equally. There was, however, a tenfold difference in response to viral antigen. SIRPα^- and SIRPα^+ subsets which had been incubated with RSV virus in culture were used to stimulate CD4 cell proliferation and the SIRPα^+ cells produced the greater response (Howard *et al.*, 1997).

DCs from other ruminants too have been studied in this way. Lymph returning from the mucosal surfaces of the head and neck has been successfully collected from sheep following removal of the cervical lymph nodes. The cell types have been investigated as potential vaccine targets (Schwartz-Cornil *et al.*, 2005). As the DCs are important for generating tolerance or immune responses to encountered antigen they may be able to

present vaccine to naive T cells. DCs could potentially act as vehicles, taking vaccine from mucosal surfaces to inductive sites in lymph nodes. Both cannulation methods (prescapular and cervical) have been used in sheep to investigate the role of the mucosal environment on DC phenotype and migration (Epardaud *et al.*, 2004). These methods too have revealed significant heterogeneity in the DC populations recovered. Although cells from both anatomical locations express MHC II and co-stimulatory molecules (CD40, CD80 and CD86) at similar levels there were a greater number of $CD26^{hi}$, $SIRP\alpha^{-}$ cells found in the lymph returning from the head mucosa than the skin. These $CD26^{+}$ cells are thought to be tolerogenic within the mucosa as they can take up apoptotic cellular fragments. When considering that these cells transport apoptotic DNA during their migration to lymph nodes it is possible that this cellular subset continually samples and migrates, presenting self antigen to T cells with the purpose of maintaining tolerance to self. Additionally in this study it was found that a lower number of total DCs circulating from the mucosa were recovered than from the skin (Epardaud *et al.*, 2004). A subset of bovine $SIRP\alpha^{-}$ DCs have been shown to be $CD26^{+}$ and have also been found in lymph node tissues and afferent lymph draining the skin (Gliddon & Howard, 2002).

From the published work in these veterinary species and the immunohistochemistry carried out in Chapter Two several molecules were chosen for study in this chapter. They were selected for their capacity to indicate dendritic cell populations and stages of maturity. See Chapter Two for a more in-depth description of each molecule, its function and relevance to bovine dendritic cell subsets.

The advantage of studying *ex vivo* cells in this way reduces the possibility of cellular modification following culture, separation, enrichment or other potentially altering laboratory techniques. The DCs isolated represent most closely their *in vivo* counterparts. However, cannulation is not a viable technique for many tissue compartments. For example, in sheep it has been possible to cannulate the lymphatic system draining the small intestine (Hein *et al.*, 2004; Pernthaner *et al.*, 2002), but this would be very difficult in cattle due to the complexity of the procedure, and has not yet been attempted.

Lymphatic drainage from the bovine abdominal organs is inconsistent, in that not all lymph nodes are found in all animals. The predominant lymph nodes draining the organs in the pelvic area are the medial iliac and sacral nodes, which are found around the bifurcation of the aorta, just caudal to the kidneys (Dyce *et al.*, 1996). These nodes do not lend themselves easily to surgical techniques due to their deep location. In addition, these nodes are likely to receive lymph from other pelvic organs such as the reproductive organs and vessels as well as bladder etc. and therefore do not contain cells which are solely intestinal in origin. However, there are scattered anorectal and caudal mesenteric nodes found on the surface of the terminal intestine. It is likely that these smaller, more variable nodes will first receive the circulating DCs coming from the terminal rectum. Unfortunately these nodes will be very difficult to surgically remove, even at post mortem, and cannulation would in all probability be impossible. To even attempt these intricate techniques were out-with the remit of time and finances of this project.

Thus, DCs have been extracted from many different tissues in different species, but intestinal DCs in cattle have not yet been collected successfully and subsequently used for downstream applications.

Cells have been elicited from intestinal tissue in various species and DCs specifically have been collected for use in downstream assays. Murine colonic DCs have been extracted using digestion followed by density gradient centrifugation and filtering/sieving techniques. These cells were then characterised based on expression and functional profiles (Pavli *et al.*, 1990; Takenaka *et al.*, 2007). It is from this work that the possibility of developing a protocol for collecting bovine DCs local to the site of interest was investigated.

Similar digest methods have been used to elicit cells from intestinal tissues in rats. DCs from intestinal tissue were compared with lymph DCs in their ability to stimulate a mixed leukocyte reaction. It was noted that culturing of the intestinal cells altered their stimulatory capabilities when compared with freshly isolated cells. Culturing of cells appeared to increase their stimulatory capacity which was proposed to correspond to a functional maturation of these cells (Liu & MacPherson, 1995).

Human DCs also have been isolated from intestinal tissues in several studies, mainly making use of surgical biopsy samples and digestion techniques (Bell *et al.*, 2001; Pavli *et al.*, 1996).

If DCs could be isolated from the terminal rectal tissue of cattle it would facilitate investigations of potential DC and *E. coli* O157:H7 interactions *in vitro.* This would negate the need for using blood-derived cell models which

may not accurately reflect *in vivo* responses. If it is possible to collect intestinal tissue DCs it would enable the comparison of the responses of these tissue DCs with the moDC in cattle. This comparison should be able to go some way to assessing the suitability of the widely used moDC model for *E. coli* O157:H7 colonisation studies. The use of post-mortem tissue would also remove the need for carrying out complex surgical techniques on live experimental animals. Furthermore, collection of these cells will enable more comprehensive characterisation of terminal rectal cells by methods such as flow cytometry for cell surface molecule expression, and functional assays; which would yield more detailed information than immunohistochemistry and other *in-situ* techniques will allow.

In this study post-mortem terminal rectal tissue from cattle was used to attempt to develop a Dendritic Cell recovery protocol. Three main methods were employed, extrapolated from published protocols for other tissues and species.

The aims of this chapter were:

1. To develop a method of extracting TRDC (Terminal Rectal Dendritic Cells)

2. To characterise TRDCs by IHC and FACS analysis

3. To carry out a phenotypic comparison of TRDC with moDC

4. To use extracted TRDCs and investigate interactions with *E. coli* O157:H7 *in vitro*.

3.2 Materials and Methods

3.2.1 Method 1: Tissue Digestion

The initial part of this method is adapted from the technique developed by Dr. A. Mahajan to establish primary cell cultures of bovine rectal epithelial cells. This method had been modified from published techniques (Booth *et al.*, 1995; Hoey *et al.*, 2003). Terminal rectal tissue including the anus was collected from the abattoir. It had been immediately placed on ice in Hank's Balanced Salt Solution (HBSS) with gentamicin (50 μg/ml) for the journey to the laboratory. Previous studies have revealed that up to 3 hours after removal, form and function of gastrointestinal tissue is essentially unchanged (Jackson *et al.*, 2004). Once returned to the laboratory the luminal contents were flushed with PBS. Excess fat and connective tissue were removed, and the lumen opened along its length. The tissue was at this stage cut into two pieces, again along the length of the piece of intestine. Each piece was placed into a Falcon tube containing HBSS with Fungizone (Invitrogen) at (5 μg/ml) for 5 min. The mucus was gently scraped from mucosal surface with a glass slide and put to waste. A sterile glass slide was then used to scrape the mucosal cells from the underlying muscular and connective layers. These scrapings containing the intestinal cells were collected into a Falcon tube containing HBSS. The cells were washed five times in HBSS by gentle centrifugation at 260 x g for 2 min. After each spin any floating debris was then removed while being careful not to disturb the cell pellet. Digestion medium (Appendix A.4.2.5) was then added to the cells to make up to a final volume of 25 ml, and incubated in a shaking incubator for 1 h 30 min to 1 h 45 min at 37°C. Following the incubation the cells were washed in PBS once and then passed through a 100 μm cell strainer (352360

BD bioscience). Cells were washed again in PBS at 260 x g for 5 min. The sample was resuspended in 20 ml PBS, underlaid with 10 ml histopaque 1083 and centrifuged (35min, 1200 x g, no brake, 20°C). The resulting cells at the interface were collected into a fresh Falcon tube. Following a final wash in PBS, the cells were strained again if required before proceeding with viable cell counting in Nigrosin, (Sigma) using a disposable chamber slide (Fast-read 102, ISL, Devon). The cells were then resuspended to desired concentration for downstream applications including immunohistochemistry and flow cytometry.

3.2.2 Method 2: GM-CSF Migration

This method was developed from the technique used at IAH, Compton to extract DCs from tracheal tissue which in turn was modified from a published technique (Ortner *et al.*, 1996) (Personal Communication, Dr. J. Hope, 2008). Terminal rectal tissue from the abattoir was stored on ice until returned to the laboratory. The epithelial layer was dissected out and was stored in TCM (Appendix A.4.2.3) for approximately 10 min at ambient temperature. The epithelium was cut into small pieces approx 1 cm x 1 cm using scissors or scalpel. The tissue pieces were transferred into a 9 cm Petri dish containing approximately 30 ml TCM with GM-CSF. (1:100 v/v, IAH, Compton) The tissue was incubated in the medium containing GM-CSF for approximately 24 h in Humidified incubator at 37°C with 5% CO_2. After incubation the TCM containing any cells were removed into a Falcon tube. Any remaining cells were washed from the dish using PBS pre-warmed to 37°C and add to the Falcon tube. These cells were pelleted by centrifugation at approximately 480 x g for 5 min, washed by adding approximately 20 ml PBS and a repeat of the pelleting step. The cells were then resuspended in 10

ml TCM, underlaid with 5 ml Histopaque and centrifuged (1200 x g for 30-40 min, no brake, 20°C). Cells were removed from the interface using a graduated pipette and placed into a fresh universal. The cells were washed 3 times, initially pelleted at 480 x g for 10 min at 4°C followed by two wash steps at 260 x g for 8 min at 4°C. Viable cell counts were made in TCM using a disposable cell counting chamber and Nigrosin stain as before. Cells were then resuspended as required for downstream procedures.

3.2.3 Method 3: Mechanical Disaggregation

This method was developed from a published method of retrieving viable cells from intestinal tissue from human biopsy samples (Olsen, 2009) Two pieces of terminal rectal tissue, including the anus, were collected from male Friesian cattle, following slaughter at Moredun Research Institute (MRI). The luminal contents were washed out using tap water. Excess connective and fatty tissue was removed and the pieces of intestine were opened along their length. Small samples of the terminal rectal mucosa, approx 1 cm^2, were dissected out using a sterile scalpel blade and rat-toothed forceps. Three samples from each animal were put into separate Petri dishes containing TCM with 5 μg/ml Fungizone, Penicillin 100 U/ml and Streptomycin 30 μg/ml added. These samples were incubated overnight at 37°C and 5% CO_2. The following day the samples were dissected further to approx $3mm^3$. Three of these pieces were placed inside a BD Medimachine Medicon (BD Medimachine™ Medicon, 50 μm Sterile) with 1 ml of PBS. Disaggregation of the tissue was carried out by running the Medicon inside the Medimachine for 120 sec. A sterile syringe was then used to collect any cell suspension. If larger tissue particles remained inside the Medicon, fresh PBS was added and the disaggregation repeated until no visible tissue was left inside the

Medicon. The resulting cells in suspension were washed twice by centrifugation at 480 x g for 5mins in PBS. The cells were then filtered through a 100 μm Filcon™ (BD) to remove any debris. The cells were resuspended in 20 ml PBS, underlaid with 10ml histopaque and centrifuged (1200 x g for 35 min, no brake, 20˚C). The cell layer resulting at the interface was collected using a graduated pipette. Three washes were then carried out; one at 480 x g for 10 min and then two washes at 210 x g for 8 min; each at 4°C. At this stage any resulting cells were counted using Nigrosin stain and a disposable counting chamber, and resuspended in the appropriate media for downstream applications.

3.2.4 Slide preparations

Treated glass slides (superfrost plus®™, Menzel-glaser, Germany) were assembled with Cytospin filter paper on the charged surface and held together with bulldog clips along the long edge. These were used for making the slide preparations on the laboratory bench without the use of the Cytospin machine. 50 μl of cell suspensions at 5 x 10^5 or greater were dropped into the holes in the Cytospin paper. The liquid was wicked away into the filter paper and this left the cells on the surface of the slide, without the need for centrifugation. These preparations were then allowed to air dry. Once dried, the slides were kept at -20°C until required for staining protocols.

3.2.5 Giemsa Staining of Slide Preparations

Giemsa Stain (Sigma) was diluted 1:10 with Sorensen's Buffer (Appendix A.4.5.1). The slides were flooded with Giemsa stain and incubated at room temperature for 30 min on a staining rack. Following the incubation the

slides were washed with Sorensen's buffer and then fully dried under a fume hood. The slides were then coverslipped with Shandon Consul-mount (Thermo-scientific). The slides were observed and imaged using the same microscope and camera set up as described in section 2.2.1.

3.2.6 Immunohistochemistry

All slides were stained as previously described (See section 2.2.1).

3.2.7 Flow Cytometry

Primary mouse anti-bovine monoclonal antibodies (mAbs) and secondary anti-mouse FITC conjugated antibodies were used to indicate the presence of cellular markers. MoDCs were seeded at 5 x 10^5 cells per well of a 96 well microtitre plate (sterilin) in 50 µl TCM. Primary mouse anti-bovine monoclonal antibodies (mAbs) and secondary anti-mouse FITC conjugated antibodies were used to indicate the presence of cellular molecules. (Appendix A.4 mAbs used and concentrations) Antibodies were diluted in PBS with 0.1% NaN_3 and 1% BSA (PBS/BSA/Azide). Antibodies were added to cell suspensions, agitated on a plate shaker (Fisherbrand® Vortex Whirlimixer) and incubated for 10-30 min at room temperature, covered to prevent drying. To wash the cells, 100 µl PBS/BSA/Azide was added to each well and cells were pelleted for 1 min at 480 x g, with supernatant flicked to waste. The secondary antibody was added and plates were agitated, incubated and washed as before. After the cells were stained they were fixed in 100 µl per well of 1% paraformaldehyde solution and stored at 4°C for a maximum of 14 days before FACS analysis. For analysis the fixed cells in 1% paraformaldehyde were resuspended in approx 250 µl of FACSFlow (BD) in a suitable tube. The samples were assayed on a FACSCalibur (Becton-

Dickinson, San Jose, CA) or a Dako CyAn and data was analysed using FCS Express software. (DeNovo Software, Ontario, Canada) The limit was set at 10,000 gated cells for each preparation, although in some cases there were not sufficient cells for this (see Results section 3.3).

3.2.8 Data Analyses

Dot plots of forward scatter against side scatter (Fig. 3.1 A and C) were used to gate out dead cells and debris, the resulting cells were then used for further gating. Next, dot plots were used to plot forward scatter against fluorescence for the molecule of interest and its corresponding isotype control samples. These plots were used to gate for MHC II^+ cells (Fig. 3.1 B & D). Within the positive population (gate 3), were cells expressing higher levels of MHC II; (gate 4) this population of cells were then gated and this final gate (gate 2) was applied to the original dot plots of forward and side scatter (Fig. 3.1 E) to indicate the location of the MHC II cells within the total population. It was this population of cells (gate 4), which were used for all analyses. Histograms were used to obtain mean fluorescence intensity (MFI) data and a marker was drawn to calculate the percentage of positively stained cells for each molecule of interest (Fig. 3.1 F and G). The MFI data for each cell surface molecule was calculated after subtracting the MFI for the specific isotype control. Graphs were plotted in Microsoft Excel. Statistical analyses were carried out in Minitab. Two sample t-tests were carried out for each cell surface molecule after assessing the data for equality of variance using an F-test. Statistical significance was defined if the p value was less than 0.05.

3.3 Results

3.3.1 Method 1: Tissue Digestion

3.3.1.1 FACS analysis of tissue cells

Bovine terminal rectal tissue was digested and the resulting cells were analysed by flow cytometry. Each preparation was very different reflecting large animal to animal variation. Some preparations had numerous cells in a single cell suspension and were able to be analysed by flow cytometry. Other preparations contained many aggregations of cells which stopped the flow cytometer running. With some of these preparations, filtering the cell suspension through sterile lens tissue would enable reading of the sample. However for some preparations this was not sufficient and incomplete FACS datasets were generated. Due to the great differences in cell numbers retrieved, not all cell surface molecules were assessed in each preparation. From 11 cows sampled only 6 cows provided complete data sets of staining profiles for comparison of DC cell surface molecule expression by FACS. This digest technique was very inconsistent with much between animal and preparation variation. Within the gated population selected for analysis there was a greatly varied number of cells that were MHC II^+. The percentage of MHC II^+ cells ranged from 18.4% to 42.04%. A representative example of the plots and gates used for analysis from one animal are shown in Fig. 3.1. This gating method was chosen to try to locate dendritic cells within the population of cells that were collected as it was assumed due to the nature of the technique, and early observations of slide preparations, that the population would not be pure.

From the first animal studied (individual data not shown) results suggested that the cells recovered from the abattoir terminal rectal tissue expressed a similar set of surface expressed molecules to the monocyte derived cells. A similar percentage of MHC II^+ cells were found and these were shown to be at a similar level of maturity (based on CD40, 80 and 86 expression) although fewer of these cells were positive for both CD1b and SIRPα which appeared to correspond with findings on tissue DCs draining the mucosa of ovine tissues where the more prominent subset is SIRPα^-. (Eperdaud, 2004) However, each time that this technique was repeated the more diverse the results were and no data sets were the same. (See Table 3.1 for ranges of results) The final data presented here is an average (+/- standard error) of six animals (Fig. 3.2).

The monocyte derived DC data used for comparison is pooled from 7 animals, four housed at IAH, Compton and three which were kept at MRI. The cell surface molecule expression profiles for these separate groups of animals was similar and so it was deemed reasonable that this data was representative of this widely used model and thus used for comparison. The moDC data is displayed fully and discussed further in Chapter Four.

The percentage of cells expressing each cell surface molecule was lower for the tissue cells than the moDCs ($p<0.05$), with the exception of CD205 and CD86 which were similar (CD205, $p=0.87$; CD86, $p=0.140$). The most significant difference observed was for SIRPα ($p<0.001$) (Fig. 3.2 A)

Mean fluorescence intensity (MFI) data for this terminal rectal tissue indicated that the level of cellular expression of the co-stimulatory molecules

CD40 and CD80 on these positive cells was lower ($p<0.05$) than the moDC suggesting a more immature DC type. (Fig. 3.2 B) However, the level of expression found was similar for MHC II, CD86 and CD205 (MHC II, $p=0.315$; CD86, $p=0.198$; CD205, $p=0.186$

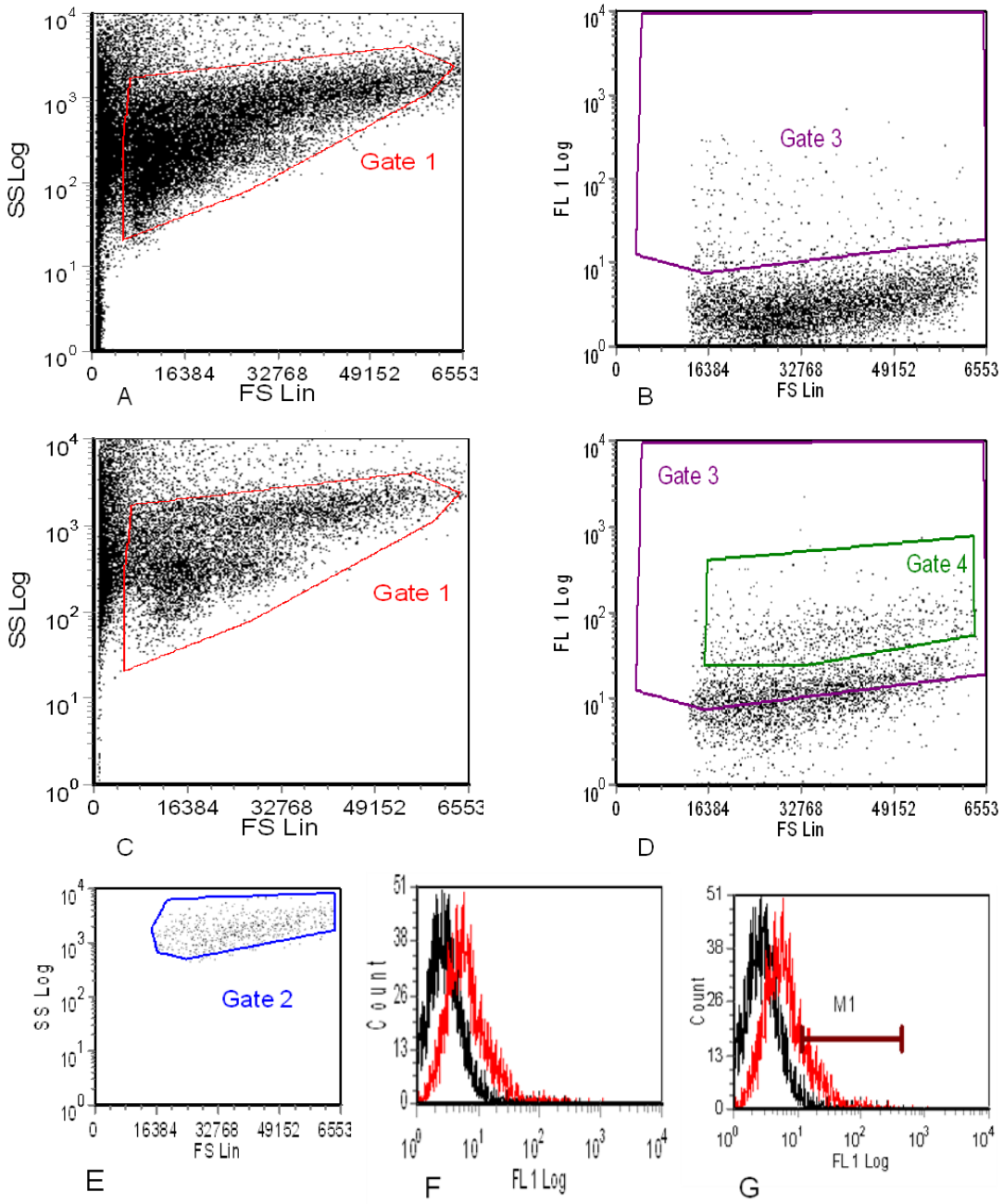


Fig. 3.1 Flow cytometry of terminal rectal tissue cells showing gating strategy. Dot plots of cells retrieved from terminal rectal tissue showing the gated populations used for analyses. A & B – Dot plots of terminal rectal cells labelled with an isotype control. The live cells were gated for analysis (gate 1). Gate 3 was used to locate the cells positively stained for MHC II. C & D – Dot plots of terminal rectal cells labelled with MHC II. Gate 1 and 3 as above, Gate 4 contains the population of live cells highly positive for MHC II. E – Dot plot showing population of cells used for analysis (gate 2); these cells were gated through gate 4, F & G - Histograms of MHC II expression (red) and isotype matched control (black) of the terminal rectal tissue cells selected for in Gate 1 showing the marker used to calculate the percentage of positively stained cells. The histogram without the marker was used for analysis of mean fluorescence intensity (MFI). Great variability in cell number was observed between the cell preparations.

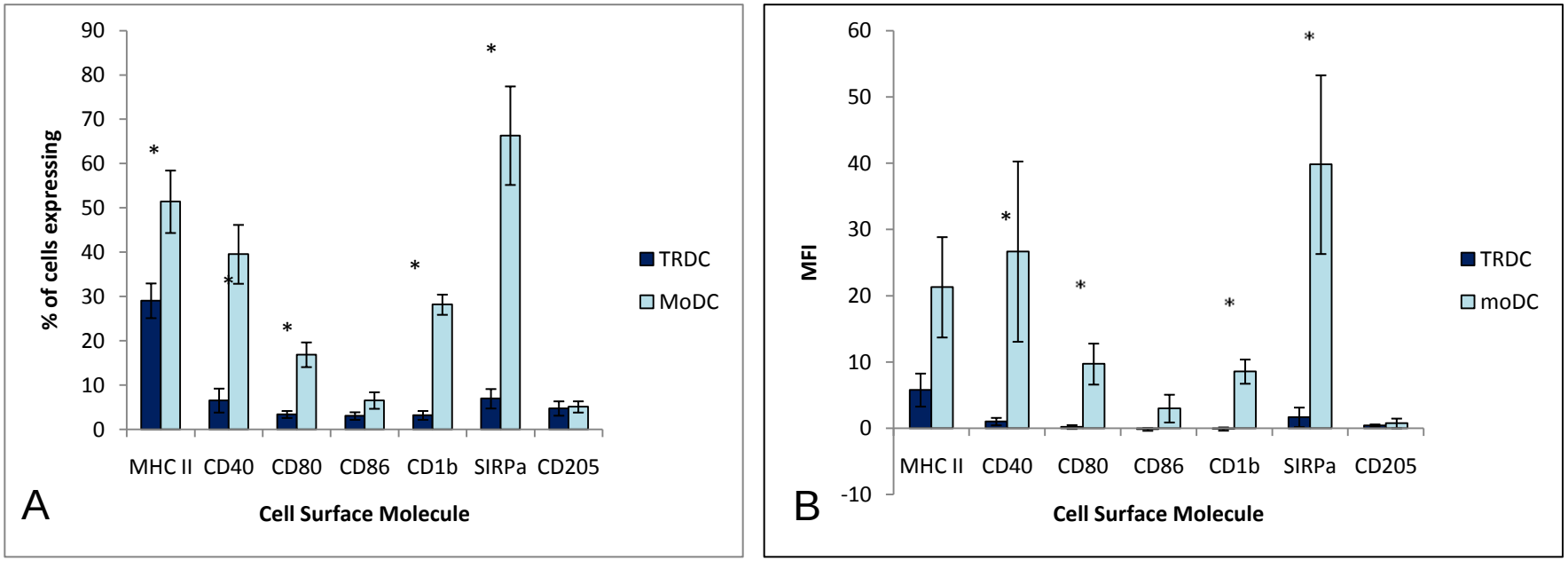


Fig 3.2 Flow cytometry of terminal rectal cells : method 1

(A) **Cellular expression of molecules on bovine moDC compared with cells recovered from bovine terminal rectal tissue using a tissue digestion method.** MoDC: average of 7 animals, tissue cells (TRDC): 6 animals. Error bars represent standard error of the mean. Cells were gated through gate 4 as described. (Section 3.2.8. & Fig. 3.1.). The percentage of tissue cells and moDC that were $CD205^{+}$ were very similar ($p=0.87$). Numbers of cells expressing CD86 were also close to the moDC ($p=0.14$). A greater percentage of the moDC expressed MHC II, CD40, CD80, CD1b and SIRPα than the tissue cells, (* $p<0.05$).

(B) **Mean Fluorescence Intensity (MFI) of cell surface molecules on moDC compared with cells recovered from bovine terminal rectal tissue using a digestion method.** MoDC: average 7 animals, Tissue Cells (TRDC): average 6 animals. Error Bars represent standard error of the mean. Cells were gated through gate 4 as described. (Section 3.2.8. & Fig. 3.1.). Mean Fluorescence intensity for CD40, CD80, CD1b, SIRPα molecules was much lower for the TRDCs than the moDCs (* $p<0.05$). MFI for MHC II, CD86 and CD205 was not significantly different in the tissue cells compared to the moDCs (MHC II, $p=0.32$; CD86, $p=0.198$; CD205, $p=0.186$)

	% positive cells	MFI
MHC II	18.4 → 42.04	0.7 → 17.88
CD40	1.01 → 19.38	0.02 → 3.31
CD80	1.21 → 5.66	-0.78 → 1.35
CD86	0.79 → 5.74	-1.04 →0.42
CD1b	0.45 → 6.8	-1.1 → 0.48
SIRPα	0.35 → 15.6	-0.36 → 9
CD205	0.74 → 10.41	-0.17 → 0.86

Table 3.1. Range of results for percentage of positive cells and MFI of tissue cells collected using a digest technique. The range of results collected was broad. Each animal gave differing results; this technique did not provide consistent results.

This was an interesting finding and the reduced number of cells expressing each molecule could be due to an impure cell population even with the efforts to gate for dendritic cells. It is also probable that there are different subsets of dendritic cells within the tissues of the terminal rectum and comparing with the moDCs which are a more homogeneous cell population will show differing results when analysed in this way. Also different subsets in the tissues would not be able to be identified by the single colour FACS staining carried out here. The similar levels of expression of the MHC II and the CD205 suggest however that some dendritic cells have been collected and positively stained, and suggest that the cells recovered are not monocytes as CD205 is not expressed by ruminant monocytes (McNeilly *et al.*, 2006). It was hoped that experimental repeats and modification of the technique would be able to confirm and increase the significance of these findings as well as further reducing the numbers of contaminating non-DC cells.

The first tissue to be used for the digest method produced a large number of cells enabling numerous cell surface molecules to be studied. From this large panel there were five molecules expressed by more cells in the TRDCs than the moDCs, and at higher levels (data not shown). These molecules were CD1w3, CD3, CD5, CD13 & CD26. These molecules have been found to be expressed on epithelium (CD13, CD26) (Hansen *et al.*, 1994; Stange *et al.*, 1996), T cells (CD3, CD5, CD13, CD26) B cells (CD5, CD1w3) (Stabel & Khalifeh, 2008; Werner-Favre *et al.*, 1989) and vascular endothelium (CD1w3) (Jorundsson *et al.*, 2000). Subsequent experiments did not yield sufficient cell numbers to study such a wide panel of molecules and so CD1w3, CD13 & CD26 were not selected for further work. CD5 and CD3 expression are investigated further in Chapter 4. This preliminary data could indicate that the cell population retrieved was not purely dendritic cells as both numbers of cells expressing each molecule and levels of expression was greater for the tissue cells when compared with moDC. However this could also point to the presence of dendritic cell subsets within the tissues of the terminal rectum. When looking at the published data available, it is known that ruminant mucosal dendritic cells express CD26 (Epardaud *et al.*, 2004; Gliddon & Howard, 2002) and CD13 (personal communication Dr. J. Hope) and furthermore that SIRPα^- dendritic cells express CD5 (Howard *et al.*, 1997).

3.3.1.2 Giemsa staining of *ex vivo* terminal rectal tissue cells

Staining of slide preparations of these cells was carried out using Giemsa stain to look for DC morphology and assess the quality of the slide preparations (Fig. 3.3).

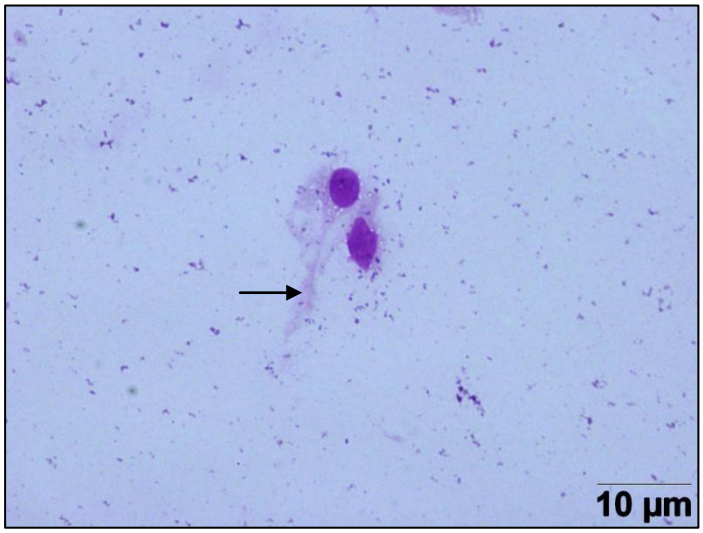


Fig. 3.3 Giemsa Stain of terminal rectal tissue cells collected using a digest method. DC morphology was observed on several of the cells including eccentric nuclei and dendrites. The slide preparations contained some considerable mucus and cellular debris. (arrow = dendrite)

Immunohistochemistry was carried out on slide preparations for several molecules known to be expressed by DC; MHC II, SIRPα and CD1b as well as isotype matched controls. The slide preparations were very variable in numbers of cells present. Some had numerous cell aggregations and mucus despite efforts to eliminate this in the technique. Varying cell types were observed indicating that this method did not produce a pure DC population. The slides were examined independently by a veterinary pathologist and dendritic type cells, epithelial cells, fibroblasts, plasma cells, macrophages and neutrophils as well as cell debris and mucus were observed.

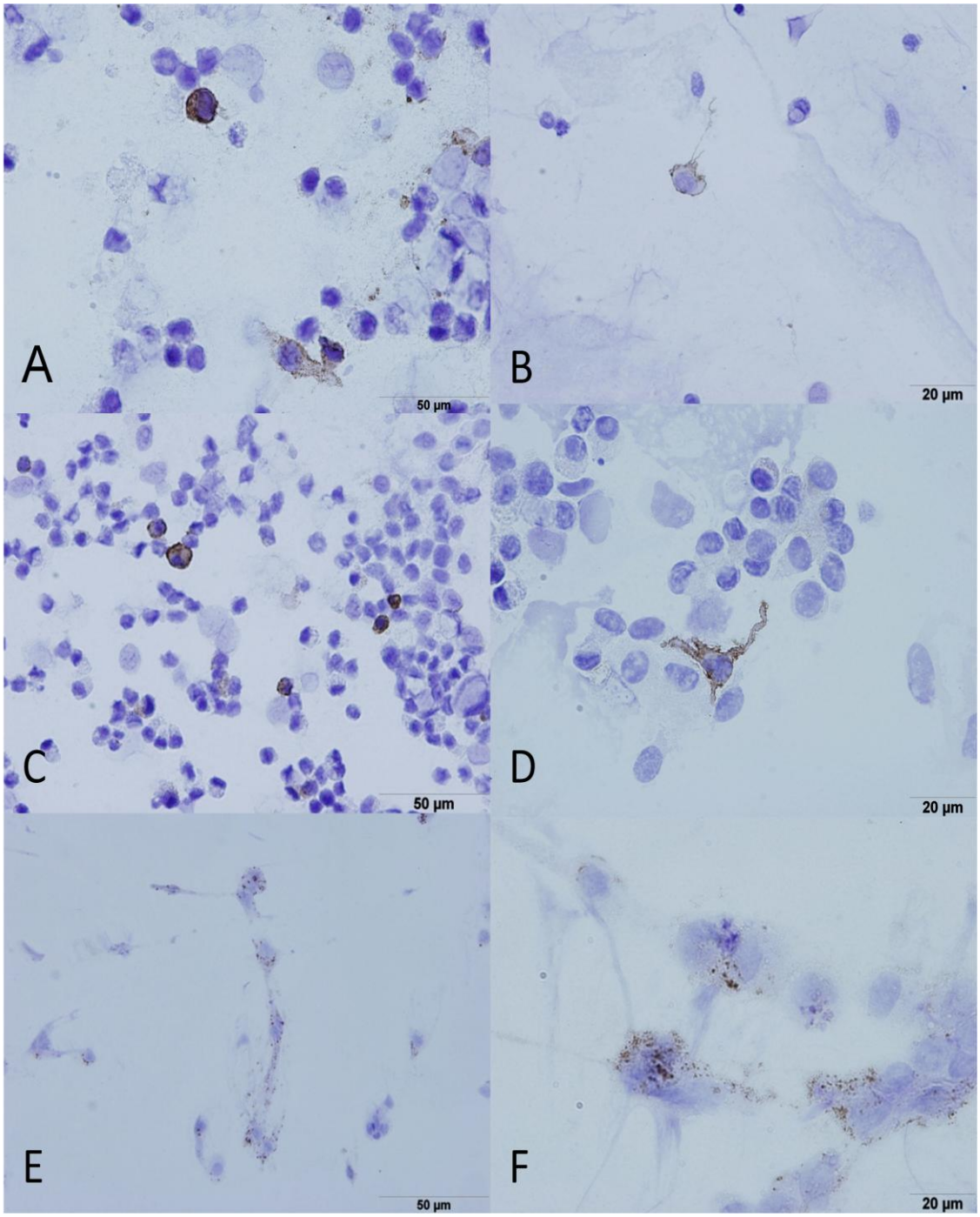


Fig. 3.4 Immunohistochemistry for cell surface expressed molecules on *ex vivo* cells from bovine terminal rectal tissue. DAB Staining for MHC II (A, B), SIRPα (C,D) and CD1b (E,F). Brown colour is positive staining. Positive cells were observed for all the molecules and cells showing distinct dendritic cell morphology were observed in all sections. Cellular Nuclei are counterstained blue.

Positive staining was observed however for MHC II, SIRPα and CD1b, which are all expressed on bovine DCs as well as other cell types. Some distinct DC morphology was noted on some of the positively stained cells, with dendrites and large eccentric nuclei being a common feature (Fig. 3.4). Several repeats of this method and variations on it did not yield the corroborating results that were hoped for. A GM-CSF migration technique was the next method to be assessed.

3.3.2 Method 2: GM-CSF Migration

GM-CSF was used to stimulate DCs to migrate from bovine terminal rectal tissue. These cells were assessed by flow cytometry for cell surface expressed molecules. Although there was animal to animal variation a generally low number of cells retrieved meant that a much reduced number of cell surface expressed molecules could be assessed and that it was not possible to carry out any further investigations. (See table 3.2 for range of results obtained)

Cells retrieved using this method did not have similar number expressing MHC II or CD1b compared to the moDCs for ($p<0.05$) but a similar number of cells were positive for CD205 in the tissue cells preparation as the moDCs. ($p=0.75$) (Fig. 3.5 A) However for such a small panel of markers and number of cells it is less likely that a pattern will be observed, or this may indicate the impurity in the cell population, or could indicate that subsets of DCs were collected using this method. Levels of expression for MHC II and CD1b were much lower on the tissue cells compared with the moDCs ($p<0.05$). CD205 expression levels were similar for both the moDC and the tissue cells obtained by this migration method. ($p=0.87$) (Fig. 3.5 B)

Fig. 3.6 shows percentage expression (Fig. 3.7 A) and MFI (Fig. 3.6 B) data for cells recovered by the digestion method and the migration method compared with the moDCs. The percentage of MHC II^+ cells and MFI was different for each method and also when compared to the moDCs. The numbers of $CD1b^+$ cells and the level of CD1b expression was similar for each TRDC method, however these were both much lower compared to the data for the moDCs. CD205 was the only molecule studied which had similar percentage of positive cells and MFI by all methods evaluated.

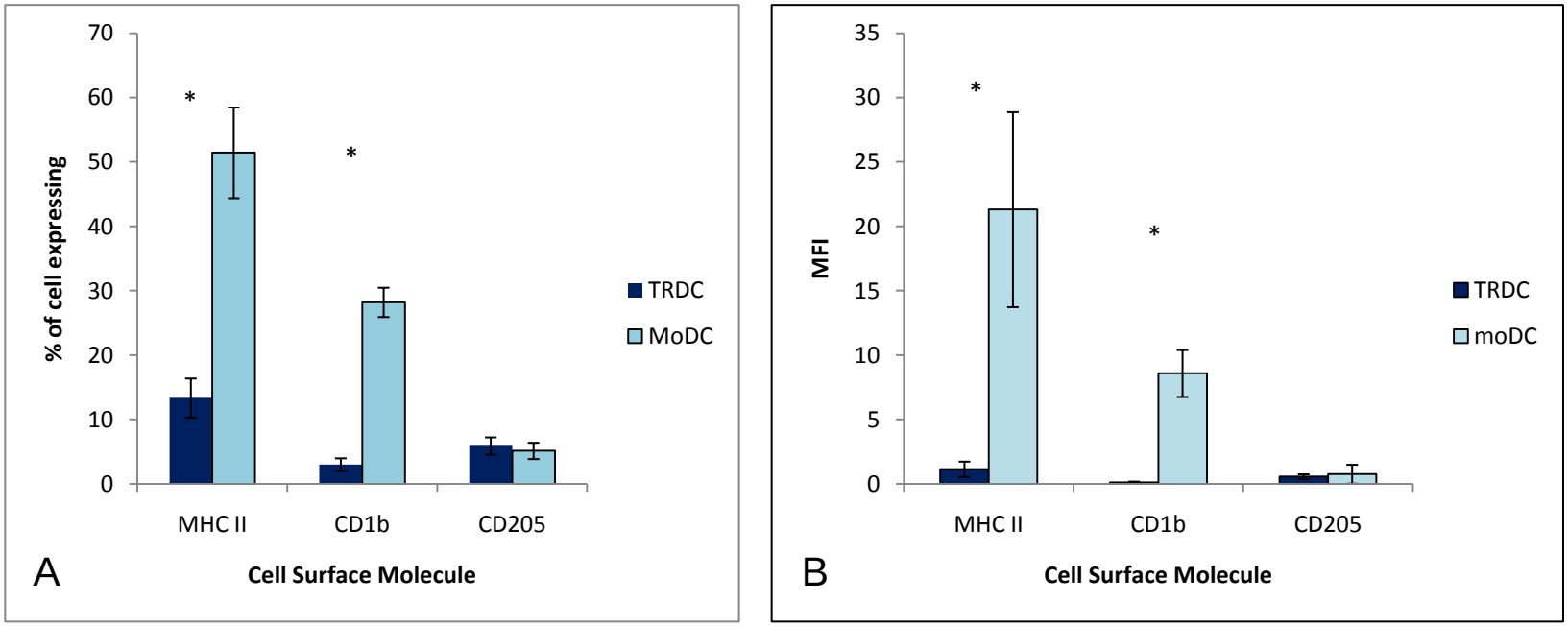


Fig 3.5 Flow cytometry of terminal rectal cells: method 2

(A) Expression profiles of terminal rectal tissue cells collected by GM-CSF migration method of extraction compared with MoDC; MoDC: 7 animals. Tissue DCs: 4 animals for MHC II and 3 animals for CD1b and CD205. Error bars represent standard error of the mean. Cells were gated through gate 4, and analysed as described (Section 3.2.8 & Fig. 3.1). A low number of cells extracted with this technique allowed only three molecules to be analysed. Some positive staining was observed and the percentage of cells expresing CD205 was very similar for the tissue cells and the moDC ($p=0.75$). The numbers of cells expressing MHC II and CD1b was much lower for the tissue cells than the moDC. (* = $p<0.05$)

(B) Mean fluorescence intensity of cell surface molecules on terminal rectal tissue cells collected by a GM-CSF migration method of extraction compared with moDC; moDC: 7 animals, Tissue DCs: 4 animals for MHC II and 3 animals for CD1b and CD205. Error bars represent standard error of the mean. The cells were gated through gate 4, and analysed as described (section 3.2.8 & Fig 3.1). Mean fluorescence intensity for the tissue cells was much lower on the tissue cells for MHC II and CD1b compared to the levels on the moDC (* = $p<0.05$), but the levels of expression for CD205 vas very similar ($p=0.87$).

