CYTOKINE GENE EXPRESSION IN NAÏVE AND PREVIOUSLY INFECTED SHEEP AND LAMBS AFTER CHALLENGE WITH THE ABOMASAL NEMATODE *TELADORSAGIA CIRCUMCINCTA*

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DECLARATION

I declare that the contents of this thesis are my own work and that they have not been presented to any university other than the University of Edinburgh.

Nicola Margaret Craig

Edinburgh, September 2009

ABSTRACT

The abomasal helminth *Teladorsagia circumcincta* is one of the most economically important parasites to affect the farming of sheep and goats. *T.circumcincta* infection is particularly detrimental to lambs, in which it can cause pronounced morbidity and severe production losses. Due to the spreading resistance of this parasite to all currently available classes of anthelmintic drugs, it is having an increasingly severe impact on the sheep industry with significant implications for sheep welfare.

Infection of sheep with *T.circumcincta* triggers local changes in the abomasum characteristic of a T helper type-2 (Th2) driven immune response, including local eosinophilia, mastocytosis and increased mucus production, which leads to expulsion of the parasite. However, this protective immunity develops slowly during repeated exposure, wanes rapidly, and does not appear to be evident in young lambs. Vaccination to provoke early onset of protective immunity has therefore been suggested as an alternative means of control in the face of spreading anthelmintic resistance. Greater understanding of the development of immunity to *T.circumcincta*, and why this is delayed in lambs, would be useful in vaccine development.

This thesis focuses on cytokine transcription profiling of the ovine abomasal mucosa and local lymphatic tissues. Changes in cytokine transcription over the course of a challenge infection with *T.circumcincta* were defined in helminth naïve sheep, and in previously infected sheep which have developed a degree of immunity during an eight week trickle infection, to clarify the mechanisms by which this immunity is orchestrated. This work demonstrated a clear Th2 cytokine response in the abomasal mucosa over the course of infection, which developed earlier and was more pronounced in the previously infected sheep; possibly owing to a population of polarised Th2-type cells built up during the previous infection. Suppression of Th1 cytokine transcription was also a prominent finding in the draining lymph node, which likewise occurred earlier in the previously infected sheep.

Repetition of this experiment using younger lambs provided a possible explanation for the reduced resistance to *T.circumcincta* in this age group. While Th2 and proinflammatory cytokine responses in the abomasal mucosa demonstrated similar trends to those found in the older sheep, little suppression of Th1 cytokine transcription was observed in the draining lymph node. It is therefore suggested that the increased susceptibility of young lambs to *T.circumcincta* is not due to an inability to generate adequate Th2 responses, but an inability to suppress transcription of antagonistic Th1 cytokines.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AAM	alternatively activated macrophage
ANOVA	analysis of variance
APC	antigen presenting cell
ATPase	adenosine triphosphatase
BME	β-mercaptoethanol
BSA	bovine serum albumin
CD	cluster of differentiation / cluster of designation
cDNA	complementary deoxyribonucleic acid
Ct	crossing threshold
DC	dendritic cell
DC-SIGN	dendritic cell specific intercellular adhesion molecule-3-grabbing nonintegrin
DNA	deoxyribonucleic acid
dNTP	dinucleotriphosphate
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme linked immunosorbent assay
ERK	extracellular signal-regulated kinase
ES-62	excretory-secretory product 62
FACS	fluorescence-activated cell sorting
FCS	foetal calf serum
FEC	faecal egg count
Foxp3	forkhead box P3
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HRPO	horseradish peroxidase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
ITLN	intelectin

IRAK	interleukin-1 receptor-associated kinase
JNK	c-Jun N-terminal kinase
L3	stage 3 larva
L4	stage 4 larva
L5	stage 5 larva
LPS	lipopolysaccharide
mAb	monoclonal antibody
MBL	mannose binding lectin
MCP	mast cell protease
MGB	minor groove binder
MGL	macrophage galactose-type lectin
MHC	major histocompatibility complex
MyD88	myeloid differentiation primary response gene 88
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NFQ	non-fluorescent quencher
NK cell	natural killer cell
Nv	naïve
OD	optical density
OvGal	ovine galectin
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	previously infected
PRR	pattern recognition receptor
PVP	polyvinylpyrrolidone
Q-PCR	quantitative / real-time polymerase chain reaction
sMCP	sheep mast cell protease
R(D)SVS	Royal (Dick) School of Veterinary Studies
RELM	resistin like molecule
RNA	ribonucleic acid
RT	reverse transcription

RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SEM	standard error of the mean
SNP	single nucleotide polymorphism
STAT	signal transducer and activator of transcription
TBE	tris-borate-ethylene diamine tetra acetic acid
Tc	cytotoxic T cell
TCR	T cell receptor
TE	tris-ethylene diamine tetra acetic acid
TGF	transforming growth factor
Th	T helper cell
TIR	toll-IL-1 receptor
TLR	toll-like receptor
TNF	tumour necrosis factor
Trif	TIR-domain-containing adapter-inducing interferon-β
UDG	uracil-DNA glycosylase
VTRI	veterinary training and research intitiative

1 Chapter One:

Ovine immune responses to *Teladorsagia circumcincta*

1.1 Introduction

The abomasal nematode *Teladorsagia circumcincta* (previously known as *Ostertagia circumcincta*) is one of the most economically important parasites to affect the farming of sheep and goats in temperate and subtropical areas (McLeod, 1995, Fox, 1997, Scott et al., 1998a, Grillo et al., 2006, Miller and Horohov, 2006). This parasite is a significant cause of production losses to the sheep industry, resulting in high lamb morbidity and mortality if not controlled. Control of teladorsagiosis currently relies upon the use of anthelmintics, and is complicated by the increasing incidence of resistance to these drugs, which has had a significant economic impact on the sheep industry and implications for sheep welfare (Jackson, 1993, Jackson and Coop, 2000, Besier and Love, 2003, Bartley et al., 2004, Wolstenholme et al., 2004, Coles et al., 2006, Jabbar et al., 2006). The spread of anthelmintic resistance has already prohibited sheep farming in some areas.

Repeated exposure to *T.circumcincta* eventually results in the development of protective immunity (Smith et al., 1983, Smith et al., 1987, McKellar, 1993), manifested as rapid expulsion of infective larvae, inhibited parasite development and growth, and reduced fecundity of adults (Gibson and Everett, 1978, Miller, 1984, Smith et al., 1984, Stear et al., 1995). Vaccination has therefore been suggested as a viable alternative to anthelmintic treatment as a means of control (Qu et al., 1998, McCririe et al., 1997, Strain and Stear, 1999, Smith et al., 2001, Redmond et al., 2006).

Development of immunity has been shown to be influenced by genetics (Shnain et al., 1973, Stear et al., 1997) and varies between breeds of sheep (Gruner et al., 1986, Burke and Miller, 2002, Gaba et al., 2006, Good et al., 2006), so selective breeding for resistance to parasites is also a possible means of salvation for the sheep industry (Sreter et al., 1994).

1.2 *Teladorsagia circumcincta*

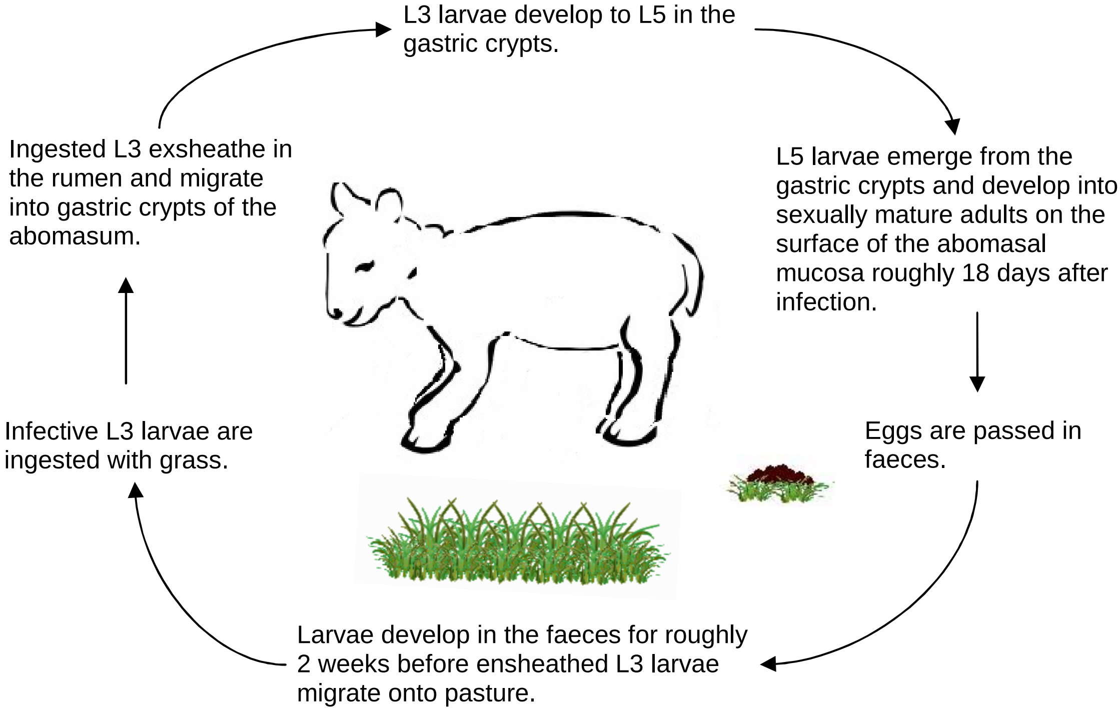
1.2.1 Life-cycle

Eggs of *T.circumcincta* are passed in the faeces of infected animals, and develop into infective third stage (L3) larvae in about two weeks. Under moist conditions, the L3 larvae move out of the faeces onto grass with which they are ingested (Callinan, 1978).

In the rumen the larvae exsheathe and the majority migrate into the gastric crypts of the abomasum within four days of infection. Once in the gastric crypts the L3 larvae develop into fourth (L4) and then fifth stage (L5) larvae over approximately ten days. The L5 larvae then leave the abomasal gland and develop into sexually mature adults on the abomasal mucosa approximately 18 days after infection (Ritchie et al., 1966, James and Johnstone, 1967, Gibson and Everett, 1978). This lifecycle normally takes about three weeks, but under adverse immunological conditions ingested larvae can undergo developmental arrest at the early L4 stage and resume maturation up to six months later (Halliday et al., 2007).

Free-living stages of *T.circumcincta* feed on bacteria. In the sheep, this parasite is thought to be a mucosal browser, feeding on mucins, epithelial cells and cellular debris, and components of the abomasal digesta (Simpson et al., 2009).

Figure 1.1: Life-cycle of T.circumcincta.



1.2.2 Pathology

Infection with *T.circumcincta* causes functional changes in the abomasum, including a rapid reduction in gastrin secretion (Scott et al., 1998b, Simcock et al., 2006) and suppression of gastric acid production, leading to increased abomasal pH (McLeay et al., 1973, Anderson et al., 1976, McKellar, 1993, Scott et al., 2000) and reduced activation of pepsinogen. This increase in pH results in bacterial overgrowth in what is normally a bacteriostatic abomasal environment (Dakkak and Khallaayoune, 1984, Simcock et al., 1999, Simpson et al., 1999). Once the larvae migrate into the gastric crypts, they cause inflammation and damage due to their antigenicity and the compression of surrounding tissues as they grow. Increased permeability of the mucosa causes increased serum gastrin and pepsinogen levels, and in severe cases can result in a protein-losing enteropathy (Anderson et al., 1981, Lawton et al., 1996, Scott et al., 1998a, Stear et al., 2003, Miller and Horohov, 2006).

Gross pathology shows areas of inflammation of the abomasal mucosa, in which parasitized glands can be located at the centre of nodular lesions. Histologically, there is epithelial hyperplasia and thickening of the mucosa, which may progress to mucosal sloughing (Ritchie et al., 1966), reduction in the prevalence of parietal cells and increased numbers of mucus neck cells (Scott et al., 1998a, Scott et al., 2000), and infiltration of mast cells, eosinophils and T cells (Gruner et al., 1994, Balic et al., 2003). Breakdown of the junctions between epithelial cells by mast cell proteases causes increased mucosal permeability, protein and fluid loss into the gut lumen and allows passage of gastrin and pepsinogen into the blood (Stear et al., 2003).

Clinical consequences of infection range from sub-optimal weight gain, to inappetence, weight loss, protein deficiency and diarrhoea (Coop et al., 1977, Stear et al., 2003).

1.3 The immune response

Due to the difficulties and expense inherent in conducting studies using large animals, much of our current understanding of immune responses in these animals has evolved from previous examination of the host-parasite interactions which take place in laboratory rodent models during infection with gastrointestinal nematodes. Here a brief overview of the immune responses to gastrointestinal nematode parasites is presented, with reference to ruminant immune systems and responses where applicable.

1.3.1 Parasite detection

Following ingestion, *T.circumcincta* larvae exsheathe in the rumen and migrate through the reticulum and omasum to the abomasum. While moving through the first three chambers of the stomach the larvae go unnoticed by the host immune system, partly because they move through the stomach contents and have minimal contact with host tissues, but mostly because these chambers are lined with a thick layer of stratified epithelium to protect the host both from abrasion by the rumen contents and from exposure to the rumen microflora. The rumen, reticulum and omasum have a good lymphatic supply to absorb volatile fatty acids produced during fermentation, but these are confined to the lamina propria mucosae and are protected by the overlying tissues. It is therefore only when the larvae enter the glandular abomasum that recognition by the immune system can occur (Schnorr et al., 1975, Hampl and Slezakova, 1977, Hampl et al., 1978).

Pathogen recognition mechanisms are a vital component of the immune system and facilitate initiation of responses appropriate to the pathogen detected. Pattern recognition receptors (PRRs) are widely expressed on the surface or endosomes of epithelial cells and antigen presenting cells among others, and bind molecules present on the surface of the parasite, or in secretory or excretory products, which have characteristics associated with a specific type of pathogen (pathogen-associated molecular patterns; PAMP) (Janeway, 1992, Medzhitov and Janeway, 1997, O'Neill, 2006, Mogensen, 2009).

The Toll-like receptor (TLR) family is so far the best characterized family of PRRs (Mogensen, 2009). TLR can act as monomers, homodimers (Takeda et al., 2003), heterodimers (Underhill, 2003) or in association with co-receptors (Gantner et al., 2003, Miller et al., 2005). Twelve functional mammalian TLRs have been identified, of which 10 are expressed in humans and 11 in mice (Beutler, 2004, Kawai and Akira, 2005, O'Neill, 2006).

Other PRRs include lectins, which bind carbohydrates (Figdor et al., 2002). This family includes C-type lectins such as dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) which binds glycoconjugates containing mannose and fucose (Figdor et al., 2002, Appelmelk et al., 2003), dectins which bind fungal β-glucans (Brown, 2006), macrophage galactose-type lectin (MGL) (Raes et al., 2005) and the mannose receptor (van Liempt et al., 2007).

In ruminants, homologues of all 10 human TLRs have been found in both cattle and sheep (McGuire et al., 2006, Menzies and Ingham, 2006, Nalubamba et al., 2007, Chang et al., 2009). Dectin 1 and 2 are also found on the sheep dendritic cells (Nalubamba et al., 2007). Ovine dendritic cells also express Fc receptors which can bind antigen-antibody complexes, allowing capture and presentation of antibody-bound antigen (Bujdoso et al., 1990, Harkiss et al., 1990, Coughlan et al., 1996a, Coughlan et al., 1996b).

Once a pathogen has been detected and recognised, this information must be passed to other immune cells through antigen presentation to generate an immune response. In the context of extracellular helminths, when pathogen molecules are taken up by antigen-presenting cells, they are processed and expressed on the surface of the cell as short peptides bound to a major histocompatibility complex (MHC) class II molecule. Molecules from bacteria and viruses are presented in a similar way by MHC class I expressed by a wide variety of cells, including non-immune cells. In this way molecules are presented to T cells to stimulate progression of the immune response. Dendritic cells, B cells, macrophages, fibroblasts and epithelial cells are all known to be capable of antigen presentation.

Dendritic cells are professional antigen presenting cells which are able to take up pathogen molecules and present them to lymphocytes. Activated dendritic cells are distinct from other antigen presenting cells in their ability to trigger adaptive immunity through activation of naïve T cells in lymphoid tissues. This is an essential function, as naïve CD4+ T cells cannot otherwise bind to antigen-binding MHC class II molecules on the surface of antigen presenting B cells to initiate B cell maturation, proliferation and antibody production. Signals from dendritic cells during the activation of CD4+ T cells can also induce these cells to produce cytokines which co-ordinate the various component cells of the immune system to optimise the appropriate response to the pathogen in question (Banchereau et al., 2000).

As well as activating naïve T cells, dendritic cells can participate in the activation of naïve and memory B cells by CD4+ T cells. It has been demonstrated in rats that dendritic cells can take up and retain unprocessed antigen, and transfer it to B cells for MHC class II processing and presentation to activated CD4+ T cells (Wykes et al., 1998). Peripheral blood derived bovine DC have also been found to promote B cell proliferation through CD40 ligation and cytokine expression independent of T cells (Bajer et al., 2003).

Dendritic cells use various pathogen recognition receptors to detect a wide variety of bacterial, viral and parasitic molecules (Reis e Sousa, 2001). They appear to be capable of discerning the threat posed by different molecules from pathogenic and non-pathogenic organisms, possibly through receptors such as the activating and inhibitory immunoglobulin-like transcripts which have been described in human immune cells (Colonna et al., 1999a, Colonna et al., 1999b, Wu and Horuzsko, 2009). Similar receptors have not yet been identified in sheep or cattle.

Further to discerning between pathogenic and non-pathogenic organisms, these cells appear to be able to differentiate between various types of pathogen, using receptors such as the various Toll-like receptors and C-type lectins, in order to invoke the most appropriate immune response (Manickasingham and Reis e Sousa, 2001, Reis e Sousa, 2001, Reis e Sousa et al., 2001, Diebold, 2008).

B cells are also capable of presenting antigen to CD4+ T cells. When antigen binds to antibody on the B cell surface, some of the bound antibody is taken back into the cell, allowing the captured antigen to be processed and presented to CD4+ T cells by MHC class II (Gupta and DeFranco, 2007). This is necessary in order for B cells to obtain “T cell help”, signals driving B cell proliferation and antibody production, from T cells specific for epitopes on the same antigen.

Ovine macrophages are also capable of acting as APCs. These cells are predominantly employed in the phagocytic killing of pathogens, but they also express MHC class II (Hein et al., 1987, Humphrey, 1986, Liu et al., 2003, Bendixsen et al., 2004) allowing them to also present pathogen molecules.

1.3.2 Dendritic cell sensitization

As discussed in section 1.3.1, dendritic cells are capable of differentiating different types of pathogen through PRR. This is an essential part of generating an appropriate and effective immune response tailored to specific pathogens, which range from viruses and intracellular bacteria to protozoa and metazoan helminths.

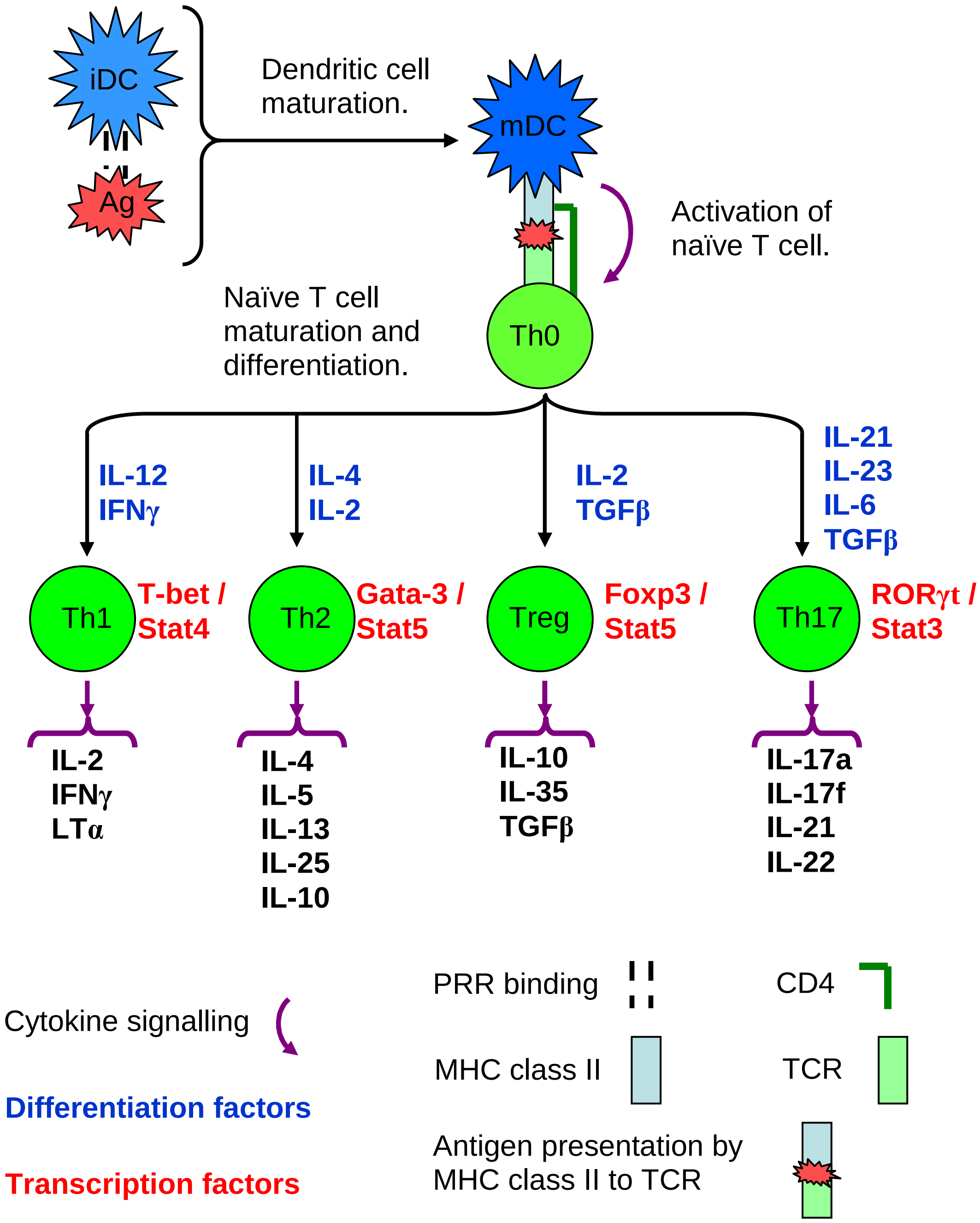
Once the pathogen has been identified, dendritic cells mature and migrate to the draining lymph node. Here they activate naïve CD4+ T cells to produce pathogen specific immune responses through a combination of antigen presentation, co-receptor molecule binding on the cell surface and production of cytokines. The activated CD4+ T cell then goes on to expand the immune response through activation of B cells and further cytokine production to enhance B cell function, stimulate switching of antibody type, and attract and enhance the activity of other immune cells. Bujdoso and colleagues have previously studied this sequence of antigen capture by ovine dendritic cells, followed by carriage to local lymph nodes and antigen-specific T cell activation in the sheep (Bujdoso et al., 1989, Bujdoso et al., 1990). Pasare and Medzhitov have also demonstrated the requirement for cytokine signalling in combination with dendritic cell antigen presentation to produce T cell activation in sheep (Pasare and Medzhitov, 2004).

The actions and cytokines produced by activated CD4+ T cells largely determine the phenotype of the immune response. These phenotypes can be separated into: T helper type-1 (Th1), which is characterized by the production of IL-2, IL-12 and IFNγ and is effective against intracellular pathogens; T helper type-2 (Th2), which is similarly characterized by production of IL-4, IL-5 and IL-13 and is effective against extracellular pathogens such as helminths; regulatory T cells (Treg), which moderate the immune response to antigens to prevent immune-mediated damage to the host and are characterized by production of IL-10, IL-35 and TGFβ; and Th17, which is characterized by production of IL-17, IL-21 and IL-22, and is effective against extracellular bacteria and fungal pathogens (Kim et al., 1985, Mosmann et al., 1986, Mosmann and Coffman, 1989, Groux et al., 1997, Bluestone and Abbas, 2003, Weaver et al., 2006, Zhu and Paul, 2008, Kim, 2009). The cytokine signalling which influences the differentiation of Th1, Th2, Treg and Th17 CD4+ T cell subsets, and the characteristic transcription factors and cytokines known to be produced by each subset are illustrated in Figure 1.2 (Zhu and Paul, 2008). More recently, a novel subset of CD4+ T cells which produce IL-9 have been discovered, which have now been termed Th9 (Dardalhon et al., 2008, Veldhoen et al., 2008, Adamson et al., 2009).

Dendritic cells play a critical role in the polarization of these CD4+ T cell responses (Moser and Murphy, 2000, Lipscomb and Masten, 2002) and can themselves undergo Th1- or Th2-like polarization (Gratchev et al., 2001). This seems to result from the maturation of immature dendritic cells in response to pathogen-specific antigen into distinct subsets, which drive the development of naive and resting T cells towards either a Th1 or a Th2 phenotype through differential cytokine expression (Pulendran et al., 1999, Maldonado-Lopez et al., 2001, Maldonado-Lopez and Moser, 2001, Halliday et al., 2007). For example, maturation of human and murine dendritic cells in response to LPS or influenza virus produces dendritic cells which release IL-12 and favour the development of Th1 cells (Macatonia et al., 1995, Cella et al., 1996, Koch et al., 1996, Cella et al., 1999). Concurrent innate immune responses also influences dendritic cell maturation; for example type-1 interferon produced in response to virus infection can enhance humoral immunity and antibody switching in mice via a dendritic cell-mediated mechanism (Le Bon et al., 2001).

It is possible that dendritic cells may even be able to induce appropriate responses to more than one antigen at the same time. Mouse dendritic cells exposed to schistosome egg antigen and *Proprionebacterium acnes* were found to segregate the bacterial and helminth antigens. Antigens from each of these pathogens were acquired via discreet pathways and kept in separate compartments, and antigen-specific Th1 and Th2 responses were induced at the same time (Cervi et al., 2004).

Figure 1.2: Polarization of CD4+ T cells in response to activation by dendritic cells and the influence of differentiation factors. Binding of antigen (Ag) by immature dendritic cells (iDC) stimulates maturation of dendritic cells. Mature dendritic cells (mDC) activate naïve T cells (Th0) through receptor /co-receptor binding and cytokine signalling to mature and differentiate into a specific phenotype to produce a suitable immune response; Th1, Th2, regulatory T cell (Treg) or Th17.



Immature monocyte-derived human dendritic cells stimulated *in vitro* with *Echinococcus granulosus*-derived molecules matured and primed naïve T cells to develop a Th2 phenotype through a mechanism involving Toll-like receptors, as indicated by activation of NF-κB and phosphorylation of IRAK (Rigano et al., 2007). Murine dendritic cells have been found to detect various helminth antigens via TLR4, such as the helminth carbohydrate lacto-N-fucopentaose III found in *Schistosoma mansoni* eggs and the glycoprotein ES-62 secreted by the nematode *Acanthocheilonema viteae*, which results in conditioning of the DC to induce Th2 responses (Whelan et al., 2000, Ferlazzo et al., 2004, Artis et al., 2005, Goodridge et al., 2005). TLR4 has also been shown to influence IL-4 production in mice infected with *Trichuris muris* (Helmby and Grencis, 2003), and TLR2 has been found to detect helminth lipids, such as lysophosphatidylserine obtained from *S.mansoni*, which has been reported to trigger DC maturation and induction of Th2 responses in humans through binding to TLR2 (van der Kleij et al., 2002, Agrawal et al., 2003).

Other, less well studied PRRs on DC are also capable of binding helminth molecules. The C-type lectins DC-SIGN (Appelmelk et al., 2003, van Die et al., 2003), macrophage galactose-type lectin (MGL) and the mannose receptor on human dendritic cells are all capable of binding extracts of *S.mansoni* and skewing the immune response towards the Th2 phenotype (van Liempt et al., 2007).

The immune response generated subsequent to processing of helminth molecules by dendritic cells appears to be distinct in being independent of MyD88/Trif signalling (Goodridge et al., 2005). Binding of TLR2 and TLR4 to schistosome egg antigens triggers both human and mouse dendritic cell maturation through phosphorylation of extracellular signal regulated kinase (ERK), but unlike the signalling of TLR triggered by other antigens does not cause phosphorylation of c-Jun N-terminal kinases (JNK) (Agrawal et al., 2003, Ferlazzo et al., 2004, Dillon et al., 2004a). Similarly, binding of DC-SIGN induces phosphorylation of ERK in human dendritic cells to preferentially evoke Th2 responses (Caparros et al., 2006). The different signalling pathways triggered by helminth antigens in comparison to other pathogens is likely to be a key factor in the maturation of dendritic cells into a distinct subsets, driving the immune response towards a Th1- or Th2-type response depending on the pathogen (Pearce et al., 2006).

Mature dendritic cells which have been primed to promote different antigen-specific immune responses can be separated according to phenotype. Maldonado-Lopez and colleagues have demonstrated in mice that administration of CD8α+ dendritic cells exposed to antigen results in the induction of a Th1-type response, however administration of antigen-exposed CD8α- dendritic cells induces a Th2-type response (Maldonado-Lopez et al., 2001). Further studies of this discrepancy showed that dendritic cell-derived IL-10 is required for induction of Th2 responses by CD8α- dendritic cells, and IL-12 and IFNγ are required for the induction of Th1 responses by CD8α+ dendritic cells (Maldonado-Lopez et al., 2001). These dendritic cell phenotypes were also found to be influenced by cytokines in the local environment; treatment *in vitro* with IL-10 reduced the viability of Th1-promoting CD8α+ dendritic cells, whereas treatment with IFNγ reduced the Th2-promoting capacity of CD8α- dendritic cells (Maldonado-Lopez et al., 2001).

In human models, monocyte-derived dendritic cells which promote Th1 responses are known as type 1 dendritic cells (DC1), and plasmacytoid-derived dendritic cells which promote Th2 responses are known as type 2 dendritic cells (DC2) (Moser and Murphy, 2000).

Several subsets of dendritic cell have been identified from cattle and sheep, defined in various studies by expression of CD5, CD11a and CD21 (Howard et al., 1997), CD11c and CD14 (Bajer et al., 2003), CD172a and CD45RA (McNeilly, 2005, Nalubamba et al., 2007), and MyD1 (Howard et al., 1999, Howard and Hope, 2000). In sheep, two dendritic cell subsets identified by differential expression of CD172a and CD45RA have been found to express different cytokines profiles and distinct sets of PRRs, which are likely to reflect distinct functions (Nalubamba et al., 2007). Ovine dendritic cells obtained from pseudoafferent lymph which express CD172a have been found to transcribe IL-10 but almost no IL-12p40 leading to the promotion of Th2 responses, whereas those which do not express CD172a transcribe IL-12p40 but not IL-10 promoting Th1 responses (McNeilly, 2005, Halliday et al., 2007).

Similarly, bovine dendritic cells expressing MyD1, a homologue of rat SIRPα (Brooke et al., 1998), induced strong proliferative responses in both CD4+ and CD8+ T cells, whereas MyD1- dendritic cells only stimulated proliferation of CD4+ T cells (Hope et al., 2001). Bovine peripheral blood derived dendritic cells have also been shown to have features similar to human DC2 cells, such as production of IL-10 and IL-13 and the ability to influence B cell activation (Bajer et al., 2003).

1.3.3 Macrophage sensitization

The "classical" role of macrophages activated in response to IFNγ and LPS is the expression of inflammatory mediators and intracellular killing of bacteria; for example through production of inducible nitric oxide synthase (iNOS). However, following exposure to IL-4, IL-10, IL-13 or IL-21 macrophages can become activated via an "alternative" pathway. These alternatively activated macrophages can be distinguished by expression of IL-4 receptor chain α (IL-4Rα), arginase-1, mannose receptor and a lack of iNOS expression (Balic et al., 2004, Nair et al., 2006, Urban et al., 2007).

Alternatively activated macrophages have been shown to accumulate in response to nematode infection (Lanier et al., 1986), and are thought to act in several ways which are distinct from classically activated macrophages. Alternatively activated macrophages suppress inflammatory responses, contribute to the repair of tissue damage caused by helminths, promote Th2 responses, and produce molecules such as Ym1 which is chemotactic for eosinophils (Lanier et al., 1986, Balic et al., 2004).

As well as responding to the surrounding cytokine milieu, alternatively activated macrophage activity may be influenced by direct exposure to helminth molecules. Macrophage galactose-type lectin (MGL) is present on alternatively activated macrophages elicited in mice in response to *Taenia crassiceps* infection, and is up-regulated on human macrophages *in vitro* in response to IL-4 (Raes et al., 2005). Schistosome egg antigens have also been shown to trigger the Jagged/Notch pathway, which has been implicated in driving Th2-type development in both murine bone marrow-derived macrophages and human monocyte-derived macrophages (Goh et al., 2008).

Another group of Th2-promoting macrophages has been produced by the classical activation stimuli IFNγ and LPS in the presence of IgG (Gerber and Mosser, 2001). These macrophages appear to be more specialised towards promotion of Th2 responses and suppression of pro-inflammatory responses, and have been termed "type 2 activated macrophages". These cells are distinct from classically activated macrophages as they produce no IL-12, but large amounts of IL-10 and induce Th2-type responses including IL-4 production and the switching of B cells to IgG production (Gerber and Mosser, 2001, Anderson and Mosser, 2002, Mosser, 2003). Type 2 activated macrophages are distinguished from alternatively activated macrophages by the absence of arginase induction (Gerber and Mosser, 2001).

Alternatively activated macrophages have been produced from the sheep blood monocyte-derived cell line MOCL7 (Olivier et al., 2001, Boscariol et al., 2006) in response to *Fasciola hepatica*, as distinguished by arginase and chitinase activity (Flynn et al., 2007), and it is likely that Th2-promoting macrophages participate in the ovine immune response to helminths *in vivo*.

1.3.4 Lymphocyte recruitment and mucosal responses

As briefly described in section 1.3.2 and illustrated in Figure 1.2, naïve CD4+ T cells can be activated by dendritic cells presenting antigen and induced to differentiate into the specific phenotype which will promote the most appropriate immune response. Non-naïve CD4+ T cells can also be activated by other APCs, such as macrophages. The activated T cell phenotype population then expands, activates B cells and produces cytokines to co-ordinate and amplify this immune response (Kim et al., 1985, Mosmann et al., 1986, Mosmann and Coffman, 1989, Groux et al., 1997, Bluestone and Abbas, 2003, Weaver et al., 2006, Urban et al., 2007, Zhu and Paul, 2008, Kim, 2009).

Activated lymphocytes which localise in the ovine mucosa and gastrointestinal tract have been shown to preferentially circulate back to the mucosa after passing through the draining lymph node (Weisz-Carrington et al., 1991, Hein and Mackay, 1991, Au et al., 2001), and have a predilection for the lamina propria due to homing receptors with a preference for capillary endothelial cells in this tissue (Cahill, 1977, Chin and Hay, 1984, Kimpton et al., 1989, Hopkins, 2000). Examples of these homing receptors in mice and humans include: integrin $\alpha_4\beta_7$, which binds to mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1) expressed on vascular endothelium in the lamina propria in humans (Briskin et al., 1997); integrin α_E(CD103)β_7, which binds to E-cadherin expressed on the basolateral surface of intestinal enterocytes in mice (Cepek et al., 1994); and CCL9, a chemokine receptor which binds to CCL25 on the surface of epithelial cells in the small intestine, also in mice (Wurbel et al., 2000). Integrin $\alpha_4\beta_7$ and MAdCAM-1 have been indentified in sheep, on the surface of lymphocytes preferentially circulating through the mesenteric lymph nodes and on the surface of endothelial venules within the same lymph nodes respectively (Abitorabi et al., 1996, Hein and Mackay, 1991).

The CD4+ T cells phenotype associated with immune responses to gastrointestinal helminths in humans and mice is characterised by production of IL-4, IL-5, IL-9, IL-10 and IL-13 and is termed Th2 (Grencis, 1997, Finkelman and Urban, 2001, Finkelman et al., 2004, Balic et al., 2004, Meeusen et al., 2005, Urban et al., 2007). For example, studies of murine infection with the gastrointestinal nematodes *Nippostrongylus brasiliensis, Trichinella spiralis* and *Trichuris muris* have demonstrated that IL-4 and IL-13 are important in host protection (Grencis et al., 1991, Else et al., 1992, Else et al., 1993, Finkelman et al., 1999, Artis et al., 1999, Garside et al., 2000). Th2 cytokines have also been found to be important in human immune responses to helminths, such as the nematode *Ascaris lumbricoides* (Cooper et al., 2000, Jackson et al., 2006).

The immune response orchestrated by Th2 cells originating from these tissues acts both locally and in the draining lymph node to produce characteristic responses, including class-switching of B cell antibody production to IgA, IgE and IgG_1, mast cell and eosinophil proliferation and recruitment, increased gut motility and mucus production (Balic et al., 2004, Meeusen et al., 2005, Urban et al., 2007).

CD8+ T cells (cytotoxic T cells; Tc cells) are primarily involved in fighting viral infections. They are influenced by cytokines produced by other T cells, and also produce cytokines themselves to help regulate the immune response. In the same way as CD4+ T cells, cytotoxic T cells can also be divided according to the type of cytokines they produce and immune responses they regulate or promote; Tc1 cells produce IFNγ which contributes to suppression of Th2 cell activity, while Tc2 cells produce IL-4 which contributes to suppression of Th1 (Sadick et al., 1991, Dickensheets and Donnelly, 1999).

T cells expressing γδ T cell receptors (γδ T cells), which are able to bind to antigen-presenting CD1 (Porcelli et al., 1989, Sieling et al., 1999, Schiefner and Wilson, 2009), are mainly associated with epithelial and mucosal tissues (Ferlazzo et al., 2004, Gyorffy et al., 1992). Sheep γδ T cells have been found to express a unique family of proteins WC1 (which is also known as T19), are much more numerous than in humans and rodents (Hein and Mackay, 1991, Evans et al., 1994), and have a larger repertoire of TcR variable genes than humans and mice, implying a more important role for these cells in this species (Hein and Mackay, 1991, Hein and Dudler, 1993, Evans et al., 1994, Cahill et al., 1996). One such role may be defence against mycobacterial infections; the population of γδ T cells has been found to increase markedly in the lymphoid tissues of the intestine in sheep infected with *Mycobacterium avium* ssp. *paratuberculosis* (Beard et al., 2000).

Natural killer cells (NK cells) are a lymphocyte subset specialised to detect and attack virally infected or tumour cells (Moretta and Moretta, 2004). NK cells have also been shown to be an important source of interleukin-13 (IL-13) in the intestinal tissue of mice infected with *Trichinella spiralis*, and there is emerging evidence that

NK cell interaction is important for the activation and maturation of dendritic cells (McDermott et al, 2005).

Work done by David Smith and colleagues in the early 1980’s on the immune responses to *T.circumcincta* has demonstrated that there is a significant increase in the numbers of reacting lymphocytes in lymph obtained from the common gastric lymph duct of naive sheep 8 days following infection (Smith et al, 1983). These lymphocytes have been shown to be able to transfer immunity between sheep (Smith et al, 1986). The changes in efferent gastric lymph following infection with *T.circumcincta* have since been further defined as increased in levels of IgA, B cells, and CD4+ and CD8+ T cells (Halliday et al., 2007, Halliday et al., 2009b).

Studies of the immune responses to *Haemonchus contortus* in the sheep abomasum have shown that there is a rapid increase in CD4+ T cells in the abomasal lymph node, and an increase in both CD4+ and γδ T cells in the abomasal mucosa, following infection. However these increases were found not to be maintained during infection, indicating these cells are involved in the early stage response to *H.contortus* in the sheep (Balic et al., 2000). This increase in γδ T cells in the abomasal mucosa following infection with *H.contortus* contrasts with the findings of the same group when studying the immune response to *T.circumcincta*; in which γδ T cells were found to be reduced in the abomasal tissue throughout a 10 day infection in immune sheep (Balic et al., 2003).

Studies of sheep infected with *H.contortus* have found CD4+ lymphocytes are significantly associated with resistance to this helminth (Pena et al, 2006), and are required for protection from *Nippostrongylus brasiliensis* in the mouse jejunum (Finkelman et al, 2001). This is consistent with the findings of Grencis and colleagues that CD4+ T cells produce a Th2 cytokine response, including IL-4, IL-5, IL-9 and IL-10, in the mesenteric lymph nodes of mice following infection with *Trichinella spiralis* (McDermott et al, 2005). However, studies of sheep infected with *Trichostrongylus colubriformis* have shown that CD4+ lymphocytes are significantly reduced in efferent abomasal lymph from 7 days following infection (Pernthaner et al, 2005).

Previous studies of infection with the abomasal nematode *Ostertagia ostertagi* in cattle have shown that the cytokine changes taking place in the gastric lymph nodes in this species are predominantly those associated with a Th2 immune response. However, the polarisation of the cytokine response taking place in the abomasal mucosa may not be as clearly delineated as responses to helminths in the mouse; though transcription of IL-4, IL-5 and IL-10 was increased, IFNγ transcription was also raised subsequent to infection (Gasbarre et al., 2001, Claerebout et al., 2005). While it is possible that the cytokine responses to helminths in ruminants is more complex than those observed in mice and humans, it is also likely that the increase in IFNγ transcription in the abomasal mucosa following challenge with *O.ostertagi* is not in fact a response to the helminth, but to increased bacterial load in the abomasum and contamination of lesions caused by *O.ostertagi*, which takes place in a similar manner to that described in section 1.2.2.

Local immune responses to gastrointestinal nematodes in sheep, such as *T.circumcincta*, the blood-feeding abomasal nematode *H.contortus* and the small-intestinal nematode *T.colubriformis* are predominantly Th2 in phenotype, involving mucosal inflammation, mastocytosis, eosinophilia and hyperplasia of goblet and mucus cells (Bendixsen et al., 2004, Gruner et al., 1994, Scott et al., 1998a, Balic et al., 2000, Scott et al., 2000, Balic et al., 2003).

Previous studies of cytokine responses to gastrointestinal nematodes in sheep have examined the response in afferent and efferent mesenteric lymph to *T.colubriformis* (Hein et al., 2004, Pernthaner et al., 2005), and in the gastric lymph node to *H.contortus* (Gill et al., 2000, Meeusen et al., 2005, Lacroux et al., 2006); these studies have demonstrated changes in cytokine transcription following infection which are indicative of a Th2-mediated response. However, although changes in draining lymph composition, antibody production and mastocytosis in responses to *T.circumcincta* infection have previously been explored (Smith et al., 1984, Stear et al., 1995, Halliday et al., 2007), the cytokine changes which orchestrate immune responses to this parasite remain to be investigated.

1.3.5 Cytokine function in gastrointestinal nematode infection

The cytokine communication that coordinates immune responses, which has been examined most comprehensively in mice, is extremely complex. Not only are there large numbers of cytokines, but one cytokine may be produced by several different types of cell and have varied effects on several different target cells. The effect of a particular cytokine on a particular target cell may also be influenced by the state of the target cell or the presence of other cytokines. This is further complicated by species variations when applied to other animals, and the continuing discovery of new cytokines and signalling processes.

The cytokines which appear to be predominantly responsible for immune responses to helminths are Th2-type cytokines such as IL-4, IL-5 and IL-13 (Finkelman et al., 2004, Urban et al., 2007). These cytokines coordinate B cell proliferation and antibody production, antibody heavy-chain switching and eosinophil and mast cell proliferation, as well as inhibiting the production of Th1-type responses (Mire-Sluis and Thorpe, 1998, Theze, 1999, Urban et al., 2007).

IL-4 is produced by activated Th2 cells, basophils, mast cells and eosinophils. One of the most significant actions of IL-4 is its involvement in the induction of B cell proliferation and heavy-chain switching of antibody production to IgE and IgG_1. This cytokine promotes mast cell proliferation and inhibits the Th1-type response through suppression of IL-12. IL-4 also has an anti-inflammatory effect via suppression of IL-1, IL-6 and TNFα production. The actions of IL-4 are antagonised by IFNγ (Mire-Sluis and Thorpe, 1998, Theze, 1999).

IL-13 is produced by activated Th2 cells, basophils, mast cells, eosinophils and NK cells. IL-13 activates B cells, stimulates antibody heavy-chain switching to IgG_1 and IgE, and has an important role in mucous cell hyperplasia and increasing mucus production (Khan et al., 2001, Wynn, 2003, Khan and Collins, 2004). It also inhibits macrophage activation and cytokine production. IL-13 acts synergistically with IL-4, but is generally produced in larger quantities and for longer than IL-4 (Mire-Sluis and Thorpe, 1998, Theze, 1999, Gessner et al., 2005, McDermott et al., 2005).

Both IL-4 and IL-13 interact with IL-4 receptors, however, IL-4 can interact with both type 1 and 2 receptors, whereas IL-13 can only interact with type 2. Type 1 receptors are linked with cells of the immune system, particularly T cells, in contrast with type 2, which are also found on non-immune cells including goblet cells, which are involved in helminth expulsion (Finkelman et al., 2004). These cytokines have overlapping effects on a range of target cells, including monocytes, epithelial cells and B cells, and combine to play an important role in anti-parasite immune responses (Trigona et al., 1999).

Experimental studies using knockout mice have shown that the Th2 cytokines IL-4 and IL-13 are essential for the expulsion of intestinal helminths in mice (McKenzie et al., 1999). This is probably due to the role of these cytokines in mast cell proliferation, for example as demonstrated in response to *T.spiralis* in the mouse (Urban et al., 2000, Helmby and Grencis, 2002), and in B cell proliferation and immunoglobulin class switching to IgE and IgG_1 (Snapper et al., 1988, Finkelman et al., 1990), for example during murine *N.brasiliensis* infection (Finkelman et al., 1988). Recombinant human IL-13, which is very similar to bovine IL-13, is able to stimulate antibody production in activated bovine B cells (Trigona et al., 1999).

Work done by Finkelman and colleagues has shown that the immune responses in mice to *N.brasiliensis* and *T.spiralis* are implemented through activation of signal transducer and activator of transcription 6 (Stat6) by IL-4 and IL-13. Activation of Stat6 is involved in the expulsion of intestinal nematodes via processes such as increased intestinal smooth muscle contractility and mucus secretion, and the induction of mastocytosis (Finkelman et al, 2001, Urban et al, 2000).

IL-5 is produced by activated Th2 cells, mast cells and eosinophils. Like IL-4 and IL-13, this cytokine also promotes B cell activation and antibody heavy-chain switching (to IgA and IgE). IL-5 also promotes proliferation and degranulation of eosinophils (Mire-Sluis and Thorpe, 1998, Theze, 1999, Justice et al., 2002).

Previous studies have shown that stem cell factor (SCF), IL-3 and IL-9 may also contribute to Th2-type immune responses through the proliferation or survival of mast cells in the intestinal mucosa of mice infected with *N.brasiliensis* or *T.spiralis* (Madden et al., 1991, Donaldson et al., 1996, Faulkner et al., 1997).

Recently the novel cytokines IL-25 and IL-33 have been implicated in Th2-type immune responses to helminths. IL-25 (Fort et al., 2001) is produced by Th2 cells and mast cells, enhances responses in human Th2 memory cells *in vitro*, promotes Th2-cytokine dependent immune responses to *T.muris* in mice through expression of IL-4 and IL-13, and suppression of Th1-type responses (Owyang et al., 2006, Wang et al., 2007). IL-33, which induces expression of IL-4, IL-9 and IL-13, has been found to be expressed early in infection of mice with *T.muris* and increased expulsion of the parasite from susceptible mice (Schmitz et al., 2005, Humphreys et al., 2008).

Regulatory cytokines, such as IL-10 and TGFβ, are also important in limiting immune-mediated pathology and promote healing (Sanjabi et al., 2009).

IL-10 is produced mainly by Th2 cells, regulatory T cells and macrophages, but also by B and other T cells, mast cells, eosinophils, endothelial cells and fibroblasts. This cytokine acts upon Th1 cells, B cells, macrophages, NK cells and mast cells, and is known to increase NK cell cytotoxicity and promote antibody synthesis by B cells. By blocking CD28 phosphorylation, IL-10 inhibits Th1 cell proliferation and production of Th1 cytokines IFNγ and IL-2. IL-10 also inhibits the production of TGFβ and pro-inflammatory cytokines such as IL-1β, IL-6, IL-8 and TNFα (Mire-Sluis and Thorpe, 1998, Theze, 1999).

TGFβ is produced in the majority of tissues, particularly in the platelets, activated macrophages, neutrophils, B cells and T cells. However, TGFβ requires activation by external factors, so levels of TGFβ expression do not necessarily correspond to levels of active TGFβ present (Munger et al., 1999). This cytokine acts on a wide variety of cell types and is a chemoattractant for mast cells, macrophages and monocytes. TGFβ has an important role in tissue repair, promotes mast cell differentiation, regulates the proliferation of lymphocytes and mast cells, and is also implicated in stimulating antibody switching to IgA. TGFβ tends to promote Th1 cells, resulting in the promotion of IL-2 production (Mire-Sluis and Thorpe, 1998, Miller et al., 1999, Theze, 1999, Knight et al., 2002, Brown et al., 2004, Faler et al., 2006, Knight et al., 2007a).

The relatively recently identified cytokine IL-31 is also produced by Th2 cells (Dillon et al., 2004b), and has been shown to limit Th2-type cytokine responses, IgE and IgG1 production, goblet cell hyperplasia and secretion of goblet cell-derived resistin-like molecule beta (RELM-β) in the intestine of mice infected with *T.muris* (Perrigoue et al., 2009).

Immune responses to helminths are also influenced by Th1-type cytokines, such as IL-2, IL-12 and IFNγ, which promote macrophage activation, antibody opsonisation and inhibit the proliferation of Th2-type responses.

IL-2 is produced primarily by Th1 and NK cells, promotes the proliferation and activation of B cells, and the proliferation of activated T cells. This cytokine also enhances NK cell cytotoxicity and production of IFNγ, killing of bacteria by macrophages and antibody production by B cells (Mire-Sluis and Thorpe, 1998, Theze, 1999). However, the Th1-promoting nature of IL-2 may not be clear cut; in the presence of IL-2, IL-18 has been found to induce Th2-dependant mastocytosis in the intestine in response to *Strongyloides venezeulensis* infections in mice (Sasaki et al., 2005).

Other Th1-type cytokines, such as IL-12 and IFNγ suppress Th2 responses through suppression of IL-4 production or antagonism of its effects (Urban et al., 1993, Mire-Sluis and Thorpe, 1998, Theze, 1999). IL-12 has been shown to reduce goblet cell hyperplasia in mice following *T.spiralis* infection (Khan et al., 2001), and transcription of this cytokine is reduced during Th2 responses and reciprocal IL-4 up-regulation in cattle following infection with *O.ostertagi* (Claerebout et al., 2005). Production of IL-12 is significantly suppressed by IL-10 and TGFβ, it is also partially suppressed by IL-4 and IL-13. IFNγ production is also suppressed by IL-10 (Mire-Sluis and Thorpe, 1998, Theze, 1999, Schopf et al., 2002).

IL-1β, IL-6, IL-18 and TNFα are generally considered to be proinflammatory, and are likely to be involved in the inflammation which accompanies many helminth infections (Mire-Sluis and Thorpe, 1998, Theze, 1999).

IL-1β is produced mainly by monocytes, macrophages and dendritic cells, but also by lymphocytes and fibroblasts. This cytokine is a co-stimulator of T cell activity and IL-2 production, enhances B cell proliferation, antibody production and response to IL-5, and induces further production of pro-inflammatory cytokines by macrophages. Production of IL-1β is suppressed by Th2 cytokines including IL-4, IL-10 and IL-13, and also by IL-6 and TGFβ (Mire-Sluis and Thorpe, 1998, Theze, 1999).

IL-6 is produced by B cells, T cells, macrophages, endothelial cells, fibroblasts, mast cells, eosinophils and smooth muscle cells, and stimulates production of acute phase proteins, B cell proliferation, maturation and, in combination with IL-4, antibody production. As a further example of the difficulties of conveniently classifying cytokines, IL-6 is also involved in the promotion of IL-2 production and Th2 differentiation (Mire-Sluis and Thorpe, 1998, Theze, 1999).

IL-18 has previously been classified as a Th1 type cytokine but is now generally regarded as proinflammatory. This cytokine is produced by many cell types, especially monocytes, macrophages, dendritic cells, B and T cells. It stimulates IFNγ production by Th1 cells, promotes NK cell cytotoxicity (Arend et al., 2008), and suppresses mast cell proliferation in *T.spiralis* infection in the mouse (Helmby and Grencis, 2002). However, in the presence of IL-2, IL-18 has been found to have a role in the induction of Th2-dependent mastocytosis in response to *S.venezeulensis* in mice (Sasaki et al., 2005).

TNFα is produced by macrophages, mast cells, fibroblasts, T cells and B cells. This cytokine promotes fibroblast proliferation and macrophages activation, leading to increased production of IL-1 and IL-6. TNFα shares many of its effects with IL-1 and has an element of synergy with IFNγ in promoting IL-2 production by T cells (Mire-Sluis and Thorpe, 1998, Theze, 1999). TNFα may also have an enhancing effect on Th2 cytokines including IL-4 and IL-13 (Artis et al., 1999).

1.3.6 The action of mast cells during gastrointestinal nematode infection

Mastocytosis is a characteristic feature of Th2-type immune responses to gastrointestinal helminth infection, and is linked with host resistance and parasite expulsion. Mast cells play a key role in the immune response to helminths, not only through production of cytokines to promote the immune response, but by releasing mediators which increase intestinal motility and permeability to aid expulsion of the parasite.

Mast cells are activated by pathogen molecules binding of PRR or cross-linking of IgE bound to Fcε receptors (Else and Finkelman, 1998, Yamasaki and Saito, 2005), triggering release of soluble mediators including histamine, heparin, leukotrienes, prostaglandins, chemokines, and enzymes such as mast cell proteases (Miller, 1993, McDermott et al., 2005, Urban et al., 2007, Bischoff, 2009). Cytokines released by activated mast cells attract lymphocytes and granulocytes to the site of infection. Ovine mucosal mast cells may also secrete compounds that directly inhibit larval migration into the mucus of the small intestine (Douch et al., 1996).

Mast cell proteases have been shown in mice to produce increased intestinal permeability and leakage of fluid into the intestine through degradation of occludin, which is a component of epithelial tight junctions (McDermott et al., 2005). Combined with the actions of mast cell mediators, such as prostaglandins and histamine, in increasing vascular permeability, mucus and electrolyte secretion, this results in an increase in fluid in the intestine (Perdue et al., 1991, Russell, 1986, Baird et al., 1985, Bischoff, 2009). Mast cell signalling via histamine, proteases, lipid mediators such as leukotrienes and prostaglandins, and by cell-cell signalling also causes the enteric nervous system to increase intestinal peristalsis (Bischoff, 2009). Together with the increase in fluid content of the intestine, this increased motility assists expulsion of gastrointestinal parasites.

The importance of mucosal mast cells in the immune response to gastrointestinal helminths appears to vary according to parasite species. Mast cell-mediated increases in gut permeability and contractility have been shown to be essential for the expulsion of *T.spiralis* in mice (Else and Finkelman, 1998, McDermott et al., 2005), and expulsion of *T.spiralis* is delayed in mice lacking mouse mast cell protease-1 (Scott et al., 2000, Knight et al., 2008). However, this is in contrast with expulsion of *T.muris* and *N.brasiliensis*, for which mast cell activity is not essential (Uber et al., 1980, Miller, 1996a, Else and Finkelman, 1998, Knight et al., 2008). Studies of immune responses to *N.brasiliensis* in mice treated with anti-stem cell factor antibody, which causes a marked reduction in mucosal mast cells, have further found that the fecundity of this nematode is reduced in treated mice compared to untreated mice (Newlands et al., 1995), consistent with previous findings that fecundity of *N.brasiliensis* is reduced in mast cell-deficient mice compared to wild-type (Arizono et al., 1993).

Mastocytosis has frequently been observed in response to infection with gastrointestinal helminths in sheep, including *T.circumcincta*, *H.contortus* and *T.colubriformis* (Salman and Duncan, 1984, Bisset et al., 1996, Harrison et al., 1999, Schallig, 2000, Henderson and Stear, 2006), and higher numbers of mucosal mast cells has been linked with greater resistance to parasites (Bisset et al., 1996, Shakya et al., 2009). This is supported by observations that reduced numbers of mucosal mast cells are linked to lower resistance to helminths in periparturient sheep (Huntley et al., 2004, Houdijk et al., 2005).

1.3.7 The action of eosinophils during gastrointestinal nematode infection

An increase in eosinophil numbers has for many years been considered a key feature of helminth infection, however the actual function of these cells during infection is still unclear. While eosinophils are known to produce Th2 cytokines which drive immune responses to helminths, they are thought also to attract dendritic cells in response to helminth infection through production of alarmins, such as eosinophil-derived neurotoxin, and to play a direct role in fighting tissue-dwelling parasites (Kazura and Aikawa, 1980, Butterworth, 1984, Nakajima et al., 1996, Balic et al., 2000, Justice et al., 2002, Klion and Nutman, 2004, Gessner et al., 2005, Oppenheim and Yang, 2005).

Eosinophils bind the complement protein C3b, and also bind IgE via Fc receptors. During helminth infection the C3b and Fc receptors on the eosinophil allow it to bind to C3b and IgE attached to the surface of the helminth, where it releases active oxygen metabolites and toxic granule proteins which damage the parasite membrane directly onto its surface (Kazura and Aikawa, 1980, Butterworth, 1984).

While eosinophils are not essential for expulsion of some parasites, such as *N.brasiliensis* or *T.spiralis* in mice, these cells still mediate damage to the parasite (Kazura and Aikawa, 1980, Vallance et al., 2000, Knott et al., 2007), and depletion of eosinophils increases susceptibility of mice to other parasites, such as *Strongyloides stercoralis* (Galioto et al., 2006). Eosinophils from sheep previously exposed to *H.contortus* L3 larvae were able to kill these larvae *in vitro* in the presence of antibody specific to L3 surface antigen through adherence and degranulation (Rainbird et al., 1998, Terefe et al., 2007). This *in vitro* killing of *H.contortus* L3 by eosinophils was augmented by the addition of complement

(Rainbird et al., 1998, Terefe et al., 2007). The killing of *H.contortus* L3 larvae by closely associated eosinophils has also been observed *in vivo* in the ovine abomasum (Balic et al., 2006). Sheep bred for resistance to gastrointestinal helminths produce more pronounced eosinophil responses to infection following previous exposure than susceptible breeds (Dawkins et al., 1989, Buddle et al., 1992, Hooda et al., 1999, Shakya et al., 2009).

The role of eosinophils in the response to tissue-dwelling helminths may be supported by neutrophils, which accumulate early on at the site of helminth infection (Anthony et al., 2006, Urban et al., 2007) and have been found to similarly damage *Heligmosomoides bakeri* and kill *S.stercoralis in vitro* (Galioto et al., 2006, Urban et al., 2007). Both eosinophils and neutrophils isolated from sheep blood have been found to adhere to *H.contortus* larvae *in vitro* and, though less effective than eosinophils, neutrophils were also shown to reduce larval motility (Terefe et al., 2007). Both eosinophils and neutrophils accumulate rapidly in the mucosa of sheep infected with *T.circumcincta* (Scott et al., 2000).