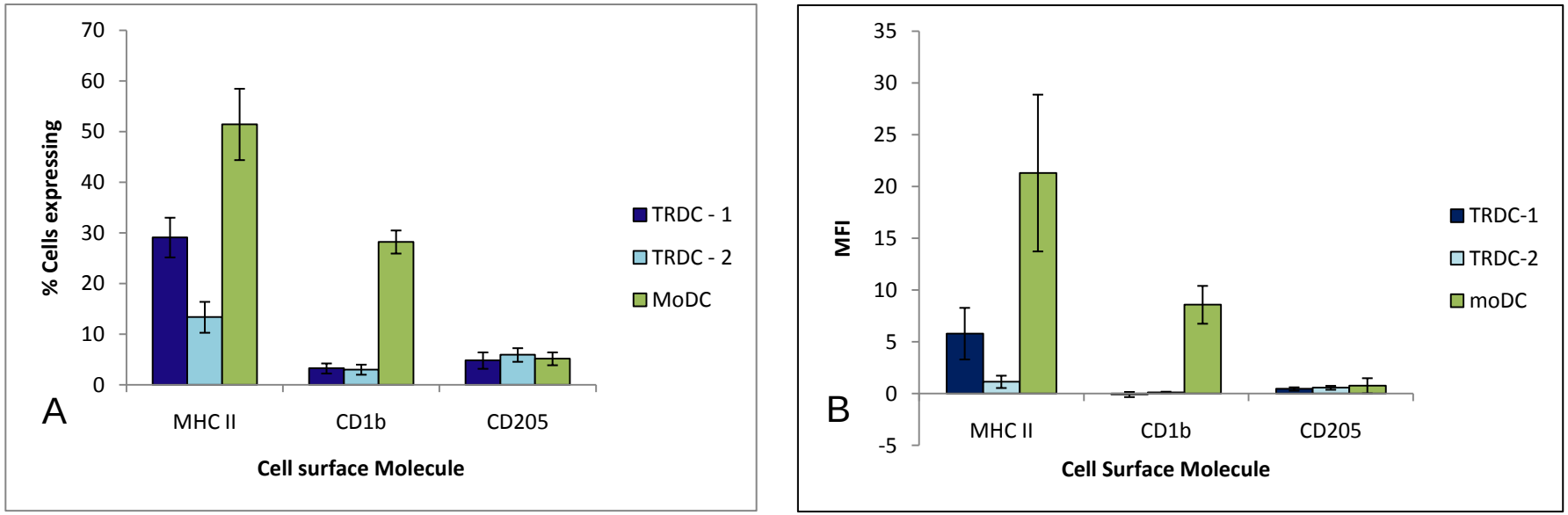


Fig. 3.6 Flow cytometry of terminal rectal tissue cells: method 1 and method 2 compared with moDCs

(A) Expression profile of cells extracted from bovine terminal rectal tissue; two methods compared with monocyte derived DCs; MoDCs: 7 animals, TDC–1: 4 animals for MHC II and 3 animals for CD1b and CD205. TDC-2: 4 animals. Error bars represent standard error of the mean. TDC-1 = digestion method; TDC-2 = migration method. The methods used produced a different expression profile from each other and the moDCs for MHC II. Both methods showed a similar percentage of cells that were positive for CD1b but this was different from the percentage seen with the moDCs. A similar percentage of cells were positive for CD205 in both methods as well as the moDCs

(B) Mean fluorescence intensity of cells extracted from terminal rectal tissue; two methods compared with moDC; moDC; 7 animals, TDC–1: 4 animals for MHC II and 3 animals for CD1b and CD205. TDC-2: 4 animals. Error bars represent standard error of the mean. TDC-1 = digestion method; TDC-2 = migration method. MHC II expression was different for both methods and the moDCs. Both methods produced cells that had a similar low expression of CD1b which was different from the higher expression observed on moDCs. Both methods used produced a similar profile to the moDC for CD205 expression.

	% positive cells	MFI
MHC II	7.85 → 19.01	0.46 → 2.92
CD1b	1.07 → 4.38	-0.04 → 0.46
CD205	3.79 → 8.4	0.2 → 0.81

Table 3.2. Range of results for percentage of positive cells and MFI of tissue cells collected using a migration technique. The range of results collected was broad. Each animal gave few cells for analysis and differing results.

3.3.3 Method 3: Mechanical Disruption using the Medimachine System

This method did not yield any viable cells at all when initially used on tissues from two animals. A small cellular layer was observed after Histopaque separation. Filtration of this layer following centrifugation resulted in no cellular pellet suggesting that it consisted of cell debris/tissue aggregation and not a single cell suspension.

3.4 Discussion

Several methods were employed in attempts to extract dendritic cells from the terminal rectal tissues of cattle. Each method had its limitations and due to time and financial constraints this area of research was eventually abandoned.

With all the methods used, despite some promising early results, it was not possible to consistently produce a cell population from the intestinal tissue that could be accurately identified as dendritic cells. The early data from the first animal studied, using the digest method, suggested that it was possible to collect terminal rectal cells in sufficient numbers to carry out FACS analysis. This data also suggested that these cells were a mixed population likely to contain DCs. A percentage of cells expressed similar cell surface molecules when compared to the monocyte-derived DC and the levels of expression were comparable for the tissue cells and the moDC. Microscopy suggested the presence of cells with DC morphology and expressing DC cell surface molecules but also indicated that the cell suspension was not a single population of cells. For example plasma cells, macrophages, fibroblasts and epithelial cell types were identified in the preparations, as well as cell debris and mucus. DC type cells that could be identified appeared to express cell surface molecules at a similar level to moDCs. Many attempts were made to improve and even reproduce this data, and a significant amount of work went into trying to develop a workable protocol. Unfortunately, these methods have not been found to be reproducible and in fact several of the attempts did not manage to yield a single cell suspension suitable for analysis. There seemed to be great animal to animal differences as each preparation had greatly differing cell numbers.

Following these experiments it became clear that to study the interaction of *E. coli* O157:H7 and dendritic cells in the bovine host it would be necessary to use the monocyte-derived DC model.

A combination of the methods described in this chapter would possibly be useful, for example use of digest medium following the Medimachine technique. Addition of an EDTA treatment step as used by Pavli *et al* (Pavli *et al.*, 1996), prior to use of digestion medium too may be useful to try to reduce any contaminating epithelial cells.

Although the GM-CSF migration technique produced some cells for analysis, the numbers were so small that it was not a practical option and again there were vast differences between the four animals studied. However if this method could be used following a digest step, it may be possible to elucidate a greater number of cells.

There are a few other points to note that could assist with the data analysis of this technique. If a single cell suspension could be fairly consistently produced it would be helpful to carry out four colour FACS analysis if a sufficient number of cells could be collected. One colour for the molecule of interest, one for MHC II, one for CD45 which would enable epithelial cells to be gated out and finally a viability indicator such as propidium iodide to accurately analyse the live cells. FITC could also be substituted for an alternative conjugate such as Alexa-Fluor 488 that is brighter due to a smaller stokes shift.

The gating process used for analysis should exclude monocytes as they are smaller and less granular than dendritic cells, however, expression of CD14 could be used in addition to ensure monocytes are not being collected and

detected by these methods. Of the molecules used for analysis in this study, CD1b and CD205 have not been found to be expressed by monocytes/macrophages (McNeilly *et al.*, 2006).

Future work would ideally further develop a protocol to extract viable DCs from bovine rectal tissue. The IHC from Chapter Two suggests that there could be many DCs in this area and a consistent method of retrieval could be of benefit to many branches of intestinal research, including Crohn's disease, inflammatory bowel diseases, gut allergies as well as food borne pathogens. Development of a protocol could be further enhanced by the work carried out in Chapter Two. The immunohistochemistry results could be used to identify a panel of cell surface molecules expressed by TRDC and the reagents that could be used with a FACSort system to collect these cells.

Given the inconsistency of the methods above in producing useable DC populations, the remainder of the laboratory work carried out investigated the interactions of *E. coli* O157:H7 with monocyte-derived dendritic cells.

Chapter 4 Characterisation & reproducibility of a bovine moDC culture system including responses of bovine moDCs to bacterial components

4.1 Introduction

Dendritic cells are of great importance for generating immune responses and are found dispersed throughout the tissues of the body in a wide variety of species. Dendritic cells (DC) are professional antigen-presenting cells (APCs) which are understood to be the only APC with the ability to interact with naive T cells. This interaction can subsequently elicit primary T cell-mediated responses (Banchereau & Steinman, 1998). Study of DCs however, has proved complex due to their low frequency in tissues and circulating blood and the relative difficulty of extracting DCs directly from tissues. The discovery of several DC subsets, which exhibit tissue and species heterogeneity as well as the migratory nature of these cells, makes them a challenge to investigate. All of these factors have prompted researchers to find novel ways of looking at these key immune cells.

DCs are common in circulating lymph where they are to be found returning from peripheral tissues, and a great deal of valuable information has arisen from the study of this cell population. However to collect these lymph DCs, complex surgical techniques must be undertaken which necessarily incur technical, ethical and cost implications. The possibility of generating DCs from proliferating progenitor cells or non-proliferating precursor cells was subsequently explored. DCs have now been successfully generated from bone marrow cells as well as blood and lymphoid organ cells, and these methods have provided valuable insights into DC populations.

Initially bone marrow was identified as a source of suitable progenitor cells from which to derive DCs. Bone marrow DCs have been generated *in vitro* for mouse (Inaba *et al.*, 1992), rat (Talmor *et al.*, 1998), human (Reid *et al.*,

1992) and bovine systems (Hope *et al.*, 2000b). Subsequently PBMCs and, more specifically, monocytes were employed as progenitor cells to generate large numbers of DCs following culture in the presence of cytokine. These moDCs have been generated and characterised in human (Sallusto & Lanzavecchia, 1994), primate species (O'Doherty *et al.*, 1997) murine (Schreurs *et al.*, 1999), equine (Hammond *et al.*, 1999), porcine (Paillot *et al.*, 2001), rat (Richters *et al.*, 2002), canine (Catchpole *et al.*, 2002; Wang *et al.*, 2007), ovine (Chan *et al.*, 2002) and bovine (Hope *et al.*, 2000a; Werling *et al.*, 2002; Werling *et al.*, 1999) systems.

Bovine moDC were first generated by Werling *et al* in 1999. This drew on a published technique for successful generation of human moDCs *in vitro* (Woodhead *et al.*, 1998). Adherent bovine PBMCs were incubated with recombinant bovine cytokines GM-CSF (at an empirically determined concentration – approx 0.2 U/ml) and IL-4 (200 U/ml) for up to seven days with the addition of fresh media with cytokine after 3 days. By day 3 in culture visible changes were observed whereby the cells exhibited a more heterogeneous morphology and displayed dendrites. These cells were no longer as adherent to the culture plates and had also formed cell clusters. By days 6-7 of culture under the continuing culture conditions the cell clusters were of maximum size. When compared to bovine monocytes these cultured cells were found to have upregulated the cell surface molecules MHC II, Mannose receptor (MR), CD80/CD86, SIRPα and CD1b when examined by flow cytometry. Down-regulation of CD14, CD32 and CD62L on these cells was also observed. Morphological assessments after 6-7 days in culture, along with the cell surface expression pattern and their capacity for stimulating lymphocyte proliferation indicated that these cells were indeed

blood-derived dendritic cells (Werling *et al.*, 1999). These moDCs were however compared to bovine ALDCs returning from the skin and were discovered to have higher expression of CD14 and lower expression of MHC II than the levels published for the *ex vivo* lymph DCs (Howard *et al.*, 1997) A modification to the technique has been widely incorporated and replaces the PBMC adherence process. Instead, $CD14^+$ cells are labelled using super-paramagnetic particles to enable their positive selection from the PBMC population, and these cells are then cultured as before (Hope *et al.*, 2000a). This same moDC generation method is now exploited in a variety of bovine research areas including Mycobacteria (Langelaar *et al.*, 2005; Price & Hope, 2009; Werling & Kaiser, 2006), *Salmonella* (Norimatsu *et al.*, 2003; Norimatsu *et al.*, 2004), bovine respiratory syncitial virus (BRSV) (Werling *et al.*, 2002) and Bovine Viral Diarrhoea Virus (BVDV) (Glew *et al.*, 2003).

One interesting point of note with this technique is that different researchers have utilised varied culture times to suit their needs. Bovine moDCs after 3 days in culture are generally thought to represent an immature DC phenotype with lower expression of MHC II and co-stimulatory molecules than cells cultured for longer. Seven day moDCs are indicated to represent a more mature phenotype. A brief comparison was carried out here, although as intestinal DCs are likely to be of an immature phenotype when they encounter *E. coli* O157:H7 it was decided that working with 3 day moDCs would be the most biologically relevant (Langelaar *et al.*, 2005).
Functional characteristics of these moDCs have been investigated, including T cell stimulatory abilities, phagocytosis of pathogens and cytokine production in response to pathogenic stimuli.

In their role as antigen presenting cells DCs are important for directing immune responses to encountered pathogens. To aid them in this task they express numerous toll-like receptors (TLR) to enable recognition of motifs expressed by various organisms. Bovine APCs have been shown to express numerous TLR *in vitro* which recognise bacterial components such as flagellin (TLR5) LPS (TLR4) and CpG (TLR9) (Werling *et al.*, 2006), and enable generation of appropriate immune responses.

It is known that bacterial LPS is a potent stimulator of DCs *in vitro* (Sallusto *et al.*, 1995) and *in vivo* (De *et al.*, 1996). *In vitro* LPS challenge of moDCs prompts cells to up regulate expression of MHC II, CD80, CD58, CD54 as well as reducing pinocytosis activity within 24-48hr (Sallusto *et al.*, 1995). DCs *in vivo* have also been stimulated to mature and migrate to tissue T cell areas following LPS administration. In this murine model, migration occurred rapidly (within 6 hr) and was accompanied by upregulation of MHC II and CD80/CD86 (De *et al.*, 1996). Kaisho *et al* (2001) have also shown murine BMDC maturation *in vitro* in response to LPS, and found this to occur in a TLR4-dependent manner (Kaisho *et al.*, 2001). Human moDCs have also been shown to mature in response to LPS *in vitro* (Benhamron *et al.*, 2006) In ovine moDCs cytokine responses to LPS stimulation have been studied using real time PCR and this has demonstrated a difference in the responses of moDCs which were dependent on the time in culture. Ovine moDCs differentiated in culture for three days produced no cytokine response to LPS; however cells which underwent five days of differentiation produced cytokine responses as detected by mRNA transcripts. TNFα responses were detected around 3 h post-stimulation and the IL-12 responses were slightly delayed at between 3 and 6 h. This study too found there to be differences in

the responses of cells from individual animals (Budhia *et al.*, 2006). It is clear that LPS is an important bacterial component which is recognised by DCs. The responses of DCs to LPS are marked and are likely to be essential for host recognition of bacterial infiltration. Bovine moDCs are also known to respond to LPS in the same way by expressing cell surface molecules indicated of a more mature phenotype. (Dr. J. Hope, personal communication) This response to LPS by production of cytokine and upregulation of cell surface molecules will be used in this study to confirm activity of the *in vitro* generated bovine moDCs.

Flagellin is an important pathogenicity factor of many bacterial pathogens including *E. coli* O157:H7 as it confers motility (Monday, 2003). As a protein located outside the bacterial cell wall it often directly contacts host cells and is termed a pathogen associated molecular pattern (PAMP) recognised by TLR5 (Hayashi *et al.*, 2001). H7 flagellin has been shown to be important during binding of EHEC to primary bovine epithelial cells *in vitro* (Mahajan *et al.*, 2009), and has been demonstrated to produce specific serum IgA responses in *E. coli* O157:H7 challenged cattle (McNeilly *et al.*, 2008), and so H7 flagellin may potentially play in important role within the bacterial-host interaction. Bovine DCs in the terminal rectum are likely to contact H7 flagellin during colonisation and so characterisation of this interaction *in vitro* would be advantageous to advance the understanding of the bovine immune responses to EHEC. Murine lamina propria DCs have been shown to produce cytokine in response to stimulation with flagellin; IL-12 production was detected with little production of IL-10 (Uematsu & Akira, 2009). Murine BMDCs *in vitro* also respond to flagellin stimulation by production of IL-12 with no detectible IL-10 secretion (Vicente-Suarez *et al.*,

2009). Flagellin has shown effects on murine DCs *in vivo.* Following injection of mice with *Salmonella* flagellin, cell surface molecules CD40, CD80 and CD86 were upregulated by splenic DCs. Variations in the responses of DC subsets was observed: the $CD11b^+$ cells responded with greater magnitude than $CD8\alpha^+$ DCs. This difference was interesting because responses stimulated by LPS were similar for both $CD11b^+$ and $CD8\alpha^+$ cell populations. Flagellin injection into mice also stimulated migration of splenic $CD11c^+$ DCs; within six hours of treatment the $CD11c^+$ cells were found in the T cell area of the white pulp and absent from other areas. A further interesting aspect of this study was that when isolated and purified the $CD11c^+$ DCs did not respond *in vitro* to flagellin, (by measurement of cytokine production and levels of co-stimulatory molecule expression) and it was concluded that these cells are activated *in vivo* by means of 'bystander' stimulation (Salazar-Gonzalez *et al.*, 2007). However other reports have indicated that murine DCs do respond *in vitro* to flagellin, and mirror the *in vivo* responses. Didierlaurent *et al.* reported that murine splenic DCs increased expression of CD40, CD80 and CD86 *in vivo* following an injection of flagellin and also that murine BMDCs were found to upregulate costimulatory cell surface molecules and MHC II as well as produce IL-12p40 in response to flagellin (Didierlaurent *et al.*, 2004). The role which DCs play in response to flagellin is therefore complex and not fully defined. Consequently studies into interactions of H7 flagellin and bovine DCs would be of value, concentrating in particular on the production of IL-12 and changes in expression of cell surface molecules highlighted by the murine studies.

Verotoxin is a significant mediator of the most serious disease progression (HUS) in human infection with EHEC, and so quite a body of research has

probed the actions of this toxin *in vitro* with various host species. The structure of VT is important in relation to function. One central A subunit inhibits protein synthesis in eukaryotic cells by acting on the cell ribosome. The A subunit is surrounded by five B subunits which specifically bind to the galactosyl-α1,4-galactose (Galα1, 4Gal) linkage of globotriaosylceramide (Gb_3 or CD77) found on endothelial cell surface. Expression of CD77, the receptor for VT, by eukaryotic cells is thought to relate to VT susceptibility (Eisenhauer *et al.*, 2001). VT has profound cytotoxic effects on vascular endothelium. Human monocytes when stimulated with Verotoxin (VT) *in vitro* promote synthesis of cytokines, in particular IL-1β, TNFα, IL-6 and IL-8, and GM-CSF (Cameron *et al.*, 2003; van Setten *et al.*, 1996). These cytokines are pro-inflammatory and TNFα in particular has been shown to contribute to the pathological disease processes involved in HUS, by increasing the sensitivity of endothelial cells to VT (Eisenhauer *et al.*, 2001; van Setten *et al.*, 1996). However studies show that VT is not cytolethal to monocytes when challenged with doses higher than those toxic to Vero cells (van Setten *et al.*, 1996). Bovine monocytes have also been investigated in terms of their responses to VT and significant animal variation was observed, however 60% of the monocytes from cattle tested responded to VT by production of 1000-2000 pg/ml of TNFα. (Dr. P. Cameron, unpublished observations) In mice the B subunit of the toxin is sufficient to cause changes in BMDCs. *In vitro* BMDCs increase expression of MHC II, CD40, CD80 and CD86 in response to incubation with VT B sub-units, as well as production of TNFα and IL-12 (Ohmura *et al.*, 2005). This finding was tested *in vivo* and splenic DCs were found to also increase expression of MHC II, CD40, CD80 and CD86 following subcutaneous injection of VT B (Ohmura *et al.*, 2005). To date no work has been published indicating responses of bovine DCs to VT, although

taking into consideration the studies already mentioned it is likely that at least a proportion of cattle DCs would respond to VT by cytokine production and alterations in expression of maturational cell surface molecules. However, the lack of pathology *in vivo* to *E. coli* O157:H7 colonisation in cattle in comparison with human disease sequelae may indicate divergent responses to VT could play a role in this although the involvement if any of DCs in these responses is unknown. Investigation of DC responses to VT is one of the aims of work in this chapter.

As important immune system cells DCs have been shown to produce various cytokines in response to pathogens, these key signals help shape the immune responses generated. The IL-12 cytokine response of DCs to microbial pathogens is possibly one of their most important determinants of downstream immune responses. IL-12 is a significant cytokine for the generation of the Th1 response (Macatonia *et al.*, 1993b). DC secretion of IL-12 in response to microbial products can occur in less than 24hrs and stimulates T cells to become interferon-γ (IFN-γ)-producing Th1 cells. Macrophages are activated by this IFN-γ and T cells can be induced to differentiate into killer cells. Thus the role that DCs play in producing IL-12 and subsequently stimulating Th1 cells promotes resistance to microbial infection (Banchereau & Steinman, 1998). IL-12 gene expression is known to be upregulated by bovine day 3 moDCs in response to killed *E. coli* as reported by Langelaar *et al* (2005) although the strain that was used is not specified; the authors suggest that responses are likely to be via TLR4 signalling. IL-10 is an important immunoregulatory cytokine producing varied effects and which shows a degree of species heterogeneity. An IL-10-deficient mouse strain shows uninhibited IL-12 and TNFα release which

causes significant pathology in the host tissues, in particular intestinal enterocolitis (Rennick *et al.*, 1997). IL-10 plays an important role in DC cellular interactions. IL-10, by its inhibition on DC as well as macrophage-induced IFNγ production *in vitro*, may play a role in regulating the class of immune response directed towards pathogens, by affecting the downstream T cell populations (Macatonia *et al.*, 1993a). In cattle IL-10 is generally considered to play an anti-inflammatory role in down regulation of responses following infection (Norimatsu *et al.*, 2003). TNFα is an inflammatory cytokine, as an early inflammatory protein it signals to attract other immune cells involved in immune responses to varied stimuli including physical, and environmental as well as immunopathological. The immune response directed by TNFα involves induction of further cytokines (e.g. IL-1 and IL-6) (Kramer *et al.*, 2007; McGee *et al.*, 1995). TNFα is produced in response to bacterial endotoxin *in vivo* and *in vitro* (Cicala *et al.*, 1997; Collart *et al.*, 1990) and is also important in the direction of immune responses to intracellular bacteria (Kwong *et al.*, 2010; Lin *et al.*, 2007) and is known to sensitise endothelial cells to the effects of VT *in vitro* (Eisenhauer *et al.*, 2001).

The work in this chapter was carried out chiefly to generate and characterise bovine moDCs, to ensure methods and reagents in our laboratory were sufficient to successfully and reproducibly provide bovine DCs for investigation of interactions with *E. coli* O157:H7 strains and bacterial components and to correlate phenotype and responses with known parameters for bovine moDCs. Following validation of culture and downstream laboratory techniques including ELISA and flow cytometry, the

response of moDCs to key pathogenic determinants of *E. coli* O157:H7 were investigated.

The aims of this chapter were:

1. To generate bovine moDC cultures in a reproducible manner
2. To characterise bovine moDCs used in this study by morphology, cytokine responses and cell surface marker expression to compare with known parameters for these cells.
3. To investigate the interaction of bovine moDCs with selected bacterial components including LPS, Verotoxin (VT) and H7 flagellin.

4.2 Materials and Methods

4.2.1 Generation of Monocyte Derived Dendritic Cells (moDCs)

4.2.1.1 Donor animals

All blood sampling was carried out according to the requirements of the Scientific Procedures Act (1986). All animals were reared conventionally either at Moredun Research Institute, Easter Bush Veterinary Centre or the Institute for Animal Health (IAH), Compton. Further animal information is detailed in Appendix A.2.

4.2.1.2 Peripheral Blood Mononuclear Cell (PBMC) Isolation

PBMC isolation was carried out following a protocol developed at IAH, Compton; and is summarised below (Werling *et al.*, 1999). Collection of bovine blood was by venepuncture and collection into sodium heparin vacutainer tubes (BD) and used within 2 h of collection. Equal volumes of PBS and blood were mixed in a 50 ml Blue Max polypropylene tube (Falcon, Oxford) and peripheral blood mononuclear cells (PBMC) were separated from whole blood by density gradient centrifugation (1.083 g/ml Histopaque, Sigma). The blood and PBS mix was underlaid with half the volume of Histopaque. These cell suspensions were centrifuged at 1200 x g for 35 min, at 20°C with the brake off. The PBMC layer was removed and these cells were pelleted in 30 ml PBS at 400 x g at 4°C for 10 min, resuspended in 10 ml PBS and counted (section 4.2.1.6). Cells were washed in 30 ml PBS by pelleting at 300 x g at 4°C for 8 min. All residual fluid was removed from the pellet prior to monocyte purification

4.2.1.3 Monocyte Purification

10^7 PBMCs were labelled with 2.5 µl αCD14-conjugated paramagnetic beads (MACS, Miltenyi Biotec). The cells and beads were gently mixed by pipetting and then incubated at room temperature for 10 min. To wash the cells, 15 ml of PBS was added and the suspension was centrifuged at 400 x g for 5 min at 4°C. The wash step was repeated then cells were re-suspended in 3 ml FACSflow (Becton Dickinson) containing 1% w/v BSA (Sigma) (FACSflow/BSA). A MACS LS separation column (Miltenyi Biotec) was assembled with the midiMACS magnet (Miltenyi Biotec). 2.5 ml of FACSflow/BSA was applied to the column and allowed to run through to a waste container. When the solution had passed the through the column the cell suspension was added and unbound cells were allowed to flow into a waste container. The bound cells were washed by addition of 2.5 ml FACSflow/BSA to the column which also flowed into the waste container. To elute the bound cells, 5 ml of Tissue Culture Medium (TCM, Appendix A.4.2.3) was applied to the column. The column was quickly removed, held over a 50 ml tube containing 5 ml TCM and labelled cells were eluted using the plunger. This solution was made up to 35 ml with PBS and then centrifuged at 400 x g for 5 min. The cell pellet was re-suspended in 1 ml of TCM and viable cells were counted (section 4.2.1.6.). The cell yield was expected to fall within the range of 5-15% of the starting cell number (based on average percentages of CD14+ cells within bovine PBMC). The purity of the cells was evaluated by flow cytometry and was greater than 83% (data not shown).

4.2.1.4 Dendritic Cell Generation and Culture

The monocyte cell pellet was re-suspended to between 8 x 10^5 and 1 x 10^6 cells / ml in Dendritic Cell Tissue Culture Medium (DC-TCM, Appendix A.4.2.4). 3 ml of this cell suspension was added to each well of a six well plate. Optimal concentrations of recombinant bovine GM-CSF (rboGM-CSF) and recombinant bovine interleukin 4 (rboIL-4) were determined empirically. GM-CSF (IAH, Compton) and recombinant bovine IL-4 (Moredun Research Institute) were added to the culture medium at final concentrations of 0.2 U/ml and 200 U/ml respectively, to stimulate differentiation of cells. The rboIL-4 was expressed from a permanently-transfected Chinese hamster ovary cell line (Hope *et al.*, 2005), and rboGM-CSF was produced from transiently-transfected COS cells. The six well plate was incubated at 37°C, 5% CO_2 for three or seven days. Cells incubated for seven days were fed after three days in culture by the removal of the culture supernatant and the addition of DC-TCM to all wells.

4.2.1.5 Harvesting Dendritic Cells

Non-adherent cells and media were removed from the 6-well plate, placed into a Falcon™ tube and stored at 4°C. 1 ml of pre-warmed cell dissociation fluid (Sigma) was added to each well and the plate was incubated at 37°C, 5% CO_2 for 30 min. Cells were detached by pipetting and were pooled with the non-adherent cells. Cells were centrifuged at 400 x g for 5 min and re-suspended in DC-TCM at the appropriate concentration for further assays.

4.2.1.6 Viable cell counting

Viable cell counts were carried out using Trypan Blue exclusion dye (0.4%) or 0.1 % Nigrosin in a disposable counting chamber (Fast-read counting chamber, ISL, Immune Systems Ltd).

4.2.2 Cytospin and slide preparations of moDC

To prepare slide preparations, two methods were used. Firstly the cells were re-suspended to 5 x 10^5 cells per ml. Superfrost™ plus slides were assembled with cytofunnels™, filters and clamps. 100 µl of the cell suspension was pipetted into the funnel and spun in the Cytospin centrifuge (Thermo Shandon) at 300 x g for 3 min. The slides were left to air dry at room temperature and then stored at -20°C. Alternatively slide preparations were generated as described previously (section 3.2.4).

4.2.3 Immunohistochemistry (IHC)

IHC was carried out as described previously (Section 2.1).

4.2.4 Challenge of moDCs with *E. coli* components

In preparation for challenge, the cells were collected on day three or seven of culture and counted. Wells of a 12 well plate were seeded with 1 x 10^6 cells with 1 ml TCM per well. Cells were challenged for 18 h at 37°C and 5% CO_2 with either purified H7 flagellin (Dr. T. McNeilly), LPS (Sigma) or Verotoxin 2 (VT2) (Sigma) at concentrations indicated in figure legends. VT2 stock was diluted into sterile PBS in low protein bind tubes. In control wells, moDCs were cultured in RPMI alone. Cell supernatants were collected and stored at -20°C and moDCs were collected for analysis of cell surface markers by FACS (section 3.2.7).

4.2.5 Enzyme Linked Immunosorbent Assay (ELISA) analysis of bovine cytokines

IL-10, IL-12 and TNFα were determined by ELISA. The protocol for these ELISAs was developed at IAH (Hope *et al.*, 2002; Kwong *et al.*, 2010; Kwong *et al.*, 2002) using reagents as in Table 4.1. Black or clear 96-well microtitre plates (Nunc Maxisorp) were coated overnight with 100 µl/well of capture mAb diluted in coating buffer. Coating buffer, 0.05 M carbonate-bicarbonate buffer, pH 9.6, was made up using Carbonate/Bicarbonate buffer capsules (Sigma) and 100 ml DDW in a suitable container. The plates were then incubated either at room temperature O/N or for 36 h at 4°C sealed in an airtight container. The plates were either washed 3 x manually or 5 times on a Skatron Skanwasher/stacker 300 Version B on an appropriate programme with wash buffer (PBS + 0.05% Tween 20). Plates were blocked for 1-2 h with 300 µl/ well of 1 mg/ml sodium casein (Sigma) in PBS at room temperature in a sealed container. Following a wash step, 100 µl of each sample to be tested in duplicate, or triplicate if volume of sample allowed, was added to the plates. Standards diluted in 1 mg/ml sodium casein in PBS were included in every plate. Internal standards were also included for some assays as a positive control and blocking buffer was added to two wells per plate as a blank. Samples and standards were incubated for 1-2 h at room temperature in sealed container and subsequently washed. Supernatants from transiently transfected COS cells were used to generate standards to be used at predetermined ranges. The internal standards were also COS cell supernatant, diluted to a concentration falling within the linear range of the standard curve and optimised to fall within a specified range of concentrations. These ensure accuracy between multiple plates or assays. 100 µl/well of biotinylated detection mAb, diluted in blocking buffer was added

and incubated for 1-2 h at room temperature in sealed container. Another wash step was followed by adding 100 µl/well of Streptavidin-HRP at 1:500 diluted in reagent diluent (PBS + 1 mg/ml sodium casein + 0.05% Tween 20) which were incubated for 45-90 min at room temperature. Plates were developed using TMB or luminol. For TMB development, clear plates were washed before addition of 100 µl/well of TMB substrate solution (Appendix A.4.1.6) and then stopped with 50 µl/well of 1M H_2SO_4 as soon as a colour change was observed in blank/control wells. The optical density was read on an Ultra microplate reader EL$_x$808iu (BIO-TEK Instruments) at 450nm using DASDAQ Stingray software, or a DYNEX Technologies MRX II ELISA plate reader linked to a computer running MRX EndPointSoftware, (Version 2.02).. For luminol development, black plates were washed 3 times with a 5 min soak in wash buffer between each wash before the contents of the wells was aspirated. The plates were developed with 100 µl/well of a 50:50 solution of luminol and peroxide, gently mixing the plates for 1 min. Relative light unit values were read immediately on an Anthos Lucy 1.0 photometer/luminometer (Anthos Labtec, Salzburg, Austria) connected to a PC using DASDAQ Stingray Software.

Cytokine	Capture mAb	Top Conc. Standards	Biotinylated mAb
IL-10	CC318 (1 µg/ml)	30 U/ml	CC320-b (1 µg/ml)
IL-12	CC301 (1 µg/ml)	1000 U/ml	CC326-b (8 µg/ml)
TNFα	CC327 (2 µg/ml)	333 ng/ml	CC328-b (1 µg/ml)

Table 4.1 IL-10, IL-12 and TNFα ELISA antibody pairs and standard concentrations

4.2.6 Flow Cytometry for Cellular Expression of Cell Surface Molecules

Flow cytometry was carried out as described in Chapter 3 (section 3.2.7).

4.2.7 Culture and Passage of Cell Lines

Vero cells (ATCC # CCL-81™) were resuscitated from liquid nitrogen stock and defrosted at 37°C, and diluted into cell culture medium (CCM, Appendix A.4.2.1) and seeded into a T75 tissue culture flask with a vented cap (Corning). For routine passage, cells were trypsinised at approximately 80% confluence. Briefly, cells were detached by incubation in 3 ml Trypsin EDTA (Lonza) for 10 min, followed by the addition of 7 ml CCM. Cells were pelleted at 170 x g for 4 min, resuspended in fresh media and used to seed flasks for further culture or plates for challenges. Caco-2 cells (ATCC # HTB-37™) were provided by Dr. Pamela Cameron, cultured in cell culture medium (CCM Appendix A.4.2.1) and passaged as above at 70-80% confluence.

4.2.8 Vero cell challenge with Vero toxin 2 (VT2)

Vero cells were challenged with Verotoxin (Sigma) at concentrations indicated in figure legends for 18 h at 37°C, 5% CO_2. Unchallenged control wells were included. For challenges where viability was to be assessed with the Vybrant MTT cell Proliferation Assay Kit, Phenol Red free CCM (Appendix A.4.2.1) was used.

4.2.9 Cell Viability Assay

To assess the viability of challenged cells, the Vybrant MTT cell Proliferation Assay Kit, (Molecular probes) was used, following the manufacturer's instructions. Following Vero cell challenge, cell supernatants were removed and replaced with 100 µl of fresh media. A 12 mM MTT stock solution was prepared by dissolving 5 mg of MTT in 1 ml of sterile PBS. 10 µl of the MTT stock solution was then added to each well and incubated at 37°C for 4 h.

Control wells included unchallenged cells, cells with media alone and wells with media and MTT only. 100 µl of SDS-HCL solution (Appendix A.4.2.6) was added per well and mixed by pipetting. A further incubation at 37˚C was carried out for 4 h in a humidified chamber. Before reading on a plate reader, each well was then mixed by pipetting. The absorbance was read at 570nm on a DYNEX Technologies MRX II ELISA plate reader linked to a computer running MRX EndPointSoftware.

4.2.10 Live Cell Imaging

Live cell images were taken using an AxioCam MRm digital camera mounted on a Ziess Axiovert microscope with an integrated cell culture incubator.

4.2.11 Endotoxin removal from H7 flagellin samples

The H7 flagellin samples were lyophillised over night in a ModulyoD-230 Freeze Dryer (Thermo), resuspended in 300 µl of ultrapure endotoxin free water (Sigma) and quantified on a NanoDrop (Thermo Scientific). The samples were then run through an endotoxin removal column (Detoxi-Gel columns, Pierce) following the manufacturer's instructions. Briefly, the column was equilibrated to room temperature. The column was washed with five resin bed volumes of 1% w/v sodium deoxycholate solution followed by five resin bed volumes of distilled water. The H7 flagellin sample was applied to the top of the column and when it was fully contained within the reservoir bed, the ends of the column were capped off and the column was incubated for 1 h at room temperature. Following incubation, the sample was washed through the column into an endotoxin-free tube with ultrapure water. The sample was placed at -70˚C for 2 h prior to lyophilising overnight.

The following day the sample was again suspended in water (Sigma) and quantified on a NanoDrop Spectrophotometer (Thermo, ND-1000, UV-Vis) to allow calculation of the quantity of H7 flagellin retained within the column (usually around 4 μg). This purified sample was used for moDC and Caco2 cell challenges.

4.2.12 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The BIO-RAD Mini-Protean III system™ was used for SDS-PAGE analysis according to principles described by Laemmli (Laemmli, 1970). 1 mm glass plates were cleaned with 70% 74OP, assembled in the casting stand and approximately 5 ml resolving gel mixture (Appendix A.4.3.1) was poured between them, 250 μl of 1% w/v SDS was added over the top to exclude air and allow a level interface between resolving and stacking gels. This was left to polymerise at room temperature for 1 h. SDS solution was removed using filter paper and the stacking gel mixture (Appendix A.4.3.2) was overlaid to the top. Plastic well-forming combs were cleaned with 70% 74OP and immediately pushed into the top of the gel. The gel was again allowed to set at room temperature. When polymerisation was complete, after approximately 10 min, the combs were removed. The fully assembled gel was placed into an electrophoresis tank. The tank reservoirs were filled with running buffer (Appendix A.4.3.3). Samples were adjusted to appropriate concentrations in 20μl reducing sample buffer (Appendix A.4.3.4) in a 1.5 ml centrifuge tube. Protein quantities are indicated in figure legends. Samples were boiled for 5 min, briefly pulsed in a microcentrifuge and gently mixed. Samples were run concurrently with a prestained broad range protein ladder (Fermentas; SM0671) and loaded into the wells using gel loading pipette tips. A current was passed across the gel at 135v (BioRad PowerPac HC) at room

temperature for approximately 60-90 min, until the bromophenol blue front reached the end of the gel.

4.2.13 Coomassie Staining

The gel was removed from the gel tank and the glass plates; the stacking gel was cut off and discarded. To visualise proteins, the gel was placed into a staining tray and washed with three washes of distilled water. Gels were immersed in Coomassie® G-250 stain (SimplyBlue ™, Invitrogen) and placed on a rocker overnight at room temperature. The following day the gel was destained in distilled water until protein bands were clearly differentiated.

4.2.14 Western Blotting

Following separation of proteins by SDS-PAGE, proteins were transferred from acrylamide gels onto nitrocellulose membranes. Six pieces of blotting paper (Hybond) and a nitrocellulose membrane (0.45 μm, Hybond-C) cut to the dimensions of the gel were soaked in electroblotting transfer buffer (Appendix A.4.4.1) for 5-10 min. The gels were assembled next to the nitrocellulose with three sheets of blotting paper on either side. The assembled gels, nitrocellulose and blotting paper were placed onto a semidry blotter (Fisherbrand) with the nitrocellulose towards the anode, and smoothed to remove any air bubbles. A small volume of transfer buffer was poured onto the blotting components to ensure saturation before assembling the blotter. A current was applied at 70 mA per mini gel for 90 min (BioRad PowerPac HC).

4.2.15 Probing Blots with Antisera

Detection of H7 flagellin on Western blots was carried out according to McNeilly *et al* 2008. To reduce non-specific antibody binding the blots were incubated with blocking buffer (Appendix A.4.4.2) for 1 h at room temperature. Blots were probed with primary antisera, either rabbit polyclonal αH7 (Mast-Assure) or naïve rabbit serum (Scottish Antibody Production Unit) at 1:500 in blocking buffer for 1 h at room temperature. The blots were then rinsed twice in blocking buffer followed by a 15 min and two 5 min washes. Blots were then incubated in goat α rabbit HRP conjugated secondary antibody diluted 1:1000 in blocking buffer for 1 h at room temperature. Wash steps were carried out as described above with an additional wash for 1 h. Bound antibody was detected using enhanced chemiluminescence (ECL) system (Pierce) according to manufacturer's instructions. The bands were visualised by incubating the blots with radiograph film (Kodak) which was developed in an X-ray developer (OPTIMAX 2010).

4.2.16 Challenge of Caco2 cells with H7 flagellin

To assess biological activity of the H7 flagellin, Caco2 cells were used because they have been shown to produce IL-8 in response to H7 (Donnelly & Steiner, 2002). Caco2 cells were cultured in CCM in 12 well plates until 2 days post confluency and then serum starved for 24 h. The cells were challenged on the third day post confluence with 500 ng/ml of H7 flagellin in fresh serum free CCM for 18 h at 37°C and 5% CO_2. Six biological replicates were used for challenged cells and unchallenged controls. Following challenge the supernatants were collected and stored at -20°C for further analysis.

4.2.17 Interleukin 8 (IL-8) ELISA

Levels of IL-8 in Caco2 cell supernatants were monitored using a human IL-8 ELISA kit (Duoset, R&D Systems) according to the manufacturer's instructions. Overnight, 96-well EIA/RIA stripwell plates (Costar) were coated with 100 µl/well of capture antibody (4 µg/ml in PBS) at room temperature and sealed to prevent drying. After incubation the wells were aspirated and each well washed three times with 400 µl of PBS containing 0.05% v/v Tween-20. Any residual washing buffer was removed by blotting on clean paper towels. Wells were blocked with 300 µl of PBS containing 1% w/v BSA for at least 1 h. All wells were aspirated and washed as before. 100 µl of samples were added in triplicate and incubated for 2 h. 100 µl of IL-8 standards were included, in doubling dilutions, in IL-8 ELISA reagent diluent (Appendix A.4.1.5). Three wells with 100 µl reagent diluent alone were also included. Wells were washed and 100 µl of 20 ng/ml detection antibody in IL-8 ELISA reagent diluent was added. A further 2 h incubation was carried out at room temperature followed by three washes. 100 µl/well of Streptavidin-HRP (diluted 1:200 in IL-8 ELISA reagent diluent) was added and incubated for 20 min in the dark and then washed as before. 100 µl of substrate solution, made up with a 1:1 mix of colour reagent A (H_2O_2) and colour reagent B (trimethylbenzidine), was added and the plate was incubated for 20 min in the dark. To stop the reaction 50 µl/ well of 2 N H_2SO_4 was added and the plate read at 450 nm using an ELx808IU Ultra Microplate Reader. (BIO-TEK Instruments Inc.)

4.2.18 Determination of Toll-Like Receptor 5 (TLR5) expression by RT-PCR

Previous studies have shown bovine moDC cultured for seven days transcribe TLR5 (Werling *et al.*, 2006); RT-PCR was employed to investigate whether moDC cultured in the conditions described in this study (4 day culture) express this receptor.

4.2.18.1 Preparation of moDC for RT-PCR

Monocyte derived dendritic cells were prepared as described above (section 4.2.1) and collected after four days in culture to correspond with the timing of challenges used in this work. Briefly, cells were collected, counted (section 4.2.1.6) and re-suspended to 1 x 10^7 / ml in PBS. 1 ml of the cell suspension was added to 1.5 ml centrifuge tubes, the cells were washed twice with PBS, each consisting of 5 min in a centrifuge at 300 x g. Supernatant was then discarded, and 50 µl of fresh PBS was added. To this suspension 500 µl RNA*later*® (Qiagen) was added and mixed by pipetting. These cell suspensions were stored at 4°C for 24 h then transferred to storage at -20°C until required. Two positive controls were also employed in this study, human derived Caco2 cells (section 4.2.7), and primary bovine epithelial cells. Primary bovine epithelial cells, stored in liquid nitrogen and defrosted at room temperature, were previously prepared by Arvind Mahajan.

4.2.18.2 Isolation of RNA using RNeasy Mini Kit (Qiagen)

All reagents used in this section, unless otherwise stated, are included in the RNeasy Mini kit (Qiagen) and the manufacturer's instructions were followed. Briefly, the cell preparations were pelleted at 3000 x g for 4 min. The supernatant was discarded and the pellet was resuspended in 300 µl of

RLT then passed through a sterile 21G needle several times. 250 µl of ethanol was added to each sample and mixed by pipetting. The samples were then placed into the RNeasy mini columns. The columns were pulsed in a microcentrifuge for 15 sec at 8000 x g and the flow-through discarded. The column was then washed with 700 µl of buffer RW1 in the microcentrifuge for a further 15 sec at 8000 x g and the flow-through again discarded. 500 µl of Buffer RPE, was applied to the column and placed in the microcentrifuge as before, again the flow through was discarded. A further 500 µl of Buffer RPE was added and microfuged at 8000 x g for 2 min. The samples were then eluted by the addition of 50 µl of RNase-free water and centrifugation at 8000 x g for 1 min. The samples were quantified by using a Nanodrop Spectrophotometer (Thermo, ND-1000, UV Vis) and stored at -20°C until required.

4.2.18.3 Reverse Transcription

Reverse transcription of extracted RNA was carried out using Superscript III Reverse Transcriptase (Invitrogen) as per manufacturer's instructions. Briefly, in a nuclease free 0.2 ml microcentrifuge tubes, 100 ng of total RNA was added to 4 µl 5x first strand buffer, 1 µl RNaseOUT, 1 µl 10 mM dNTP mix (10mM each) 0.5 µl random primers and 1 µl SuperscriptIII (200 U/µl) reverse transcriptase and made up to a total of 20 µl with nuclease free water. The tubes were then incubated at 50°C for 60 min, 70°C for 15 min followed by a final denaturation step of in a TC-512 thermocycler (Techne). The samples were then stored at -20°C until required for PCR.

4.2.18.4 Polymerase chain reaction (PCR)

Forward and reverse TLR5 Primers were obtained from MWG-Biotech and were designed based on published work (Menzies & Ingham, 2006). PCR

amplification was carried out using Platinum Taq (Invitrogen) in a TC-512 thermocycler (Techne).

Target		Sequence	Reference
mRNA transcript for TLR5	Forward Reverse	5′-AACGCTTTGCTCAAACACCT-3′ 5′-ACCCTCTGATGGACTGATGC-3′	Menzies & Ingham, 2006

Table 4.2 Primer sequences used for TLR5 PCR

4.2.18.5 PCR Mix & PCR cycling conditions

PCR mix was made up with 5 µl 10x buffer, 1.5 µl MgCl, 1 µl each of forward and reverse primers (see table 4.2), 1 µl dNTPs and 0.2 µl Taq. 5 µl of template was then added and made up to a total volume of 50 µl. RNA which had not been reverse transcribed was also subjected to PCR as a control to demonstrate that any amplification was from DNA and not RNA.