1.3.8 Additional factors in the rejection of gastrointestinal helminths

The mechanisms of immune detection, orchestration and application of response described in sections 1.3.1-1.3.7 combine to produce immunity to gastrointestinal helminths. These mechanisms are augmented by additional local responses which take place in the abomasum of sheep in response to *T.circumcincta*.

Mucous cell hyperplasia occurs in the abomasal mucosa in response to changes in cytokines such as IL-13 following infection, leading to increased production and altered composition of mucus (Anderson et al., 1988, Else and Finkelman, 1998, Scott et al., 1998a, Scott et al., 2000, McDermott et al., 2005). As well as inhibiting the movement of larvae into the gastric crypts due to its viscosity, this mucus also contains anti-parasitic substances which damage or paralyse the parasites (Miller and Horohov, 2006). Examination of intestinal mucus obtained from sheep following infection with *T.colubriformis* has shown increased levels of parasite-specific antibody and direct anti-parasite activity, demonstrated by larval migration inhibition assays, compared to mucus from naïve sheep (Harrison et al., 1999).

Transcription of sheep intelectins 1, 2 and 3, while undetectable in naïve sheep, has been found to be induced in the mucous neck cells following infection with *T.circumcincta* (French et al., 2009), possibly in response to IL-4 (French et al., 2007). Sheep intelectin 2 expressed by mucous neck cells has also been found in the abomasal mucus following infection with *T.circumcincta* (Athanasiadou et al., 2008, French et al., 2008). Transcription of sheep intelectin 2 occurs earlier and to a greater extent in challenged sheep following previous exposure than in naïve challenged sheep (French et al., 2008). Similarly, sheep intelectin 2 is also up-regulated early in sheep following infection with *H.contortus* (Rowe et al., 2009). Though the precise function of intelectin is not yet clear, it may have a role in altering the properties of mucin and increasing mucus viscosity, and has been implicated in the protective immune responses of mice to *T.spiralis* and *T.muris* (Tsuji et al., 2001, Arranz-Plaza et al., 2002, Pemberton et al., 2004, Datta et al., 2005, Miller and Horohov, 2006, Dann and Eckmann, 2007).

Ovine galectin-14 has been found to be present in the gastric mucus of sheep infected with *T.circumcincta* (Athanasiadou et al., 2008). Ovine galectin-14 is produced by activated eosinophils (Dunphy et al., 2002) and appears to have multiple functions, binding to eosinophils, neutrophils, activated lymphocytes, basement membrane, mucous cells and mucus (Young et al., 2009). This molecule also binds various glycans, such as those with terminal N-acetyllactosamine residues which have been found on N-glycans from *Echinococcus granulosus* hydatid cyst membrane and protoscoleces (Khoo et al., 1997), and lacto-N-neotetraose which is present on schistosome eggs and has been found to drive Th2-type responses (Terrazas et al., 2001, Young et al., 2009). Young and colleagues recently discovered that ovine galectin-14 also binds liver fluke (Young et al., 2009). The extent to which ovine galectin-14 is expressed in mucus following infection with gastrointestinal helminths implies a significant role in the innate response, for example changing the properties of gastrointestinal mucus during helminth infection (Kemp et al., 2009, Young et al., 2009).

Several additional proteins have been identified in mice which are thought to have direct effects on gastrointestinal helminths, such as chitinases/chitinase-like molecules and resistin-like molecules. For example, resistin-like molecule beta is secreted by murine intestinal goblet cells in response to IL-13 following infection with *T.muris*. This molecule has been observed to bind to chemosensory organs associated with the helminth cuticle and inhibit chemotactic migration (Steppan et al., 2001, He et al., 2003, Artis et al., 2004, Nair et al., 2006, Urban et al., 2007, Knight et al., 2008). Resistin-like molecule beta has not been identified in ruminants to date (Knight, 2009, personal communication). Chitinase-like molecules have been identified in sheep, and are up-regulated in the abomasum and gastric lymph node following repeated infection with *T.circumcincta* (Knight et al., 2007b). However the function of these molecules in the sheep is unknown. It has been suggested that chitinase-like molecules and resistin-like molecule beta may have a role in binding mucus and helminth cuticle to trap parasites in the mucus layer (Miller and Horohov, 2006).

Antibody responses are also important in the immune response to *T.circumcincta* (Strain and Stear, 1999). Previous studies of the development of immunity to this parasite have demonstrated increased levels of IgA and B cells in efferent gastric lymph (Smith et al., 1983, Halliday et al., 2007, Halliday et al., 2009b) and abomasal mucus (Athanasiadou et al., 2008) following repeated infection. The scale of this IgA response has been correlated with reduced worm fecundity and the degree of larval stunting in the abomasum (Smith et al., 1985, Stear et al., 1995, Strain and Stear, 1999). The significance of secreted antibodies present in abomasal mucus has been confirmed by the finding that exsheathed *T.circumcincta* larvae coated in IgG obtained from the gastrointestinal mucus of repeatedly infected sheep produced worm burdens 76% lower than uncoated larvae when injected into the abomasum of naïve sheep (Harrison et al., 2008). Increased mucosal permeability during helminth infection may assist transfer of antibodies across the mucosa (Miller, 1996a).

The Th2-driven effects of mast cells and eosinophils, local production of antibodies and anti-parasitic molecules, increased mucus volume and altered mucus composition combine to damage and immobilise the invading parasites (Grencis, 1997, Jackson et al., 2004b, Miller and Horohov, 2006). The compromised parasite is then dislodged and expelled by contractions of the gastrointestinal smooth muscle and increased peristalsis (Castro et al., 1976, Bendixsen et al., 2004, Jackson et al., 2004b), possibly under the influence of IL-13 (Akiho et al., 2007). The role of gastrointestinal motility in the expulsion of *T.circumcincta* is supported by the observation that abomasal smooth muscle obtained from previously infected sheep contracts in response to *T.circumcincta* antigen *in vitro* (Scott and McKellar, 1998).

The overall effect of these immune responses is a combination of exclusion of infective larvae from reaching their tissue niche and invading gastric crypts, reduced growth or arrested development of larvae which do reach the crypts, and reduced fecundity or expulsion of adult parasites (Strain and Stear, 1999, Balic et al., 2000, Jackson et al., 2004a, Kemp et al., 2009, McNeilly, 2005).

For reasons which are not yet known, immune responses to gastrointestinal helminths take far longer to develop, and develop to a lesser extent, in young lambs than immune responses to other pathogens. This is demonstrated by the increased level of morbidity, mortality, faecal egg counts and worm burdens in younger lambs infected with parasites compared to older lambs (Smith et al., 1985, Stear et al., 1999, Good et al., 2006). This is illustrated by studies of *H.contortus* and *T.colubriformis* infection, wherein lambs less than 6 months of age failed to develop immunity following exposure which resulted in protective immune responses in older sheep (Manton et al., 1962, Ritchie et al., 1966, Gregg et al., 1978, Dobson et al., 1990, Good et al., 2006). In contrast, young lambs are able to recruit eosinophils and mast cells in response to *H.contortus* challenge and generate immune responses to *H.contortus* intestinal antigen, which results in a significant reduction in pasture contamination with *H.contortus* larvae (Lacroux et al., 2006, LeJambre et al., 2008).

The reduced capacity of young lambs to develop resistance to gastrointestinal helminths following exposure compared to older sheep may be due to differences in the immune system between the two age groups. At birth 60% of a lamb's circulating T cells are γδ T cells, falling to 30% by the time the animal is one year old (Hein and Mackay, 1991, Washington et al., 1992) and to 10-20% in the adult sheep (Eriksson et al., 1999). Similarly, Merino lambs 4-8 months old have also been found to have a lower proportion of CD4+ cells in their lymph and blood than older sheep 3-6 years old (Watson et al., 1994). It is possible that the comparatively low percentage of CD4+ T cells in young lambs is insufficient to generate the required Th2-type response, or that γδ T cells somehow inhibit its development. Previous studies have demonstrated that young lambs are less able to generate some of the phenotypic changes associated with Th2 responses. Sheep over 12 months old were found to have greater numbers of mucosal mast cells and fewer connective tissue mast cells than lambs 4 months old following the same period of exposure to infected pasture (Douch and Morum, 1993). Similarly, mucus production in lambs 2-3 months old has also been found to be less than in lambs at 9-10 months (Anderson et al., 1988).

1.4 Novel control strategies for gastrointestinal nematodes in sheep

The rising incidence of anthelmintic resistance in gastrointestinal helminths, and the economic impact of this phenomenon on the sheep industry, has made the genetic selection for disease resistance an attractive prospect for farmers. Numerous studies have demonstrated that different breeds are more or less susceptible to infection (Bahirathan et al., 1996, Good et al., 2006, Gonzalez et al., 2008), and that the ability of sheep to develop immunity to gastrointestinal helminths is influenced by the individual's genotype (Gill, 1991, Gauly and Erhardt, 2001, Miller and Horohov, 2006, Stear et al., 2009).

Efforts have been made to artificially develop sheep breeds which are resistant to parasites with some success, and nematode-resistance estimated according to faecal egg count has been shown to be heritable. Further to this, animals from the Wallaceville nematode-resistant Romney sheep line, selectively bred for low faecal egg counts when exposed to natural challenge, consistently have faecal egg counts 36-fold lower, express higher levels of IL-5 and IL-13, and produce small intestinal mucus with significantly more larval migration inhibitory activity than the nematode-susceptible Romney sheep line when infected with *T.colubriformis* (Rabel et al., 1994, Pernthaner et al., 2005). Merino sheep bred for resistance to helminths did not perform better than random-bred sheep in response to primary infection with *H.contortus,* but had significantly higher levels of antibody, mucosal mast cell hyperplasia, mucosal eosinophilia and lower faecal egg counts and worm burdens after a second infection (Gill, 1991). However, selecting sheep for breeding on the basis of faecal egg counts requires treatment for helminth infection to be suspended, thus incurring production losses during this time.

Selective breeding for more specific genetic traits is also a possibility in the development of helminth-resistant sheep breeds, which does not require helminth infection of potential breeding stock for assessment. For example, certain sheep major histocompatibility complex alleles are associated with improved resistance to gastrointestinal helminth infection (Schwaiger et al., 1995, Paterson et al., 1998, Sayers et al., 2005, Stear et al., 2005, Stear et al., 2007).

As sheep do eventually acquire immunity following repeated or prolonged exposure to gastrointestinal helminths, vaccination has been suggested as another possible alternative means of control in the face of growing anthelmintic-resistance. Thus far there are no commercially available vaccines to gastrointestinal helminths, though there is an effective vaccine for bovine lungworm.

Studies of *T.circumcincta* infection of sheep have discovered that the scale of antibody response is correlated with larval stunting and reduced worm fecundity (Smith et al., 1985, Stear et al., 1995, Strain and Stear, 1999). The significance of secreted antibodies present in abomasal mucus has been further confirmed by the finding that exsheathed *T.circumcincta* larvae coated in IgG obtained from the gastrointestinal mucus of repeatedly infected sheep produced worm burdens 76% lower than uncoated larvae when injected into the abomasum of naïve sheep (Harrison et al., 2008).

While attempts to develop vaccines to gastrointestinal helminths using native antigens have had some success, for example *H.contortus* intestinal antigen, which can significantly reduce pasture contamination (Lacroux et al., 2006, LeJambre et al., 2008), commercial production of native antigen from helminths is not a viable means of production. However cloned antigens have not been able to reproduce the efficacy required of a commercial vaccine (Redmond et al., 2006). This problem is likely to be overcome through the use of adjuvants to amplify stimulation of the correct immune response phenotype, probably in combination with mucosal vaccine delivery systems (Lightowlers, 2006). More detailed understanding of the mechanisms by which immunity to gastrointestinal helminths is generated is essential in the development of vaccines capable of maximising the host response (Miller, 1996b, Redmond et al., 2006).

1.5 Objectives

The work described in this thesis is based upon the hypothesis that there will be significant changes in cytokine transcription following infection of sheep with *T.circumcincta*, and that these changes will be different in helminth-naïve sheep compared to those which have developed a degree of immunity, and in young lambs compared to older animals.

Previous studies of gastrointestinal helminth infection in sheep and cattle have found that immune responses to these infections are predominantly Th2 in phenotype, with associated up-regulation of Th2-type cytokines (Gill et al., 2000, Claerebout et al., 2005, Pernthaner et al., 2005, Lacroux et al., 2006). However, the polarisation of Th1- and Th2-type immune responses in ruminants may not be as clearly defined as in rodent models of helminth infection (Gasbarre et al., 2001, Claerebout et al., 2005). Better understanding of the nature and mechanics of the immune responses to gastrointestinal parasites of ruminants is essential to the development of both genetic resistance and potential vaccines.

While the immune response to *T.circumcincta* has many features associated with a phenotypic Th2-type response, the cytokine profile generated in response to infection has not yet been examined. This has become a priority in the study of the development of immunity to *T.circumcincta*, in order to direct the development of potential vaccines towards an optimal effective immune response.

The advantage of examining immune responses to abomasal parasites, compared to those of the intestine, is that the abomasum is a naturally bacteriostatic environment, reducing the potential immunological effect of background bacterial load when examining the response to helminths (Nicholls et al., 1987). *T.circumcincta* is an ideal candidate for this work, as it causes considerably less trauma to the abomasal mucosa in comparison to *H.contortus*, so inflammation is more likely to be a component of the immune response to infection rather than due to trauma of the mucosa (Salman and Duncan, 1984, Crab et al., 2002, Gaugler and Bilgrami, 2004).

The aim of the work described in this thesis was to analyse the cytokine changes which coordinate the immune response to *T.circumcincta*, in particular:

1. Evaluation of cytokine changes in the abomasum, gastric lymph node and gastric efferent lymphocytes of helminth-naïve yearling sheep, and how this changes over the course of a primary infection with *T.circumcincta* (chapters 3, 4 and 5).

2. Comparison of the cytokine changes taking place in the abomasum, gastric lymph node and gastric efferent lymphocytes following a primary infection of helminth-naïve yearling sheep with *T.circumcincta*, and following challenge infection of yearling sheep which had developed partial immunity through

previous infection, in order to define differences in the cytokine profiles of sheep with differing immune status (chapters 3, 4 and 5).

3. Evaluation of cytokine changes in the abomasum and gastric lymph node of naïve and previously infected 5 month old lambs, and how this changes over the course of infection with *T.circumcincta,* in comparison with yearling sheep, to define age-related differences in the cytokine profile following infection (chapter 6).

The overall findings of this project are summarised in chapter 7.

2 Chapter Two: Materials and Methods

2.1 Production of *Teladorsagia circumcincta* L3 larvae

This work was done by Steven Smith and colleagues at the Moredun Research Institute.

Infective third stage (L3) *T.circumcincta* larvae were obtained by faecal culture from previously helminth-free 7-12 month-old sheep harbouring a pure experimental infection at the Moredun Research Institute. The parasites were then washed five times in 100 vols of sterile 0.1M phosphate buffered saline (PBS), pH 7.4, to which had been added 1% (w/v) glucose, 500IU/ml penicillin and 5mg/ml streptomycin (Knox and Jones, 1990). These L3 were stored for up to a month at 4°C before use, and challenge doses administered to each group of sheep were derived from the same batch.

2.2 Experimental animals and sample preparation

This section of the work was carried out by David Smith, Aileen Halliday and colleagues at the Moredun Research Institute, Pam Knight, Anne French, Judith Pate, Elisabeth Thornton and Steven Wright. The animal experiments referred to in this thesis were all subject to approval by the Moredun Research Institute's Animal Experiments and Ethics Committee, and performed according to the regulations of a UK Home Office Project Licence.

These trials were carried out as part of a wider study funded by the VTRI (Veterinary Training and Research Initiative) project (DEFRA/SFC (VT0102)) to investigate the diversity of the immune mechanisms generated to *T. circumcincta* larvae. Much of the work in this section is described in papers submitted for publication by Aileen Halliday, David Smith and colleagues (Halliday et al., 2007, Halliday et al., 2009c, Halliday et al., 2009a). Samples generated during these trials were also used during examination of intelectin (French et al., 2007, French et al., 2008), ovine chitinase-like molecules (Knight et al., 2007b) and changes in the proteome of gastric lymph following infection with *T.circumcincta* (Goldfinch et al., 2008). Samples from these trials have also been a significant component of the PhD theses of Anne T. French and Gillian M. Goldfinch, and the MSc thesis of Julie A. Bethune.

2.2.1 Yearling sheep

Thirty-six Blackface × Leicester and thirty-six Dorset × Suffolk cross-breed sheep 10-12 months old were housed from birth at the Moredun Research Institute under conditions to prevent accidental infection with helminths, and were considered to be helminth-naïve. These sheep were used in a series of experiments described in Table 2.1.

Six Blackface × Leicester and six Dorset × Suffolk sheep were killed while still helminth-naïve. Another six Blackface × Leicester sheep were orally administered a primary infection of 50,000 L3 *T.circumcincta* larvae produced as described in section 2.1 and killed 2 days following infection.

The common gastric lymph ducts of eighteen Dorset × Suffolk and six Blackface × Leicester naïve sheep were cannulated following the procedure developed by Smith and colleagues (Smith et al., 1981) as specified in section 2.2.3; four days later these were orally administered a primary infection of 50,000 L3 larvae; six Dorset × Suffolk sheep were then killed 5 days following infection, six Dorset × Suffolk and six Blackface × Leicester sheep were killed 10 days following infection and the remaining six Dorset × Suffolk sheep were killed 21 days following infection.

Eighteen Blackface × Leicester and twelve Dorset × Suffolk naïve sheep were orally administered a trickle infection of 2,000 benzimidazole-susceptible L3 larvae three times per week for eight weeks, which was then cleared using levamisole at the recommended dose rate of 7.5mg/kg. Seven days later six of the Blackface × Leicester sheep were killed following clearance of the trickle infection, another six Blackface × Leicester sheep were administered a secondary challenge dose of 50,000 L3 larvae, then killed 2 days after that. The common gastric lymph duct of the remaining eighteen sheep was cannulated as before; seven days later these sheep were administered a challenge dose of 50,000 L3 larvae; six of the Dorset × Suffolk sheep were killed 5 days following challenge, the remaining six Blackface × Leicester and six Dorset × Suffolk were killed 10 days following challenge.

The sheep were otherwise treated identically. Naïve sheep were treated with Levamisole (7.5mg/kg) 7 days before challenge or euthanasia as a control, with the exception of experimental group 1. Kill method was by captive-bolt stunning and exsanguination. Post-mortem samples were collected as specified in section 2.2.5.

Table 2.1: 10-12 month old sheep experimental design.

BL = Blackface ewe/Leicester ram; DS = Dorset ewe/Suffolk ram.

Nv = Naïve; PI = Previously Infected; Day 0 = unchallenged.

Experimental Group	Kill date	Trickle infection[a]	Levamisole (7.5mg/kg)	Cannulation of common gastric lymph duct	Challenge infection[b]	Kill day following challenge[c]				
						0	2	5	10	21
1 – Nv (DS)	July 2005	-	-	-	-	6	-	-	-	-
2 – Nv (BL)	Feb 2005	-	+	+	+	-	-	-	6	-
2 – PI (BL)	Feb 2005	+	+	+	+	-	-	-	6	-
3 – Nv (DS)	Jan 2006	-	+	+	+	-	-	6	6	6
3 – PI (DS)	Feb 2006	+	+	+	+	-	-	6	6	-
4 – Nv (BL)	April 2007	-	+	-	+	6	6	-	-	-
4 – PI (BL)	April 2007	+	+	-	+	6	6	-	-	-

[a] 2000 *T.circumcincta* L3 administered three times per week for 8 weeks.

[b] One dose of 50,000 *T.circumcincta* L3, given 7 days after Levamisole treatment.

[c] Quoted figure is the number of sheep in the group killed on the corresponding day following challenge.

2.2.2 Five month old lambs

Thirty-five Blackface × Leicester and forty-four Dorset × Suffolk cross-breed lambs 4-5 months old were housed from birth at the Moredun Research Institute. These lambs were raised under conditions to prevent accidental infection with helminths, and were considered to be helminth-naïve. The common gastric lymph duct of these lambs was cannulated as described in section 2.2.3, and these animals were used in a series of experiments described in Table 2.2.

Four Blackface × Leicester and four Dorset × Suffolk lambs were killed while still helminth-naïve.

Fifteen naïve Blackface × Leicester lambs were orally administered a primary infection of 50,000 L3 larvae; six were killed 5 days following infection, five were killed 10 days following infection and four were killed 22 days following infection. Eighteen naïve Dorset × Suffolk lambs were also orally administered a primary infection of 50,000 L3 larvae; six each were killed 5, 10 and 21 days following infection.

Sixteen naïve Blackface × Leicester lambs were orally administered a trickle infection of 2,000 benzimidazole-susceptible L3 larvae three times per week for eight weeks, which was then cleared using levamisole at the recommended dose rate. Seven days later these lambs were administered a secondary challenge dose of 50,000 L3 larvae; six of these lambs were killed 5 days following challenge, five were killed 10 days following challenge the remaining five were killed 22 days following challenge. Twenty-two naïve Dorset × Suffolk lambs also underwent trickle infection and anthelmintic treatment using the same protocol as the Blackface × Leicester group; four of these lambs were killed on day 0, and six each were killed on days 5, 10 and 21.

The common gastric lymph ducts of the lambs killed on days 5, 10 and 21 had previously been cannulated as described in section 2.2.3. The lambs were otherwise treated identically. Naïve lambs were treated with Levamisole (7.5mg/kg) 7 days before challenge or euthanasia as a control. Kill method was by captive-bolt stunning and exsanguination. Post-mortem samples were collected as specified in section 2.2.5.

Table 2.2: 4-5 month old lamb experimental design.

BL = Blackface ewe/Leicester ram; DS = Dorset ewe/Suffolk ram.

Nv = Naïve; PI = Previously Infected; Day 0 = unchallenged.

Experimental Group	Kill date	Trickle infection[a]	Levamisole (7.5mg/kg)	Cannulation of common gastric lymph duct	Challenge infection[b]	Kill day following challenge[c]				
						0	5	10	21	22
5 – Nv (BL)	Aug 2006	-	+	+	+	4	6	5	-	4
5 – PI (BL)	Aug 2006	+	+	+	+	-	6	5	-	5
6 – Nv (DS)	June 2007	-	+	+	+	4	6	6	6	-
6 – PI (DS)	June 2007	+	+	+	+	4	6	6	6	-

[a] 2000 *T.circumcincta* L3 administered three times per week for 8 weeks.

[b] One dose of 50,000 *T.circumcincta* L3, given 7 days after Levamisole treatment.

[c] Quoted figure is the number of sheep in the group killed on the corresponding day following challenge.

2.2.3 Cannulation of the common gastric lymph duct

The sheep were bedded on sawdust and starved for twenty-four hours prior to anaesthesia to help prevent gastric reflux during surgery. Anaesthesia was induced via facemask and isofluorane gas, or using intravenous thiopentone and maintained during surgery using a gaseous combination of oxygen, isofluorane and nitrous oxide delivered through a cuffed endotracheal tube using a closed circuit apparatus.

Surgical approach to the peritoneal cavity was through a flank incision caudal to the last rib on the right hand side of the animal. The abomasum was located and exposed, and approximately 0.2ml of 1% Evans blue in 0.9% saline was injected under the serosa near the greater curvature. The Evans blue allowed visualisation of the lymph vessels draining the abomasum and the common gastric lymph duct.

The incision was held open using a Cleland retractor set pulled cranially to retract the ribs, the liver retracted cranially and the stomachs moved caudally. The vena cava was retracted dorsally and blunt dissection was used to separate it from the dorsal surface of the pancreas. The common gastric lymph duct, highlighted by the Evans blue within it, could then be seen running from behind the portal vein caudally and dorsally over the pancreas.

Two simple ligatures of were tied loosely around the common gastric lymph duct. The cranial ligature was tightened and a polyvinyl chloride (PVC) cannula, with an external diameter of 1.2mm to 1.27mm, was inserted running caudally into the duct, having previously been flushed with saline. The caudal ligature was then tightened to secure the cannula within the duct (Lascelles and Morris, 1961). The cannula was led out through a small incision on the dorsal flank and secured using elastoplast stitched to the skin. Muscle layers and skin were closed using simple continuous sutures.

A catheter was also inserted into either the jugular vein or the carotid artery, also secured using elastoplast secured to the skin, and run deep under the fleece to the surgical site.

A harness incorporating a bag containing a sterile, heparinized urine collection bag was attached to the sheep’s torso and the lymphatic cannula was run into the urine bag. The distal end of the jugular/carotid catheter was also placed in the harness bag to keep it clean and out of the way.

The sheep was extubated when a satisfactory swallow was observed and returned to its pen to recover from the anaesthetic.

This technique has also been described by David Smith and colleagues (Smith et al., 1981).

2.2.4 Collection of efferent lymph samples

Samples of efferent lymph were collected every day from the day of surgery until either the lymph stopped flowing or the animal was killed.

The lymphatic cannula was removed from the urine bag and the flowing lymph was collected for about an hour into a clean heparinized (<10IU heparin) falcon tube. During this time the accumulated lymph was re-infused via the jugular/carotid catheter. When the fresh lymph collection was complete the lymphatic catheter was returned to the now empty urine collection bag, which was put back in the sheep harness.

Collected efferent lymph samples were centrifuged for 10 minutes at 1200rpm and 4°C, the supernatant was removed and the efferent lymphocytes were resuspended in PBS to remove plasma which can form an insoluble precipitate with RNAlater®. The cells were again centrifuged for 10 minutes at 1200rpm and 4°C, before being resuspended in a small volume of PBS and 5-10 times the volume of RNAlater®. The cells were allowed to sit at room temperature for 15-30 minutes or 1-2 hours at 4°C to increase uptake of the fixative, then stored at -20ºC until processing. (On the day of challenge lymph was collected within an hour of challenge, which is not long enough for larvae to reach the abomasum.)

Aliquots of whole lymph were also taken, which were stored at -20°C for use in proteomic analysis at a later date.

2.2.5 Collection of post-mortem samples

Immediately post-mortem, samples consisting several cubes roughly $5mm^3$ were taken from the gastric lymph node and the mucosa of 2-3 abomasal folds. One set of samples was collected for RNA isolation into pre-labelled tubes and fixed in RNAlater®, incubated at 4°C overnight, then stored at -20˚C until processing. A second set of samples for proteomic analysis was wrapped in labelled aluminium foil, snap-frozen on dry ice, then stored at -70°C. A third set of samples for histological examination was incubated overnight in 4% paraformaldehyde with agitation, then transferred to 70% ethanol and stored at 4°C before trimming and embedding in paraffin.

The abomasum was removed and opened along the lesser curvature, and the contents collected. The abomasum was bisected longitudinally. One half was washed twice with warm saline and the washings added to the abomasal contents. This half was used for the collection of gastric fold samples. The mucosa of the other half of the abomasum was also washed twice with warm saline, and the washings added to the abomasal contents. This half was then submerged in saline at 37°C for 4 hours to recover the larvae from the mucosa (Jackson et al., 1984). The recovered larvae and abomasal contents were separately made up to 5L and fixed using 2% formalin. These solutions were vigorously stirred and a 250ml sample was taken from each for *T.circumcincta* counting, staging and measuring.

2.2.6 *T.circumcincta* counting and measurement

This work was done by Steven Smith and colleagues at the Moredun Research Institute.

Each 250ml sample of abomasal contents or recovered larvae was stained with concentrated iodine solution, and the numbers of adult males, females and sexually undifferentiated worms were recorded.

A random sample of approximately 50 worms was obtained from the abomasal contents of each animal, and these were measured using a camera lucida with ×10 magnification. Sexually undifferentiated worms longer than 1.5mm were classified as ‘developing worms’, shorter ones were classified as L4. Linear regression of the mean lengths of worms recovered from naïve challenged sheep on days 5 and 10 was used to estimate the normal growth rates for each sex; as these can be assumed to grow at a linear rate (Denham, 1969). The resulting formula was used to calculate the ‘equivalent age’ of the worms recovered from the abomasal contents of the previously infected sheep, to assess the extent to which the growth of the worms had been stunted in this group.

Significantly higher numbers ($P<0.01$) of *T.circumcincta* were recovered from the abomasum of naïve yearling sheep on days 2, 5 and 10 (day 2 mean 8,323, day 5 mean 5,574, day 10 mean 12,492) than from the previously infected yearling sheep (day 2 mean 2248, day 5 mean 936, day 10 mean 2,760) (Halliday et al., 2007, Halliday et al., 2009b). Details of day 2 worm counts are contained in a manuscript by Halliday et al, accepted by Parasite Immunology. In some previously infected animals far higher percentages of L4 were recovered, indicating an increase in larval stunting in these animals. These data indicate that parasite loss was occurring by day 2 in the previously infected sheep, and confirms increased resistance in these animals compared to naïve sheep. This is in accordance with previous observations by Smith, Jackson and colleagues (Smith et al., 1983, Smith et al., 1984).

Worm counts obtained from the lambs in experimental group 5 were also significantly higher in the naïve lambs compared to the previously infected (Halliday et al, manuscript submitted to Parasite Immunology). However, the worm counts for experimental group 6, while higher in the naïve challenged groups, were not significantly different.

A small number of adult or L4 worms were recovered from the previously infected yearling sheep killed on day 0 and day 2 following challenge (experimental group 4). It is uncertain why these worms were present, possibly due to a failing of biosecurity during the week following anthelmintic treatment or emerging resistance to Levamisole in the *T.circumcincta* isolate used during trickle infection in the case of the previously infected groups.

2.3 Molecular biology protocols

2.3.1 Homogenisation of tissue stored in RNAlater®

Stratech 2ml screw-top vials were $^1/_3$ filled with 1mm diameter zirconia/silica beads which were incubated in 500µl RLT/BME solution for five minutes at room temperature with inversion to wash them, then drained. Sections of tissue approximately 4mm^3 were then finely chopped under aseptic conditions into pieces roughly 0.5-1mm^3, these were added to the beads with another 1ml RLT/BME. The sample was homogenised in a Stratech Beadbeater-8 three times for one minute.

This protocol was adapted from manufacturer's recommendations and provided by Tracey Coffey, further optimised by Steven Wright and Pam Knight.

2.3.2 Isolation of mRNA from homogenised tissue

Homogenised tissue suspended in RLT/BME solution was applied 700µl at a time to a Qiashredder column and centrifuged for two minutes at 13,000rpm (13793G) using a Biofuge fresco centrifuge at 4°C. The decanted run-off was then mixed with 600µl 70% ethanol, applied 700µl at a time to an RNeasy column (RNeasy Mini Kit™) and centrifuged for one minute at 13.000rpm. 350µl buffer RW1 (RNeasy Mini Kit™) was then added to the column and centrifuged for one minute at 13,000rpm. The sample was treated with 80µl DNAse/RDD applied to the column membrane and incubated at room temperature for 30 minutes, this was then washed using 350µl buffer RW1 centrifuged for one minute at 13,000rpm. The column was transferred to a new collection tube and washed twice with 500µl buffer RPE centrifuged for one minute then two minutes at 13,000rpm. The column was transferred to a 1.5ml eppendorf and centrifuged for one minute at 13,000rpm as an additional drying step. The column was then transferred to a new 1.5ml eppendorf and the mRNA was eluted by twice adding 100µl RNAse-free H_2O to the column and centrifuging for one minute at 13,000rpm.

To fully remove native DNA contamination additional DNAse treatment in solution, using a DNA-free™ kit according to the manufacturer's protocol, was required; probably owing to the high concentration of proliferating cells in the tissue.

Following extraction total mRNA was stored at -70°C until use. This protocol was adapted from manufacturer's recommendations and provided by Tracey Coffey, further optimised by Steven Wright and Pam Knight.

2.3.3 Isolation of mRNA from cells stored in RNAlater®

Cells preserved in RNAlater® were centrifuged at 6,000rpm (2938G), using a Biofuge fresco centrifuge at 4°C, for five minutes to form a pellet. The supernatant was removed and saved until mRNA had been successfully extracted. The pellet was disrupted by the addition of 600µl RLT/BME and agitated for 30 seconds.

Cells in RLT/BME solution were then applied to a Qiashredder column 700µl at a time and centrifuged for two minutes at 13,000rpm. The decanted run-off was mixed with 600µl 70% ethanol then applied 700µl at a time to an RNeasy column (RNeasy Mini Kit™) and centrifuged for one minute at 13.000rpm. 700µl buffer RW1 (RNeasy Mini Kit™) was then added to the column and centrifuged for one minute at 13,000rpm. The sample was treated with 80µl DNAse/RDD applied to the column membrane and incubated at room temperature for 15 minutes, this was then washed using 700µl buffer RW1 centrifuged for one minute at 13,000rpm. The column was transferred to a new collection tube and washed twice with 500µl buffer RPE centrifuged for one minute then two minutes at 13,000rpm. The column was then transferred to a 1.5ml eppendorf and centrifuged for one minute at 13,000rpm as an additional drying step. The column was then transferred to a new 1.5ml eppendorf and the mRNA was eluted by twice adding 30µl RNAse-free H_2O to the column and centrifuging for one minute at 13,000rpm.

Following extraction total mRNA was stored at -70°C until use. This protocol was adapted from manufacturer's recommendations by Pam Knight.

2.3.4 Isolation of mRNA from FACS sorted cells stored in RNAlater®

Fluorescence-activated cell sorter (FACS) sorted cells in RNAlater® were centrifuged using a Biofuge fresco centrifuge at 4°C for 5 minutes at 6,000rpm to form a pellet, and the supernatant removed. The cell pellet was loosened and 350µl RLT/BME was added before agitating thoroughly.

The cells in RLT/BME were then mixed with 350µl 70% ethanol and added to a MiniElute column (RNeasy® Micro Kit), then centrifuged at 13,000rpm for 15 seconds and the flow-through discarded. The column was washed with 350µl buffer RW1, centrifuged at 13,000rpm for 15 seconds and the flow-through discarded, then DNAse treated using 80µl DNAse/RDD and incubated for 15 minutes at room temperature. The column was then washed again using 350µl buffer RW1, centrifuged at 13,000rpm for 15 seconds and the flow-through discarded. The collection tube was replaced and the column was washed with 500µl buffer RPE, centrifuged at 13,000rpm for 15 seconds and the flow-through discarded, then washed with 500µl 80% ethanol and centrifuged at 13,000rpm for 2 minutes. The column was then transferred to a new collection tube and centrifuged with the cap open at 13,000rpm for 5 minutes as an additional drying step. The column was then transferred to a 1.5ml eppendorf and eluted twice using first 14µl then 12µl RNAse-free H_2O, centrifuging at 13,000rpm for 1 minute each time. As 2µl are left behind in the membrane, this results in a total elution volume of 22µl.

Following extraction total mRNA was stored at -70°C until use.

2.3.5 Quantification of mRNA by UV spectrophotometer

When using a Beckman DU 650 spectrophotometer, a 1:50 dilution of the mRNA was produced by adding 2μl of sample RNA to 98μl RNAse-free water (a dilution factor of 50). When using a Cecil CE2021 2000 series spectrophotometer, a 1:5 dilution was produced by adding 3μl mRNA to 12μl RNAse-free H_2O (a dilution factor of 5).

The 260nm absorbance (A_{260}) and the 280nm absorbance (A_{280}) of the diluted sample were measured, and these values were then used to calculate the concentration of mRNA in the sample and as an indication of mRNA quality and purity. The spectrophotometric conversion factor of mRNA is 40.

$$[\text{mRNA}]\ \text{ng}/\mu\text{l} = \text{Dilution Factor} \times A_{260} \times \text{Spectrophotometric Conversion Factor}$$

The OD ratio A_{260}/A_{280} indicates nucleic acid quality. A ratio of 1.8−2.0 is desired, indicating highly purified nucleic acid. A lower ratio indicates protein or phenol contamination (Sambrook et al., 1989).

2.3.6 Reverse transcription of mRNA into single-stranded cDNA

Reverse Transcription (RT) of 1μg total RNA was set up on ice and performed in 20μl volumes using a Techgene thermocycler. Each 20μl reaction volume consisted of 5mM $MgCl_2$, 10mM Tris-HCl (pH 8.8), 1mM deoxynucleotide triphosphate (dNTP) mixture, 20u RNAse inhibitor, 15u AMV reverse transcriptase, 0.5μg oligo dT primer (all from Reverse Transcription Kit) and 1μg total RNA. Following incubation for one hour at 42ºC, the enzymes were deactivated by heating to 99ºC for 5 minutes then the RT-mix was chilled on ice. The resulting single-stranded cDNA was diluted in 80μl of RNAse-free water, to a concentration of 50ng/μl, before being stored at -20ºC.

RNA controls were set up by diluting 0.5μg of sample RNA in 50μl of RNAse-free water. These control samples were also stored at -20ºC and were used to check for native DNA contamination.

2.3.7 Oligonucleotide primer design

Polymerase chain reaction (PCR) assays were used to detect cDNA specific for a set of ovine Th1- and Th2-type cytokines, regulatory cytokines and proinflammatory cytokines. The cytokines chosen were those for which there were ovine gene or RNA sequences available, and which were thought to be good indicators of each type of immune system activity. The number of cytokines to be examined was limited by the time available. The Th1-type cytokines to be examined were IL-2, IL-12 (P_{40} fraction) and IFNγ; the Th2-type cytokines were IL-4, IL-5 and IL-13; the regulatory cytokines were IL-10 and $TGF\beta_1$; the proinflammatory cytokines were IL-1β, IL-6, IL-18 and TNFα.

The oligonucleotide primers specific for ovine IL-1β, IL-6, IL-10, IL-$12p_{40}$, IL-18, $TGF\beta_1$ and TNFα cDNA have been previously published (McNeilly, 2005). Primers were designed to detect ovine IL-2, IL-5, IL-13 and IFNγ cDNA using the published nucleic acid sequences available from the NCBI Reference Sequence Collection (Pruitt et al., 2007) and Primer 3 (Rozen, 2000). Primer sequences for detection of IL-4 cDNA were designed by Pam Knight. Primer sequences produced were checked for secondary structure (Zuker, 2003) and NCBI BLAST searched to check specificity (http://www.ncbi.nlm.nih.gov/BLAST/). Primer sequences selected had low secondary structure with approximately 50-60% guanine/cytosine content, similar melting temperatures but no stable primer interactions, if possible no combination of guanine and cytosine longer than 3bp, less than 2 guanine/cytosine in the last 3bp of the 3' end, and produced a 100-350bp amplicon.

ATPase, a constitutively expressed 'housekeeping' gene, was used as a control to assess the quality of the RT product and as a reference for quantification of cytokine mRNA. Primers sequences for ovine ATPase were kindly provided by Tom

McNeilly. Primer sequences produced are listed in Table 2.3 and were checked again for specificity using PCR product sequencing (see section 2.3.11). The reference sequences used are listed in Table 2.4.

Table 2.3: Primer sequences used for cytokine expression profiling.

Primer		Sequence	Product Size (base pairs)
ATPase	Fw	GCT GAC TTG GTC ATC TGC	167bp
	Rv	CAG GTA GGT TTG AGG GGA TAC	
IL-1β	Fw	CCT TGG GTA TCA GGG ACA A	317bp
	Rv	TGC GTA TGG CTT TCT TTA GG	
IL-2	Fw	GTG AAG TCA TTG CTG CTG GA	202bp
	Rv	TGT TCA GGT TTT TGC TTG GA	
IL-4	Fw	AAC GCC GAA CAT CCT CAC AT	171bp
	Rv	AGT CCG CCC AGG AAT TTG TT	
IL-5	Fw	TGG CAG AGA CCT TGA CAC TG	168bp
	Rv	TTT TCA CAG CAT CCC CTT GT	
IL-6	Fw	TCC AGA ACG AGT TTG AGG	236bp
	Rv	CAT CCG AAT AGC TCT CAG	
IL-10	Fw	AGC TGT ACC CAC TTC CCA	305bp
	Rv	GAA AAC GAT GAC AGC GCC	
IL-12p_{40}	Fw	TCA GAC CAG AGC AGT GAG GT	241bp
	Rv	GCA GGT GAA GTG TCC AGA AT	
IL-13	Fw	GCC AAC TTC AGC TGC CCT GT	210bp
	Rv	AAT GTG CCT GGG CTG CTC TC	
IL-18	Fw	GAG CAC AGG CAT AAA GAT GG	241bp
	Rv	TGA ACA GTC AGA ATC AGG CAT A	
IFNγ	Fw	CTC CGG CCT AAC TCT CTC CT	175bp
	Rv	AGG CCC ACC CTT AGC TAC AT	
TGFβ_1	Fw	GAA CTG CTG TGT TCG TCA GC	169bp
	Rv	GGT TGT GCT GGT TGT ACA GG	
TNFα	Fw	GAA TAC CTG GAC TAT GCC GA	238bp
	Rv	CCT CAC TTC CCT ACA TCC CT	

Table 2.4: Reference sequences used to produce primer sequences.

Cytokine	Sequence Name	NCBI Accession Number (RefSeq)
ATPase	Ovis aries ATPase, Na+/K+ transporting, alpha 1 polypeptide (ATP1A1), mRNA	NM_001009360.1
IL-1β	Ovis aries interleukin 1 beta (IL-1B), mRNA	NM_001009465.2
IL-2	Ovis aries interleukin 2 (IL2), mRNA	NM_001009806.1
IL-4	Ovis aries interleukin 4 (IL4), mRNA	NM_001009313.2
IL-5	Ovis aries interleukin 5 (IL5), mRNA	NM_001009783.1
IL-6	Ovis aries interleukin 6 (IL6), mRNA	NM_001009392.1
IL-10	Ovis aries interleukin 10 (IL10), mRNA	NM_001009327.1
IL-12p_{40}	Ovis aries interleukin-12 p40 subunit precursor (IL-12), mRNA	NM_001009438.1
IL-13	Ovis aries interleukin 13 (IL13), mRNA	NM_001082594.1
IL-18	Ovis aries interleukin 18 (IL18), mRNA	NM_001009263.1
IFNγ	Ovis aries interferon gamma (IFNG), mRNA	NM_001009803.1
TGFβ_1	Ovis aries transforming growth factor, beta 1 (TGFB1), mRNA	NM_001009400.1
TNFα	Ovis aries tumor necrosis factor alpha (TNF-alpha), mRNA	NM_001024860.1

2.3.8 Polymerase chain reaction amplification of cDNA

Polymerase chain reactions (PCR) were set up on ice using PCR Taq DNA Polymerase. These reactions were made up in thin-walled 0.2ml PCR tubes containing a total volume of 50µl; consisting of 250ng cDNA, 10mM Tris-HCl, 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.2µM each forward and reverse primers and 2.5u Taq DNA Polymerase. Mastermix only controls containing no cDNA were used to check for contamination of the PCR reaction mixture.

Techne Gradient and PerkinElmer GeneAmp PCR System 2400 thermocyclers were used to heat the samples to 94°C for 2 minutes for the initial denaturation. Temperature cycling then consisted of a further 40 seconds at 94ºC, annealing at an appropriate temperature for 40 seconds and an extension at 72°C for 2 minutes, this was concluded by a final extension of 5 minutes at 72ºC.

Table 2.5: Conditions used for RT-PCR.

Primer Set	Annealing Temperature	Cycle Number
ATPase	60°C	31
IL-1β	55°C	40
IL-2	52°C	32
IL-4	60°C	40
IL-5	60°C	35
IL-6	55°C	40
IL-10	60°C	35
IL-12p_{40}	54°C	32
IL-13	68°C	37
IL-18	55°C	30
IFNγ	58°C	33
TGFβ_1	61°C	25
TNFα	58°C	30

Optimal annealing temperatures and number of cycles had to be determined for each of the cytokines. Using an annealing temperature which was too low resulted in non-specific primer binding, too high a temperature resulted in an unsuccessful reaction. The annealing temperature of a primer is influenced by its melting temperature (Tm) which is determined by its components, and by the thermodynamics of the reaction between primer and target which is more difficult to predict. The temperature gradient function of the Techne Gradient was used to optimise primer annealing temperature. Cycle numbers were optimised by running repeated PCRs of increasing cycle numbers, then assessing the product using densitometry. Cycle numbers were selected which resulted in sufficient product during the exponential phase of production, but before the reaction became saturated.

Optimisation of cytokine assays is detailed in chapter 3 section 3.2, and the final conditions used are listed in Table 2.5.

2.3.9 Agarose gel electrophoresis

A 10µl aliquot of each PCR product was migrated with 2µl loading dye through a 1.3% agarose-TBE gel stained with 0.1% ethidium bromide, for 30 minutes at 130V alongside 6µl 1Kb Plus DNA ladder as a molecular weight marker. The gel was then photographed using a Kodak® Digital Science Image Station 440CF. Ethidium bromide was diluted to 500µg/ml in a fume cupboard before use, as specified by COSHH risk analysis, and disposed of according to the University of Edinburgh's waste disposal policies.

Ethidium bromide was later replaced with 0.01% GelRed™, which is less carcinogenic, simpler to dispose of and less harmful to the environment. GelRed™ also has the advantage that it does not migrate up the gel during electrophoresis, reducing the risk that samples running in the lower portion of the gel would produce an artificially low reading if they are allowed to migrate into an area of the gel with a reduced concentration of dye.

The Kodak® Digital Science Image Station 440CF used for sections 3.3.1 and 3.3.2 was replaced during later work with a Bio-Rad Molecular Imager® FX, which is capable of producing higher definition images.

Staining technique, photography equipment and settings were consistent within each section of work.

2.3.10 Densitometry of PCR products

To quantify the level of PCR product, densitometry using the 1D Analysis software supplied with the Kodak® Digital Science Image Station 440CF was used to determine the density of bands following migration in agarose-TBE gel. To compare the relative cytokine mRNA expression levels for each of the samples, the values were presented as a ratio of the band density of the cytokine PCR product over that of the corresponding ATPase PCR product. ATPase PCR products used for comparison with each cytokine were produced from the same sample of RT product to further reduce experimental variation.

2.3.11 PCR product sequencing

Before sequencing RT-PCR products were first purified using a High Pure PCR product purification kit according to the manufacturer's protocol. RT-PCR products were sequenced in both directions using di-deoxy chain termination cycle sequencing (Functional Genomics Unit, Moredun Research Institute), sequences were then checked again for identity using an NCBI BLAST search. This technique was used to confirm product specificity of cytokine RT-PCR assays optimised in chapter 3 section 3.2.

2.3.12 Real-time polymerase chain reaction using SYBRgreen

External primers were designed to detect all the cytokines previously listed using the published nucleic acid sequences available from the NCBI Reference Sequence Collection (Pruitt et al., 2007) and Primer 3 (Rozen, 2000). These primers were designed to bind mRNA sites outside the primers listed in Table 2.3, and specificity was again checked by PCR product sequencing as previously described in section 2.3.11. External primer sequences for IL-1β, IL-6, IL-10, IL-12p_{40}, IL-18, TGFβ_1 and TNFα have been previously published (McNeilly, 2005). All external primer sequences are listed in Table 2.6.

Table 2.6: External primer sequences used for production of cDNA stock.

External Primer		Sequence	Product Size (base pairs)	Annealing Temperature
IL-1β Ex	Fw	CTG TGT TCT TCC CTT CCC TT	518bp	60°C
	Rv	CAA AAA TCC CTG GTG CTG		
IL-2 Ex	Fw	CGG GGA ACA CAA TGA AAG AAG TG	250bp	59°C
	Rv	TTG TCC ATT GAA TCC TTG ATC TCT C		
IL-4 Ex	Fw	CCA TGG ACA CAA GTG TGA CAT TAC C	246bp	61°C
	Rv	GTC TTG CTT GCC AGG CTG CT		
IL-5 Ex	Fw	TGC CTA TGT TTG TGC CAA TGC	290bp	57°C
	Rv	TCC ATC TTT CTC CTC CAC ACT TCC		
IL-6 Ex	Fw	GCT TCC AAT CTG GGT TCA	347bp	57°C
	Rv	CCA CAA TCA TGG GAG CCG		
IL-10 Ex	Fw	AGC TGT ACC CAC TTC CCA	305bp	60°C
	Rv	GAA AAC GAT GAC AGC GCC		
IL-12p_{40} Ex	Fw	CTG CTG CTT TTG ACA CTG AA	452bp	57°C
	Rv	CTG GTT TTC CCT GGT TTT G		
IL-13 Ex	Fw	CCT GTG ATG CCT GCC TTG AG	295bp	60°C
	Rv	CCC CAG TAC AGA CAA AAA CAC TTG		
IL-18 Ex	Fw	TCA GAT CAC GTT TCC TCT CC	348bp	63°C
	Rv	GAT GGT TAC AGC CAG ACC TC		
IFNγ Ex	Fw	CAC AGG AGC TAC CGA TTT CAA	248bp	54°C
	Rv	TCT TTT TGT CAC TCT CCT CTT TCC A		
TGFβ_1 Ex	Fw	GAA CTG GAC ACC AAC TAC TG	338bp	60°C
	Rv	TCA GCT GCA CTT GCA GGA G		
TNFα Ex	Fw	TCC TTG GTG ATG GTT GGT	548bp	57°C
	Rv	CAC TGA CGG GCT TTA CCT C		

External primers were used to produce stock PCR product using 40 cycles at optimised annealing temperatures. These PCR products were then quantified by using a Cecil CE2021 2000 series spectrophotometer to measure the 260nm absorbance (A_{260}); double-stranded cDNA has a spectrophotometric conversion factor of 50. The A_{260} of cDNA should be 0.15-1.0 and the A_{260}/A_{280} should be 1.9-2.1.

$$[\text{cDNA}]\ \text{ng}/\mu\text{l} = \text{Dilution Factor} \times A_{260} \times \text{Spectrophotometric Conversion Factor}$$
$$= 1 \times A_{260} \times 50$$

Stock cDNA was then diluted to serial dilutions of 10ng/µl, 1ng/µl, 0.1ng/µl, 0.01ng/µl, $1x10^{-3}$ng/µl, $1x10^{-4}$ng/µl, $1x10^{-5}$ng/µl, $1x10^{-6}$ng/µl, $1x10^{-7}$ng/µl and $1x10^{-8}$ng/µl and stored at -20°C. These standard cDNA concentrations were used during real-time PCR (Q-PCR) to produce standard curves from which the initial concentration of the cytokine cDNA in the sample could be evaluated. Single-stranded sample cDNA was quantified in the same fashion using a spectrophotometric conversion factor of 33.

Real-time polymerase chain reactions (Q-PCR) were set up in a Hoefer™ UVC 500 UV cross-linker using 0.2ml thin-walled, low-profile, white Q-PCR tubes. Each 20µl reaction consisted of 10µl SYBR Green (containing HotStarTaq® DNA polymerase, QuantiTect SYBR Green PCR buffer, dNTP mix including dUTP, SYBR Green I, ROX passive reference dye and 5mM $MgCl_2$), 0.3µM each forward and reverse primers and 50ng single-stranded sample cDNA. Eight blank reactions, which did not have cDNA added, were set up with the samples to be examined and standard cDNA concentrations, both in triplicate.

An Opticon 2 DNA Engine was used to heat the samples to 95°C for 15 minutes to activate the DNA polymerase in the SYBR green. Temperature cycling then consisted of 15 seconds denaturation at 94ºC, annealing at an appropriate temperature for 30 seconds and an extension at 72°C for 30 seconds. After 50 cycles the reaction was concluded with a melting curve analysis to check for primer dimerisation and product specificity, a peak with a low melting-point indicating primer dimerisation. Product specificity was also checked using agarose gel electrophoresis for a sample of products as described in section 2.3.9.

Once Q-PCR was completed, Opticon Monitor 3.1.32 software was used to quantify the samples. Baseline fluorescence indicated by the blanks was subtracted from the sample fluorescence, then amplification curves of fluorescence against cycle number were used to calculate PCR copy numbers. PCR reaction efficiency was indicated by the gradient of the standard curve, which should be approximately -3.3, and correlation coefficient (R^2) should be approximately 1.

$$\text{Efficiency} = \left((10^{\,\mathbf{-1/standard\ curve\ gradient}}) - 1\right) \times 100$$

Product quantity (ng) was derived from the number of cycles at which fluorescence increased above a specified threshold (Ct value). Samples were considered negative if they failed to cross the threshold after 40 cycles of amplification. The average molecular weight of one base pair was assumed to be 650 Daltons, and the number of cDNA copies produced was calculated using the formula:

$$\text{Copy number} = \frac{(\text{PCR Product (ng)} \times 6.022\text{x}10^{\mathbf{23}})}{(\text{Amplicon Length (bp)} \times 1\text{x}10^{\mathbf{9}} \times 650)}$$

2.3.13 Real-time polymerase chain reaction using ΔΔCt Taqman assays

Sheep mRNA sequences listed in Table 2.4 were compared by pairwise alignment (http://www.ebi.ac.uk/emboss/align) with the bovine exons for the relevant gene (http://www.ensembl.org), sequence differences were masked and exon-exon junctions labelled. The masked sequence was checked using an NCBI BLAST search to ensure that it still matched the original sheep mRNA. This sequence was then checked for repeated sequences (http://www.repeatmasker.org) (Smit, 2008) and single nucleotide polymorphisms (SNPs) (http://www.ncbi.nlm.nih.gov/SNP/snpblastByChr.html), and any found were masked. Areas within 5 bases of a masked region or less than 30bp from 5’/3’ end were masked and an appropriate unmasked site remaining at an exon-exon junction was selected. This sequence and selected site were sent to Applied Biosystems for the production of a custom Taqman assay.

Applied Biosystems custom Taqman assays are designed and optimised to work using standard Q-PCR conditions and primer/probe concentrations. The assay consists of primers at a final concentration of 900nM each, and a Taqman MGB (6-FAM dye-labelled) probe at a final concentration of 250nM. The Taqman probe incorporates a 6FAM™ reporter dye at the 5' end and a minor groove binder (MGB) non-fluorescent quencher (NFQ) at the 3' end. The MGB type of NFQ increases melting temperature (T_m) without increasing probe length (Afonina et al., 1997, Kutyavin et al., 1997). Primer and probe sequences used for ATPase were designed by Gillian Goldfinch. These primers and probe were diluted in 1x TE and the concentrations of primer and probe were individually optimised for low Ct and standard deviation before use. Sequences of Taqman primers and probes are listed in Table 2.7.

Taqman Q-PCR reactions were set up in quadruplicate in a Hoefer™ UVC 500 UV cross-linker. For cytokine detection each 20µl reaction consisted of 1µl Taqman gene expression assay (20x), 10µl Taqman gene expression mastermix (2x) and 99ng single-stranded sample cDNA in RNAse-free H_2O. ATPase assays were set up as above, but the Taqman gene expression assay was replaced with separate primers (600nM each) and probe (400nM). Blank reactions, which did not have cDNA added, were also set up for both cytokine and ATPase with the samples to be examined. These reactions were set up in ABgene Thermo-fast 96 detection plates and covered with optical adhesive covers. For each sample, both cytokine and ATPase assays were run on the same plate.

An ABI Prism 7500 was used to heat the samples to 50°C for 2 minutes for optimal Uracil-DNA Glycosylase (UDG) activity (which prevents amplification of contamination from other PCR products incorporating uracil), then 95°C for 10 minutes to activate the AmpliTaq Gold® DNA Polymerase in the assay. Temperature cycling then consisted of 15 seconds denaturation at 95ºC and annealing/extension for 1 minute at 60°C for 45 cycles.

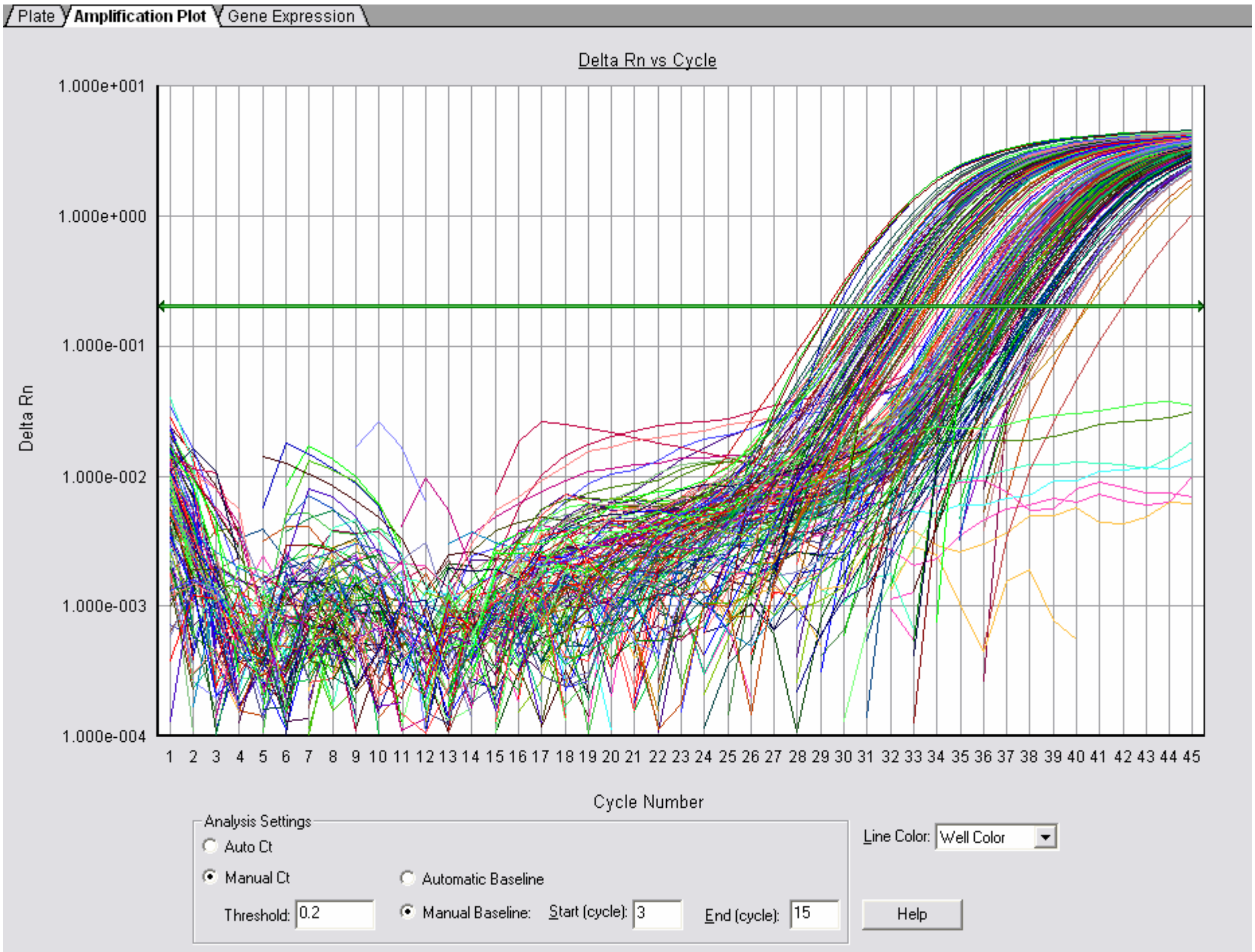
Table 2.7: Taqman assay sequences.

Assay	Forward Primer	Reverse Primer	Probe	Reporter dye	Quencher
ATPase	AAC GGC TTC CTC CCT AAT CAC	GTC GTT GAT CCA ACG GTC ATC	TGC TGG GCA TCC GAG TGA CCT G	6FAM™	TAMRA™
IL-1β	CCT GTC TTG TGT GAA AAA AGG TGA TAC	GGG TAC AGG ACA GAC TCA AAT TCA A	CTG GAG GAA GTA GAC CCC A	6FAM™	NFQ
IL-4	GCG GAC TTG ACA GGA ATC TCA	GTC TTT AGC CTT TCC AAG AGG TCT	CAA GCA AGA CCT GTT CTG TG	6FAM™	NFQ
IL-13	GAA GAG CTG GTT AAT ATC ACC CAG AA	CAC CAT GCT GCC ATT GCA	CAG CGG CAC CTT CT	6FAM™	NFQ
IFNγ	CTC TGA GAA ACT GGA GGA CTT CAA A	GGC TTT GCG CTG GAT CTG	ATC CAC CGG AAT TTG	6FAM™	NFQ

Taqman gene expression mastermix contains ROX™ passive reference dye, which was used to normalize the 6FAM™ reporter signal from each well (Rn), reducing fluctuations in fluorescence between wells due to pipetting error. Applied Biosystems Sequence Detection Software version 1.4 (7500 System SDS software), was used to plot Rn against cycle number. Ct values were then calculated for each reaction: the cycle number at which Rn crossed a predetermined threshold. The threshold used for analysis was 0.2, which fell within the exponential phase of all amplification curves. Samples were considered negative if fluorescence failed to cross the threshold after 40 cycles of amplification.

The efficiency of the assays was calculated by creating a standard curve using serially diluted cDNA. The difference in the gradient of the standard curves for each cytokine compared to ATPase was ≤0.1, indicating the efficiencies of the assays are similar enough to be used for ΔΔCt analysis. ΔCt was calculated by subtracting the mean ATPase Ct value from the mean cytokine Ct value for each sample. Mean ΔCt was calculated for each group of sheep, and the mean ΔCt of an assigned calibrator group was subtracted from that of the other groups to give ΔΔCt. The fold difference in mRNA concentration between each group and the calibrator group was calculated using the formula $2^{-\Delta\Delta Ct}$ (Applied Biosystems User Bulletin #2, ABI PRISM 7700 Sequence Detection System, December 11, 1997) (Winer et al., 1999, Schmittgen et al., 2000, Livak and Schmittgen, 2001).

Figure 2.1: Taqman Q-PCR amplification plot of fluorescence (Rn) against cycle number (green line indicates threshold Rn).



2.4 Immunoassay protocols

2.4.1 Cytokine detection in tissue homogenates by ELISA

Enzyme-linked immunosorbant assay (ELISA) was used to assess levels of cytokine protein present in samples of snap-frozen tissue and tissue preserved in RNAlater. Antibodies used in this protocol were suggested by Sean Wattegedera and Colin McInnes and the protocol was adapted from theirs.

Homogenates were made from 0.2g of tissue finely diced and added to 1ml fresh extraction buffer in a QBiogene Lysing Matrix D tube, then homogenized twice for 40 seconds using a Thermo Electron Bio101 Fastprep (FP120) machine on speed 6. This homogenate was then processed by centrifuging through a Qiashredder to reduce viscosity, using a Biofuge fresco centrifuge at 4°C for 2 minutes at 13,000rpm. The homogenate was then centrifuged for a further 30 minutes at 15,000rpm and 4°C using a Costar® Model 10 centrifuge, and the supernatant aliquotted while leaving the top layer of fats behind; this was repeated three times to remove the majority of the fats and debris in the sample. Equilibrated PHM-L Liposorb was then added to the homogenate at 1 part Liposorb to 1.5 parts homogenate, and allowed to stand for 15 minutes at room temperature. The mixture was then vortexed for 1 minute before being centrifuged for 10 minutes at 3,000rpm and 4°C using a Costar® Model 10 centrifuge. The resulting supernatant consisted of the tissue homogenate with the majority of the lipids and lipoproteins removed. Homogenates were then stored at -80°C.

An M129B ELISA plate was coated with 50µl/well coating antibody diluted to 5µg/ml in 0.1M carbonate buffer, covered with clingfilm and incubated overnight at 4°C. The following day the coating antibody was splashed off, and the plate washed six times with ELISA washing buffer. The plate was then blocked by adding 50µl ELISA blocking buffer, covered with clingfilm and incubated at room temperature for 1 hour. During this incubation recombinant protein was made up to standard concentrations in blocking buffer, and any samples requiring dilution were diluted in PBS/Tween20, both on ice. Blocking buffer was then splashed off and the plate washed twice before loading the plate with 50µl/well sample, recombinant standard or blocking buffer, all in duplicate, covered with clingfilm and incubated at room temperature for 1 hour. Standards and samples were then splashed off and the plate washed again six times before 50µl/well secondary antibody, diluted according to the manufacturer's recommendations, was added and the plate was covered in clingfilm and incubated for 1 hour at room temperature. Secondary antibody was then splashed off and the plate washed six times before 50µl/well of either donkey anti-rabbit antibody conjugated with horseradish peroxidase (HRPO) 1:10000, or streptavidin peroxidase 1:2000, diluted in PBS/Tween20 was added and the plate wrapped in clingfilm incubated at room temperature for 1 hour. The plate was then washed another six times and 50µl/well TMB peroxidase substrate at room temperature was added. Once a graduation of colour was seen in the recombinant standards, but before colour appeared in the blanks, the reaction was stopped by adding 50µl/well 0.18M H_2SO_4. The 450nm absorbance of each well on the plate was read within 30 minutes using a Bio-Rad model 550 microplate reader. This ELISA protocol is summarised in Table 2.8 and antibodies used are described in Table 2.9.

Table 2.8: ELISA protocol summary.

Step	Wash	Add 50µl/well	Incubate
1	-	Coating antibody	Overnight 4°C
2	6 times	Blocking buffer	1 hour, room temperature
3	2 times	Sample/standard/blank	1 hour, room temperature
4	6 times	Secondary antibody	1 hour, room temperature
5	6 times	Donkey anti-rabbit HRPO/ Streptavidin peroxidase	1 hour, room temperature
6	6 times	TMB peroxidase substrate at room temperature	Until colour develops then stop reaction using 50µl/well H_2SO_4

Table 2.9: Antibodies used for cytokine ELISA.

Cytokine	Coating Antibody	Secondary Antibody	Detector
IL-1β	MCA1658 Mouse anti-ovine IL-1β IgG1 Mc 5μg/ml	AHP423 Rabbit anti-ovine IL-1β IgG Pc 1:500	Donkey anti-rabbit IgG HRPO 1:1000
IL-4	MCA2371 Mouse anti-bovine IL-4 IgG2a Mc 5μg/ml	MCA 2372B Mouse anti-bovine IL-4 IgG2b Biotin-conjugated Mc 5μg/ml	Streptavidin HRPO 1:2000

Mc = Monoclonal, Pc = Polyclonal, HRPO = Horseradish peroxidase-conjugated

The mean 450nm absorbance of the blank wells was subtracted from the mean 450nm absorbance of each recombinant standard to give OD units for each concentration. The standard OD units were plotted on a scatter diagram and a trend line was added. The equation of the trend line was used to find the concentration of cytokine in each sample from the OD units of each sample repeat, the mean cytokine concentration for each sample was then used. The correlation coefficient (R^2) of the line should be near to 1 and greater than 0.95. Background OD should be less than 0.2 units, and standard curve height should be 1.0-3.0 OD units.

Protein concentration of each homogenate was calculated using a BCA protein assay. Homogenate samples were diluted 1:5 in extraction buffer, then examined in comparison to a standard curve generated using known concentrations of bovine serum albumin in extraction buffer. BCA protein assay reagent A was mixed with reagent B at a ratio of 50:1, then 100μl was added to each well of an M129B ELISA plate with 10μl homogenate or standard in duplicate. The plate was wrapped in clingfilm and incubated at 37°C for 1 hour, before the 570nm absorbance was read. Protein concentrations were calculated as described above.

Cytokine concentration was then expressed as pg cytokine / g total protein.

2.4.2 Fluorescence activated cell sorting of efferent lymphocytes

Freshly collected efferent lymph samples were centrifuged at 1200rpm for five minutes at 4ºC with brake and the supernatant discarded. The pellet was resuspended in 5ml fluorescence activated cell sorting (FACS) buffer then centrifuged again at 1200rpm for five minutes and the supernatant discarded, this was repeated and the cells washed a second time with 5ml FACS buffer. Following the second wash with FACS buffer, the pellet was resuspended in 6ml FACS buffer resulting in a solution of approximately $1x10^7$ cells/ml.

The washed cells were placed in round-bottomed tubes, 1ml ($1x10^7$ cells) per tube, and incubated with primary monoclonal antibody in the dark at 4ºC for 20 minutes as described in Table 2.10. The cells were then washed again with FACS buffer as described above and incubated for 15 minutes in the dark at room temperature in 1ml FACS buffer containing a 1:400 dilution of secondary mAb; goat anti-mouse IgG r-phycoerythrin, a fluorochrome conjugated antibody which binds to the primary antibody. After incubation the cells were washed twice with FACS buffer as before and resuspended in 2ml PBS containing 0.5−1% FCS ($5x10^6$ cells/ml). Cell only controls, isotype controls containing mouse anti-ovine IgG1and mouse anti-ovine IgG2a primary mAb, and secondary mAb only controls were used. Isotype controls are test antibodies which are not fluorochrome conjugated.

Table 2.10: Monoclonal antibodies used for FACS cell labelling.

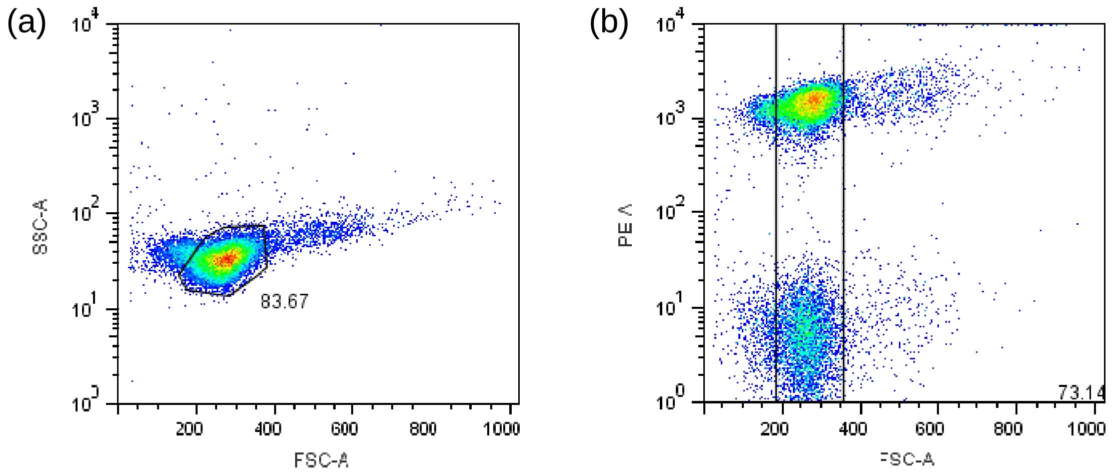
Tube	Primary mAb	Dilution
1	Cell only control	-
2	Isotype control mouse anti-pestivirus IgG1and IgG2a	1:500
3	Secondary mAb only control	-
4	mouse anti-ovine CD4 IgG1	1:1000
5	mouse anti-ovine CD8 IgG2a	1:1000
6	mouse anti-bovine γδ TcR IgG1	1:1000

The cells were then sorted into CD8+ve, CD8-ve, CD4+ve, CD4-ve, γδ+ve and γδ-ve cells by Shonna Johnston at The University of Edinburgh's Queen's Medical Research Institute, using a FACSVantage SE with DiVa (Becton Dickinson). Each population of 10^4-10^5 cells was gated to include lymphocytes and exclude dead cells on the basis of forward-scatter and side-scatter. This sub-population was then further sorted into labelled positive and unlabelled negative cells on the basis of r-phycoerythrin fluorescence.

Sorted cells were collected into PBS containing 10% FCS. These cells were washed once in PBS to remove FCS and resuspended in a 200µl PBS to which 1ml RNAlater® was added. The cells were incubated for 15-30 minutes to increase uptake of the fixative, then stored at -20ºC until processing.

This protocol was developed by David W. Smith and adapted by Pam Knight and Aileen Halliday.

Figure 2.2: Fluorescence activated cell sorting of efferent lymph.
Cell populations gated on the basis of (a) forward-scatter (FSC-A) and side-scatter (SSC-A), then further divided on the basis of (b) r-phycoerythrin fluorescence (PE-A).



2.5 Histology

Mucosal mast cells, and their derivatives globule leucocytes, contain granules which store serine esterase. This enzyme can be specifically stained using a chloroacetate staining solution (Huntley et al., 1985).

Post-mortem samples of gastric fold were collected as detailed in section 2.2.5 and fixed using 4% paraformaldehyde. These samples were later trimmed and embedded in paraffin, longitudinally sectioned, de-waxed in ethanol, then washed and stained for 30 minutes using chloroacetate esterase staining solution. Stained sections were washed using dH_2O and mounted using Polyvinylpyrrolidone (PVP). Embedding was carried out by the pathology department at the R(D)SVS, Easter Bush Veterinary Centre, sectioning and staining was done by Judith Pate.

Longitudinal sections were examined using a Leitz Laborlux S microscope (Leica Microsystems GmbH, Wetzlar, Germany) and mast cells were identified by their content of dark red/purple stained granules. The number of mast cells was counted using a $1cm^2$ eyepiece graticule, as closely as possible orientated with the top edge along the epithelial surface, under $\times 250$ magnification. Counts were repeated ten times across each section of abomasal mucosa and the mean count for each sample was recorded. The mean counts for each of the samples were used to find the group mean. Digital images were collected using an ExwaveHAD 3CCD colour video camera (Huntley et al., 1984, Huntley et al., 1985, Li, 2001).

2.6 Statistical analyses

Minitab® version 14 (Minitab Inc.) was used to analyse data and Prism® 4 (GraphPad Software Inc) was used to graph the data. Advice on statistical analysis was kindly given by Darren Shaw.

Multiple groups of data (cytokine transcription relative to ATPase or mast cell counts) were checked for normal distribution using a One-Way Analysis of Variance (ANOVA) and assessment of the plot of residual values. A linear residual plot indicated a normal distribution, however deviations from a linear plot indicated the data were not normally distributed. A 95% confidence interval was used, and significance was indicated by a P-value less than 0.05.

If data were found not to be normally distributed, excluding the use of parametric tests, non-parametric tests were applied. Kruskal-Wallis test was used to look for significant differences between naïve-challenged or previously infected-challenged data, an H-value of more than 3 and a P-value less than 0.1 indicating significance and the use of post-hoc tests (Kruskal, 1952). Degrees of freedom for each H-value are indicated as subscripts.

Mann-Whitney U test with 95% confidence intervals was also used to check for significant differences from the day 0 groups, and between naïve challenged and previously infected groups on the same day. Significant differences between two data sets were indicated by $P<0.05$. This prevented significant differences being masked by the production of a non-significant P-value when using Kruskal-Wallis. Dunn's test was then carried out using InStat® 3 (GraphPad Software Inc.) to look for significant differences from day 0 groups and confirm that a significant difference found using Mann-Whitney U test was not an artefact of the large number of test being run (type I error) (Mann, 1947, Dunn, 1964, Zar, 1999).

Worm count and measurement analyses were done by Aileen Halliday and colleagues at the Moredun Research Institute. Worm counts and the percentage of L4 larvae were compared by Student's t-test. Frequency distributions of male and female worm lengths were calculated for each sheep, from which group mean distributions were derived. Correlations between worm burden and cytokine levels were examined using scatter plots and Pearson's correlations on ranked data, with significance indicated by $P<0.05$ (Halliday et al., 2007).

3 Chapter Three:

Cytokine Responses in the Gastric Lymph Node

3.1 Introduction

Studies of *O.ostertagi* infection in cattle have shown that the cytokine changes taking place in the gastric lymph nodes in this species are predominantly those associated with a Th2 immune response, though the polarisation of the response may not be as clearly delineated as in the mouse (Gasbarre et al., 2001, Claerebout et al., 2005). Similar studies in the sheep have examined the cytokine response in afferent and efferent mesenteric lymph to the small-intestinal mucosal browser *T.colubriformis* (Pernthaner et al., 2005), and in the gastric lymph node to the abomasal blood-feeding nematode *H.contortus* (Gill et al., 2000), demonstrating that the immune response to these parasites is predominantly Th2 mediated. However, the cytokine response to *T.circumcincta* which takes place in the ovine gastric lymph node remains to be examined.

The gastric lymph node in sheep drains the rumen, reticulum, omasum and abomasum. However, the rumen, reticulum and omasum are lined with thick stratified epithelium, and lymphatic vessels are confined to the layers of the lamina propria mucosae (Schnorr et al., 1975, Hampl and Slezakova, 1977, Hampl et al., 1978). Therefore the immune exposure of this lymph node is almost exclusively to the contents of the abomasum. The normal environment of the abomasum is bacteriostatic due to the activity of digestive enzymes and low pH; which implies that in an animal infected with abomasal helminths, the observed immune response would be provoked by the presence of the helminth not background bacterial populations (Nicholls et al., 1987).

The cytokine responses taking place in the gastric lymph nodes of naïve-challenged and previously infected-challenged sheep were examined following exposure to

T.circumcincta. These were compared with those taking place in naïve and unchallenged previously infected sheep, to gain a greater understanding of the protective immune mechanisms involved in parasite expulsion and resistance, and how these develop over time following exposure.

The advantages of this study over its predecessors is that because the abomasum is normally a bacteriostatic environment, the immunological changes observed following infection with abomasal helminths are less likely to be influenced by the presence of gut microbes, which may have been a factor in previous studies of intestinal helminths such as *T.columbriformis*. *T.circumcincta* also causes far less trauma than *H.contortus*, reducing the probability of observed proinflammatory responses being subsequent to mucosal damage. *H.contortus* feed on blood by lacerating the abomasal mucosa and producing anti-coagulant (Crab et al., 2002, Gaugler and Bilgrami, 2004), which causes inflammation, oedema, congestion of the mucosal blood vessels and small haemorrhages (Salman and Duncan, 1984). In contrast *T.circumcincta* is a mucosal browser, with larvae developing within gastric crypts before emerging as adults on the surface of the mucosa. Though damage is caused to the mucosa as the *T.circumcincta* larvae outgrow their crypt and compress the surrounding tissue, they would not be expected to cause the same level of trauma as *H.contortus* does.

Differences in the cytokine responses of naïve, naïve-challenged, previously infected-unchallenged and previously infected-challenged sheep possessing partial immunity may provide clarification on the development of immunity to *T.circumcincta*, and why this development takes a relatively long time.

The main objectives of this section of work were:

(1) To optimise RT-PCR assays for the detection of cytokine transcription.

(2) To assess whether each cytokine is being transcribed in the gastric lymph node.

(3) To define how transcription of these cytokines changes following *T.circumcincta* infection in naïve-challenged compared with previously infected-challenged groups of sheep.

3.2 Optimisation of cytokine RT-PCR assays

A range of Th1, Th2, proinflammatory and regulatory cytokines was chosen to be examined following challenge of sheep with *T.circumcincta*, as well as the "housekeeping" gene ATPase. This range was limited by the availability of ovine gene sequences and by the number of cytokines which reasonably be examined. Ten cytokines were initially chosen: the Th1-type cytokines IL-2, IL-12 (p_{40} fraction) and IFNγ; the Th2-type cytokine IL-4; the proinflammatory cytokines IL-1β, IL-6, IL-18 and TNFα; and the regulatory cytokines IL-10 and TGFβ_1.

RT-PCR assays were initially tested using three groups of sheep. This was done both to determine whether ATPase, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p_{40}, IL-18, IFNγ, TGFβ_1 and TNFα transcripts were detectable in the lymph node using these assays, and to optimise the assays to produce semi-quantitative results. These assays were designed as described in section 2.3.7.

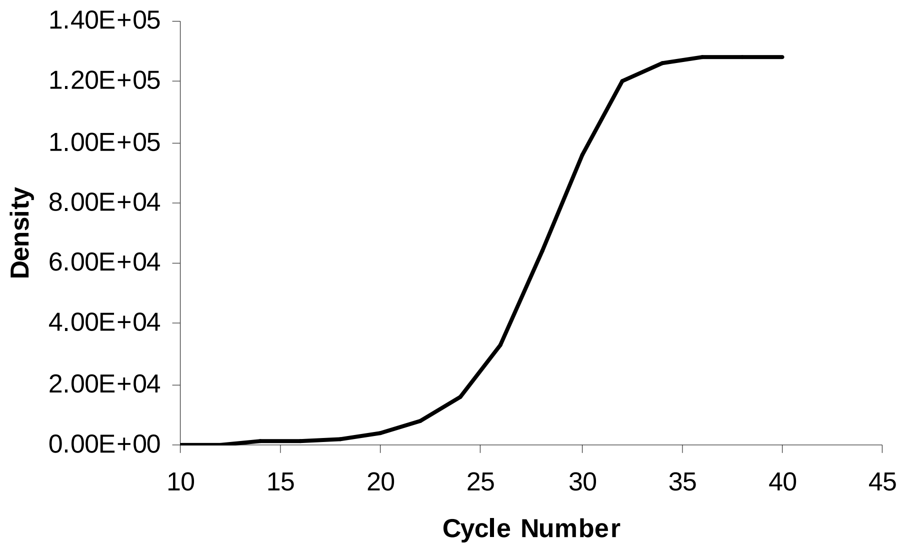
Eighteen gastric lymph node samples were taken at post mortem from naïve sheep, and naïve-challenged and previously infected-challenged sheep 10 days after challenge in experimental groups 1 and 2 as described in sections 2.2.1 and 2.2.5, and Table 2.1. These samples were homogenised and RNA extracted and quantified as specified in sections 2.3.1, 2.3.2 and 2.3.5. This RNA was then examined for transcription of the housekeeping gene ATPase by RT-PCR according to sections 2.3.6 – 2.3.10.

Initially RNA extraction, which includes DNAse treatment, did not include an additional DNAse treatment in solution. However, RNA-only control samples included in the RT-PCR for ATPase produced an amplified product, indicating native DNA contamination of the RNA. This contamination was probably due to the large amount of DNA in the tissue overwhelming the DNAse treatment included in the RNeasy Mini Kit™, and was removed by incorporating additional DNAse treatment in solution using a DNA-free™ kit as specified in section 2.3.2.

For each set of primers, the annealing temperature used during RT-PCR was optimised by running identical reactions for 40 cycles across a temperature gradient using the temperature gradient function of the Techne Gradient thermocycler. These samples were imaged using agarose gel electrophoresis and quantified by densitometry. The highest temperature which produced a single distinct band of the correct molecular weight was selected. At higher annealing temperatures the primers would anneal less efficiently, limiting the reaction. At lower temperatures multiple bands are more likely to be produced due to non-specific primer binding.

The optimal number of cycles for each assay was found using repeated PCR with increasing cycle numbers and quantifying the product by densitometry. A graph was then made of band density against cycle number, as illustrated in Figure 3.1.

Figure 3.1: RT-PCR product band density increases with cycle number.



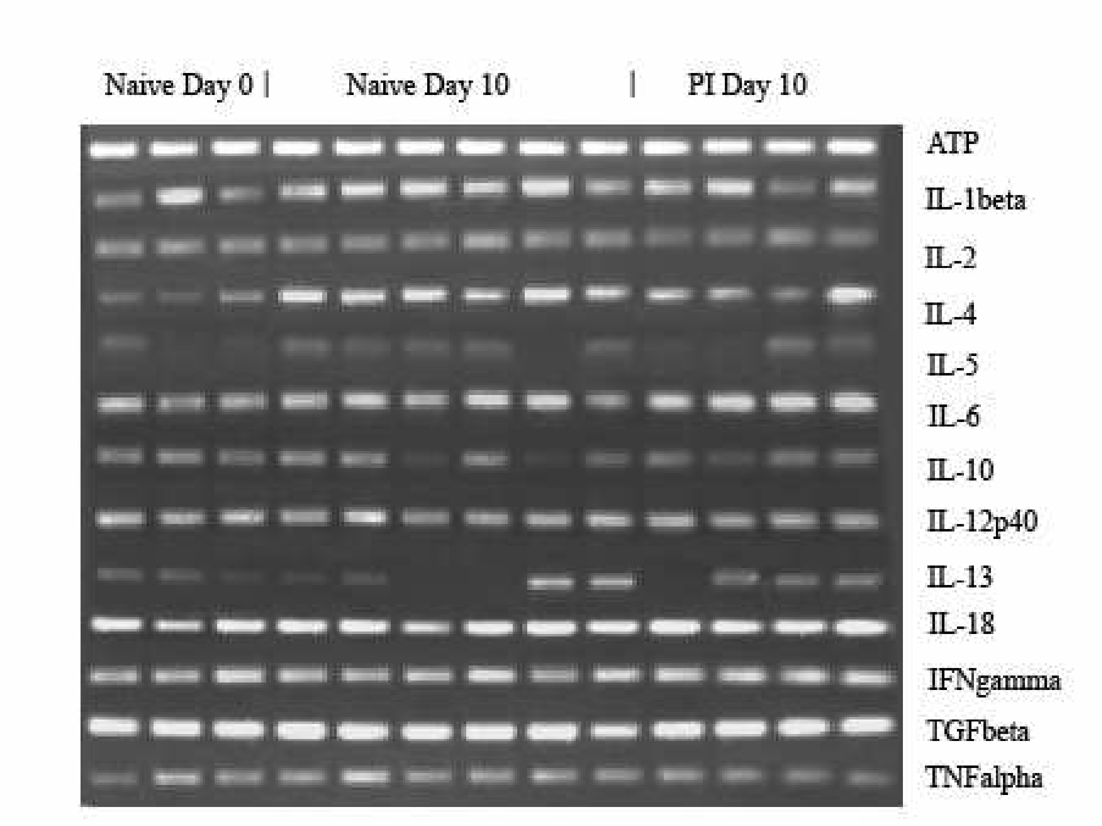
In the early stages of PCR, product is produced almost exponentially. During later cycles the conditions within each reaction are altered by the consumption of reaction components, reducing the reaction efficiency. Eventually product concentration reaches a plateau once all the components have been used up. The optimal cycle number places the end of the PCR reaction within the "exponential" phase of the reaction for each particular primer set.

Annealing temperature and PCR cycle numbers were optimised in this way for each cytokine primer set, and are listed in Table 2.5. Assay specificity was confirmed by PCR product sequencing as described in section 2.3.11.

At the start of this project a complete sequence for ovine IL-13 was unavailable. Attempts to optimise primers generated from bovine IL-13 or from partial ovine sequences were unsatisfactory as they produced either multiple bands of incorrect molecular weights, or did not produce any detectable product after 40 cycles of PCR (data not shown). Ovine IL-13 primers used by Hein and colleagues (Hein et al., 2004) were also tested, however the PCR product produced by these primers was too small to be reliably differentiated from the primers on an agarose gel, so this assay was not used.

New ovine mRNA sequences for the Th2-type cytokines IL-5 and IL-13 were released during 2006, which were used to produce successful RT-PCR assays during study 2.

Figure 3.2: Composite image of cytokine assay PCR products visualised on agarose gel.



3.3 Cytokine transcription in the gastric lymph node

3.3.1 Study 1 – Preliminary studies of cytokine transcription in the gastric lymph node

Following optimisation, assays were used to examine the transcription of ATPase, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p_{40}, IL-18, IFNγ, TGFβ_1 and TNFα in gastric lymph node samples. These samples were taken from unchallenged naïve, previously infected-challenged and naïve-challenged day 10 yearling sheep as detailed in Table 3.1. Cytokine transcription in the naïve-challenged and previously infected-challenged sheep 10 days after challenge was compared, with the naïve group included as a comparison.

Table 3.1: Experimental design – Gastric lymph node study 1.

BL = Blackface ewe/Leicester ram; DS = Dorset ewe/Suffolk ram.
Nv = Naïve; PI = Previously Infected; Day 0 = unchallenged.

Experimental Group	Kill date	Trickle infection[a]	Challenge infection[b]	Kill day following challenge[c]	
				0	10
1 – Nv (DS)	July 2005	-	-	6	-
2 – Nv (BL)	Feb 2005	-	+	-	6
2 – PI (BL)	Feb 2005	+	+	-	6

[a] 2000 *T.circumcincta* L3 larvae administered 5 times per week for 8 weeks.
[b] One dose of 50,000 *T.circumcincta* L3, given 7 days after Levamisole treatment.
[c] Quoted figure is the number of sheep in the group killed on the corresponding day following challenge.

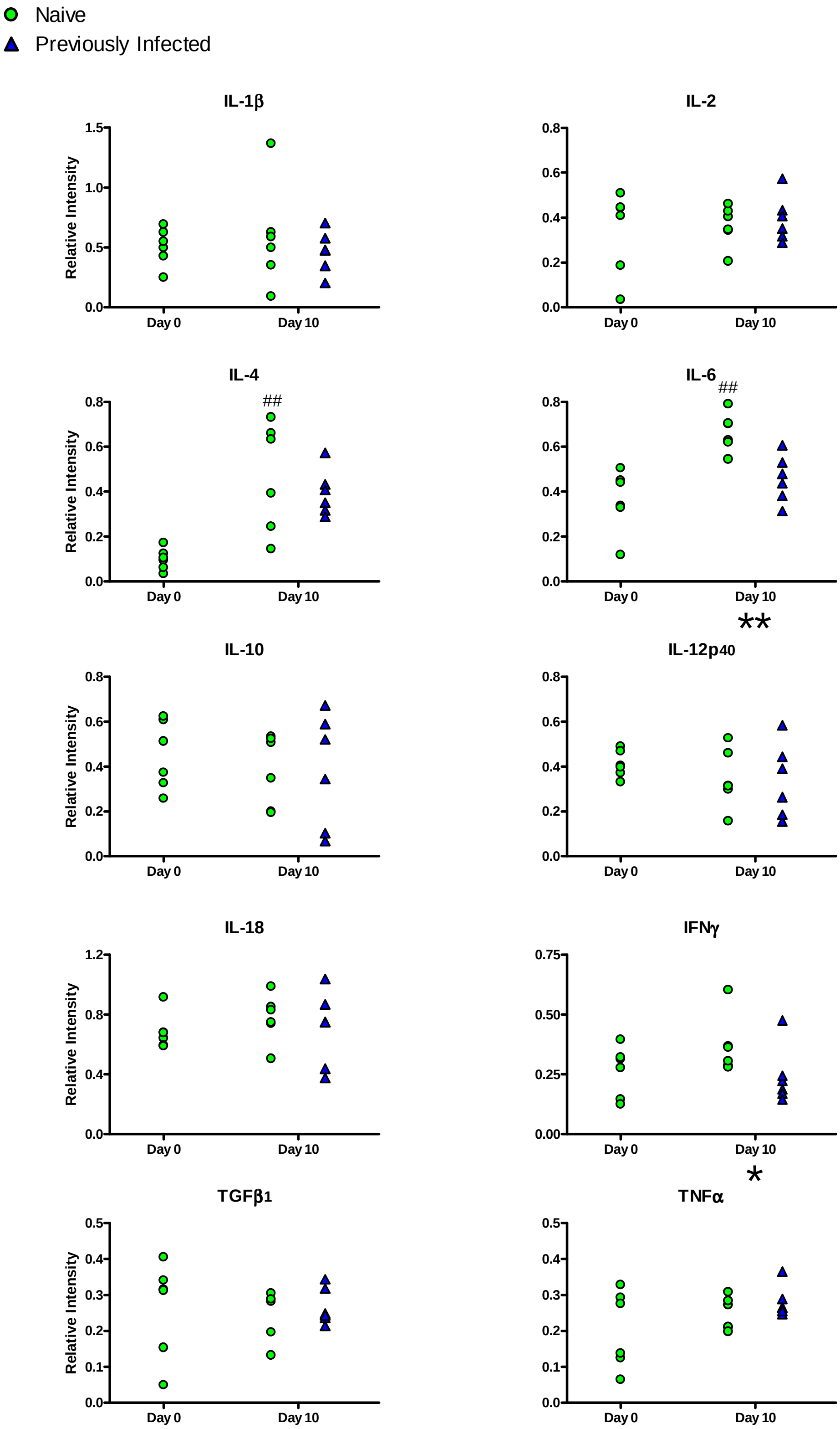
The ATPase primers used for this section of work were derived from the bovine ATPase sequence and designed by Pam Knight; these were replaced in subsequent studies with primers derived from the ovine ATPase sequence, as detailed in section 2.3.7. A Kodak® Digital Science Image Station 440CF was used to image agarose gels prior to densitometry during this section of work.

To compare the relative cytokine expression levels for each of the samples, cytokine expression was presented as a ratio of the band density of the cytokine PCR product over that of the corresponding ATPase PCR product, as described in section 2.3.10. This reduced experimental variation by normalising for the amount of RNA in each RT reaction. ATPase PCR products used for comparison with each cytokine were produced from the same sample of RT product to further reduce experimental variation.

All cytokines were detectable in the lymph node samples at both day 0 and day 10. Statistical analysis was carried out using ANOVA residual plot to check for normality. As all the cytokines were found not to be normally distributed according to the ANOVA residual plot, with the exception of IL-2 which was normally distributed, non-parametric tests were considered to be appropriate for analysis of these data. Transcription of IL-2 was also analysed using non-parametric tests so that the statistical analysis was consistent across the range of cytokines. Kruskal-Wallis test was used to look for significant differences between groups, indicated by a P-value of <0.01 only in the case of IL-4 ($P=0.005$, $H=10.77_2$) and IL-6 ($P=0.004$, $H=11.24_2$). Mann-Whitney U test was also used to make specific comparisons between groups or time-points as described in section 2.6.

Significant increases in IL-4 ($P=0.0082$) and IL-6 ($P=0.0051$) transcription were seen following infection in the naïve-challenged compared to the naïve group. IL-6 ($P=0.0082$) and IFNγ ($P=0.0453$) transcription was significantly higher in the naïve-challenged group than the previously infected-challenged group at day 10. These results are illustrated in Figure 3.3.

Figure 3.3: Cytokine transcription in the gastric lymph node relative to ATPase – Study 1. Analysed using Mann-Whitney U-test for non-parametric data with a 95% confidence interval, n = 6 sheep per group for each time point; significant difference between naïve- and previously infected-challenged groups on day 10: ✱ *(P<0.05),* ✱✱ *(P<0.01); significant difference between naïve and naïve-challenged: # (P<0.05), ## (P<0.01). Note different ranges on Y-axes.*



3.3.2 Study 2 – Temporal changes in cytokine transcription in gastric lymph nodes at day 0, 5, 10 and 21

Following the success of the preliminary experiment detailed in section 3.3.1, this work was repeated using samples from a second trial, which included previously infected-challenged day 5 and 10, and naïve-challenged day 5, 10 and 21 sheep. As well as confirming the previous experiment using new sheep killed on day 10, this allowed the inclusion of additional day 5 and day 21 time-points. Results were compared to those of the unchallenged naïve animals in experimental group 1- Nv.

Additional Th2 cytokine assays were also included in this study. The availability of new ovine IL-13 sequences allowed development of an additional IL-13 assay, and a new IL-5 assay was also included. Both these assays were developed as described in section 2.3.7 and optimised as in section 3.2. This study is detailed in Table 3.2 and the results are illustrated in Figure 3.4. A Kodak® Digital Science Image Station 440CF was used to image agarose gels during this section of work.

Table 3.2: Experimental design – Gastric lymph node study 2.

BL = Blackface ewe/Leicester ram; DS = Dorset ewe/Suffolk ram.

Nv = Naïve; PI = Previously Infected; Day 0 = unchallenged.

Experimental Group	Kill date	Trickle infection[a]	Challenge infection[b]	Kill day following challenge[c]			
				0	5	10	21
1 – Nv (DS)	July 2005	-	-	6	-	-	-
3 – Nv (DS)	Jan 2006	-	+	-	6	6	6
3 – PI (DS)	Feb 2006	+	+	-	6	6	-

[a] 2000 *T.circumcincta* L3 larvae administered 5 times per week for 8 weeks.

[b] One dose of 50,000 *T.circumcincta* L3, given 7 days after Levamisole treatment.

[c] Quoted figure is the number of sheep in the group killed on the corresponding day following challenge.

Figure 3.4(a): Cytokine transcription in the gastric lymph node relative to ATPase – Study 2. Analysed using Mann-Whitney U-test for non-parametric data with a 95% confidence interval, n = 6 sheep per group for each time point; significant difference between naïve- and previously infected-un/challenged groups on the same day: ★ (P<0.05), ★★ (P<0.01); significant difference between naïve and naïve-challenged: # (P<0.05), ## (P<0.01). Note different ranges on Y-axes.

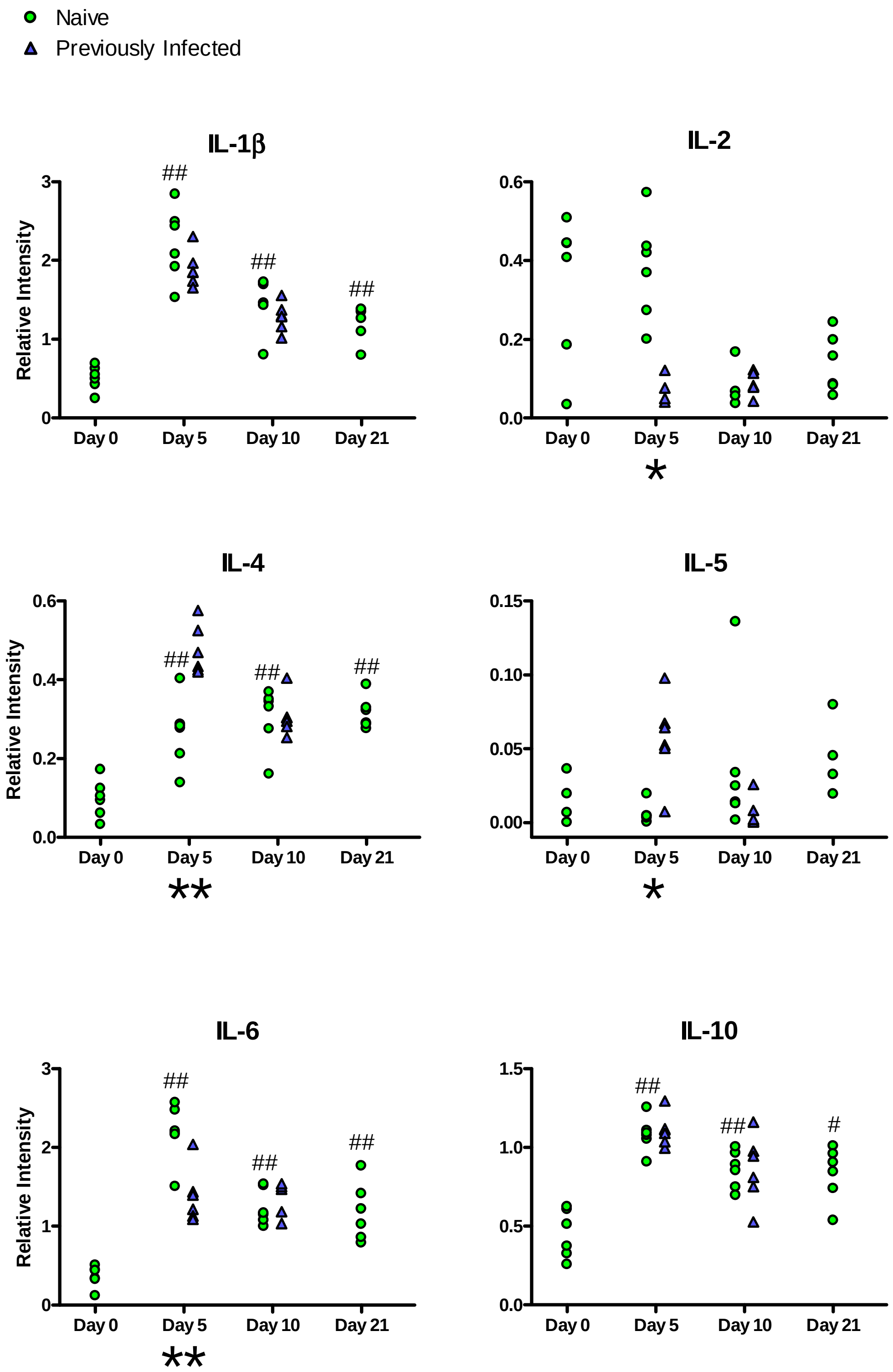
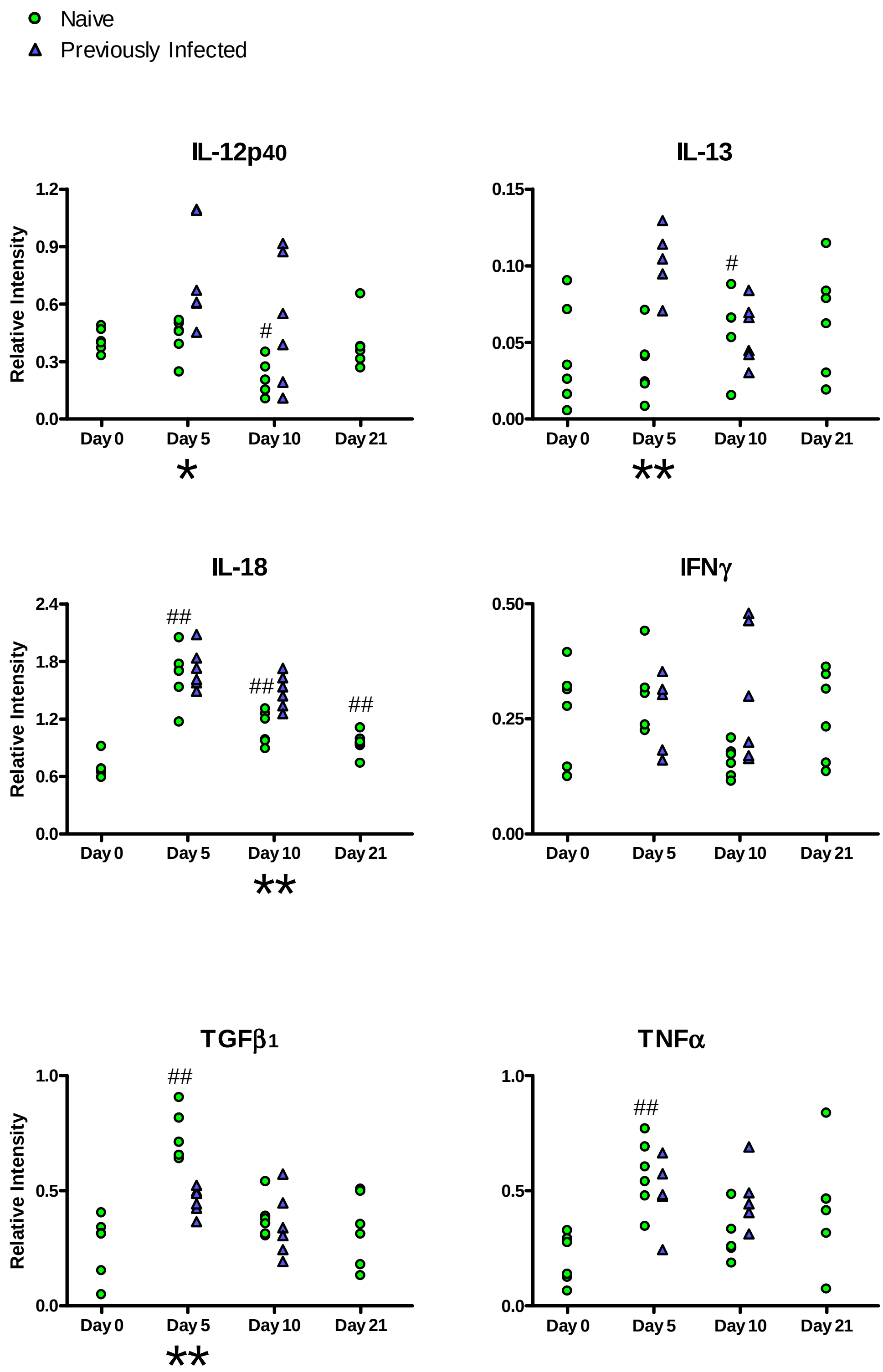


Figure 3.4(b): Cytokine transcription in the gastric lymph node relative to ATPase – Study 2. Analysed using Mann-Whitney U-test for non-parametric data with a 95% confidence interval, n = 6 sheep per group for each time point; significant difference between naïve- and previously infected-un/challenged groups on the same day: ✱ *(P<0.05),* ✱✱ *(P<0.01); significant difference between naïve and naïve-challenged: # (P<0.05), ## (P<0.01). Note different ranges on Y-axes.*



As all the cytokines were found not to be normally distributed according to the ANOVA residual plot, non-parametric test were considered to be appropriate for analysis of these data. Kruskal-Wallis test indicated a significant difference between groups in the case of IL-1β ($P<0.001$, $H=28.51_5$), IL-2 ($P<0.001$, $H=16.87_5$), IL-4 ($P<0.001$, $H=24.75_5$), IL-6 ($P<0.001$, $H=23.46_5$), IL-10 ($P<0.001$, $H=24.03_5$), IL-18 ($P<0.001$, $H=28.96_5$), $TGF\beta_1$ ($P<0.001$, $H=19.62_5$) and TNFα ($P=0.002$, $H=14.54_5$). Mann-Whitney U test was also used to make specific comparisons between groups or time-points as described in section 2.6.

Significant ($P<0.01$) up-regulation of IL-1β, IL-4, IL-6, IL-10, IL-18, $TGF\beta_1$ and TNFα was observed in the naïve-challenged day 5 group compared to the naïve. A significant increase in IL-1β ($P<0.01$), IL-4 ($P<0.01$), IL-6 ($P<0.01$), IL-10 ($P<0.05$) and IL-18 ($P<0.01$) transcription compared to the naïve group was also found in the naïve-challenged day 10 and 21. IL-12p_{40} was significantly down-regulated ($P<0.05$), and IL-13 up-regulated ($P<0.05$), in the naïve-challenged group by day 10 compared to the naïve. Transcription of IL-2, IL-5 and IFNγ did not significantly change following challenge of naïve animals.

When comparing naïve-challenged and previously infected-challenged groups at the same time-point, significantly higher transcription of IL-2 ($P<0.05$), IL-6 ($P<0.01$) and $TGF\beta_1$ ($P<0.01$) was observed in the naïve-challenged group compared with previously infected-challenged samples on day 5. In contrast, transcription of IL-4 ($P<0.01$), IL-5 ($P<0.05$), IL-12p_{40} ($P<0.05$) and IL-13 ($P<0.01$) was significantly higher in the previously infected-challenged samples on day 5 than in the naïve-challenged. No significant difference in these cytokines was observed between the two challenged groups on day 10. Transcription of IL-18 was significantly higher in the previously infected-challenged group compared to naïve-challenged animals on day 10 ($P<0.01$).

The results of this study were published in Craig NM, Miller HR, Smith WD and Knight PA (2007) “Cytokine expression in naïve and previously infected lambs after

challenge with *Teladorsagia circumcincta*." Veterinary Immunology and Immunopathology 120(1-2): 47-54.

3.3.3 Study 3 – Temporal changes in cytokine transcription in gastric lymph nodes at day 0, 2 and 5

Subsequent to the work detailed in section 3.3.2, an additional experiment was run to establish the early phase cytokine responses occurring on day 2 following infection. This study used samples from a further experiment trial comprising the naïve and previously infected animals in experimental group 4.

Previously used experimental group 1 samples, from sheep which had not been challenged but had not been confirmed naïve by faecal egg count or treated with Levamisole, were replaced with group 4 samples which were confirmed naïve by faecal egg count, treated with Levamisole as a control and unchallenged.

Experimental group 4 also provided a previously infected-unchallenged control group, which had been administered an 8 week trickle infection then killed without challenge on day 0, as a baseline control for the immune response in previously infected-challenged sheep. Day 2 samples provided information on the early cytokine responses occurring as the larvae were moving into the gastric crypts. These samples had not been available during earlier studies as sheep from experimental group 4 were killed as part of a separate experiment a year later than the animals used in previous studies. Samples collected from naïve-challenged and previously infected-challenged animals in experimental group 3 at 5 days post-challenge were included as a comparison.

Sheep in experimental group 4 were killed during the same month. Sheep from experimental group 3 had been killed a year earlier and were included for day 5 comparison.

RNA was extracted from the experimental group 4 samples by Judith Pate. This study is detailed in Table 3.3 and the results are illustrated in Figure 3.5. During this and future sections of work, a Bio-Rad Molecular Imager® FX was used to image agarose gels prior to densitometry.

Table 3.3: Experimental design – Gastric lymph node study 3.

BL = Blackface ewe/Leicester ram; DS = Dorset ewe/Suffolk ram.
Nv = Naïve; PI = Previously Infected; Day 0 = unchallenged.

Experimental group	Kill date	Trickle infection[a]	Challenge infection[b]	Kill day following challenge[c]		
				0	2	5
3 – Nv (DS)	Jan 2006	-	+	-	-	6
3 – PI (DS)	Feb 2006	+	+	-	-	6
4 – Nv (BL)	April 2007	-	+	6	6	-
4 – PI (BL)	April 2007	+	+	6	6	-

[a] 2000 *T.circumcincta* L3 larvae administered 5 times per week for 8 weeks.
[b] One dose of 50,000 *T.circumcincta* L3, given 7 days after Levamisole treatment.
[c] Quoted figure is the number of sheep in the group killed on the corresponding day following challenge.

As in the previous experiment, statistical analysis to check for normality was carried out by evaluation of an ANOVA residual plot. As transcription of all the cytokines was found not to be normally distributed, non-parametric tests were considered appropriate for data analysis. Kruskal-Wallis test indicated a significant difference between groups in the case of IL-1β (P=0.001, $H=22.04_5$), IL-4 (P=0.006, $H=16.31_5$), IL-10 (P=0.003, $H=18.19_5$), IL-13 (P=0.003, $H=17.72_5$), IL-18 (P<0.001, $H=24.72_5$), IFNγ (P<0.001, $H=25.17_5$) and $TGF\beta_1$ (P<0.001, $H=22.34_5$). Mann-Whitney U test was also used to make specific comparisons between groups or time-points as described in section 2.6.

Up-regulation of IL-4 and IL-10 was observed in naïve-challenged samples on day 2 ($P<0.05$) compared to the naïve group; in contrast to a reduction in transcription of IL-12p_{40}, IL-18 and IFNγ found in the previously infected-challenged samples ($P<0.05$) at this time-point compared to the previously infected-unchallenged group. Transcription of IL-1β and TGFβ_1 was significantly ($P<0.05$) increased in both the naïve-challenged and the previously infected-challenged groups on day 5 compared to their respective unchallenged groups, whereas IFNγ transcription was reduced in both groups ($P<0.01$). Transcription of IL-2, IL-4, IL-18 was significantly reduced in the previously infected-challenged group on day 5 ($P<0.01$), whereas IL-10 was increased ($P<0.05$), compared to the previously infected-unchallenged animals. Transcription of IL-5, IL-6, IL-13 and TNFα did not significantly change following challenge.

When comparing the two unchallenged groups, significantly higher transcription of IL-4 was observed in the previously infected-unchallenged group compared with naïve group ($P<0.05$), whereas transcription of IL-10 was higher in the naïve group than in the previously infected-unchallenged ($P<0.05$). On day 2 following challenge transcription of IL-10 ($P<0.05$), IL-12p_{40} ($P<0.05$), IL-18 ($P<0.01$) and IFNγ ($P<0.05$) was significantly higher in the naïve-challenged samples than in the previously infected-challenged, whereas IL-13 transcription was higher in the previously infected-challenged group ($P<0.01$). On day 5 significantly ($P<0.01$) higher transcription of IL-1β, IL-2 and IL-18 was found in the naïve-challenged samples than the previously infected-challenged, whereas IL-13 transcription was again higher in the previously infected-challenged group ($P<0.05$).

There was some inconsistency in the significance of the differences in cytokine transcription between the naïve- and previously infected-challenged day 5 groups, and between the naïve and naïve-challenged day 5 groups, when the findings of this study are compared to study 2. In the case of differing levels of significance when evaluating the changes in transcription between the naïve and the naïve-challenged day 5 groups, as for IL-4, IL-6, IL-10, IL-18, IFNγ and TGFβ_1, this is most likely to be due to the replacement of the original naïve group with that from experimental group 4. Where there are differing levels of significance when evaluating the changes in transcription between the naïve- and the previously infected-challenged day 5 groups, as for IL-1β, IL-4, IL-5, IL-6, IL-12p_{40}, IL-18 and TGFβ_1, this is probably due to experimental variation between the different RT-PCR reactions run during each study. Quantification of RT product and dilution to a standard concentration prior to PCR may have reduced this type of error, however this was not done.

Figure 3.5(a): Cytokine transcription in the gastric lymph node relative to ATPase – Study 3. Analysed using Mann-Whitney U-test for non-parametric data with a 95% confidence interval, n = 6 sheep per group for each time point; significant difference between naïve- and previously infected-un/challenged groups on the same day: ★ *(P<0.05),* ★★ *(P<0.01); significant difference between challenged groups and their respective unchallenged groups: # (P<0.05), ## (P<0.01). Note different ranges on Y-axes.*

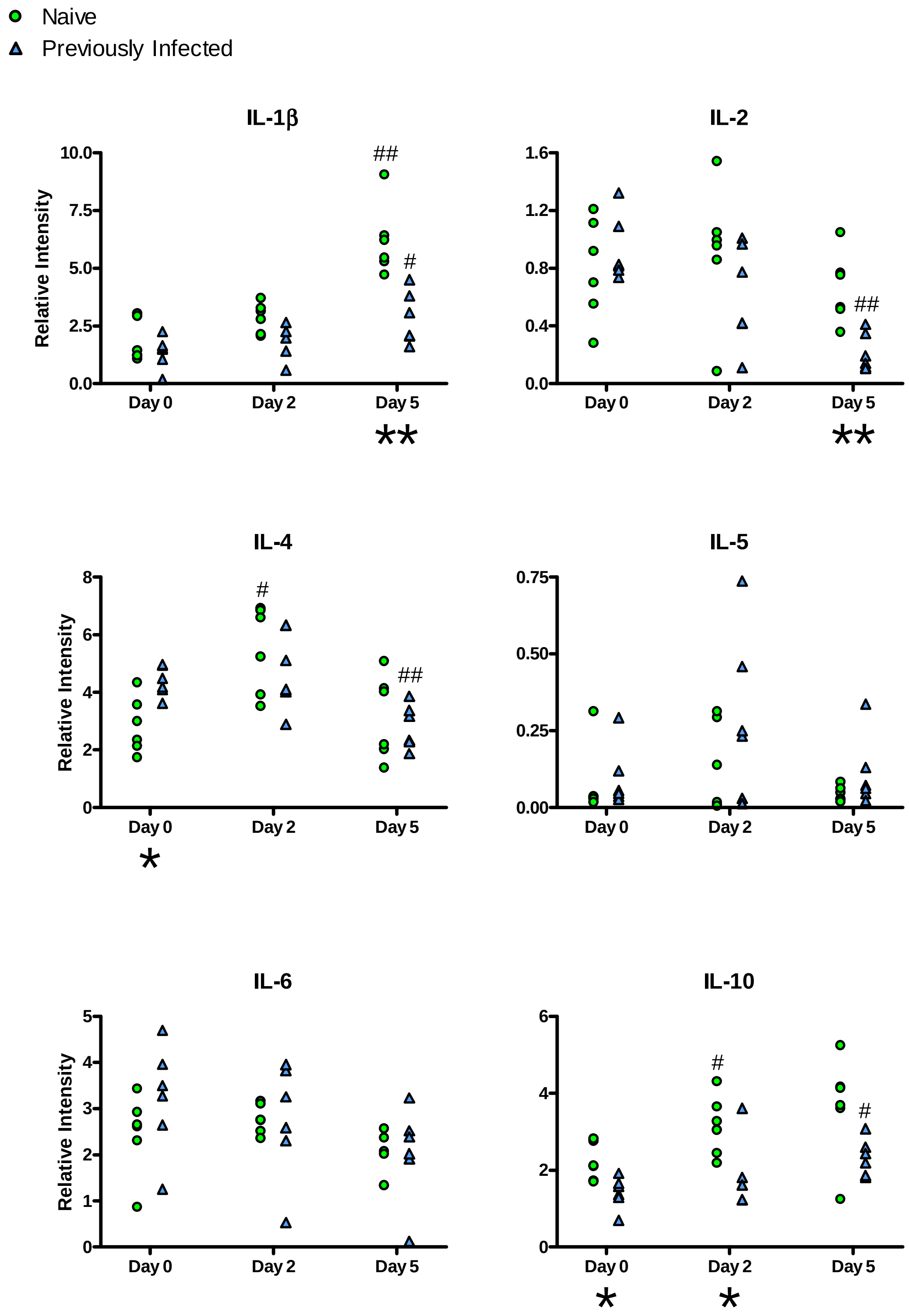
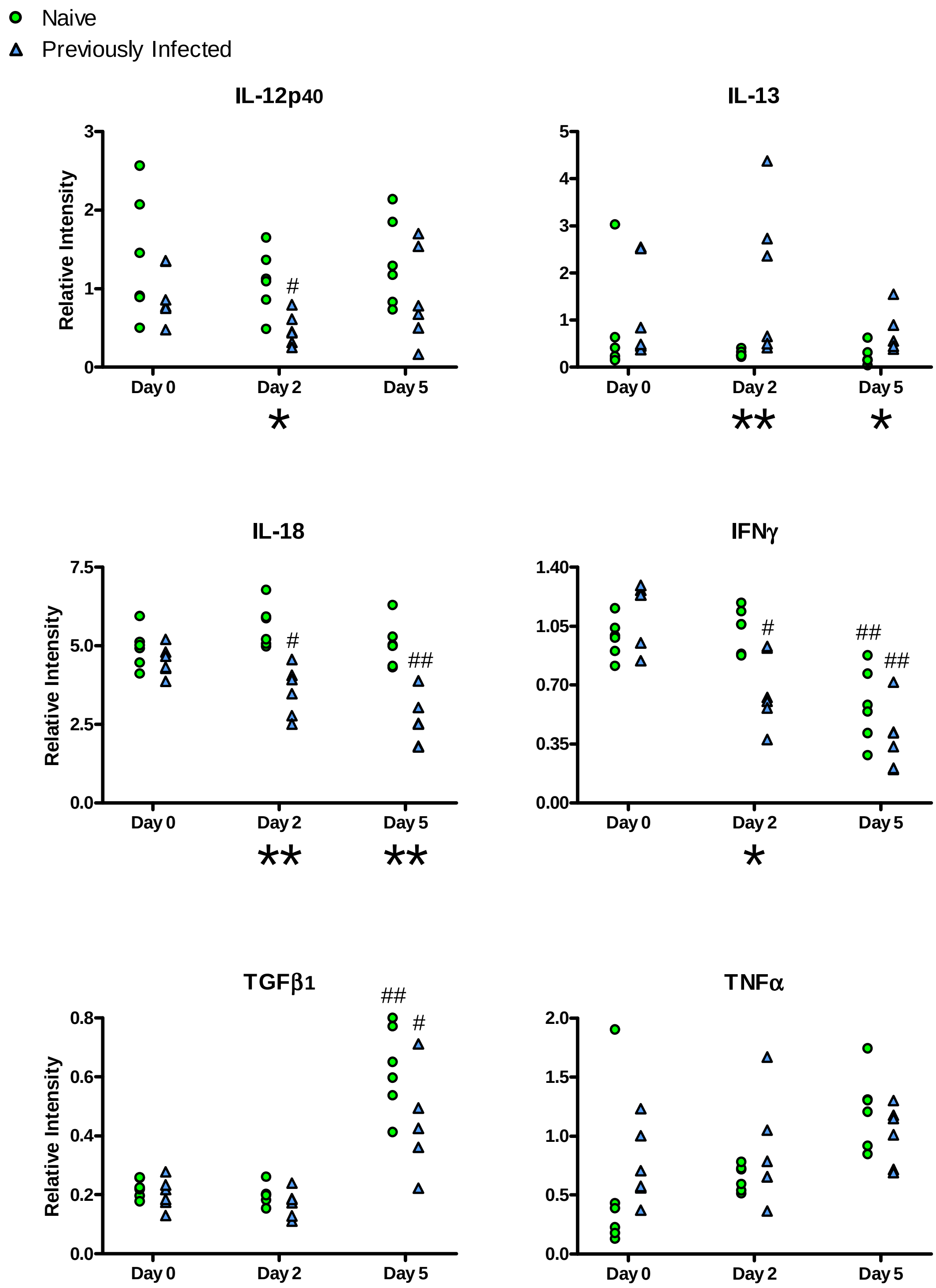


Figure 3.5(b): Cytokine transcription in the gastric lymph node relative to ATPase – Study 3. Analysed using Mann-Whitney U-test for non-parametric data with a 95% confidence interval, n = 6 sheep per group for each time point; significant difference between naïve- and previously infected-un/challenged groups on the same day: * *(P<0.05),* ** *(P<0.01); significant difference between challenged groups and their respective unchallenged groups: # (P<0.05), ## (P<0.01). Note different ranges on Y-axes.*



3.3.4 Study 4 – Cytokine transcription in gastric lymph nodes at day 0, 2, 5, 10 and 21

The trends established in section 3.3.2 and 3.3.3 were confirmed in subsequent assays incorporating unchallenged naïve and previously infected, naïve-challenged day 2, 5, 10 and 21, and previously infected-challenged day 2, 5 and 10. The design for this experiment is detailed in Table 3.4 and the results are illustrated in Figure 3.6.