Temperature	Time	Cycles
95°C	15 min	1 cycle
94°C	1 min	40 cycles
60°C	1 min	
72°C	1 min	
72°C	5 min	1 cycle

Table 4.3. PCR cycling conditions used for TLR5 RT-PCR

4.2.18.6 Agarose gel electrophoresis

The samples were subject to gel electrophoresis on a 1.8% agarose gel containing 1x Gel Red (Biotium Inc.) according to manufacturer's instructions. Gels were made by melting 0.9 g agarose in 50 ml of 0.5x Tris Acetate EDTA (TAE) buffer (Appendix A.4.6.1) in a microwave oven (PRO*line* micro chef ST44); following melting of the agarose, 5 µl of Gel Red (10000 x stock) was added prior to allowing the molten agarose to cool to 56°C in a water bath (Grant, SUB28). Following cooling to 56°C, the molten agarose was poured into a submarine gel casting tray with appropriate comb. The gel was left at room temperature on a flat surface to set. The gel was then placed into a tank (Bio Rad) containing 0.5x TAE buffer. 50 µl of sample was mixed with 5 µl 6x Blue/Orange loading dye (Promega), 15 µl of each sample was then loaded per well. Each end well contained 10 µl of 100bp ladder (Promega). The electrodes were fitted onto the tank with the lid and 80v was applied to the gel for approximately 80 min using a Bio Rad PowerPac 200/2.0. The gel was then visualised by UV transillumination on a Bio Rad imager.

4.2.19 Data Analyses

Flow Cytometry data was analysed using FCSExpress software (DeNovo software, Canada), the limit was set at 10,000 live cells, these were gated and histograms of fluorescence were generated. Isotype controls were included. MFI levels were calculated by subtracting the MFI for the relevant isotype control from the MFI of the molecule of interest for the selected cell population. Graphs were plotted in Microsoft Excel, with error bars to represent the standard error of the mean. All ELISA data was analysed using linear regression analysis, a standard curve was constructed from the data

points using Microsoft Excel and concentration figures were calculated by inputting the absorbance readings into the equation of the line. Statistical analysis was carried out using Minitab (v13) software. Paired t-tests were used for ELISA and FACS results of challenged and unchallenged cells where only two conditions were tested. The flow cytometry profiles of the 3 day and 7 day moDCs were analysed using a 2 sample t-test with unequal variance for each cell surface molecule. If more than two conditions were to be examined a one-way ANOVA was carried out. Significance for all data sets was determined when $p<0.05$.

4.3 Results

4.3.1 Bovine moDCs: phenotype of cultured cells

Bovine moDCs were generated as described (Price, 2009; section 4.2.1). The cells had differentiated by three days in culture to exhibit dendritic cell morphology (Fig 4.1 A) when compared with the same population of $CD14^+$ cells concurrently cultured without cytokines (Fig. 4.1 B) Cytospin samples of the cultured cells were subjected to IHC to investigate expression of DC cell surface molecules MHC II, SIRPα, CD40, CD1b and CD205. The results from IHC staining are shown in Fig 4.2. The cells showed positive staining for all the antibodies tested. MHC II expression was widespread at a high level throughout the majority of cells. (Fig. 4.2 A) SIRPα expression was strong and ubiquitous within the cell population. (Fig. 4.2 B) CD1b expression was also widespread and strong. (Fig. 4.2 C) CD205 was expressed on very few cells but those cells which showed positive expression of CD205 were generally large cells with an irregular morphology. (Fig. 4.2 D) CD14 expression was very strong on a small sub-population of cells, and weak on a larger population. A further population was negative for CD14. (Fig. 4.2 E) CD40 expression was moderate in strength and observed on the majority of the cells. (Fig. 4.2 F). In all of the cytospin preparations the majority of the cells were large with eccentric nuclei and cells with dendrites of varying length were observed. Flow cytometry for cell surface molecules was carried out with day three moDCs from seven animals. Four animals were housed at IAH and three were at MRI. The pooled data is shown in Fig 4.3. The results were relatively consistent between animals. The majority of cells showed expression of MHC II, MHC I, and SIRPα with further cell surface molecules CD1b, CD40, CD80, CD14 and CD11c also expressed. The percentage of cells

expressing B cell (CD5) and T cell (CD3) molecules was low (<5%), and although not specific for these cell types, they provide indication of moDC cell population characteristics. The percentage of cells expressing CD205 was also low, which correlates with known parameters for bovine moDCs. There were no differences in the percentage of cells expressing each molecule when comparing the 3 and 7 day moDCs. The MFI for most of the molecules was higher for the 7 day moDCs when compared to the 3 day moDCs ($p<0.05$), except for MHC I, MHC II, CD205 and CD3. For these molecule there was a trend for increase in MFI of the seven day moDCs although no statistical significance was observed ($p>0.05$), this indicates an increase in autofluorescence.

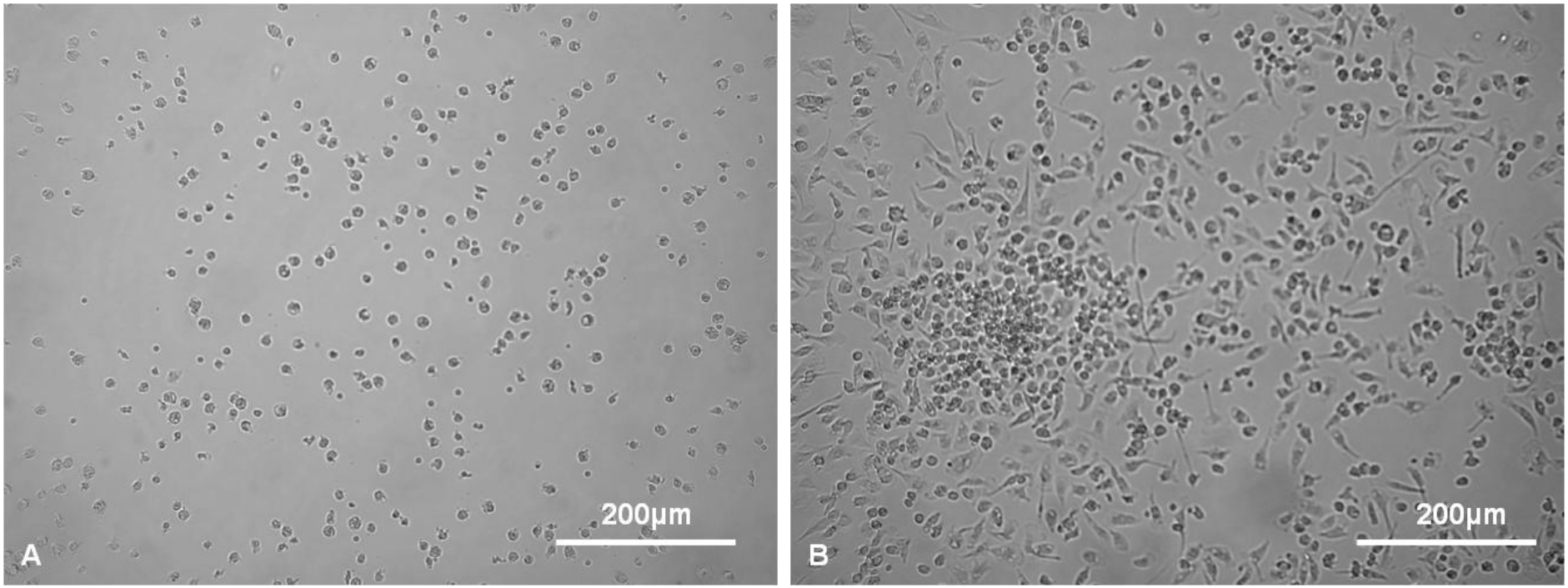


Fig. 4.1 Live cell images of bovine CD14$^+$ cells cultured for 3 days (A) in TCM which has no GM-CSF or IL-4 or (B) DC-TCM with 0.2 U/ml of GM-CSF and 200 U/ml IL-4. Representative images of cells from the same animal. Culturing cells in medium with the addition of GM-CSF and IL-4 promotes differentiation of the cells into a DC phenotype. The cells in panel (B) are much more heterogeneous, dendrites are easily observable on many of the cells and an area where cells have clustered together, as described in Werling *et al* (1999) is shown.

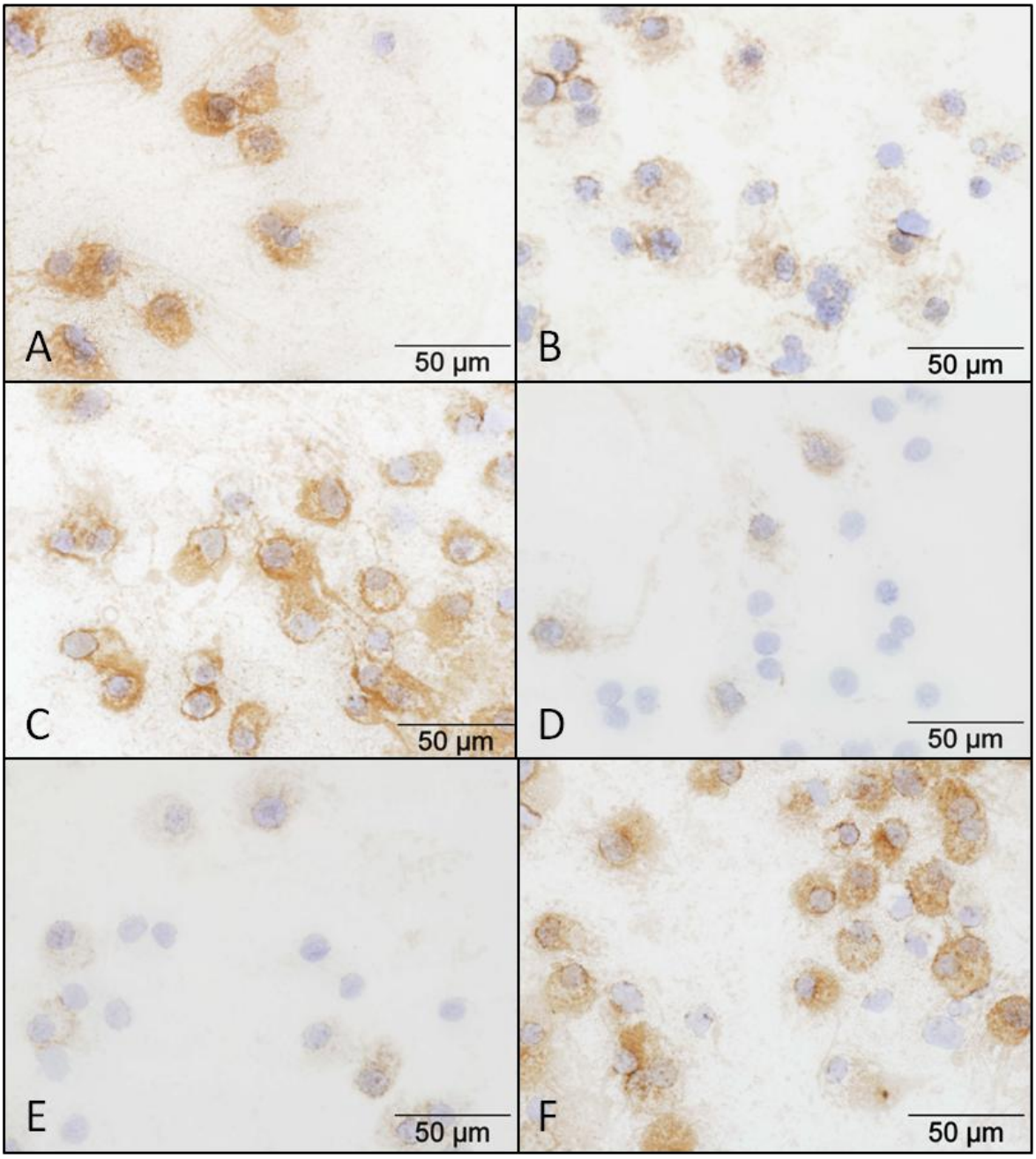


Fig. 4.2 Results of moDC IHC on cytospin preparations. (A) MHC II, (B) SIRPα, (C) CD1b, (D) CD205, (E) CD14, (F) CD40. Positive staining is indicated by brown colour, cell nuclei are counterstained blue. Positive staining was observed for all molecules. Corresponding Isotype controls were negative (data not shown) (A) The majority of the cells were positive for MHC II, this image shows large positively stained cells with eccentric nuclei and expression of dendrites of varied length. (B) SIRPα staining was widespread throughout the cell population at a moderate level. (C) CD1b staining was observed in most of the moDCs. Dendritic morphology is clear. (D) There were a small population of cells which stained positively for CD205. Staining was not as strong as that observed with MHC II or CD1b. (E) CD14 expression was varied throughout the population. Some cells were strongly stained, a significant population were more weakly stained and a further population were negative. (F) CD40 staining was strong and prevalent in the moDC population.

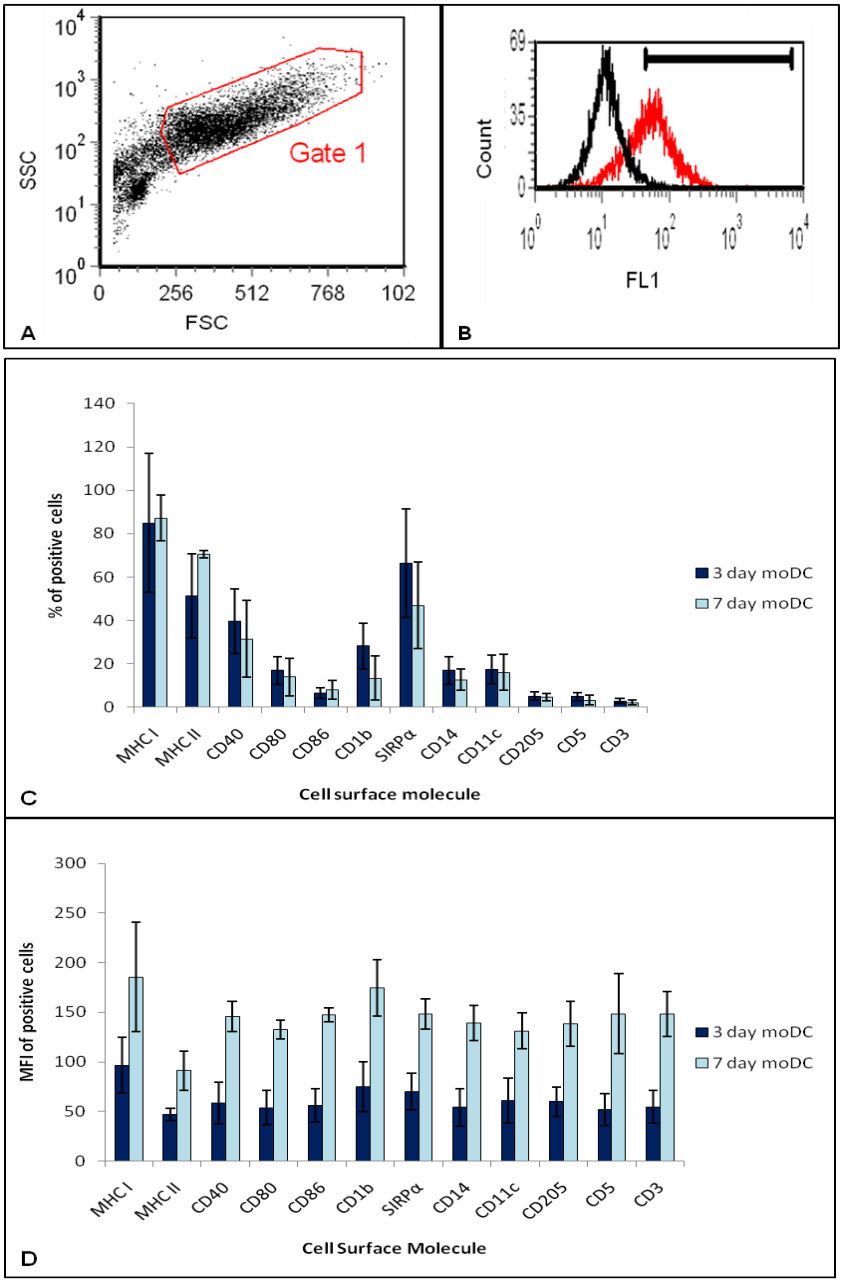


Fig. 4.3 Phenotyping of bovine moDCs by flow cytometry (A) A representative dot plot of moDC forward (FSC) and side scatter (SSC) showing the gated population of cells used for analyses (one animal). (B) Histogram of Fluorescence vs cell number showing isotype control (black) and MHC I (red) staining and the marker used for analyses. (C) Results of FACS analysis of 3 day and 7 day moDCs; percentage of positive cells (in gate 1) of cells which are positive for each cell surface molecule. (D) Results of FACS analysis of 3 day and 7 day moDCs; MFI of the positive cells with the MFI for the isotype control subtracted. Pooled data from seven animals – 3 day moDCs, pooled data for 3 animals – 7 day moDCs. Error bars represent standard error of the mean. The FACS data for the moDCs was relatively consistent between animals and corresponded to known expression for bovine moDCs. The percentage of the population positive for each molecule was not statistically significantly different between 3 day and 7 day moDCs (2 sample t-test, $p>0.05$). The mean MFI was higher for each molecule tested which was due to increased autofluorescence. For the 7 day moDC this difference was significant compared to the 3 day moDCs for all molecules (2 sample t-test, $p<0.05$) except MHC I, MHC II, CD205 and CD3 (2 sample t-test, $p>0.05$).

4.3.2 Challenge of moDCs with LPS

Bovine moDCs (3 day and 7 day cultures) were challenged with 1 μg/ml of LPS to measure cytokine response and changes in cell surface molecules. ELISA for IL-10, IL-12 and TNFα were carried out on cell supernatants from 3 day moDCs from six animals and 7 day moDCs from 3 animals (Fig. 4.4). IL-10, IL-12 and TNFα were all produced in response to LPS challenge by both 3 day (Fig. 4.4 A, C & E) and 7 day (Fig. 4.4 B, D & E) moDCs. The graphs also give an indication of the large spread of responses by individual animals, for example, the 3 day moDCs results show that cow 5 did not produce any IL-10, IL-12 or TNFα in response to LPS. Statistical analysis of the ELISA data using paired t-tests show a significant difference in IL-10 production by the 3 day moDCs in response to LPS challenge, compared to non-challenged controls. ($p<0.05$) No significant difference was observed for IL-12 ($p=0.09$) or TNFα ($p=0.22$) compared to unchallenged cells. The 7 day moDCs stimulated with LPS showed a significant increase in TNFα production ($p<0.05$) but no significant differences for IL-10 ($p=0.10$) or IL-12 ($p=0.06$) production compared to unchallenged cells. Flow cytometry was carried out for MHC II, CD1b and CD40 on 3 day moDCs (Fig. 4.5) and 7 day moDCs (Fig. 4.6) from three animals. FACS results show a trend for increase in MFI for MHC II (Fig 4.5 A), CD1b (Fig 4.5 B) and CD40 (Fig 4.5 C) in response to LPS challenge with the 3 day moDCs. For the 3 day moDCs only three animals were challenged to provide this data and of these three, two animals (cow 401 and cow 501) had greatly increased expression of all three cell surface molecules in response to LPS stimulation compared to unchallenged cells however the third animal (cow 600) showed no such response. This diversity in animal responses provided data that, when pooled, showed no statistically significant differences measured by a paired

t-test. ($p>0.05$) For the 7 day moDCs, again only three animals were used to produce the data. There was a trend for increase in expression of MHC II, but the expression of CD1b and CD40 showed even greater animal variation in response to LPS where cells from one animal showed increased expression, one decreased expression and the third remained relatively unchanged (Fig. 4.5).

The results from these early LPS challenges showed that the moDCs were able to produce cytokines IL-10, IL-12 and TNFα, and that these cells were also able to up-regulate cell surface molecules in response to LPS challenge. However, some considerable animal to animal variation was observed in the magnitude of responses, and this combined with the smaller sample sizes, meant that statistically significant differences were not observed. The aim of the LPS challenges was to observe and measure cytokine responses and changes in cell surface molecule expression to confirm the activity of the generated cells and the efficacy of the detection methods. The results shown here when taken into account with the morphological and expression profile data confirm that bovine moDCs have been successfully generated. If these results were to constitute a discrete study in itself then larger sample sizes would have been employed and a greater emphasis placed on statistical significance of the data. However, these results were sufficient to progress with further moDC challenges with confidence in phenotype and function of the generated cells.

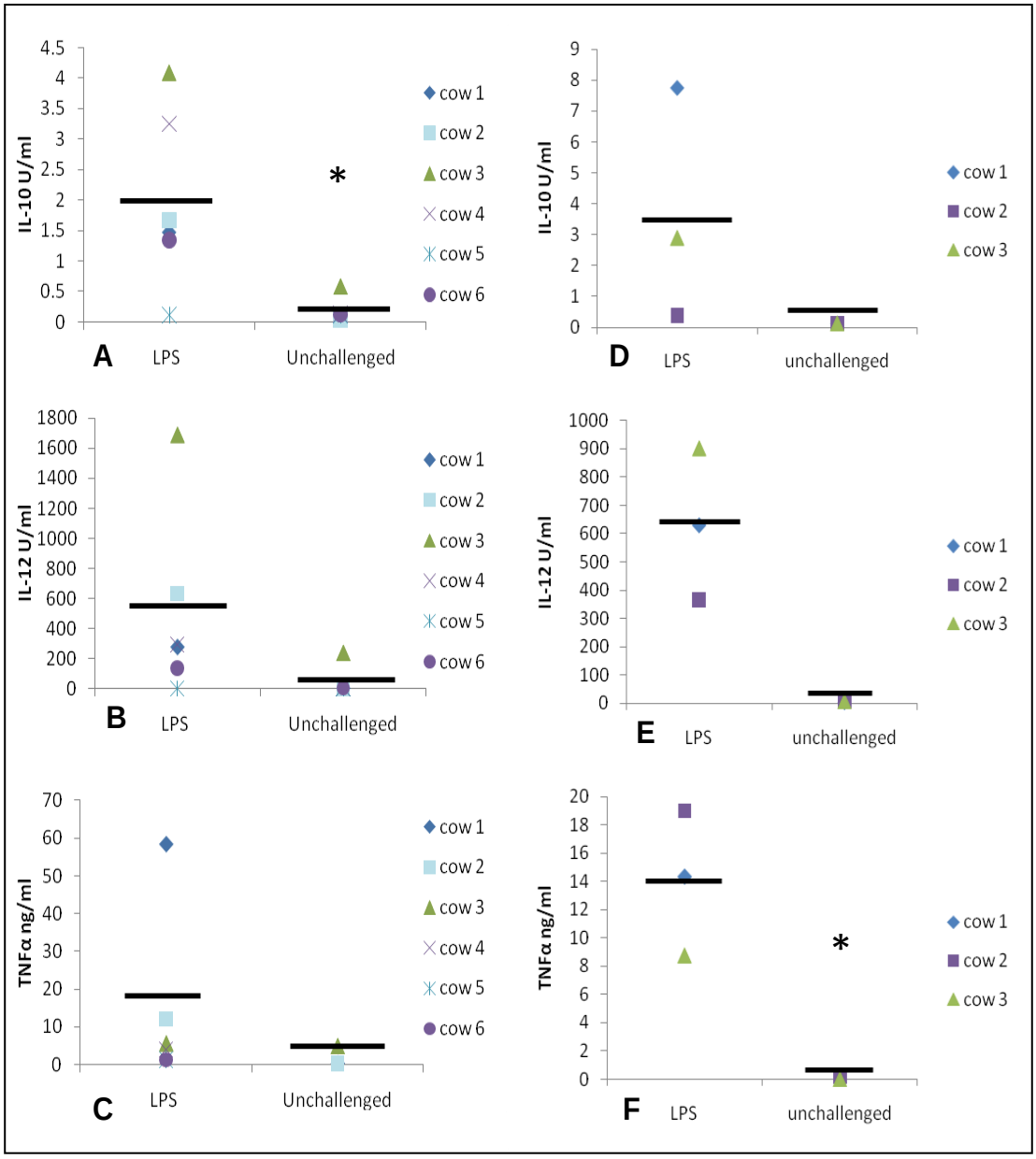
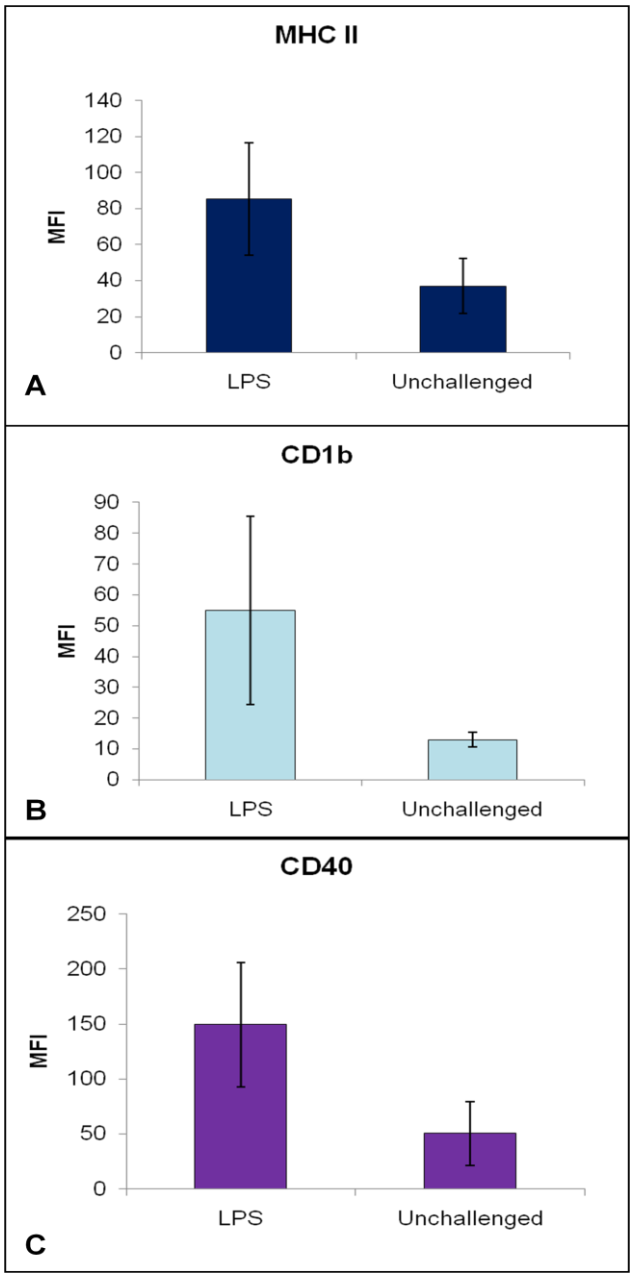


Fig. 4.4 Results of ELISA for cytokines in cellular supernatant following moDC challenge with LPS (1 µg/ml, for 18 h) (A) IL-10 3 day moDC, (B) IL-12 3 day moDC, (C) TNFα 3 day moDC, (D) IL-10 7 day moDC, (E) IL-12 7 day moDC, (F) TNFα 7 day moDC (A), (B) & (C) pooled data from six animals. (D), (E) & (F) pooled data from three animals The presence of IL-10, IL-12 and TNFα were assessed and compared to levels in supernatant from unchallenged cells. Mean = Black Marker. Stimulation of production of all three cytokines was observed, however there was found to be animal variation in the magnitude of responses as shown by the spread of the data points in panels (A) and (B) in particular. The data were analysed by paired t-tests. P values of less than 0.05 were found (*) for IL-10 production by 3 day moDCs (A), and TNFα production by 7 day moDCs (F). All other p values were greater than 0.05.

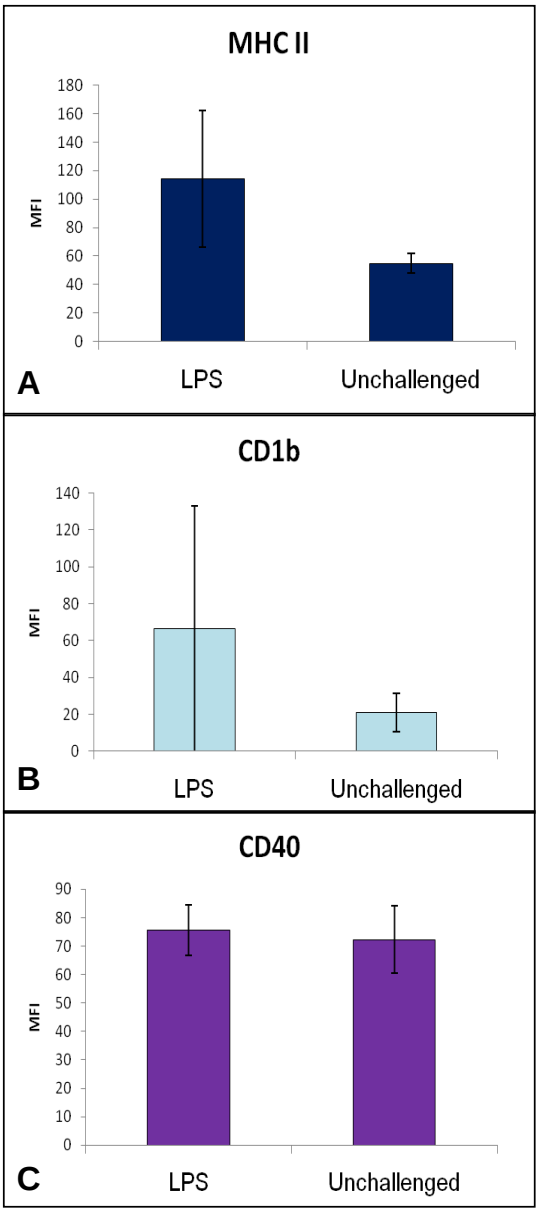


	MFI MHC II expression	
cow no.	LPS 1 µg/ml	Unchallenged
401	80.56	22.02
501	141.68	22.02
600	34	67.27

	MFI CD1b expression	
cow no.	LPS 1 µg/ml	Unchallenged
401	108.27	15.41
501	53.79	15.41
600	2.85	8.26

	MFI CD40 expression	
cow no.	LPS 1 µg/ml	Unchallenged
401	244.39	27.59
501	153.98	15.41
600	49.79	108.4

Fig. 4.5 FACS analysis of 3 day moDCs challenged with LPS (1 µg/ml). MFI for (A) MHC II, (B) CD1b and (C) CD40 are shown. All graphs show pooled data from three animals. Error bars represent standard error of the mean. moDCs were challenged with LPS for 18 h. The tables show the individual values obtained for three animals. Two of the three animals responded by increasing expression of MHC II, CD1b and CD40 (Cows 401 and 501) One animal did not respond to LPS challenge (cow 600) by changes in cell surface molecule expression. The changes in cell surface molecule expression were not significant ($p>0.05$) when compared to the levels on unchallenged control cells. However, this value will have been affected by cow 600, and the small sample size. A larger sample size would be required to clarify the heterogeneity in animal responders.



	MFI MHC II expression	
cow no.	**LPS 1 µg/ml**	**Unchallenged**
401	96.29	61.81
501	205.09	61.81
600	41.41	40.88

	MFI CD1b expression	
cow no.	**LPS 1 µg/ml**	**Unchallenged**
401	-10.96	30.79
501	205.17	31.79
600	5.03	0.39

	MFI CD40 expression	
cow no.	**LPS 1 µg/ml**	**Unchallenged**
401	58.02	84.05
501	83.21	84.05
600	85.99	48.73

Fig. 4.6 FACS analysis of 7 day moDCs challenged with LPS (1 µg/ml). MFI for (A) MHC II, (B) CD1b and (C) CD40 are shown. All graphs show pooled data from three animals. Error bars represent standard error of the mean. moDCs were challenged with LPS for 18 h. The tables show the individual values obtained for three animals. MHC II expression was increased on cells from two animals following LPS challenge (cow 401 and cow 501) cow 600 was relatively unchanged. Overall, no significant difference in MHC II expression was observed between LPS challenged and unchallenged cells. (p=0.30) CD1b expression was varied following LPS challenge. One animal increase expression, (501) one decreased expression (401) and one was relatively unchanged (600). No statistical difference was observed. (p=0.56) CD40 expression showed a similar pattern to CD1b; One animal increased expression in response to LPS (600), one decreased (401) and one was relatively unchanged (501) A larger sample size would be required to clarify the heterogeneity of animal responses. All data was analysed using paired t-tests.

4.3.3 Challenge of bovine moDCs with H7 flagellin

Purified *E. coli* O157:H7 flagellin was supplied by Dr. T. McNeilly (McNeilly, 2008) for use in moDC challenges. The samples had been made by acid dissociation, neutral-pH re-association and ammonium sulphate precipitation (Ibrahim *et al.*, 1985). therefore LPS could have been present in the samples. To investigate the response of the moDCs to H7 flagellin it was desirable to remove LPS from the samples as it has already been shown that moDCs in this study produce cytokine in response to LPS (see section 4.3.2 and Fig. 4.5) The moDCs showed no cytokine response to the H7 flagellin challenge, or any significant change in expression of MHC II and CD1b (Fig. 4.7) so the presence and activity of the H7 in the samples was assessed. The column-treated sample and an untreated control sample (from the same batch) were first subjected to SDS-PAGE analysis to visualise protein, followed by Western blot to confirm any bands on the gels were H7 flagellin. The results of the SDS-PAGE and Western blot analysis are shown in Fig 4.8. The untreated sample was visualised on the coomassie stained gel and was also detected by the αH7 antibody in the Western Blot. The sample which had pre-treatment in the endotoxin removal column was not observed on a gel or detected by probing with αH7 antibody. These results suggested that the H7 flagellin had been adversely affected by laboratory manipulations and was no longer active or present in the sample used to challenge the moDCs. To confirm any activity of the H7 flagellin, the treated sample was used to challenge Caco2 cells which are known to produce IL-8 following challenge with *E. coli* flagellin (Donnelly & Steiner, 2002). The results of an ELISA to detect production of IL-8 by Caco2 cells are shown in Fig. 4.9. There was no significant IL-8 production by the challenged cells compared to

unchallenged controls (p=0.376) and the H7 flagellin sample was concluded to be inactive.

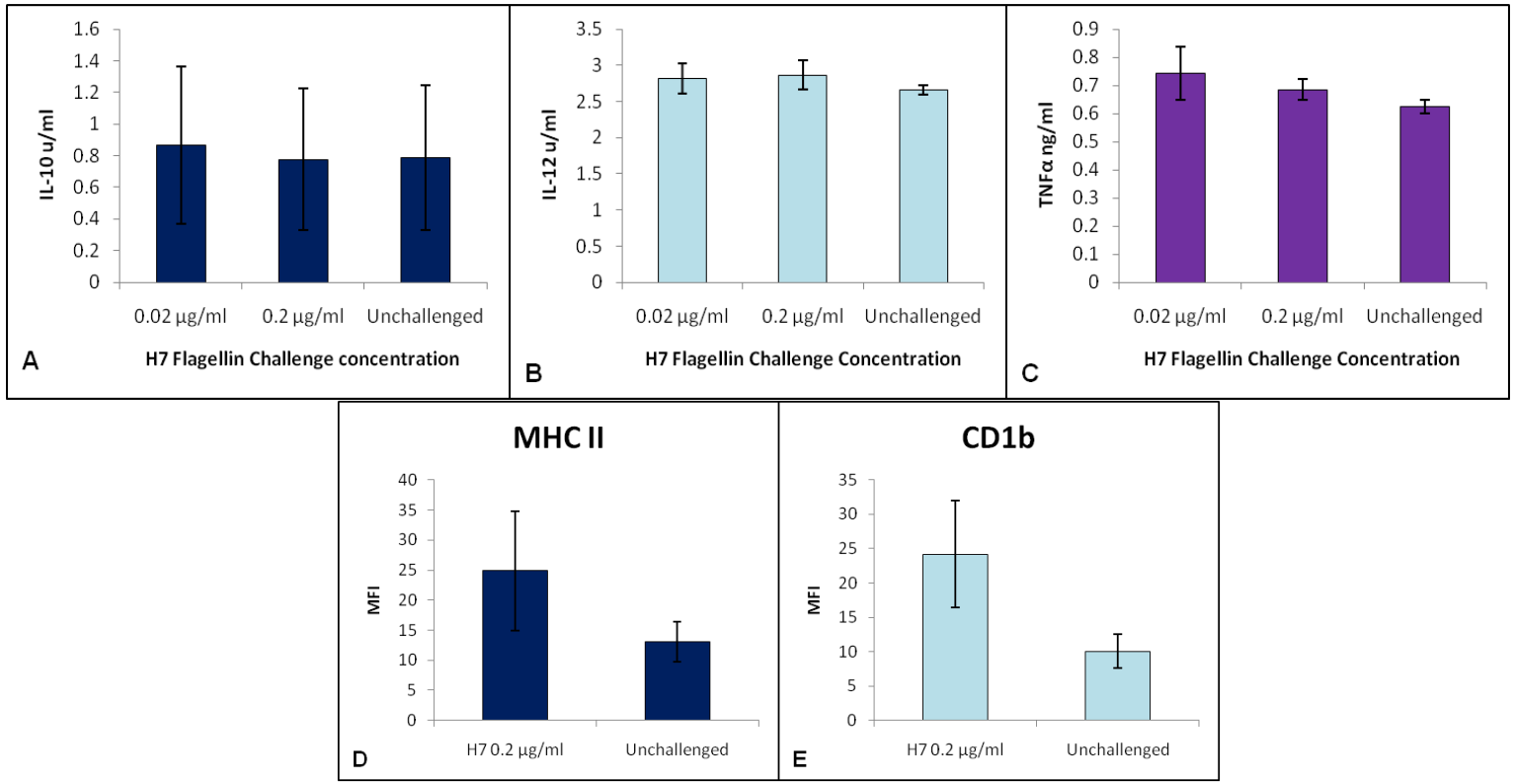


Fig. 4.7 Results from moDC challenge with a purified H7 flagellin sample

ELISA for (A) IL-10, (B) IL-12, (C) TNFα in cell supernatant following challenge with a purified H7 flagellin sample and FACS analysis of these cells showing (D) MHC II expression and (E) CD1b expression. The H7 sample had been pre-treated by endotoxin removal. The concentration of H7 used for challenge as quantified by nanodrop is indicated on the Y axes. Each graph represents pooled data from three animals; error bars represent the standard error of the mean. The cells for FACS analysis were gated through gate 1 (Fig. 4.3) Range of the number of cells in gate: 300-5000. There was no significant cytokine response to challenge with this purified H7 flagellin preparation. The levels of IL-10, IL-12 and TNFα were not significantly different from the levels found in the supernatant of unchallenged cells. (one-way ANOVA; p=0.987, p=0.698 & p=0.439 respectively) There was no significant change in the mean fluorescence intensity (MFI) of cell surface molecules MHC II or CD1b on the moDCs following H7 flagellin challenge (paired t-test; p=0.232 & p= 0.136).

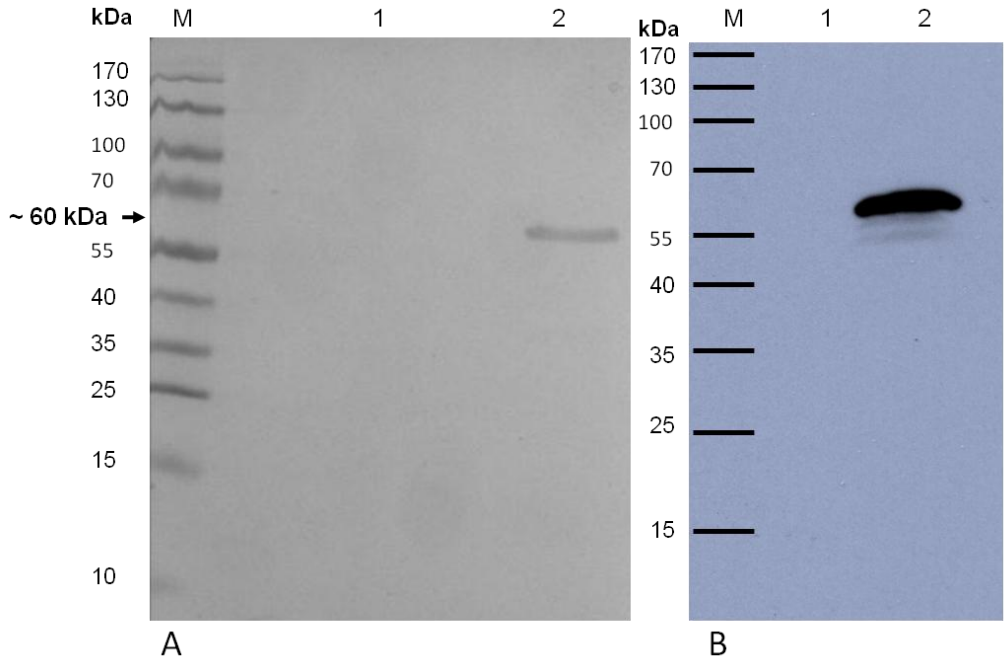


Fig. 4.8 Investigation of the effect of an endotoxin removal column on H7 samples (A) Coomassie Blue stained gel of H7 flagellin samples & (B) Western Blot of H7 flagellin samples probed with polyclonal anti-H7 antibody. M: Marker, Lane 1: H7 flagellin sample treated with an endotoxin removal column, Lane 2: untreated H7 flagellin sample.

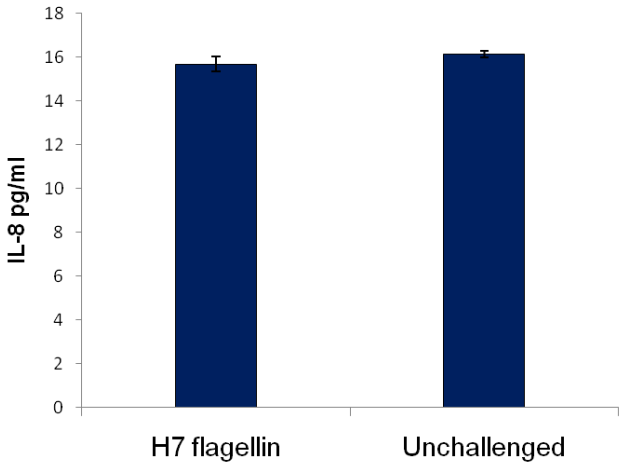


Fig. 4.9 Results of an IL-8 ELISA on Caco2 cell supernatants. Results are the means of six biological replicates. Error bars represent standard error of the mean. Caco2 cells were challenged with 500 ng/ml of H7 flagellin diluted in serum–free Cell culture medium (CCM) or serum-free CCM alone. There was no production of IL-8 in response to challenge with the H7 flagellin which had been passed through an endotoxin removal column. There was no significant difference between the IL-8 levels with challenged cells and unchallenged controls when tested with a paired t-test. (p=0.38)

4.3.4 RT-PCR for TLR5 on bovine moDCs

RT-PCR was carried out for TLR5 on bovine moDCs (Fig 4.10). Cells from three animals after 4 days in culture were tested. The human cell line Caco-2, and bovine primary epithelial cells were used as positive controls. All the moDCs were PCR-negative for TLR5, as were the human Caco-2 cells. The bovine primary epithelial cells were PCR-positive. (Fig. 4.10 lane 9) The bovine primary cells were run on a separate gel as there were not sufficient lanes for all the samples on one gel. Furthermore, the viability of the primary cells was uncertain because earlier attempts to resurrect vials of these cells from liquid nitrogen storage for culture purposes had proved unsuccessful. After 24 h in culture, many non-viable cells and large amounts of cell debris were observed. The results shown here indicate that although the primary cells may not have been viable for culture following liquid nitrogen storage, the RNA was still preserved and provided the positive control required for this experiment.