Table 3.4: Experimental design – Gastric lymph node study 4.

BL = Blackface ewe/Leicester ram; DS = Dorset ewe/Suffolk ram.

Nv = Naïve; PI = Previously Infected; Day 0 = unchallenged.

Experimental group	Kill date	Trickle infection[a]	Challenge infection[b]	Kill day following challenge[c]				
				0	2	5	10	21
3 – Nv (DS)	Jan 2006	-	+	-	-	6	6	6
3 – PI (DS)	Feb 2006	+	+	-	-	6	6	-
4 – Nv (DS)	April 2007	-	+	6	6	-	-	-
4 – PI (DS)	April 2007	+	+	6	6	-	-	-

[a] 2000 *T.circumcincta* L3 larvae administered 5 times per week for 8 weeks.

[b] One dose of 50,000 *T.circumcincta* L3, given 7 days after Levamisole treatment.

[c] Quoted figure is the number of sheep in the group killed on the corresponding day following challenge.

In order to compare and confirm the earlier results described in section 3.3.2 and 3.3.3, it was necessary to examine the previously infected-unchallenged samples and both naïve-challenged and previously infected-challenged day 2 samples with the previous samples from days 5, 10 and 21 in the same assay. This reduced error caused by variation between PCR thermocycler runs.

Statistical analysis was carried out as before, using an ANOVA residual plot to check for normality. Transcription of all the cytokines was found not to be normally distributed, therefore non-parametric tests were considered appropriate for data analysis. Kruskal-Wallis test indicated a significant difference between groups in the case of IL-2 ($P=0.001$, $H=29.58_9$), IL-4 ($P=0.007$, $H=22.73_9$), IL-5 ($P<0.001$, 30.88_9), IL-6 ($P=0.002$, $H=26.33_9$), IL-10 ($P<0.001$, $H=34.38_9$), IL-13 ($P<0.001$, $H=32.86_9$), IL-18 ($P<0.001$, $H=30.29_9$), IFNγ ($P<0.001$, $H=39.57_9$), $TGF\beta_1$ ($P<0.001$, $H=38.14_9$) and TNFα ($P<0.001$, $H=38.14_9$). Mann-Whitney U test was also used to make specific comparisons between groups or time-points, in this study with the addition of Dunn's test as described in section 2.6. Dunn's test was added to determine whether significance according to Mann-Whitney U test was an artefact of the large number of test being done, rather than a genuinely significant difference. Scatter plots and Pearson's correlations on ranked data were used to look for correlations between worm burden and cytokine levels, however none were found.

Significant increases were found using Mann-Whitney U test in transcription of IL-4 ($P<0.05$) and IL-5 ($P<0.01$) in previously infected-challenged samples on day 2 following challenge when compared to the previously infected-unchallenged group. This is in contrast to the reduction in IL-4 transcription found in the previously infected-challenged samples on day 5 ($P<0.05$). These findings were not significant when using Dunn's test, possibly reflecting the features of this statistical test discussed in section 2.6, or because the biological variation between individual sheep rendered the difference between groups insignificant using Dunn's test. Transcription of IL-5 was found to be higher in previously infected-challenged groups than the naïve-challenged on days 0, 2 ($P<0.01$) and 5 ($P<0.05$) after challenge, and that of IL-13 was found to be higher in previously infected-challenged groups than the naïve-challenged on days 2 ($P<0.01$) and 5 ($P<0.05$).

Figure 3.6(a): Cytokine transcription in the gastric lymph node relative to ATPase – Study 4. Analysed using Mann-Whitney U-test for non-parametric data with a 95% confidence interval, n = 6 sheep per group for each time point; significant difference between naïve- and previously infected-un/challenged groups on the same day: ✱ *(P<0.05),* ✱✱ *(P<0.01); significant difference between challenged groups and their respective unchallenged groups: # (P<0.05), ## (P<0.01). Note different ranges on Y-axes.*

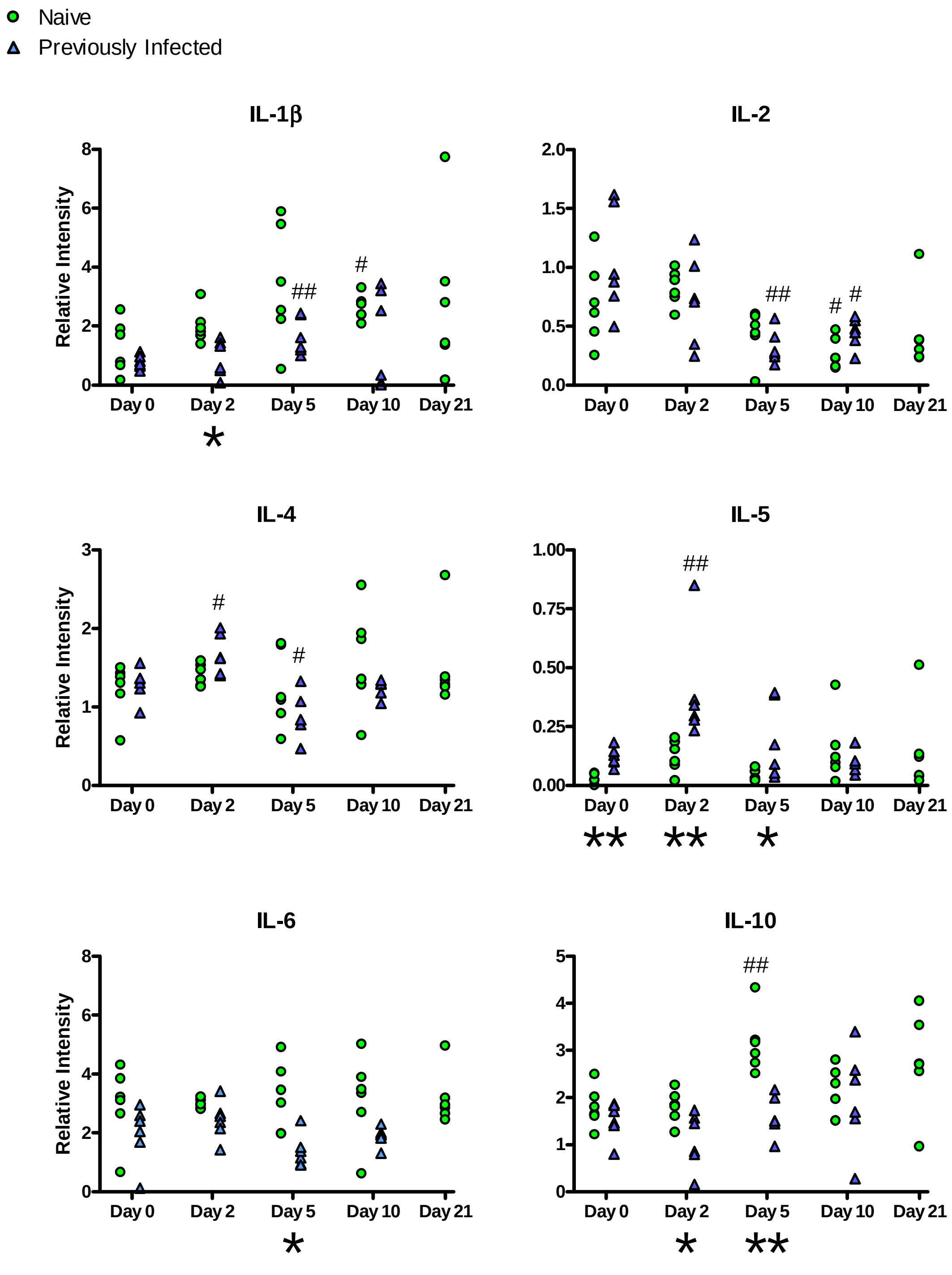
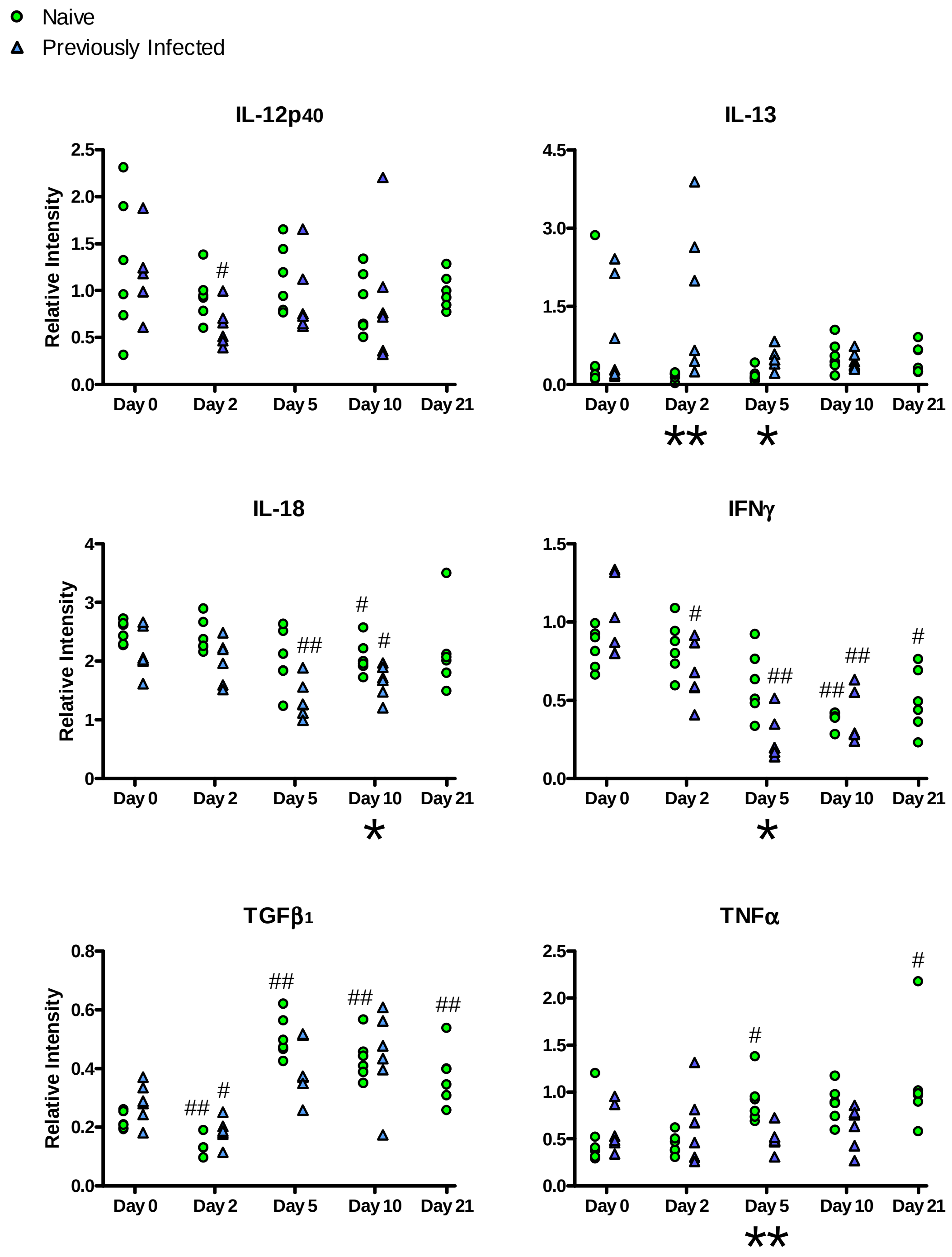


Figure 3.6(b): Cytokine transcription in the gastric lymph node relative to ATPase – Study 4. Analysed using Mann-Whitney U-test for non-parametric data with a 95% confidence interval, n = 6 sheep per group for each time point; significant difference between naïve- and previously infected-un/challenged groups on the same day: ★ *(P<0.05),* ★★ *(P<0.01); significant difference between challenged groups and their respective unchallenged groups: # (P<0.05), ## (P<0.01). Note different ranges on Y-axes.*



Statistical analysis using Dunn's test found a significant down-regulation of IL-2 in the previously infected-challenged sheep on days 5 ($P<0.01$) and 10 ($P<0.05$) following challenge, and in the naïve-challenged group on day 10 ($P<0.05$). Transcription of IL-12p_{40} was also reduced in the previously infected-challenged group on day 2 ($P<0.05$). Significant reduction according to Dunn's test was found in transcription levels of IFNγ; in the previously infected-challenged group on days 5 ($P<0.001$) and 10 ($P<0.01$), and in the naïve-challenged group on day 10 ($P<0.05$). Significant ($P<0.05$) reductions in IFNγ transcription were also found in the previously infected-challenged group on day 2 and the naïve-challenged group on day 21 using a Mann-Whitney U test, however these were not confirmed using Dunn's. IFNγ transcription was higher in naïve-challenged than previously infected-challenged samples on day 5 ($P<0.05$). Significant reductions in the proinflammatory/Th1 cytokine IL-18 were also found in the previously infected-challenged group on day 5 using Dunn's test ($P<0.05$) and in both challenged groups on day 10 using Mann-Whitney U test ($P<0.05$). While IL-18 was higher in naïve-challenged samples on days 2, 5 and 10, this was not quite significant ($P=0.0656$) except on day 10 ($P<0.05$).

Transcription of IL-1β was increased in previously infected-challenged samples on day 5 ($P<0.01$) and in naïve-challenged samples on day 10 ($P<0.05$), and of TNFα in naïve-challenged samples on days 5 and 21 ($P<0.05$), as indicated by Mann-Whitney U test. Of these results, only the increase in TNFα in the naïve-challenged samples on day 21 was confirmed using Dunn's test ($P<0.05$). When comparing naïve- and previously infected-challenged samples using Mann-Whitney U test, transcription of IL-1β was found to be significantly higher in naïve-challenged than previously infected-challenged samples on day 2 ($P<0.05$). TNFα transcription was higher in naïve-challenged than previously infected-challenged samples on day 5 ($P<0.01$). Transcription of IL-6 was higher in naïve and naïve-challenged than previously infected and previously infected-challenged samples throughout the experiment, however this was not quite significant ($P=0.0656$) other than on day 5 ($P<0.05$).

Transcription of IL-10 was found to be higher in naïve-challenged than previously infected-challenged samples on days 2 ($P<0.05$) and 5 ($P<0.01$) using Mann-Whitney U test, and was significantly increased in naïve-challenged samples on day 5 when compared to the naïve group using Dunn's test ($P<0.01$).

Transcription of $TGF\beta_1$ was increased in naïve-challenged samples on days 5 ($P<0.01$) and 10 ($P<0.05$) and 21 ($P<0.01$) when analysed using Dunn's test. According to Mann-Whitney U test, $TGF\beta_1$ transcription was also significantly down-regulated in both naïve-challenged ($P<0.01$) and previously infected-challenged ($P<0.05$) samples on day 2, but these findings were not confirmed by Dunn's test.

Although there were some differences in the levels of significance of the changes in cytokine transcription found between this study and study 3, similar to the differences discussed previously in section 3.3.3, the patterns of cytokine transcription in the unchallenged and challenged day 2 and 5 groups observed in this study were very similar to those found in study 3.

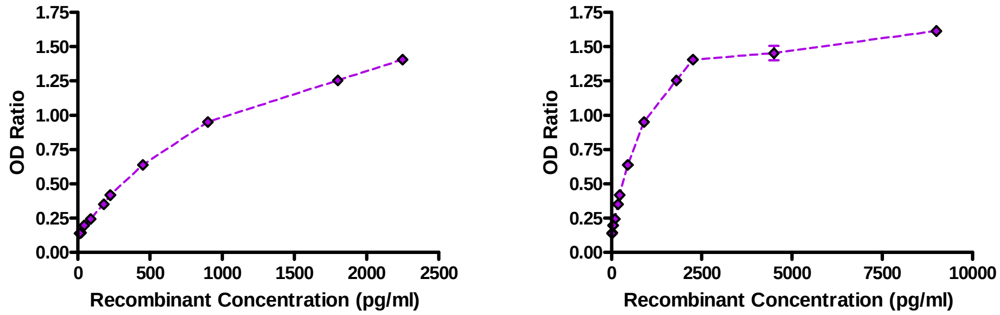
3.4 Cytokine detection in the gastric lymph node using ELISA

The use of ELISAs to assess the levels of key cytokines in gastric lymph node tissue homogenates was attempted, as detailed in section 2.4.1, in order to determine whether changes in transcription observed in the gastric lymph node correlated with changes in cytokine production *in vivo*. However, problems were encountered due to matrix effects in the samples and confounding factors.

ELISAs for the detection of IL-1β and IL-4 have been developed by Jim Rothel of the CSIRO Division of Animal Health, Animal Health Research Laboratory, Parkville, Australia and Jayne Hope of the Moredun Research Institute respectively (Rothel et al., 1997, Wattegedera et al., 2008). Recombinant ovine IL-1β and IL-4 were provided by Sean Wattegedera of the Moredun Research Institute. These recombinants are included in the online Immunological Toolbox resource database of veterinary immunology reagents (http://www.immunologicaltoolbox.com/). These ELISAs have previously been used for the examination of cytokine production by cells *in vitro*; the protocol used is detailed in sections 2.4.1 and was developed from a protocol by Sean Wattegedera and Colin McInnes.

Before using the ELISA on tissue homogenates, the dynamic range and sensitivity of the assay was evaluated by plotting the OD ratio of a wide range of recombinant standard concentrations. Where the plot flattened at the top was deemed to be above the dynamic range, and where the standard absorbance was below that of the mean blank was considered below the sensitivity of the assay. The dynamic range and sensitivity of the IL-4 ELISA is illustrated in Figure 3.7, showing the assay has a dynamic range of 90 – 2,250pg/ml. This assay is sensitive above 45pg/ml. Tissue homogenates of gastric lymph node samples which had been found to have high levels of IL-1β and IL-4 transcription were then used to optimise the assays.

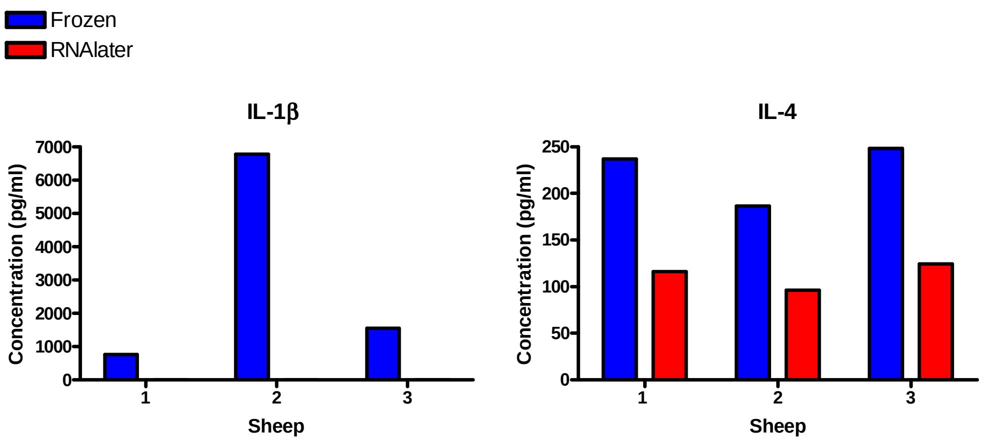
Figure 3.7: Evaluating sensitivity and dynamic range of the IL-4 ELISA. OD ratio of recombinant standards 12.5 – 2,250pg/ml and 12.5 – 9,000pg/ml, mean and SEM, n=2.



Due to the limited availability of snap-frozen tissue for this work, samples of snap-frozen abomasal mucosa and gastric lymph node were compared with samples of the same tissue from the same animal preserved in RNAlater®, to assess whether RNAlater-preserved samples would also be suitable for ELISA. Homogenates were produced as described in section 2.4.1, using 0.2g of tissue homogenized in 1ml fresh extraction buffer in a QBiogene Lysing Matrix D tube. Both IL-1β and IL-4 were detectable by ELISA in the snap-frozen samples, however in both tissues levels of IL-4 were greatly reduced in the RNAlater®-preserved samples and IL-1β was rendered undetectable, therefore only snap-frozen samples were used for ELISAs. Results of frozen and RNAlater-preserved gastric lymph node IL-1β and IL-4 ELISA are illustrated in Figure 3.8.

The assays were then checked for the presence of matrix effects; inhibition by other components of the sample, such as lipids, glycolipids and cytokine receptors, which could affect results when using tissue homogenate samples. This was done by examining several samples spiked with a specific amount of recombinant standard, along with the original samples and PBS spiked with the same concentration of recombinant. The difference in concentration, calculated using a standard concentration curve, between the sample and the spiked sample should be the same as that of the spiked PBS. However, when this was done it was found that the spiked samples produced lower concentration readings than expected. This was likely due to matrix effects inhibiting cytokine detection, most probably caused by lipids in the sample. Therefore the original samples may also have produced under-estimates due to matrix effects.

Figure 3.8: IL-1β and IL-4 protein concentration assessed by ELISA in frozen and RNAlater-preserved gastric lymph node samples.



Matrix effects were minimized by dilution of the samples, and processing of tissue homogenates to reduce lipids and glycolipids. This homogenate processing was optimised by serial treatment of a single batch of gastric lymph node homogenates, then comparison between spiked and un-spiked samples produced using each of the different protocols. The various protocols tested, which were run using homogenates produced using extraction buffer with and without Triton-X, are illustrated in Figure 3.10, and the results obtained from the samples including Triton-X are illustrated in Figure 3.9.

Triton-X was found to be essential in homogenate processing for detection of IL-1β, so its use was continued in production of future homogenates. Sample D, which was centrifuged to remove lipids, passed through a Qiashredder to reduce viscosity and treated with PHM-L Liposorb to remove remaining lipids, produced the largest difference in spiked concentration compared to un-spiked sample concentration (so the greatest recovery of the spiked recombinant concentration) in both the IL-1β and IL-4 ELISAs. These processes were incorporated into the homogenization protocol detailed in section 2.4.1.

Processing of the samples in this way reduced, but did not eliminate the apparent matrix effects. To add further complication the estimated concentration of the PBS spiked with IL-1β recombinant was higher than it should have been, even though the increase in the spiked samples compared to the un-spiked was not as high, indicating inhibition due to remaining matrix effects. This unexpected increase in the spiked PBS indicated a false positive error was occurring in the assay. Increases in the blocking and washing steps of the ELISA protocol failed to resolve this problem, so the technical support of the antibody manufacturer Serotec was consulted. It transpired that the rabbit anti-sheep IL-1β polyclonal secondary antibody (AHP423) used in the IL-1β ELISA may have been contaminated with anti-mouse IgG antibodies, which would cause a false positive result by binding to the mouse anti-sheep IL-1β (MCA1658) primary antibody (Martin Hill, Technical Advisor, AbD Serotec, personal communication, August 2008).

Due to the continuing problems with the cytokine ELISAs and failure to optimise them sufficiently to allow quantification of cytokines in tissue homogenates, or comparison between samples or to previous PCR results, an alternative means of assessing cytokine protein concentration was required.

Figure 3.9: IL-1β and IL-4 protein concentrations from spiked samples after subtraction of un-spiked sample protein concentration. Samples are gastric lymph node samples prepared by serial treatment illustrated in Figure 3.10 with the inclusion of Triton-X. IL-1β samples were spiked with 222pg/ml recombinant IL-1β, IL-4 samples were spiked with 750pg/ml recombinant IL-4.

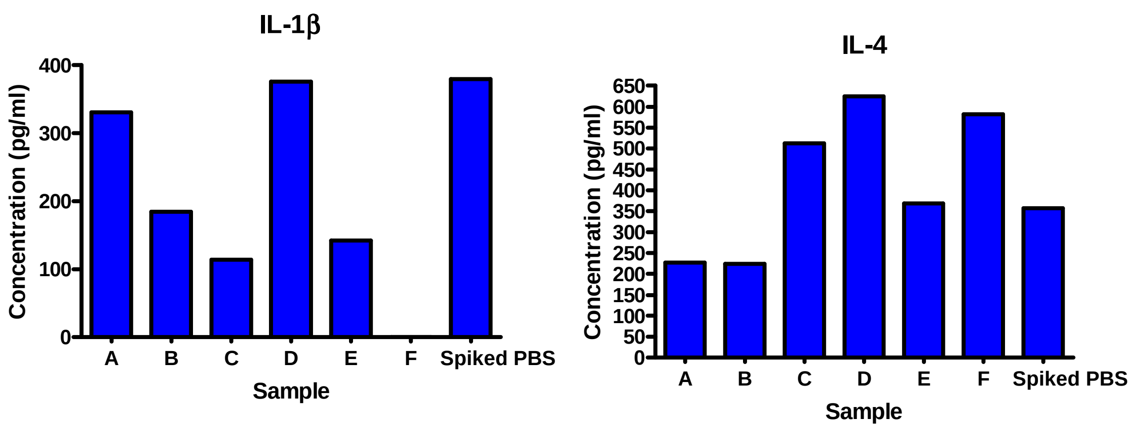


Figure 3.10: Protocols tested for minimal inhibition by matrix effects.

Sample A = Pooled homogenate of snap-frozen gastric lymph node tissue.

Sample B = Sample A centrifuged for 30 minutes at 15,000rpm and 4°C using a Costar® Model 10 centrifuge, and the supernatant aliquotted while leaving the top layer of fats behind.

Repeat 3 times.

Sample C = Sample B passed through a Qiashredder to reduce viscosity, using a Biofuge fresco centrifuge at 4°C for 2 minutes at 13,000rpm.

Sample D = Sample C treated with equilibrated PHM-L Liposorb at 1 part Liposorb to 1.5 parts homogenate, allowed to stand for 15 minutes at room temperature, vortexed for 1 minute then centrifuged for 10 minutes at 3,000rpm and 4°C using a Costar® Model 10 centrifuge and the supernatant collected.

Sample E = Sample D centrifuged for 30 minutes at 15,000rpm and 4°C using a Costar® Model 10 centrifuge, and the supernatant aliquotted to remove any remaining debris or fat.

Sample F = Sample B treated with equilibrated PHM-L Liposorb at 1 part Liposorb to 1.5 parts homogenate, allowed to stand for 15 minutes at room temperature, vortexed for 1 minute then centrifuged for 10 minutes at 3,000rpm and 4°C using a Costar® Model 10 centrifuge and the supernatant collected.

Western blotting was then tried as an alternative technique for detection of IL-4 to ameliorate the matrix effects which were proving difficult to remove during ELISA. Protein in the homogenized samples was denatured by diluting 6μl homogenate in 24μl SDS-PAGE reducing buffer containing DTT, and heating to 95°C for 3 minutes. Western blots were then run using 10μl of denatured homogenate migrated through a Ready Gel 12% Tris-HCl premade gel suspended in SDS-PAGE running buffer at 200V for 35 minutes alongside 3μl of molecular weight marker. Once run, the gel was washed in CAPS with 10% methanol, and placed in a Bio-Rad semi-dry blotter device on top of an Immobilon-P membrane soaked in 100% methanol with two layers of blotting paper soaked in CAPS with 10% methanol underneath. Two more layers of blotting paper soaked in CAPS with 10% methanol were placed on top, and the blotter was run at 10V for one hour. Once blotted, the Immobilon-P membrane was removed and soaked in western blot blocking buffer with agitation for 2 minutes, then incubated overnight at 4°C. The Immobilon-P membrane was then soaked in mouse anti-bovine IL-4 monoclonal antibody MCA2371 diluted to 5μg/ml in western blot blocking buffer with agitation for one hour and washed five times for 1-2 minutes in ELISA wash buffer. After washing, the membrane was soaked in donkey anti-mouse antibody conjugated with horseradish peroxidase (2μl diluted in 20ml western blot blocking buffer) for one hour, then washed another five times for 1-2 minutes in ELISA wash buffer. Antibody binding to the membrane was imaged using 0.9ml chemiluminescent reagent diluted in 2 volumes of reagent buffer and a Kodak® Digital Science Image Station 440CF. The membrane was stained for protein and molecular weight marker on the membrane using 0.25% Coomasie Blue R250, de-stained twice then rinsed in water and dried. Unfortunately western blotting was not successful using mouse anti-bovine IL-4 monoclonal antibody MCA2371.

Further development of techniques for cleaning up protein extracted from tissue samples appears to be necessary before successful cytokine quantification using ELISA will be possible.

3.5 Discussion

Some differences were found in the levels of significance of the results of the different studies described in this chapter. This may have been due to biological variation between the groups of sheep used during the different studies. Between studies 1 and 2 a change was made in the breed of the sheep used for the experiment. The unchallenged naïve samples used in study 1 were Dorset-Suffolk crosses, whereas the challenged day 10 sheep used were Blackface-Leicester. However, during study 2 all the sheep used were Dorset-Suffolk crosses. This may account for the changes in transcription by day 10 which were found to be significant during study 2, but not significant in study 1. However, these two breeds of sheep produced similar day 10 parasitology results, implying that there was no significant difference in resistance to *T.circumcincta* due to the difference in breeds (David Smith, 2008, personal communication) (Halliday et al., 2007).

Between studies 2 and 3, the unchallenged naïve sheep in experimental group 1 were replaced by those in experimental group 4, which included challenged day 2 and previously infected-unchallenged groups killed at the same time. Although experimental groups 1 and 4 were of different breeds, both unchallenged naïve groups were unchallenged and helminth naïve. It is possible that individual biological variation between animals in these groups prior to helminth challenge may have been a factor in the differing results between the studies.

However, despite differences in significance, the trends in cytokine transcription observed were broadly conserved during repetition of experiments. This discussion therefore focuses on the results of section 3.3.4, which included all the samples except the first naïve group, and confirmed the findings of previous sections. Figure 3.11 illustrates the trends in cytokine transcription observed in the gastric lymph node following challenge of naïve and previously infected sheep.

Figure 3.11(a): Schematic illustration of the trends in transcription of Th1- and Th2-type cytokines in the gastric lymph node of naive-challenged and previously infected-challenged sheep. Note ranges on Y-axes. Day 0 =unchallenged.

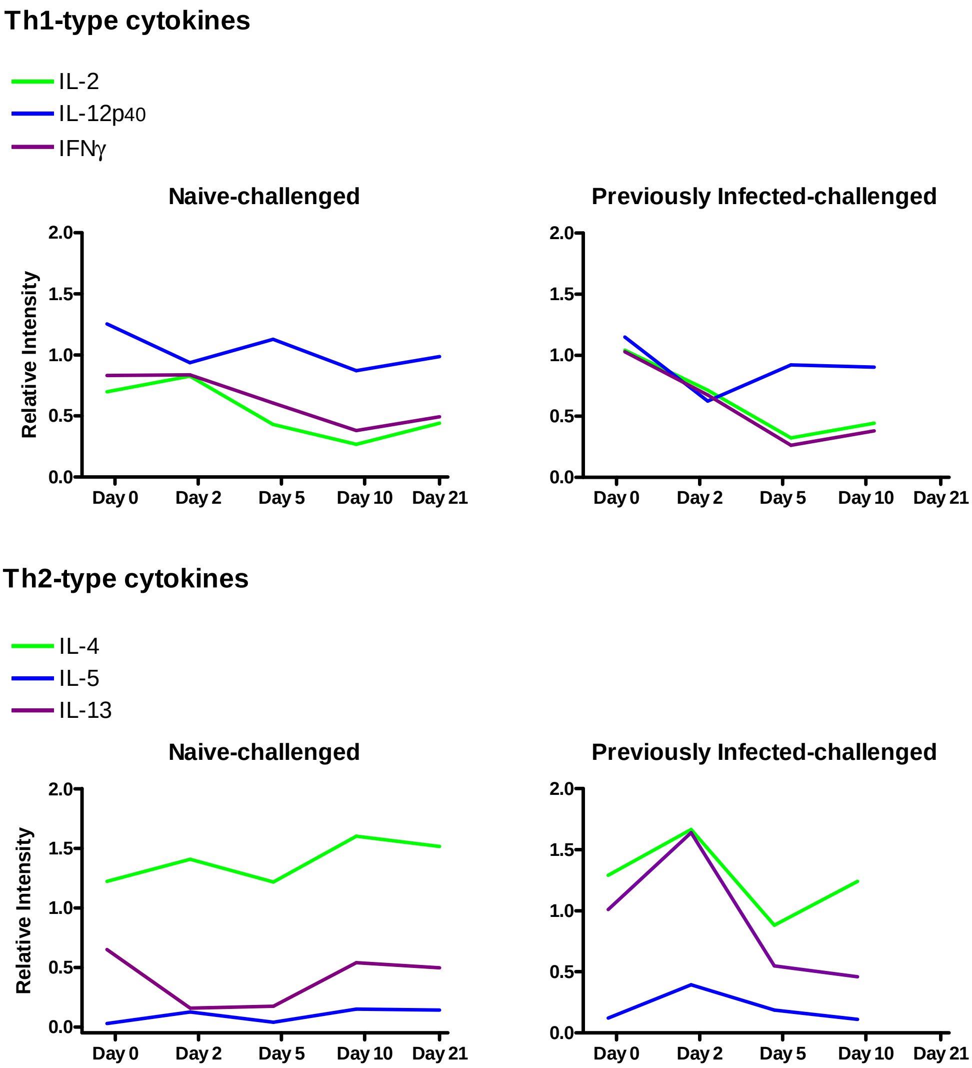
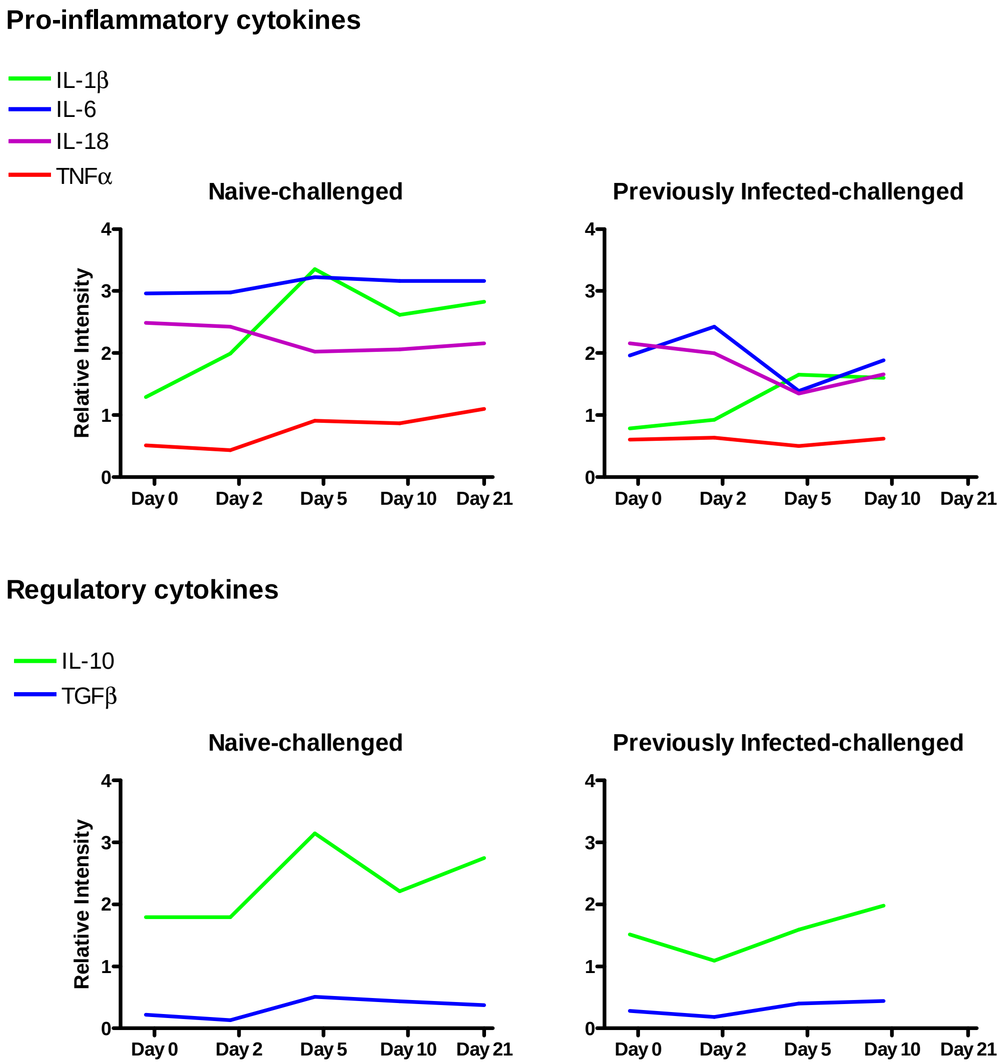


Figure 3.11(b): Schematic illustration of the trends in transcription of pro-inflammatory and regulatory-type cytokines in the gastric lymph node of naive-challenged and previously infected-challenged sheep. Note ranges on Y-axes. Day 0 =unchallenged.



The role of a Th2-type cytokine response was indicated by the increase in transcription of IL-4 and IL-5 found during the early stages of infection, and the finding that transcription of both IL-5 and IL-13 was higher at certain time-points in the previously infected-challenged animals following challenge than in the naïve-challenged.

A pro-inflammatory influence was also found, which was more pronounced in the naïve-challenged groups than the previously infected-challenged. This may be more marked in the naïve-challenged group because these animals have not developed the adaptive immune response present in the previously infected sheep, which would help to limit the mucosal damage caused by larvae living in the gastric crypts by causing their expulsion and stunting their growth. Examination of *T.circumcincta* recovered at post mortem by Steven Smith as described in section 2.2.6 demonstrated significantly reduced worm burdens and increased larval stunting and arrest in the previously infected-challenged than the naïve-challenged animals (Halliday et al., 2007).

However, the most pronounced finding was not the increases in Th2 cytokine transcription, but the dramatic suppression of Th1 cytokine transcription which took place in the draining lymph node following infection with *T.circumcincta*. This effect took place earlier in the previously infected-challenged sheep, possibly due to a pre-existing population of Th2 or regulatory (Tr1) T cells accumulated during the previous 8 week trickle infection and capable of causing rapid suppression of Th1 lymphocyte activity.

The observed increase in transcription of IL-10 is unlikely to be due to the activity of Th2 cells; as though these cells have been found to produce IL-10, they also produce IL-4 and IL-5 (Bottomly, 1999, Rissoan et al., 1999), transcription of which was not increased in these groups. However, regulatory Tr1 cells have been demonstrated in both mice and humans to suppress antigen-specific immune responses *in vivo* through the production of IL-10 with little IL-2 and no IL-4 (Groux et al., 1997, Vieira et al., 1991). Inducible CD4+CD25+Foxp3+ regulatory T cells (Treg) have also been shown to be involved in the immune response to *Litomosoides sigmodontis* infection in mice (Taylor et al., 2009).

Interesting changes were found in transcription of TGFβ_1. The increase in transcription found in the naïve-challenged groups is possibly a reflection of the greater extent of the tissue damage occurring in naïve-challenged sheep following infection with *T.circumcincta* compared to previously infected-challenged sheep which already have a degree of resistance. The increased transcription of TGFβ, which has been found to have an important role in tissue repair (Faler et al., 2006), on day 5 following infection corroborates the presence of a pro-inflammatory element to *T.circumcincta* infection caused by damage to the mucosa due to the growth of larvae. However, as TGFβ requires activation by external factors, levels of TGFβ expression do not necessarily correspond to levels of active TGFβ present in the abomasal lymph node or recirculating to the abomasal mucosa (Crawford et al., 1998, Munger et al., 1999).

The increased expression of TGFβ_1 found on day 5 in the naïve-challenged group may, like IL-10, also be indicative of regulatory T cell activity. Activated TGFβ is essential in the activation of regulatory T cells in mice (Travis et al., 2007) through induction of Foxp3 transcription (Chen et al., 2003, Davidson et al., 2007). It is also important in driving production of IL-10 from T cells (McGeachy et al., 2007).

Abomasal samples from the sheep used in this study have been examined by Tom McNeilly at the Moredun Research Institute for expression of Foxp3 (forkhead box P3), which is known to be expressed by regulatory T cells. Foxp3 was found to be significantly increased in the abomasal mucosa 10 days after infection of the naïve-challenged sheep, but did not significantly change in the previously infected-challenged sheep (Tom McNeilly, 2009, personal communication). This implies that a more pronounced regulatory response is taking place in the naïve-challenged sheep following infection with *T.circumcincta* than in those that have previously been exposed. This supports the hypothesis that the higher levels of IL-10 and TGFβ_1 transcription in the gastric lymph node are due to regulatory T cells (McNeilly, 2005). Whether these cells are Tregs preventing immunity to autologous antigen, Th3 suppressing immunity to gut flora and oral antigen, or Tr1 preventing pathological immune responses remains to be defined.

A subset of ovine dendritic cells distinguished by expression of CD172a (McNeilly, 2005) has also been shown to produce IL-10 (Halliday et al., 2007). These, or a similar subset of dendritic cells, may contribute to the changes in IL-10 transcription seen in the gastric lymph node as they migrate to the lymph node in order to present antigen to T cells (Bujdoso et al., 1990, Torgerson and Lloyd, 1993), and direct the immune response towards a Th2 type response through production of IL-10 as has been demonstrated in mouse CD11b+CD8α- dendritic cells (Iwasaki and Kelsall, 2001, Maldonado-Lopez et al., 2001) and human DC2 (Moser and Murphy, 2000). This hypothesis is supported by the observation that IL-1β and IL-18, which are also produced by ovine CD172a+ dendritic cells (Halliday et al., 2007), also tended towards higher transcription in the lymph node of naïve-challenged sheep on day 5 after infection.

IL-1β in particular may be indicative of dendritic cell activity, as this cytokine is involved in several dendritic cell activation pathways. For example, Fas ligation-induced maturation of murine dendritic cells via ERK1/2 activation is mediated by autocrine feedback of IL-1β (Granucci et al., 2001, Guo et al., 2003). Downstream signalling pathways initiated by ligation of TLR7 and TLR8 in human dendritic cells also incorporate synthesis of IL-1β (Hurst et al., 2009).

Human dendritic cells have been shown to produce TNFα (McCall et al., 1989, Granucci et al., 2001), and murine dendritic cells to produce IL-6 in the lymph node (Hope et al., 1995, Schramm et al., 2007), both of which showed a similar pattern of transcription to IL-10 in being transcribed at significantly higher levels in the naïve-challenged sheep on day 5 than in the previously infected-challenged group.

These results demonstrate a predominantly Th2 type response taking place in the gastric lymph node of sheep following exposure to *T.circumcincta*. This is highlighted by the observed increase in transcription of the Th2 cytokines IL-4 and IL-5, and particularly by the observed suppression of the Th1 cytokines IL-2 and IFNγ.

These responses are much more evident in the previously infected-challenged sheep than in the naïve-challenged groups; up-regulation of IL-4 and IL-5 was not observed in the naïve-challenged animals, and suppression of IL-2, IL-18 and IFNγ occurs earlier in the previously infected-challenged groups. These differences in the cytokine responses to *T.circumcincta* may reflect the absence of antigen-specific memory T cells in the naïve-challenged animals; resulting in a slower response to infection, which is more prone to suppression by antagonistic cytokines or regulatory T cells. This may be a reason for the lower resistance to helminth infection in naïve-challenged animals compared to those which have built up partial immunity following previous exposure (Pernthaner et al., 2005).

The data also suggest a greater influence of dendritic cells and regulatory T cells in directing and regulating the immune response in the naïve-challenged sheep than in the previously infected-challenged groups. Dendritic cell activation of the Th2 response and generation of Th2 and regulatory cells from naïve T cells will have already occurred in the previously infected sheep during trickle infection with *T.circumcincta*. Activation of dendritic cells and generation of regulatory T cells would therefore be less necessary in this group following challenge due to the pre-existing population of Th2 cells which can be activated by a variety of APCs and would contribute to the suppression of Th1 activity.

The suppression of IL-12p_{40} and IFNγ, and increased transcription of IL-4, IL-5 and IL-10 are consistent with the findings of Claerebout and colleagues' examination of cytokine responses in the gastric lymph nodes of cattle infected with *Ostertagia ostertagi* (Claerebout et al., 2005). However, Claerebout's study demonstrated a more pronounced up-regulation of IL-13, which was present as a trend but not significant in this work. Up-regulation of IL-4, IL-5 and IL-13 is also consistent with observations in the abomasal lymph nodes during the ovine response to *H.contortus*

(Lacroux et al., 2006) and in intestinal lymph following infection with *T.colubriformis* (Hein et al., 2004).

These results show, for the first time, that the changes in cytokine expression in the draining lymph node in response to *T.circumcincta* infections in sheep are similar to those elicited by *Haemonchus contortus* in sheep and *Ostertagia ostertagi* in cattle*;* being predominantly Th2 driven. Importantly, we have demonstrated that sheep immunised by trickle infection increase transcription of the Th2 cytokines IL-4 and IL-5 early in response to challenge, and suppress transcription of the Th1 cytokines IL-2, IL-12p_{40} and IFNγ earlier and to a greater extent than unimmunised, naïve-challenged animals.

4 Chapter Four: Cytokine Responses in the Abomasal Mucosa

4.1 Introduction

As described in section 1.3, local immune responses to gastrointestinal nematodes in sheep are predominantly Th2 in phenotype, involving mucosal inflammation, mastocytosis, eosinophilia and hyperplasia of goblet and mucus cells. However, though humoral and innate responses to *T.circumcincta* infection have previously been explored (Smith et al., 1984, Stear et al., 1995, Halliday et al., 2007), the cytokine changes which orchestrate this response locally have not yet been examined.

Lacroux (2006) and colleagues have previously found that *H.contortus* infection of 12 week old lambs stimulated early recruitment of eosinophils, mast cells and globule leukocytes, and that this was significantly correlated with transcription levels of IL-4. Murine models of helminth infection have demonstrated that anti-IL-4 antibody can partially inhibit mast cell accumulation in the mucosa of mice infected with *N.brasiliensis* (Madden et al., 1991) and that mast cell-derived IL-4 is important in the expulsion of *Trichinella spiralis* (Ierna et al., 2008). The role of IL-4 suggested by these studies corroborates the opinion that mucosal infiltration by mast cells is an important component of the Th2 immune response in some gastrointestinal helminth infections. Mast cells have an important role in the expulsion of gastrointestinal helminths, as discussed in section 1.3.6, and mastocytosis is considered a marker for phenotypic Th2-type responses. Differences in the rate and extent of mast cell accumulation are likely to be a factor in the inconsistency of helminth resistance between groups of sheep.

The work described in chapter 3 demonstrated a predominantly Th2 type response taking place in the gastric lymph node in response to infection with *T.circumcincta*.

This was defined by increased transcription of Th2 cytokines and marked suppression of Th1 cytokine transcription, particularly in the previously infected groups. The question now is whether these changes have parallels in the mucosa, in close proximity to the parasites, and whether they are reflected in effector responses such as mastocytosis.

The objectives of this section of work were:

(1) To establish whether the cytokines previously examined in the gastric lymph node are also transcribed in the abomasum.

(2) To define how this transcription changes following infection with *T.circumcincta* in naïve-challenged compared with previously infected-challenged sheep.

(3) To examine and compare the extent of mastocytosis in the abomasal mucosa in naïve, naïve-challenged, previously infected-unchallenged and previously infected-challenged sheep as an indicator of phenotypic Th2-type changes in these groups before and after challenge.

Figure 4.1: Cross-section of a T.circumcincta larva growing within a gastric crypt. The slide is stained using chloroacetate esterase stain and the bar indicates 200µm.

4.2 Cytokine transcription in the abomasal mucosa

Abomasal gastric fold samples taken immediately post mortem, as described in section 2.2.5, from yearling sheep in experimental groups 1, 3 and 4, as detailed in section 2.2.1 and Table 4.1.

RNA from these samples was extracted by Judith Pate and quantified according to sections 2.3.1 and 2.3.2. RT-PCR and densitometry were then used to examine cytokine transcription in the samples as described in sections 2.3.6-2.3.10. The RT-PCR assays used to detect transcription of ATPase, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p_{40}, IL-13, IL-18, IFNγ, TGFβ_1 and TNFα were developed and optimised during examination of gastric lymph node samples, as described in section 3.2. Gastric lymph node samples were used as a positive control during each PCR.

Table 4.1: Experimental design – Abomasal mucosa.

BL = Blackface ewe/Leicester ram; DS = Dorset ewe/Suffolk ram.
Nv = Naïve; PI = Previously Infected; Day 0 = unchallenged.

Experimental group	Kill date	Trickle infection[a]	Challenge infection[b]	Kill day following challenge[c]				
				0	2	5	10	21
1 – Nv (DS)	July 2005	-	-	6	-	-	-	-
3 – Nv (DS)	Jan 2006	-	+	-	-	6	6	6
3 – PI (DS)	Feb 2006	+	+	-	-	6	6	-
4 – Nv (BL)	April 2007	-	+	-	6	-	-	-
4 – PI (BL)	April 2007	+	+	6	6	-	-	-

[a] 2000 *T.circumcincta* L3 larvae administered 5 times per week for 8 weeks.

[b] One dose of 50,000 *T.circumcincta* L3, given 7 days after Levamisole treatment.

[c] Quoted figure is the number of sheep in the group killed on the corresponding day following challenge.

All cytokines examined were detectable in the abomasal samples from each group. Statistical analysis was carried out using ANOVA residual plots to check for normality. Transcription of all the cytokines was found not to be normally distributed, with the exception of IL-6 and IL-10. Non-parametric tests were therefore considered to be appropriate for analysis of these data, including IL-6 and IL-10 so that the statistical analysis was consistent across the range of cytokines. Kruskal-Wallis test indicated a significant difference between groups in the case of IL-1β ($P<0.001$, $H=32.06_8$), IL-4 ($P<0.001$, $H=34.65_8$), IL-5 ($P<0.001$, $H=33.27_8$), IL-6 ($P<0.001$, $H=31.47_8$), IL-13 ($P<0.001$, $H=36.77_8$), IL-18 ($P<0.001$, $H=45.68_8$), $TGF\beta_1$ ($P<0.001$, $H=28.69_8$) and TNFα ($P=0.001$, $H=27.10_8$). Mann-Whitney U test was also used to make specific comparisons between groups or time-points as described in section 2.6. The findings of this work are illustrated in Figure 4.2. No correlation was found between transcription of any of the cytokines and total worm burdens for each sheep.

Significant increases in the transcription of the Th2 cytokines IL-4, IL-5 and IL-13 were found in the abomasal mucosa following infection with *T.circumcincta* when compared to the unchallenged groups. Transcription of IL-4 was significantly increased in the previously infected-challenged group on day 2 ($P<0.05$), and in the naïve-challenged groups on days 10 ($P<0.001$) and 21 ($P<0.05$) when analysed using Dunn’s test. Significant increases in IL-4 transcription were also indicated by Mann-Whitney U test in the naïve-challenged samples on day 2 ($P<0.05$) and in the previously infected-challenged samples on day 10 ($P<0.05$) compared to their respective unchallenged groups, however these were not significant according to Dunn’s test due to the different properties of this test. Significant up-regulation of IL-5 was found in previously infected-challenged groups on days 2 and 5 according Dunn’s tests ($P<0.05$), however the apparent increase in naïve-challenged samples on day 21 was only significant using Mann-Whitney U test ($P<0.05$). Significant increases in the transcription of IL-13 were found on day 5 in the previously infected-challenged samples ($P<0.05$), and on days 10 ($P<0.001$) and 21 ($P<0.01$) in the naïve-challenged using Dunn’s test when compared to their respective unchallenged groups.

When comparing naïve-challenged to previously infected-challenged samples at the same time-point using Mann-Whitney U test, transcription of IL-4 was found to be significantly higher in the previously infected-challenged groups on days 2 ($P<0.05$) and 5 ($P<0.01$), as was IL-5 on days 2 and 5 ($P<0.01$) and IL-13 on day 5 ($P<0.05$). In contrast transcription levels of IL-4 and IL-13 were higher in naïve-challenged groups by day 10 ($P<0.05$).

In contrast to the marked changes in Th2 cytokine transcription, no significant changes were found in the Th1 cytokines IL-2, IL-12p_{40} or IFNγ when compared to the unchallenged groups. No significant differences were found in transcription of IL-2 or IL-12p_{40} when comparing naïve-challenged and previously infected-challenged groups at the same time-point, however IFNγ transcription was significantly higher in previously infected-challenged samples than the naïve-challenged on day 5 according to Mann-Whitney U test ($P<0.05$). Marked differences were found in the transcription of IL-18 during the experiment, both over the course of infection and between naïve-challenged and previously infected-challenged groups. IL-18 transcription was found to be significantly ($P<0.01$) higher in naïve-challenged sheep than in the previously infected-challenged animals throughout the study, and down-regulated in the previously infected-challenged group on day 2 ($P<0.05$) and the naïve-challenged groups on days 10 ($P<0.05$) and 21 ($P<0.05$) according to Mann-Whitney U test when compared with their respective unchallenged groups. However this observed down-regulation was not confirmed by Dunn's test.

A proinflammatory component to the local immune response to *T.circumcincta* was indicated in the naïve-challenged group by an increase in the transcription of IL-1β on days 10 ($P<0.01$) and 21 ($P<0.05$), and of IL-6 on days 2, 10 and 21 ($P<0.05$) according to Dunn's test when compared to their respective unchallenged groups. Transcription of IL-1β was also found to be higher in the naïve-challenged sheep than the previously infected-challenged group on day 10 ($P<0.01$), and IL-6 was higher in the naïve-challenged group than the previously infected-challenged on days 2 and 10 ($P<0.01$) using Mann-Whitney U test. Interestingly, TNFα transcription was initially reduced in the naïve-challenged sheep on day 2 ($P<0.05$), then increased in the previously infected-challenged group by day 5 ($P<0.05$), according to Dunn's test when compared to their respective unchallenged groups. A further increase in TNFα transcription, found in previously infected-challenged samples on day 10 compared to the previously infected-unchallenged group using Mann-Whitney U test ($P<0.05$).

No significant changes in transcription of IL-10 were found using Dunn's test, however a reduction in transcription in naïve-challenged samples on day 2 compared to the naïve group was indicated using Mann-Whitney U test ($P<0.05$).

Significant up-regulation of TGBβ_1 was found in previously infected-challenged samples on days 5 and 10 ($P<0.05$) compared to the previously infected-unchallenged group using Dunn's test.

*Figure 4.2(a): Cytokine transcription in the abomasal mucosa relative to ATPase. Analysed using Mann-Whitney U-test for non-parametric data with a 95% confidence interval, n = 6 sheep per group for each time point; significant difference between naïve- and previously infected-un/challenged groups on the same day: * (P<0.05), ** (P<0.01); significant difference between challenged groups and their respective unchallenged groups: # (P<0.05), ## (P<0.01). Note different ranges on Y-axes.*

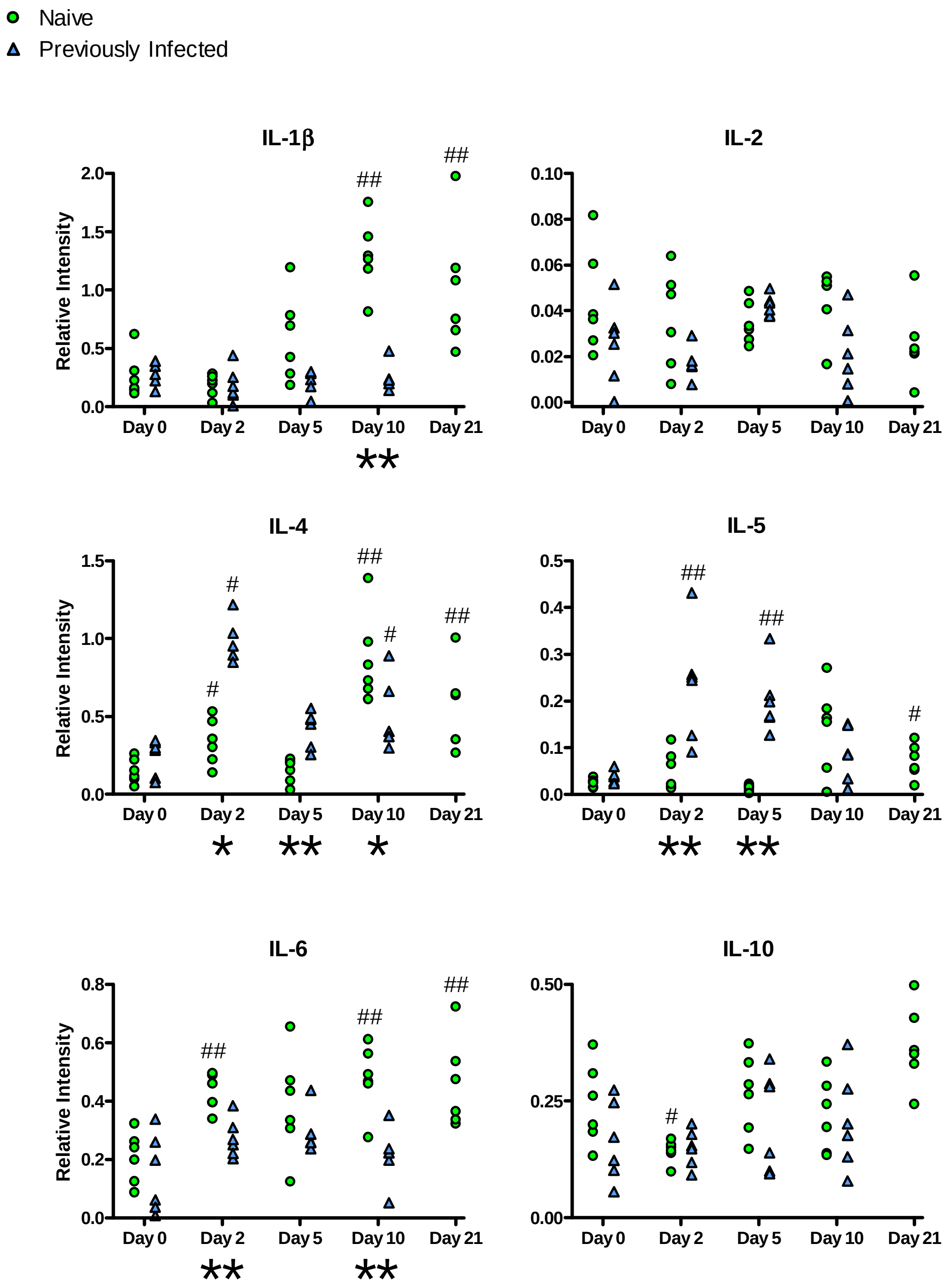
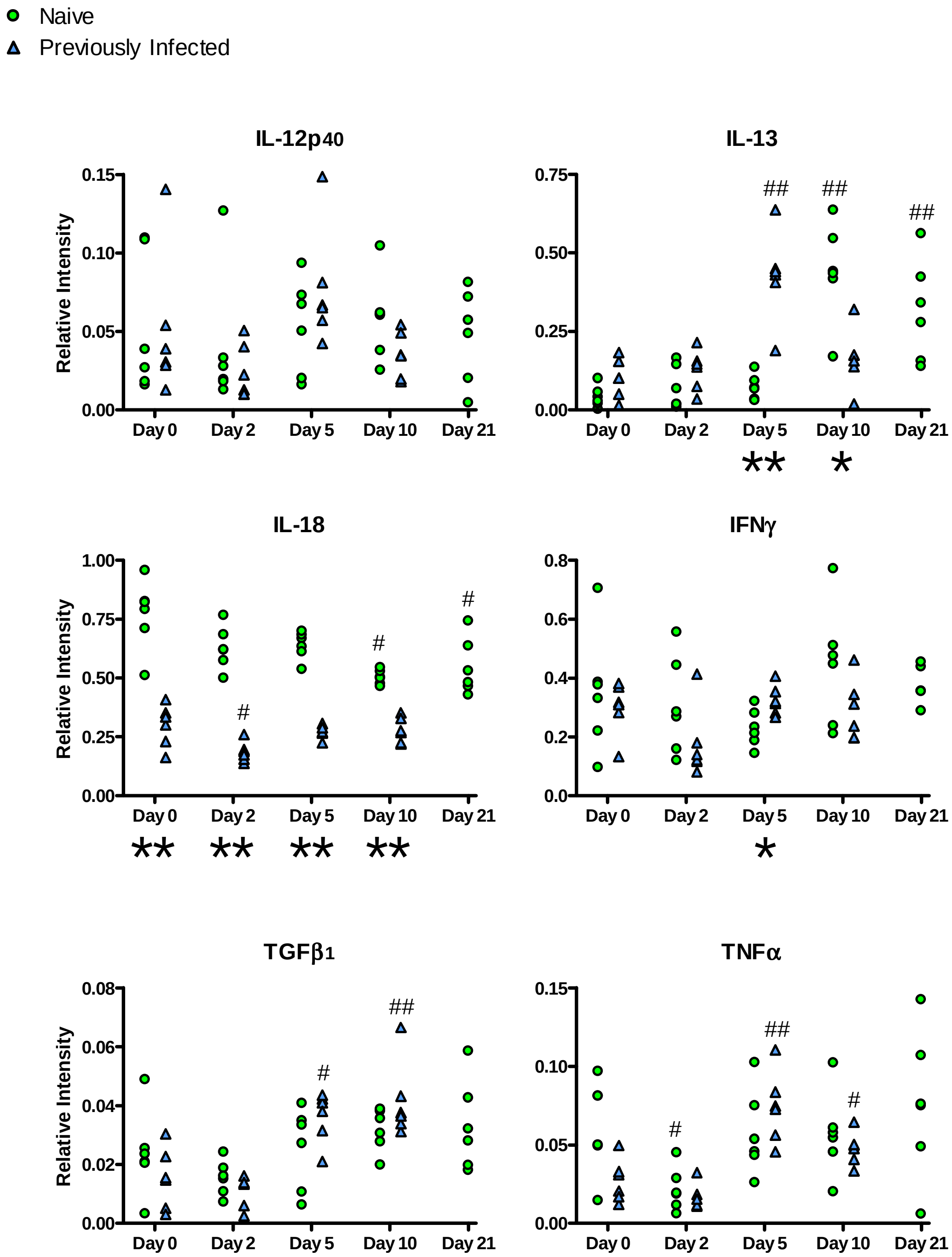


Figure 4.2(b): Cytokine transcription in the abomasal mucosa relative to ATPase. Analysed using Mann-Whitney U-test for non-parametric data with a 95% confidence interval, n = 6 sheep per group for each time point; significant difference between naïve- and previously infected-un/challenged groups on the same day: * *(P<0.05),* ** *(P<0.01); significant difference between challenged groups and their respective unchallenged groups: # (P<0.05), ## (P<0.01). Note different ranges on Y-axes.*



4.3 Confirmation of altered cytokine transcription using Q-PCR

The changes in transcription of IL-1β and IL-4 detected by RT-PCR were re-examined using Taqman Q-PCR assays, as described in section 2.3.13. This was done to examine the changes in cytokine transcription with greater sensitivity, and to verify that the trends observed using RT-PCR were repeatable when using this more sensitive technique. The experimental group 1 – naïve samples used during RT-PCR, were replaced with experimental group 4 – naïve samples during this section of work.

Quantification of cDNA prior to Q-PCR analysis reduced variation in initial cDNA concentration which could be introduced due to differences in efficiency of the RT reaction between samples when using RT-PCR. Q-PCR is more sensitive than RT-PCR because the amount of cDNA PCR product is recorded after each cycle of heating and cooling. In the case of Taqman assays this cDNA specifically comprised transcripts of the target gene, providing an assay which is far more specific than both RT-PCR and SYBR green Q-PCR. Because the amount of cDNA PCR product is quantified repeatedly during the process using light emissions, a considerable amount of the experimental error introduced during the imaging of DNA using agarose gels following RT-PCR is also removed, producing far more accurate quantification.

As described in section 2.3.13, a Ct value of 40 or more was considered to be negative. The cytokine Ct values were normalised by subtracting the Ct value for ATPase, to produce the value ΔCt for each sample. ATPase assays for each sample were run on the same plate as the cytokine assay. The mean ΔCt for a nominated calibrator group was subtracted from the mean ΔCt of each of the other groups to produce the value ΔΔCt. The fold difference between each group and the calibrator group was then calculated using the equation:

$$\text{Fold difference} = 2^{-\Delta\Delta Ct}$$

The calibrator (baseline) groups used were the naïve and previously infected-unchallenged groups; these groups have a fold difference of 1 indicating there has been no change. These data are illustrated in Figure 4.3, in which Ct values are displayed as 40-Ct for clarity, as lower Ct indicates higher levels of transcription.

Statistical analysis was carried out on the 40-Ct values as had been done previously, using ANOVA residual plots to check for normality. Transcription of both cytokines was found not to be normally distributed, therefore non-parametric tests were considered to be appropriate for analysis of these data. Kruskal-Wallis test indicated a significant difference between groups for both IL-1β ($P<0.001$, $H=39.51_8$) and IL-4 ($P<0.001$, $H=39.30_8$). Mann-Whitney U test was then used to make specific comparisons between groups or time-points as described in section 2.6.

IL-1β transcription increased 2.5-3 fold on days 5 and 10 in the naïve-challenged sheep compared to the naïve group, indicating a proinflammatory response was taking place. This response appeared to be waning on day 21 when transcription was reduced. A transient increase in transcription was also seen on day 2 in the previously infected-challenged sheep compared to the previously infected-unchallenged group, but this was not evident on day 5. This transient up-regulation was not found using RT-PCR.

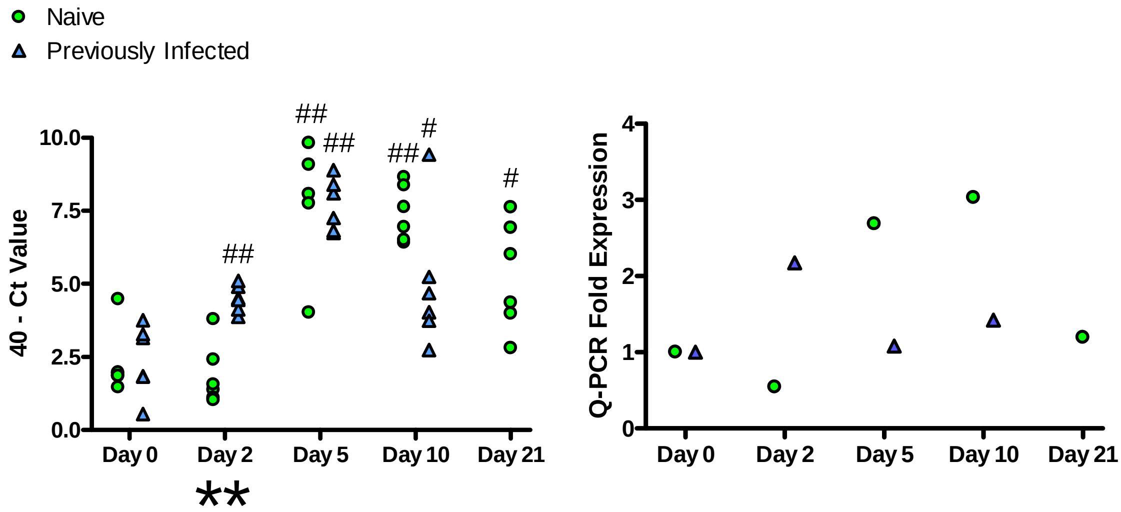
Transcription of IL-4 increased 5 fold in previously infected-challenged sheep on day 2 following challenge compared to the previously infected-unchallenged group, before falling on days 5 and 10. In contrast, in the naïve-challenged abomasum transcription was initially reduced on day 5, then increased on day 10 compared to the naïve group.

The results of these assays confirmed the trends found using RT-PCR in both a Th2 and a proinflammatory/Th1 cytokine.

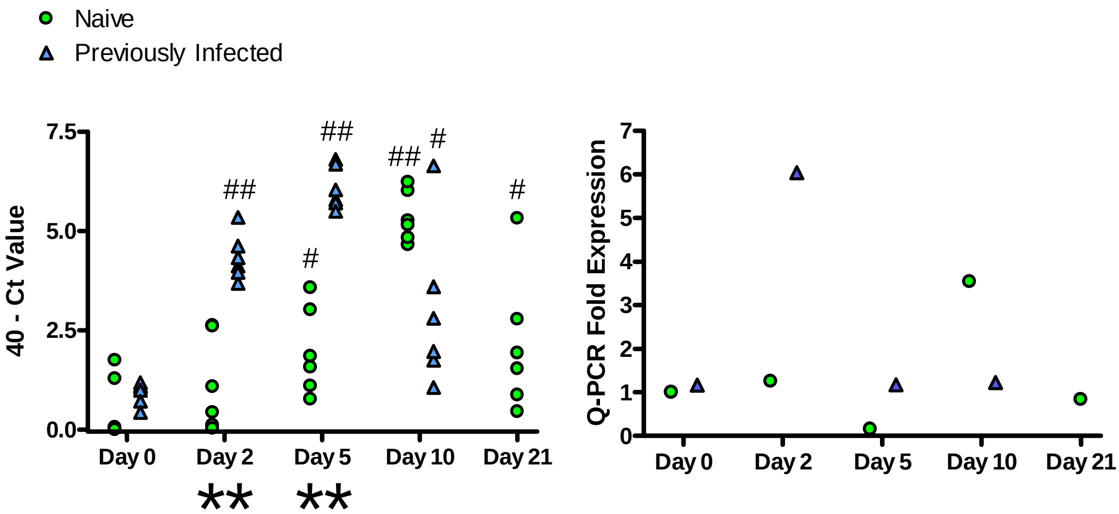
While RT-PCR produced a good assessment of the range of cytokines being transcribed in the tissues and the major changes in transcription taking place, the increase in IL-1β detected on day 2 in the previously infected-challenged group, which was not found using RT-PCR, illustrates the increased sensitivity of Q-PCR assays in comparison to RT-PCR.

Figure 4.3: Cytokine transcription in the abomasal mucosa assessed using 40 – Ct value and Q-PCR group fold difference from unchallenged groups. 40 – Ct values analysed using Mann-Whitney U-test for non-parametric data with a 95% confidence interval, n = 6 sheep per group for each time point; significant difference between naïve- and previously infected-un/challenged groups on the same day: ∗ *(P<0.05),* ∗∗ *(P<0.01); significant difference between challenged groups and their respective unchallenged groups: # (P<0.05), ## (P<0.01). Note different ranges on Y-axes.*

(a) IL-1β



(b) IL-4



4.4 Mast cell counts in the abomasal mucosa

Mast cells in the abomasal mucosal epithelium were stained using chloroacetate esterase stain, which stains mast cell granules for serine esterase, as described in section 2.5.1, and counted as described in section 2.5.2.

Mean mast cell counts for each sample are illustrated in Figures 4.4 and 4.7, and are listed in Table 4.2. Worm counts per 250ml sample of abomasal contents are also listed in Table 4.2 for each of the corresponding animals; these were carried out by Steven Smith and colleagues as described in section 2.2.6, and are illustrated in Figure 4.5. Examples of chloroacetate esterase stained samples are illustrated in Figure 4.6.

Throughout the course of infection, including the unchallenged groups, previously infected-un/challenged sheep were found to have significantly higher numbers of mast cells in the abomasal mucosa than naïve/naïve-challenged sheep. Very low numbers of mast cells were found in naïve-challenged sheep on days 0, 2 and 5. Mast cells were more numerous in the naïve-challenged samples on day 10 and 21, but did not reach the levels seen in the previously infected-un/challenged sheep. The latter showed an increase in the numbers of mast cells in the abomasal mucosa on days 2 and 5 after challenge, much earlier than in the naïve-challenged sheep, but mast cell numbers had decreased again on day 10.

Following a one-way ANOVA, a normality plot of residuals showed that mast cell numbers were not normally distributed overall. Therefore a non-parametric Kruskal-Wallis test was then used to search for differences within the naïve/naïve-challenged and the previously infected-un/challenged groups. This test produced a P-value of 0.139 ($H=5.50_3$) in the previously infected-un/challenged group, indicating no significant differences in this group, and 0.004 ($H=15.61_4$) ($P=0.003$, $H=15.77_4$ when adjusted for ties) in the naïve/naïve-challenge group, indicating highly significant differences and the need for more detailed statistical analysis. More detailed analysis of the relationships between the groups was carried out using a Mann-Whitney U test, the results of which are detailed in Table 4.3. Comparisons of naïve/naïve-challenged and previously infected-un/challenged groups at the same time-point demonstrated that mast cell counts were significantly higher in the previously infected animals throughout the study.

Figure 4.4: Mean mast cell counts per 0.24mm^2 of sectioned abomasal mucosal epithelium. Analysed using Mann-Whitney U-test for non-parametric data with a 95% confidence interval, n = 6 sheep per group for each time point; significant difference between naïve- and previously infected-un/challenged groups on the same day: ★ (P<0.05), ★★ (P<0.01); significant difference between challenged groups and their respective unchallenged groups: # (P<0.05).

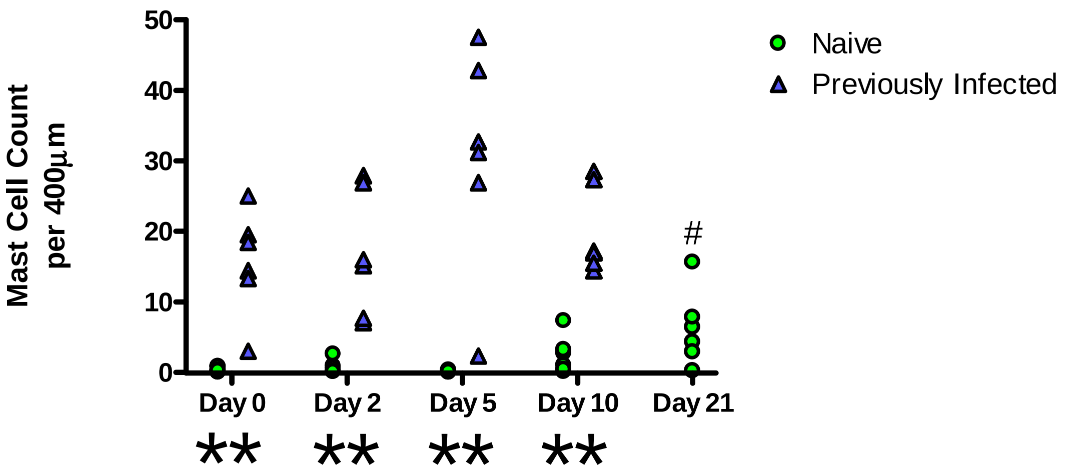


Figure 4.5: Worm counts per 250ml of abomasal contents (mean and SEM, n=6).

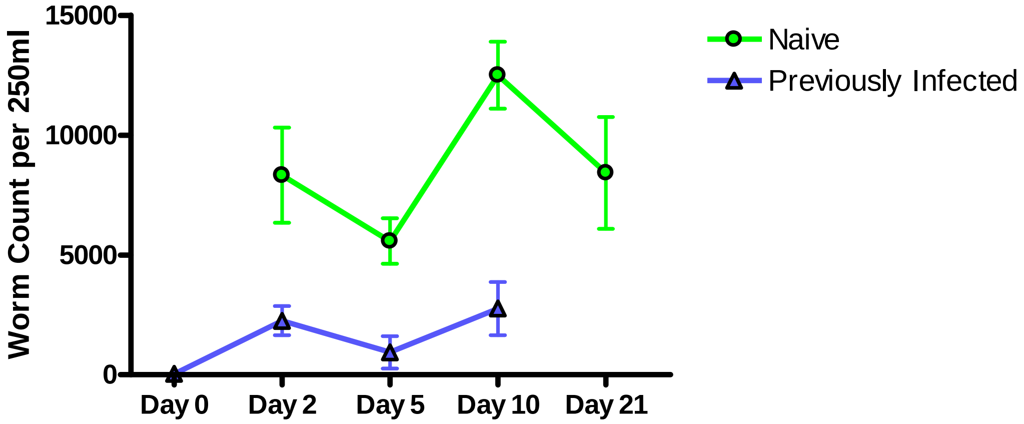
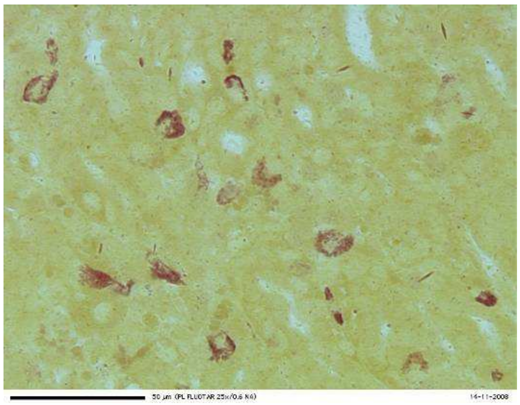


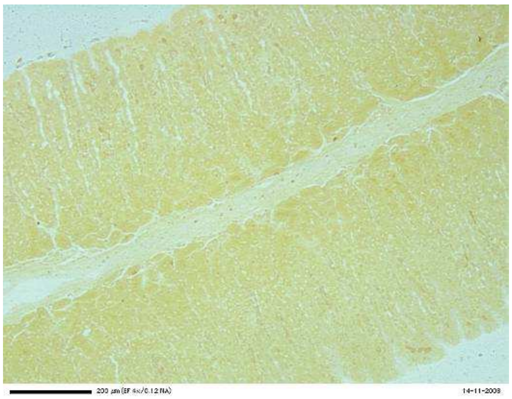
Figure 4.6: Representative images of mast cells in the abomasal mucosa stained using chloroacetate esterase stain (a,b), and chloroacetate esterase stained sections taken from naïve day 0 (c,d), naive day 21 (e,f), previously infected day 0 (g,h) and previously infected day 10 (i,j). Note mucosal thickening and increased mast cell numbers in previously infected animals compared to worm free and naïve (g,i).

(a) Bar indicates 50µm

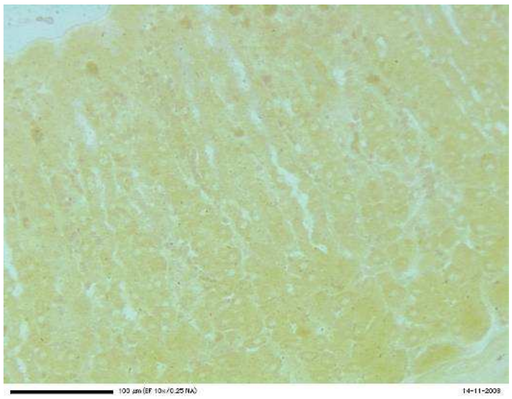
(b) Bar indicates 50µm



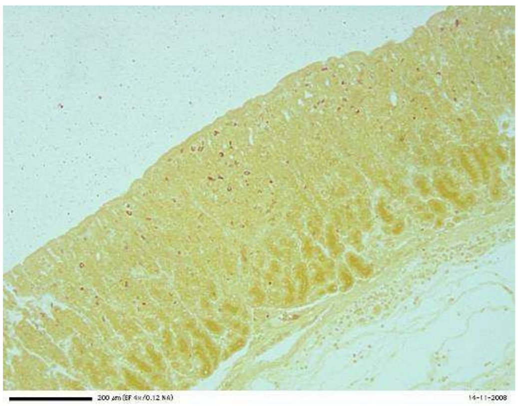
(c) Bar indicates 200µm



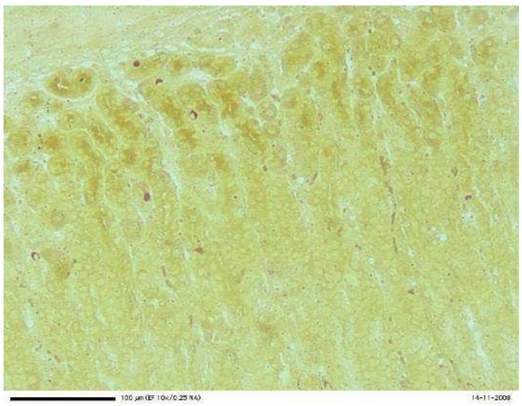
(d) Bar indicates 100µm



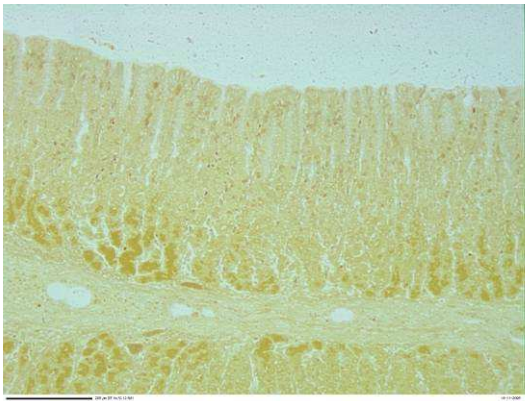
(e) Bar indicates 200µm



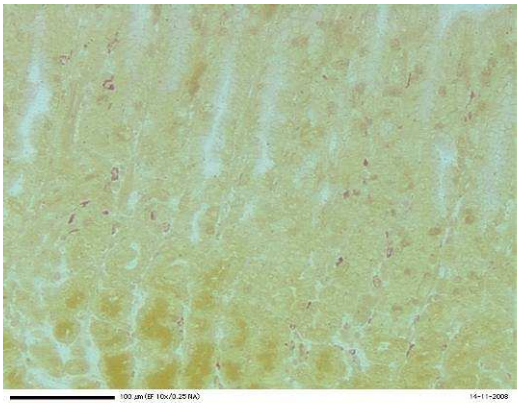
(f) Bar indicates 100µm



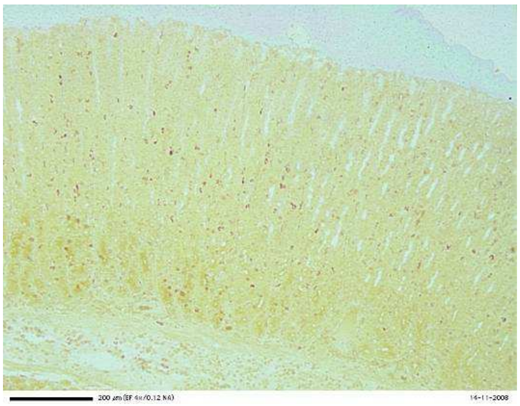
(g) Bar indicates 200μm



(h) Bar indicates 100μm



(i) Bar indicates 200μm



(j) Bar indicates 100μm

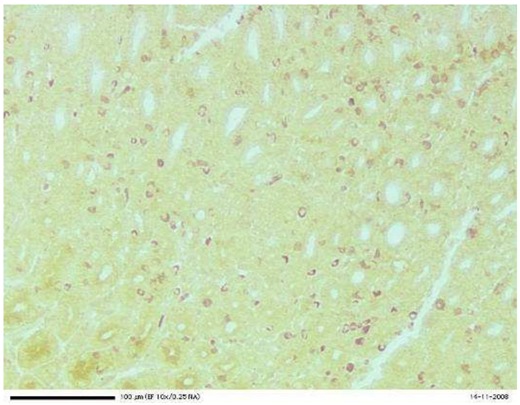


Table 4.2: Mean abomasal mast cell counts and corresponding worm counts.

Group	Naïve-Challenged			Previously Infected-Challenged		
	Sample Mean	Group Mean (± SE)	Worm count	Sample Mean	Group Mean (± SE)	Worm Count
Unchallenged	0.8	0.35	-	14.4	15.60	38
	0	(±0.15)	-	19.5	(±3.04)	0
	0		-	18.4		0
	0.8		-	3		0
	0.3		-	25		70
	0.2		-	13.3		73
Day 2	0.2	0.75	8285	27.9	16.77	2618
	0.9	(±0.39)	12305	26.9	(±3.69)	1324
	0.1		3200	7		3085
	2.6		15429	15.1		578
	0.6		3478	7.7		1252
	0.1		7242	16		4629
Day 5	0	0.08	2923	32.7	30.57	0
	0.3	(±0.05)	7220	26.9	(±6.46)	652
	0		5818	2.3		4273
	0.1		8591	31.2		451
	0		6073	47.5		40
	0.1		2821	42.8		198
Day 10	7.3	2.45	9871	14.4	19.97	5185
	2.7	(±1.10)	8471	17.2	(±2.55)	6642
	3.2		11853	16.9		19
	0.1		11564	15.5		36
	1		15836	28.5		2967
	0.4		17355	27.3		1707
Day 21	6.4	6.20	11855			
	7.8	(±2.17)	2791			
	4.3		16647			
	15.6		3780			
	0.2		3863			
	2.9		11564			

Table 4.3: Comparisons between mast cell counts of naïve and previously infected groups at the same time-point using Mann-Whitney U test with a 95% confidence interval. Values in brackets have been adjusted for ties. * $P \leq 0.05$, ** $P \leq 0.01$.

P-value Day 0	P-value Day 2	P-value Day 5	P-value Day 10
0.0051** (0.0049**)	0.0051** (0.0050**)	0.0051** (0.0047**)	0.0051**

One sheep in the previously infected-challenged group killed on day 5 was found to have unusually low numbers of mast cells in the abomasal mucosa compared to the other five sheep in this group. When this animal's mast cell count was removed, the P-value produced by a Mann-Whitney U test comparison of this group with the previously infected-unchallenged group changed from 0.0656, which was considered not quite significant, to 0.0081, indicating a significant increase in mast cell numbers on day 5 in the previously infected-challenged sheep compared to the previously infected-unchallenged group.

Huntley and colleagues have previously suggested that the influx of intraepithelial mast cells during the inflammatory response to *T.circumcincta* is focal, involving local migration and proliferation of cells rather than an organ-wide response (Huntley et al., 2004). Therefore, it is possible that the sample used for histology in this sheep was taken from an area of the abomasum which was less affected by the parasite infection. However, the number of worms found to be present in this sheep were far higher than in the rest of the group, indicating that this animal diverged from the other group members, exhibiting an unusually low resistance to *T.circumcincta*. This observation supports the findings of previous studies of periparturient sheep, which indicated that reduced mast cell numbers were linked to lower helminth resistance (Huntley et al., 2004, Houdijk et al., 2005).

Statistical analysis using Mann-Whitney U test was also carried out to identify differences in worm counts between the naïve and previously infected groups. The result of this analysis is detailed in Table 4.4. In contrast to mast cell numbers, worm counts were found to be significantly higher in the naïve-challenged groups than in the previously infected-challenged animals. However, worm counts and mast cell counts were not found to be significantly correlated in this study.

Table 4.4: Comparisons between worm counts of naïve and previously infected groups at the same time-point using Mann-Whitney U test with a 95% confidence interval. * P≤0.05, ** P≤0.01.

P-value Day 2	P-value Day 5	P-value Day 10
0.0131*	0.0131*	0.0051**

4.5 Discussion

These results indicate that cells within the abomasal mucosa itself appear to be a major source of cytokine production, and show evidence of a marked Th2-type immune response occurring during infection with *T.circumcincta*. Transcription of the Th2 cytokines IL-4, IL-5 and IL-13 increased earlier and more markedly in the previously infected-challenge groups than in the naïve-challenged groups. A proinflammatory component was evident in the naïve-challenged sheep, demonstrated by the significantly increased transcription of IL-1β and IL-6 in the naïve-challenged groups following infection. The trends in two selected cytokines, one proinflammatory and one Th2-type, were confirmed by Q-PCR. The patterns of cytokine transcription observed were pronounced despite the variety of cell types present. Figure 4.7 illustrates the trends in cytokine transcription observed in the abomasal mucosa following challenge of naïve and previously infected sheep.

IL-4 and IL-5 were up-regulated earlier in the previously infected-challenged animals, possibly due to mastocytosis and eosinophilia during trickle infection leading to increased populations of these cells in the abomasal mucosa at prior to challenge compared to naïve sheep (Seaton et al., 1989, Scott et al., 2000, Balic et al., 2003). Both of these cytokines have been found to be produced by mast cells in response to IgE cross-linking of Fc receptors in mouse and human mast cell lines (Burd et al., 1989, Plaut et al., 1989, Sanmugalingham et al., 2000, Bressler et al., 1997). IL-4 and IL-5 have also been shown to be produced by human eosinophils (Bjerke et al., 1996, Lalani et al., 1999). It is likely that the large eosinophil and mast cell populations in the mucosa of previously infected-challenged sheep rapidly increase transcription of IL-4 and IL-5 following detection of the parasite, producing a significant increase earlier in previously infected-challenged sheep than in the naïve-challenged sheep, which have lower numbers of eosinophils and mast cells in the abomasum. This hypothesis is supported by the findings of a study by Athanasiadou (2008) and colleagues. Athanasiadou's study examined the proteins produced by abomasal explants from naïve and previously infected sheep before and after challenge infection with *T.circumcincta*. Sheep mast cell protease 1 (SMCP-1) and galectin-14, produced by mast cells and eosinophils respectively, were found in the mucosal wash from previously infected, but not naïve tissue, before challenge. This indicates higher numbers of mast cells and eosinophils in the previously infected than naïve tissue (Sture et al., 1995, Dunphy et al., 2002). A previous study by Stevenson (1994) and colleagues also demonstrated significant levels of eosinophil potentiating activity and sheep mast cell protease in efferent gastric lymph within 48 hours of *T.circumcincta* challenge of previously infected sheep. These substances were not detected in the efferent lymph of naïve-challenged animals. These findings also support a rapid mobilisation of eosinophils and mast cells in the previously infected-challenged animals which is not available to the naïve-challenged sheep.

The probable production of IL-4 and IL-5 by mast cells and eosinophils in the abomasal mucosa is in contrast to the lymph node, in which the predominant sources of IL-4 and IL-5 are considered to be T cells (Takatsu et al., 1987, Brown et al., 1998, King and Mohrs, 2009). Other possible sources of IL-4 and IL-5 in the abomasal mucosa include basophils and NK cells respectively (Seder et al., 1991a, Seder et al., 1991b, MacGlashan et al., 1994, Warren et al., 1995).

IL-13 was found to be up-regulated in the previously infected-challenged groups later than IL-4 and IL-5. This may be because an accumulation of IL-4 is necessary to prime mast cells to produce IL-13, delaying up-regulation compared to IL-4 and IL-5; human mast cells have been found to produce IL-13 in response to IgE following stimulation by IL-4 (Toru et al., 1998).

Another possibility is that the observed increase in IL-13 transcription is due to the influx of other cell populations producing IL-13. Studies of NK cell activity in mice have found that they accumulate in response *Litomosoides sigmodontis* infection (Korten et al., 2002), and produce IL-13 in response to *Trichinella spiralis* infection (McDermott et al., 2005). It is therefore possible that NK cells accumulate and produce IL-13 in response to helminth infection in sheep. If so, this may occur earlier in previously infected-challenged sheep due to differences in abomasal cell populations in these animals, as suggested for IL-4 and IL-5. This hypothesis is supported by the finding that transcription of granulysin increased dramatically following infection with *T.circumcincta* (Knight et al, paper in preparation, Griffith et al, paper in preparation); in humans, this protein has been found to be produced by NK cells (Obata-Onai et al., 2002) and to kill *Leishmania major in vitro* (Stenger et al., 1998). However, to date NK cell markers have not been successfully generated for sheep, so this hypothesis remains untested.

The timing of the observed up-regulation of IL-13 also coincides with establishment of *T.circumcincta* larvae in the gastric crypts of the abomasum, which occurs after day 3-4 (Sommerville, 1953). This may be an alternative stimulus for the production of IL-13, which has been demonstrated in murine models to be critical in driving the increased epithelial turnover essential to the expulsion of *Trichuris muris* (Cliffe et al., 2005). If increased epithelial turnover is involved in the expulsion of *T.circumcincta* larvae, it may be a contributing factor in the mucosal hyperplasia (Ritchie et al., 1966) and epithelial changes, such as the reduction in parietal cells, which occur during infection (Scott et al., 2000). Transplantation of adult *Ostertagia ostertagi* into the abomasum of cattle does not produce the hyperplastic epithelial changes characteristic of larval infection (McKellar et al., 1986, McKellar et al., 1987, McKellar, 1993), indicating that this is provoked by substances produced only by larval stages of the parasite, or by the presence of larvae in the gastric crypts. IL-13 my also be involved in the immune-mediated arrested development of *T.circumcincta* larvae which has been observed during infection of sheep (Halliday et al., 2007).

The observed increase in transcription of IL-4 and IL-5 on day 2 in the previously infected-challenged group demonstrates a similar trend to that which occurred in the gastric lymph node described in chapter 3. However no up-regulation of IL-13 was observed in the gastric lymph node. This may be because parasite-specific T cells generated in the lymph node have migrated to the abomasal mucosa before producing IL-13 to drive local responses such as mastocytosis. IL-13 may also be being produced by other cells involved in local immune responses in the abomasum, such as mast cells, basophils and NK cells.

Figure 4.7(a): Schematic illustration of the trends in transcription of Th1- and Th2-type cytokines in the abomasal mucosa of naive-challenged and previously infected-challenged sheep. Note ranges on Y-axes. Day 0 =unchallenged.

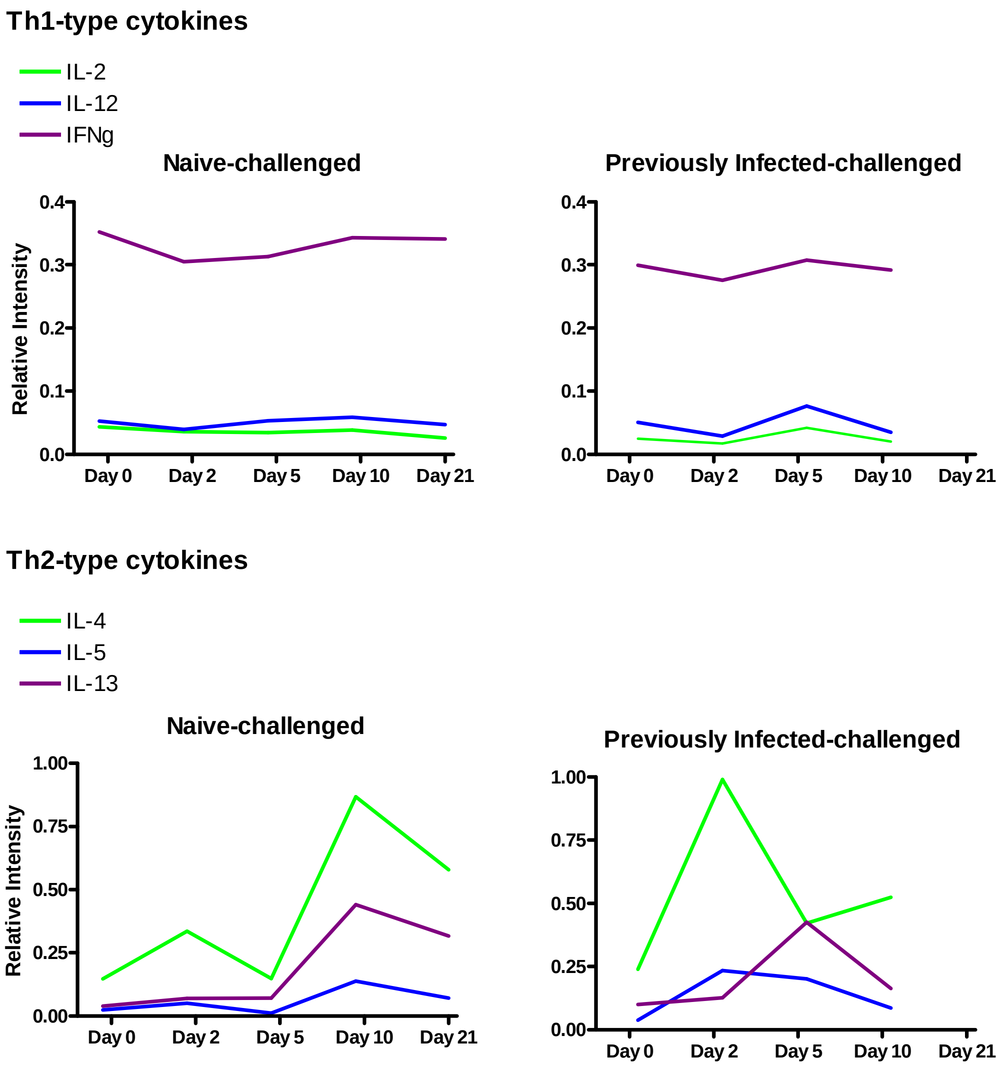
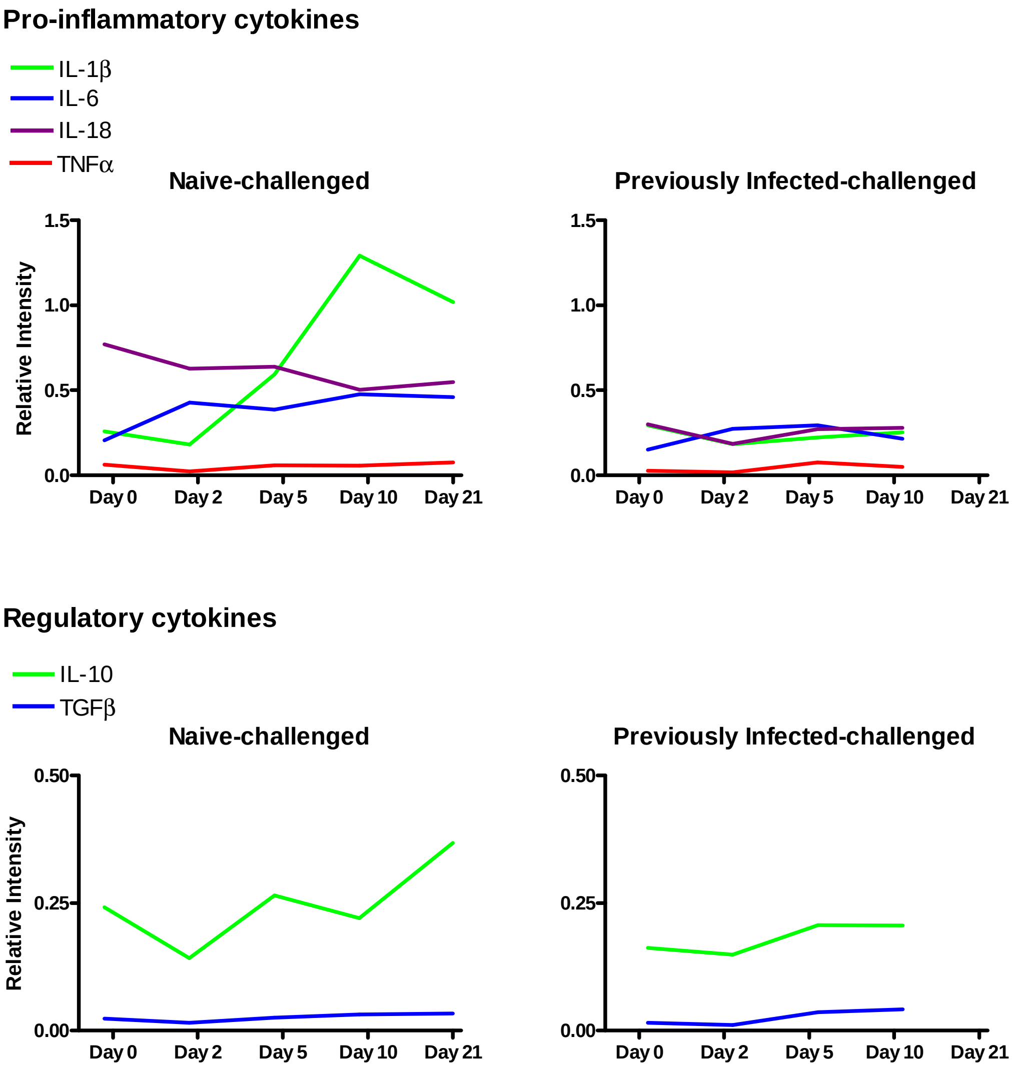


Figure 4.7(b): Schematic illustration of the trends in transcription of pro-inflammatory and regulatory-type cytokines in the abomasal mucosa of naive-challenged and previously infected-challenged sheep. Note ranges on Y-axes. Day 0 =unchallenged.



While the observed increase in transcription of Th2 cytokines in the abomasal mucosa shows similarity to the anti-parasitic response found in the draining lymph node, the suppression of Th1 cytokines observed in the gastric lymph node was not evident in the mucosa. No significant changes in transcription of IL-2, IL-12p_{40} or IFNγ were found in the abomasum following infection with *T.circumcincta*. This is in contrast to the findings of Claerebout and colleagues' examination of cytokine responses in the abomasal mucosa of cattle infected with *O.ostertagi* (Claerebout et al., 2005), in which an up-regulation of IFNγ was found in the abomasal mucosa. It is possible that this increase was due to a more pronounced increase in abomasal pH and bacterial load in the study of *O.ostertagi* infection compared to *T.circumcincta*, however these parameters were not examined.

IL-1β was found to be up-regulated on days 10 and 21, and IL-6 was up-regulated on days 2, 10 and 21 in the naïve-challenged animals. In contrast only a transient increase in transcription of IL-1β was found in the previously infected-challenged group on day 2 using Q-PCR. The proinflammatory response observed in the naïve-challenged sheep on days 10 and 21 is likely to be due to damage to the mucosa caused by growth of the larvae in the gastric crypts and bacterial infection of these lesions. However, the up-regulation found on day 2 of IL-6 in the naïve-challenged sheep and IL-1β in the previously infected-challenged group may reflect the initiation of immune responses to larval challenge.

Type 2 activated macrophages similar to those produced from mouse bone marrow derived macrophages may be involved in generating the early stage IL-1β response observed in the previously infected-challenged sheep. Distinct from classically IFNγ/LPS-activated, or alternatively IL-4-activated macrophages, these cells are primed by ligation of Fcγ receptors by IgG and activated via TLRs (Sutterwala et al., 1997, Anderson and Mosser, 2002). These type 2 macrophages continue to produce IL-1β, IL-6, TNFα, and increased levels of IL-10, but do not produce IL-12 (Gerber and Mosser, 2001, Anderson and Mosser, 2002). When acting as antigen presenting cells, type 2 macrophages also induce a Th2 phenotype (Anderson and Mosser, 2002). Cells with a similar phenotype have been produced in mice with chronic helminth infections (Lanier et al., 1986, Rodriguez-Sosa et al., 2002).

It is possible that production of IgG during trickle infections of the previously infected group produces similar type 2 activated macrophages in sheep, which generate proinflammatory cytokines and promote Th2 responses at the early stages of helminth infection. Granulysin, which increased significantly during infection with *T.circumcincta* (Knight et al, paper in preparation, Griffith et al, paper in preparation), has been shown to be chemoattractant for human monocytes (Deng et al., 2005), and monocyte-derived ovine macrophages and dendritic cells have been shown to produce IL-1β, IL-4, IL-12p_{40}, IFNγ and TNFα within 3-6 hours of stimulation with LPS (Budhia et al., 2006). Type 2 activated macrophages, if found in sheep, may have a role in the rapid expulsion of *T.circumcincta* larvae before they enter the gastric crypts in immune sheep.

Up-regulation of IL-1β found in the previously infected-challenged sheep may have been suppressed on day 5 due to the increasing levels of IL-13; as this cytokine has been shown to reduce IL-1β production by human monocytes (Scotton et al., 2005).

Macrophages may also be responsible for the temporary increase in IL-6 transcription observed in the naïve-challenged sheep on day 2. Alternatively activated macrophages have been produced *in vitro* by stimulating ovine macrophage cell lines with proteins derived from *Fasciola hepatica* (Flynn et al., 2007), so it is possible that they could have a role in the early immune response to *T.circumcincta*. Activated macrophages taken from mice chronically infected with *Brugia malayi* have been shown to produce IL-6 independent of IL-4 (Lanier et al., 1986), and macrophages from mice infected with *Toxocara canis* have been found to produce IL-6 in response to *T.canis* antigen (Kuroda et al., 2001).

The increase in transcription of IL-1β observed following infection may also be an indication of dendritic cell activation. As described in sections 1.3 and 3.4, binding of pathogen molecules to dendritic cell pattern recognition receptors results in the initiation of activation pathways, many of which involve IL-1β (Granucci et al., 2001, Guo et al., 2003, Hurst et al., 2009). This is supported by the increased transcription of IL-6 in the mucosa of naïve-challenged sheep 2 days following challenge. IL-6 has been shown to be produced by murine dendritic cells during maturation in response to *Nippostrongylus brasiliensis* ES products (Balic et al., 2004).

In contrast to the changes observed in the abomasal mucosa of cattle infected with *O.ostertagi* (Claerebout et al., 2005), no increase was observed in the transcription of IL-10 following infection with *T.circumcincta*. In fact, a transient suppression of IL-10 transcription was found on day 2 in the naïve-challenged group. IL-10 prevents the differentiation of human cultured monocytes into dendritic cells *in vitro*, instead promoting maturation into macrophages (Allavena et al., 1998), therefore a reduction in IL-10 could be instrumental in promoting production of dendritic cells in the abomasal mucosa.

Significantly higher levels of IL-18 transcription were observed in the naïve and naïve-challenged sheep than in previously infected-un/challenged animals throughout the study. However, this transcription was reduced in the naïve-challenged sheep on days 10 and 21. This indicates suppression of IL-18 transcription was initiated following challenge of naïve sheep and during trickle infection of the previously infected sheep, and that this was maintained through the clearance of infection and subsequent challenge of previously infected group. This would be expected given the Th1-type functions of IL-18; IL-18 deficiency in mice has been shown to suppress IFNγ production (Dinarello, 1999) and promote IgE production (Salagianni et al., 2007), and Helmby and colleagues found that IL-18 inhibited mastocytosis and production of IL-4 and IL-13 in mice infected with *T.muris* independent of IFNγ (Helmby et al., 2001, Helmby and Grencis, 2002).

However, the role of IL-18 in response to helminths may be more complex than simply that of a classical Th1 or proinflammatory cytokine. IL-18 in combination with IL-2, another traditionally Th1 cytokine, has been shown to induce IL-4 production by naïve murine T cells *in vitro* (Sasaki et al., 2005) and intestinal mastocytosis in mice (Sasaki et al., 2005), and activate intestinal mucosal mast cells in STAT6-independent manner (Sasaki et al., 2005). These data imply a role for IL-18 in innate responses to parasitic helminths, however, the patterns observed during this study do not support IL-18 having this role in the sheep. No change in IL-2 transcription was found in the abomasum, IL-18 transcription was gradually reduced during the course of infection in naïve-challenged sheep, and was significantly lower in the previously infected-un/challenged sheep which would be expected to have undergone mastocytosis.

The increased transcription of both $TGF\beta_1$ and TNFα in the abomasum of the previously infected-challenged group from day 5 onwards is interesting. This trend in $TGF\beta_1$ was observed in both the naïve-challenged and previously infected-challenged sheep in both the gastric lymph node and the mucosa, however the changes were only significant in the gastric lymph node of the naïve-challenged group and the mucosa of the previously infected-challenged group.

Though $TGF\beta_1$ and TNFα have wide ranging actions, both have been found to be produced by neutrophils during the local response to *N.brasiliensis* (Pesce et al., 2008). Pesce and colleagues have speculated that this neutrophil response may be due to the helminth acting as a vector for potentially pathogenic bacteria (Pesce et al., 2008). TGFβ has also been found to recruit neutrophils and macrophages (Faler et al., 2006). A similar neutrophil response may be occurring in *T.circumcincta* infection; not only could *T.circumcincta* larvae be bringing bacteria into the gastric crypts, but the increased bacterial content of the abomasum due to the raised pH encourages bacterial colonisation of mucosal lesions caused by the parasite. TNFα may also be being up-regulated as part of a proinflammatory dendritic cell response, however in that case a greater increase would be expected in the naïve-challenged animals, as was found for IL-1β and IL-6. An alternative function for the increase in $TGF\beta_1$ transcription may be the generation of Treg cells, as discussed in section 3.4.

The observed increases in intraepithelial mast cells confirm a more rapid Th2-type phenotypic response taking place in the previously infected-challenged animals. These observations are consistent with previous studies of ovine *T.circumcincta* infection, in which an increase was observed 10 days following challenge of previously infected sheep (Balic et al., 2003) and 21 days following infection in naïve sheep (Stevenson et al., 1994). However, the increase in mast cell numbers found in Balic's study were observed 10 days following challenge, whereas in the current study an increase was found 5 days following challenge (when the single anomalous result is excluded). This difference could be due to the far longer helminth-free period between trickle-infection and challenge used in Balic's study; 12 weeks compared to only one week in this study. This implies that the rapid mastocytosis in response to infection with *T.circumcincta* in a partially immune sheep is a response which wanes with time if exposure is removed.

Earlier recruitment of immune cells in previously infected 3-month old lambs was also observed in studies of *H.contortus* infection carried out by Lacroux (2006) and colleagues. Not only were there significantly higher numbers of eosinophils and mast cells in the abomasal mucosa of previously exposed-challenged animals compared to naïve-challenged individuals 3 and 7 days following challenge, but numbers of these cells increased earlier in the previously exposed-challenged group.

The fall in mast cell numbers observed in the abomasum of the previously infected-challenged group between days 5 and 10 following challenge is interesting. As detailed in section 2.2.6, evidence from worm count and worm measurement data indicates that parasite loss was occurring by day 2 in the previously infected sheep (Halliday et al., 2009b). It is likely that in this group mast cells are involved in the early stages of the hypersensitivity response, and that their role in the rejection of *T.circumcincta* in previously infected-challenged sheep is concluded in less than 10 days following infection, as indicated by the reduction in mast cell numbers on day 10.

Transcription studies of gastric mucosal mast cell marker sheep mast cell protease-1 (sMCP-1) (Pemberton et al., 2000) and eosinophil marker ovine galectin 14 (OvGAl14) (Dunphy et al., 2002) in the abomasum of these sheep have previously been carried out by Anne French (French et al., 2008). These studies demonstrated a significant increase in transcription of sMCP-1 in naïve-challenged sheep on days 2, 10 and 21 following challenge. Transcription of sMCP-1 was also significantly higher in previously infected-unchallenged sheep compared with naïve individuals. In contrast, no increase in sMCP-1 transcription was found in previously infected-challenged sheep following challenge. However, because sMCP-1 is stored in granules before release, transcription levels do not necessarily correlate with protein release. A significant increase in transcription of OvGal14 was also shown in naïve-challenged sheep on days 10 and 21, and in previously infected-challenged sheep on days 2, 5 and 10. This increase in transcription of both sMCP-1 and OvGal14, and in mast cell numbers, in the abomasum of naïve-challenged sheep complements Stevenson's study of *T.circumcincta* infection, which found accumulation of eosinophils and mast cells 21 days after challenge of naïve sheep (Stevenson et al., 1994). These are in contrast with the findings of Balic's study of local cellular responses to *H.contortus* infection, in which mast cells are described as being inversely related to the number of eosinophils (Balic et al., 2006). However eosinophils have previously been associated with the killing of *H.contortus* larva (Rainbird et al., 1998, Balic et al., 2006) and may have a role in stunting the growth of *T.circumcincta*. Therefore it is possible that the evidence for the accumulation of mast cells and eosinophils in response to *T.circumcincta* infection, in combination with this parasite's potent chemoattractive effect on bone marrow derived eosinophils *in vitro* (Wildblood et al., 2005), could indicate that these cells act in synergy to control *T.circumcincta* infection.

IL-4 has been found to promote mast cell proliferation in combination with stem cell factor or IL-3 in human and mouse models (Hamaguchi et al., 1987, Lorentz and Bischoff, 2001), and has been shown to be chemoattractant for human mast cells (Olsson et al., 2004). The significant increase in transcription of IL-4 in the abomasal mucosa observed on day 2 in the previously infected-challenged sheep, which was reduced again on day 5, could account in part for the spike in mast cell numbers in these sheep; increasing IL-4 could provoke mast cell infiltration and proliferation, once sufficient mastocytosis is achieved IL-4 production may be reduced again. IL-4 may also be in effect in the naïve-challenged group; the significant increase in IL-4 transcription on days 10 and 21 correlates with the beginning of mastocytosis in this group. However it is unclear whether this is purely a case of IL-4 causing the observed increase in mast cell numbers (Perdue et al., 1991), or whether the mast cells in the abomasal mucosa are proliferating in response to an alternative stimulus and producing IL-4 (Brown et al., 1987, Huels et al., 1995), or a combination of the two.

The fluctuations in mast cell numbers may also account for the changes in transcription of IL-13 found in the abomasal mucosa. The pattern of this transcription was similar to the pattern of mast cell changes found in the previously infected-challenged sheep, and was increased on days 10 and 21 in the naïve-challenged group. Human mast cell lines have been found to produce IL-13 after IgE receptor cross-linking, particularly if sensitized by IL-4 (Burd et al., 1995, Toru et al., 1998).

Up-regulation of $TGF\beta_1$ in the abomasal mucosa 5 days after challenge of previously infected sheep also coincided with the increase in intraepithelial mast cell numbers. This increase was maintained to day 10, whereas mast cell numbers declined. However, $TGF\beta_1$ transcription levels do not necessarily correlate with levels of active $TGF\beta_1$ due to the post-translational modifications required to activate latent $TGF\beta_1$ (Nunes et al., 1995, Munger et al., 1999). Lindstedt and colleagues demonstrated that rat mast cells not only produce latent $TGF\beta_1$, but also chymase-1, which is a potent activator of $TGF\beta_1$ (Lindstedt et al., 2001). $TGF\beta_1$ has been found to promote the release of fibroblast growth factors (Qu et al., 1998) and proteases (Miller et al., 1999, Funaba et al., 2005) from mouse mast cell lines, and is an important component in the mucosal mast cell response to *Nippostrongylus brasiliensis* in mice due to the release of these proteases (Knight et al., 2002). $TGF\beta_1$ has also been shown to be a potent chemoattractant for mouse and human mast cells (Gruber et al., 1994, Olsson et al., 2000). It is therefore possible that $TGF\beta_1$ plays an important part in the orchestration of an immune response appropriate for helminth infection, as well as having a role in repairing the damage caused during inflammation. This is supported by the finding that $TGF\beta_1$ can also inhibit the release of mast cell mediators such as histamine and TNFα (Bissonnette et al., 1997).

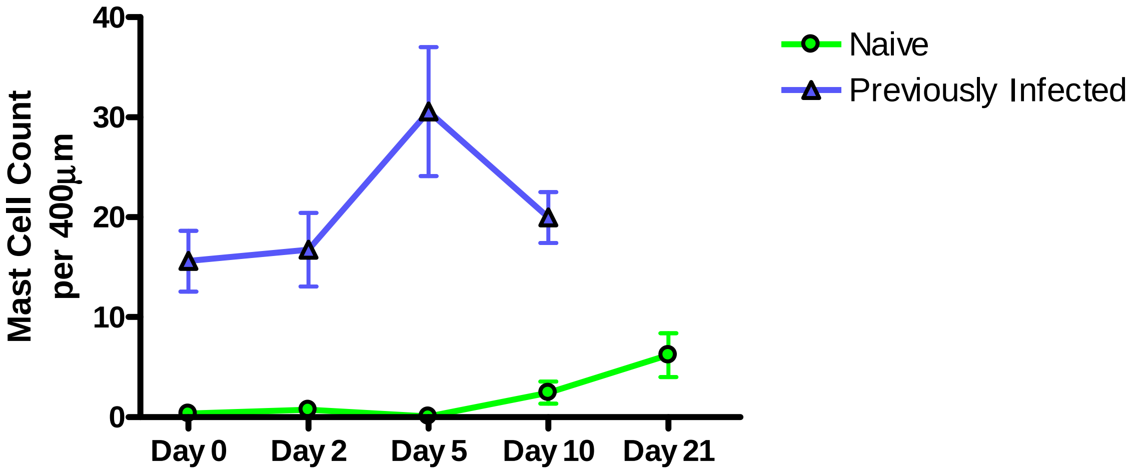
Mast cell numbers and transcription of IL-4, IL-13 and $TGF\beta_1$ are illustrated for comparison in Figure 4.8.