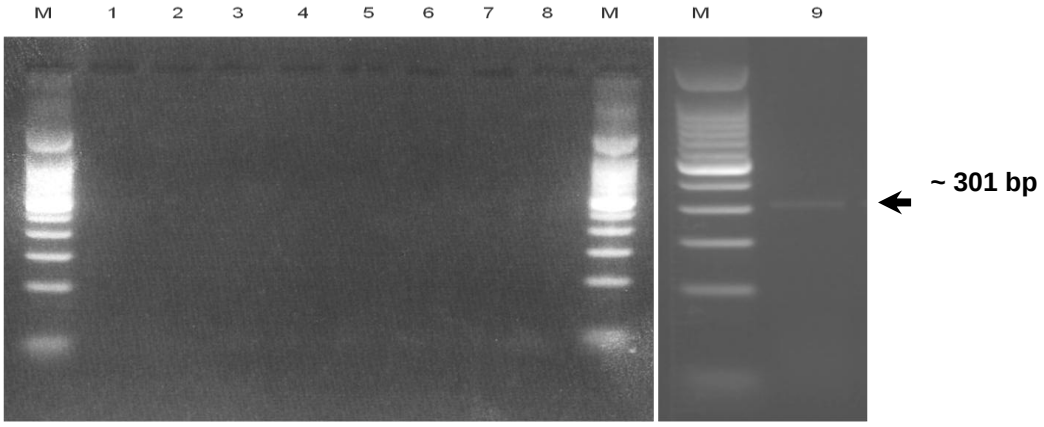


Fig. 4.10. Results from Reverse Transcription PCR for TLR5 with bovine moDCs and controls. M: 100bp molecular weight marker. Lanes 1-3: moDC cDNA from three animals, 5-7: moDC RNA controls (same three animals), lane 4: Caco2 cell cDNA, lane 8: Caco2 cell RNA control, lane 9: primary bovine epithelial cell cDNA positive control. All moDCs from the three animals tested were negative for TLR5, the human Caco2 cell line were also negative. The bovine primary epithelial cells were positive for TLR5, as shown by a band (arrow) of expected size at approximately 301 bp.

4.3.5 Challenge of moDCs with VT2

Bovine moDCs were challenged with 25, 50 and 100 ng/ml of VT2 for 18 hours and supernatants were examined for IL-10, TNFα and IL-12 by ELISA. No difference in the production of these cytokines was observed compared to the unchallenged controls for 3 day (Fig. 4.11) or 7 day (Fig. 4.13) moDCs. The results were analysed by means of a one-way ANOVA and no significant differences were observed. ($p=0.96$, $p=0.99$ & $p=0.97$ respectively) The challenged moDCs were also examined for changes in cell surface markers, MHC I, MHC II, CD1b, SIRPα, CD205, CD40, CD80 and CD86 by flow cytometry. No change in the levels of expression of these molecules was found for the 3 day moDCs from three animals, the results are shown in Fig. 4.12. The MFI was calculated by subtracting the MFI for an isotype matched control from the MFI of the molecule of interest. This flow cytometry data for MHC II was analysed by one-way ANOVA and no significant differences from the levels on unchallenged cells were observed. ($p=0.86$) The expression levels for the remaining cell surface molecules could not be analysed statistically due to cell numbers only providing sufficient cells for two animals, however, no changes in expression were observed. The 7 day moDCs were from one animal only, due to limited cell numbers. The results are shown in Fig. 4.14. No change in expression of cell surface molecules was observed and again no statistical analysis was possible. The activity of the VT2 was then examined because some effect on the moDCs was expected and it is known that human monocytes as well as murine macrophages and BMDCs respond to VT or VT subunits (Ohmura *et al.*, 2005; Tesh *et al.*, 1994; van Setten *et al.*, 1996). In addition to these studies, work carried out by a colleague, which included monocytes from the same animals used in this study, showed that at least 60% of bovine monocytes responded to VT by

production of TNFα (Dr. P. Cameron, unpublished data). Vero cells were challenged with VT2 from three batches, one which had been aliquotted and stored at -20˚C, one aliquot from a store at -70˚C and a new, previously unopened batch which had also been stored at -70˚C. The Vero cells were assessed by microscopy and by MTT assay for viability following an 18 h challenge. A range of concentrations of VT2 up to 1 μg/ml were used and no effect on viability or cell morphology was observed (Fig. 4.15). This indicated that all the aliquots of VT2 were inactive. Several published studies have shown the 50% lethal dose for Vero cells can be variable but 5 ng/ml (Cameron *et al.*, 2002), 10-100 pg/ml (Tesh *et al.*, 1994), or even as little as 0.8 pg/ml (Stamm *et al.*, 2008) have all been reported, therefore concentrations of 1 μg/ml (i.e. 200 to 100000 times the reported LD50 values) should have produced a marked effect on Vero cell viability. The three aliquots were tested to investigate whether the storage conditions of the toxin had any effect. Storage of the toxin at -70°C is recommended by the supplier, and one of the batches had been aliquotted and stored at -20°C. However because none of the batches was active it seemed unlikely that storage conditions were the cause. In the absence of a reliable source for biologically-active VT this work was then abandoned; if the VT2 had shown activity and therefore indicated that moDCs did not respond in ways detectible by these methods then experimental replicates would have been carried out to enable statistical analysis to be carried out for all the data sets. Six animals in total would have been used.

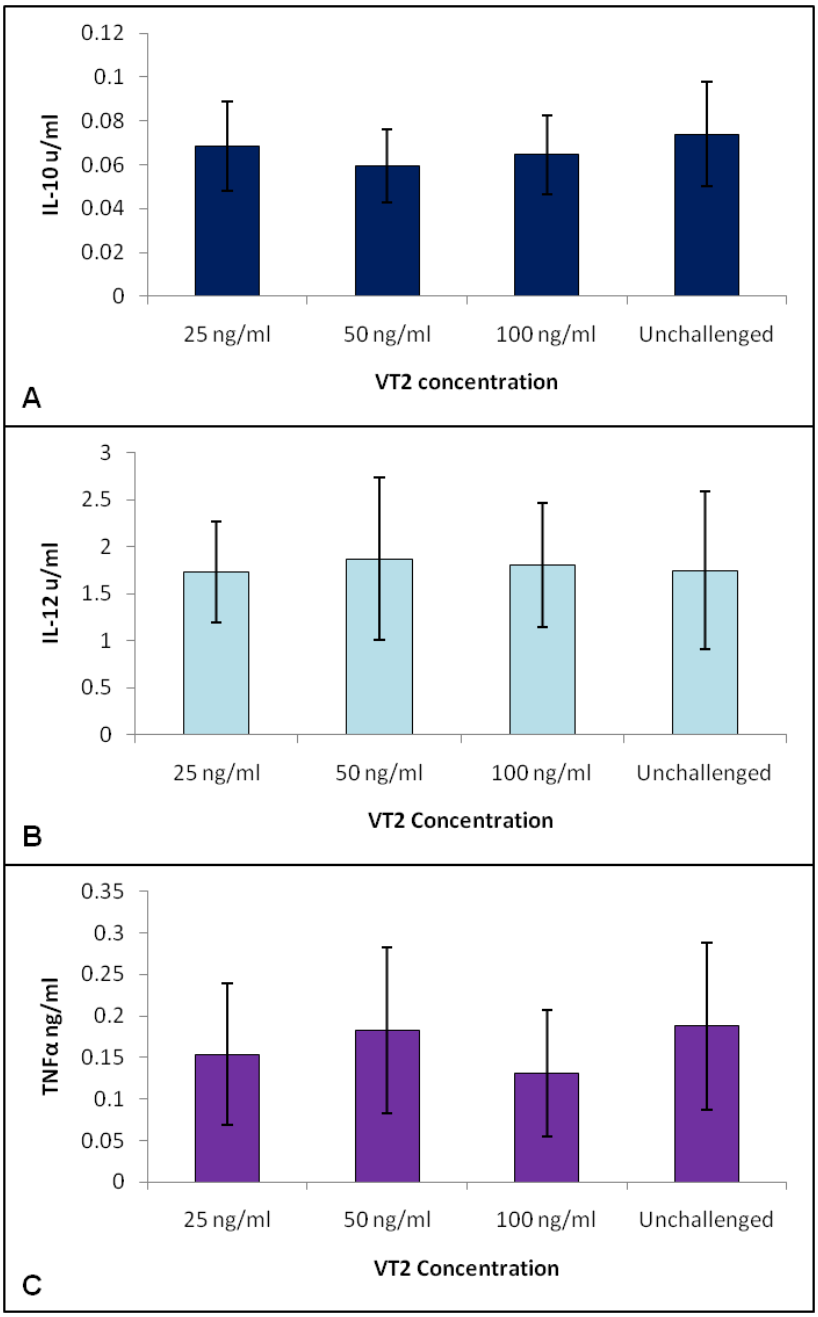


Fig. 4.11 Results of ELISA with 3 day moDC supernatant following challenge with VT2 (A) IL-10, (B) IL-12, & (C) TNFα. Cells challenged at concentrations indicated. Each graph represents pooled data from three animals; error bars represent the standard error of the mean. There was no significant cytokine response to challenge with VT2. The levels of IL-10, IL-12 and TNFα were not significantly different from the levels in the supernatant of unchallenged cells. (one-way ANOVA, p=0.06, p=0.99 & p=0.97 at 95% confidence interval)

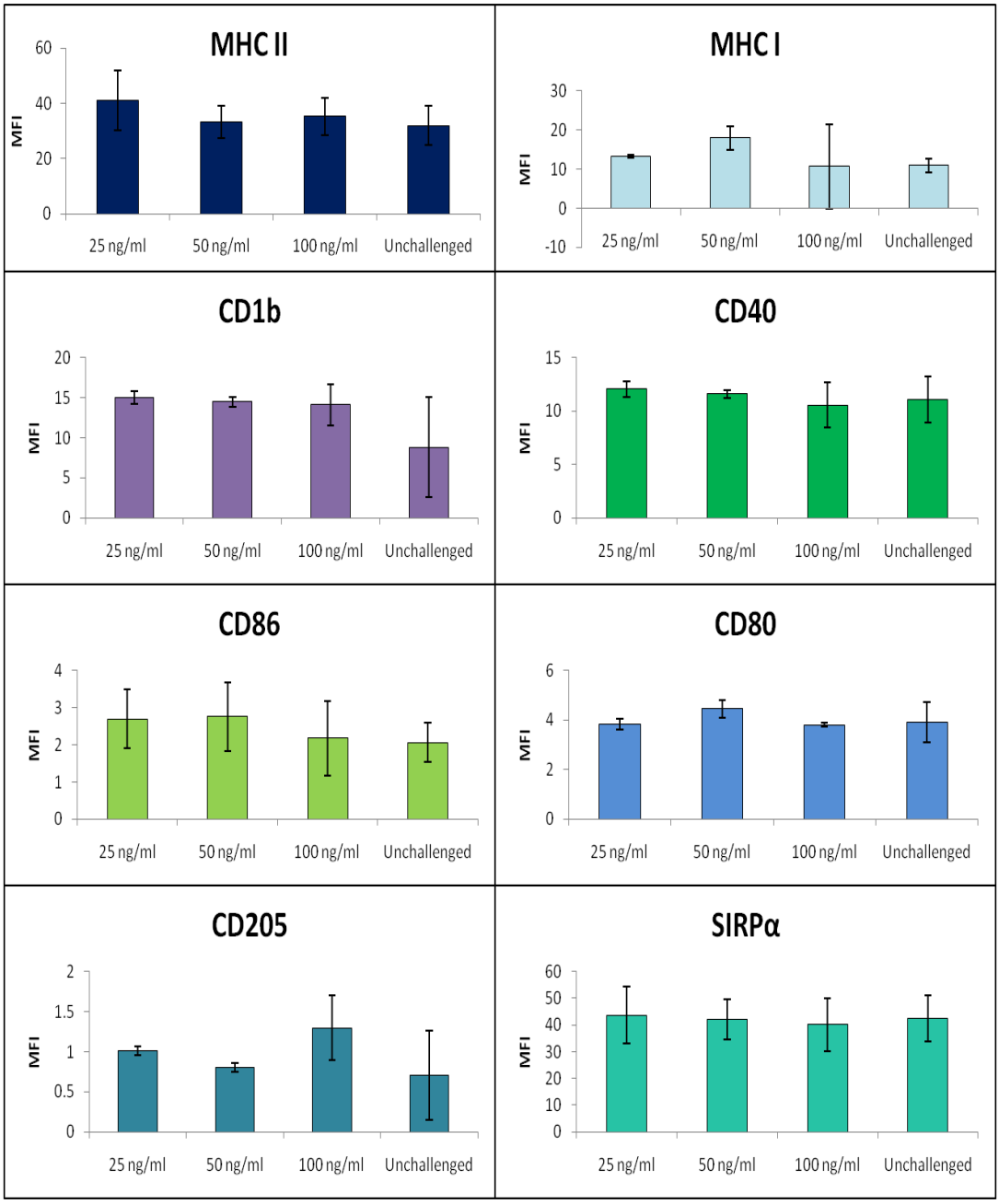


Fig. 4.12 FACS analysis of 3 day moDCs challenged with a range of doses of VT2. Concentrations of VT as indicated on the graphs: 25 ng/ml, 50 ng/ml and 100 ng/ml. MFI for various cell surface molecules are shown. The MHC II panel shows pooled data from three animals; all other molecules show duplicate data sets. Error bars represent standard error of the mean. moDCs were challenged with VT2 at the concentrations indicated for 18 h. No changes in cell surface molecule expression were observed compared to unchallenged control cells. Statistical analysis of MHC II expression showed no significant differences. (one-way ANOVA, p=0.86, 95% confidence interval) The duplicate data sets for the remaining molecules were unable to be analysed statistically.

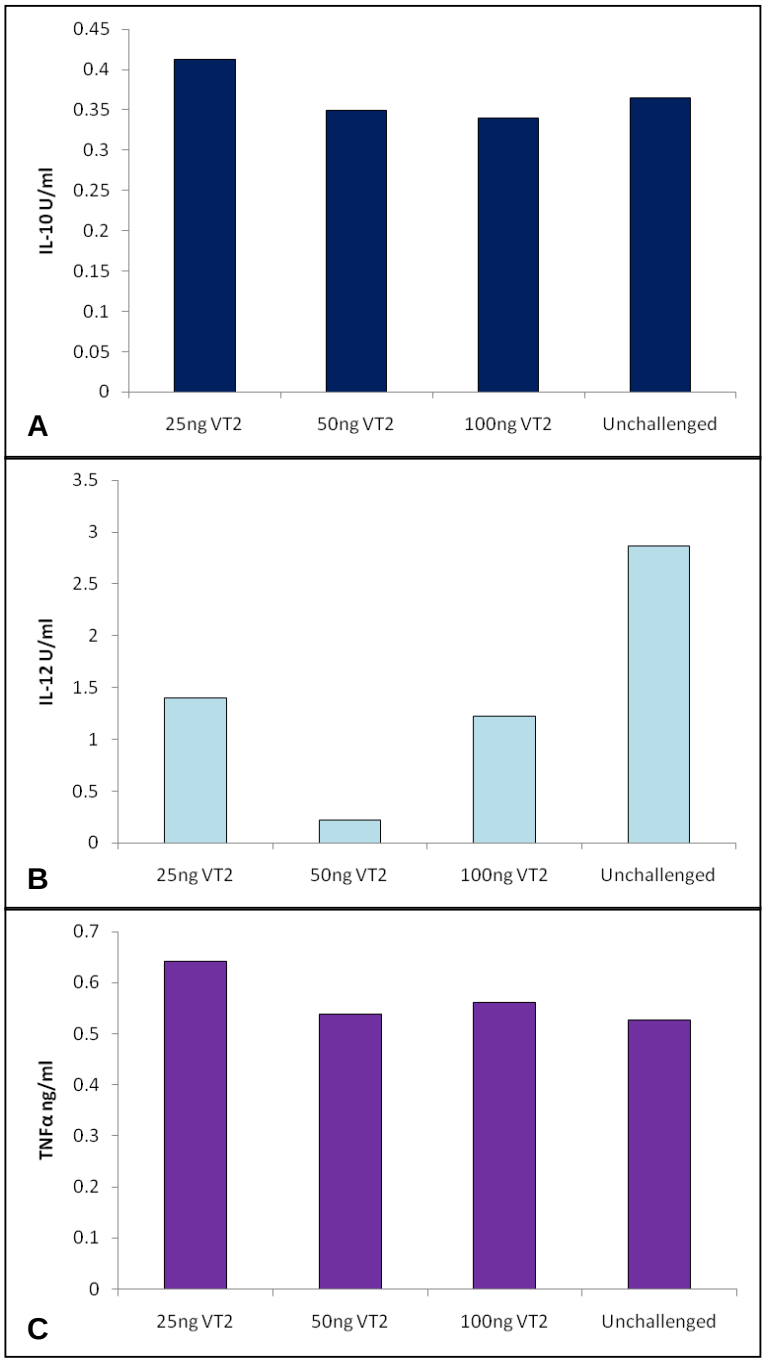


Fig. 4.13 Results of ELISA with 7 day moDC supernatant following challenge with VT2 (A) IL-10, (B) IL-12, (C) TNFα. Cells were challenged at the concentrations indicated. Each graph shows data from only one animal due to limits of the numbers of cells generated. There was no cytokine response to challenge with VT2. Although it appears that there were differences in panel (B) for IL-12, the actual levels, (represented here on the Y axes) are so small that it can be concluded that there was no cytokine production. No statistical analyses were possible due to sample size.

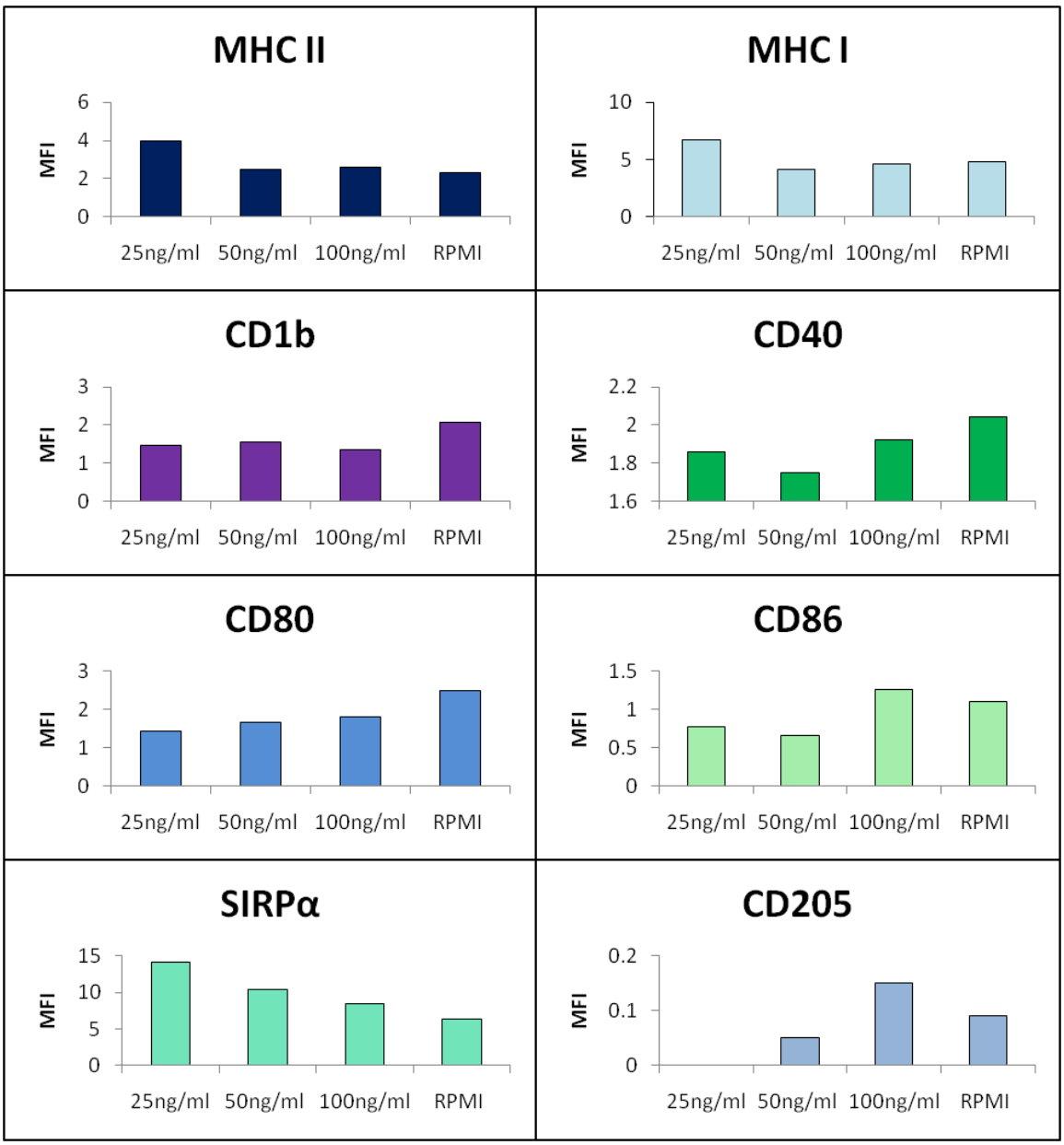


Fig. 4.14 FACS analysis of 7 day moDCs challenged with a titration of VT2. MFI for various cell surface molecules are shown. The MHC II panel shows pooled data from three animals; all other molecules show duplicate data sets. Error bars represent standard error of the mean. moDCs were challenged with VT2 at the concentrations indicated for 18 h. No significant changes in cell surface molecule expression were observed compared to unchallenged control cells.

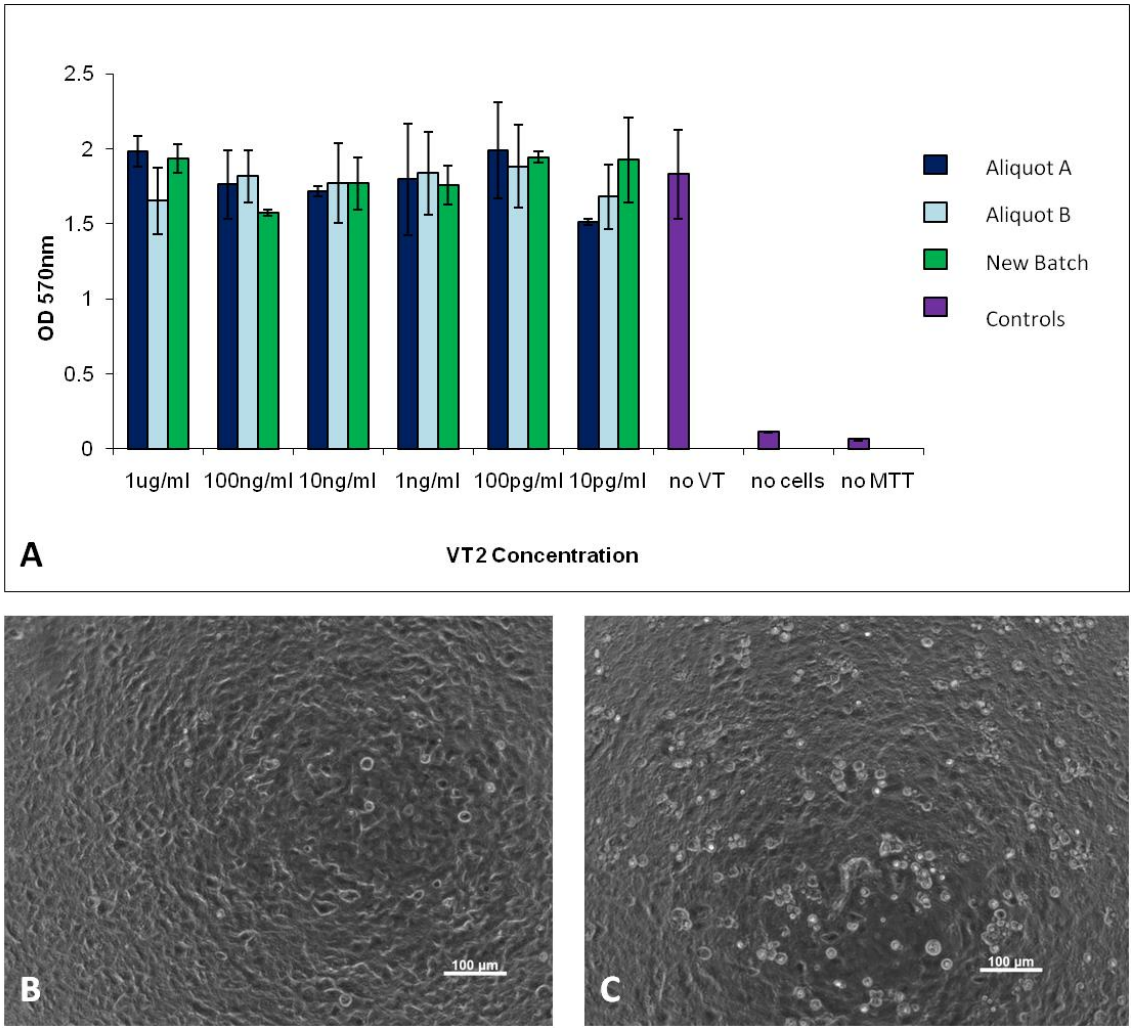


Fig. 4.15 Vero cell viability following challenge with VT. Results of an MTT assay (A) and live cell imaging of Vero cells in 96-well plates (B) & (C) to show viability of Vero cells following challenge with a titration of VT2 (Sigma) samples. The mean values from a duplicate experiment are shown. Error bars represent standard error of the mean. Aliquot A: stored at -20˚C, Aliquot B: stored at -70˚C, New Batch: unopened new batch of VT2 stored at -70˚C. Images taken 18 h post challenge (A) The MTT assay indicates no loss of viability for any of the VT2 samples at any of the concentrations tested. (B) Representative live cell image of unchallenged Vero Cells showing a monolayer of viable cells. (C) Representative live cell image of Vero cells, challenged with 1 µg/ml of VT2 shows slight rounding of a very small number of cells, but overall morphology of the monolayer is unaffected. No statistical analysis was possible as this was only a single duplicate experiment.

4.4 Discussion

Monocyte derived – DCs (moDCs) are a widely used model for investigation of the interactions of various pathogens with the host immune system. Since they were first generated bovine moDCs have been well characterised and are a key element used by various research groups. These cells have been well characterised both phenotypically and in their responses to various pathogens. However, this cell generation technique while providing a reliable method to create numerous DCs for *in vitro* investigations, nonetheless is still a complex process which may be affected by variations in techniques, reagents and equipment as well as animal factors. The aim of this chapter was to follow published methods for generation of moDCs and assess the resulting cells according to published parameters. Day three moDCs were chosen primarily as they are generally accepted as representing immature DCs of the type likely to encounter *E. coli* O157:H7 *in vivo*.

Bovine moDCs were successfully generated from several groups of donor animals as defined by cell surface molecule expression, morphology and cytokine response to LPS stimulation, and were used throughout the remainder of this work. Early work also presented here also generated seven day moDCs for comparison of cell surface molecule expression and there was no significant difference in percentages of cells expressing DC molecules from the 3 day moDCs. Mean fluorescence intensity (MFI) of the positive cell populations were also compared and the 7 day moDCs expressed most of the molecules at a higher level which was due to increased autofluorescence of the 7 day cells (Fig. 4.3). A pilot study comparing the responses of 3 day moDC and 7 day moDC to LPS was carried out and the responses by the 7 day moDCs were very disparate, showing much animal variation and for

such a small sample size it is difficult to compare this data to the 3 day moDCs. However, there was a trend for production of cytokines (IL-12, IL-10 and TNFα) and upregulation of cell surface molecules (MHC II, CD1b & CD40) in response to LPS challenge. One point of interest, and a further area of animal variation was noted when animal 600 responded to LPS challenge by production of cytokine (Fig. 4.4 cow 3) but with no changes in expression of cell surface molecules (Fig. 4.5), whereas cow 501 responded by increased expression of cell surface molecules (Fig. 4.5) but not production of cytokine (Fig. 4.4 cow 5). Larger sample sizes would be required to clarify this result and further probe the prevalence of animal variation. This was not the purpose of this chapter but rather it was to characterise and validate DC phenotype and investigate interactions with bacterial components.

To assess interactions of moDCs with various pathogenicity factors of *E. coli* O157:H7, challenges of cells with first H7 flagellin and then Verotoxin (VT2) were planned. These were both hampered by technical constraints and the nature of the cellular response requires further work for full characterisation.

First, H7 flagellin challenges were carried out. H7 flagellin is known to stimulate IL-12 production from murine DCs *in vitro* (Uematsu & Akira, 2009; Vicente-Suarez *et al.*, 2009), and upregulation of cell surface costimulatory molecules *in vivo* (Salazar-Gonzalez *et al.*, 2007). It has also been published that bovine moDCs express TLR5 (Werling *et al.*, 2006), which is the pattern recognition receptor for flagellin, thus it was expected that bovine moDCs would respond to H7 flagellin challenge by changes in cell surface molecules and production of cytokine. However, no response was observed (Section 4.3.3) prompting further work to determine whether a technical issue was the reason for this or whether this was a confirmed

finding. It seemed possible that the endotoxin removal treatments affected the H7 flagellin sample in some way, although LPS removal from Flagellin samples has been published by Donnelly *et al* (2002) and no detrimental effects were reported (Donnelly & Steiner, 2002), and no loss of activity of the flagellin was noted following use of an endotoxin-removal column by McDermott *et al* (McDermott *et al.*, 2000). Despite this, the postulate appeared to be supported by SDS-PAGE analysis and Western blotting of untreated and treated H7 flagellin samples, whereas H7 protein was not detectible in the treated sample by either of these methods, a strong band was observed for the untreated sample (Fig. 4.7). The lyophilisation of the H7 flagellin is another area where alteration of structure or function could have occurred, although this seems unlikely as these methods were used by Dr. McNeilly following the initial preparation of the sample and activity was evident. However, there could be an additional factor involved which is worth mentioning. It is also possible that the quantification of the treated sample was inaccurate. The sample was quantified using a Nanodrop Spectrophotometer (ND-1000, UV-Vis) before and after endotoxin-removal treatment. Colleagues have since observed potential discrepancies in the accuracy of the Nanodrop measurements when compared to a method of quantification used by an external laboratory. This could indicate that challenges and SDS-PAGE analysis was carried out on a much smaller quantity of H7 than was detectible by these methods. Although the Nanodrop too was the method used to first quantify the H7 flagellin when it was purified and was the method that had been used to quantify the untreated sample used as a control. The treated H7 sample was also unable to induce IL-8 secretion in Caco2 cells, thus it appeared to be not only structurally but also functionally altered and inactive. As these experiments

were unable to definitively elucidate the nature of the response of bovine moDCs to H7 flagellin, PCR was carried out on day 3 moDCs from three animals for TLR5. This was intended to facilitate the appraisal of potential cellular responses to H7 flagellin, had the sample remained active. As mentioned previously, earlier work has indicated that moDCs do express TLR5 (Werling *et al.*, 2006), however the study by Werling *et al* was carried out with moDCs cultured for seven days. It was interesting to note that the day 4 moDCs used in this study were RT-PCR negative for TLR5 using published methods (Menzies & Ingham, 2006) although Werling *et al*. (2006) found that bovine monocytes expressed TLR5 using RT-PCR. Also of interest was that the human cell line Caco2, a widely-reported flagella-responsive cell, which were used as a positive control, and known to express TLR5 (Sierro *et al.*, 2001) which has 83% nucleotide similarity to bovine TLR5 (Werling *et al.*, 2006) however the primer sequences used here did not amplify RNA from these cells. It would be of interest to carry out further investigation of TLR5 expression of bovine moDCs, to investigate whether the negative results generated here are due to animal heterogeneity or a feature relating to the length of time of culture or some factor relating to laboratory technique. Bovine TLR heterogeneity is an area of active research at present and has not been fully characterised. The results of these studies may be able to shed some light on variations in bovine responses to micro-organisms *in vitro* and *in vivo* (Jann *et al.*, 2009; Werling *et al.*, 2009) (http://www.roslin.ac.uk/research/people.php/Liz.Glass/).

The next moDC challenge to be carried out was with a titration of VT2. It is known that VT affects immune cells in other species including human monocytes (van Setten *et al.*, 1996) murine macrophages (Tesh *et al.*, 1994),

and murine DCs (Ohmura *et al.*, 2005) by stimulating production of cytokines, in particular TNFα, as well as an upregulation of maturational cell surface molecules on the DCs. However, no studies had investigated the responses of bovine DCs to VT, and because cattle are a major reservoir of *E. coli* O157:H7, which localises to DC-rich intestinal sites, and VT is one of this pathogen's main virulence factors it is of great interest to assess the *in vitro* responses of these cells to VT. As with the H7 flagellin challenges, no responses were observed, and although the VT2 was ordered from a reputable supplier (Sigma), further testing to establish the efficacy of this toxin was carried out, to establish whether this was a tangible result showing no cellular response or whether it stemmed from some form of methodological difficulty. Two aliquots of an opened, previously used batch of VT2, one stored at -20˚C and one stored at -70˚C, as well as an unopened new batch (-70˚C stored) were tested for activity on Vero cells by visual examination for cell death and by MTT assay. A titration of VT2 was employed up to a maximum concentration of 1 μg/ml. A study on cellular signalling by Vero cells following challenge has been published and although it was carried out to investigate cellular signalling factors in the first few hours following VT2 challenge, a cytotoxicity study was performed and it was shown that 50% cytolethal dose of toxin was 5 ng/ml (Cameron *et al.*, 2002)(Toxin sourced from Toxin Technologies) Other studies too have published 50% lethal doses of VT or Vero cells as 0.8 pg/ml (Stamm *et al.*, 2008) and 10-100 pg/ml (Tesh *et al.*, 1994). Verotoxin purchased from Sigma failed this basic test of VT2 bioactivity as it showed no toxicity against cultured Vero cells. No effects on Vero cell viability were observed with any of the batches of VT2 and it was concluded that they were inactive. At the time the VT2 was purchased the Sigma website listed several publications

which had used purified VT2 (Nakao & Takeda, 2000; Sugatani *et al.*, 2000; Suzuki *et al.*, 2000). Each of these studies examines the bioactivity of VT2, and demonstrated activity with both *in vitro* and *in vivo* experiments. Sigma customer services were contacted regarding this matter and the response received suggested that that activity was not guaranteed by their testing procedures prior to sale, only toxin purity, which did not provide a satisfactory explanation. Due to time constraints these experiments were not repeated, although VT effects were addressed in some way when VT^+ strains were used for challenges with moDC and compared with VT^- strains (Chapter 5). The loss of activity was not ever accounted for, however at the time of writing Sigma have discontinued sales of VT2. Further work would source a toxin with proven efficacy against Vero cells for challenging moDCs to characterise responses *in vitro*.

In Summary, bovine moDCs were successfully and reproducibly generated for use in investigations with *E. coli* O157:H7, this was shown by dendritic morphology and cell surface molecule expression profiles as well as cytokine responses to LPS. Challenges of moDCs with *E. coli* O157:H7 with both H7 and VT2 were complicated by issues with efficacy of these components. These challenges would ideally be repeated with components of confirmed efficacy. These experiments while not providing the expected insights into bovine immune responses to *E. coli* O157:H7 pathogenicity factors were still valuable as technical problem solving exercises and highlight that purchased products may be subject to varying quality. The bovine moDCs used in this study were found not to express TLR5, and further work to examine whether this is an animal factor or a condition of the culture duration would be of interest.

The following two chapters investigate the bovine moDCs as characterised here and their interaction with various *E. coli* strains. The VT effects on moDCs are in some way addressed by challenges with Verotoxin positive and negative strains; however, a challenge with active purified toxin and moDCs would be an important area for future work.

Chapter 5 Challenge of bovine moDCs with various *E. coli* strains

5.1 Introduction

Bovine moDCs are a useful model for investigating *in vitro* the interactions of pathogens with cells central to the development of immune response. They have been well characterised both in this study (Chapter 4) and elsewhere (Hope *et al.*, 2000a; Werling *et al.*, 1999). The aim of work in this chapter was to investigate the possible contribution of DCs in the asymptomatic carriage of *E. coli* O157:H7 in cattle by challenging bovine moDCs with various *E. coli* strains; subsequently measuring cytokine response, cell surface molecule expression, phenotype and intracellular bacterial numbers. Interactions between bacteria and DCs are initiated through the interactions of a number of ligands and their receptors, principally PAMPs (e.g. flagella and LPS) and PRRs, (e.g. TLRs) (See Chapter 4). Many of these bacterial signals will be available to moDC receptors following *in vitro* challenge with live *E. coli* strains, and the subsequent cellular responses have not been characterised to date.

E. coli are a diverse species of bacteria of which many strains are beneficial to the host and indeed *E. coli* strains occur as part of the normal gut flora in numerous species of animals and man. However, other strains are pathogenic, and this pathogenicity can often be host specific. One interesting feature of *E. coli* O157:H7 in particular is its significant pathological effects on man, including potentially fatal outcomes; while these bacteria can colonise ruminant hosts with no clinical signs and little or no pathological consequences. A/E lesions, which are a feature of human disease, have been found in animal tissues but without the ensuing gastrointestinal symptoms. These differences are likely to be due to host factors and an understanding of which features are involved in these disparate responses would not only

advance understanding of bovine immunopathology but would potentially allow novel intervention methods to be developed by which outbreaks of human disease can be reduced. This study focused on *in vitro* EHEC challenges and bovine DC responses, as key immune cells found at the site of O157 colonisation, which could be key to the different responses observed *in vivo* between human and bovine hosts.

Several *E. coli* strains were selected to challenge moDCs. The rationale behind the selection of strains was to use a group of bovine commensal *E. coli* strains and a group of bovine pathogenic *E. coli* strains (entrotoxigenic *E. coli*, ETEC) to compare to *E. coli* O157:H7 strains both in terms of bacterial characterisation and in the responses generated by moDCs following challenge. This would enable the characterisation of bovine moDC responses to *E. coli* O157:H7 and assess whether the responses generated were more comparable to those generated by harmless commensal bacteria or those of pathogenic *E. coli*, and facilitate the further investigation of any differences observed. This comparison of EHEC, ETEC and commensal strains has not been undertaken with bovine DCs to date.

The motility phenotype of the panel of bacterial strains used for moDC challenges was assessed. Motility is conferred on *E. coli* strains by the presence of flagella. These filament-like appendages rotate to facilitate bacterial movement (Berg & Anderson, 1973). Motility is an important aspect of bacterial pathogenicity as it enables bacteria to travel into areas of plentiful nutrients or away from locations of harmful substances. Flagella have also been shown to be important in the formation of biofilm by *E. coli*, which is a further important facet of bacterial virulence (Pratt & Kolter, 1998). Flagella

are comprised of polymerised flagellin monomers, and these sub-units are ligands for host cell TLR5 (Hayashi *et al.*, 2001). It is important to be aware of the motility phenotype of bacterial strains because this factor may contribute to the responses of moDCs to *E. coli*. The presence of flagella within EHEC strains has been shown to be an important factor for adherence to bovine intestinal tissues *in vitro* (Erdem *et al.*, 2007; Mahajan *et al.*, 2009). Therefore the presence or absence of this important PAMP may also affect interactions with bovine moDCs.

A recent study has compared the responses of bovine primary colonic cells to various bacterial strains including EHEC, EPEC, ETEC and commensal *E. coli in vitro* (Bridger *et al.*, 2010). The study by Bridger *et al*. does suggest some strain differences but also acknowledges the limitations of concentrating solely on epithelial cells and recognises the importance of immune cells in the ultimate response to encountered bacteria *in vivo*. That study suggested that co-cultures of epithelial cells and immune cells could help further characterise reactions of the intestinal micro-environment to these various *E. coli* strains. Gene transcripts for inflammatory factors such as IL-8 were increased in response to EHEC when compared to EPEC, and interestingly this response was also observed in response to a commensal strain (Aktan *et al.*, 2004; Bridger *et al.*, 2010). However, the 'commensal' strain used in the Bridger *et al.* study was isolated from a calf displaying diarrhoeic symptoms, and although was found to be negative for the tested virulence factors, it is possible that it may not be a true commensal strain. Indeed several of the commensal strains in this study were collected from clinically normal cattle and as well as a lack of virulence factors, these strains were usually collected from more than one animal and had been localised to several intestinal sites.

Bovine moDC cytokine responses to bacterial challenges *in vitro* were investigated in this study. Cytokines are produced by DCs in response to various bacteria and bacterial agonists to signal to other cell types and initiate the generation of immune responses. IL-10, IL-12 and TNFα production were measured as important cytokines in Th1 responses (IL-12, TNFα) and tolerance (IL-10) as well as inflammation and pathology (TNFα). These cytokines, or probably a combination of these cytokines, as well as other factors are important *in vivo* for initiating immune responses against pathogenic bacteria and tolerance to commensal bacteria. For further information on the functions of these cytokines in cattle see section 4.1.

In response to bacterial challenge the expression of cell surface molecules MHC II, CD1b, CD205 and SIRPα on moDCs was measured. As key molecules expressed on, and with important roles for the function of DCs any differences in expression following challenge with various *E. coli* strains could indicate variation in cellular functions such as antigen presentation for example. See section 2.1 for further information on the functions of these molecules.

Many of the more severe clinical sequelae of EHEC infection in humans have been shown to be due to the effects of VT. VT does not have the same pathological effects on bovine hosts *in vivo* although has been shown to stimulate production of TNFα in bovine monocytes *in vitro* from a proportion of animals sampled. (Dr. P Cameron, personal communication) Problems with the activity of purified toxin discussed in Chapter 4 prevented investigation of its effects on moDCs *in vitro* so the work in this chapter compared moDC responses to VT^+ and VT^- O157 strains. Murine BMDCs

have been shown to respond to VT (Ohmura *et al.*, 2005)(discussed further in Chapter 4 section 4.1) and these responses are blocked following incubation with an antibody treatment for the VT receptor Gb_3 indicating expression by this cell type. Bovine moDCs have not been investigated for the presence of Gb_3. The aim of this work was to discover any role for VT in bovine moDC responses.

As a pathogen which is primarily extracellular within the host, the fate of *E. coli* which become phagocytosed have not been fully characterised. DCs *in vivo* are efficient in antigen uptake by numerous methods including phagocytosis. This study investigated the uptake of *E. coli* strains by moDCs to investigate whether intracellular bacterial survival was possible and whether any differences of bacterial uptake between the strains was evident. As an extracellular pathogen it was assumed that *E. coli* could be phagocytosed by moDCs and that its antigens would be processed and presented for generation of appropriate immune responses. The fact that *E. coli* is not an obligate intracellular pathogen suggests that any intracellular survival mechanisms which it does express are likely to differ from those mechanisms which are known for pathogens which are dependent on this method of virulence.

Although the phagocytosis of *E. coli* by DCs has not so far been investigated, phagocytosis of *E. coli* by human macrophages has been studied and one strain (*E. coli* strain EDL933) was shown both to be internalised by human macrophages and to resist killing by these cells (Poirier *et al.*, 2008). The numbers of intracellular viable bacteria were measured up to 24 h and intracellular bacteria were still viable at this time. It was also found that *E.*

coli O157:H7 could replicate within the macrophages, as although fewer infected cells were detected at 24 h, each infected cell had higher numbers of internal bacteria than those detected at 2 h. VT was also found to play an important role in this interaction, the presence of the toxin reduced bacterial uptake by the macrophages as well as promoted bacterial survival (Poirier *et al.*, 2008). In contrast *E. coli* O157:H7 was rapidly killed when taken up by murine macrophages *in vitro*. Around 15 minutes after infection the bacteria were killed within the cells, and these remained alive for up to 24 h (Shimada *et al.*, 1999). A search of the literature reveals no comparable studies with EHEC strains and bovine macrophages.

Intracellular bacterial studies have been carried out with DC for numerous intracellular pathogens in various species. Bovine moDCs in particular have been studied in greater depth with intracellular pathogens such as *M. bovis* (Denis, 2008) and *Salmonella typhimurium* (Norimatsu *et al.*, 2003; Norimatsu *et al.*, 2004). Non-pathogenic *E. coli* strains have been used in some human studies as control strains for *Salmonella* infections (Schoppet *et al.*, 2000) however, no discrete studies investigating the uptake of pathogenic *E. coli* strains by DC have been carried out. Phagocytosis of *E. coli* by murine BMDCs has been demonstrated but the strain was not identified and was unlikely to be O157:H7 (Holt *et al.*, 2007). DCs are known to be phagocytic, and have been shown to internalise several bacterial species *in vitro*. However no studies have been carried out to date which investigate phagocytosis of EHEC or ETEC strains by DCs in their bovine host.