The increased transcription of IL-4 and IL-13, and the observed mast cell recruitment are all consistent with previous observations of the local immune response to *H.contortus* (Meeusen et al., 2005, Lacroux et al., 2006). The up-regulation of IL-4 and IL-5 is also consistent with the previous study of *O.ostertagi* infection (Claerebout et al., 2005). However, as discussed previously, changes in IFNγ and IL-10 observed in response to *O.ostertagi* infection of cattle were not evident following *T.circumcincta* infection in sheep.

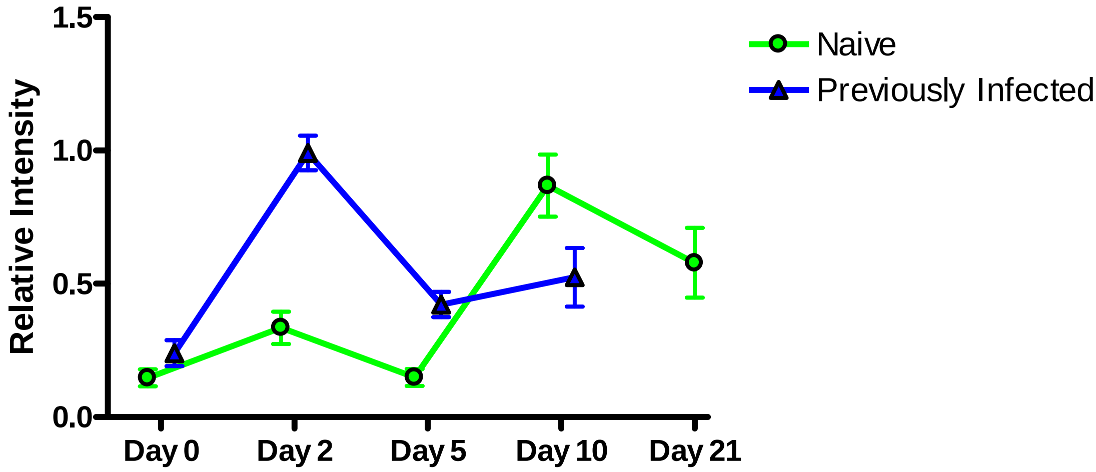
These results confirm the findings of the previous studies of the gastric lymph node in demonstrating that the immune responses to *T.circumcincta* infection in sheep are similar to those elicited by *H.contortus* in sheep and *O.ostertagi* in cattle. Importantly, the observed differences in cytokine and mast cell responses to *T.circumcincta* between naïve-challenged and previously infected-challenged sheep indicate that the increased anti-parasitic responses built up during trickle infection of previously infected sheep may be responsible for the greater resistance to *T.circumcincta* infection in partially immune sheep, in conjunction with specific antibody responses (Halliday et al., 2007, Halliday et al., 2009b).

Figure 4.8: Mean mast cell counts (a), IL-4 (b), IL-13 (c) and TGFβ₁ transcription (d) in the abomasal mucosa.

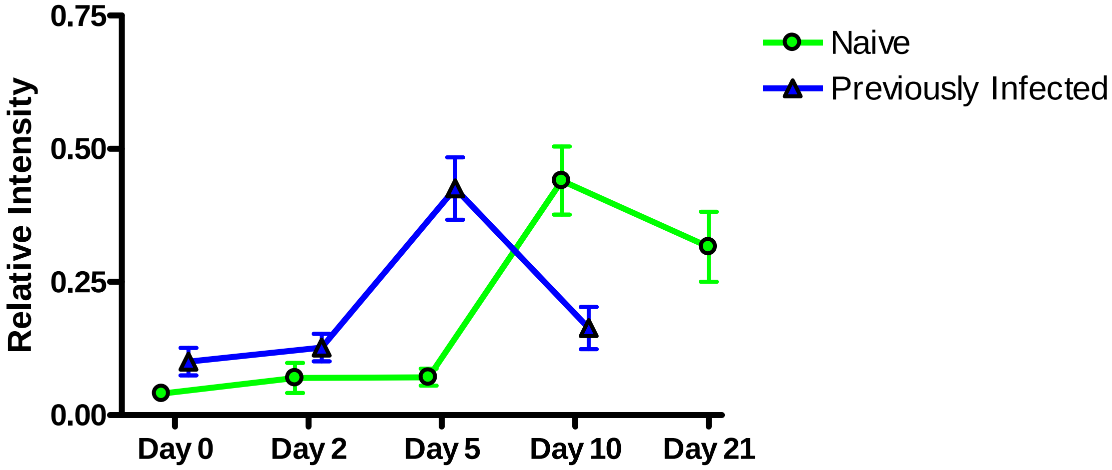
(a) Mast cell counts per 400μm of sectioned abomasal mucosal epithelium (mean and SEM, n=6).



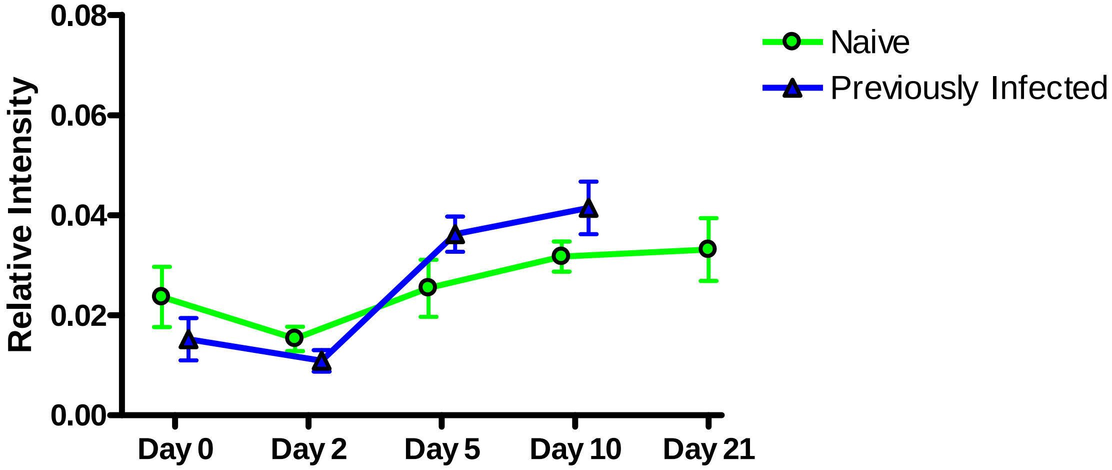
(b) IL-4 transcription in the abomasal mucosa RT-PCR relative to ATPase (mean and SEM, n=6).



(c) IL-13 transcription in the abomasal mucosa RT-PCR relative to ATPase (mean and SEM, n=6).



(d) TGFβ_1 transcription in the abomasal mucosa RT-PCR relative to ATPase (mean and SEM, n=6).



5 Chapter Five:
Cytokine Responses in Gastric Efferent Lymph

5.1 Introduction

The findings of chapter 3 and chapter 4 illustrate the orchestration of the immune response at the site of infection and in the draining lymph node, however, the signals passing between these sites have not been examined. Unfortunately due to the nature of the afferent lymphatics of the abomasum as a multitude of small vessels, it has not been possible to cannulate these vessels to obtain samples of afferent lymph. However, cannulation of the common gastric lymph duct as described in section 2.2.3 has allowed collection of efferent lymph over the course of infection with *T.circumcincta* in both naïve-challenged and previously infected-challenged sheep.

As described in section 1.3, activated lymphocytes originating in the gastrointestinal tract preferentially circulate back to the mucosa after passing through the draining lymph node and joining the venous circulation (Weisz-Carrington et al., 1991, Hein and Mackay, 1991, Au et al., 2001). Lymphocytes present in efferent lymph may therefore be instrumental in carrying information from the draining lymph node back to the site of infection during immune responses.

Cytokine transcription in efferent lymphocytes was examined to see which cytokines were detectable in these cells, and how this compared to those detectable in the abomasal mucosa and the gastric lymph node. Efferent lymph also presented an opportunity to investigate how cytokine transcription in these lymphocytes changed over the course of infection within the same sheep, which had not previously been possible using only post-mortem samples, and how this also compared to the cytokine changes in the abomasal mucosa and the gastric lymph node. Efferent lymphocyte cytokine transcription over the course of infection in naïve-challenged and previously infected-challenged individuals was compared to see if differences between these two groups could contribute to understanding the disparity in resistance to *T.circumcincta* between naïve-challenged and previously infected-challenged animals.

Examination of the phenotypic lymphocyte populations present in the gastric efferent lymph of the animals used in this study has been completed by Aileen Halliday and colleagues at the Moredun Research Institute (Halliday et al., 2009b). This examination has shown that numbers of CD4+, CD8+ and γδ+ T cells increase in the efferent lymph early following challenge of previously infected animals. Levels of CD4+ and CD8+ T cells also increased in the naïve individuals, but later than in the previously infected. Fluorescence activated cell sorting (FACS) of these different phenotypes, with the development of more sensitive real-time PCR assays, allowed transcription profiling of specific lymphocyte subsets to see which of these cell types were producing cytokines of interest.

The objectives of this section of work were:

(1) To establish which cytokines are transcribed in efferent lymphocytes from the gastric lymph node.

(2) To examine how this transcription compares with that taking place in the gastric lymph node.

(3) To define how cytokine transcription in gastric efferent lymphocytes changes over the course of infection in naïve-challenged compared with previously infected-challenged sheep.

(4) To examine which cytokines are transcribed by particular subsets of lymphocyte phenotypes, and how this changes following challenge of naive and previously infected sheep.

5.2 Comparison of cytokine transcription in abomasal efferent lymphocytes and gastric lymph node

Initially, in order to verify whether cytokine transcription was detectable in samples of the efferent lymphocytes, and to compare this with that in the reacting lymph node of the same sheep, samples of lymph taken close to slaughter were compared with post-mortem samples of gastric lymph node.

RNA extracted from efferent lymphocytes taken on day 9 following challenge from three previously infected-challenged sheep in group 2 – PI killed on day 10. Sufficient RNA was not available from lymphocytes taken from the other sheep in this group on day 9, or from these sheep on day 10. RNA was also extracted by Judith Pate from lymph samples taken from four naïve-challenged sheep in group 3 – Nv killed 21 days following challenge. Extracted RNA reverse transcribed to produce cDNA. New cDNA samples were also produced using RNA from the corresponding gastric lymph nodes from each of these sheep (used previously in sections 3.3.1 and 3.3.2). Details of these samples are listed in Table 5.1.

These samples were examined for transcription of ATPase, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p_{40}, IL-13, IL-18, IFNγ, TGFβ_1 and TNFα using PCR as described in sections 2.3.3-2.3.10. The results of these assays are illustrated in Figure 5.1. All the cytokines examined were found to be transcribed in the efferent lymphocytes, with the exception of IL-12p_{40}, which was not detected in any of the efferent lymphocyte samples. Transcription of IL-6 and IL-18 also appeared to be generally lower in the efferent lymphocytes than in the gastric lymph node.

Table 5.1: Time-points relative to challenge used for comparison of efferent lymphocytes and gastric lymph node in naïve-challenged (group 3-Nv) and previously infected-challenged (group 2-PI) groups.

Group	n	Efferent lymphocytes	Gastric lymph node
2 – PI	3	Day 9	Day 10
3 – Nv	4	Day 21	Day 21

Figure 5.1(a): Cytokine transcription in efferent lymphocytes and gastric lymph node relative to ATPase in naïve-challenged sheep from group 3 – Nv on day 21, and in previously infected-challenged (PI-challenged) sheep from group 2 – PI on day 9.

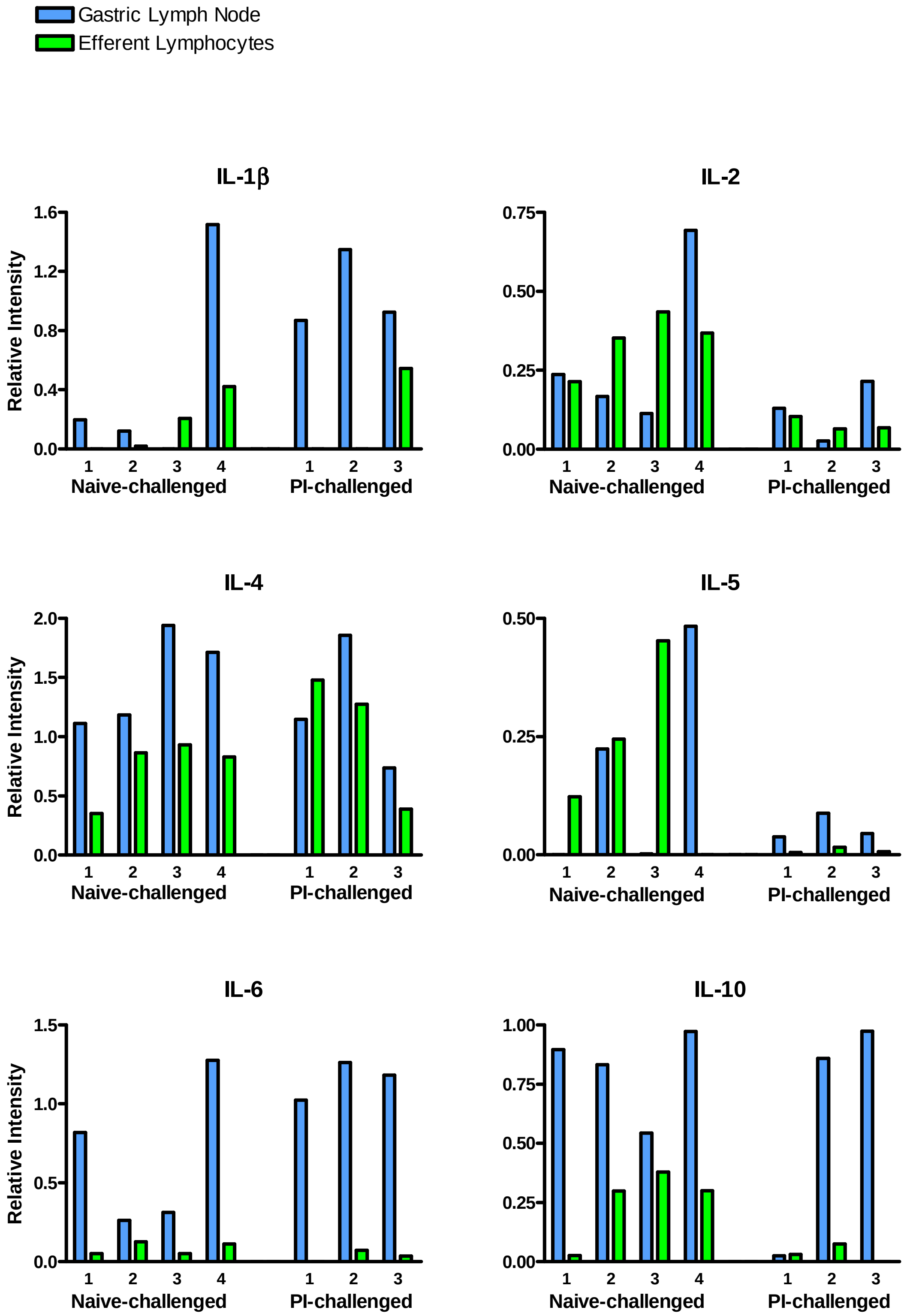
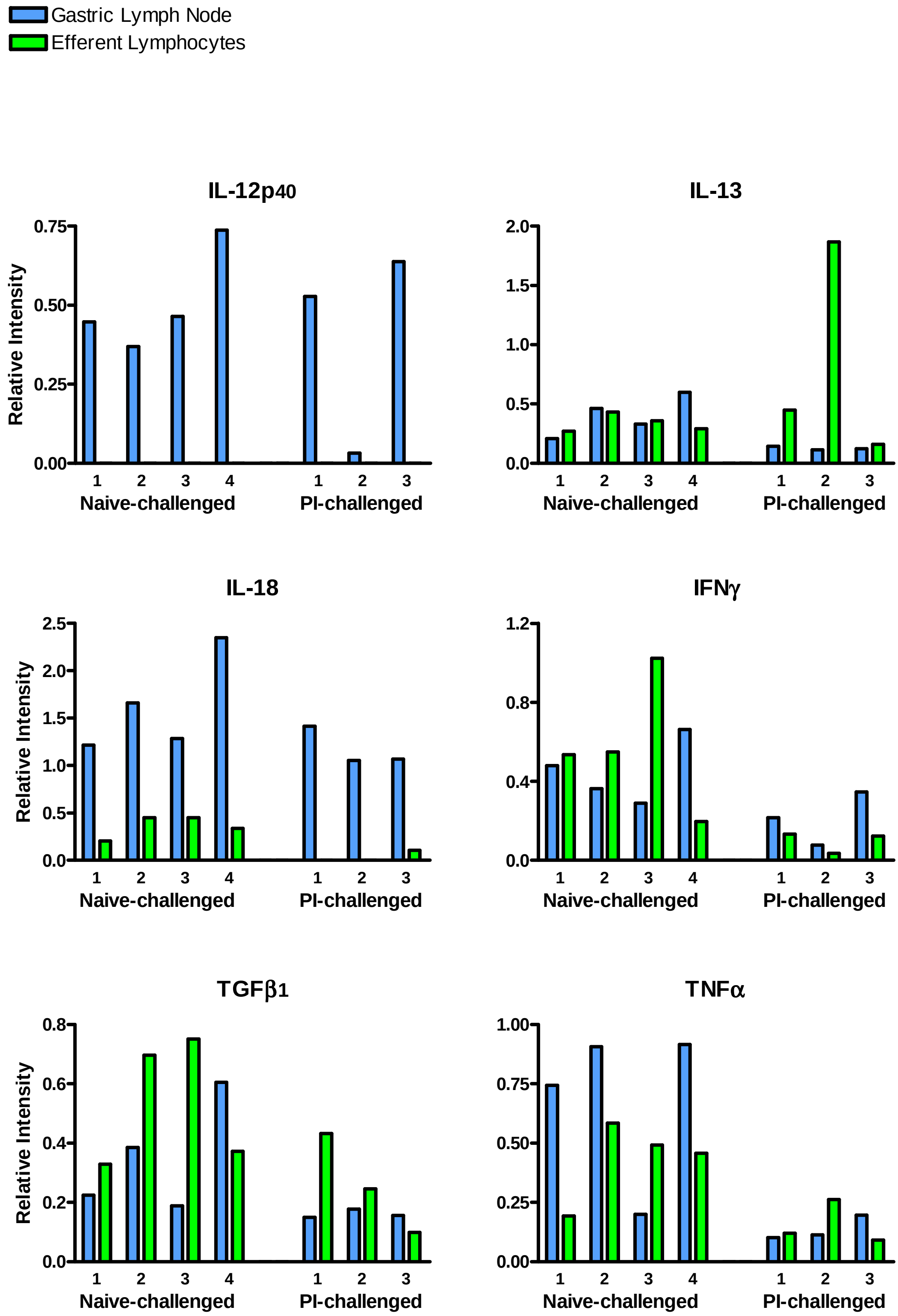


Figure 5.1(b): Cytokine transcription in efferent lymphocytes and gastric lymph node relative to ATPase in naïve-challenged sheep from group 3 – Nv on day 21, and in previously infected-challenged (PI-challenged) sheep from group 2 – PI on day 9.



5.3 Quantification of cytokine transcription in abomasal efferent lymphocytes by SYBR green Q-PCR

Due to the low levels of RNA extracted from efferent lymphocytes, Q-PCR was considered to be a more suitable technique than RT-PCR by which to assess cytokine transcription, as it is more sensitive, and so should be able to pick up more subtle changes over time, and can be completed using smaller amounts of starting RNA.

Extensive optimisation is required when using SYBR green Q-PCR, as the dye binds double stranded DNA in a non-specific manner. This may result in amplification of fluorescence due to the formation of primer-dimers, or of erroneous product due to non-specific primer annealing, which can result in overestimation of target expression.

Temperatures used during SYBR green Q-PCR were those identified as optimal for each primer pair previously using RT-PCR, as described in section 2.3.8. Because SYBR green mastermix includes 5mM $MgCl_2$, optimisation of Mg^{2+} concentrations was not attempted.

Initially Q-PCR using SYBR green was developed as described in section 2.3.12, tested using efferent lymphocyte RNA and the PCR product identity confirmed using agarose gel electrophoresis. However, problems were encountered due to poor reaction efficiency. Overall assay efficiency, as determined using a standard concentration gradient was within 95-105%. Ideally efficiency should be 98-102%, which produces a concentration gradient slope of -3.3, but 95-105% is acceptable. However, the efficiency within each reaction, as recorded by the Opticon Monitor 3.1.32 software, was unacceptably highly variable between triplicates (for example 97.8%, 57.5% and 110.3%) and a product concentration frequently could not be calculated. Attempts to improve efficiency were made by optimising primer concentrations individually, as the optimal concentration may not be the same for both forward and reverse primers. This was done by running forward and reverse primer concentrations ranging from 0.1μM to 0.8μM in a grid arrangement with the same cDNA sample. Primer optimisation failed to significantly stabilize efficiency between triplicates. This indicated that there was a problem with this type of assay run on the Opticon 2 DNA Engine Q-PCR machine, and it was concluded they were inappropriate for assessing cytokine transcription in efferent lymphocytes and that a more specific and reliable Q-PCR technique was required.

It is also noteworthy that on discussion with two researchers from different research groups, it became apparent that others were encountering similar problems when running SYBR green Q-PCR on this Opticon 2 DNA Engine. Each researcher had been using different Q-PCR assays and had found optimisation of individual primer pairs failed to significantly improve their results. This indicated that a basic problem with the equipment could also have been an issue. Due to staff relocation the Opticon 2 DNA Engine was removed from Easter Bush Veterinary Centre, so these problems were not further addressed in this study.

5.4 Quantification of cytokine transcription in abomasal efferent lymphocytes by Taqman Q-PCR

To more closely examine the influence of recirculating efferent lymphocytes, samples of these cells taken from four groups of sheep over the course of infection were examined using Taqman Q-PCR assays developed as described in section 2.3.13 and 4.3. Transcription of IL-4 and IL-13 was examined as markers of Th2-type responses, IFNγ as a marker of Th1-type, and IL-1β as a marker of proinflammatory-type cytokine responses.

Taqman Q-PCR was considered more suitable because it is more sensitive and more specific than both RT-PCR and SYBR green Q-PCR. In contrast to SYBR green, which fluoresces when bound non-specifically to double stranded DNA, Taqman probes are produced to bind specifically to defined DNA sequences, as described in section 2.3.13. The probes are constructed with a 6FAM™ fluorophore reporter at the 5’ end and a non-fluorescent quencher at the 3’ end. When the probe is intact the reporter and quencher are close enough together that the fluorescence emitted by the reporter when excited by light from the PCR machine is absorbed by the quencher. However, when the probe anneals to the DNA region being amplified, it is cleaved by the action of Taq polymerase. This releases the reporter and it moves away from the quencher, at which time the fluorescence emitted by the reporter is detected by sensors in the Q-PCR machine. The use of a minor groove binder (such as dihydrocyclopyrroloinole tripeptide) as the non-fluorescent quencher at the 3’ end of the Taqman probes also increased the melting temperature without increasing the probe length, by folding into the minor groove formed by the terminal 5-6 base pairs and forming a very stable hybrid with complementary cDNA, thus further reducing non-specific binding (Urban et al., 2007).

Because Taqman assays produced by Applied Biosystems are designed and optimised to work using standard Q-PCR conditions, the ATPase assay could be run on the same plate as the target cytokine for each sample. The ABI Prism 7500 real-time PCR thermocycler at the Moredun Research Institute, which was used for this section of work, also incorporates Applied Biosystems Sequence Detection Software version 1.4 which allows for normalisation and comparison between plates. This feature was important as the number of samples to be compared was more than would fit on one plate. Comparison between plates is not recommended when using other Q-PCR techniques due to inter-assay variation.

Due to limitations on sample availability, lymphocytes taken from sheep in groups VTRI-1 and VTRI-7 were used in addition to lymphocytes from sheep in groups 3 – Nv and 3 – PI. Sheep in group VTRI-1 were helminth naïve prior to infection using the same protocol as for other naïve-challenged groups, and were killed in September 2004. Sheep in groups VTRI-7 were trickle infected and challenged as for previous groups, and were killed in November 2006. These samples are detailed in Table 5.2. This produced a total of six naïve-challenged sheep from which lymphocyte samples were taken at intervals up until day 21, and six previously infected-challenged sheep from which lymphocyte samples were taken at intervals up until day 10.

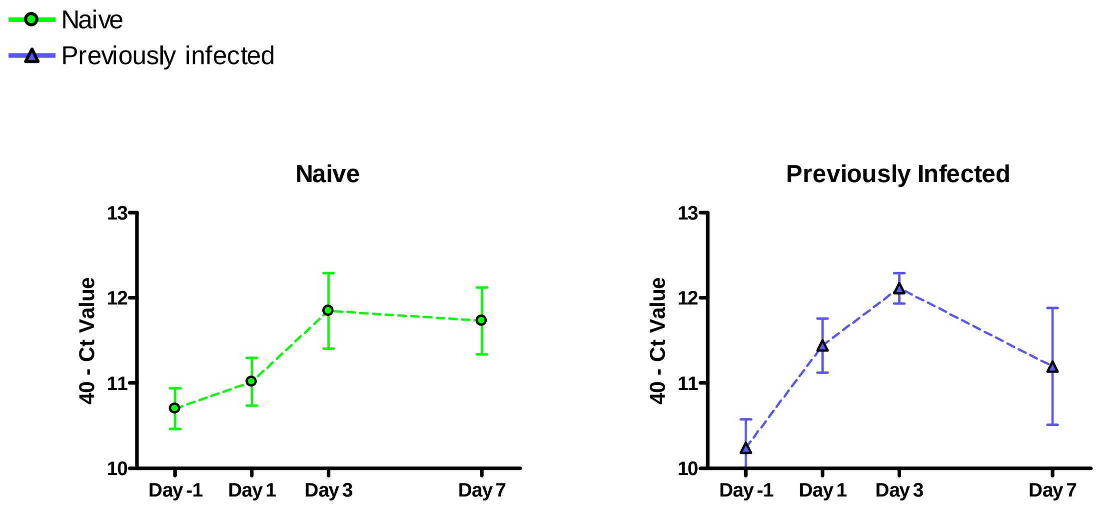
Results of these Taqman assays are illustrated in Figures 5.2 and 5.3. Statistical analysis of these data was performed by David Waddington of the Roslin Institute. This analysis was complicated by emergence of variation in the ATPase transcription, which was consistently evident during examination of the ATPase assays run concurrently with each of the four target cytokines.

Table 5.2: Efferent lymphocyte samples used for Taqman Q-PCR from naïve/-challenged (VTRI-1 Nv and 3-Nv) and previously infected-un/challenged (3-PI and VTRI-7 PI) groups at various time-points before and after challenge..

Group	n	Sample time-point (Days relative to challenge date)					
VTRI-1 Nv	3	-1	1	3	7	14	21
3 – Nv	3	-1	1	3	7	14	21
3 – PI	2	-1	1	3	5	7	9
VTRI-7 PI	4	-1	1	3	5	7	9

A basic analysis of all replicates in the naïve-challenged and previously infected-challenged groups found the mean Ct of ATPase to be higher in the naïve-challenged group, demonstrating an increased level of ATPase transcription in the previously infected-challenged group. Scatter-plot examination of ATPase transcription in naïve-challenged and previously infected-challenged sheep over time revealed trends over time which were consistent across the four replicated assays of the complete experiment (ATPase assays having been repeated on different plates corresponding to each cytokine). This trend is illustrated in Figure 5.2. When the ATPase assay Ct values of each individual sheep were plotted over time, some individuals were found to have consistently low or high transcription over the 4 replicates, indicating biological variation between sheep. This variation in ATPase transcription, which was not apparent when examining RT-PCR data, is likely to be due to changes in the overall level of transcriptional activity within the cells as would be expected during an active immune response.

Figure 5.2: Trends in ATPase transcription in efferent lymphocytes on days -1, 1, 3 and 7 relative to challenge, assessed using Taqman Q-PCR. Displayed as 40 – Ct value mean and SEM, n=4-6.



Variation in a housekeeping gene due to experimental treatment effects, in this case *T.circumcincta* infection, renders it unsuitable for normalising target genes, as it would introduce additional error and more importantly bias in estimates of gene transcription. However, none of the other potential housekeeping genes which have been examined previously have been more consistent (McNeilly, 2005). Pam Knight and colleagues have also examined transcription of other commonly used housekeeping genes, including β-actin and GAPDH, of which ATPase was the most consistent (unpublished observations). Recently, this group also examined three housekeeping genes, ATPase, and transmembrane proteins RW1 and TM57, which were found not to vary in these tissues when using microarray analysis. However, when transcription of these genes was examined over the course of infection using a sensitive multiplex RT-PCR all three were found to vary to some extent at some time-points, even when using a mean of all three (unpublished observations). The problem in this case is that of finding a gene which does not vary in transcription in lymphoid and mucosal tissues which are very active and contain a large proportion of proliferating cells at different time-points following infection.

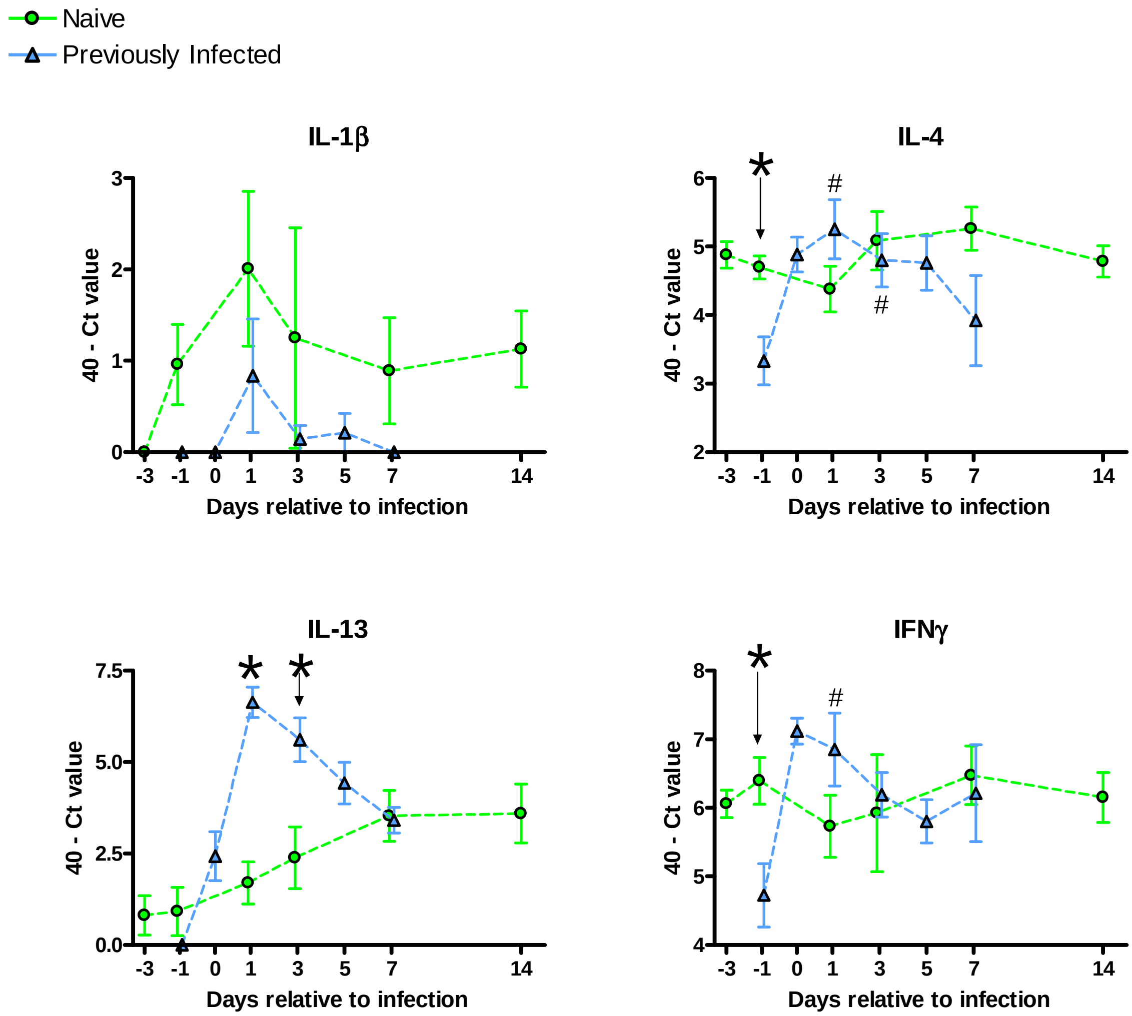
Housekeeping gene normalisation should correct for differences in cDNA concentration used in the Q-PCR assay, reducing the random variability between samples and giving increased precision for comparisons. As described above, when the housekeeping gene is affected by experimental treatments it becomes characteristic of an individual animal's response, producing bias in gene transcription estimates. In consideration of this, it was recommended that the ATPase transcription be analysed without housekeeping gene normalisation. This analysis relies on the cDNA concentration in the assay varying randomly, and thus only affecting comparisons between treatment groups by chance. As treatment group sample-size increases, this becomes increasingly unlikely. The sample-size of 6 animals per time-point used in this experiment should be sufficient for this problem not to arise. Cytokine Ct values were analysed on days -1, 1, 3 and 7, for which there was both naïve-challenged and previously infected-challenged sample data, using split-plot ANOVA to allow for the repeated time sampling of individual sheep, followed by t-tests between treatment groups. Cytokine Ct values are illustrated in Figure 5.3.

IL-4 and IFNγ were readily detectable in all the lymphocyte samples. Transcription of both IL-4 and IFNγ was significantly lower in the previously infected-unchallenged sheep than in the naïve on day -1 relative to challenge ($P<0.05$). This trend was reversed in direction between day -1 & day 1 ($P<0.05$), but there was not a significant difference between the two groups on day 1. Transcription of both cytokines was also significantly increased between day -1 and day 1 in the previously infected-un/challenged sheep (IL-4 $P<0.01$; IFNγ $P<0.05$).

Very low levels of IL-1β transcription were found, and this cytokine was only detectable in five naïve-challenged and two previously infected-challenged sheep. This may be due to a very small number of IL-1β-producing lymphocytes present in the efferent lymph. Where IL-1β transcription was detectable, it appeared to be higher in the naïve-challenged sheep, which is supportive of the previous findings demonstrating a greater degree of inflammation occurring in the abomasum of the naïve-challenged sheep. However, this difference was not significant when tested by a t-test.

IL-13 transcription was detectable in the majority of the efferent lymphocyte samples, and was significantly higher in the naïve-challenged sheep than in the previously-challenged infected on day 1 (P<0.001), and in the previously infected-challenged than in the naïve-challenged on day 3 (P<0.01).

Figure 5.3: *Cytokine transcription in efferent lymphocytes from naïve sheep on days -3, -1, 1, 3, 7 and 14 relative to infection, and in previously infected sheep on days -1, 0, 1, 3, 5 and 7. Assessed using Taqman Q-PCR, displayed as 40 – Ct value mean and SEM, n=4-6. Significant difference from day -1 (#) or between naïve and previously infected groups (*) using t-test following ANOVA.*



5.5 Cytokine transcription in abomasal efferent lymphocyte subsets

In order to assess which particular types of lymphocytes in the efferent lymph could be the source of some of the cytokine transcription described in section 5.4, lymphocyte subsets were extracted using fluorescence activated cell sorting and assessed separately using the IL-4 and IL-1β Taqman Q-PCR assays described in section 5.4.

Efferent lymph samples were used from four naïve/naïve-challenged and four previously infected-un/challenged sheep in group 3, as described in section 2.2.3 – 2.2.4. In both the naïve/naïve-challenged and the previously infected-un/challenged groups, lymph was collected from two sheep prior to challenge infection, from one sheep subsequent to infection, and from one sheep both before and after infection. Details of the efferent lymphocytes collected and sorted are listed in Table 5.3.

Efferent lymphocytes from these samples were labelled for the presence of surface antigens CD4 and CD8, and for γδ T-cell receptor, and sorted by FACS into positive and negative samples as described in section 2.4.2. CD8- and γδ-labeled efferent lymphocytes from the three previously infected-challenged sheep prior to challenge were not sorted for technical reasons, so only CD4 positive and negative cells were available from these sheep prior to challenge.

RNA was extracted from sorted efferent lymphocytes using an RNeasy® Micro Kit as described in section 2.3.4. This kit has been developed for extraction of RNA from low yield samples such as these using specialised MiniElute columns. The ATPase RT-PCR assay optimised in section 5.2 was used to check for successful reverse transcription of cDNA.

These cDNA samples were then examined for transcription of IL-4 and IL-1β using Taqman assays as described in section 5.4. Results of these Taqman assays are illustrated in Figures 5.4, 5.5 and 5.6.

As shown by Figure 5.4, IL-4 was detectable only in the CD4+ve lymphocyte samples. There also appeared to be a greater increase in the transcription of IL-4 by CD4+ lymphocytes in the naïve-challenged animals following challenge than in the previously infected-challenged group, as illustrated in Figure 5.5. However, due to the small sample size and the fact that the lymphocytes are only extracted from the same animal on one occasion, this can only be considered as an indication and is not sufficient to draw conclusions.

IL-1β was only detectable in various lymphocyte subsets from four of the eight sheep, the results of which are illustrated in Figure 5.6. Of these four sheep, three were sampled prior to challenge, and very low levels of IL-1β transcription were observed in the samples from the remaining post-challenge sheep.

Table 5.3: Labelled lymphocytes sorted by FACS into positive and negative samples of CD4, CD8 and γδ cells.

n	Group	Sorted efferent lymphocytes		
	Pre-challenge	CD4	CD8	γδ
3	3 – Nv	+	+	+
3	3 – PI	+	-	-
	Post-challenge			
2	3 – Nv	+	+	+
2	3 – PI	+	+	+

Figure 5.4: IL-4 transcription in efferent lymphocyte subsets from naïve and previously infected sheep pre- and post-challenge. Assessed using Taqman Q-PCR, displayed as 40 – Ct value mean and SEM, n=3-6.

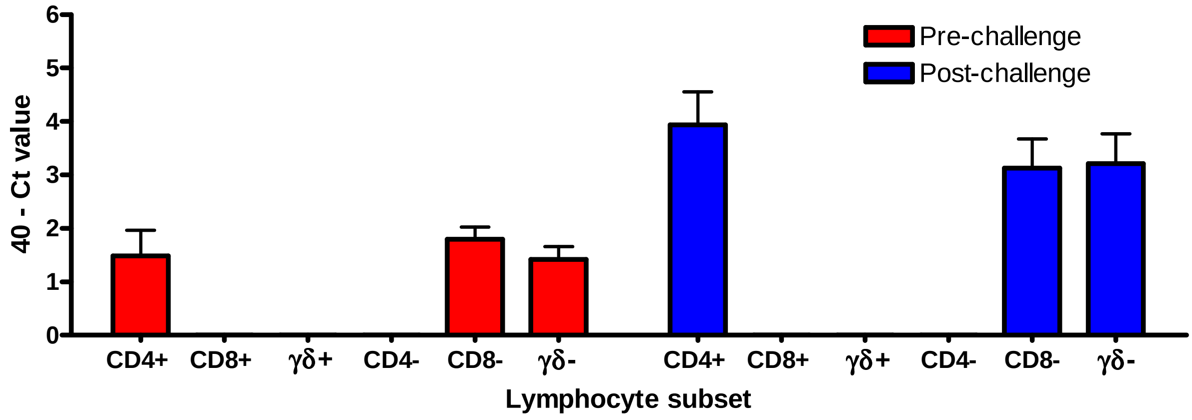


Figure 5.5: IL-4 transcription in CD4+ve efferent lymphocytes from naïve, naïve-challenged (Nv-chall), previously infected-unchallenged (PI-unchall) and previously infected-challenged (PI-chall) sheep. Assessed using Taqman Q-PCR, transcription displayed on the Y-axis as 40 – Ct value, sheep group and identification number is displayed on the X-axis .

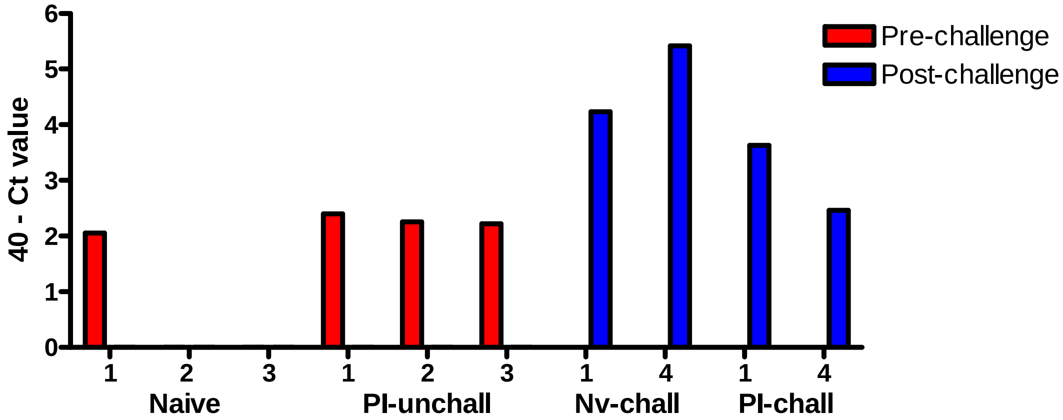
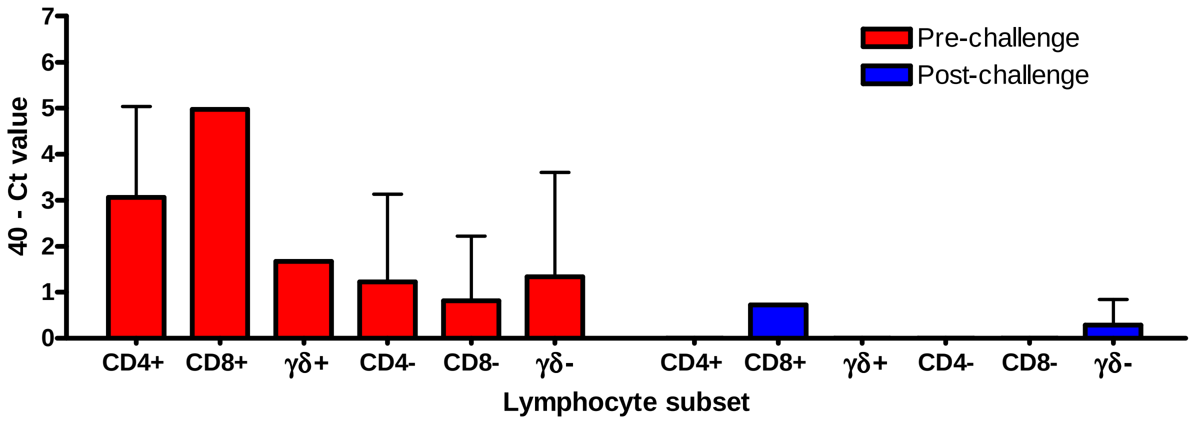


Figure 5.6: IL-1β transcription in efferent lymphocyte subsets from naïve/naïve-challenged and previously infected-un/challenged sheep pre- and post-challenge. Assessed using Taqman Q-PCR, displayed as 40 – Ct value mean and SEM, n=3-6.



5.6 Cytokine detection in efferent lymph using ELISA

A commercial ELISA for the detection of bovine IFNγ has been successfully used at the Moredun Research Institute to detect ovine IFNγ in blood serum. Samples of efferent lymph were examined by Sean Wattegedera for detection of IFNγ using this commercial bovine IFNγ ELISA. Efferent lymph was also examined for detection of IL-4 using the ELISA protocol described in section 2.4.1. However, IL-4 and IFNγ were not detectable in ovine efferent lymph.

5.7 Discussion

The lack of IL-12p$_{40}$ transcription observed in the efferent lymphocyte samples examined is interesting. Though the predominant sources of this cytokine are monocytes, macrophages and dendritic cells, which would not be expected in efferent lymph, this cytokine is also produced by B cells in cattle (Schramm et al., 2007). B cells have been found in the efferent lymph of these sheep during infection with *T.circumcincta* (Halliday et al., 2009b). It is possible that production of IL-12p_{40} by B cell migrating in response to *T.circumcincta* is being suppressed by TGFβ, which has been found to suppress IL-12p_{40} transcription in murine macrophages (Du and Sriram, 1998) and human lymphocytes (Pardoux et al., 1997), and was strongly transcribed in efferent lymphocytes. It is also possible that IL-12p_{40}, if it is produced by ovine B cells migrating in response to *T.circumcincta*, is only produced earlier in infection (Trinchieri, 1995). This lack of IL-12p_{40}, in combination with the observed transcription of IL-4, IL-5 and IL-13 in the efferent lymphocytes, is supportive of a Th2 response being orchestrated in the draining lymph node in naïve-challenged and previously infected-challenged sheep. This Th2 response, triggered by lymphocytes originating in the gastric mucosa and amplified in the gastric lymph node, may then be circulated via efferent lymphatics back to the gastric mucosa (Hein and Mackay, 1991).

Interestingly, transcription of both IL-4 and IFNγ was significantly lower in the previously infected-unchallenged sheep than in the naïve on day -1 relative to challenge. The lower levels of these cytokines detected in the previously infected-unchallenged group may be attributed to regulation of the immune response developed during trickle infection, which may also be suppressing inflammation due to cannulation surgery. The naïve sheep, which would not have previously developed a regulatory immune response, may have comparatively higher levels of IL-4 and IFNγ as a response to surgery and damage to the lymph duct. Both IL-4 and IFN have been implicated in wound healing in mice (Salmon-Ehr et al., 2000, Schaffer et al., 2006).

The observed increase in both IL-4 and IFNγ transcription in the efferent lymphocytes from one day before challenge to one day after in the previously infected-challenged sheep is also interesting. A rapid increase in IL-4 transcription would be expected as part of the early Th2 response; however the observed increase in IFNγ is less easily explained. This increase is in contrast to the reduction in IFNγ transcription found in ovine afferent mesenteric lymphocytes following infection with *Trichostrongylus colubriformis* by Hein and colleagues (Hein et al., 2004). It is possible that the increase in IFNγ is part of an initial Th1 response to bacteria and viruses introduced to the gastric crypts by migration of exsheathed *T.circumcincta* L3 (Pesce et al., 2008). The effect of *T.circumcincta* as a vector could be more pronounced in the abomasal mucosa than in the intestine, as the gastric crypts are normally protected by the abomasal mucus layer, and the environment in the abomasum is normally bacteriostatic in contrast to the gut flora present in the intestine. However, if this was the case, an increase in IFNγ transcription would be expected in either the abomasal mucosa or the gastric lymph node at day 2 following challenge, but no such increase was found. It is therefore likely that IFNγ is involved as part of a negative feedback mechanism, controlling the effects of IL-4 and the initial Th2 response generally in the lymph or possibly through recirculation of these lymphocytes back to the site of infection.

The earlier and more pronounced increase in IL-13 transcription found in the efferent lymphocytes of the previously infected-challenged sheep in comparison to the naïve-challenged group supports the hypothesis that a population of Th2 lymphocytes is built up during the course of the trickle infection. These results mirror the change in IL-13 transcription found in the gastric lymph node two days following challenge in the previously infected-challenged sheep.

The observed increases in both IL-4 and IL-13 following infection is consistent with the findings of Hein (2004) and colleagues demonstrating increased transcription of these cytokines in pseudoafferent intestinal lymphocytes subsequent to infection with *Trichostrongylus colubriformis*.

Due to the small sample size available, it was not possible using this data to draw firm conclusions regarding the cytokines produced by CD4+, CD8+ and γδ+ lymphocytes in the efferent lymph. There was, however, a clear indication that IL-4 is only produced by CD4+ lymphocytes, and to a lesser extent that this transcription increased following challenge. This possible up-regulation of IL-4 in CD4+ lymphocytes was not reflected in the efferent lymphocytes as a whole, wherein IL-4 was not notably increased from day -1 on either day 14 in the naïve-challenged sheep or day 7 in the previously infected-challenged group. This may be because the CD4+ lymphocytes were transcribing significantly more IL-4, but this did not make a noticeable impact on the proportion of IL-4 RNA in the efferent lymphocyte total RNA due to a general increase in RNA transcription, or because the trend implied in these findings was an artefact of the small sample size.

The appearance of a greater increase in the transcription of IL-4 by CD4+ lymphocytes found in the naïve-challenged animals following challenge than in the previously infected-challenged group would be consistent with the Th2 polarisation of the immune response in naïve-challenged sheep, which would require large amounts of IL-4 transcription; in contrast to the previously infected-challenged animals in which a large population of Th2 lymphocytes is already in place.

Transcription of IL-1β by CD4+ lymphocytes found in three of the efferent lymphocyte samples collected prior to challenge indicates a proinflammatory component to the immune response was taking place in the previously infected-unchallenged and naïve sheep, possibly in response to the cannulation surgery. However, the very low levels of IL-1β detected in the sorted lymphocytes after challenge reflects the findings from un-sorted efferent lymphocytes in section 5.4, and may indicate that these proinflammatory responses are being suppressed at later stages of infection.

Detailed monitoring of CD4+, CD8+ and γδ+ lymphocyte subsets in efferent lymph from the sheep used in these trials over the course of infection has been carried out by Aileen Halliday and colleagues at the Moredun Research Institute. This examination found that CD4+ and CD8+ lymphocyte output peaked earlier in the previously infected-challenged animals, 2-3 days following challenge, than in the naïve-challenged group which peaked at day 10 (Halliday et al., 2009b). The lymphocyte output at day 2-3 in the previously infected-challenged animals also consisted mostly of CD4+ lymphocytes, in contrast to the peak output of the naïve-challenged sheep at day 10, which was a more mixed population of CD4+ and CD8+ cells. This trend in CD4+ cells population is likely to be responsible for the observed peak in IL-4 transcription in the unsorted efferent lymphocytes from previously infected-challenged sheep at day 1, and in the naïve-challenged animals at day 7.

The early peak in CD4+ lymphocytes observed in the efferent lymph of previously infected-challenged sheep may also be an indication of a more widespread increase in CD4+ lymphocytes; experimental immunization of mice with alum-precipitated OVA demonstrated that IL-4-producing CD4+ T cells developed in the draining lymph node, and a proportion of these became recirculating cells with a central memory phenotype, migrating to distant lymph nodes and more rapidly interacting with B cell follicles than naïve T cells, while still capable of producing IL-4 (Serre et al., 2009). A similar series of events, wherein IL-4-producing memory CD4+ T cells develop in the draining lymph node with some recirculating to the mucosa, may be responsible for the early increase in IL-4 transcription observed in the abomasal mucosa and gastric lymph node at day 2 in the previously infected-challenged sheep. As both IL-13 and IFNγ also showed similar patterns of transcription in the efferent lymphocytes, it could be implied that these cytokines were also being produced by CD4+ lymphocytes. The implication from these data is that CD4+ lymphocytes are important in anti-parasite responses in immune sheep, as these cells produce IL-4 and possibly IL-13, and peak at day 2 in previously infected-challenged sheep, at which time these sheep are demonstrating anti-parasite responses. Neither the early peak in CD4+ lymphocytes nor the early anti-parasite responses have been found in naïve-challenged sheep after challenge.

6 Chapter Six: Cytokine Expression in 5 Month Old Lambs

6.1 Introduction

As discussed in section 1.1, infection with *T.circumcincta* is having an increasingly significant economic impact for sheep farmers due to the spreading resistance of this parasite to all the classes of anthelmintic drugs currently available. *T.circumcincta* infection is especially detrimental to younger lambs, in which it can cause pronounced morbidity and severe production losses.

It appears that the enhanced morbidity due to helminth infection in lambs under 6 months of age is due to an inability to generate significant immunity to gastrointestinal parasites in early life. This has been demonstrated by a number of studies in which lambs less than 6 months of age failed to develop immunity following exposure to helminths including *H.contortus* and *T.colubriformis,* whereas the same exposure resulted in protective immune responses in older sheep (Manton et al., 1962, Ritchie et al., 1966, Gregg et al., 1978, Dobson et al., 1990, Good et al., 2006).

Paradoxically, while apparently deficient in generating immune responses to helminth infection, young lambs have been shown to produce immune responses to vaccines containing *H.contortus* intestinal antigen which resulted in a significant reduction in pasture contamination with *H.contortus* larvae (LeJambre et al., 2008) and develop protective immunity in response to vaccines for a variety of bacterial diseases. Recruitment of eosinophils and mast cells in response to *H.contortus* challenge has also been observed in 3-month-old lambs, and this occurred earlier in lambs which had been previously exposed than in naïve-challenged animals (Lacroux et al., 2006). Not only were there significantly higher numbers of eosinophils and mast cells in the abomasal mucosa of previously infected lambs compared to the naïve-challenged 3 and 7 days following challenge, but numbers of these cells increased earlier in the previously infected-challenged group.

Previous studies of the ovine immune response to *T.circumcincta* have demonstrated that lambs at 4.5 months of age were capable of generating resistance to infection, as shown by increased larval stunting and arrest following later challenge. However, this resistance was measurably less than that generated using the same infection protocol in 10-month-old sheep, in which almost all larvae were arrested early in development (Smith et al., 1983, Smith et al., 1984, Smith et al., 1985).

In order to further examine the differences in immune responses between young lambs and yearling sheep challenged with *T.circumcincta*, the study described in section 2.2.1 (samples from which were used during the work described in chapters 3-5) was repeated using 4- to 5-month-old lambs as described in section 2.2.2. This provided an opportunity to evaluate how the orchestration of the immune response in younger lambs differs from that of older animals, using resources which were not available during the studies described in previous chapters. This may lead to clarification of the reasons why susceptibility to *T.circumcincta* infection appears to be age-related and is enhanced in young lambs which are capable of generating protective immune responses to vaccines. The experiment was also repeated using a different breed of lambs to assess whether there were any notable differences in the cytokine responses between breeds. This knowledge may be useful in the development of an effective *T.circumcincta* vaccine for young lambs and in the development of helminth resistant breeds of sheep.

The objectives of this section of work were:

(1) To examine whether the cytokines previously examined in the abomasum and gastric lymph node of yearling sheep are transcribed in these tissues in 4- to 5-month-old lambs.

(2) To define how the response to infection with *T.circumcincta* in naïve-challenged and previously infected-challenged 4- to 5-month-old lambs compares with that of yearling sheep.

(3) To examine whether previous exposure to *T.circumcincta* produced detectable resistance to infection compared to naïve-challenged lambs, and compare the cytokine responses of naïve-challenged and previously infected-challenged sheep.

(4) To compare the cytokine responses of naïve-challenged and previously infected-challenged Blackface × Leicester lambs with those of Dorset × Suffolk lambs.

6.2 Cytokine responses in 5-month-old Blackface × Leicester lambs

As described in section 2.2.2 and Table 2.2, the previous study using yearling sheep in experimental groups 3 and 4 was repeated using thirty-five Blackface × Leicester 5-month-old lambs in experimental group 5, as illustrated in Table 6.1. As in the previous study, RT-PCR assays were used to examine cytokine responses in the abomasal mucosa and gastric lymph node at different time-points over the course of infection. Unfortunately limitations on the number of animals available in this group resulted in variable numbers of lambs killed at each time-point, and no previously infected lambs were killed prior to challenge.

As was found in the older animals, transcription of all twelve cytokines examined was detectable in both the abomasal mucosa and the gastric lymph node. These results are illustrated in Figure 6.1 and 6.2. Statistical analysis was carried out as before, using an ANOVA residual plot to check for normality. Transcription IL-5, IL-6, IL-10, IL-12p_{40}, IL-13 and IL-18 in the gastric lymph node, and IL-6, IL-10, IL-13, IFNγ, TNFα and TGFβ_1 in the abomasal mucosa, was found not to be normally distributed. Non-parametric tests were therefore considered appropriate for data analysis. Cytokines with normally distributed transcription were also analysed using non-parametric test so that the statistical analysis was consistent across all the cytokines. Kruskal-Wallis test indicated a significant difference between groups in the case of IL-18 (P=0.002, $H=20.76_6$) in the gastric lymph node and IL-12p_{40}

(P=0.003, $H=19.78_6$) in the abomasal mucosa. Mann-Whitney U test was also used to make specific comparisons between groups or time-points, with the addition of Dunn’s test as described in section 2.6. The smaller number of lambs in some of the groups, where n<6, reduces the statistical power of this experiment and increases the impact of biological variation between individuals.

Table 6.1: Experimental design – Blackface × Leicester 5-month-old lambs.

Nv = Naïve; PI = Previously Infected; Day 0 = unchallenged.

Experimental group	Kill date	Trickle infection[a]	Challenge infection[b]	Kill day following challenge[c]			
				0	5	10	22
5 – Nv	Aug 2006	-	+	4	6	5	4
5 – PI	Aug 2006	+	+	-	6	5	5

[a] 2000 *T.circumcincta* L3 larvae administered 5 times per week for 8 weeks.

[b] One dose of 50,000 *T.circumcincta* L3, given 7 days after Levamisole treatment.

[c] Quoted figure is the number of sheep in the group killed on the corresponding day following challenge.

In the gastric lymph node limited significant changes were found over the course of infection. Some proinflammatory activity was indicated by increased transcription of IL-18 in the naïve-challenged lambs on days 10 and 22 compared to the naïve group, which was significant using Mann-Whitney U test (P<0.05); however only the increase on day 22 was significant using Dunn’s test (P<0.01). Transcription of IL-18 was also higher in the naïve-challenged than in the previously infected-challenged group on day 22 (P<0.05). No changes were found in transcription of IL-1β, IL-6 or TNFα.

An increase in transcription of IL-4 was found in the naïve-challenged groups on days 5 and 10 when compared to the naïve group according to Mann-Whitney U test (P<0.05); however these increases were not significant according to Dunn’s test. IL-5 transcription was also found to be significantly up-regulated in the naïve-challenged group on days 5 and 22 according to Mann-Whitney U test ($P<0.05$) when compared with the naïve group. The increase in IL-5 transcription on day 5 compared to the naïve group was confirmed using Dunn's test ($P<0.05$). No significant differences in transcription of either IL-4 or IL-5 were found in the lymph node between the naïve-challenged and previously infected-challenged groups on the same day. No significant changes were found in the transcription of IL-13 in the gastric lymph node.

No significant change was found in the transcription of the Th1 cytokines IL-2, IL-12p_{40} or IFNγ in the lambs when compared to the naïve group or between the naïve-challenged and previously infected-challenged groups at the same time-point.

Transcription of the regulatory cytokine IL-10 was significantly increased in the gastric lymph node of the naïve-challenged group on day 22 compared the naïve group ($P<0.05$), and at this time-point was also significantly higher in the naïve-challenged group than the previously infected-challenged ($P<0.05$). However, this increase compared to the naïve group was not confirmed using Dunn's test. No significant changes in the transcription of TGFβ_1 were found in the gastric lymph node.

Transcription of IL-4 was significantly increased in the abomasal mucosa of the naïve-challenged lambs on day 5 when compared to naïve group using Mann-Whitney U test ($P<0.05$), however this was not confirmed using Dunn's test. No significant differences were found between IL-4 transcription in the naïve-challenged and previously infected-challenged lambs at the same time-point. No change in the transcription of IL-5 was found when comparing naïve and naïve-challenged groups; but transcription of this cytokine was significantly higher on day 5 in the previously infected-challenged lambs than in the naïve-challenged ($P<0.05$). Significant up-regulation of IL-13 was found in the naïve-challenged lambs on day 22 compared to naïve group using Mann-Whitney test ($P<0.05$), however this was not confirmed using Dunn's test. Transcription of IL-13 was also significantly higher in the previously infected-challenged lambs than in the naïve-challenged group on day 10 ($P<0.05$).

IL-1β transcription was increased in the naïve-challenged group on days 5 and 10 when compared to the naïve group according to Dunn's test ($P<0.05$). Transcription of IL-6 was also increased in the naïve-challenged groups on days 5 and 10 compared to the naïve group according to Mann-Whitney U test ($P<0.05$), however this was only confirmed using Dunn's test on day 10 ($P<0.01$). IL-6 transcription was significantly higher in the naïve-challenged lambs on day 10 compared to the previously infected-challenged group ($P<0.05$), however no difference in transcription of IL-1β was found between the naïve-challenged and previously infected-challenged groups. IL-18 transcription in the naïve-challenged group was significantly higher than in the previously infected-challenged on day 22 ($P<0.05$), but did not differ from the naïve group following challenge. No significant differences were found in the transcription levels of TNFα in the abomasal mucosa.

No significant change in transcription of IL-2, IL-12p_{40} or IFNγ was found in the abomasal mucosa following challenge. Transcription of IL-12p_{40} was found to be significantly higher in the previously infected-challenged than in the naïve-challenged lambs on days 5 ($P<0.01$) and 22 ($P<0.05$).

No significant changes in IL-10 transcription was found in the abomasal mucosa, except for an increase on day 10 in the naïve-challenged group compared to the naïve group according to Mann-Whitney U test ($P<0.05$), however this was not confirmed using Dunn's test. No significant changes in transcription of TGFβ_1 were found in the abomasal mucosa following challenge.

Figure 6.1(a): Cytokine transcription in the gastric lymph node of 5-month-old Blackface × Leicester lambs in experimental group 5 relative to ATPase. Analysed using Mann-Whitney U-test for non-parametric data with a 95% confidence interval, n = 4-6 sheep per group for each time point; significant difference between naïve-challenged and previously infected-challenged groups on the same day: ★ *(P<0.05); significant difference between naïve and naïve-challenged groups: # (P<0.05). Note different ranges on Y-axes.*

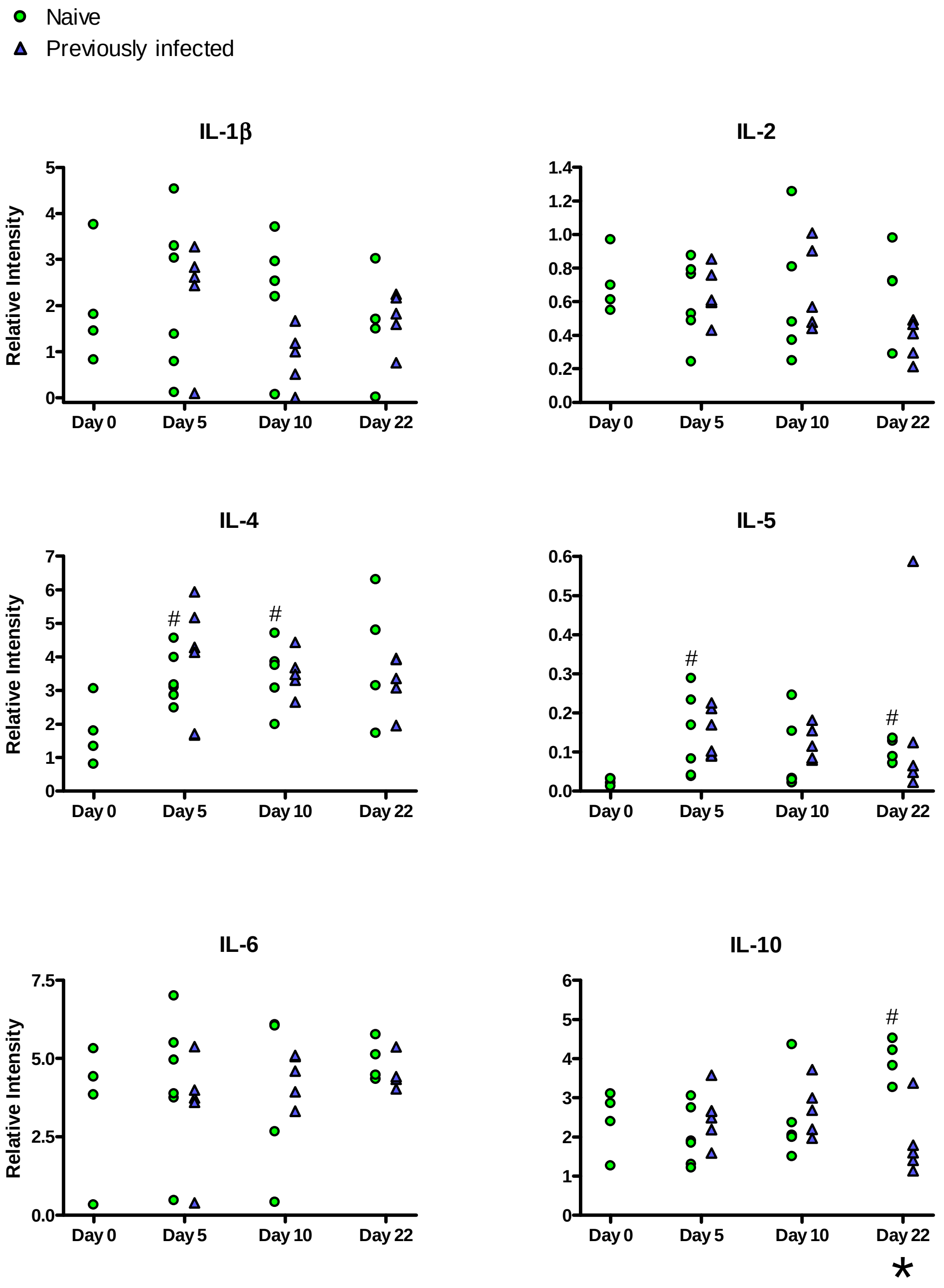


Figure 6.1(b): Cytokine transcription in the gastric lymph node of 5-month-old Blackface × Leicester lambs in experimental group 5 relative to ATPase. Analysed using Mann-Whitney U-test for non-parametric data with a 95% confidence interval, n = 4-6 sheep per group for each time point; significant difference between naïve-challenged and previously infected-challenged groups on the same day: ✱ *(P<0.05); significant difference between naïve and naïve-challenged groups: # (P<0.05). Note different ranges on Y-axes.*

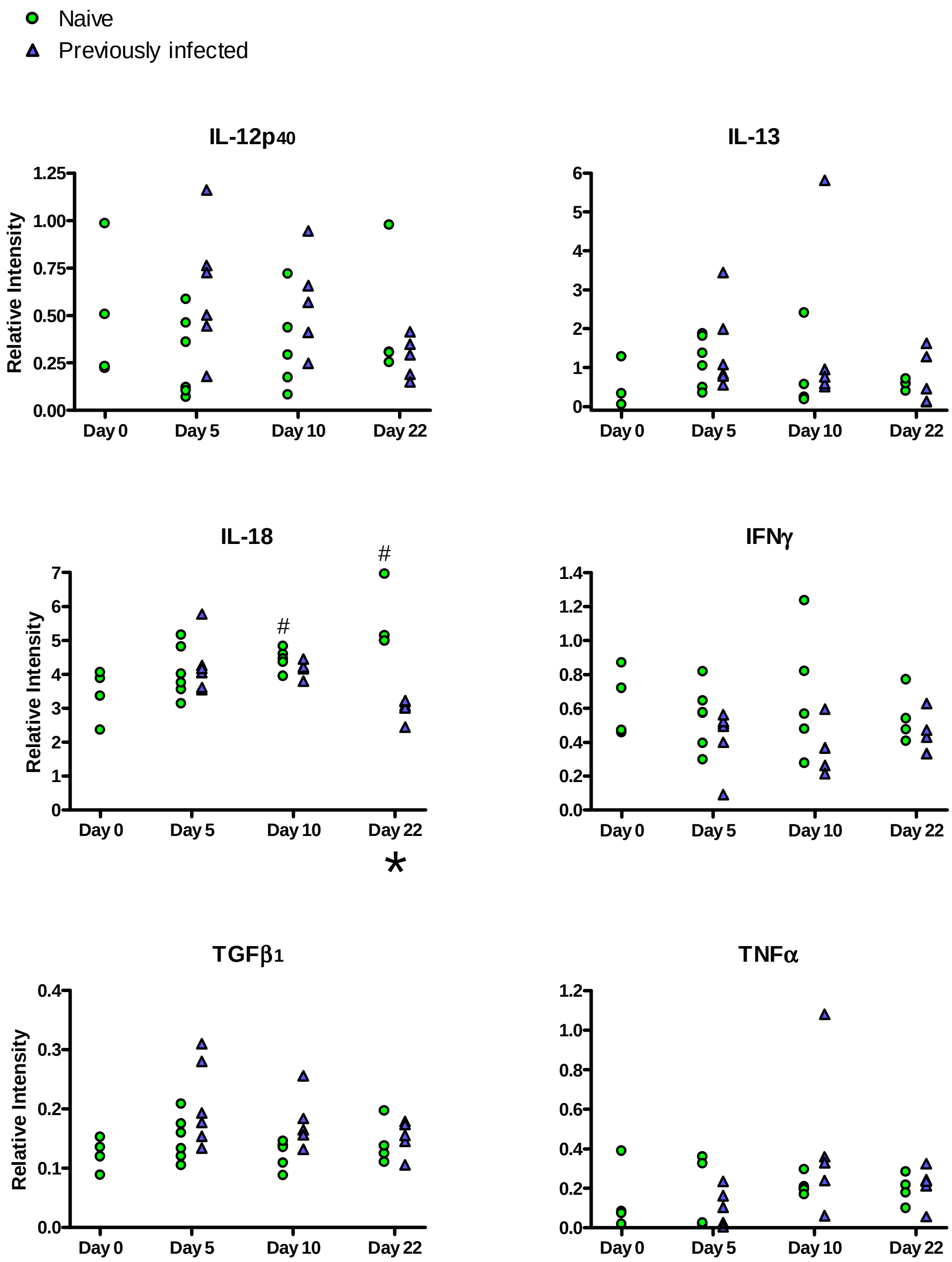


*Figure 6.2(a): Cytokine transcription in the abomasal mucosa of 5-month-old Blackface × Leicester lambs in experimental group 5 relative to ATPase. Analysed using Mann-Whitney U-test for non-parametric data with a 95% confidence interval, n = 4-6 sheep per group for each time point; significant difference between naïve-challenged and previously infected-challenged groups on the same day: * (P<0.05); significant difference between naïve and naïve-challenged groups: # (P<0.05). Note different ranges on Y-axes.*

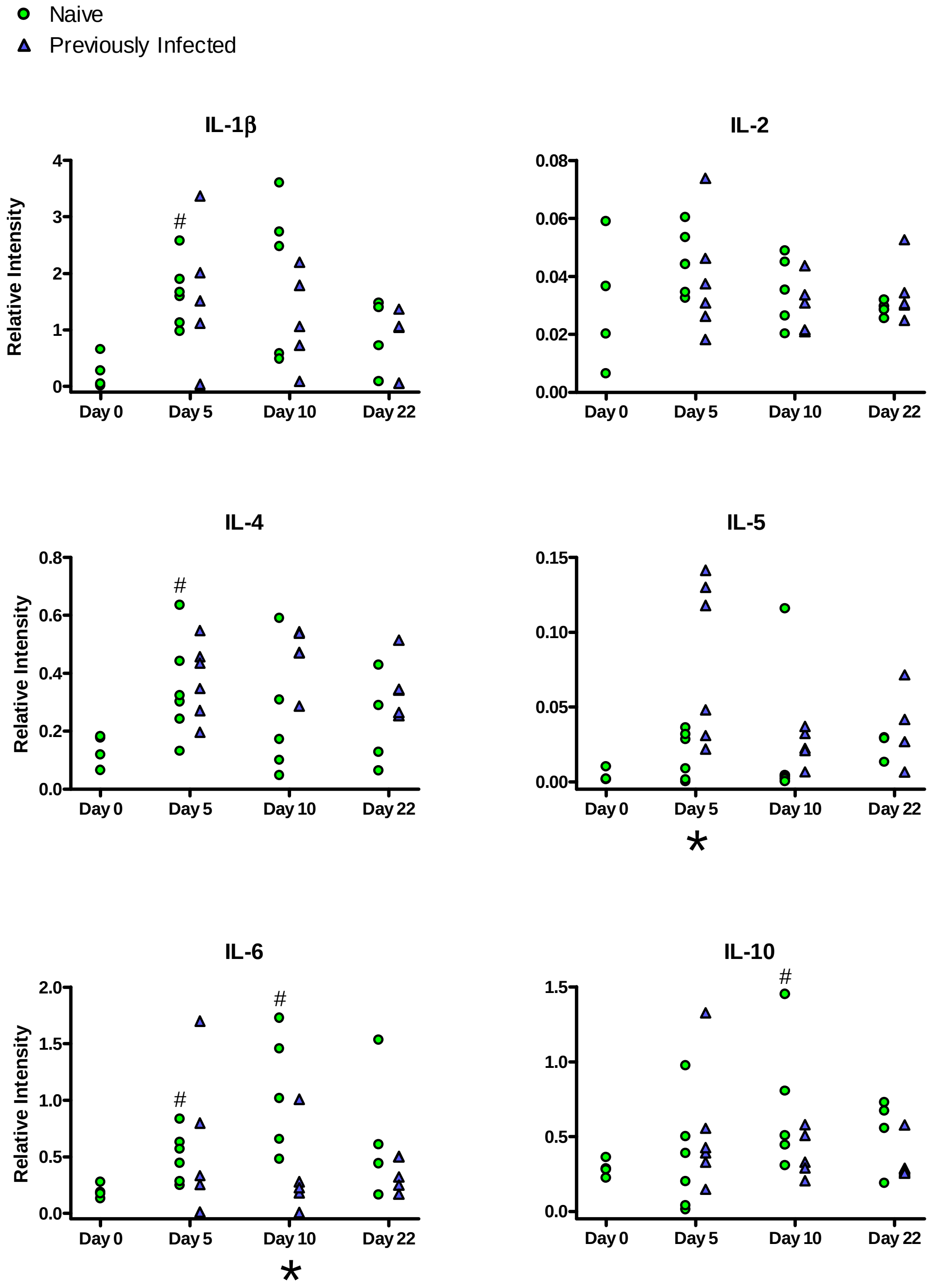
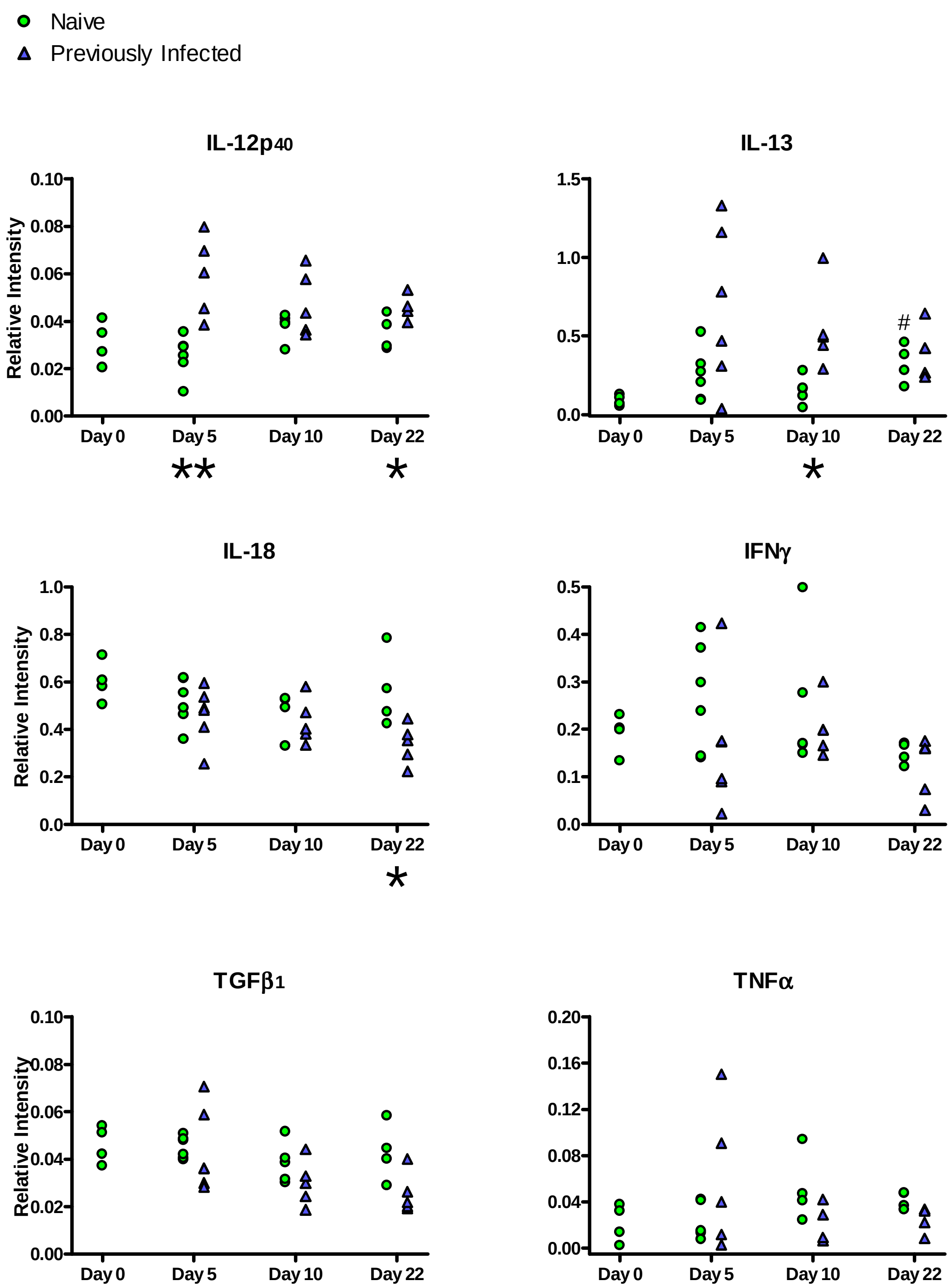


Figure 6.2(b): Cytokine transcription in the abomasal mucosa of 5-month-old Blackface × Leicester lambs in experimental group 5 relative to ATPase. Analysed using Mann-Whitney U-test for non-parametric data with a 95% confidence interval, n = 4-6 sheep per group for each time point; significant difference between naïve-challenged and previously infected-challenged groups on the same day: ★ (P<0.05), ★★ (P<0.01); significant difference between naïve and naïve-challenged groups: # (P<0.05). Note different ranges on Y-axes.



6.3 Cytokine responses in 5-month-old Dorset × Suffolk lambs

The anti-parasite responses observed in the lambs used in section 6.2 were similar to those of the yearling sheep (Halliday et al, paper in preparation), so this experiment was repeated to confirm that this finding was not due to breed differences between the experiments. For this experiment a larger number of Dorset × Suffolk 5-month-old lambs from experimental group 6 were used, as described in section 2.2.2, Table 2.2 and Table 6.2. As previously, RT-PCR assays were again used to examine cytokine responses in the abomasal mucosa and gastric lymph node at different time-points over the course of infection. More lambs were available in this group than in the previous experiment, allowing four naïve and four previously infected-unchallenged animals to be included. This was important as it allowed baseline levels following trickle infection and prior to challenge to be assessed. The larger number of animals also allowed there to be six individuals in each group of challenged lambs killed on days 5, 10 or 21.

Table 6.2: Experimental design – Dorset × Suffolk 5-month-old lambs.

Nv = Naïve; PI = Previously Infected; Day 0 = unchallenged.

Experimental group	Kill date	Trickle infection[a]	Challenge infection[b]	Kill day following challenge[c]			
				0	5	10	21
6 – Nv	June 2007	-	+	4	6	6	6
6 – PI	June 2007	+	+	4	6	6	6

[a] 2000 *T.circumcincta* L3 larvae administered 5 times per week for 8 weeks.

[b] One dose of 50,000 *T.circumcincta* L3, given 7 days after Levamisole treatment.

[c] Quoted figure is the number of sheep in the group killed on the corresponding day following challenge.

As was found previously, transcription of all twelve cytokines was detectable in both the abomasal mucosa and the gastric lymph node. These results are illustrated in Figure 6.3 and 6.4. Statistical analysis was carried out as before, using an ANOVA residual plot to check for normality. Transcription of all the cytokines was found not to be normally distributed, with the exception of IL-12p_{40} in the gastric lymph node. Non-parametric tests were therefore considered appropriate for data analysis in all cases, so that the statistical analysis was consistent across all the cytokines in both tissues. Kruskal-Wallis test indicated a significant difference between groups in the case of IL-4 ($P=0.003$, $H=21.77_7$), IL-5 ($P=0.001$, $H=25.59_7$) and IL-13 ($P=0.002$, $H=23.30_7$) in the gastric lymph node and IL-1β ($P=0.009$, $H=18.67_7$), IL-4 ($P<0.001$, $H=27.11_7$), IL-5 ($P<0.001$, $H=32.35_7$), IL-6 ($P=0.003$, $H=21.61_7$), IL-13 ($P<0.001$, $H=29.04_7$), IL-18 ($P=0.004$, $H=21.13_7$) and TGFβ1 ($P=0.006$, $H=19.89_7$) in the abomasal mucosa. Mann-Whitney U test was used to compare naïve-challenged and previously infected-challenged groups at the same time-point and to compare each group to the corresponding unchallenged group. Dunn's test was then also used to compare each group to the corresponding unchallenged group to check for type I error.

No change in IL-18 transcription was found in the gastric lymph node of the Dorset × Suffolk lambs over the course of infection; it was, however, significantly higher in the naïve-challenged lambs than in the previously infected-challenged group on day 21 ($P<0.05$). No significant changes were found in either transcription of IL-1β or TNFα, though the level of IL-1β transcription was significantly higher in the naïve-challenged lambs than the previously infected-challenged group on day 22 ($P<0.01$). Transcription of IL-6 was significantly increased in the previously infected-challenged lambs compared to the previously infected-unchallenged group on day 10 using both Mann-Whitney U test and Dunn's test ($P<0.05$).

Increased transcription of IL-4 was found in the gastric lymph nodes of both the naïve-challenged and previously infected-challenged Dorset × Suffolk lambs on day 10 according to Mann-Whitney U test ($P<0.05$), and in the naïve-challenged lambs on days 10 ($P<0.001$) and 21 ($P<0.01$) according to Dunn's test, when compared with the respective unchallenged groups. IL-4 transcription was also significantly higher in the previously infected-unchallenged lambs than in the naïve group ($P<0.05$), and in the naïve-challenged than the previously infected-challenged on day 10 ($P<0.01$). IL-5 transcription was increased in the previously infected-challenged group on day 5, and in both groups on days 10 and 21 when compared with the respective unchallenged groups, according to Mann-Whitney U test and Dunn's test ($P<0.05$).

A significant increase in transcription of IL-13 was found in the gastric lymph node of the naïve-challenged lambs on days 10 and 21 compared to the naïve group according to Mann-Whitney U test ($P<0.05$). This was confirmed using Dunn's test on day 21 ($P<0.01$), but not on day 10. IL-13 transcription was also significantly higher in the previously infected-unchallenged lambs than in the naïve group ($P<0.05$), and in the previously infected-challenged lambs than the naïve-challenged group on day 5 ($P<0.01$).

No significant changes in the transcription of IL-2, IL-12p_{40} or IFNγ were observed in the gastric lymph node of the Dorset × Suffolk lambs following challenge; though transcription of IL-12p_{40} was significantly higher in the previously infected-challenged group than in the naïve-challenged on day 21 ($P<0.05$), and IFNγ transcription was higher in the naïve-challenged lambs than in the previously infected-challenged on day 10 ($P<0.05$).

No change in IL-10 transcription was found in the gastric lymph node of the Dorset × Suffolk lambs following challenge. However, significantly higher transcription levels of both IL-10 ($P<0.01$) and TGFβ_1 ($P<0.05$) were found in the naïve-challenged lambs than in the previously infected-challenged groups on days 10 and 22.

Transcription of IL-1β was increased in the abomasal mucosa of the naïve-challenged Dorset × Suffolk lambs on day 10 compared to the naïve group according to Mann-Whitney U test and Dunn's test ($P<0.05$), and was significantly higher in the naïve-challenged than in the previously infected-challenged lambs on day 5

(P<0.05). IL-6 transcription was increased in the abomasal mucosa of both the naïve-challenged and previously infected-challenged lambs on days 5 and 10, and in the naïve-challenged lambs on day 21, compared to their respective unchallenged groups according to Mann-Whitney U test (P<0.05). This significance in IL-6 up-regulation was confirmed using Dunn's test for the naïve-challenged lambs on days 5 (P<0.05) and 10 (P<0.01), and for the previously infected-challenged lambs on day 10 (P<0.05). Transcription of IL-18 was increased in the naïve-challenged group on day 10 compared to the naïve group according to Mann-Whitney U test (P<0.05), however this was not confirmed using Dunn's test. IL-18 transcription was significantly higher in the naïve-challenged group than in the previously infected-challenged on days 5 (P<0.01) and 10 (P<0.05). TNFα was significantly up-regulated in the naïve-challenged lambs on day 10 compared to the naïve group using Dunn's test (P<0.01).

Transcription of IL-4 in the abomasal mucosa was significantly higher in the previously infected-challenged Dorset × Suffolk lambs than in the naïve-challenged on day 5 (P<0.01), was increased in the previously infected-challenged lambs compared to the previously infected-unchallenged group on days 5 and 10, and in the naïve-challenged lambs compared to the naïve group on days 10 and 21 according to Mann-Whitney U test (P<0.05). However, these increases were only confirmed in the naïve-challenged group compared to the naïve on days 10 and 21 using Dunn's test (P<0.01). IL-5 transcription was increased in the previously infected-challenged lambs on days 5, 10 and 21, and in the naïve-challenged lambs on days 10 and 21, compared to their respective unchallenged groups using Mann-Whitney U test (P<0.05). However, only the up-regulation in the previously infected-challenged lambs was confirmed using Dunn's test on days 5 (P<0.01), 10 and 21 (P<0.05). Transcription of IL-5 was significantly higher in the abomasal mucosa of the previously infected-challenged lambs than in the naïve-challenged on days 5 and 10 (P<0.05). IL-13 transcription in the abomasal mucosa was higher in the previously infected-unchallenged lambs than in the naïve-unchallenged (P<0.05), and in the previously infected-challenged lambs than in the naïve-challenged on day 5 (P<0.01); and was significantly up-regulated in the previously infected-challenged lambs on days 5, 10 and 21, and in the naïve-challenged lambs on days 10 and 21, compared to their respective unchallenged groups according to Mann-Whitney U test ($P<0.05$). However, the up-regulation of IL-13 was only confirmed using Dunn's test in the naïve-challenged group on days 10 ($P<0.01$) and 21 ($P<0.05$), and in the previously infected-challenged group on day 10 ($P<0.05$).

Little significant change in Th1 cytokine transcription was found in the abomasal mucosa of the Dorset × Suffolk lambs. No changes were found in the levels of IL-2 or IL-12p_{40} transcription, however transcription of IFNγ was significantly higher in the naïve-challenged group than in the previously infected-challenged on day 10 ($P<0.05$), and was up-regulated in this group on day 10 compared to naïve lambs according to both Mann-Whitney U test and Dunn's test ($P<0.05$).

Transcription of IL-10 in the abomasal mucosa was significantly increased on day 10 in the naïve-challenged group compared to the naïve according to Dunn's test ($P<0.05$). TGFβ_1 transcription in the abomasal mucosa was found to be up-regulated in both the naïve-challenged and previously infected-challenged groups on days 10 and 21 according to Mann-Whitney U test ($P<0.05$), however this was not confirmed by Dunn's test.

Figure 6.3(a): Cytokine transcription in the gastric lymph node of 5-month-old Dorset × Suffolk lambs in experimental group 6 relative to ATPase. Analysed using Mann-Whitney U-test for non-parametric data with a 95% confidence interval, n = 6 sheep per group for each time point; significant difference between naïve- and previously infected-un/challenged groups on the same day: ***** *(P<0.05),* ****** *(P<0.01); significant difference between challenged groups and their respective unchallenged groups: # (P<0.05). Note different ranges on Y-axes.*

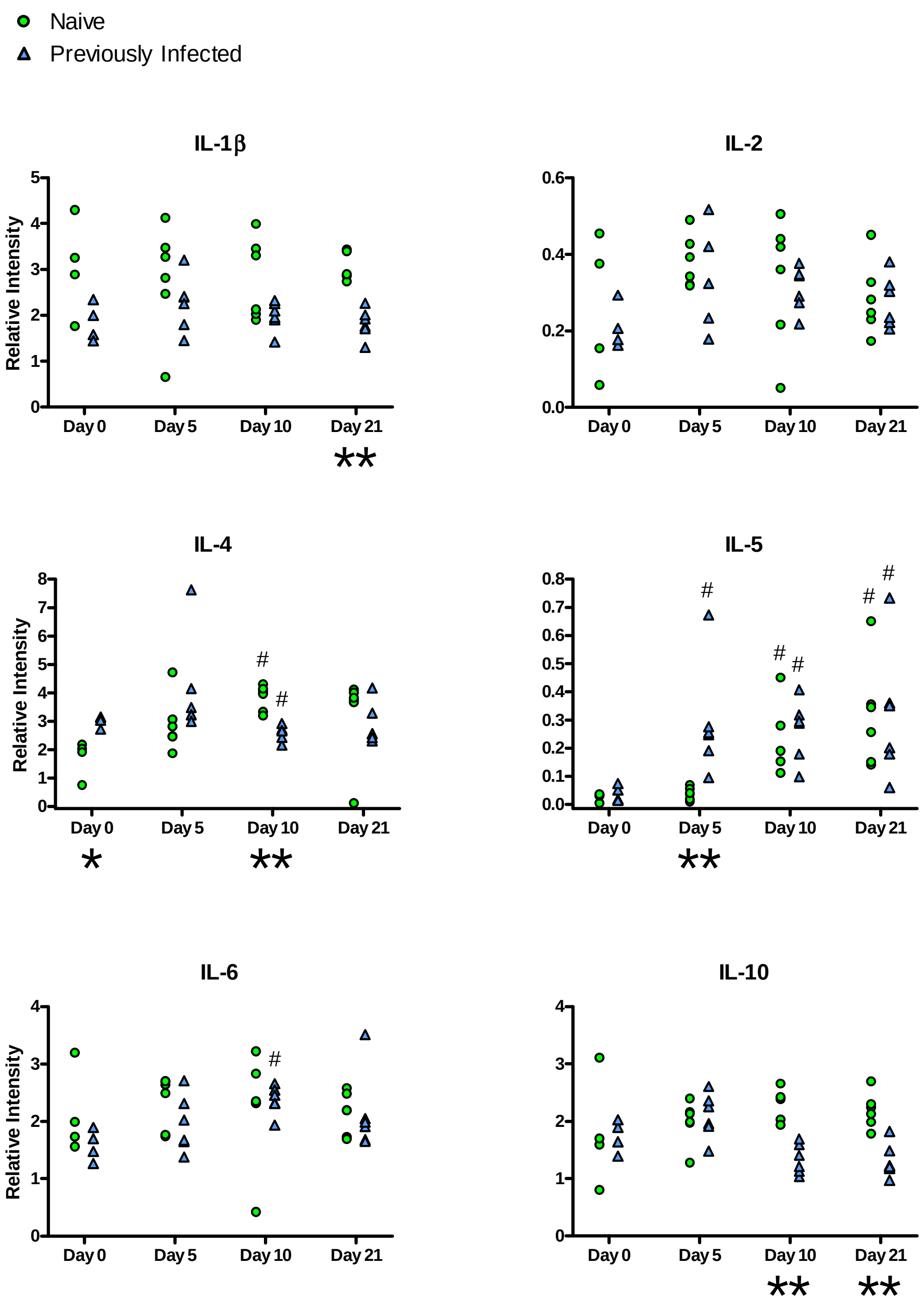


Figure 6.3(b): Cytokine transcription in the gastric lymph node of 5-month-old Dorset × Suffolk lambs in experimental group 6 relative to ATPase. Analysed using Mann-Whitney U-test for non-parametric data with a 95% confidence interval, n = 6 sheep per group for each time point; significant difference between naïve- and previously infected-un/challenged groups on the same day: ★ *(P<0.05),* ★★ *(P<0.01); significant difference between challenged groups and their respective unchallenged groups: # (P<0.05). Note different ranges on Y-axes.*

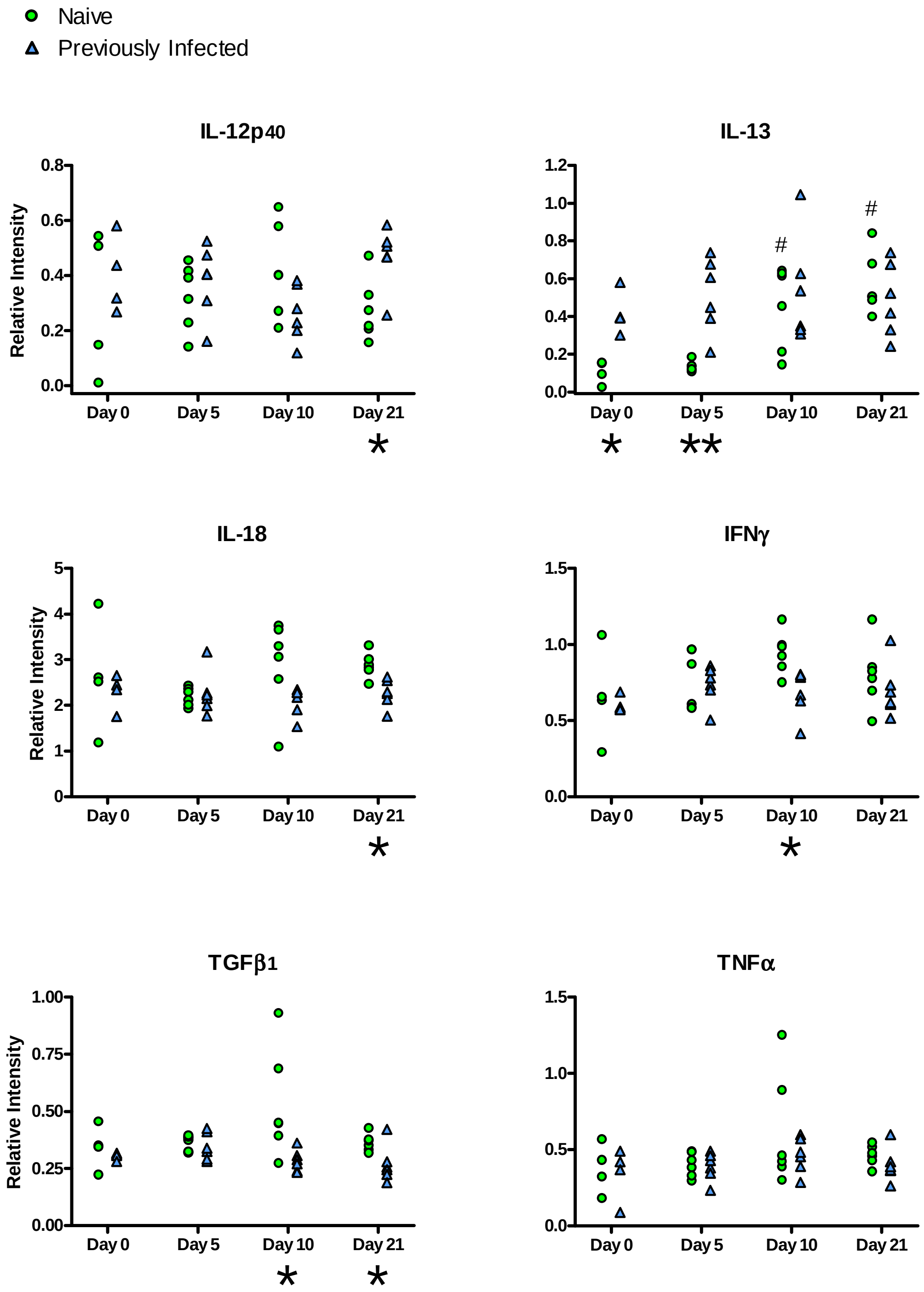


Figure 6.4(a): Cytokine transcription in the abomasal mucosa of 5-month-old Dorset × Suffolk lambs in experimental group 6 relative to ATPase. Analysed using Mann-Whitney U-test for non-parametric data with a 95% confidence interval, n = 6 sheep per group for each time point; significant difference between naïve- and previously infected-un/challenged groups on the same day: ✱ *(P<0.05),* ✱✱ *(P<0.01); significant difference between challenged groups and their respective unchallenged groups: # (P<0.05). Note different ranges on Y-axes.*

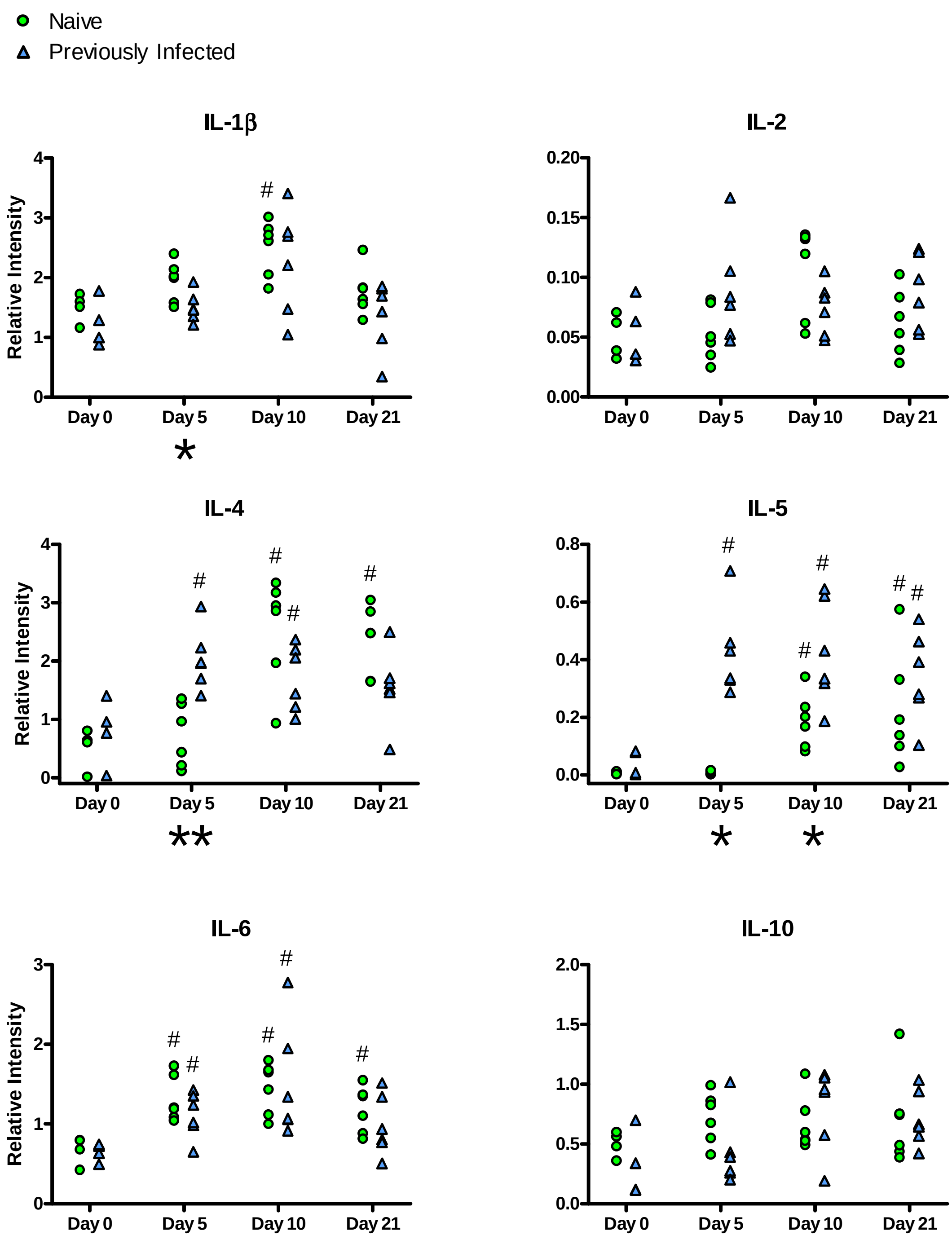
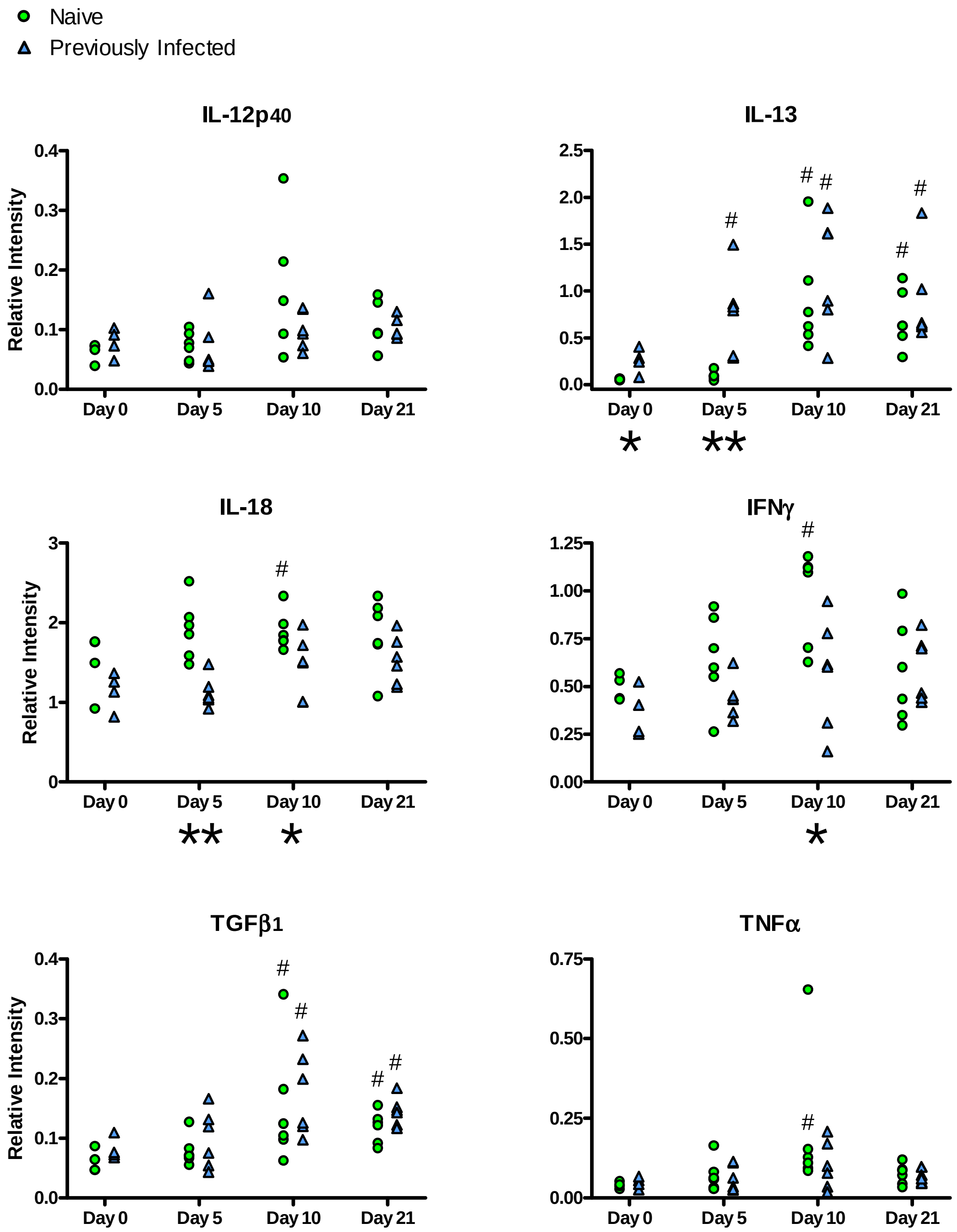


Figure 6.4(b): Cytokine transcription in the abomasal mucosa of 5-month-old Dorset × Suffolk lambs in experimental group 6 relative to ATPase. Analysed using Mann-Whitney U-test for non-parametric data with a 95% confidence interval, n = 6 sheep per group for each time point; significant difference between naïve- and previously infected-un/challenged groups on the same day: ✱ *(P<0.05),* ✱✱ *(P<0.01); significant difference between challenged groups and their respective unchallenged groups: # (P<0.05). Note different ranges on Y-axes.*



6.4 Discussion

The findings of the first study of Blackface × Leicester 5-month-old lambs generally echoed the proinflammatory and Th2 responses found in the yearling sheep and described in chapter 3; proinflammatory responses occurred more notably in the abomasal mucosa of the naïve-challenged group, and Th2 responses were generally earlier and more pronounced in the gastric lymph node of the previously infected-challenged group, with no change in Th1-type cytokines found in the abomasal mucosa of either group following challenge. The major contrast between the findings of the two studies concerned the suppression of Th1-type cytokine transcription in the gastric lymph node of the yearling sheep, of which there was no evidence in the gastric lymph node of the younger lambs. The up-regulation of IL-18 in the gastric lymph node of the younger animals also contrasted with its suppression in the older group. This suggests that the deficiency in the immune response to helminths in young lambs is not due to an inability to generate Th2 responses, but may be due to inadequate suppression of Th1 responses in the lymph node. However, it is difficult to draw conclusions regarding the changes in cytokine transcription taking place over the course of infection in the tissues of the previously infected-challenged lambs, as there was no previously infected-unchallenged group to act as a baseline to compare this group to.

The addition of a previously infected-unchallenged group during the Dorset × Suffolk lamb experiment allowed interpretation of the previously infected group results in comparison to a baseline. This study confirmed that the cytokine responses taking place in the abomasal mucosa of the Dorset × Suffolk lambs was similar to those found in this tissue in the previous Blackface × Leicester lambs and in the yearling sheep; indicating a Th2 response which occurred earlier and more prominently in the previously infected-challenged animals, and an inflammatory response which was more pronounced in the naïve-challenged group. Both breeds of lambs also demonstrated changes indicative of a Th2 response taking place in the gastric lymph node, as had been previously found in the yearling sheep; however, in contrast to the yearlings, no suppression of Th1-type cytokine transcription after challenge was evident either in the Dorset × Suffolk lambs or the Blackface × Leicester group. Figures 6.5 and 6.6 illustrate the trends in cytokine transcription observed, in the gastric lymph node and abomasal mucosa respectively, following challenge of naïve and previously infected sheep over both 5-month-old lamb experiments. Transcription of IL-2 and IFNγ in the gastric lymph node of yearling sheep compared with Dorset × Suffolk lambs is illustrated in Figure 6.7 (a) and (b) respectively. Transcription of IL-4, IL-5 and IL-13 in the abomasal mucosa of yearling sheep compared with Dorset × Suffolk lambs is illustrated in Figure 6.8 (a), (b) and (c) respectively.

However, some differences were found in the results of the Dorset × Suffolk lamb experiment compared to the Blackface × Leicester lambs. No change in IL-18 transcription was found in the gastric lymph node of the Dorset × Suffolk lambs over the course of infection, which contrasts with the findings in the gastric lymph nodes of both the Blackface × Leicester lambs, in which this cytokine was increased in the naïve-challenged group following challenge, and the yearling sheep, in which it was suppressed in both naïve-challenged and previously infected-challenged groups following challenge. Transcription of IL-6 was also significantly increased in the previously infected-challenged Dorset × Suffolk lambs compared to the previously infected-unchallenged group, a change which was not previously found in the gastric lymph node of the yearling sheep.

Another notable difference from the results of the previous lamb and yearling studies was the significantly increased transcription of IL-13 in the gastric lymph node of the naïve-challenged Dorset × Suffolk lambs following challenge, which was not found in the Blackface × Leicester lambs or the yearling sheep groups. IL-4 and IL-5 responses in the gastric lymph node both occurred earlier in the Blackface × Leicester lambs than in the Dorset × Suffolk lambs.

In contrast with the up-regulation of IL-10 in the gastric lymph node of the naïve-challenged Blackface × Leicester lambs on day 22 compared to the naïve group, no change in IL-10 transcription was found in the gastric lymph node of the Dorset × Suffolk lambs following challenge. The increased transcription of IL-10 in the gastric lymph node of the naïve-challenged Dorset × Suffolk lambs on days 10 and 22 compared to the previously infected-challenged groups is consistent with the findings in yearling sheep; however in the yearlings this difference in IL-10 was found on days 2 and 5 compared to days 10 and 21 in these lambs. This suggests that a delay or reduction in the development of regulatory responses, which either reduce immune-mediated pathology or aid in the suppression of Th1, may also have an influence on the reduced resistance and increased morbidity due to helminth infection in young lambs. Transcription of IL-10 in the gastric lymph node of yearlings compared with Dorset × Suffolk lambs is illustrated in Figure 6.7 (c).

Proinflammatory cytokine responses in the abomasal mucosa of the Dorset × Suffolk lambs were similar to those found in the Blackface × Leicester and in the yearlings, suggesting a more pronounced inflammatory response was taking place in the abomasal mucosa of the naïve-challenged individuals during infection than in the previously infected-challenged group. Th1-and Th2-type cytokine transcription in the abomasal mucosa of the two lamb breeds was also similar in the two lamb breeds and consistent with the results from the yearling sheep, with no significant changes in Th1-type cytokine transcription following challenge, and a Th2-type response occurring earlier in the previously infected-challenged lambs.

Though some differences were found in the cytokine responses between the Blackface × Leicester lambs and the Dorset × Suffolk group, such as the more pronounced Th2 responses in the Dorset × Suffolk lambs than in the Blackface × Leicester, the general trends were similar. No significant differences in the anti-parasite responses as measured by worm counts and lengths were found between the two groups, so differences in cytokine responses between the two lamb experiments are likely to be due to individual biological variation rather than differences in response to *T.circumcincta* infection due to breed (Halliday et al, paper in preparation). This implies that the use of different breeds at different time-points in the previous studies of yearling sheep will not have had a significant effect on the observed immune responses.

These studies present a clear indication that the lower resistance and increased morbidity due to helminth infection in young lambs is not due to an inability to produce Th2 or local inflammatory responses. This is consistent with the findings of previous studies of *H.contortus* infection of 3-month-old lambs, which demonstrated a clear Th2 response indicated by increased transcription of IL-4, IL-5 and IL-13 in the abomasal fundic mucosa (Lacroux et al., 2006).

The most dramatic and significant difference in the cytokine responses between the two age groups is the almost total lack of suppression of Th1 cytokine responses in the gastric lymph node in the younger lambs. Again, this finding is consistent with infection of 3-month-old lambs with *H.contortus*, in which no significant suppression of IL-12 or IFNγ was found in the gastric lymph node (Lacroux et al., 2006). It is possible that Th1 cytokines may be inhibiting anti-parasitic immune responses due to suppression of Th2 cytokine production and/or antagonism of their effects.

The reason for this evident lack of Th1 suppression is unclear. Merino lambs 4-8 months old have been found to have a lower proportion of CD4+ cells in their lymph and blood than older sheep 3-6 years old (Watson et al., 1994). As IL-4 was transcribed in the efferent lymph by CD4+, but not CD8+ or $\gamma\delta$+ lymphocytes (see section 5.5), it is possible that the influence of the available CD4+ lymphocyte population in young lambs is insufficient to generate enough Th2 cytokines to significantly suppress Th1. However responses to other pathogens involving CD4+ T cells are not stunted in young lambs in the same way as the responses to gastrointestinal helminths appears to be; therefore the delay in developing resistance to helminth infection is more likely to be due to some other aspect of the immune response.

Figure 6.5(a): Schematic illustration of the trends in transcription of Th1- and Th2-type cytokines in the gastric lymph node of naive-challenged and previously infected-challenged 5-month-old lambs. Note ranges on Y-axes. Day 0 =unchallenged.

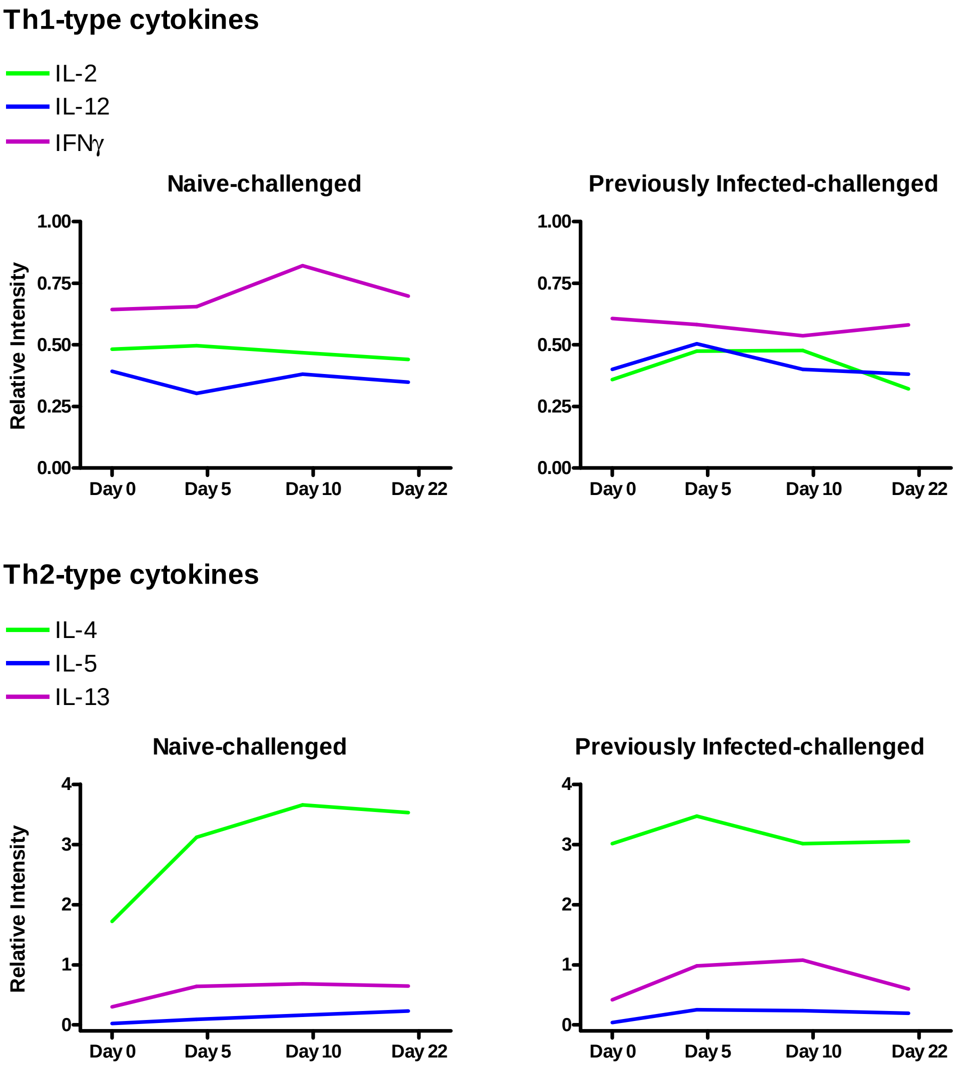


Figure 6.5(b): Schematic illustration of the trends in transcription of pro-inflammatory and regulatory-type cytokines in the gastric lymph node of naive-challenged and previously infected-challenged 5-month-old lambs. Note ranges on Y-axes. Day 0 =unchallenged.

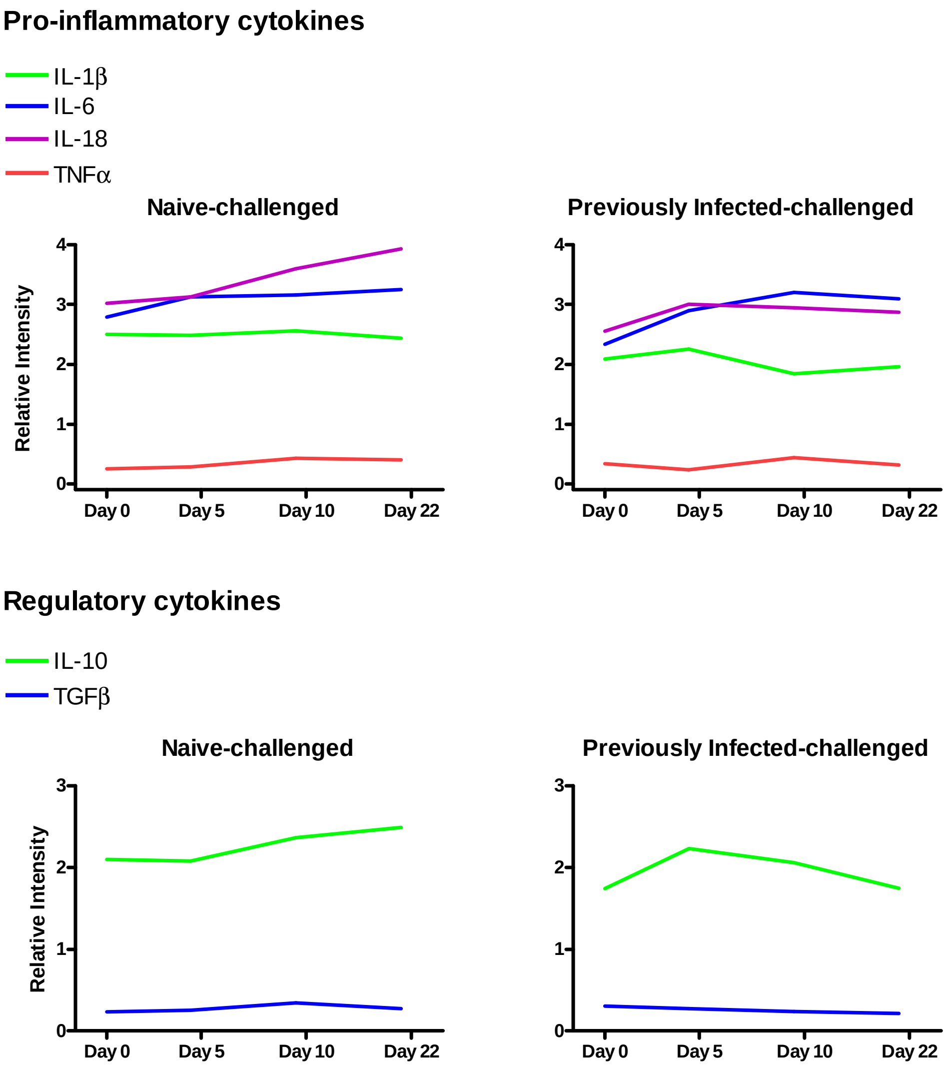


Figure 6.6(a): Schematic illustration of the trends in transcription of Th1- and Th2-type cytokines in the abomasal mucosa of naive-challenged and previously infected-challenged 5-month-old lambs. Note ranges on Y-axes. Day 0 =unchallenged.