The aims of this chapter were:

1. To characterise the growth and motility phenotypes of the bacterial strains selected for investigation.
2. To investigate the effects of *E. coli* strains on the cytokine production and cell surface expression profiles of bovine moDCs.
3. To compare the effects of $VT2^{+}$ *E. coli* O157:H7 strains and a VT^{-} strain on the cytokine production and cell surface expression profile of bovine moDCs.
4. To compare characteristics of phagocytosis of *E. coli* strains by moDCs, to include frequency of occurrence and investigation of duration of intracellular survival.

5.2 Materials and Methods

5.2.1 Background

The bacterial challenges were developed from published moDC bacterial challenge techniques with *Salmonella* strains (Norimatsu *et al.*, 2003; Norimatsu *et al.*, 2004). (Norimatsu *et al* 2003 & 2004)

5.2.2 Selection of Bacterial Strains

The *E. coli* O157:H7 category 2 containment level (CAT 2) strains selected were chosen for study because the corresponding category 3 containment level (CAT 3) strains were also available. For strain information see Appendix A.3. The ETEC strains were kindly gifted by Roberto LaRagione (VLA, Weybridge) and were isolated from cases of bovine diarrhoea (Turner *et al.*, 2006). Commensal strains were collected by Dr. E Clark and were selected from the in-house collection. These commensal strains were chosen based on prevalence within the population of sampled animals, the numbers of bacteria at selected intestinal sites and absence of known virulence factors. The commensal strains were selected if they were isolated from more than one animal and had been present in significant numbers at more than one intestinal site. A lack of virulence factors was also a requirement for classification as a commensal in this study. The absence of virulence factors was confirmed by various laboratory techniques including PCR (Dr. E. Clark, Ph.D. Thesis, http://theses.gla.ac.uk/706/).

5.2.3 Bacterial Characterisation

5.2.3.1 Motility Assay

Phenotyping of *E. coli* strains, by means of stab cultures were carried out to assess motility. The bacterial stocks were kept in glycerol or in cryobank vials (Mast Diagnostics) at -80°C and were grown up on nutrient agar plates (Oxoid). Two to five colonies were used for overnight bacterial culture in 5 ml MEM/HEPES (Sigma) and placed in a shaking incubator at 37°C and 200 rpm. Universal containers were filled with 20 ml of 0.3% agar containing 0.01% of TTC (Fluka) pre-heated to 50°C, and placed on a level surface at 4°C to set. Following the overnight incubation a sterile inoculating needle was coated with bacterial culture and used to inoculate the agar in a single vertical stab, through the agar to the bottom of the universal container. The stab cultures were incubated overnight at 37°C, 5% CO_2. TTC reduction reflects bacterial growth and is indicated by a red colour change. Following 16-18 h incubation the cultures were visually examined for areas of bacterial growth. Motility was confirmed if red colour had spread throughout the agar and the strain was non-motile if the red colouring was restricted to the stab line.

5.2.3.2 Bacterial growth assay

The Bioscreen C system (Oy Growth Curves Ab Ltd.) allows investigation of bacterial growth dynamics using optical density measurements. Culture conditions, wavelengths and frequency of data recording may all be user-defined. Cultures of bacterial strains were set up in triplicate in MEM/ HEPES, in a shaking incubator at 200 rpm and 37°C. Following overnight incubation, triplicate 1:10 dilutions were made in MEM/HEPES. These cultures were incubated for a further two hours in the same conditions.

Further 1:10 dilutions were made in MEM/HEPES or DC-TCM and 250 μl of these cultures were added in triplicate to wells of a Bioscreen micro-plate. Media alone was included as a reference sample. Further serial 1:10 dilutions were made in sterile PBS to allow determination of the numbers of bacteria present at the start of the growth curve measurements. 100 μl of dilutions $10^{\wedge 5}$ and $10^{\wedge 6}$ were plated out on nutrient agar plates (Oxoid) in triplicate and incubated over night at 37°C. The bioscreen provided static incubation at 37°C and took $O.D._{600}$ measurements every 15 min for defined durations, either 2 h, or 24 h. The plate was shaken for 15 sec before each O.D. reading. When culture and sampling was complete serial dilutions of the cultures were made in sterile PBS and dilutions $10^{\wedge 7}$ and $10^{\wedge 8}$ were plated out in triplicate to determine bacterial numbers. Bacterial colonies were counted and used to calculate cfu/ml at the start and end points of the growth curves. The O.D. readings from the Bioscreen C were used to generate growth curves for each bacterial strain over different time periods.

5.2.4 Calculation of bacterial MOI based on $O.D._{600}$ measurements

Bacterial strains were grown overnight in 10 ml MEM/HEPES (Sigma) in a shaking incubator set to 200 rpm and 37°C. A 1:10 dilution in 9 ml of fresh MEM/HEPES was made and cultured under the same conditions for approximately 3 h. At this point, serial 1:10 dilutions of bacterial cultures, for three strains, were plated out to calculate challenge cfu. The cultures for all three bacterial strains contained approximately 3×10^8/ml cfu (data not shown) at optical density of between 0.25 and 0.35 $O.D._{600}$ (typically 0.31), this data was used to calculate the MOI for further challenges.

5.2.5 Preparation of bacterial cultures for moDC challenge

After overnight growth at 200 rpm and 37°C in 10 ml MEM/HEPES, bacteria were diluted 1:10 into 9 ml of fresh MEM/HEPES. When the $O.D._{600}$ of these cultures was between 0.25 and 0.35, serial 1:10 dilutions was made into RPMI 1640 (Invitrogen) and 100 µl of the appropriate dilution was used to challenge the cells. Usually 100 µl of dilution 10^3 was added per well of a 12 well plate for each strain. This corresponded to an MOI of approximately 0.5. Optical density was measured on an Amersham Pharmacia Novaspec II spectrophotometer.

5.2.6 Preparation of moDC

Bovine moDC were generated as described (section 4.2.1.4). Cells were harvested after 3 days in culture (section 4.2.1.5). The harvested cells were washed with three washes of gentamicin-free DC-TCM by pelleting at 1000 x g for 4 min, then counted (section 4.2.1.6) and diluted to 1 x 10^6 cells per ml in gentamicin-free DC-TCM. 1 ml of this cell suspension was added to one well of a twelve well plate. These cells were incubated overnight at 37°C and then used for bacterial challenges on day 4.

5.2.7 Bovine moDC challenges

100 µl of bacterial culture at the appropriate O.D. and dilution for the experiment was added per well of the 12 well plate. Where cell numbers allowed each different strain or condition was carried out in triplicate. 100 µl of pre-warmed RPMI was added for control wells. Bacteria were also added to wells containing gentamicin-free TCM only as a control for bacterial growth in the medium. The challenged cells were incubated for 2 h at 37°C and 5% CO_2 then gentamicin was added (50 µg/ml) to each well to kill

extracellular bacteria. Gentamicin was used as it has been shown to have little uptake by eukaryotic cells and therefore unlikely to affect the viability of intracellular bacteria. (Vaudaux, 1979) The cell culture plates were replaced in the incubator and incubated at the same conditions for a further 22 h. (total culture time 24 h, pulse-chase experiment) For CAT 2 organisms, following this incubation the plates of challenged cells were placed in the centrifuge for 2 min at 480 x g. The supernatants were carefully removed and stored individually at -20°C until used for Enzyme Linked Immunosorbent Assays (ELISA). For CAT 3 organisms the plates were removed from the incubator and placed on the level work surface in the hood for three minutes to let non-adherent cells settle to the bottom. The supernatants were very gently removed and then individually filter-sterilized with a 1 ml syringe and a 0.2 μm filter before removal to CAT 2 and stored at -20°C. To harvest the challenged cells, 500 μl of pre-warmed Cell Dissociation Medium (Sigma) was added per well (section 4.2.1.5). The cells were then washed in TCM, counted (section 4.2.1.6) and resuspended for FACS analysis. CAT 3 organisms underwent an additional 15 min incubation in FACS buffer (Appendix A.4.2.7) containing ampicillin 100 mg/ml; this was required before the cells were removed to the CAT 2 laboratory for further procedures.

5.2.8 Enzyme Linked Immunosorbent Assay (ELISA) analysis of bovine cytokines

ELISA for IL-10, IL-12 and TNFα were carried out as described previously (section 4.2.5).

5.2.9 Flow Cytometry for Cellular Expression of Cell Surface Molecules

Flow cytometry was carried out as described previously (section 3.2.7).

5.2.10 Determination of viable intracellular bacterial numbers following moDC challenges

One *E. coli* O157:H7 strain (MCI 0010), one bovine ETEC (MCI 0132) and one bovine commensal (MCI 0430) strain were selected for this experiment as representative for each group of bacteria (O157, ETEC & commensal). The experiment was carried out using cells from three animals on two separate occasions. Following the 2 h challenge, the media was removed and replaced with pre-warmed media containing 50 μg/ml gentamicin to kill extracellular bacteria. After further incubation two wells of cells were harvested (section 4.2.1.5) for each strain and pooled. Time points for cell collection were 4, 8, 12, 24 and 36 h after commencement of challenge. Cells were then collected into universal tubes and washed three times by pelletting at 800 x g for 4 min and resuspending in sterile PBS. After the first wash the cells were transferred into centrifuge tubes, and all supernatants were discarded. To lyse the cells 1 ml of 1 % v/v Triton-X 100 (Sigma) in PBS was added to the cell pellet and mixed by pipetting. Serial 1:10 dilutions for each strain were made in sterile PBS and plated out in triplicate on nutrient agar plates. The dilutions used for plating out were neat, $10^{\wedge 1}$ and $10^{\wedge 2}$. The agar plates were incubated overnight at 37°C.

5.2.11 Live Cell images

Live cell images were obtained as described previously (section 4.2.10).

5.2.12 Data Analysis

Data analysis for FACS and ELISA were carried out as described previously (4.1.19). Graphs were plotted in Microsoft Excel and statistical analysis was carried out using Minitab (v13).

5.3 Results

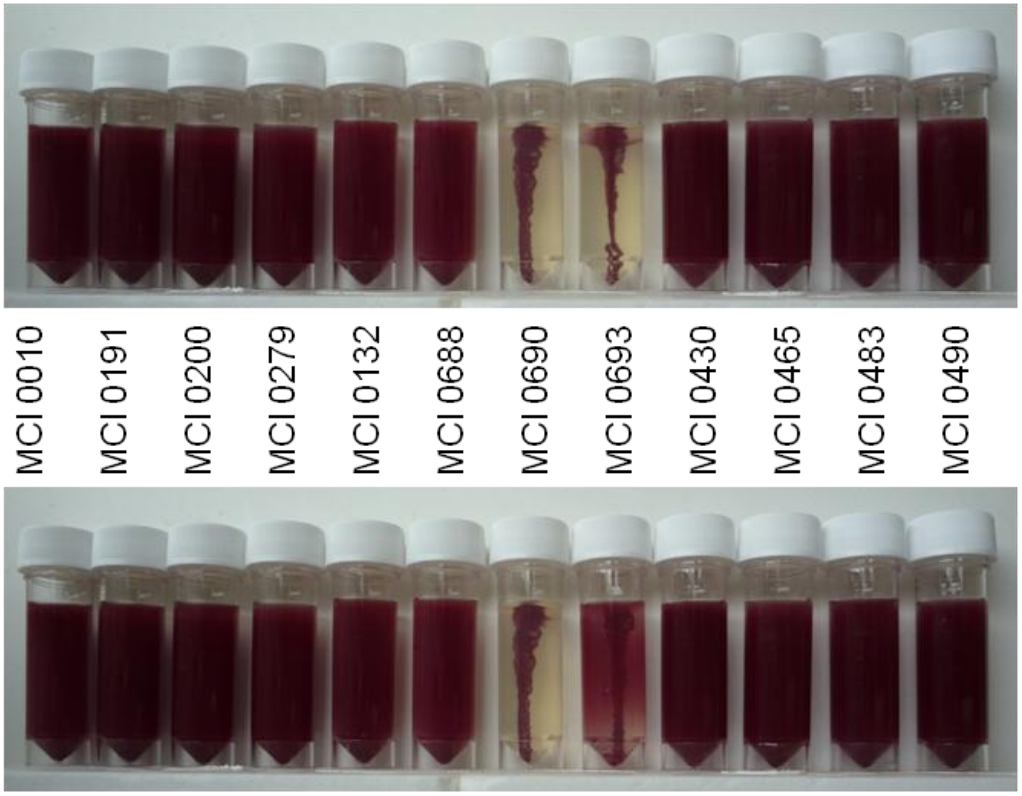
5.3.1 Bacterial Motility Assay

Stab cultures in 0.3% agar were used to assess the motility of the panel of bacterial strains selected for interactions with moDCs. The addition of TTC to the agar provided a red colour change indicating the location of viable bacteria (Fig. 5.1 A). The resulting motility phenotypes of the strains are shown in a table (Fig. 5.1 B) All of the *E. coli* O157:H7 strains were motile as were all the bovine commensal *E. coli* strains. Two of the bovine ETEC strains were also motile (MCI 0132 & MCI 0688). One bovine ETEC strain (MCI 0690) was non-motile while the final bovine ETEC strain (MCI 0693) gave inconclusive results, as it demonstrated non-motility in one experiment and partial or reduced motility in a repeat experiment.

5.3.2 Bacterial growth characteristics in two culture media

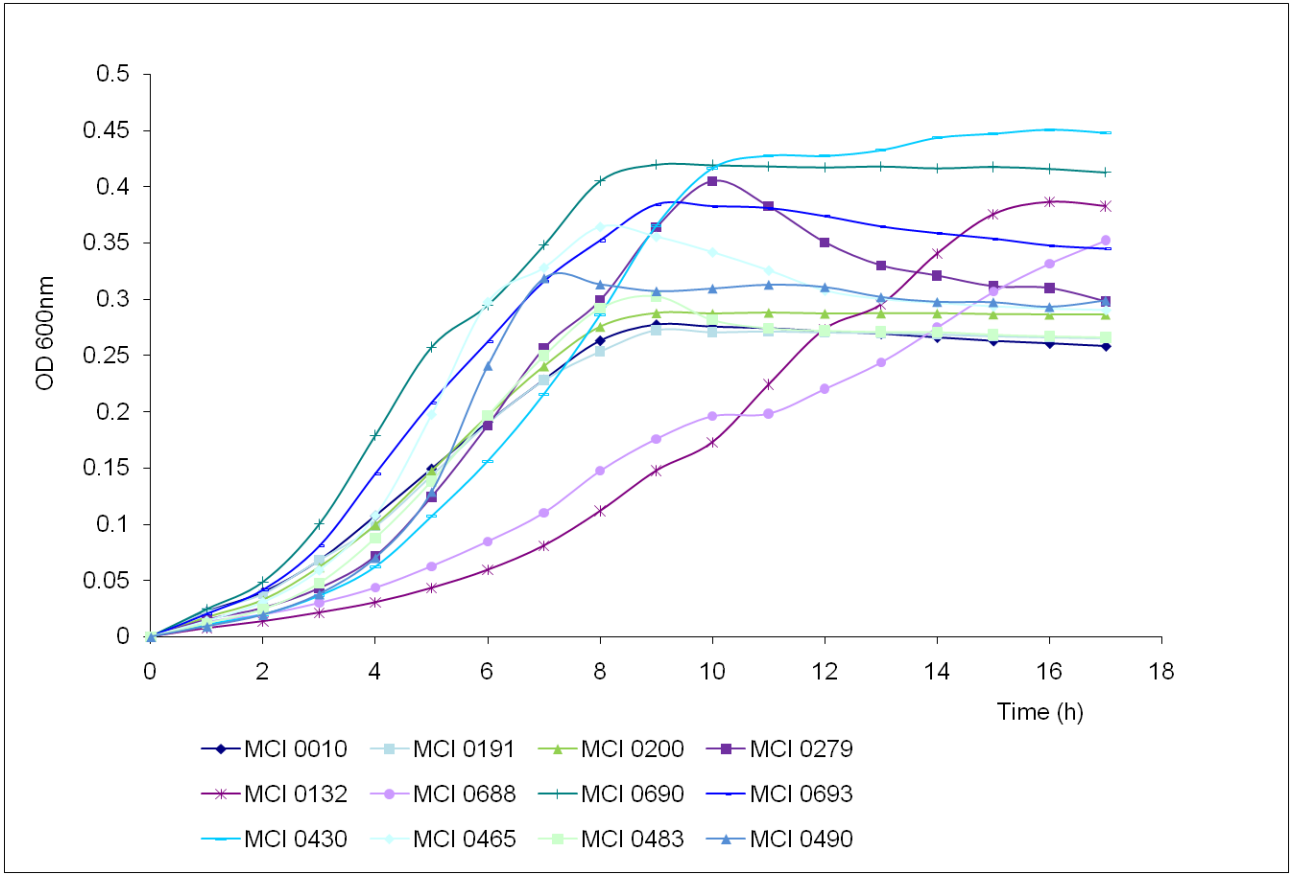
The growth curves of all of the bacterial strains were obtained over an 18 h period in both MEM/HEPES (Sigma) (Fig. 5.2) and dendritic cell tissue culture medium (DC-TCM) (Fig. 5.3). The aim of these experiments was to compare bacterial growth rates for the different strains to determine whether the growth of the strains was similar enough to be used in experiments with moDCs. Also growth rates were assessed in the DC-TCM to observe the presence and nature of any changes in growth rates which would need to be considered in experimental planning and in the interpretation of downstream results. The growth rates of all the strains were faster and much more consistent in DC-TCM than the MEM/HEPES and this suggests that during the challenge period all the bacteria will be exhibiting similar growth characteristics. The bacteria all reached a stationary phase of growth by 6 h in

DC-TCM, but this took approximately 10 h in MEM/HEPES for most strains, one strain reached stationary phase at around 15 h (MCI 0132, an ETEC) and a further strain (MCI 0688, an ETEC) did not reach a stationary phase by the end of the experiment (18 h). The bacteria also reached higher final $O.D._{600}$ readings in the DC-TCM (0.65 – 0.9) compared to the MEM/HEPES (0.25 – 0.45). This corresponded with higher final cfu/ml calculated for the bacterial strains grown in DC-TCM (between 5×10^{10} and 1×10^{11}) and MEM/HEPES (approximately 1×10^{7} – 1×10^{8}). The challenges were planned to be carried out at an approximate MOI of 0.5; this was to be obtained by $O.D._{600}$ measurements in MEM/HEPES. At an $O.D._{600}$ of 0.31 the bacterial strains were to be diluted before adding to the cells to provide this approximate MOI. Dynamics of the bacterial growth in the cellular supernatant should not be a variable factor affecting the results of the challenge experiments because a similar number of bacteria will have been added to each well as determined by $O.D._{600}$ readings and this data shows that the bacteria will all grow at similar rates in the DC-TCM. Growth to a particular $O.D._{600}$ is used as an approximation of cfu, and although not exact, O.D. readings are commonly used in bacterial challenges for this purpose, but as can be seen from the cfu/ml data here, there is some variability in numbers of bacteria at each final $O.D._{600}$ reading.



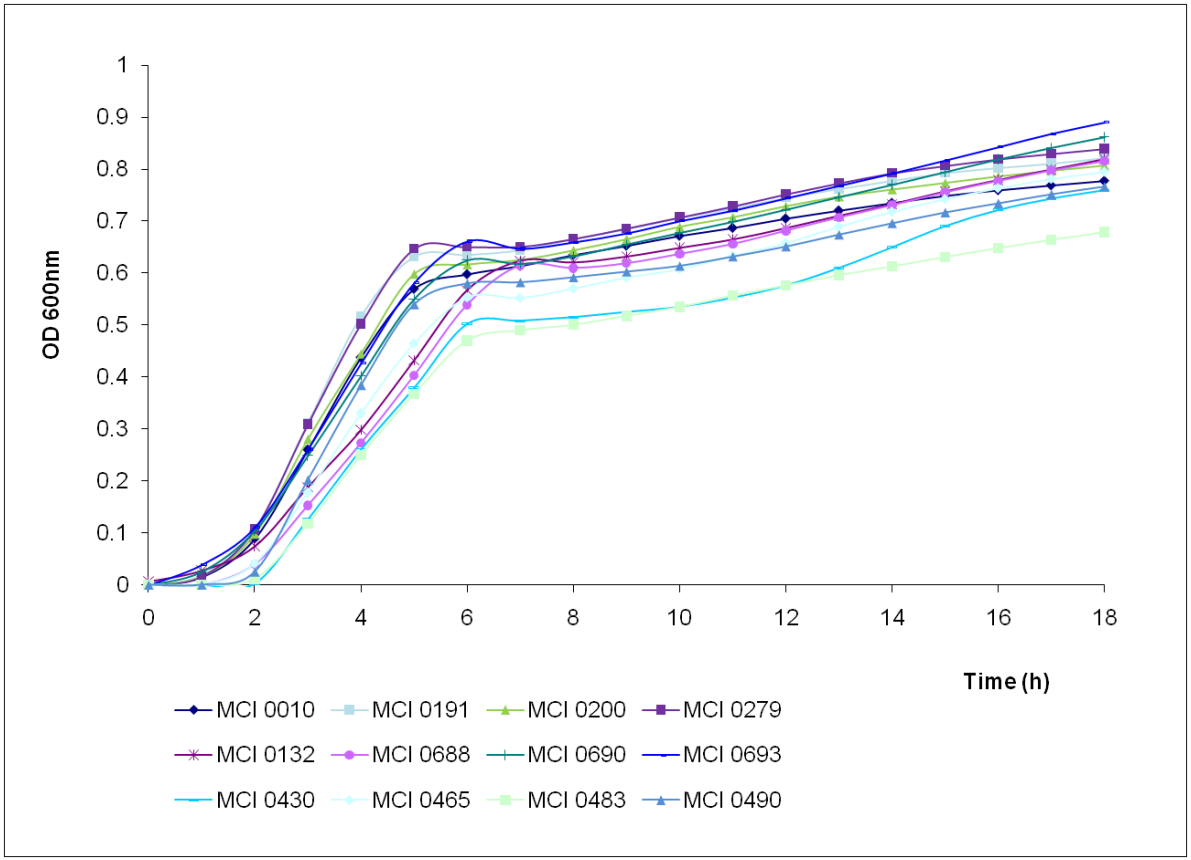
Serotype	Strain	Motility Phenotype
E. coli O157:H7	MCI 0010	+
	MCI 0191	+
	MCI 0200	+
	MCI 0279	+
Bovine ETEC	MCI 0132	+
	MCI 0688	+
	MCI 0690	-
	MCI 0693	-/+
Bovine Commensal *E. coli*	MCI 0430	+
	MCI 0465	+
	MCI 0483	+
	MCI 0490	+

Fig. 5.1 Motility assay for selected panel of bacteria. (A) Image of stab cultures for all the strains showing results from a duplicate experiment. Red colour represents bacterial growth. (B) Table of results from the motility assay. All the strains displayed a motile phenotype except MCI 0690 which was non-motile and MCI 0693 which gave inconclusive results.



Strain	cfu/ml at 0 h	cfu/ml at 18 h
MCI 0010	1.96×10^7	1.00×10^8
MCI 0191	2.65×10^7	3.07×10^8
MCI 0200	3.27×10^7	3.87×10^8
MCI 0279	4.49×10^7	2.49×10^8
MCI 0132	1.83×10^7	3.40×10^8
MCI 0688	1.73×10^7	8.11×10^7
MCI 0690	4.52×10^7	5.63×10^8
MCI 0693	3.27×10^7	1.87×10^8
MCI 0430	2.62×10^7	5.22×10^7
MCI 0465	3.59×10^7	2.74×10^8
MCI 0483	2.60×10^7	2.23×10^8
MCI 0490	2.35×10^7	3.01×10^8

Fig. 5.2 Growth of bacterial strains in MEM/HEPES, Data is pooled from a triplicate experiment**.** After 10 hours all the strains had reached a stationary phase of growth except two of the ETEC strains: MCI 0688 and MCI 0132. MCI 0688 did not reach stationary phase before the end of the experiment but was at a similar $O.D._{600}$ to the rest of the strains at this time. MCI 0132 reached stationary phase by around 15 h. The final $O.D._{600}$ of the bacterial cultures at 18 h ranged from 0.25 to 0.45, and there were some variations in growth pattern during the experiment. The cfu/ml at 0 h and 18 h were calculated by serial dilution on Agar plates and these are shown in the table.



Strain	cfu/ml at 0 h	cfu/ml at 18 h
MCI 0010	5.73×10^{7}	1.31×10^{11}
MCI 0191	6.77×10^{7}	1.22×10^{11}
MCI 0200	7.62×10^{7}	1.09×10^{11}
MCI 0279	8.27×10^{7}	1.28×10^{11}
MCI 0132	4.56×10^{7}	6.39×10^{10}
MCI 0688	4.30×10^{7}	6.73×10^{10}
MCI 0690	1.27×10^{8}	9.80×10^{10}
MCI 0693	9.93×10^{7}	9.34×10^{10}
MCI 0430	8.51×10^{7}	7.02×10^{10}
MCI 0465	1.25×10^{8}	9.98×10^{10}
MCI 0483	7.74×10^{7}	7.44×10^{10}
MCI 0490	7.49×10^{7}	1.19×10^{11}

Fig. 5.3 Growth of bacterial strains in DC-TCM. Data is pooled from a triplicate experiment. After 7 hours all the strains had reached a stationary phase of growth; however the $O.D._{600}$ of the strains continued to climb slowly after this time. The final $O.D._{600}$ of the bacterial culture at 18 h ranged from 0.65 to 0.90. The growth of MCI 0483 (a commensal strain) was consistently lower than the other strains. The growth of all the strains was quicker and more consistent in DC-TCM than MEM/HEPES, and reached higher final $O.D._{600}$ readings. The cfu/ml at 0 h and 18 h were calculated by serial dilution on Agar plates and these are shown in the table.

5.3.3 Phenotype of moDCs challenged with viable bacteria

Bovine moDCs challenged with viable *E. coli* for 2 h and cultured for a further 22 h took on an activated morphology with many more elongated dendrites visible. No differences in morphology were observed when different strains were used for challenge. Cells from some animals showed less pronounced morphological changes, however the challenged cells always displayed increased frequency of longer dendrites than unchallenged cells from the same animal. Representative images are shown in Fig. 5.4.

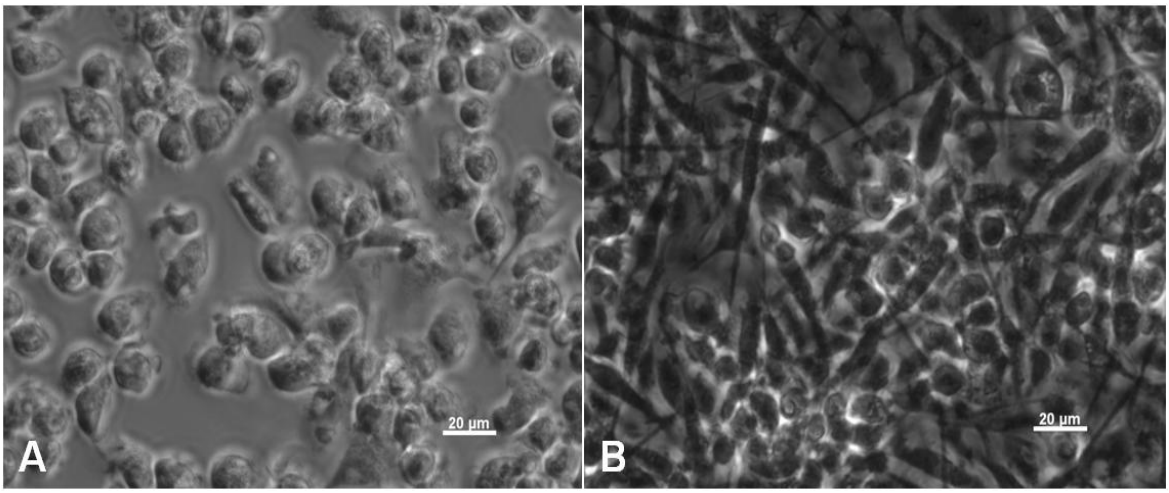


Fig. 5.4 Representative live cell images of bovine moDCs challenged with live bacteria. Representative images from one animal are shown. Changes in morphology were observed from unchallenged cell phenotype (A) and cells challenged for 2 h with viable *E. coli,* at an MOI of 0.5 (B) (strain MCI 0430). The cells took on an activated morphology with many long dendrites observable. The changes in morphology were observed for all the *E. coli* strains.

5.3.4 Cytokine responses of moDCs challenged with *E. coli* strains

Following the 24 h total challenge time, supernatants were collected and subjected to ELISA for IL-10, IL-12 and TNFα. All three cytokines were produced in response to challenge with all of the bacterial strains compared to unchallenged cells ($p<0.001$). When the data for all the strains and all the animals is pooled, no statistically significant difference is observed (tested at the 95 or 99% confidence intervals) (Fig. 5.5). However, the magnitude of responses of cells from individual animals is subject to great variability, and therefore individual animal and strain data is also presented here (Fig. 5.6 – 5.8). When considering the data from individual animals there are some trends that appear. IL-10 is produced by moDCs from all the animals studied following challenge with all strains. There is a great animal variation in magnitude of cytokine response to bacterial challenge, which makes it difficult to pool data. However, Fig. 5.6 indicates that there may be a trend for increased IL-10 production in response to commensal strains when compared to ETEC or EHEC strains. This pattern can be observed in many of the graphs in Fig. 5.6 (A, C, D, F & K) although commensal strain MCI 0483 did not stimulate this increased IL-10 production in any of the animals (G, H & I), and a selection of data sets do not indicate any strain differences. (B, E & H) IL-12 was produced in response to all the *E coli* strains with cells from all the animals when compared to unchallenged cells (Fig. 5.7). Again, great variations in the magnitude of responses were observed between animals. There appeared to be no differences related to strain; in the majority of the panels the levels of IL-12 for each strain is comparable. (Fig. 5.7 A, D, E, F, G, H & J). TNFα is also produced in response to challenge with all the strains, compared to levels produced by unchallenged cells. (Fig. 5.8) A trend is

observed is several of the panels where TNFα production is lower for the ETEC strain compared to O157 and commensal strains (Fig. 5.8 A, D, E, F, J & L).

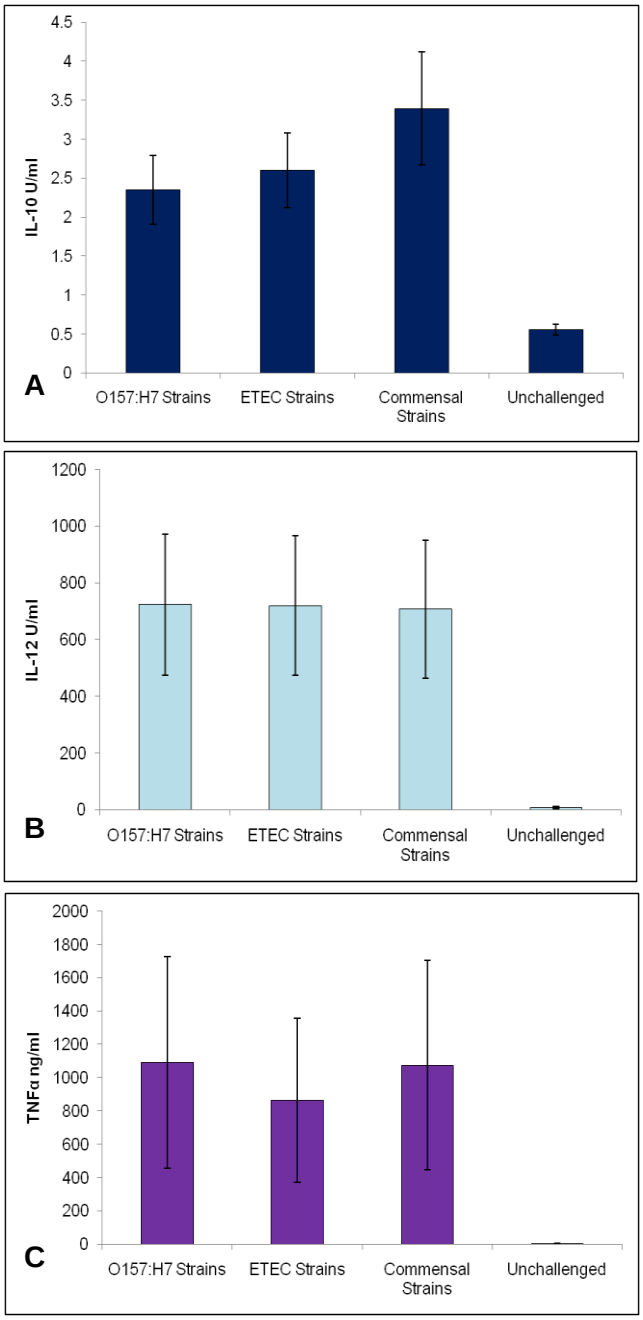


Fig. 5.5 Results of ELISA of moDC supernatant following challenge with *E. coli* strains. IL-10 (A), IL-12 (B) and TNFα (C) ELISA of moDC supernatant following challenge with four O157:H7 strains, four ETEC strains and four commensal strains. Results for each strain group are pooled. Data is pooled results of individual challenges carried out each time with moDCs from three animals. Each challenge was also carried out in duplicate or triplicate if cell numbers allowed. Error bars represent the standard error of the mean. Statistically significant levels of IL-10, IL-12 and TNFα were produced in response to challenge with bacteria from all three groups compared to the levels for unchallenged cells. Analysis using a one-way analysis of variance (ANOVA) gave a main p value <0.001, significant at the 99% confidence interval, There were no significant differences noted between cytokine levels produced in response to the strain pathotypes however, also tested at the 99% confidence interval.

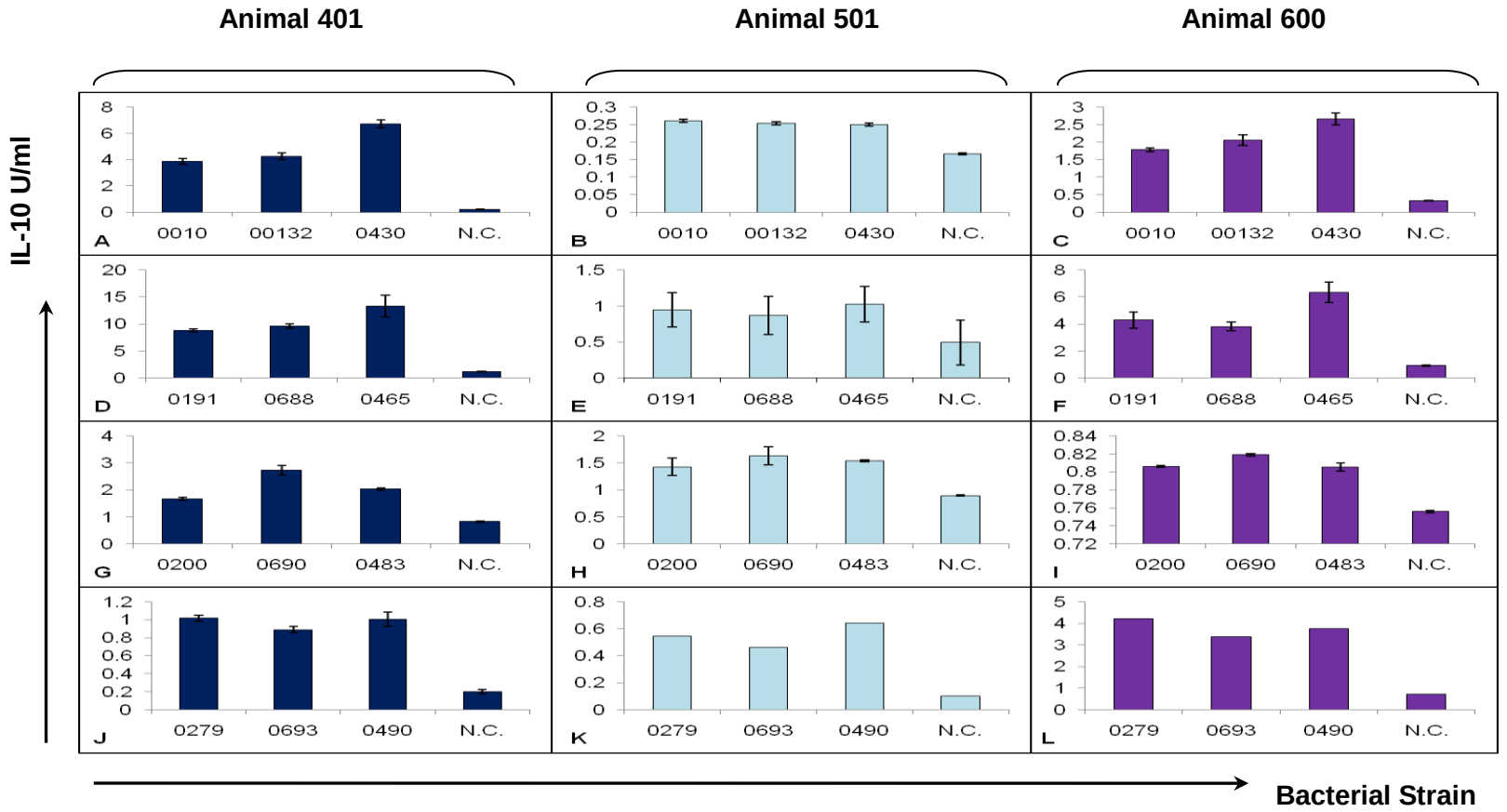


Fig. 5.6 Results of ELISA for IL-10 production by moDCs in response to challenge with *E. coli* strains. Each histogram shows data from one animal. Error bars represent the standard error of the mean where duplicate or triplicate experiments were carried out. N.C. = No Challenge. IL-10 was produced in response to all of the bacterial strains. A trend is observed greater IL-10 production in response to commensal strains compared to ETEC and O157:H7 strains, however, this trend was not observed with all the animals for all the commensal strains. The magnitude of response also varied greatly between animals (note the difference in scale of the axes). In each histogram the first column is an O157:H7 strain, the second column is an ETEC, the third column is a commensal and the fourth is unchallenged.

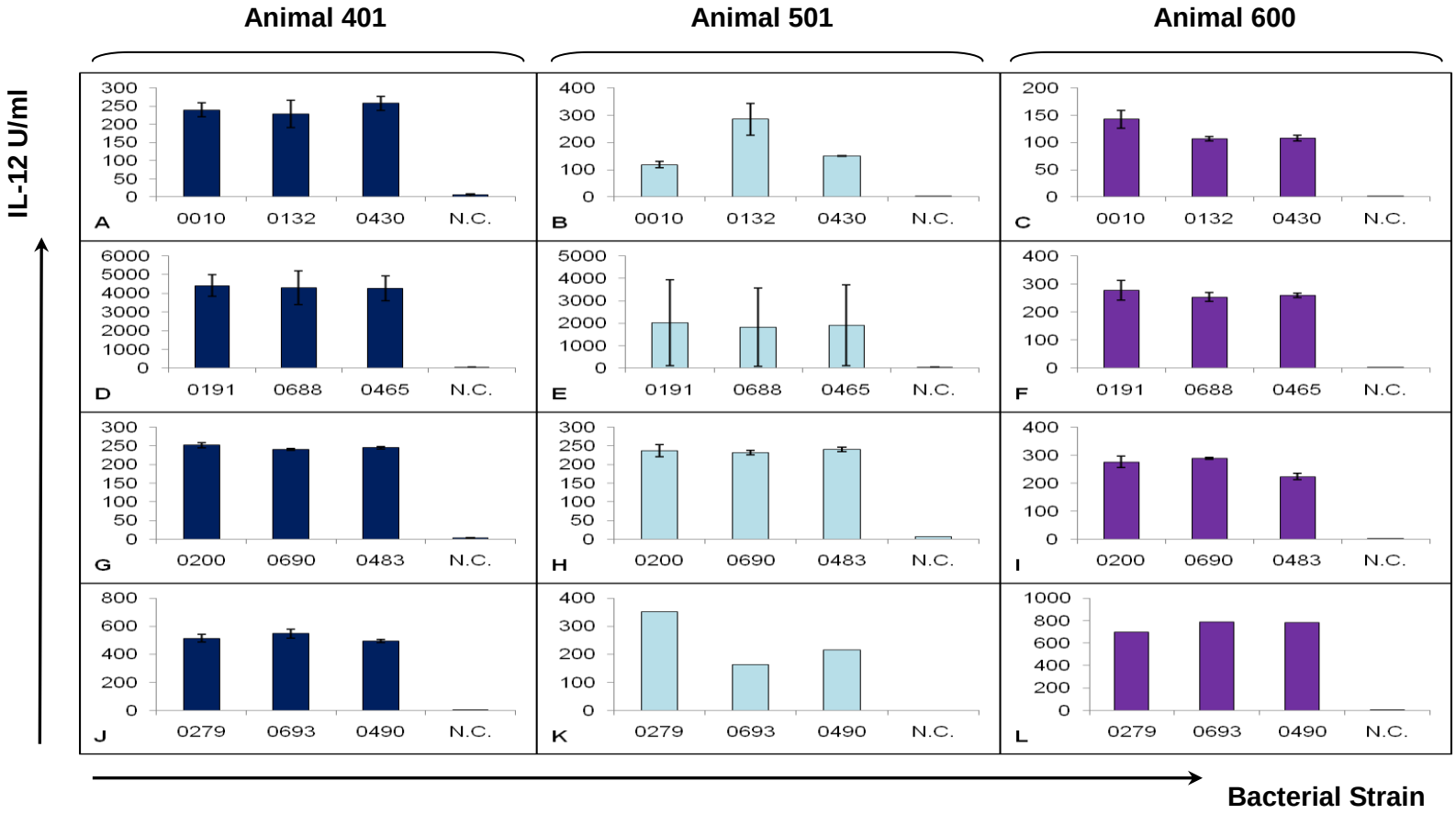


Fig. 5.7 Results of ELISA for IL-12 production by moDCs in response to challenge with *E. coli* strains. Each histogram shows data from one animal. Error bars represent the standard error of the mean where duplicate or triplicate experiments were carried out. N.C. = No Challenge. IL-12 was produced in response to all of the bacterial strains. There were no trends observed for differences in cytokine production between the strains – within each histogram the levels of IL-12 production were similar for each strain, and above that of the unchallenged cells. The magnitude of IL-12 response varied between animals. (note the difference in scale of the axes). In each histogram the first column is an O157:H7 strain, the second column is an ETEC, the third column is a commensal and the fourth is unchallenged.

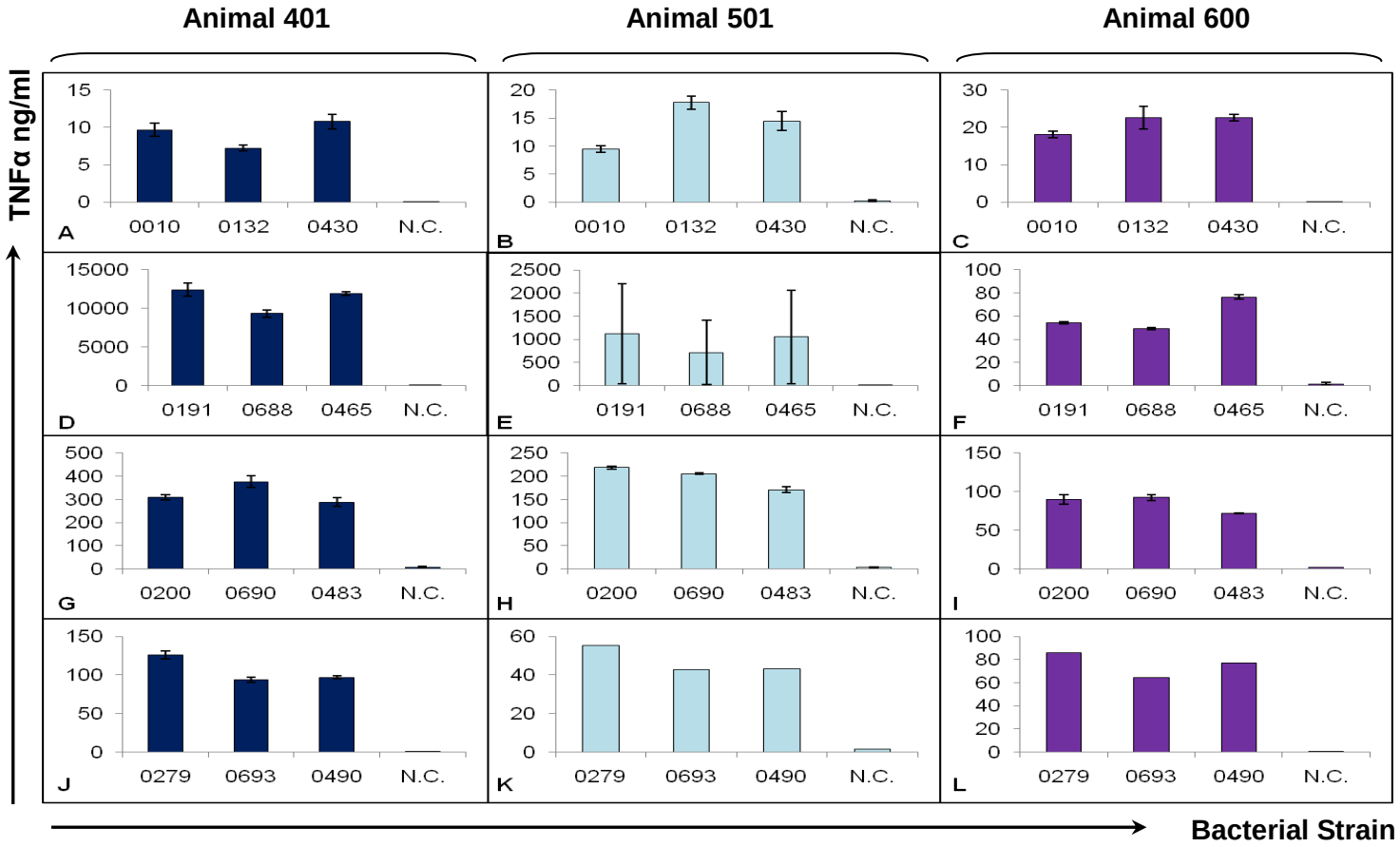


Fig. 5.8 Results of ELISA for TNFα production by moDCs in response to challenge with *E. coli* strains. Each histogram shows data from one animal. Error bars represent the standard error of the mean if duplicate or triplicate experiments were carried out. N.C. = No Challenge. TNFα was produced in response to all of the bacterial strains. A trend, which many of the histograms show, was observed. Slightly lower levels of TNFα were produced in response to the ETEC strains compared to the O157 and commensal strains. The magnitude of TNFα response varied between animals. (note the difference in scale of the axes) In each histogram the first column is an O157:H7 strain, the second column is an ETEC, the third column is a commensal and the fourth is unchallenged.

5.3.5 Expression of cell surface molecules on moDCs challenged with *E. coli* strains

Bovine moDCs were challenged with a panel of 12 *E. coli* strains in total, and the levels of expression of cell surface molecules MHC II, CD1b, CD205 and SIRPα were measured. The pooled data is shown (Fig. 5.9 C) Each experiment consisted of one O157 strain, one ETEC strain, one commensal strain and unchallenged cells. Each experiment (consisting of three strains and unchallenged cells) was carried out with moDCs from three animals on the same day in 12 well plates. Often cell numbers limited the number of replicate wells which could be used for each condition, but three wells for each strain were challenged if numbers allowed, and the wells were then pooled for FACS labelling. One experiment was repeated in full, (Fig. 5.10 D) giving data from six animals. The complete pooled data for all 12 strains (Fig. 5.9 C) shows no significant differences in levels of expression for any of the molecules either between the pathotypes themselves or the levels expressed by the pathotype challenged moDCs compared to the unchallenged cells. (MHC II, p=0.759; CD1b, p=0.843; CD205, p=0.991; SIRPα, p=0.963) Fig 5.10 shows data from individual challenges. Challenge 1 (Fig. 5.10 A) shows there was little difference between the strains for expression of MHC II, CD1b, CD205 or SIRPα compared to unchallenged cells. The differences were not statistically significant for MHC II (p=0.759) Challenge with MCI 0132 (ETEC) appeared to downregulate SIRPα expression; however a sample size of one for CD1b, CD205 and SIRPα would make it impossible to draw conclusions from this. The data from this challenge was not repeated as the level of expression observed in this experiment was much greater compared to the other three challenges (Fig. 5.10 B, C & D) and in fact when the data from challenge 1 is removed from the pooled data the upregulation of MHC

II in response to all the pathotypes becomes clearer but is still not statistically significant (p=0.2) (data not shown). Experiment 2 (Fig 5.10 B) shows that there was no difference in expression levels of MHC II (p=0.911), CD1b (p=0.936), or CD205 (p=0.806) compared to unchallenged cells. SIRPα appeared to be down regulated in response to MCI 0465 but this difference was not significant (p=0.753) Experiment 3 (Fig. 5.10 C) showed no differences in expression of MHC II (p=0.135), CD1b (p=0.494), CD205 (p=0.977) or SIRPα (p=0.531) compared to unchallenged moDCs. MHC II expression appeared to be upregulated by challenge with MCI 0200 and MCI 0483 but not MCI 0690 when compared to unchallenged cells and expression of SIRPα seemed to increase in response to MCI 0200 and MCI 0690 but not MCI 0483. However, these differences were not significant in comparison to the expression levels on unchallenged moDCs. Experiment 4 (Fig. 5.10 D) indicated that MHC II was upregulated in response to all three strains (MCI 0279, MCI 0693, and MCI 0490) compared to unchallenged cells but this change was not significant (p=0.706), and no differences were evident between the pathotypes. There were no significant differences observed for expression of CD1b (p=0.998), CD205 (p=0.479), or SIRPα (p=0.889). Experiment 4 comprised pooled data from six separate animals and is therefore the most robust data set. To summarise these results, challenge of bovine moDCs with *E. coli* strains tends to lead to increased expression of MHC II, which is an expected finding, showing that the moDCs are responding to bacteria by increasing their antigen presenting capacity in order to initiate immune responses. However there were no differences observed between the levels of response to the different pathotypes suggesting that this is a generalised MHC II upregulation, not specific to

individual strains or pathotypes. No other cell surface molecule was consistently altered by bacterial challenge.

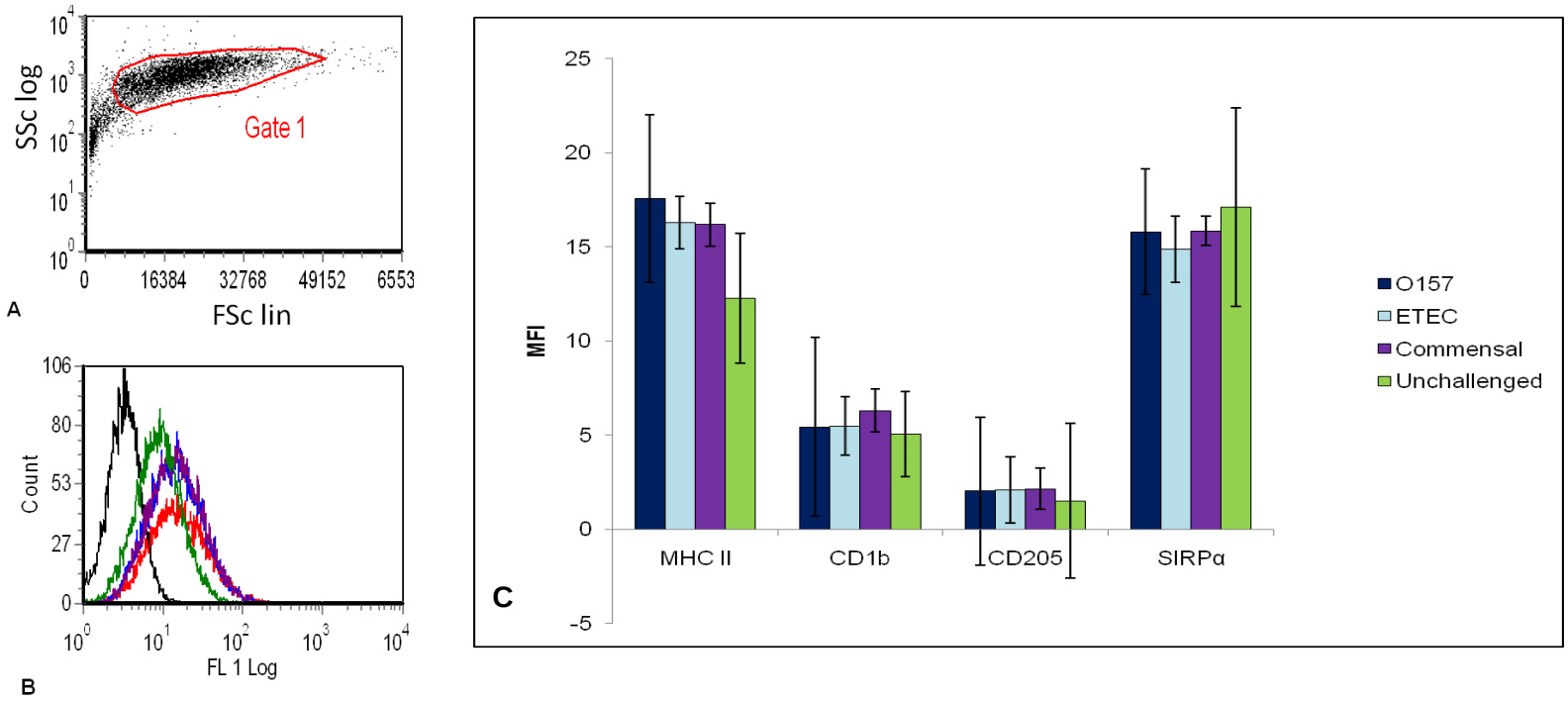


Fig. 5.9. Flow cytometry for cell surface molecules expressed by moDCs following challenge with various *E. coli* strains. (A) Dot plot of forward and side scatter of moDCs showing the gated population of cells used for analysis (gate 1) The limit on the gated population was set at 10,000 cells but due to limits on cell numbers the actual number of cells in the gate ranged from 3,000 to 10,000. (B) A representative histogram generated by the flow cytometry data of fluorescence vs cell count: Black: isotype control AV37; red, blue, purple and green: MHC II expression of cells challenged with various *E. coli* strains (Red: MCI 0010, Blue: MCI 0132, Purple: MCI 0430, Green: unchallenged).The MFI for the relevant isotype control was subtracted from the MFI for each molecule to give the values presented here and the results shown are pooled from 15 data sets for MHC II and 12 data sets for CD1b, CD205 and SIRPα (C). These data sets consisted of moDCs challenged with four O157:H7 strains, four ETEC strains and four commensal strains. Error bars represent standard error of the mean. There was a trend observed in which MHC II was upregulated in response to challenge with all strains compared to unchallenged cells, but this difference was not significant (p=0.759) There were no change in expression for CD1b, CD205 or SIRPα (p=0.843, p=0.991 & p=0.963 respectively) compared to unchallenged cells and there was no difference observed between the pathotypes when analysed this way at the 95% confidence interval.

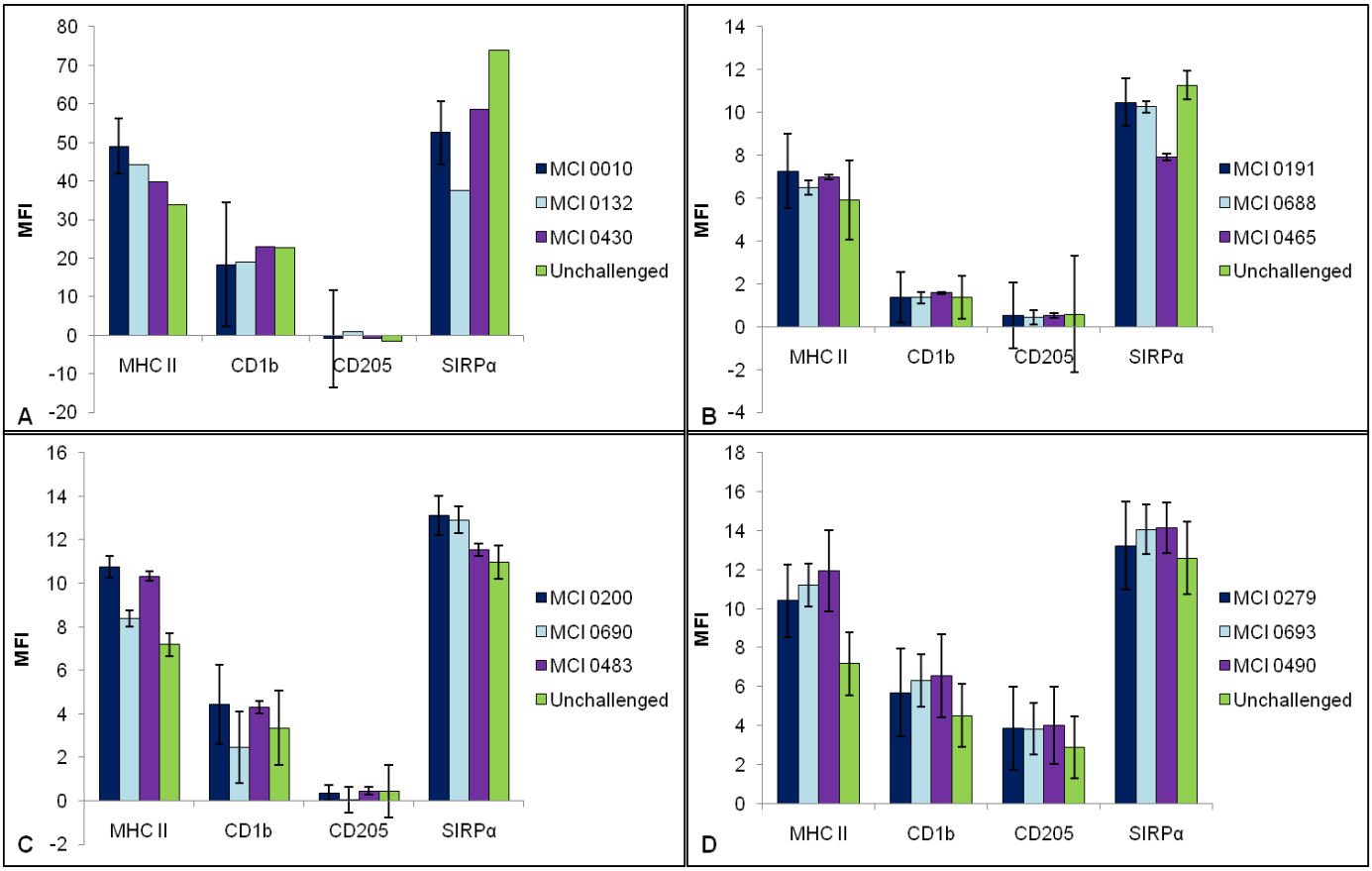


Fig. 5.10 Flow cytometry for cell surface molecules expressed by moDCs following challenge with various *E. coli* strains. Data for each experiment used to generate Fig. 5.9. The cells were gated as in Fig. 5.9. (A). The MFI for the relevant isotype control was subtracted from the MFI for each molecule to give the values presented here. The results shown are data from (A) 3 animals for MHC II, one animal only for CD1b, CD205 and SIRPα, (B) 3 animals, (C) 3 animals for MHC II and 2 animals for CD1b, CD205 and SIRPα, (D) 6 animals. Error bars represent standard error of the mean. (A) shows little differences between the strains for expression of MHC II (p=0.759), CD1b or CD205 compared to unchallenged cells, except possibly challenge with MCI 0132 to down regulate SIRPα compared to control and other strains. As this represents only a sample size of one it would be difficult to draw conclusions from this. (B) There was no difference in expression levels of MHC II (p=0.911), CD1b (p=0.936), CD205 (p=0.806) or SIRPα (p=0.753) compared to unchallenged cells. (C) There were no differences in expression of any of the molecules compared to unchallenged cells MHC II p=0.135, CD1b p=0.494, CD205 = 0.977 and SIRPα p=0.531 (D) MHC II is upregulated in response to all three strains (MCI 0279, MCI 0693, and MCI 0490) compared to unchallenged cells but this difference is not significant (p=0.706) There are no significant differences observed for expression of CD1b, CD205, or SIRPα either. (p=0.998, 0.479 & 0.889 respectively) Also, note the difference in the scale of the axes between (B), (C) & (D) which are similar and compare with (A) which is much larger. This experiment (A) was not repeated. In each graph dark blue represents the O157:H7 strain, light blue represents the ETEC strain and purple represents the commensal, green is unchallenged moDCs.

5.3.6 Cytokine responses and cell surface molecule expression of moDCs challenged with VT^+ and VT^- *E. coli* O157:H7 strains

Three *E. coli* O157:H7 VT^+ strains (CAT 3) were selected to challenge the moDCs to assess the effects of VT on moDCs during challenge. These strains produce VT2 only (MCI 0066) or both VT1 & VT2 (MCI 0045 and MCI 0218). These were chosen over strains that produce only VT1 as challenges of moDCs with purified VT2 were planned (Chapter 4) and it was intended that the results would be complimentary. These strains were also chosen as they are the 'parent' strains for the CAT 2 strains selected, in that they are the same strains but lack VT producing capabilities. The challenges with the three VT^+ strains were carried out concurrently with a representative VT^- strain (MCI 0010, CAT 2) to compare any differences in cytokine production stimulated by these strains. Three experiments were carried out with cells from three animals each time; technical replicates were also included where cell numbers allowed. ELISA was carried out on the cell supernatant for IL-10, IL-12 and TNFα. The results of the ELISA are shown in Fig. 5.11. All three cytokines were produced in response to bacterial challenge when compared to levels in unchallenged supernatant (ANOVA, $p<0.05$). No differences were observed between the different VT^+ strains themselves or between the levels of cytokine stimulated by the VT^+ strains and the VT^- strains when tested at the 95% confidence interval. FACS analysis of these cells was also carried out to assess any changes in cell surface molecule expression. The molecules selected for examination by this method were MHC II, CD1b, CD205 and SIRPα. The results are shown in Fig. 5.12. There was an increase in MHC II cell surface expression for cell challenges with all strain studied, but this data was not significantly different ($p=0.058$) compared to unchallenged cells. There was also a trend for an upregulation of CD1b for all strains however

the difference was not significant. (p=0.118). There was no statistical difference observed in cellular expression of SIRPα (p=0.877) or CD205 (p=0.594). There were no significant differences observed within the VT^+ strains or between the VT^+ and VT^- strains when tested at the 95% confidence interval.

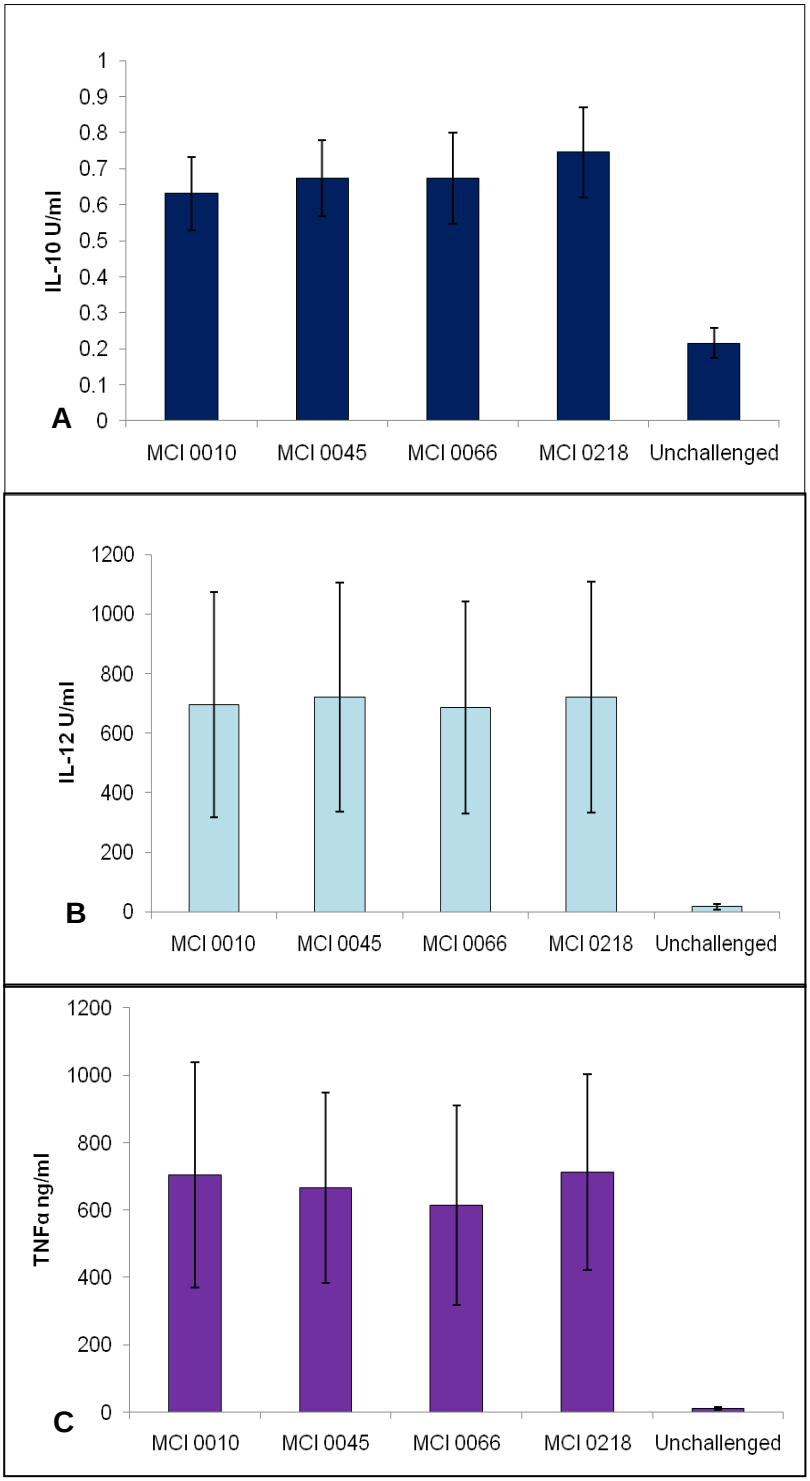


Fig. 5.11 Results of ELISA of moDC supernatant following challenge with VT$^+$ strains. (A) IL-10, (B) IL-12 and (C) TNFα ELISA results are shown following challenge with three VT$^+$ strains (MCI 0045, 0066 and 0218) and one VT$^-$ strain (MCI 0010). Results are pooled from three individual experiments; error bars represent the standard error of the mean. IL-10, IL-12 and TNFα were all produced in response to bacterial challenge (ANOVA $p<0.05$). Using an ANOVA and a post hoc Tukey's test, no differences were observed between the cytokine responses to the strains when tested at the 95% confidence interval.

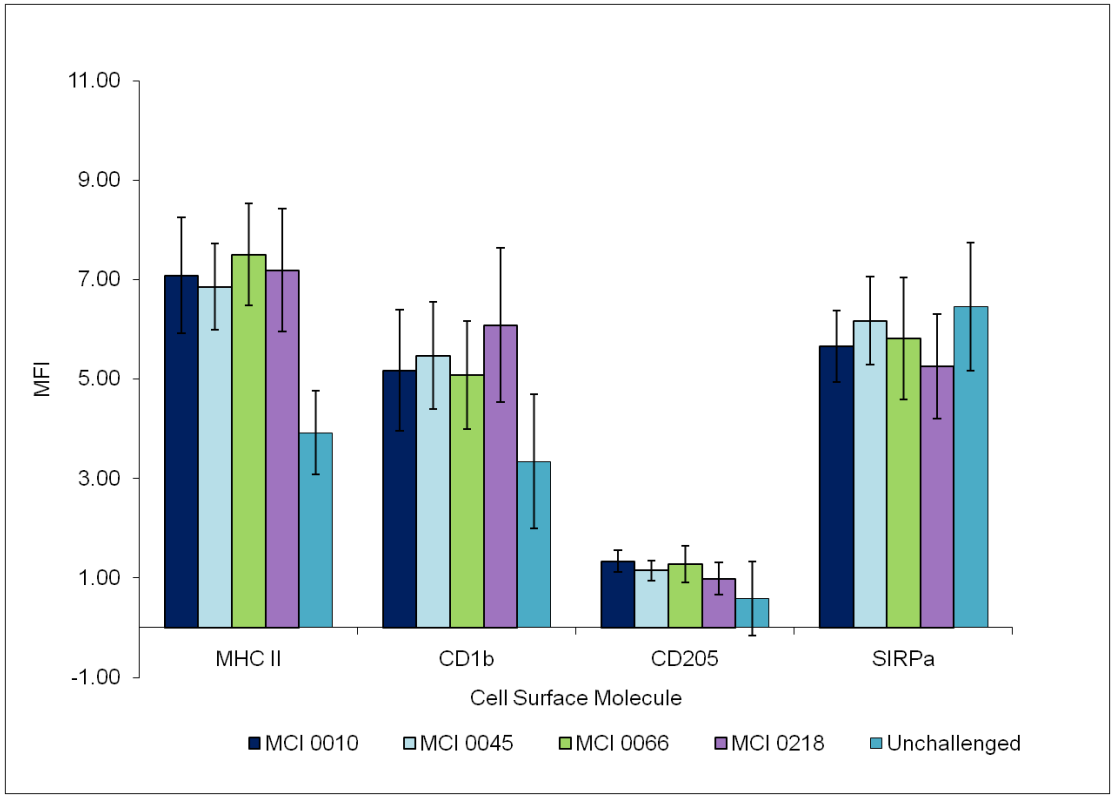


Fig. 5.12 Flow cytometry for cell surface molecules expressed by moDCs following challenge with various *E. coli* strains. The results shown are pooled from three animals collected from three separate experiments. Error bars represent standard error of the mean. The MFI for the relevant isotype control was subtracted from the MFI for each molecule to give the values presented here. Cells were gated as shown in gate 1 in Chapter 4 (Fig. 4.3 A) MHC II was upregulated in response to challenge with all strains compared to unchallenged cells, although the differences were not significant. (p=0.058) There was a trend for upregulation of CD1b for all strains however any difference was not significant. (p=0.118) There was no change in expression for CD205 (p=0.594) or SIRPα (p=0.887). There were no significant differences in responses to challenge with VT^+ or VT^- strains. All data was subjected to AVOVA and a post hoc Tukey's test at the 95% confidence interval.

5.3.7 Determination of numbers of intracellular bacteria following moDC challenge over a time-course up to 36 h: comparison of an O157:H7 strain with an ETEC and commensal *E. coli* strain

Numbers of intracellular bacteria following 2 h challenge were determined at various time-points up to 36 h by lysing cells and subsequent spreading on agar plates of the cell lysate. To compare differences between O157 strains, ETEC and commensal *E. coli* strains a representative strain for each was chosen for study. (MCI 0010, MCI 0132 & MCI 0430) Two separate experiments were carried out, each with three animals. The results are shown in Fig. 5.13 where some interesting strain differences can be observed. There were very few viable intracellular O157:H7 bacteria at any of the time points selected for measurement and these levels did not change over time. The ETEC strain yielded moderate numbers of viable intracellular bacteria, and these numbers remained fairly constant at all the time points. The commensal strain proved to have many more numerous viable intracellular bacteria than either the ETEC or the O157:H7 strains for time points up to 12 h. After 12 h the numbers of viable commensal bacteria retrieved started to reduce and by 36 h the numbers were comparable to those of the ETEC strain. The numbers of viable intracellular O157:H7 bacteria were always significantly lower than that of the ETEC or the commensal. Overall the numbers of intracellular bacteria were small, ranging from one bacterium per 500 cells for the commensal strain to one bacterium per 700,000+ cells for the O157:H7 strain. It is not known whether single cells had several internalised bacteria or whether only one bacterium was internalised per cell. This would require further investigation with additional techniques such as IHC or flow cytometry.

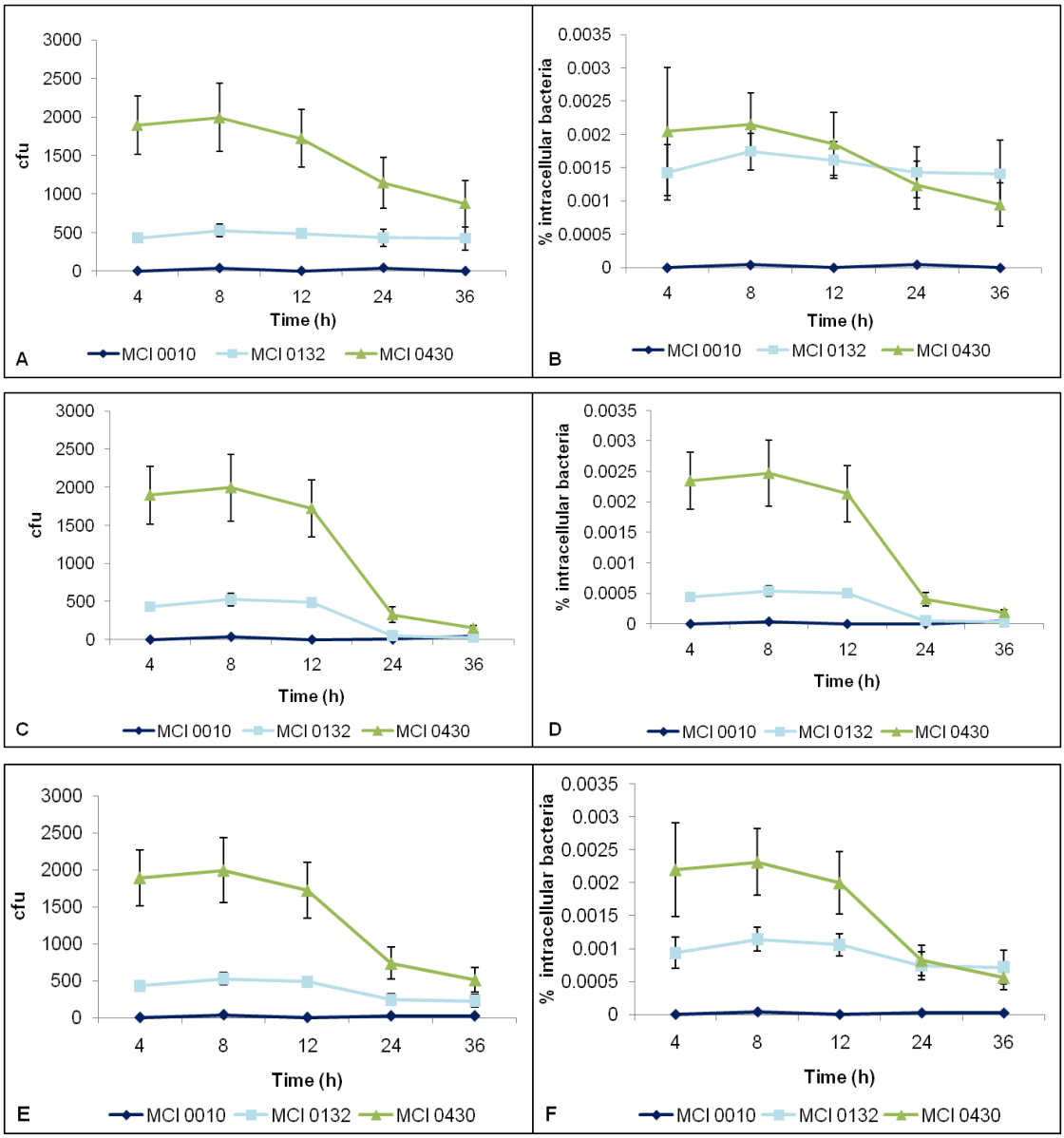


Fig. 5.13 Results of experiments to investigate viable intracellular bacterial numbers. moDCs were challenged and subsequently collected at various time points up to a maximum time of 36 h. Challenges were carried out with an O157 strain (MCI 0010), an ETEC strain (MCI 0132) and a bovine commensal strain (MCI 0430). Two experiments were carried out; experiment 1 results are shown in (A) & (B) and experiment 2 results are shown in (C) & (D). Pooled data, for both experiments is also shown (E) & (F). Challenges were carried out at an MOI of between 60 and 200 with three animals. Panels (A), (C) and (E) show cfu recovered per ml of cellular supernatant at each time point. Panels (B), (D) and (F) show the intracellular bacteria as a percentage of the numbers of bacteria added for each strain (i.e. takes into account any variation in MOI between the strains). Error bars represent the standard error of the mean. Infection with MCI 0430 showed the highest numbers of intracellular bacteria, these numbers started to fall after 12 h. Infection with MCI 0010 showed very low numbers of intracellular bacteria at close to zero and was consistent for all the time points examined. Infection with MCI 0132 resulted in fewer intracellular bacteria than MCI 0430 and more than MCI 0010, the levels of this strain remained relatively constant over time.

5.4 Discussion

The experiments carried out in this chapter were designed to characterise any role of DCs in the interactions of EHEC with the bovine host. Characterising moDC reactions as pathogenic in type, represented by ETEC strains, or non-pathogenic as represented by commensal strains and then attempt to assess to which group the responses to O157:H7 strains most closely resembled proved difficult.

Growth characteristics were assessed in this study as it is likely that phase of growth and culture medium may have important effects on bacterial expression of various virulence factors and pathogenicity of EHEC *in vitro*. (discussed in (Law, 2000)) Using the various strains at defined $O.D._{600}$ measurements was used in order to minimise differences.

In terms of cytokine responses and changes in cell surface molecule expression there were no statistically significant differences observed between the pathotypes in the moDC responses with pooled data. All three cytokines, IL-10, IL-12 and TNFα were produced in response to bacterial challenge, compared to unchallenged cells; however there were great variations in the magnitude of responses generated by individual animals. When individual animal data was considered some trends for differences in cytokine production emerge. Observation of the individual animal data, IL-10 production was possibly greater in response to most of the commensal strains compared to ETEC and EHEC strains. This data was difficult to tease apart as the IL-10 production to commensal strain MCI 0483 did not follow the trend. There are potential reasons for this which are discussed in Chapter 6, where virulence factors were identified within MCI 0483 which had effects

on moDC viability at higher MOI. Therefore if the data for MCI 0483 is not considered here, the trend for greater production of IL-10 in response to commensal strains compared to ETEC and EHEC becomes somewhat more evident. TNFα production also indicated a trend for differences between the pathotypes, where the levels in response to ETEC strains was lower than those in response to EHEC or commensal. It appeared that IL-12 was indeed produced equally in response to all the strains as this was observed for the majority of the individual animals studied. However, it must be remembered that, when pooled, this data showed no significant differences and therefore little weight can be attributed to these trends, however, they are useful to dissect in this way as they could indicate areas for further study. A further point of interest in the ELISA data is IL-12 production by challenged cells shows a greater fold change over levels produced by unchallenged cells when compared with IL-10. This was observed in all the viable bacteria challenges in this chapter and suggests that IL-12, a Th1 cytokine, is more dominant in the host response to *E. coli* strains. Following this work it would appear to be preferable to carry out challenges with fewer strains for example; possibly a representative strain from each group. Also, to carry out challenges on cells from larger numbers of animals would be of value to see if the trends observed here are indeed characteristic of *in vitro* responses.

Cell surface molecule expression of the moDCs showed no differences in response to the three pathotypes. Challenge with all the *E. coli* strains tended to increase the level of expression of MHC II, indicating a change in the cells from a phagocytic cell type to an antigen processing cell type as expected. No other cell surface molecule studied (i.e. SIRPa, CD205 or CD1b) was significantly affected. There was a corresponding change in morphology of

the challenged moDCs to an activated phenotype following bacterial challenge and although not directly measured there appeared to be no detrimental effect observed on moDC viability; these factors were similar for all the strains.

There was no difference in the responses of the cell to three VT^+ and a corresponding VT^- O157:H7 strain. Cytokines were produced at similar levels in response to all strains indicating that the presence of VT does not significantly affect the cytokine responses of moDCs. MHC II was upregulated in response to the VT^+ strains, but no other cell surface molecule in this study was altered.

All of this data indicates that the moDCs tend not to detect differences between various strains of *E. coli.* This could be due to the presence of such high levels of similar PAMPs (LPS, H7 flagellin and CpG) that any smaller differences are not able to be observed. No differences between responses to EHEC strains, ETEC strains and commensal strains were observed using the methods described above. It is possible however that there were differences in parameters that were not measured here. It could be that investigation of different cell surface molecules or cytokines may have clarified differential responses. Norimatsu *et al.* (2003) studied expression of co-stimulatory molecules (CD40, CD80 & CD86) and transcripts of proinflammatory cytokines (e.g. IL-6) of moDCs following *Salmonella* challenge and study of those parameters could be considered for studies with the *E. coli* strains employed here.

The trends observed in the ELISA data would require further elucidation to see if these indicate detectable differences. Flow cytometry protocols using two-colour (or more) staining protocols would help to retrieve the additional data from these populations of limited cell numbers, and may help to uncover any differences. Challenge of moDCs with purified VT would be of use to assess any cellular responses to this important bacterial virulence factor. As the main role of DCs is to present antigen and generate immune responses it would also be interesting to observe any differences in the T cell responses to moDCs challenged with these various strains, by employing commonly used techniques such as T cell proliferation studies; unfortunately time constraints precluded the inclusion of these experiments in this study.

The most striking differences observed in this chapter between the three pathotypes were discovered when measuring intracellular bacteria. A time course experiment was carried out to detect viable intracellular bacteria following challenge of moDCs, up to a time of 36 h. A representative strain from each group was chosen for study. The commensal strain was internalised by the moDCs at greater numbers than the ETEC or the EHEC strains. Viable bacteria were detected for all strains during the full course of the experiment up to 36 h post challenge. The EHEC strain did not readily enter the moDCs and there were very few EHEC recovered at any of the time points, compared to the ETEC and commensal strains. A further difference was observed with the intracellular numbers. The commensal strain entered the moDCs at higher numbers and these numbers of viable bacteria retrieved remained at these high levels until 12 h post challenge when they began to decline. The numbers of both the ETEC and the EHEC remained relatively constant during the sampling period. This suggests that the commensal

strains are easily phagocytosed and begin to be killed around 12 h post challenge, whereas the ETEC strains are less easily phagocytosed, but when they become intracellular, they are able to resist intracellular killing for periods up to 36 h. The EHEC strain is even less likely to be phagocytosed and, although only small numbers of bacteria become intracellular this strain too shows extended periods of survival within the moDCs, as the small number of viable bacteria retrieved did not change during the sampling period, and could still be detected at 36 h. It is important to be aware that the ETEC and EHEC strains could be taken up and quickly killed by the moDC and studies to investigate this possibility would be valuable. Extracellular counts from parallel cultures would be required to validate moDC uptake and/or killing of these strains. However, by evading phagocytosis it is possible that *E. coli* O157:H7 strains are able to colonise cattle and induce scant immune response to clear them from the intestinal environment. By resisting intracellular killing for extended periods, this too reduces generation of specific immune responses to bacteria. DCs are able to migrate to local lymph organs to present antigens within hours following uptake to initiate immune responses. If bacteria are able to resist intracellular killing and therefore specific antigen presentation for prolonged periods, then generation of immune responses too will be delayed permitting multiplication of these organisms and potential colonisation of the host. The intracellular bacteria would be interesting to study further with repeats of this experiment using other strains to see if these differences are specific to pathotype or merely specific to these particular strains. Observation of intracellular bacteria would also be interesting, using IHC techniques or electron microscopy.

The results presented here for the first time indicate intracellular survival of low numbers of EHEC in bovine moDCs and how this differs from the numbers and survival time observed for commensal and ETEC strains. This has relevance for the *in vivo* situation where, evasion of phagocytosis and survival for extended periods inside immune cells could both be an advantage to colonisation of the host. This data could indicate an important role for DCs in colonisation of cattle, and highlights areas for further investigation. This study progressed to investigate the effect of MOI on moDC challenges, to see if higher MOI would elucidate further differences between the pathotypes observed here.

Chapter 6 Investigation of the effects of a high multiplicity of infection on the responses of bovine moDCs to bacterial challenge

6.1 Introduction

Challenges of the moDCs with *E. coli* strains showed differences in phenotype and functions depending on the challenge strain. Within this chapter the role that multiplicity of infection plays in bacterial interactions with moDCs was investigated.

Multiplicity of infection is an area where it is difficult to predict accurately values that reflect the *in vivo* state. In Chapter 5 an approximate calculation was carried out based on colonisation information of the bovine intestinal tract with *E. coli* strains and the approximate number of tissue DCs that could be present. The DC numbers were based on work that had been carried out in ovine mucosal tissues (specifically lung) which indicated numbers of DCs per mm^2 of tissue within the airways (McNeilly *et al.*, 2006). These numbers led to early challenges being carried out at low MOIs of 0.05, 0.5 and 5. These MOI were tested in a pilot study and there was little difference in effect on cytokine responses thus an MOI of 0.5 was selected for the majority of the challenges in Chapter 5. Chapter five showed that moDCs responded to challenge with viable bacteria at low MOI by cytokine production and changes in cell surface molecules, but without any observable detrimental effects on moDC viability. Similar MOI to those used in Chapter 5 have been used with other bacterial challenges on moDCs. A very recent human study using moDCs challenged with *S. typhimurium,* to investigate downstream signalling pathways used challenges at MOIs of 1, 5 and 10 (Pietila *et al.*, 2010). As this project progressed it became clear that there are many more DCs within the tissues of the terminal rectum than that found within lung tissues, and that there is also great animal to animal variation in the numbers of *E. coli* carried in the bovine intestine (Dr. E.

Clark, Thesis: http://theses.gla.ac.uk/706/). Although it is known that DCs can actively sample antigen from the intestinal lumen by means of dendrites extending between the epithelial cells, it is unclear what proportion of DCs within the tissues will be contacting the bacteria within the lumen at any time. In mice, the number of trans-epithelial DC extensions into the intestinal lumen is known to change with intestinal location and presence of bacteria (Chieppa *et al.*, 2006). The presence of trans-epithelial DC extensions was significantly reduced *in vivo* by treatment with a broad-spectrum antibiotic, indicating that DC lumen sampling is affected by the bacterial load of the intestine, and it is not necessarily pathogenic bacteria alone but commensal organisms too which are able to induce this response. Chieppa *et al*. also proposed that this increase in sampling following bacterial challenge was mediated by TLR signalling via epithelial cells and DCs. Signalling via TLRs 2, 4 and 9 when initiated by their ligands peptidoglycan (PG), LPS and CpGs all increased frequency of DC trans epithelial extensions, As the selected *E. coli* strains used in this study contain LPS, CpGs and PG they too must be expected to signal through these same TLR on bovine epithelial cells and DCs and could lead to increased bacterial interactions with DCs via the resulting trans-epithelial extensions. However, it was interesting to note that signalling by TLR 5 and its ligand flagellin showed no such increase and was reported to be due to the low or lack of TLR5 expression of the epithelium at the intestinal site and species tested in this study (murine ileum). TLR 1-10 have been detected in ovine intestinal tissues using quantitative PCR (Menzies & Ingham, 2006) and the presence of a wide range of mRNA transcripts for TLR have been found on bovine antigen presenting cells (Werling *et al.*, 2006). Primary bovine colonic cells have also been examined for TLR mRNA expression and it was found that TLR1, TLR3, TLR4 and

TLR6 were all detectible, although the authors postulate whether further TLR mRNA would be identified using a technique with greater sensitivity (Bridger *et al.*, 2010). Therefore it is likely that there will be many TLR present within the tissues of the bovine intestinal mucosa which can recognise PRR of *E. coli* O157:H7. Any disruption in epithelial integrity due to injury or inflammation is also likely to lead to higher numbers of lamina propria DCs contacting the luminal contents directly.

All of these factors indicate that there are many issues which affect the *in vivo* ratios of bacteria to DCs. These ratios are also dynamic and have potential to be high at certain times and at certain intestinal locations, thus *in vitro* studies at a range of MOI are of relevance to host situations.

To investigate the effect of MOI on moDCs a high MOI of approximately 100 was selected, this being a multiplicity of infection used quite frequently for *in vitro* experiments with other cell types and pathogens. EPEC and EHEC strains have both been used at MOI of 100 with epithelial cells to investigate attachment of bacteria to the cells and concurrent protein signalling. (Kenny, 1995) The pathogenesis of *Listeria monocytogenes,* which initiates infections in human and animal hosts by entering GALT, was also investigated at MOI of 100 with a murine splenic DC line (CB1 cells) (Guzman *et al.*, 1995). A study using heat-inactivated *E. coli* strain DH5α to challenge human moDCs has employed a wide range of MOI from 10 to 1250 to investigate sepsis in humans (Falcone *et al.*, 2004). Sepsis is a syndrome whereby, among other cellular and signalling effects, DCs are found to be profoundly reduced in number by apoptosis in affected patients (Hotchkiss *et al.*, 2002).

The effects of challenge at high MOI were intended to be investigated using similar assays to those used in Chapters 4 and 5. These would include FACS, ELISA and intra- and extracellular bacterial counts. However, the initial challenge immediately illustrated effects of bacteria on moDC viability and the decision was made instead to explore this interesting discovery further.

A search of the literature has yielded only sparse information concerning moDC viability during challenge with viable bacteria. The study mentioned earlier by Falcone *et al* (2004) however, has investigated apoptosis of DCs following challenge with *E. coli* laboratory strain (DH5α) as a model for studying sepsis in human patients. Apoptosis of human moDCs was observed in that study but only at MOIs of 500 or greater and the killing effect was maximal and plateaued at an MOI of 1000. The Falcone study predominantly carried out challenges with heat-inactivated bacteria in contrast to the work presented here which used live *E. coli* strains isolated from the bovine intestinal tract. Some bacterial components too have been shown to have effects on DC viability. *Bacillus anthracis* lethal toxin (LT) has been shown to kill both murine and human moDCs *in vitro* and murine DCs *in vivo*. Observation of morphological changes and MTT assays were used to characterise apoptotic cell killing (Alileche *et al.*, 2005). Listeriolysin, the main virulence factor of the bacterial pathogen *Listeria monocytogenes* is also able to induce DC apoptosis when used to challenge murine DCs of the cell line CB1 *in vitro* as well as *ex vivo* bone marrow derived DCs (BMDCs) (Guzman *et al.*, 1996). Viral challenges too have been found to effect DC viability *in vitro*: herpes simplex virus 2 (HSV-2) induces rapid death of murine BMDCs by apoptosis (Jones *et al.*, 2003). MHC II has been implicated in DC cell death pathways. Immature human moDCs have been stimulated to mature when

cultured with an anti-MHC II antibody, whereas mature moDCs were induced to apoptose; these outcomes were found to be mediated by independent cell signalling pathways (Lokshin *et al.*, 2002). This indicates a role *in vivo* for MHC II mediated DC death. Rapid DC death following T cell interactions *in vitro* has been observed (Matsue *et al.*, 1999), and suggests that mature DCs *in vivo* are likely to have a very limited lifespan following migration from peripheral tissues. So, although DC apoptosis in response to challenge is not a completely new finding, few other studies have reported DC killing by bacterial pathogens.

Salmonella and its interactions with moDCs have been studied by Norimatsu *et al*. (2003 & 2004) *Salmonella*, although it has notable differences from *E. coli* O157:H7, is worthy of note here as some similarities can be drawn. On a basic bacteriological level both are Gram-negative rods which can use flagella for motility. Both *Salmonella* and *E. coli* are intestinal pathogens with their effects in human and bovine hosts (among others) and cause diarrhoeal disease. Although *Salmonella* is an intracellular pathogen, initially it too makes use of a TTSS, similar to O157:H7, to introduce virulence factors to host cells and it is this action which then induces uptake of the bacterium by the cell (Brumell *et al.*, 1999; Collazo & Galan, 1997). Live salmonellae have been found to elicit different moDC cell responses following challenge when compared to killed salmonellae (Norimatsu *et al.*, 2003). Both live and killed *S. Typhimurium* induced moDC maturation, cytokine expression, and T cell proliferation. The responses observed with the live bacteria however were generally greater. Both live and killed challenges upregulated MHC I and MHC II, and CD80 to similar extents showing a maturation of the moDCs. The live bacteria stimulated a greater up regulation of CD40 and CD86 than

the killed bacteria. A greater magnitude of cytokine response was observed with the live salmonella challenge, where up-regulation of the transcription of IL-6, IL-12p40 and GM-CSF were all increased over the killed challenge, which in turn was greater than the minimal levels observed with unchallenged cells. The authors suggest that these differences indicate the important role that DCs play in priming the immune response, in particular when discussed in terms of vaccination; this is because live bacterial vaccines tend to provide greater protection against subsequent infection than killed (Lindberg & Robertsson, 1983; Mastroeni *et al.*, 2001; Robertsson *et al.*, 1983). This live vaccine perspective may not have such an impact with EHEC, as current vaccine research is not centred on whole bacteria but rather combinations of bacterial components such as TTSS and outer membrane proteins (Khare *et al.*, 2010; McNeilly *et al.*, 2010b). However, the underlying immune responses whereby killed bacteria tend to prime a Th2 response and live bacteria tend towards a Th1 response very much corresponds to the known key roles that DCs play in directing immune responses (Haeberle *et al.*, 1997; Thatte *et al.*, 1993) and could be integral to the carriage of O157:H7 by cattle. If these responses could be fully characterised, it may open up additional areas of research for reduction of colonisation and spread by these bovine hosts.

Given the role of DCs in directing immune responses and the possibility for bovine intestinal DCs to interact with O157:H7; *in vitro* methods to explore these interactions were employed.

The aims of this chapter were:

1. To investigate the effect of high MOI (100) during moDC challenges.
2. To investigate any differences in the effect of moDC challenge with gentamicin-killed bacteria and live bacteria.
3. To investigate whether the cattle used in this study were colonised by *E. coli* O157:H7.

6.2 Materials and Methods

6.2.1 Animals

The animals used in this chapter were clinically healthy adult cattle conventionally managed and housed at MRI. They were all male Holstein Friesian (HF) between 2 and 5 years old. Blood sampling was carried out according to the requirements of the Animal Scientific Procedures Act (1986).

6.2.2 Preparation of moDCs

Bovine moDCs were prepared for challenges as described in Chapter 5 (section 5.2.6). Briefly, moDCs were generated from bovine blood and cultured for 3 days in culture medium containing GM-CSF and IL-4 (DC-TCM Appendix A.4.2.4). On day three the cells were collected, counted and washed three times in antibiotic-free TCM (Appendix A.4.2.3, no Gentamicin). They were then seeded into a suitable well plate for challenges (usually 12 well or 96 well) cultured overnight and challenged on day four. The choice of day for cell challenge is discussed in Chapter 4 and was selected to represent immature DCs

6.2.3 Preparation of bacterial strains

The bacterial panel selected for use in this chapter are as described in Chapter 5 section 5.2.2 and Appendix A.3 Four bovine ETEC strains, four bovine commensal strains and four *E. coli* O157:H7 (CAT 2) strains were selected for investigation. In addition moDC challenge with MCI 0200 was compared with its corresponding type three secretion system knockout strain. (ΔTTSS, MCI 0201). Bacteria for challenges were grown overnight in MEM/HEPES (Sigma) at 37°C in a shaking incubator set to 200 rpm. Sub-

cultures were made (1:10 v/v) into fresh MEM/HEPES and grown at the same conditions for a further three hours until the O.D.$_{600}$ nm reading was between 0.25 and 0.35 (approximately 0.31) to correspond to the required MOI (bacterial numbers at this O.D.$_{600}$ had been confirmed in Chapter 5). Where killed bacteria were required for challenge a 1 ml sample of the bacterial culture was removed and incubated at 37°C for 1 h with gentamicin (50 μg/ml). Effective bacterial killing was confirmed by plating out on nutrient agar plates.

6.2.4 Bacterial Challenge of moDCs

The bacterial culture was used undiluted to challenge the cells at relative volumes of 100 μl of culture to 1 ml of cell culture medium (cells adjusted to 1 x 10^6). Plating out serial dilutions of the challenge media allowed the MOI to be calculated and this was approximately 100, where unchallenged cells were required as controls, an appropriate volume of warmed MEM/HEPES was added to the moDCs in place of the bacterial culture. Killed bacterial challenges were carried out concurrently with the corresponding live bacterial challenge. All challenges were pulse chase as follows: after 2 hours of challenge gentamicin was added to all wells (50 μg/ml) and then cultured for a further 22 hours. Challenges were carried out in an incubator set to 37°C and 5% CO_2 Following this time, viability counts or MTT assays were performed, or images were obtained as required.

6.2.5 Viability counts

Following challenge with bacteria the numbers of live and dead moDCs were counted and percentages were compared for each challenge. The cells were diluted in Nigrosin (final concentration of 0.1% w/v) stain (Sigma) and were

counted using disposable chambers (Fast-read 102, ISL, Devon). Nigrosin darkly stains dead cells and is excluded from live cells. Live cells can be observed clearly as light or clear against the dark Nigrosin milieu.

6.2.6 MTT Viability Assay

An MTT viability assay (Molecular Probes) was carried out on the moDCs following bacterial challenge. The MTT assay (Mossman, 1983) detects live cells and the response depends on the activation level of the cells on test, although it is widely used as a proliferation test to indicate numbers of viable cells in a population because it indicates mitochondrial function. At the end of the challenge the cellular supernatant is removed and fresh pre-warmed phenol red-free TCM (Appendix A.4.2.2) is added to the cells in the 96 well plates. The MTT assay is then carried out as described (section 4.2.9).

6.2.7 Images of Cells

Live cell images were taken using an AxioCam MRm digital camera mounted on a Ziess Axiovert microscope with an integrated cell culture incubator.

6.2.8 Determination of *eae* and *escN* by PCR

To determine which strains contained the *eae* and *escN* genes PCR was carried out for all strains (Appendix A.3) Template DNA was provided by Dr E. Clark (Moredun Research Institute). Briefly, for strains MCI 0688, MCI 0690 and MCI 0693: one bacterial colony was suspended in 50 μl of Instagene Chelex matrix (BioRad). This was vortexed for 10 sec, heated to 100˚C for 10 min, briefly cooled and vortexed for a further 10 sec. These samples were stored at -20˚C until required, at which time the samples were centrifuged to

pellet to allow the supernatant to be used for the template. For the other strains template DNA had been obtained by using the Wizard genomic DNA kit (Promega), following the manufacturer's instructions. Primers were designed based on published work (see table 6.1) The PCR reaction was made up as follows: 1µl of template was added to 2.5 µl of 10x buffer, (included with the Hotstart kit (Promega)) 0.25 µl each of forward and reverse primers (100 µM each), 0.25 µl of dNTPs (10 µM), 0.25 µl of Hotstart Taq (Promega) and made up to 25 µl with dH_2O. The samples were placed into a Thermo-Hybaid thermal cycler set for conditions detailed in table 6.2.

Target Gene		Sequence	Reference
eae	*eae*F	CTGAACGGCGATTACGCGAA	Aranda *et al.* 2004
	*eae*R	CCAGACGATACGATCCAG	
*esc*N	*esc*N F	CGCCTTTTACAAGATAGAAC	Kyaw *et al.* 2003
	*esc*N R	CATCAAGAATAGAGCGGAC	

Table 6.1 Primers used for PCR analysis of *E. coli* strains.

Temperature	Time	Cycles
95°C	15min	1 cycle
94°C	1min	40 cycles
54°C	1min	
72°C	1min	
72°C	5min	1 cycle

Table 6.2 PCR cycling conditions used for *eae* and *escN*

6.2.9 Agarose gel electrophoresis

The samples were run on a 1.1% agarose gel with Gel Red (Biotium Inc.) added according to the manufacturer's instructions to visualise the bands.

Gels were made up using 0.8 mg agarose and 75 ml TAE buffer (Appendix A.4.6.1); 7.5 µl of Gel Red (10000 x stock) was added after heating in a microwave to dissolve the agarose. This solution was allowed to cool for approximately 15 min before pouring into a suitable gel casting tray. The gel was left at room temperature to set. The gel was then placed into a tank (Bio Rad) and 1 x TAE buffer was added up to the manufacturers fill level. 3µl of sample was mixed into 1 µl Blue/Orange loading dye, 6 x (Promega) and these were loaded into the wells. The far left well contained 3 µl of 100 bp ladder. The electrodes were fitted onto the tank with the lid and 100 v were applied to the gel for 40 min using a Bio Rad PowerPac. The gel was then visualised with a Bio Rad imager using the UV light settings.

6.2.10 ELISA for H7-specific IgA in Bovine Serum

This method is taken from published work and the reagents were kindly gifted by Dr T. McNeilly (McNeilly *et al.*, 2010a). See Appendix section A.4.1 for recipes for all ELISA solutions and buffers.

An ELISA plate (Immulon 2HB, thermoelectron 3455) was coated with 50 µl H7 antigen/well at 1 µg/ml in coating buffer. The plate was then sealed and incubated at 4^{o}C overnight. All wash steps were carried out with five manual washes of wash buffer (PBST20). Blocking buffer at 100 µl/well was added and incubated for 1 hr at 37^{o}C. The plate was then washed again before the addition of the samples diluted in dilution buffer. 50 µl/well final volume was used of four doubling dilutions from 1:5 to 1:40 followed by further 1hr incubation at 37^{o}C. The plate was washed as before. The plate was incubated with 100 µl/well anti-ovine/bovine IgA (AbDSerotec MCA628) diluted 1:1000 in dilution buffer, for 1 hr at 37^{o}C and washed. An incubation with 50 µl/well of rat anti-mouse IgG1-HRP tertiary antibody (AbDSerotec MCA336P)

diluted 1:1000 in dilution buffer was carried out for 1 hr at 37^{o}C and followed with a final wash. The plate was developed with 100 µl/well of OPD substrate (SigmaFast OPD, P9187) at room temperature for 5-10 minutes and the reaction stopped by the addition of 25 µl 2.5 M H_2SO_4. The plate was read OD 492 nm using an ELISA plate reader (DYNEX Technologies MRX II) linked to a computer running MRX EndPointSoftware, Version 2.02) An average of the reading for the blank wells was calculated and subtracted from all the readings before plotting the data in Excel as a line graph.

6.2.11 Data Analyses

To ensure an approximately normal distribution of values, MTT assay data from the full bacterial panel challenge was log_{10} transformed before analysis. This data was then analysed using a one-way analysis of variance (ANOVA). The ANOVA was followed by a Tukey's post hoc test for pair-wise comparisons of means (data was tested at 95% and 99% confidence intervals). A p value of <0.05 were considered significant. Percentage viability data for the WT vs. ΔTTSS challenge was subjected to a paired t-test. P values of <0.05 were considered significant. Statistical analyses were carried out using Minitab (v13) software.

6.3 Results

6.3.1 Investigation of MOI on moDCs challenged with bacterial strains

Bovine moDCs were challenged with bacterial strains as described in section 6.2.4. Four animals were challenged with the panel of bacteria in 96 well plates. 4-6 wells were challenged for each strain. The cells were imaged and subjected to an MTT viability assay following challenge (see section 6.3.2 for MTT assay results). Results of the imaging can be seen in Figs. 6.1 – 6.4.

The *E. coli* O157:H7 strains all had an effect on the morphology of the moDCs following challenge, compared to control cells (Figure 6.4). Three O157:H7 strains visibly affected the viability of the cells as there was a great deal of cell debris, rounded, small cells and cellular particles present. Few, if any cells were observed displaying a dendritic morphology (Fig. 6.1 A, B & C). This was not the case for the cells challenged with strain MCI 0279, which did have a proportion of cells with normal morphology among some rounded dead cells and debris (Fig. 6.1 D). None of the ETEC strains had a marked affect on the morphology of the moDCs when compared to control cells; an activated morphology was observed with some cells which exhibited longer dendrites following challenge (Fig. 6.2). Three of the commensal strains gave results very similar to the ETEC strains, (MCI 0132, MCI 0465 & MCI 0490), where little detrimental effect on the viability or morphology was recorded, and only some activation phenotypes were observed (Fig. 6.3 A, B & D) However, the fourth commensal strain, MCI 0483, produced results similar to the O157:H7 strains where all the cells appeared non-viable and no dendritic morphology was observed following challenge (Fig. 6.3. C). Unchallenged cells are displayed in Fig. 6.4 for comparative purposes.

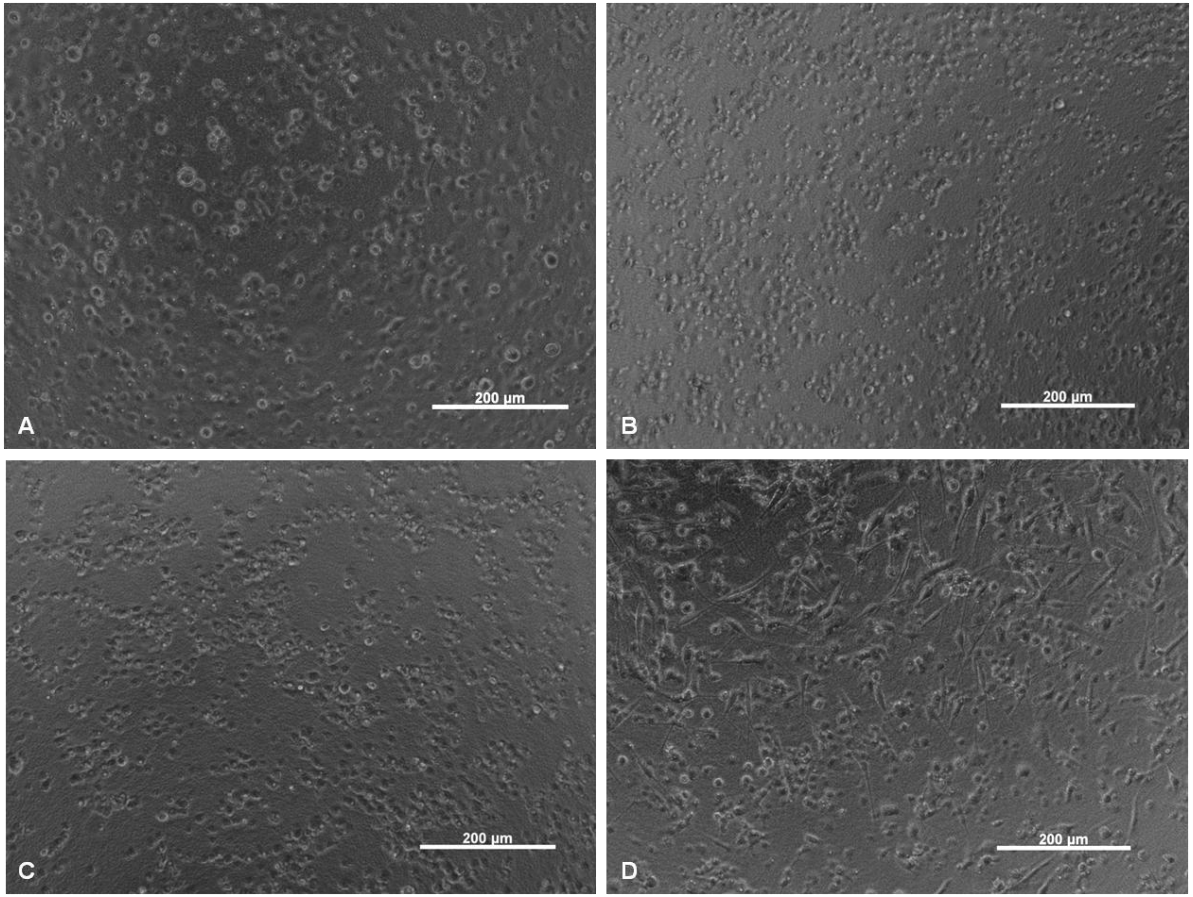


Fig. 6.1 Representative images of moDCs from four animals challenged with *E. coli* O157:H7 strains. (A) MCI 0010, (B) MCI 0191, (C) MCI 0200, (D) MCI 0279. Cells challenged for 2 h at MOI 100 followed by the addition of gentamicin to the culture medium. Images were taken approximately 24 h after challenge. Cells were challenged in 96 well plates and each strain was added to 4 – 6 wells. All strains had a pronounced effect on the moDCs. Visual inspection revealed many non-viable cells and a widespread loss of dendritic cell morphology. The changes were less obvious with strain MCI 0279 (D) where a mixed population of cells, some with normal activated DC morphology expressing long dendrites as well as a proportion of non-viable cells was observed.

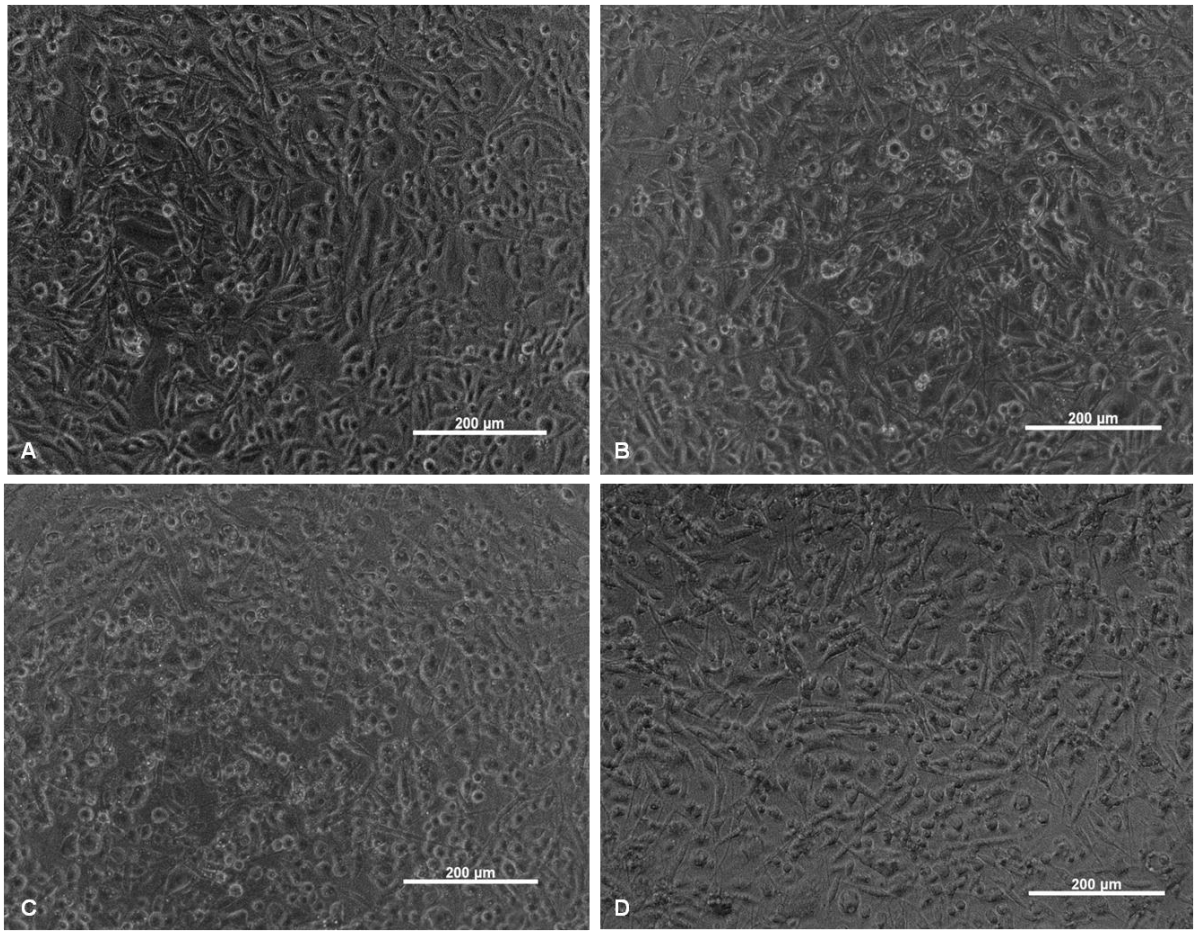


Fig. 6.2 Representative images of moDCs from four animals challenged with ETEC strains. (A) MCI 0132, (B) MCI 0688, (C) MCI 0690, (D) MCI 0693. Cells challenged for 2 h at MOI 100 followed by the addition of gentamicin to the culture medium. Images were taken approximately 24 h after challenge. Cells were challenged in 96 well plates and 4 – 6 wells per strain were challenged. All strains showed similar results. Visual inspection revealed the majority of cells showed basic dendritic morphology. The numbers of cells with long dendrites increased and the length of dendrites are also increased in comparison to control cells (Fig. 6.4).

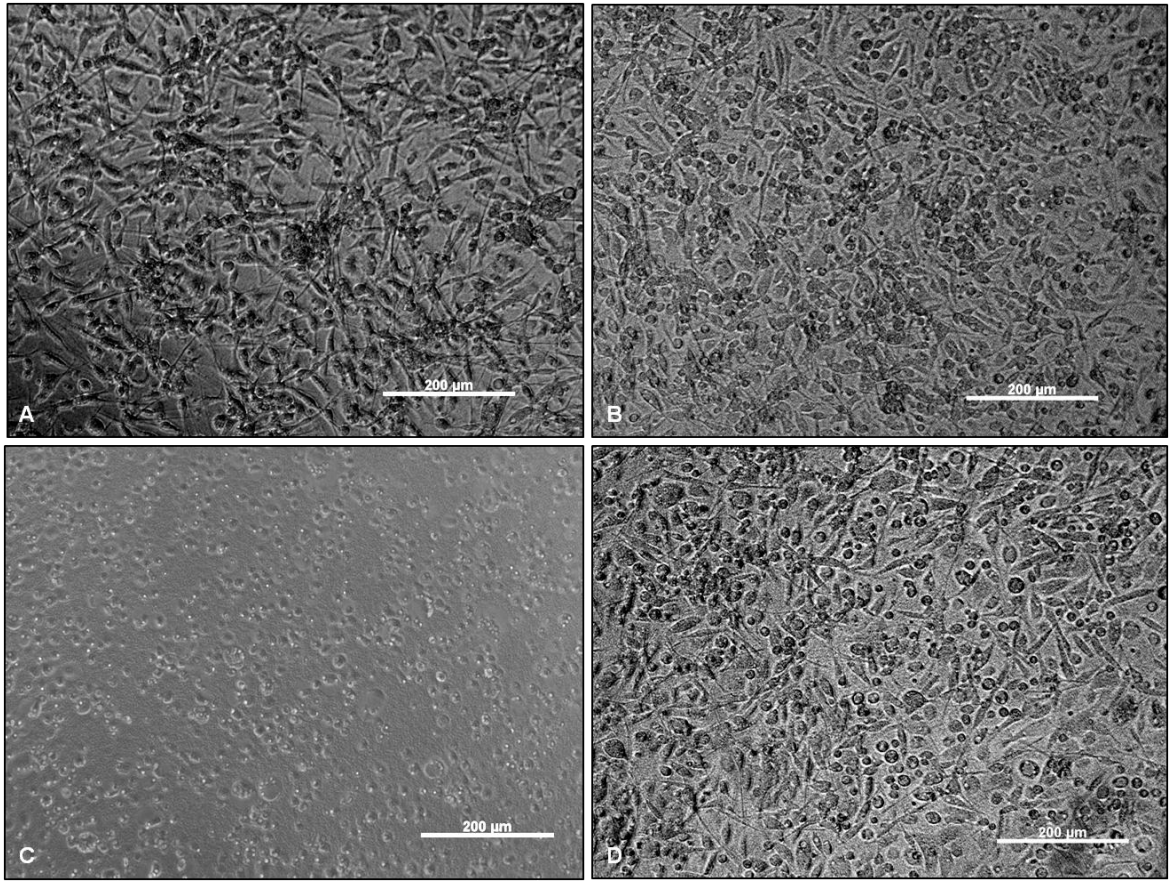


Fig. 6.3 Representative images of moDCs from four animals challenged with *E. coli* commensal strains. (A) MCI 0430, (B) MCI 0465, (C) MCI 0483, (D) MCI 0490. Cells challenged for 2 h at MOI 100 followed by the addition of gentamicin to the culture medium. Images were taken approximately 24 h after challenge. Cells were challenged in 96 well plates and 4 – 6 wells per strain were challenged. Three strains had little effect on the viability of the moDCs: MCI 0430, MCI 0465 & MCI 0490. Visual inspection of cells challenged with these strains revealed many with long thin dendrites of activated DC morphology. The changes in the cells challenged with strain MCI 0483 (C) were marked with the majority of cells appearing non-viable, with generalised loss of dendritic cell morphology.

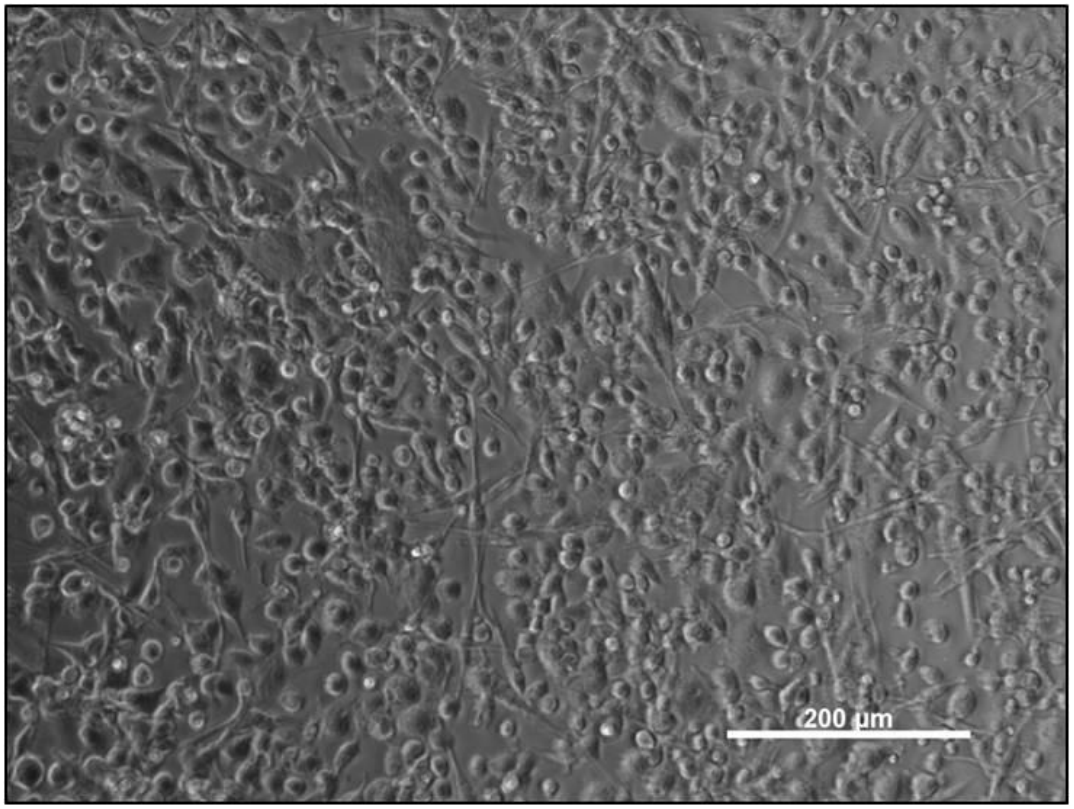


Fig. 6.4 Unchallenged moDC live cell image. A representative image of unchallenged bovine moDCs (similar images were obtained from four animals). These cells were cultured concurrently with challenged cells for the same duration and conditions. Warmed MEM/HEPES was added to the wells in place of bacterial culture. Images were taken approximately 24 h after challenge. Cells were challenged in 96 well plates and 4 – 6 wells remained unchallenged. Normal appearance of moDCs on day 4 after generation. Some rounded cells with short 'hairy' dendrites and some cells elaborating long cytoplasmic processes were observed.

An MTT assay (Molecular Probes) was employed to investigate cell viability following challenge with *E. coli* strains. This test assesses mitochondrial function and in this way indicates cellular viability. The principal of this test involves the MTT being converted to formazan by the mitochondria, and this is associated with a colour change. The change in optical density that occurs can be measured, and the higher values obtained in turn correspond to higher levels of cell viability.

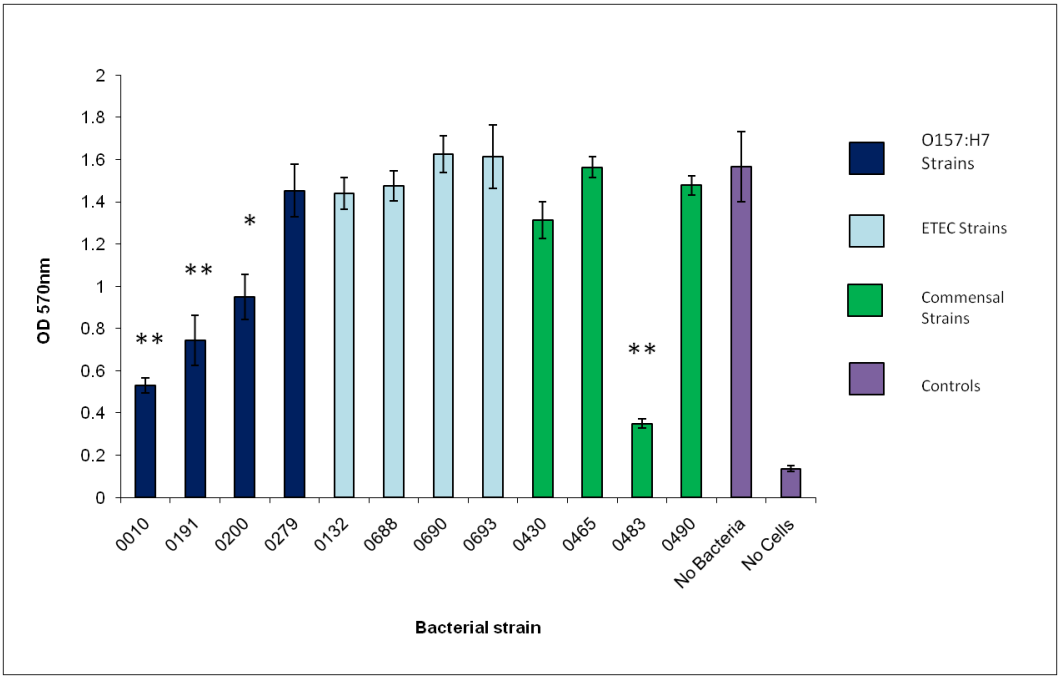
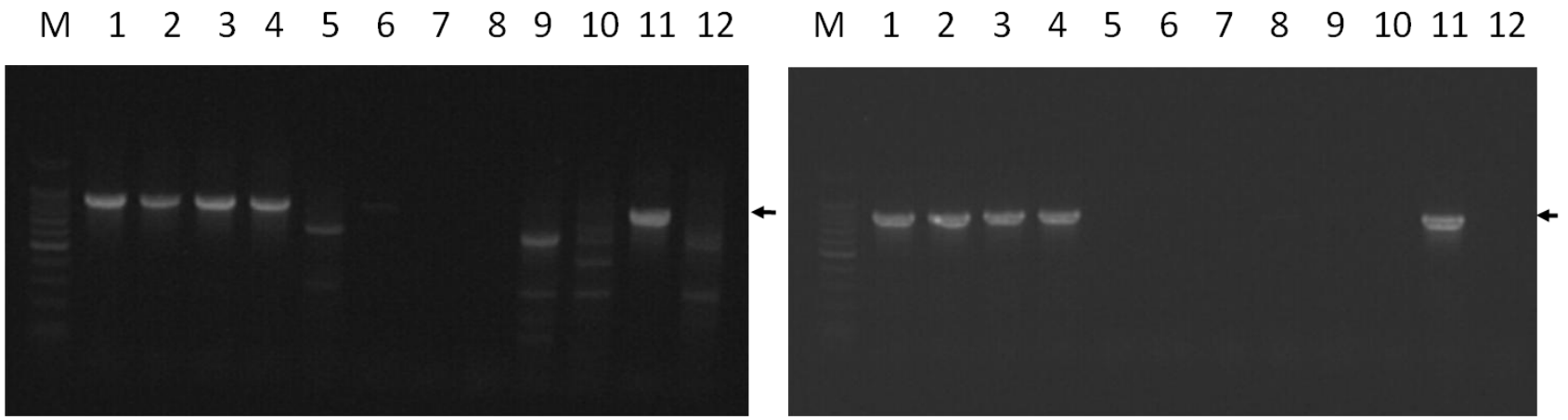


Fig. 6.5 Results of MTT viability assay of moDCs challenged with *E. coli* strains. The graph shows absorbance measured at O.D. 570nm, lower values represent reduced cellular viability. Data shows the mean of four animals, Error bars represent Standard error of the mean. (**) significance at the 99% confidence interval; (*) significance at the 95% confidence interval. Analysis using a one-way analysis of variance (ANOVA) gave a main p value <0.001. Three O157:H7 strains (0010, 0191 & 0200) produced significantly reduced moDC viability compared with unchallenged cells (0010 & 0191 significant at the 99% confidence interval, and (*) 0200 significant at the 95% confidence interval). Strain 0279 had no significant effect on moDC viability. None of the ETEC strains significantly reduced moDC viability. Of the commensal strains, 0483 significantly reduced moDC viability (at the 99% confidence interval), Strains 0430, 0465 and 0490 had no significant effect on moDC viability.

6.3.2 Investigation of bacterial virulence factors by PCR

PCR was carried out for virulence factors associated with the TTSS of EHEC on the strains from the bacterial panel, as it was postulated that the TTSS may play a role in the moDC killing observed in 6.3.1. All the O157:H7 strains were positive by PCR for *eae* and *escN*. None of the ETEC strains were positive for *eae* or *escN* using this method as expected. Of the commensal strains only MCI 0483 was PCR positive for both *eae* and *escN*. (Fig. 6.6, results summarised in table) The strains expressing eae and escN were those shown to induce reductions in moDC viability, measured by MTT assay as found in the experiments in section 6.3.1 with the exception of the O157:H7 strain MCI 0279, which was PCR positive but did not significantly reduce viability when measured with an MTT assay (section 6.4).



A **B**

Lane	Strain	*eae*	*escN*	*E. coli* Group
1	MCI 0010	+	+	O157:H7
2	MCI 0191	+	+	
3	MCI 0200	+	+	
4	MCI 0279	+	+	
5	MCI 0132	-	-	Bovine ETEC
6	MCI 0688	-	-	
7	MCI 0690	-	-	
8	MCI 0693	-	-	
9	MCI 0430	-	-	Bovine Commensal
10	MCI 0465	-	-	
11	MCI 0483	+	+	
12	MCI 0490	-	-	

Fig 6.6 PCR for *eae* (A) and *escN* (B) genes in bovine *E. coli* strains. M: 100 bp ladder. Strains were analysed for *eae* and *escN* by PCR and run in separate lanes of an agarose gel. The strains in each lane and the results obtained are summarized in the table. All O157:H7 strains were positive for both *eae* and *escN* and all of the bovine ETEC strains were negative. The bovine commensal strains, with the exception of strain MCI 0483, were negative for both *eae* and *escN*. MCI 0483 gave the same positive results for both *eae* and *escN* as the O157:H7 strains. Positive bands for *eae* and *escN* are indicated with an arrow (at expected sizes of approximately 907 bp and 856 bp respectively) (Aranda *et al.*, 2004; Kyaw *et al.*, 2003).

6.3.3 Comparison of viability of moDCs following challenge with *E. coli* O157:H7 and a corresponding ΔTTSS strain

Paired bacterial strains MCI 0200 (*E. coli* O157:H7) and MCI 0201 (ΔTTSS) were used to challenge moDCs at MOI of 100 to confirm whether the differences observed in moDC viability (section 6.3.1) was related to the bacterial type three secretion system (TTSS) as indicated by the PCR results (section 6.3.2). Initially, moDCs from three animals were challenged (401, 501, 600; group C) as previously described. Following challenge viable cells were counted and the percentage of viable cells was calculated. Using a paired T test there was a significant difference between the viability of the cells challenged with the *E. coli* O157:H7 WT strain and the ΔTTSS strain. ($p<0.01$; individual data not shown). However, these moDCs, challenged with the W.T. *E. coli* O157:H7 strain (MCI 0200) were not visibly affected to the same extent as observed in previous experiments (section 6.3.1) This was attributed, at the time, to the challenge $O.D._{600}$ nm of the bacterial cultures because this experiment had been carried out at a slightly lower $O.D._{600}$ nm of 0.27 and not 0.31. It was postulated that this lower O.D. represented a reduction in MOI close to a threshold which at which the viability of the entire population of moDCs are affected, although the bacterial numbers were not confirmed at this absorbance. These experiments were repeated with the aim of replicating the original O.D. and the effect on viability observed in the previous experiments (section 6.3.1) With this aim, the experiment was repeated with three animals from group D. The O.D. readings for the bacteria at the time of challenge were 0.32 – 0.39, to ensure an MOI of at least 100 was employed. Using Nigrosin as a vital stain, the results of this experiment showed that there was still a significant difference in numbers of viable moDC following challenge with MCI 0200 when

compared with MCI 0201. ($p<0.05$) (pooled data for all six animals is shown in Fig. 6.7) However, the previously observed effect on the morphology and viability of the total population of moDCs still was not observed. It is unclear whether this is due to differences in OD of the challenge strains or some animal factor, however, these differences are explored further in section 6.4. Although these results indicate a role for the TTSS in moDC viability further work is required to obtain more robust results.

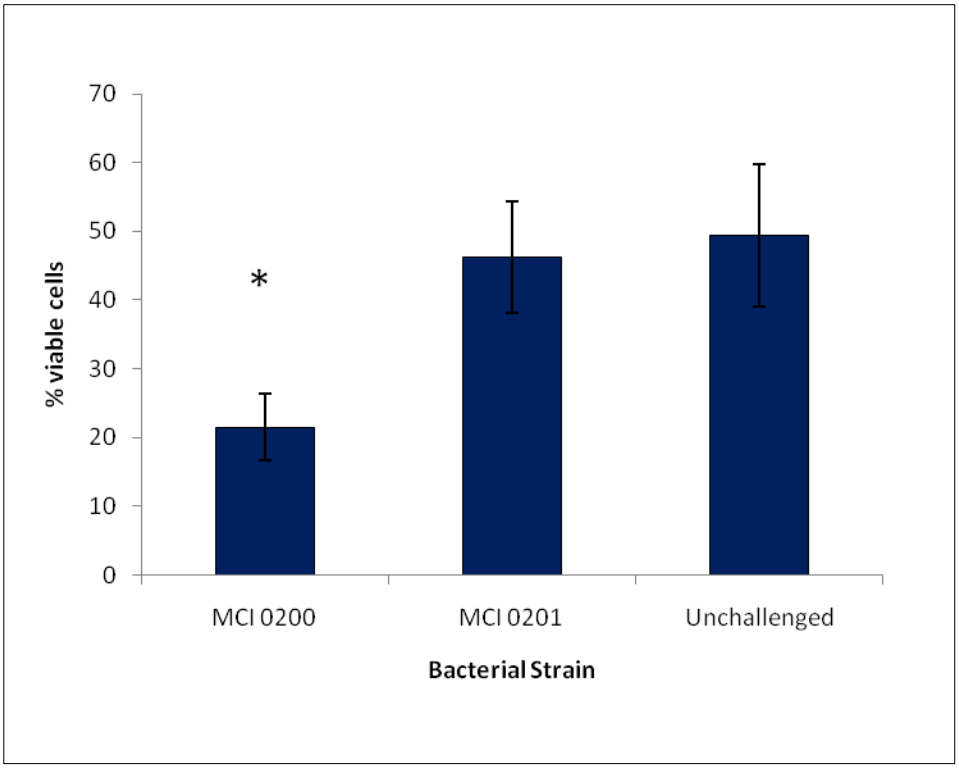
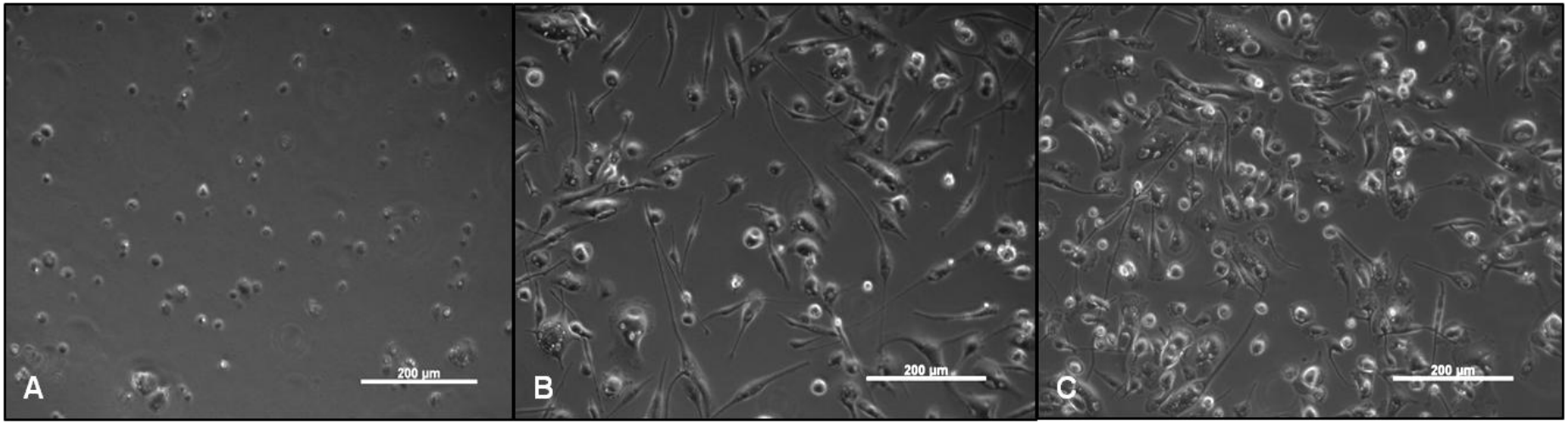


Fig. 6.7 Viability of moDCs following challenge with an *E. coli* O157:H7 strain (MCI 0200) and a corresponding ΔTTSS strain (MCI 0201). Viability was measured using Nigrosin as a vital stain. Results are pooled from six animals and error bars represent standard error of the mean. There was a significant reduction (*) in the numbers of viable moDCs challenged with MCI 0200 compared to MCI 0201. ($p<0.05$)

6.3.4 Investigation of the effect of a gentamicin-killed *E. coli* O157:H7 strain on moDC viability

Bovine moDCs were challenged using MCI 0010, as a representative *E. coli* O157:H7 strain, at an MOI of 100. A challenge with live bacteria was run concurrently with a bacterial challenge using a sample of the same challenge culture in which the bacteria had been killed. Bacterial killing was carried out during a one hour incubation with gentamicin (50 μg/ml). The pilot experiment was carried out on one animal (cow 002, group B; one of the animals from which cells were obtained for the experiment in 6.3.1) and the results showed that the gentamicin-killed bacteria had little effect on the moDC morphology or viability, while the live bacteria killed 100% of the moDCs (Fig. 6.8). Due to time constraints and limits to the number of cells that could be generated, further repeats were unable to be completed. The loss of viability of the moDCs following challenge with live and killed *E. coli* O157:H7 at MOI 100 therefore requires repeats to confirm the results from the pilot study.



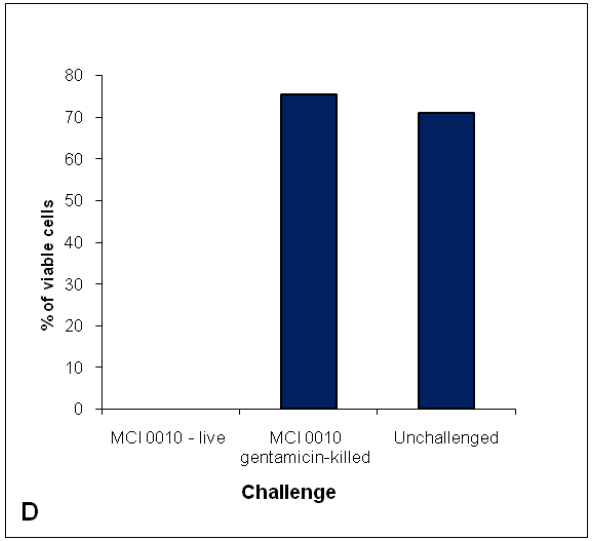


Fig. 6.8 Results from a single experiment to investigate the effect of killed bacteria on moDCs. Live cell images were taken approximately 24hrs after challenge. (A) moDCs challenged with live MCI 0010 at MOI 100 (B) moDCs challenged with a gentamicin-killed sample of MCI 0010, also at MOI 100. (C) Control moDCs: no bacterial challenge. (D) Graph of percentage of viable cells following challenge. Cell counts were carried out using the Nigrosin negative staining method and disposable counting chambers. Challenge of the moDCs with Live *E. Coli* O157:H7 clearly affected the morphology and viability of the moDCs compared to unchallenged cells. moDCs challenged with the gentamicin-killed bacteria expressed more elongated dendrites, and an active morphology compared to the unchallenged cells, This indicates that only live *E. coli* O157:H7 can affect the changes observed in section 6.3.1.

6.3.5 Investigation of H7-specific IgA responses in bovine serum

Serum samples from two of the groups of cattle housed at MRI and used throughout this study to generate moDCs, (groups C and D, see Appendix A.2 for animal details) were subjected to an ELISA for serum IgA specific for H7. The results are shown in Fig. 6.9. All animals tested were negative by this method, suggesting at the time of sampling there was no systemic immune response to H7, and therefore that it was unlikely that the donor animals were colonised with *E. coli* O157:H7 at this time. The IgA immune response is discussed further in section 6.4.

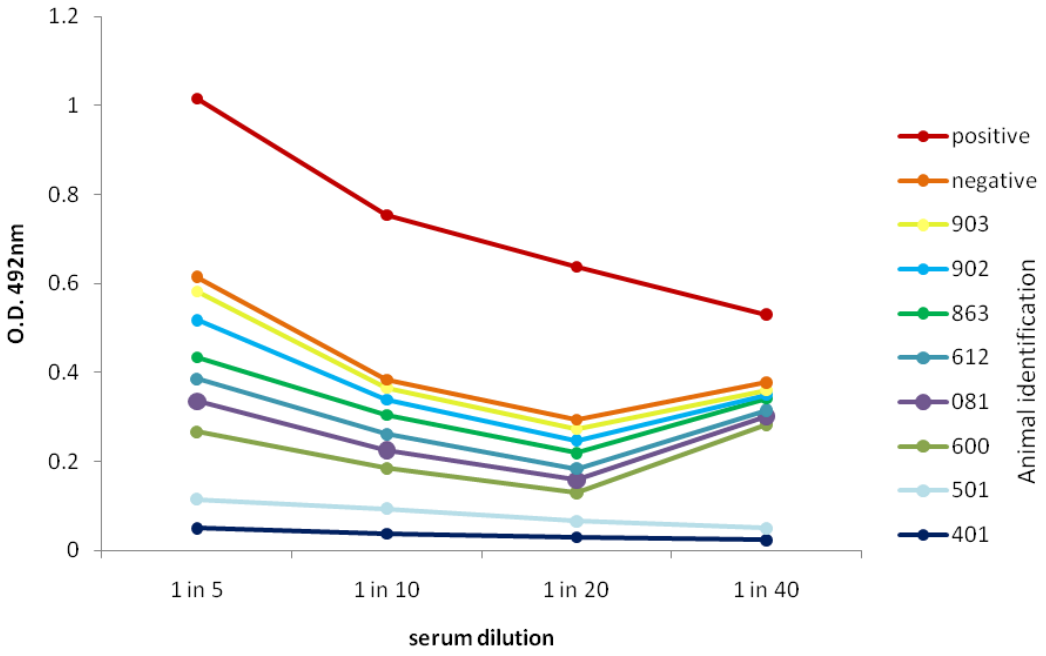


Fig. 6.9. Dilution series of samples from cattle tested for serum H7 - specific IgA by ELISA. All cows tested by this method were negative for serum H7 - specific IgA responses. The O.D. at 492 nm for all the animals tested was below the level of the negative control. Negative and positive serum samples, gifted by Dr. T. McNeilly were from challenged animals (McNeilly *et al.*, 2008).

6.4 Discussion

Using four strains each of *E. coli* O157:H7, ETEC and commensal *E. coli* strains, the results following challenge of moDCs yielded some interesting differences. At this MOI the *E. coli* O157:H7 strains and one commensal strain (MCI 0483) appeared to affect the viability of the moDCs when visually examined. With all other strains no effect on viability by visual examination was discovered. In order to investigate this phenomenon in more depth, the challenge was repeated in four animals in total, images of the cells were taken and the cells were subjected to a viability test using the MTT assay. The results were very similar for all the animals studied. Visually the cells challenged with the O157:H7 strains and also commensal strain MCI 0483 were affected, while the remaining strains left the moDCs activated but still viable, and recognisable as DCs. One difference within the O157:H7 strains became clear. MCI 0279, originally known as the TT12B strain (Feng *et al.*, 2001), did produce changes in the moDC morphology and viability, although the effects were not as pronounced as with the other O157:H7 strains and did not affect the entire cell population. A proportion of cells retained normal activated morphology. The MTT assay in practice confirmed the microscopy results. Significant reductions in viability were found for O157:H7 strains, MCI 0010, 0191 and 0200 as well as the commensal strain MCI 0483. No significant reduction in viability was noted for MCI 0279, the commensal strains MCI 0430, 0465 & 0490, or any of the ETEC strains.

This suggests that interaction with DCs is substantially affected by *E. coli* pathotype. O157 strains are typified by TTSS and it has been reported that some ruminants are colonised asymptomatically by LEE-positive *E. coli* pathotypes (Aktan *et al.*, 2004). Therefore, it is possible that MCI 0483, despite

possessing known virulence factors, could still function as a bovine avirulent strain and the rigid parameters for bovine 'commensal' strains employed by this study may be too narrow to represent the true *in vivo* situation.

Intimin is encoded by the gene *eae* which is found within the LEE5 operon of the LEE. Intimin is an outer membrane protein of EHEC which is important for attachment to the host cells by its interaction with its receptor Tir. Tir is secreted by the bacterium through the TTSS apparatus and embeds within the host cell membrane enabling the intimate attachment with A/E lesions to occur, and the formation of microcolonies (Kenny *et al.*, 1997; Kenny & Finlay, 1997). Intimin and Tir are two of the bacterial components currently being studied with respect to production of an O157:H7 vaccine (Amani *et al.*, 2010; McNeilly *et al.*, 2010b). EscN is a protein encoded for by the LEE3 operon. It functions by hydrolysis of ATP to produce energy for the TTSS (Garmendia & Frankel, 2005). The structure and function of the TTSS is discussed further in Chapter 1.

To investigate further a potential role for the TTSS in these moDC phenotype differences, PCR was carried out for the genes for intimin (*eae*), and TTSS (*escN*) in all the bacterial strains. The PCR results went some way to confirming the hypothesis. All of the O157:H7 strains and the commensal strain 0483 were PCR-positive for both *eae* and *escN.* This indicates that the reduced moDC viability following challenge is related to the TTSS.

The differing moDC viability that were discovered between the TT12B strain (MCI 0279) and the other O157:H7 strains could also be caused by TTSS effects. When preparations of secretomes from all the O157:H7 strains used

in this work were made by TCA precipitation of *E. coli* secreted proteins the TT12B strain appeared to have reduced secretion of the TTSS components EspA and EspB. (Dr. D. Fraser-Pitt, Personal Communication) This lends further weight to the argument that the TTSS is of great importance during the interactions of *E. coli* strains and bovine DCs.

The commensal strains were selected from a concurrently running study in which a colleague sampled and characterised *E. coli* strains from the bovine intestinal tract and carried out laboratory tests for numerous virulence factors, including VT, Intimin and TTSS to assign strains into commensal and pathogenic groups. At the time the commensal panel of bacteria were selected for this study, no virulence factors had been positively identified in any of these strains; it was only after the moDC challenges had commenced that the strain 0483 was found to be positive for *eae* and *escN* by PCR (confirmed by this study). So by the definition used for this study, *E. coli* strain MCI 0483 does not belong in the group alongside avirulent, commensal strains. The definition of a commensal strain for this study was: an *E. coli* strain present in moderate numbers in several intestinal locations in more than one animal which has not been identified to be carrying any virulence factor genes. During the rest of this discussion, strain MCI 0483 is excluded from the group of commensal strains.

The fact that comparable numbers of ETEC and commensal strains did not cause the cellular killing seen with the O157:H7 strains at this MOI, suggests that it is not merely the numbers of bacteria, or quantity of bacterial components (LPS or flagellin for example) or the bacterial growth rate during the challenge time that overwhelms the moDCs as these were comparable for

all the strains. The bacterial growth rates during challenge were investigated in Chapter 4 and although the O157:H7 strains tended to reach O.D. readings nearer the top of the range recorded during the two hour challenge period, the strain that reached the highest O.D. overall was a commensal strain MCI 0490. MCI 0483 which produced a pronounced killing effect had one of the lower final O.D. readings. So although it is important to consider the varied growth rates of the bacterial strains during the challenge period, it cannot account for the results generated here. From the results presented here it is most likely that the presence of bacterial TTSS accounts for the differences observed in moDC viability. The role of the TTSS in the viability of the moDCs at this MOI is very interesting and could be the key to our understanding of the immune responses of cattle to *E. coli* O157:H7. The EHEC TTSS has been confirmed to be essential for colonisation in this species (Dean-Nystrom *et al.*, 1998; Naylor *et al.*, 2005a) and although this strain does not cause clinical disease in cattle it clearly makes use of this virulence factor to colonise and persist in this host and therefore disseminate further. The finding that TTSS PCR-positive strains induced killing of moDCs at MOI 100, whereas TTSS PCR-negative strains did not indicates the potential role for DCs in the colonisation of cattle by O157 *in vivo*. It is clear that the TTSS modulates DC function and viability demonstrating the potential significance of DCs in O157 colonisation and perhaps other LEE-positive *E. coli*. DCs are integral in generating immune responses to pathogens (Steinman, 1991), and if these vital immune cells can be killed by bacteria *in vivo*, as demonstrated here *in vitro* it could greatly reduce the rate of bacterial elimination and promote persistence within the hosts. This possibility would be difficult to test however and beyond the scope of this project.

In the pilot experiment with the live bacteria compared to killed EHEC the different effect observed during challenge was marked. Replicates would enable this result to be confirmed, nonetheless, it does indicate that a live bacterium is required to effect the changes in viability observed in the moDCs. This could also be related to the TTSS because a killed bacterium is unable to secrete proteins via the TTSS structure. The killed bacteria are still 'sensed' by the moDCs as they take up a more activated morphology. This correlates with research into *Salmonella* where the moDCs still recognise killed *Salmonella* as characterised by phenotypic changes in expression of cell surface molecules (Norimatsu *et al.*, 2004).

The method of moDC death in this study is presumed to be apoptotic as this has been found in other studies (Alileche *et al.*, 2005; Falcone *et al.*, 2004). Apoptosis also indicated by the changes in morphology noted on visual examination (rounding and loss of dendrites) however, further more definitive tests would be required for confirmation such as electron microscopy and FACS analysis for annexin V labelling.

Research has indicated that around 10% of cattle could be asymptomatically colonised with *E. coli* O157:H7, (discussed in (Gansheroff & O'Brien, 2000)), and that serum response (IgA) to H7 can be used as a correlate of colonisation (McNeilly *et al.*, 2008). Selected donor animals were assessed serologically for IgA specific to H7. Excretion of *E. coli* O157:H7 in the majority of colonized animals usually occurs for less than a month (Besser *et al.*, 1997), and the half-life of serum IgA is a matter of days so it is unlikely that serum responses occur for weeks after colonization is resolved (Butler *et al.*, 2006). A proportion of animals may be colonized for longer periods of

time however and discussion of the consequences of *E. coli* colonization and the outcomes for affected animals is discussed in further depth in Chapter 1.

Where it was practical to do so, serum samples were tested for H7 specific IgA, and all were negative. IgA responses from all the animals could have been checked at time points throughout the study possibly with concurrent faecal sampling for *E. coli* O157:H7 strains to assess whether colonisation affected the *in vitro* results. In reality this was not a practical option though and also was not the aim of this study, although it is a factor which should be taken into consideration. This type of sampling also would not rule out concurrent infections or pathologies caused by other organisms, and it would be impractical to carry out numerous clinical tests before each set of moDCs was generated. As a general rule the animals were only used for sampling if they appeared clinically healthy as determined by non-invasive methods. During the length of time over which this study was carried out, several animals were euthanized for unrelated reasons and therefore serum samples from all animals were not available at the time of testing. However, because the presence of serum H7 specific IgA is a transient response, the possibility that the animals had been exposed to and colonised with *E. coli* O157:H7 at some point during this study cannot be excluded. The other two groups of animals that were unable to be tested in this way equally could have been colonised with EHEC at some time during the study and it is possible that this could account for the varied responses of the moDCs to *E. coli* strains *in vitro*. Concurrent colonisation of the donor animals with EHEC, or indeed infection with other pathogens could potentially affect the responses of the moDCs *in vitro* and attempts should be made to eliminate this where possible although much further work in this field would be required to confirm the

precise nature of effects, and would be beyond the scope of a project such as this.

The results from the WT and ΔTTSS challenge in this chapter indicate that bovine moDC responses are subject to animal variation. Research using bovine hosts can be complex in several ways. Firstly, compared with the availability of reagents for murine or human research there are many fewer existing reagents and protocols with confirmed efficacy for the veterinary researcher. Also, in many ways there can be greater diversity in responses from animal species than with human or inbred murine strains. As shown in Chapter 3, there was great animal to animal variation in the quality of the tissue cell preparations and cell numbers recovered and although these differences were not confirmed to be due to individual animal factors, it must be a likely consideration. Also, Chapters 4 and 5 indicate that although trends in responses between cows may be similar, that the magnitude of responses can be very disparate. Furthermore, concurrent research has shown that bovine monocytes from approximately 60% of cattle tested responded to VT challenge by producing cytokine, with the remaining 40% not responding in this way. These animals were sourced from different geographical locations and rearing systems and were of varied breed, age and sex, and none of these factors correlated with the divergent responses observed. (Dr. P. Cameron, personal communication) In addition those results were obtained from numerous donor animals which included some of the same animals used in this study. It has been put forward that the differences noted could be due to genetic differences and to the heterogeneous genetic nature of integral immune components such as pattern recognition receptors (PRR) in bovine hosts (Seabury *et al.*, 2010).

Prior exposure to unknown stimuli, or concurrent sub-clinical infections in some of the donor animals could also be a consideration. A definitive reason for these animal differences would be difficult to confirm. None of the MRI cows were positive for H7-specific IgA suggesting that they were free from EHEC colonisation. It would be an advantage to replicate the study here with larger numbers of donor animals selected from outbred populations of cattle which have a more defined immune status.

To summarise the findings in this chapter, DC responses vary considerably with *E. coli* pathotypes. This study shows that *E. coli* O157:H7 strains kill moDCs *in vitro.* One *E. coli* strain, isolated from a clinically normal animal indicating strain avirulence, was VT-negative and LEE positive and was also found to kill moDCs. These results indicate that the TTSS is important and may play a significant role *in vivo* in modulating DC activity and promoting colonisation by LEE positive strains, at DC-rich sites. It has been published that macrophages can be killed in a TTSS dependant manner by other organisms *in vitro* including *Yersinia, Psudomonas* and *Shigella* strains (Hauser & Engel, 1999; Mills *et al.*, 1997; Schroeder *et al.*, 2007). No publications to date have been found which extend these findings to dendritic cells or *E. coli* strains. Therefore the results presented here corroborate the importance of TTSS with respect to the viability of APCs following infection but more importantly show a novel consequence of DC and EHEC interactions *in vitro.* The work presented here strongly indicates a role for the EHEC TTSS in colonisation of the bovine host by affecting the viability of the DCs and potentially subverting immune responses to and clearance of *E. coli* O157:H7.

Chapter 7 Conclusions

7.1 Summary of Project Objectives

E. coli O157:H7 is a pathogen which has serious effects on human health. Ruminants have been identified as a significant reservoir for this strain and within the bovine intestine it shows tropism for the terminal rectal tissues (Naylor *et al.*, 2003). These terminal rectal tissues are characterised by the presence of numerous lymphoid follicles which contain important immune cell types. Cattle are not clinically affected by colonisation with this organism, and thus they can contribute to spread of EHEC into the environment, directly to humans following contact and also into food and water sources. Both bacterial and host factors may play significant roles in *E. coli* O157:H7 colonisation persistence and excretion by cattle. Bacterial factors have been examined extensively but there is much less literature regarding potential host factors involved. In particular, there are few reports describing the interactions of *E. coli* O157:H7 with systemic or mucosal immune cells. As significant mucosal immune cells, DCs play a key role in directing immune responses towards Th1, Th2 or tolerance and these cells are the focus of this project. The aims of this study were to:

1. Characterise the immune cells in the tissues of the bovine large intestine at several locations, including the terminal rectum, with a focus on DC populations (Chapter 2).
2. Develop a protocol to extract DCs from the terminal rectum tissues for further characterisation and downstream investigations (Chapter 3).
3. Characterise a bovine moDC culture system and investigate the reactions of these cells with bacterial virulence factors including LPS, VT2 and H7 flagellin (Chapter 4).

4. Investigate the responses of moDCs to various *E. coli* strains at low and high MOI, to include measuring cytokine responses, changes in cell surface molecule expression, viability, phenotype and bacterial phagocytosis. (Chapters 5 & 6)

7.2 General Discussion

The tissue tropism of *E. coli* O157:H7 to the bovine terminal rectum has been documented (Naylor *et al.*, 2003) but the reasons for this colonisation pattern have not yet been fully elucidated. This project aimed to investigate bovine DCs, as a cell type expected to be found in this anatomical location, which could provide insight into the asymptomatic carriage of EHEC by cattle. As cells which have central roles in directing immune responses to encountered organisms and which are closely associated with epithelial layers, DCs are very likely to interact with *E. coli* O157:H7 within the terminal rectum of the bovine host, and therefore it is likely that the intestinal DCs play a key role in the persistence and colonisation of ruminants by this organism. It is important to characterise the cell types which are potentially involved in the interaction with bacteria *in vivo* to try to develop methods of intervention which can ultimately reduce carriage by the cattle population and subsequent human infections. *In vitro* work characterising the intestinal DC populations and DC interactions with cultured cells and *E. coli* O157:H7 is the first step to understanding the *in vivo* situation, and is of importance to supplement knowledge of bovine cellular immunology as well as to provide a basis for future studies, which could generate these effective control strategies.

The work in this thesis has provided several main findings of potential significance to factors affecting bovine colonisation with *E. coli* O157:H7.

Firstly, the cell types within the intestinal tissues including the terminal rectum of cattle were investigated. IHC at several sites showed widespread immune cells throughout the intestine, and these occurred with greater

frequency at areas populated by lymphoid follicles. Using the working definition of large irregular cells expressing MHC II as DCs, as used by numerous other studies, many DCs were identified within the bovine intestine. A number of molecules which have been reported to be expressed by DCs were also investigated. High level expression of MHC II, and CD205 and CD40 in particular, was observed within all tissues and notably within the follicles, suggesting numerous immune cell types in this area. Considering the morphology of the positively stained cells it was clear that a significant proportion of these cells were DCs. Other molecules demonstrated different expression patterns within the tissues; SIRPα and CD1b were found to be expressed at much lower frequency in follicle cell populations but strong expression of both of these molecules were observed around the follicle periphery. It is not clear whether these cells were co-expressing CD1b and SIRPα and further co-localisation studies would be required to confirm this possibility. However co-localisation of MHC II was observed on large irregular cells expressing CD205, CD1b and SIRPα, and significant differences were observed when expression patterns were investigated with respect to intestinal site. The significance of different cell populations found at these anatomical sites suggests the host's microenvironment including antigen availability; cellular receptors and chemokines are indeed varied at these sites and could impact on the ability of various pathogens to colonise at these different areas within the intestine. Although specific subsets of DCs could not be definitively characterised, the varied expression patterns suggests that subsets do exist in the bovine intestine as has been identified in numerous other species and tissues. This was the first study of these cell types in the tissues of the bovine large intestine and has provided working protocols and a preliminary

characterisation of these cells, which future work will be able to successfully build upon.

Some intriguing findings resulted from the experiments which investigated the dynamics of intracellular bacteria. It was revealed that an *E. coli* O157:H7 strain was found in greatly reduced numbers within moDCs compared to both an ETEC and a commensal strain, at all times during a period of study (36 h). The highest numbers of viable intracellular bacteria were found for the commensal strain, and these numbers remained high until 12 h post challenge after which time the numbers declined to near comparable levels with the ETEC strain. The ETEC and O157 strain both maintained their intracellular numbers up to the 36 h limit of the study. This could have important implications for the interaction of *E. coli* O157:H7 and DCs *in vivo* as discussed (Section 5.4). These findings indicate that O157 appears to suppress its own uptake by DCs as well as resist intracellular killing. Resistance of phagocytosis as well as prolonged intracellular survival could both be important factors promoting persistence within hosts. Further work is required to confirm these novel findings and to assess any correlation with the presence and dynamics of intracellular of *E. coli* O157:H7 *in vivo.*

Bacterial killing of immune cells is an important mechanism whereby it may be possible for pathogens to evade immune responses. The work in chapter six revealed that *E. coli* strains were able to kill moDCs *in vitro* in a dose-dependent manner. This killing also appeared to be linked to the presence of TTSS within these strains. Although the TTSS has been shown to be important in colonisation with EHEC (Dean-Nystrom *et al.*, 1998; Naylor *et al.*, 2005b) this is the first time that the TTSS has been implicated in this way

in the bovine host with respect to the viability of DCs and points to a potential role for DCs in the colonisation of cattle by O157 *in vivo*. It is clear that the TTSS modulates DC function and viability and because DCs are key cells directing immune responses (Steinman, 1991) the possibility that these immune cells can be killed by bacteria *in vivo*, as demonstrated in this work *in vitro* it could significantly affect bacterial carriage within the intestine by potentially reducing the rate of bacterial elimination.

Throughout this work animal to animal variation affected the reliability of the results. Not only were tissues variable between cows, with respect to the ease of collecting *ex vivo* cells, but also in the responses of the blood-derived cells to bacterial agonists and whole bacteria. Often moDCs responses followed the same trend but with greatly differing magnitude, however there were also occasions where even the responses were disparate. This variation meant that although sometimes trends could be observed, statistical significance could not be placed on a number of these results. The small sample sizes also reduce the significance of any data collected. It would be important to take these animal variations into account if at all possible when planning future experiments. This could be possible in a number of ways, either to use larger sample sizes to try to observe more consistent trends in data sets, or to assess the responses of the animals and select animals for further study which tend to respond similarly. Although this second method does exclude data, it could reduce the number of animals used for experiments in which data sets are so disparate that nothing relevant can be concluded. Problems with disparate animal data have been experienced by many researchers working in the field of ruminant research; however this

does not tend to be a problem encountered by colleagues working in murine and human research fields.

A further practical aspect affecting the generation of large quantities of data from bovine systems is the relative lack of specific reagents and protocols. Although numerous groups are involved in the investigation of bovine cellular responses, new reagents only infrequently become available. It is hoped that in the future bovine research will become an even more active area as further reagents and protocols are developed.

An important point of note is that epithelial cells will play an important role in the interactions between DCs and bacteria *in vivo* both as a structural barrier between these cells and as secretors of various signalling molecules, and these factors have not been taken into account in these experiments. It would be important to develop the moDC culture further to include co-culture techniques or challenges with epithelial cell supernatants or migration assays. However, there is to date no well-characterised bovine intestinal cell line with which to co-culture the moDCs so the use of complex primary cell extraction techniques would have to be employed, this no doubt would require significant time to be spent optimising suitable conditions, before it could be used for challenges. Indeed, previous attempts within the group to generate immortalised bovine intestinal epithelial cells proved complex and were ultimately unsuccessful.

In conclusion, the data presented within this thesis puts forward several novel findings relevant to the interaction of *E. coli* O157:H7 with DCs and

opens up several important areas of work for future studies that could lead to the design of vaccines or immunotherapeutic agents.

7.3 Conclusions and future areas for study

In Chapter 2, Immunohistochemistry (IHC) techniques were carried out in bovine intestinal tissues. Cells expressing surface molecules characteristic of dendritic cells (DCs) were identified. This was the first IHC survey of these cell types within the bovine intestine. Cells with DC characteristics were distributed throughout the intestinal tract, and occurred more frequently in lymphoid follicle-dense regions. These observations were consistent with those obtained in other studies in other species. The terminal rectal mucosa was richly-populated with cells of DC phenotype, and co-localisation of immune cell molecules was observed. However, further investigation perhaps utilising three colour IHC techniques could be used to confirm further co-localisation of surface molecules characteristic of DCs. A relevant future IHC study would be to compare DC populations in the terminal rectum of O157:H7 colonised cattle with those within O157:H7 negative tissues. This would be useful to observe any effect that colonisation has on the cellular expression and anatomical locations of the DCs.

Chapter 3 appraised enrichment and culture protocols for *ex vivo* terminal rectal DCs to facilitate characterisation of these cells as well as the study of bacterial DC interactions. However, these techniques produced inconsistent cell yields which were insufficient to use routinely. Further work is required to build upon the techniques developed here. If cells could be collected from these tissues consistently then a FACSort system for collected cells could prove to be a useful tool.

Chapter 4 established derivation of DCs *in vitro* from blood monocytes (moDCs) as a reproducible procedure, and confirmed the phenotypic characteristics of these cells to conform to published parameters. The bovine moDCs were also challenged with various bacterial agonists; however these experiments were hampered by technical difficulties. In contrast to published work detectable TLR5 transcripts were not found in the moDCs and further investigation of this feature should be carried out to compare the effects of culture time and also to collect data from more animal donors.

In Chapter 5 the *in vitro* cultured moDCs were challenged with various *E. coli* strains. O157:H7, bovine ETEC and bovine commensal *E. coli* challenges of bovine moDCs *in vitro* all led to cellular changes consistent with DC activation. These bacterial pathotypes showed diverse outcomes with respect to internalisation and intracellular survival. An O157:H7 isolate was least efficiently internalised by moDCs whereas a commensal strain was internalised at significantly higher numbers. Viable bacteria of all strains survived for extended periods within moDCs, however, only the numbers of intracellular commensal *E. coli* began to decline after 12 h, in contrast to the numbers of O157:H7 and ETEC which remained relatively constant up to 36 h. This was an important feature of moDC-bacterial interaction and an important area for further work would be to address determinants of resistance to phagocytosis and intracellular killing. The TTSS should be one such target, to assess any role for TTSS specific effectors in these cellular events. In addition to adding biological replicates to the data sets it would also be useful to employ techniques to investigate the intracellular bacteria further. IHC and FACS would be useful for visualisation and localisation of the intracellular bacteria and to assess the numbers of cells affected at each

time point and whether bacterial multiplication can occur within moDCs. A longer time course would also be of interest to assess how long *E. coli* strains are able to survive the moDC intracellular environment. Repetition of this experiment with other strains may be able to validate attribution of these differences to pathotype rather than purely an effect of strain selection.

The work in Chapter 6 investigated the role of MOI in bacterial challenges and discovered that *E. coli* O157:H7 showed dose-dependent killing of bovine moDCs in what appeared to be a TTSS-dependent manner. These experiments were affected by animal variations and further work would be required to fully elucidate the moDC killing by *E. coli* O157:H7.

To conclude, this work has shown that the contact of *E. coli* O157:H7 with DCs *in situ* is likely and furthermore that O157 has systems to overcome DCs by inhibiting cellular uptake and preventing or delaying intracellular killing. Manipulation of the interaction between O157 and DCs *in vivo* represents a means by which host response could be targeted to reduce or eradicate *E. coli* O157:H7 in cattle and in consequence reduce the public health impact or disease caused by this organism.

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Appendices

A.1 Antibodies used in this study

A.1.1 Primary Antibodies

Antibody	Molecule Recognised	Isotype	Reference	Application	Working concentrations	Source and target species	Supplier (Cat. Number)
IL-A88	MHC I	IgG2a	Toye *et al.*, 1990	FACS	1 in 10	Mouse anti-bovine	IAH
CC158	MHC II	IgG2a	Howard *et al.*, 1993	FACS	1 in 10	Mouse anti-bovine	IAH
IL-A156	CD40	IgG1	Whelan *et al.*, 2003	FACS	1 in 1000	Mouse anti-bovine	IAH
IL-A158	CD80	IgG1	Whelan *et al.*, 2003	FACS	1 in 1000	Mouse anti-bovine	IAH
IL-A190	CD86	IgG1	Norimatsu *et al.*, 2003	FACS	1 in 1000	Mouse anti-bovine	IAH
CC14	CD1b	IgG1	Howard *et al.*, 1991	FACS	1 in 10	Mouse anti-bovine	IAH
CC149	SIRPα	IgG2b	Howard *et al.*, 1999	FACS	1 in 10	Mouse anti-bovine	IAH
CC98	CD205	IgG2b	Howard *et al.*, 1993	FACS	1 in 10	Mouse anti-bovine	IAH
CC43	CD1w3	IgG2b	Howard *et al.*, 1993	FACS	1 in 10	Mouse anti-bovine	IAH
MM1A	CD3	IgG1	MacHugh *et al.*, 1998	FACS	1 in 1000	Mouse anti-bovine	IAH
CC17	CD5	IgG1	Howard *et al.*, 1988	FACS	1 in 10	Mouse anti-bovine	IAH
CC81	CD13	IgG1	Howard *et al.*, 1997	FACS	1 in 10	Mouse anti-bovine	IAH
CC69	CD26	IgG1	Gliddon *et al.*, 2002	FACS	1 in 10	Mouse anti-bovine	IAH
AV20	Bu-1 on chicken Bursal Cells	IgG1	Rothwell *et al.*, 1996	FACS (Isotype)	1 in 100	Mouse anti-chicken	IAH
AV29	Chicken CD4+ cells	IgG2a	Werling *et al* 1999	FACS (Isotype)	1 in 100	Mouse anti-chicken	IAH
AV37	Chicken spleen cell subset	IgG2a	Gliddon *et al.*, 2004	FACS (Isotype)	1 in 100	Mouse anti-chicken	IAH

A.1.1 Continued

Antibody	Molecule Recognised	Isotype	Reference	Application	Working concentrations	Source and target species	Supplier (Cat. Number)
SW73.2	MHC II	IgG2b	Hopkins *et al.*, 1986	IHC	1 in 500 - 1 in 10000	Rat anti-ovine	MRI
IL-A156	CD40	IgG1	Whelan *et al.*, 2003	IHC	1 in 20	Mouse anti-bovine	MRI
CC20	CD1b	IgG2a	Howard *et al.*, 1993	IHC	1 in 10	Mouse anti-bovine	MRI
MCA831 (CC14)	CD1b	IgG1	MacHugh *et al.*, 1988	IHC	1 in 20	Mouse anti-bovine	AbD Serotec (MCA831)
CC149	SIRPα	IgG2b	Howard *et al.*, 1999	IHC	1 in 100	Mouse anti-bovine	AbD Serotec (MCA2041S)
MCA1651G (CC98)	CD205	IgG2b	Howard *et al.*, 1993	IHC	1 in 10 - 1 in 100	Mouse anti-bovine	AbD Serotec (MCA1651G)
IL-A16	CD11c	IgG1	Howard *et al.*, 1991	IHC	1 in 10	Mouse anti-bovine	IAH
VPM65	CD14	IgG1	Gupta *et al.*, 1996	IHC	1 in 50	Mouse anti-ovine	MRI
K84 2F9	IgA	IgG1	McNeilly *et al.*, 2008	H7 ELISA	1 in 1000	mouse anti-bovine/ovine	AbD Serotec (MCA628)
LO-MG1-2	IgG1 Heavy Chain	IgG1	McNeilly *et al.*, 2008	H7 ELISA	1 in 1000	rat anti-mouse	AbD Serotec (MCA336P)

A.1.2. ELISA Antibody pairs

Cytokine	Capture mAb	Isotype	Working Conc.	2° Biotinylated mAb	Isotype	Working Conc.	Source and target species	Supplier
IL-10	CC318	IgG2b	1 µg/ml	CC320-b	IgG1	1 µg/ml	mouse anti-bovine	IAH
IL-12	CC301	IgG2a	1 µg/ml	CC326-b	IgG2b	8 µg/ml	mouse anti-bovine	IAH
TNFα	CC327	IgG2b	2 µg/ml	CC328-b	IgG2a	1 µg/ml	mouse anti-bovine	IAH

A.1.3. Fluorochromes

Fluorochrome	Specificity	Supplier (Cat. Number)	Application	Working Concentration
Alexa Fluor 568 tyramide	HRP signal amplification	Invitrogen (T20914)	IHC	1 in 100
Alexa Fluor 488	donkey anti-rabbit IgG (H + L)	Invitrogen (A21206)	IHC	1 in 1000
Flurescein	rabbit anti-rat IgG (H + L)	Vector (FI-4001)	IHC	1 in 500
Peroxidase-labelled polymer	goat anti-mouse Ig	DakoCytomation (K4007)	IHC	Neat
FITC	goat anti-mouse Ig	Sotuhern Biotech (1012-02)	FACS	1 in 400

A.2 Animal Details

Identification	Location	Breed	Date of Birth	Sex	Group
100439	IAH	Holstein Friesian	October 2000	M	A
500009	IAH	Holstein Friesian	July 1999	M	
600010	IAH	Holstein Friesian	August 1999	M	
700320	IAH	Holstein Friesian	October 2000	M	
002	EBVC	Holstein Friesian	August 2006	M	B
481	EBVC	Holstein Friesian	August 2006	M	
851	EBVC	Holstein Friesian	April 2006	M	
332	EBVC	Holstein Friesian	February 2007	M	
475	EBVC	Holstein Friesian	March 2007	M	
401346	MRI	Holstein	September 2006	M	C
501334	MRI	Holstein Friesian	September 2006	M	
600564	MRI	Ayrshire	September 2006	M	
081	MRI	Holstein	May 2007	M	D
612	MRI	Holstein Friesian	May 2007	M	
863	MRI	Holstein Friesian	May 2007	M	
902	MRI	Holstein Friesian	April 2007	M	
903	MRI	Holstein Friesian	April 2007	M	

A.2 Details of animals used as blood donors for moDC cultures in this study. IAH: Institute of Animal Health, Compton; M: Male (castrated); EBVC: Easter Bush Veterinary Centre; MRI: Moredun Research Institute

A.3 Bacterial Strains

Strain Number	Serotype (Original Designation)	Original Supplier	Verotoxin	Pathotype
MCI 0010	*E. coli* O157:H7 (WallaWalla3)	M. Reynolds, Emory University, Atlanta	-	EHEC
MCI 0045	*E. coli* O157:H7 (EDL933)	G. Pósfai, Institute of Biochemistry, Szeged, Hungary	VT1 & VT2	EHEC
MCI 0066	*E. coli* O157:H7 (WallaWalla1)	M. Reynolds, Emory University, Atlanta	VT2	EHEC
MCI 0132	*E. coli* O9:K30 (EC46/04)	R. M. La Ragione, VLA Weybridge	-	ETEC
MCI 0191	*E. coli* O157:H7 (85-170)	M. Stevens, IAH Compton	-	EHEC
MCI 0200	*E. coli* O157:H7 (TUV933-0)	D. Miller, Bristol University	-	EHEC
MCI 0201	*E. coli* O157:H7 (ΔOI148A)	D. Miller, Bristol University	-	EHEC
MCI 0218	*E. coli* O157:H7 (84-289)	M. Stevens, IAH Compton	VT1 & VT2	EHEC
MCI 0279	*E. coli* O157:H7 (TT12B)	D. Gally, Edinburgh University	-	EHEC
MCI 0430	*E. coli* Or4:H37	E. Clark, MRI	-	Commensal
MCI 0465	*E. coli* O103:H36	E. Clark, MRI	-	Commensal
MCI 0483	*E. coli* O26:H47	E. Clark, MRI	-	Commensal
MCI 0490	*E. coli* Or7:H+	E. Clark, MRI	-	Commensal
MCI 0688	*E. coli* O9:K+ (EC219/06)	R. M. La Ragione, VLA Weybridge	-	ETEC
MCI 0690	*E. coli* O101:K30 (ED357/06)	R. M. La Ragione, VLA Weybridge	-	ETEC
MCI 0693	*E. coli* O101:K30 (EC546/05)	R. M. La Ragione, VLA Weybridge	-	ETEC

A.3 Bacterial strains used in this study. MCI: Microbial and cellular interactions; EHEC: Enterohaemorrhagic *E. coli*; VT: Verotoxin; ETEC: Enterotoxigenic *E. coli*; IAH: Institute of Animal Health; MRI: Moredun Research Institute; VLA: Veterinary Laboratories Agency.

A.4. Solutions & Buffers

A.4.1 ELISA Reagents

A.4.1.1 H7 ELISA Coating buffer

0.5 M Carbonate buffer (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$)
Make up in 1L dH_2O, pH to 9.6

A.4.1.2. H7 ELSIA Wash buffer

PBS
0.05% Tween 20 (PBST20)

A.4.1.3 H7 ELISA Blocking buffer

PBS
3% v/v fish gelatin (Sigma G7765)

A.4.1.4 H7 ELISA Reagent Dilution buffer

PBS
0.5% Tween 80
0.5 M NaCl (PBST80NaCl)

A.4.1.5 IL-8 ELISA Reagent Diluent

0.1% w/v BSA
0.05% v/v Tween 20
Make up in Tris-buffered saline (20 mM Trizma base, 150 mM NaCl)
pH to 7.2 – 7.4

A.4.1.6 TMB substrate solution

0.1 M Sodium Acetate solution (titrated to pH 6 with 0.1 M citric acid solution)
1% v/v 10mg/ml TMB in DMSO
0.005% v/v Hydrogen Peroxide

A.4.2 Cell and Tissue Culture solutions

A.4.2.1 Cell culture medium – CCM

DMEM (D6546, Sigma)
2 mM Glutamine
10% v/v FBS

A.4.2.2 Phenol Red-free cell culture medium

Iscove's Modified Dulebcco's Medium (IMDM, Invitrogen, 21056-023)
2 mM Glutamine
10% v/v FBS

A.4.2.3 Tissue culture medium – TCM

RPMI 1640 with Glutamax I & HEPES buffer 25 mM (Invitrogen),
100 U/ml Gentamicin (Sigma)
10% v/v FBS
5×10^{-5} M β-Mercaptoethanol (β-ME)

A.4.2.4 Dendritic cell tissue culture medium - DC-TCM

TCM
0.2 U/ ml GM-CSF (IAH, Compton)
200 U/ml recombinant bovine IL-4 (Moredun Research Institute)

A.4.2.5 Digestion Medium

DMEM (D6546, Sigma)
1% v/v FBS
50 μg/ml Gentamicin,
100 U/ml Penicillin
30 μg/ml Streptomycin
75 U/ml Collagenase &
20 μg/ml Dispase I
30 μg/ml DNase I (optional)

A.4.2.6 SDS-HCl

100 g/L Sodium dodecyl sulfate (SDS) in 0.01 M HCl and 1 g SDS

A.4.2.7 FACS Buffer

PBS
1% w/v BSA
0.1% Sodium Azide (NaN_3)

A.4.3 SDS-PAGE Reagents

A.4.3.1 SDS-PAGE Resolving Gel

10% w/v Acrylamide:*N,N'*-methylenebisacrylamide (37.5:1)
375 mM Tris (, pH8.8),
0.1% w/v SDS
0.025% w/v ammonium persulphate (APS)
0.005% v/v *N,N,N',N'*-tetramethylethyldiamine (TEMED)

A.4.3.2 SDS-PAGE Stacking gel

10% w/v Acrylamide:*N,N'*-methylenebisacrylamide (37.5:1),
125 mM Tris (pH6.8),
0.1% w/v SDS
0.1% w/v APS
0.14% v/v TEMED

A.4.3.3 SDS-PAGE Running Buffer

250 mM Tris, pH 6.8,
192 mM glycine,
0.1% SDS,
Make up in dH_2O

A.4.3.4 SDS-PAGE Reducing sample buffer

250 mM Tris, pH 6.8,
40% v/v glycerol,
8% w/v SDS,
520 mM dithiothreitol (DTT),
0.01% w/v bromophenol blue

A.4.4 Western Blotting Reagents

A.4.4.1 Electoblotting Transfer Buffer

25 mM Tris,
192 mM Glycine,
20% methanol
Make up required volume in dH_2O

A.4.4.2 Western Blot Blocking Buffer

PBS
0.5 M NaCl
0.5% v/v Tween 80 (Sigma)

A.4.5 Cellular stains and Tissue Fixatives

A.4.5.1 Sorensen's buffer

49ml of Na_2HPO_4 (0.06 M) and
51 ml of KH_2PO_4 (0.06 M)
Combine prior to use and titrate to pH 7.2.

A.4.5.2 Zinc Salts Fixative - ZSF

0.1 M Tris base buffer
0.05% w/v calcium acetate (pH 7.0 – 7.4)
0.5% w/v zinc acetate and zinc chloride.

A.4.6 Solutions for Molecular Techniques

A.4.6.1 TAE Buffer

40 mM tris-acetate pH 8.0
1 mM EDTA
Make up required volume in dH_2O