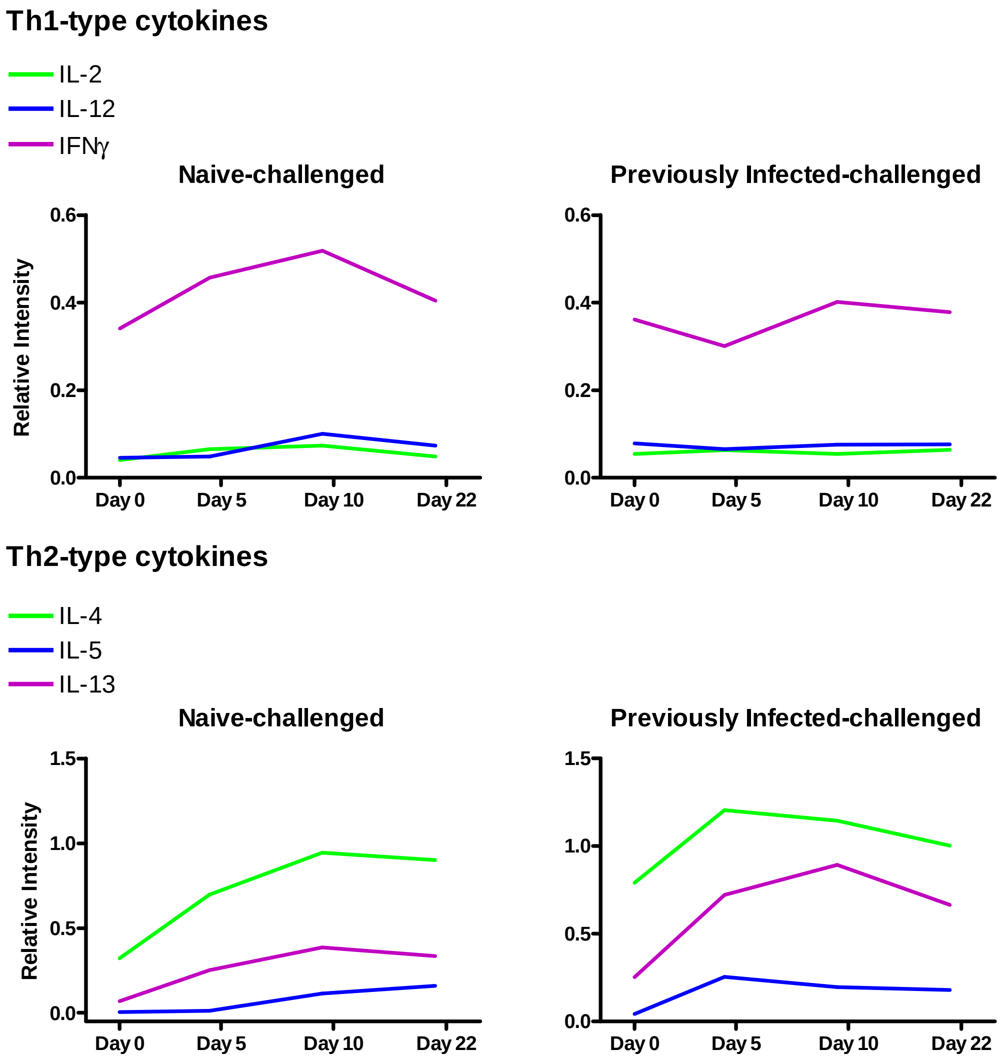


Figure 6.6(b): Schematic illustration of the trends in transcription of pro-inflammatory and regulatory-type cytokines in the abomasal mucosa of naive-challenged and previously infected-challenged 5-month-old lambs. Note ranges on Y-axes. Day 0 =unchallenged.

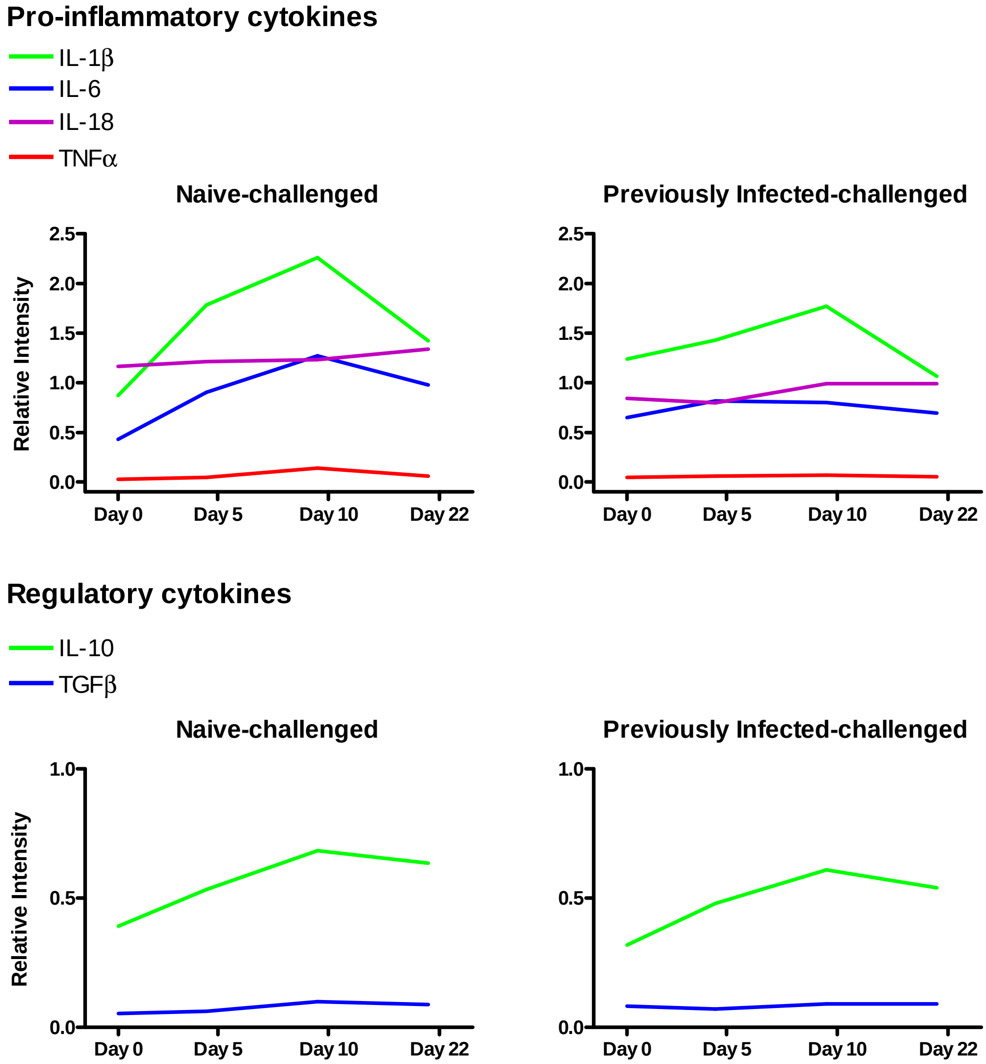


Figure 6.7: Transcription of IL-2, IFNγ and IL-10 in the gastric lymph node of yearlings (as also illustrated in Figure 3.5) in comparison with 5-month-old Dorset × Suffolk lambs (as also illustrated in Figure 6.3) relative to ATPase. Mean and SEM, n = 6 sheep per group for each time point. Note different ranges on Y-axes.

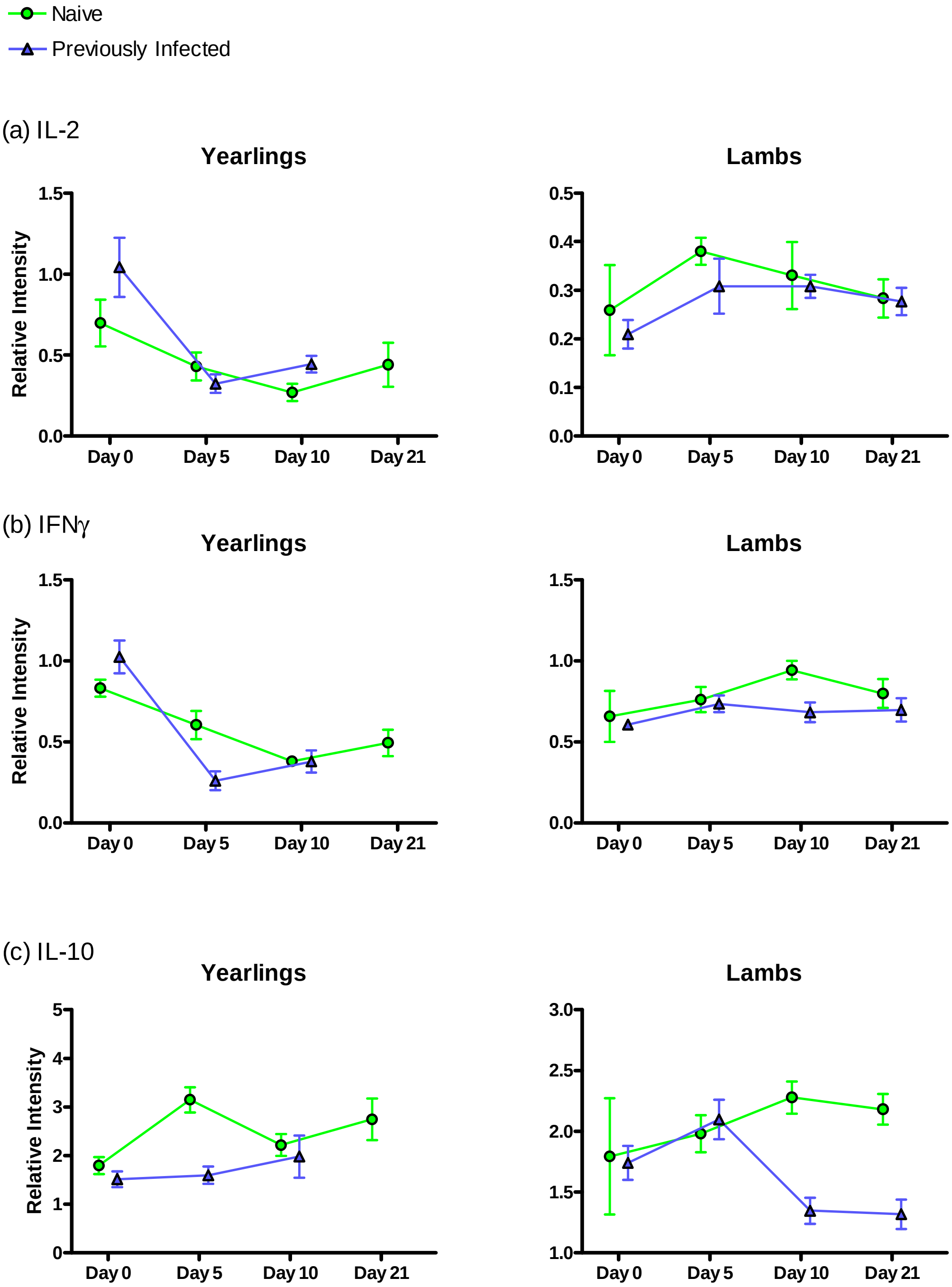
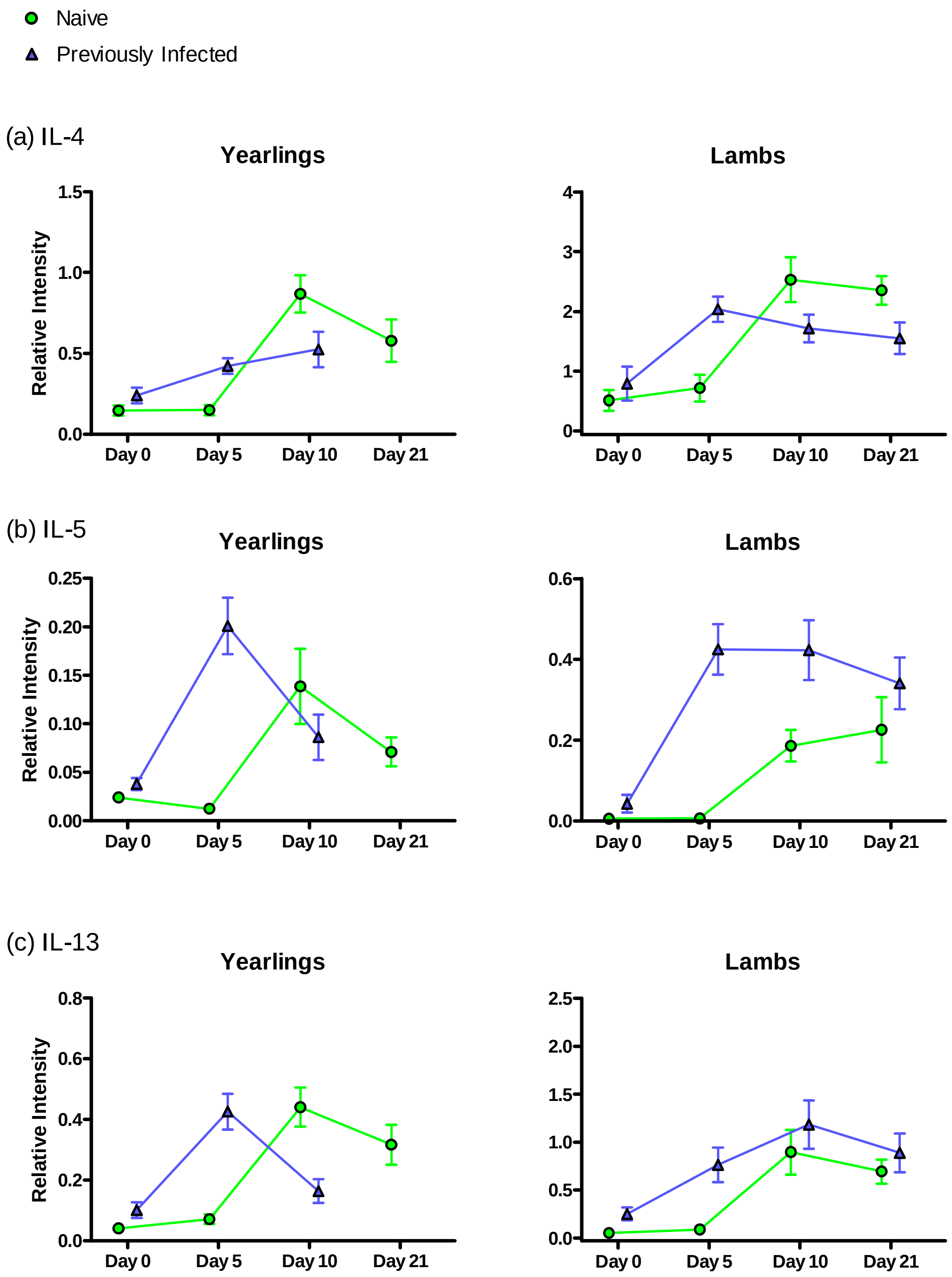


Figure 6.8: Transcription of IL-4, IL-5 and IL-13 in the abomasal mucosa of yearlings (as also illustrated in Figure 4.1) in comparison with 5-month-old Dorset × Suffolk lambs (as also illustrated in Figure 6.4) relative to ATPase. Mean and SEM, n = 6 sheep per group for each time point. Note different ranges on Y-axes.



There is a notable contrast between the outcomes of the studies detailed here, in combination with chapters 4 and 5, and the outcomes of the studies of anti-parasite responses to *T.circumcincta* in yearlings and young lambs carried out by Smith and colleagues between 1981 and 1985 (Smith et al., 1981, Smith et al., 1983, Smith et al., 1984, Smith et al., 1985). The recent studies detailed here failed to produce the differences in the blast cell response and worm counts between lambs 4-5 months old and 10-12 months old (Halliday et al, paper in preparation) which were evident in the older experiments. The lower dose of larvae administered during trickle infection of the previously infected sheep in the recent study, 2000 L3 larvae three times per week compared to five times per week in the older experiments, may explain the difference in results between the two experiments; perhaps a higher dose would have produced a more pronounced immunity in the yearling sheep, as suggested by the "threshold" hypothesis of helminth immunity wherein newly ingested larvae encounter immune responses which occur once a threshold level of antigen exposure has been reached (Dineen and Wagland, 1966), but not in the younger lambs.

This hypothesis is supported by the observation that a much higher percentage of arrested larvae was retrieved from the yearlings which had been trickle infected five times per week (74.5 and 80.0%) than from those which had been infected three times per week (31.0 and 38.2%), whereas the percentage of arrested larvae retrieved from the 4- to 5-month-old lambs (infected five times per week, 6.3 and 22.0%; infected three times per week 13.6 and 28.4%) was similar between the two series of experiments (Smith et al., 1983, Smith et al., 1984, Smith et al., 1985, Halliday et al., 2007). This has also been indicated in studies of *Brucella abortus* infection in young lambs compared to adult sheep, in which "the serum antibody responses of adult sheep to the T cell-independent antigen *Brucella abortus* lipopolysaccharide were greater over a range of antigen doses, suggesting that an apparent excess of antigen could not overcome the relative immune deficiency of young sheep" (Watson et al., 1994).

Frequency of infection may also be a factor in the discrepancy between the two sets of *T.circumcincta* infection studies. Experimental infections of mice with

Heligmosomoides bakeri have demonstrated that mice infected more frequently expelled the parasites more rapidly than mice infected less frequently, but with doses amounting to the same total number of larvae (Brailsford and Behnke, 1992).

The differences in susceptibility and morbidity between young lambs and older sheep is unlikely to be due to differences in cytokine responses in isolation. A study of changes in abomasal histology in response to *H.contortus* by Salman and colleagues (1984) produced the incidental finding that a worm free ewe had higher numbers of mast cells, eosinophils, plasma cells and lymphocytes than an equivalent worm free lamb. Over the course of infection the adult ewes also produced a more marked increase in mast cells, globule leukocytes, eosinophils and IgA-producing plasma cells than the young lambs. This disparity between the cellular profiles of lambs and older sheep is likely to have an impact on helminth resistance and to be linked to a difference in cytokine responses.

7 Chapter Seven: Summary and Conclusions

The immune response to *T.circumcincta*, as discussed in section 1.3, was known to have many features associated with a phenotypic Th2-type response; such as mastocytosis, eosinophilia, mucous cell hyperplasia and increased mucus production. However the cytokine profile generated in response to infection, which would be useful in directing the development of potential vaccines towards an effective immune response, had not yet been examined. The aim of this study was to investigate the local cytokine changes which coordinate the immune response to *T.circumcincta* and the development of acquired immunity.

7.1 Cytokine responses in yearling sheep to *Teladorsagia circumcincta* infection

Sheep which had previously been exposed to a trickle infection of 2,000 *T.circumcincta* L3 larvae three times per week for eight weeks were cleared of infection and challenged with 50,000 L3, then examined in comparison with helminth-naïve sheep which had received only a 50,000 L3 challenge dose. This study of cytokine responses following challenge of these two groups is described in chapters 3-5. The significantly increased numbers of mast cells observed in the abomasal mucosa of the previously infected animals compare to the naïve sheep on day 0, described in section 4.4, confirmed that a Th2-phenotype response had taken place during the trickle infection. Mastocytosis was also evident in the naïve sheep following challenge, as mast cell numbers were significantly increased on day 21 compared to day 0. Previously infected animals were also found to have a more effective anti-parasite response, demonstrated by lower post-mortem worm counts and a greater degree of larval stunting, IgA response and an earlier blast cell response in efferent lymph than the naïve challenged animals (Halliday et al., 2007, Halliday et al., 2009b).

Increased transcription of the Th2 cytokines IL-4, IL-5 and IL-13 was observed in the abomasal mucosa of yearling sheep during infection with *T.circumcincta*, as discussed in section 4.5. This increase is consistent with studies of *H.contortus* in sheep (Lacroux et al., 2006) and *O.ostertagi* infection in cattle (Claerebout et al., 2005), and occurred earlier and to a greater extent in the previously infected sheep than the naïve following challenge, consistent with the responses observed during *H.contortus* infection (Lacroux et al., 2006). In contrast to observations of *O.ostertagi* infection in cattle (Claerebout et al., 2005), transcription of IFNγ was not significantly increased in the abomasum of sheep infected with *T.circumcincta*. Concurrent inflammation was indicated in the abomasal mucosa of the naïve challenged animals by increased transcription of IL-1β and IL-6 compared to day 0, which was not found in the previously infected sheep. Increased transcription of $TGF\beta_1$ found in the ovine abomasum following challenge was significant compared to day 0 in the previously infected sheep, but not the naïve. Increased transcription of IL-6 and $TGF\beta_1$ was not observed during *O.ostertagi* infection in cattle (Claerebout et al., 2005).

The timing of these responses is interesting. The early up-regulation of IL-4 two days following challenge, and of IL-5 two and five days following challenge, in the abomasal mucosa of the previously infected group compared to day 0 corresponds with expulsion of *T.circumcincta* in this group. This expulsion occurred by day 2 in the previously infected group (Halliday et al., 2009b), resulting in significantly lower worm burdens compared to the naïve challenged sheep (Halliday et al., 2007). A parasite-specific IgA blast cell response also occurred in the previously infected group, and may be instrumental in stunting larval development, however this IgA response occurred too late to be implicated in worm expulsion (Halliday et al., 2007).

Increased transcription of IL-4 and IL-5 from day 0 was also found in the gastric lymph node of previously infected sheep during the early stages of the immune response to infection, as discussed in section 3.4, along with significant suppression of transcription of the Th1 cytokines IL-2 and IFNγ in both the naïve and previously infected groups. This is consistent with the Th2-type response and concurrent suppression of Th1 cytokines observed in the gastric lymph node during *O.ostertagi* infection of cattle (Claerebout et al., 2005), however no suppression of Th1 cytokines had previously been found during *H.contortus* infection of sheep (Lacroux et al., 2006). This difference may be due to differences in the immune responses of the host to *H.contortus*, which causes far more trauma to the abomasal mucosa during blood/tissue feeding than *O.ostertagi* and *T.circumcincta*. This suppression of Th1 cytokines in the lymph node again occurred earlier and to a greater extent in the previously infected sheep than in the naïve.

Increased transcription of IL-4 and IL-13 from day -1, found in efferent lymphocytes following challenge of previously infected sheep, is also consistent with that found in pseudoafferent intestinal lymphocytes following infection with *Trichostrongylus colubriformis* (Hein et al., 2004), as discussed in section 5.7. The increased transcription of IL-4 by CD4+ efferent lymphocytes in the previously infected animals peaked at day 1, earlier than the CD4+ blast cell response which peaked at day 3 in this group (Halliday et al., 2009b). A significant increase in IFNγ compared to day -1 was also observed in the efferent lymphocytes at day 1 after challenge of the previously infected sheep, possibly due to the concurrent CD8+ blast cell response which peaked at day 3 (Halliday et al., 2009b).

The earlier and more pronounced increase in Th2 cytokine transcription, and suppression of Th1 cytokines, in the previously infected sheep emphasises the importance of these mechanisms in the immune response to *T.circumcincta* infection and the development of resistance to infection. This is supported by observations that sheep intelectin 2, which is Th2-induced, was produced earlier in the previously infected sheep (Athanasiadou et al., 2008, French et al., 2008, French et al., 2009). Other Th2-induced molecules, such as calcium activated chloride channel (Knight et al, paper in preparation), ovine galectin-14 and sheep mast cell protease-1, were also increased following challenge (Zhou et al., 2001, Athanasiadou et al., 2008, French et al., 2008).

The most significant findings from the study of yearling sheep are that the Th2-type response observed in the abomasal mucosa of sheep infected with *T.circumcincta* is more clearly defined than that observed in the abomasal mucosa of cattle infected with *O.ostertagi* (Claerebout et al., 2005), occurring earlier and to a greater extent in previously infected-challenged animals than naïve-challenged, and that transcription of Th1 cytokines in the gastric lymph node is significantly suppressed following infection with *T.circumcincta*.

7.2 Cytokine responses in 5 month old lambs to *Teladorsagia circumcincta* infection

The study of cytokine responses in naïve and previously infected yearling sheep described in chapters 3-5 was then repeated using 5 month old lambs, as described in chapter 6. It was expected that the younger animals would fail to mount effective anti-parasite responses, resulting in higher post-mortem worm burdens and reduced larval stunting in comparison to the yearlings, as had been found in previous studies using a similar experimental model (Smith et al., 1985). However in this case no significant difference was found in blast cell response or worm counts between 5 months old lambs and yearlings (Halliday et al, paper in preparation). This may be due to the fact that a lower total dose of larvae was administered at a reduced frequency during trickle infection of the previously infected sheep in the recent study, as discussed in section 6.4.

This experiment was duplicated using different breeds of lambs to assess the influence of breed on cytokine responses to *T.circumcincta* in this experiment. While some differences were found in the cytokine responses between the two breeds, such as the more pronounced Th2 responses in the Dorset x Suffolk lambs than in the Blackface x Leicester, the general trends were similar and no significant differences in worm counts or larval stunting were found between the two groups (Halliday et al, paper in preparation).

The changes in cytokine transcription in 5 month old lambs following infection with *T.circumcincta* were similar to the proinflammatory and Th2 responses found in the yearling sheep. Proinflammatory responses, indicated by increased transcription of IL-1β and IL-6 following infection, were more pronounced in the abomasal mucosa of naïve than previously infected lambs. Th2 cytokine responses following infection, indicated by increased transcription of IL-4, IL-5 and IL-13 compared to day 0, were again earlier and more pronounced in the abomasum of the previously infected lambs, but not the gastric lymph node.

The most interesting contrast between the two age groups concerned the suppression of Th1 cytokine transcription in the gastric lymph node of the yearling sheep following infection, of which there was no evidence in the gastric lymph node of the 5 month old lambs. Increased transcription of IL-18 in the gastric lymph node of the younger animals compared to day 0 also contrasted with suppression of this cytokine in the older group.

This suggests that the increased susceptibility of young lambs to *T.circumcincta* may not be due to an inability to generate Th2 responses, but an inability to adequately suppress transcription of antagonistic Th1 cytokines in the draining lymph node. The suppression of Th2-type responses by ongoing production of Th1 cytokines during gastrointestinal helminth infection may, in part, explain why younger lambs do not produce significant increase in mast cells, globule leukocytes, eosinophils and IgA-producing plasma cells in the abomasal mucosa compared to adult ewes (Salman and Duncan, 1984).

The most significant findings from the study of 5-month-old lambs is that these animals, while capable of producing a Th2-type cytokine response following infection with *T.circumcincta*, were not as capable of suppressing Th1-type cytokine transcription in the draining lymph node as older sheep.

7.3 Summary of cytokine changes influenced by immune status

To summarise, transcription of the Th2-type cytokines IL-4, IL-5 and IL-13, the proinflammatory cytokines IL-1β and IL-6, and the regulatory cytokine $TGF\beta_1$ was increased in the abomasal mucosa of the yearling sheep following infection with *T.circumcincta*, whereas no significant changes were found in the Th1-type cytokines IL-2, IL-12p_{40} and IFNγ. IL-4 and IL-5 were up-regulated during the early stages of the immune response in the abomasum, in contrast to IL-13 which was up-regulated later on. Transcription of all three Th2-type cytokines increased earlier and to a greater extent in the previously infected-challenged animals than in the naïve-challenged. The proinflammatory cytokine IL-6 was up-regulated in the abomasum of the naïve-challenged group early following infection, but transcription did not increase in the previously infected-challenged group. This contrasts with transcription of IL-1β, which was increased in both the naïve- and the previously infected-challenged groups, but increased earlier in the previously infected-challenged; IL-18, which was suppressed in the naïve-challenged group following infection and was consistently lower in the previously infected-challenged group than the naïve-challenged; and TNFα, which was initially suppressed in the naïve-challenged group, then increased in the previously infected-challenged group during the later stages of infection.

No change in transcription of IL-13 was found in the gastric lymph node following infection of yearling sheep. IL-4 transcription was initially increased in the gastric lymph node during the early stages of infection in the previously infected group before being reduced at a later time-point. IL-5 transcription was transiently increased, and was higher in the previously infected-challenged group than the naïve-challenged during the early stages of infection. In contrast, transcription of IL-2 and IFNγ was markedly suppressed in the gastric lymph node of both the naïve-challenged and the previously infected-challenged yearlings; this suppression occurring earlier in the previously infected-challenged group. Transcription of IL-12p_{40} was transiently reduced during the early stages of challenge, but only in the previously infected-challenged group. A proinflammatory response was suggested in the gastric lymph node of the yearling sheep by an increase in transcription of IL-1β and TNFα, however IL-18 transcription was again suppressed in both the naïve-challenged and previously infected-challenged groups. $TGF\beta_1$ was initially suppressed in the gastric lymph node of both groups, but was later increased in the naïve-challenged group.

Increased transcription of IL-4 and IL-13 in response to *T.circumcincta* infection was also found in the efferent gastric lymph of the previously infected-challenged sheep following infection, as was an increase in transcription of IFNγ during the early stages of infection.

Infection of 5-month-old lambs produced similar changes in cytokine transcription in the abomasum to those found in the yearling sheep. However, a major contrast to the yearlings was observed in the gastric lymph node of the lambs, wherein no suppression of Th1-type cytokine transcription was observed.

7.4 Limitations

Many of the limitations of these experiments were those that are inherent in all large animal experimental trials. The need for statistically significant numbers of animals is countered by the necessity to reduce the number of animals used in research, the difficulty of producing and maintaining specific pathogen-free large animals and the expense of housing them to appropriate hygiene and welfare standards. In addition, large animals take far longer to reproduce and mature than traditional laboratory animals, and are still of variable and mixed genetics compared to inbred strains of rodents and rabbits, leading to significant variation between individuals, even within the same breed or sire-line. However, these difficulties are outweighed by the benefits of studying the immune responses to parasites in the natural host as opposed to rodent models.

Due to the limited availability of helminth-naïve yearling sheep during the early stages of these trials, it was not possible to include previously infected unchallenged control animals in the experiments described in sections 3.3.1 and 3.3.2 while still having statistically significant numbers of sheep in the challenged groups. However, as the main aim of these initial trials was to examine the differences in response to *T.circumcincta* challenge between helminth-naïve and previously infected sheep, this was not considered a major limitation at this stage. Subsequent trials were then run to address the differences in immune responses of naïve and previously infected sheep during the course of *T.circumcincta* infection, for which baseline day 0 controls were included. These trials are described in sections 3.3.3, 3.3.4 and 4.2.

As a study of cytokine transcription in sheep, this study was also limited by the availability of sheep gene/mRNA sequences for comparatively more recently identified cytokines such as IL-25 and IL-33. More genetic sequence information is becoming available for ruminants, which will be useful for similar studies in the future. More reagents for use in ruminants are also becoming available, for example through the Veterinary Immunology Committee (VIC) Toolkit (Entrican et al., 2009). However the range of reagents and gene sequence information available for ruminants is still far less than for more traditional laboratory animals.

The identification of housekeeping genes which have constant levels of transcription in all tissues and disease states in sheep is also a recurring problem when using these animals; thus far a perfect candidate has not been found.

7.5 Implications and future work

Breeding of sheep for resistance to helminth infection has been in progress for a number of years, as discussed in section 1.4; however, progress in large animals is slow, possibly because resistance to helminth infection is defined by a large number of genes which each have a relatively small effect (Crawford et al., 1998). Breeding lambs for high Th2 responses and low Th1 on the basis of the findings of this thesis and other studies may also be counterproductive to the sheep industry. While lambs bred for Th2-skewed immune responses may be more resistant to helminth infection, they may also be more susceptible to viral and bacterial diseases due to suppression of the Th1-type responses normally produced in response to these types of pathogen (Hein et al., 2004), suffer from increased Th2-mediated tissue damage, or be less productive than current commercial breeds. Vaccination is therefore a more applicable option, and does not require stock replacement or cross-breeding.

The findings of this thesis indicate that future vaccine research should focus equally on the suppression of Th1 responses in the host as on the generation of Th2 responses. This could be done by searching for helminth molecules which particularly provoke Th2 and regulatory T cell activation. Adjuvants could be incorporated to promote this effect, or helminth antigens could be conjugated with immunomodulatory molecules. For example conjugation of lacto-N-fucopentaose III, a glycan derived from schistosome eggs, has been shown to drive Th2-biased immune responses in mice independently or conjugated with human serum albumin (Goodridge et al., 2005).

The pronounced local cytokine responses in the abomasal mucosa found in this thesis also suggests that any vaccine would be most effective if delivered locally at the gastrointestinal mucosa to drive preferentially mucosal immune responses. Development of mucosal vaccine delivery systems is ongoing, for example intranasal administration of antigen which is conjugated to anti-MHC class II antibody (Snider et al., 1997).

Further work arising from this thesis could be directed towards the further optimisation of assays to detect cytokines in ovine tissues, which would focus on methods of cleaning up samples to remove matrix effects, and expansions of these experiments to examine cytokine responses in resistant and susceptible sheep exposed to natural challenge.

Examination of changes in the abomasal mucosa of naïve and previously infected individuals over the course of infection using repeated abomasal biopsy taken by means of an abomasal fistula has been done in studies of *H.contortus* (Rowe et al., 2009). This technique would be useful in studies of immune responses to *T.circumcincta*, as it would allow observation of the local immune responses to infection within the same animal; avoiding the biological variation introduced by using different animals for each time-point. This technique would also provide the opportunity to examine the changes in pH and bacterial load of the abomasal contents, and bacterial contamination of the gastric crypts, which may be contributing to the inflammatory response observed. However, the potential for interference from surgery effects should be considered.

Staining of specific cell types followed by in situ hybridisation to examine which cells are responsible for producing specific cytokines over the course of infection would also be very informative; for example dendritic cells and Foxp3-expressing regulatory T cells in the abomasal mucosa and gastric lymph node, and lymphocytes in the abomasal mucosa. Further examination of cytokine transcription in the efferent lymphocytes, in particular IL-10, and staining for Foxp3-expressing regulatory T cells in efferent lymph would be interesting to investigate whether regulatory T cells are present in efferent lymph and whether this population is increased following challenge. These cells have recently been found in the abomasal mucosa and draining lymph node (McNeilly, 2005) and have been implicated in immune modulation by helminths (Maizels and Yazdanbakhsh, 2003).

Further examination of the immune cell populations present in the afferent lymph would be very interesting, as these cells are communicating information between the local response in the abomasum and the draining lymph node, and are likely to include Th2 dendritic cells and regulatory T cells which could be influencing the response taking place in the lymph node. It is possible to produce pseudoafferent abomasal lymph by removing the draining lymph nodes, as has been done by Hein and colleagues to extract pseudoafferent mesenteric lymph (Hein et al., 2004); however, this surgical technique is very complex.

Further examination of the factors that influence the nature of the immune response to gastrointestinal helminths in sheep, and the mechanisms through which these responses are orchestrated and controlled, would be extremely useful in informing the development of novel control methods for these parasites.

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Appendix 1: Suppliers of materials

1a. Chemicals

Unless otherwise stated, all chemical used were supplied by Thermo Fisher Scientific Inc, Waltham, MA, USA.

1b. Molecular biology

<u>Applied Biosystems (UK), Warrington, Cheshire, UK:</u>

ABI Prism 7500 real-time PCR thermocycler

Custom Taqman assay: Sheep IL-1β

Custom Taqman assay: Sheep IL-4

Custom Taqman assay: Sheep IL-13

Custom Taqman assay: Sheep IFNγ

Custom Taqman primer and probe: Sheep ATPase

DNA-free™ DNAse treatment and removal reagents

Optical adhesive covers and applicator

10x TE (Tris-EDTA) buffer (10mM Tris, 1mM EDTA, pH 7.0)

RNAlater®

Taqman gene expression mastermix

<u>Axygen Scientific, Union City, CA, USA:</u>

Eppendorf tubes (1.5ml and 0.6ml)

Thin-walled 0.2ml PCR tubes

<u>Beckman Coulter, High Wycombe, UK:</u>

Beckman DU 650 spectrophotometer

<u>Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK:</u>

Bio-Rad Molecular Imager® FX
Bio-Rad Power Pac 200 gel electrophoresis power supply

Biotium, Inc., Hayward, CA, USA
GelRed™ Nucleic Acid Gel Stain

Corning Ltd., Hemel Hempstead, Hertfordshire, UK:

Costar® Model 10 centrifuge

Finnpipette®, Milford, USA:

Multichannel pipette

Pipettes

Pipettes tips

Functional Genomics Unit, Moredun Research Institute, Edinburgh, UK:

Sequencing of PCR products

Gibco™ Invitrogen Corporation, Paisley, UK:

Life Technologies™ Horizon® 11.14 gel electrophoresis apparatus

Life Technologies™ Horizon® 58 gel electrophoresis apparatus

Heraeus Instruments GmbH, Hanau, Germany:

Biofuge fresco centrifuge

Hoefer Inc., San Francisco, USA:

Hoefer™ UVC 500 UV cross-linker

Invitrogen™ Ltd, Paisley, UK:

Electrophoresis grade agarose

Foetal Calf Serum

1Kb Plus DNA Ladder quantitative DNA ladder

MJ Research, Waltham, MA, USA:

Opticon 2 DNA Engine

0.2ml thin-walled low profile white Q- PCR tubes

Operon Biotechnologies GmbH, Cologne, Germany:

PCR primers

PerkinElmer, Boston, USA:

Kodak® Digital Science Image Station 440CF

PerkinElmer GeneAmp PCR System 2400

Pierce, Rockford, Illinois, USA:

BCA protein assay

Promega, Southampton, UK:

Deoxynucleotide triphosphates (dATP, dGTP, dTTP and dCTP) 100mM

Gel Loading Dye

Reverse Transcription Kit

RNAse-free water

Qbiogene, Cambridge, UK:

Template Tamer™ UV cross-linker

Qiagen, Crawley, West Sussex, UK:

Qiashredder

QuantiTect SYBR green PCR kit

RNAse-free DNAse

RNeasy® Micro Kit including MiniElute columns

RNeasy® Mini Kit

Roche Diagnostics GmbH, Mannheim, Germany:

High Pure PCR Product Purification Kit

PCR Taq Polymerase including 10x Buffer solution

Sigma-Aldrich Co Ltd., Gillingham, Dorset, UK:

Ethidium bromide (500µg/ml)

Gel loading solution

Starlab, Ahrensburg, Germany:

Pipette tips

Stratech Scientific, Soham, UK:

Stratech Beadbeater-8

2ml screw-top vials

Swann-Morton, Sheffield, UK:

Fixed-blade scalpels

Techne, New Jersey, USA:

Techgene thermocycler

Techne Gradient thermocycler

Thermo Fisher Scientific Inc, Waltham, MA, USA:

ABgene Thermo-fast 96 detection plate

Topmix FB15024 vortex

Thistle scientific Ltd, Glasgow, UK:

1mm3 zirconia/silica beads

1c. Immunoassays

Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK:

Bio-Rad model 550 microplate reader

Ready Gel 12% Tris-HCl premade gel

Trans-Blot SD Semi-Dry Transfer Cell

Dynex Technologies:

96 well ELISA plates

eBioscience, San Diego, USA:

TMB (3,3',5,5'-Tetramethylbenzidine) substrate solution

Merck KGaA, Darmstadt, Germany:

PHM-L Liposorb™ Absorbent

Millipore, Billerica, USA:

Immobilon-P

Moredun Animal Health, Edinburgh, UK; Immunological Toolbox:

Recombinant ovine IL-1β

Recombinant ovine IL-4

Recombinant ovine IL-10

MP Biomedicals Europe, Illkirch, France:

QBiogene Lysing Matrix D tubes (2ml tubes containing 1.4mm ceramic spheres)

Sigma-Aldrich Co Ltd., Gillingham, Dorset, UK:

Bovine serum albumin, fraction V, minimum 96% lyophilised powder

Streptavidin peroxidase polymer (1.1mg protein/ml)

Triton X-100

Chemiluminescent reagent

Roche Diagnostics GmbH, Mannheim, Germany:

Complete mini, EDTA-free, protease inhibitor cocktail tablets

Thermo Fisher Scientific Inc, Waltham, MA, USA:

Thermo Electron Bio101 Fastprep (FP120)

Tween20 (Sorbitan Monolaurate)

1d. Antibodies

AbD Serotech, Kidlington, UK:

AHP423 (rabbit anti-ovine IL-1β IgG polyclonal)

MCA1658 (mouse anti-ovine IL-1β IgG1 monoclonal)

MCA2371 (mouse anti-bovine IL-4 IgG2a monoclonal)

MCA2372B (mouse anti-bovine IL-4 IgG2b biotinylated monoclonal)

Jackson ImmuoResearch Laboratories Inc, West Grove, USA:

Donkey anti-rabbit horseradish peroxidase (200μg/ml)

Donkey anti-mouse horseradish peroxidase (200μg/ml)

Invitrogen Ltd, Paisley, UK:

P852 (goat anti-mouse IgG r-phycoerythrin)

Moredun Animal Health, Edinburgh, UK:

7C2 (mouse anti-ovine CD8 IgG2a monoclonal)

17D (mouse anti-ovine CD4 IgG1 monoclonal)

86D (mouse anti-bovine γδ TcR IgG1 monoclonal)

VPM22 (mouse anti-ovine pestivirus IgG2a monoclonal)

VPM21 (mouse anti-ovine pestivirus IgG1 monoclonal)

1e. Histology

Leica Microsystems GmbH, Wetzlar, Germany:

Leitz Laborlux S microscope

Sony Corporation, Tokyo, Japan:

ExwaveHAD 3CCD colour video camera

Appendix 2: Preparation of solutions, buffers and substrates

RLT/BME:

40ml RLT buffer (RNeasy® Mini Kit)

400µl β-Mercaptoethanol (BME)

Solution made up in a fume hood using protective clothing as specified in the lab risk assessment.

DNAse/RDD:

50µl DNAse 1 stock solution (Qiagen RNAse-free DNAse set)

350µl Buffer RDD (Qiagen RNAse-free DNAse set)

Mix solutions gently. This solution can be kept at 4˚C for up to one month.

Buffer RPE:

Four volumes of 100% ethanol are added to Buffer RPE (Qiagen RNeasy® Mini Kit) to obtain a working solution according to the manufacturers' instructions.

70% Ethanol:

70% Ethanol in de-ionised water.

2mM dNTP mixture:

2mM each of: dATP , dGTP, dTTP and dCTP in RNAse-free water,

130µl aliquots stored at -20°C.

Primer solution:

100µM forward and reverse primers in RNAse-free water stored at -70°C.

Make up a 2µM mixture of forward and reverse primer in RNAse-free water before use and store at -20°C.

0.5M EDTA (Ethylenediaminetetraacetic acid):

93.05g Ethylenediaminetetraacetic acid

400ml De-ionised water

Adjust solution to pH8 using NaOH, then make up to 500ml in de-ionised water.

TBE (Tris-Borate-EDTA):

10.9g Tris

5.56g Boric acid

4ml 0.5M EDTA

Make up solution to 1L in de-ionised water.

PBS (Phosphate buffered saline):

8.5g NaCl

2.7g $Na_2HPO_4 \cdot 12H_2O$

0.39g $NaH_2PO_4 \cdot 2H_2O$

Make up solution to 1L in de-ionised water.

FACS buffer:

5% foetal calf serum (FCS) in PBS.

0.1M carbonate buffer:

0.84g $NaHCO_3$

100ml De-ionised water

Titrate to pH 9.6 with a solution of 1.06g Na_2CO_3 in 100ml de-ionised water.

ELISA blocking buffer:

0.1g Bovine Serum Albumin

50µl Tween20

100ml PBS

Keep frozen or keep at 4°C for one week. Check not cloudy before use.

PBS/Tween20:

50μl Tween20

100ml PBS

ELISA washing buffer:

90g NaCl

5g Tween20

10L De-ionised water

Check pH is approximately 7 and buffer if necessary.

0.18M H2SO4

Tris-buffered saline:

2.42g Tris

29.24g NaCl

800ml De-ionised water

Adjust to pH 7.5 using 1M HCl, then make up to 1L using distilled H_2O.

Extraction buffer:

14ml Tris-buffered saline

14μl Triton X-100

2 Complete mini, EDTA-free, protease inhibitor tablets

Keep at 4°C and use the same day.

Liposorb:

Equilibrate 1g Liposorb in 15ml TBS and allow to stand for 15 minutes. Store equilibrated Liposorb at 4°C.

4% Paraformaldehyde:

4% Paraformaldehyde in de-ionised water.

Chloroacetate esterase staining solution:

Solution A – 0.1M potassium phosphate in de-ionised water, pH to 6.0 using NaOH.

Solution B – Fast Garnet GBC salt, diluted 2mg/ml in Solution A and filter.

Solution C – Naphthol AS-D Chloroacetate, diluted 5mg/ml in DMSO.

Make up Solutions A, B and C, then mix in the ratio 60:20:1 and filter before use. Do not store.

SDS-PAGE reducing buffer with DTT:

0.31g DTT

0.4g SDS

1mg Bromophenol blue (1mg)

2ml Giycerol(2mls)

2.5ml 2M Tris-HCl pH 6.8

95.5ml Water

SDS-PAGE running buffer:

3g Tris

14.4g Glycine

1g SDS

100ml Distilled water, pH 8.5-8.6

CAPS with 10% methanol:

CAPS:makes 500mls

1.1065g CAPS,

50ml Methanol

450ml Distilled water

Western blot blocking buffer:

2% reduced fat milk powder in Tris-buffered saline with 0.5% Tween 80 added.

Store at 4°C for up to three days.

Appendix 3: Sequenced obtained from PCR products to check identity

ATPase

CGT CTT CAG CAG GGG ATG AAG AAC AAG ATC TTA ATA TTT GGC CTC TTC GAA GAG ACA GCC CTT GCT GCT TTC CTT TCC TAC TGC CCT GGA ATG GGT GTT GCC CTG AGG ATG TAT CCC CTC AAA CCT ACC TGA

IL-1β

GGA TAT CAN GNA TNA TGC TGT GAA AGA AAT GGT GAT GCA CTC GAC CCT GCA GAC GTG GAG GAA GCC AGA CCC CAN TNT CTA CCC CAA GAG GAA TAT GGA AAA GCG ACT TCG TCT TCT ACA CGA CAG AGA ATT TAG AAC ACA GTT GAA TTT GAG TCT GTC CTG TAC CCT AAC TGG TAC ATC AGC ACT TCT CAA ATC GAA GAA AAG CCC GTC TTC CTG GGA CGT TTT AGA GGT GGC CAG GAT ATA ACT GAC TTC AGA ATG GAA ACC CTC TCT CCC TAA AGA AAG CCA TAC GC

IL-2

TAC GNG GAA CAC AAT GAA AGA AGT NAA GTC ATT GCT GCT GGA TTT ACA GTT GCT TTT GGA GAA AGT TAA AAA TCC CGA GAA CCT CAA GCT CTC CAG GAT GCA TAC ATT TAA CTT CTA CNT GCC CAA GGT TAA CGC TAC AGA ATT GAA ACA TCT TAA GTG TTT ACT AGA AGA ACT CAA ACT TCT AGA GGA AGT GCT AGA TTT AGC TCC AAG CAA AAA CCT GAA CAC CAG AGA GAT CAA GGA TTC ANT GGA C

IL-4

GCA TGG AGC TGC TGT AGC AGA CGT CTT TGC TGC CCC AAA GAA CAG CAA CTG AGA AGG AAA CCT TCT GCA GGG GCT GGA ATT GAG CTT AGG CGT ATC TAC AGG AGC CAC ATG TGC TTG AAC AAA TTC CTG GGC GAG ACT A

IL-5

GGC AGA GAC CTT GAC ACT GCT CTC CAC GCA TCA AAC TCT GCT GAT AGG TGA TGG GAA CTT GAN GAT TCC TAC TCC TCA GCA TAC AAA TCA CCA ACT ATG CAT TGA AGA AGT CTT TCA GGN AAT AGA CAC ATT GAA GAA TCA AAC TGC ACA AGG GGA TGC TGT GAA AA

IL-6

CCT TTT AGA GTT GAA CAN ACA AGA TNC NNT AGC GTG CTA ATG AAC CAC TCC GAG CCA CGA CAC ACT AGA CAT GCT GGA GAA GAT GCA GTC CTC AAC ACG ACG TGG TGA TGA ANA GNA ACG CAA GAA GGT TAT CAT CAT CCT GAG AAG CCT ATG ACG AAN TTT CCT GCA GTT CAC GCC TGA ACG AAC GGC CTG AAT GTG CGA GGA TTG AGN GCG GCA CAG CGA GNA GCA GCA GNG GAG CGT GCT GNG CGA GGC GGT GCG AAC GGA ACG AAG AAT GAC NGA GCG AGG AGT GAG GAG AAG AAC GAA GAG NAA GAC GAN GGT GCC GGC GAG GCG AGC GNA AGG AAC GGA AGG ACA GCA GCC GGA GGG GAG AGA GGA AGG AAA CAA ACC GAA GGA AAG GAC

IL-10

TGG GGA GCT CGA GCT GCT TCG GCA AGT GAA GAC TTT CTT TCA AAT GAA GGA CCA ACT GAA CAG CAT GCT GTT GAC CCA GTC TCT GCT GGA TGA CTT TAA GGG TTA CCT GGG TTG CCA AGC CTT GTC GGA AAT GAT CCA GTT TTA CCT GGA GGA GGT GAT GCC ACA GGC TGA GAA CCA TGG GCC TGA CAT CAA GGA GCA CGT GAA CTC GCT GGG GGA GAA GCT GAA GAC CCT CCG GCT GCG GCT GCC GGG CGC TGT CAT CGT AAN CAC

IL-12p_{40}

TNN ACA TCC AAG TCA TAG AGT TGG AGA TGC TGG GCA GTA CAC CTG TCA CAA AGG AGG CGA GGT TCT GAG TCG TTC ACT CCT CCT GCT GCA CAA AAA GGA AGA TGG AAT TTG GTC CAC TGA TAT TTT AAA GGA TCA GAA AGA ACC CAA AGC TAA GAG TTT TTT AAA ATG TGA GGC AAA GGA TTA TTC TGG ACA CTT CA

IL-13

GCC AAC TTC AGC TGC CCT GTG CCT TGA TAT CCG GGA CTC AGC CCG GCA GGC CTC CTC CAT CCA GGA CTC CGA GCT CGC TGG ACA AGG GTG GAC CTT GCC CAC ATG ACC TCC CCC TCC TCA GAA CAA ACT GTA GTG TTG ATT AGA CAC CTG GTG GAG GAG ACA CCT GCT TTG CAC AGG GGC AAC TGA GGC AGA GAG CAG CCC AGG CAC AT

IL-18

CNT GAG TCG ACG CAA GCG CAA GGG TCA AAT GAA TGN CTG GCC ATG CAA ACG CCA ACG CTA GAT AGA CAA GAC TTG CAT TCA GTC ATG TTG TGG GAT AGA GTG AAN NGT TAT ATT TTA AAC TAA ACT ACA ATC ACT ATC TAT TTT ATT GTT ATG CTA TGC ATG ANT GGN GNC AGA

CCT GGA ATC AGA TCA CTT TGG CAA GCT TGA ACC TAA GCT CTC AAT CAT ACG AAT TTG ACG CCA CAT TCT TNC CAG GGC AGC CC

IFNγ

GCA AGC ACT TCT ACC TTA CTG CTC TGT GTG CTT TTG GGT TTC TGG TTC TTA TGG CCA GGG CCC ATT TTT TAA AGA AAT AGA AAA CTT AAA AGG AGT ATT TTA ATG CAA GTA ACC AGA TGT AGC TAA GGG TGG GCC TA

TGFβ$_1$

CAT TTG ACT TCG GAA GGA CTG GGC TGG AAG TGG ATT CAC GAA CCC AAG GGG TAC ACG CCA ATT TCT TGC CTG GGG CCC TGC CCT TAC ATC TGG AGC CTG GAC ACG CAG TAC AGC AAG GTC CTG GCC CTG TAC AAC CAG CAC AAC CA

TNFα

TGG CGG TNN ACT TTG GGA TCA TCG CCC TGT GAG GGC GCA GGA CAT GCA TCC TCT CCC ACC TCA GTT ACC TTA TTA TTT ACT CCT TCA GAC CCT CCT CAT CCC CTT CTG GTT TAG AAA GGG AAT TAG GGG CTC AGG GCT GGG CTC CAA GCG TCC AAC TTT AAA CAA CAG CTG CAC TTA GAA ATT AGG GAT GTA GGG AAG TGA GGT GGG AGA GGA TGC ATG TCT GCG CTC ACA GGG GCG ATG ATC A

IL-1β Ex

CCC GGG TAT CNC GCC ACG TGG ACT CCT GCG TAT GGC TTT CTT TAG GAG AGA GGG TTT CCA TTC TGA AGT CAG TTA TAT CCT GGC CAC CTC TAA AAC GTC CCA GGA AGA CGG GCT TTT CTT CGA TTT GAG AAG TGC TGA TGT ACC AGT TAG GGT ACA GGA CAG ACT CAA ATT CAA CTG TGT TCT TGA TTT CTG TCT TGT AGA AGA CGA ATC GCT TTT CCA TAT TCC TCT TGG GGT AGA CTT TGG GGT CTA CTT CCT CCA GCT GCA GGG TCG GTG TAT CAC CTT TTT TCA CAC AAG ACA GGT ATA GAT TCT TGT CCC TGA TAC CCA AGG CCA CAG GAA TCT TGT TGT CTC TTT CCT CTC CTT GTA CGA AGC TCA TGC AGA ACA CCA CTT CTC GGC TCA TTT CCT GTG AGA GGA GGT GGA GAG CCT TCA GCA CAC ATG GGC TAT CCA GCA CCA G

IL-2 Ex

CTA TTG CTG CTG GAT TAC AGT TGC TTT TGG AGA AAG TTA AAA TCC CGA GAA CCT CAA GCT CTC CAG GAT GCA TAC ATT TAA CTT CTA CAT GCC CAA GGT TAA CGC TAC AGA ATT GAA ACA TCT TAA GTG TTT ACT AGC AAG AAC TCA AAC TTC TAG AGG AAG TGC TAG ATT TAG CTC CAA GCA AAA ACC TGA ACA CCA GAG AGA TCA AGG ATT CAA TGG ACA ANN ACC CCC CCC CCC CCC CCC CNG GNN GGA CCG CCG GCC ANG NGA NAG CGA GNC GNG AGG NCC NNC NGN ACC GGN NTC GCN CAN GNN NNC NAG NGC CGG GNG NGN GCC GNA NNG AGC NNA CGN GAG CGG CGC NGN CGN NNG CNG GCC GGC GAN GNN ANN GAN NGN TCN GAN NNN CNC GCG ACG CGG ACG GCN GGA GCG CGA NCN CGC GNG NNG NGA GGG GCA AAC GCC NCG GCC GGC CCN CCN CCC TCC NCN NGG CCC CNG CGA AAA AAG CCA AGG AGG CAA AAA AAC CNG CCC AGA NAC GCC CCG ANG CCC NAA CCA CCC AGC GCG CGG GGA CAG GGC GCA CGG CGC GGC GGC CGC ANC GGC NAC CNN GAC GGN CGG CNC NNC CGC CNC CCA GCG CAG CAN CGA CGC NCC NGC GCG CAG CNG NCC CGG GTC AGC GAC NCG CGC GCA GAA NAC CCC ACC CGC NGN CAG CAC CGC GCA CCT CGC GCG TCG GGG CGA CNG NNC NCC GCG CGC GCC CGT ACC GCC GCG CGC CGG CCC CCG AAN GGG TCG GAC ANC GCC AGN GCA ANC GCG CAA TGC AGG ACG CGC TGA NGC GCC GAG CGC CGA ACG ACG CNA CAC NAA CAA NGN ANC GAC ATA GCA GGA NAA NCG GCC GCC GAN CNG CGC AGC CCG TTG NNG CCC CCC GGC CCC CGG CCC GCG NGC TCG CAN CGC ACT CCC GGN CGC GCG CTC CCC CNN TGG NCN GNA TCN CAG GGC NNC GCT CGC TCG CGC GNN TCA CGC GCG CTC GNC CGG GCG CGC GCA NTC GGC NCG CGC CAC CNN GCN CTC CCC CGT CGC CGG NCC CCT TNG GGC NNC GCG GCT CNC TCG CNA TGC GTC CGC TGC TCG CGT CTG NCG GCN TCT GTG NCA GCG CNC TCG CTC GCT GCT NGT CTC TCT GCT CCT NCG GCT NNG CGT NCT GCT GCT CTG CAT GCG CGT CNN CTG CCT GGN CNC CAC CGC GGC GGC GCC GGC TCC TGC CCG NCG CCG CGG CCG NCC GCG CCC GCN GCN GCT NCC CCG GGG NTG GGN CCN CAT TCC GCC CNC NTT TGC CCC GCG CCC TCC CCT CNC GCC GNG CGC NCG CCC ATC CAG CCC GNG ATC GGN CCC GCC CCT TGC CCC GTC TCN CGC CCG NCT CCG CTT CGN CCT NCC AGC NCT CNC CCT GCC CCT ACC TNC CTG TGC CTC GCT GNN CGC TGC CNC GCC GCG CGA GTC CCG CCC CGG

IL-4 Ex

TTG CAC AGA AGA CTA ATT AAA AAA CGG CTT GAA CAT TCC TCA ACA TCC GAC GAA AGG AAT TCA TGC ATG GGA GCT TGC CTT GGT AGC AGA CAG TCT TTG CTA GCC CCT AAA GGA ACG ACG TAA CTG AGC AAG CGA AAC CTT CTG CAG CGG CTC TAG CGC AAT TGA GCC TTA GCG CGT ATC GTA CAG GAG CCA CNA TGT AGC TTG GAA CAA

GAT TCC TGG GCG GAC TTG ACA GGA ATC TCA GCC AGC CTG GCA AGC AAG ACA G

IL-5 Ex

GAA GCA CAT GAA TAG ACT GGT GGC AGA GTA CTT GAC ACT GCT CTC CAC GCA TCA AAC TCT GCT GAT AGG TGA TGG GAA CTT GAT GAT TCC TAC TCC TCA GCA TAC AAA TCA CCA ACT ATG CAT TGA AGA AGT CTT TCA GGG GAA TAG ACA CAT TGA AGA ATC AAA CTG CAC AAG GGG ATG CTG TGA AAA AAA TAT TCC GAA ACT TGT CTT TAA TAA AAG AAT ACA TAG ACC TCC AAA AAA GGA AGT GTG GAG GAG AAA GAT GGA A

IL-6 Ex

GAT GTG ATC AAA CAC TGC TGG TCT TTA GGA GTA TCA GAT ATA CTG GCC TTC CGT CCT TAA ACG AGT TTG AGG GAA ATC AGG AAA CTG TCA TGG AGT TGC AGC CNC AGT AGT ACA TTN ACA CTG ATC CAG ATC CTG AAG GAA AAG ATC GCA GGT CTA ATA ACC ACT CCN CCC CAC ACA TTT CTG ACA TGC TGG AGA AGA TGC AGT CCT CAA ACG AGT GGG TAA AGA ACG CAA AGG TTA TCA TCA TCC TGA GAA GCC TTG AGA ATT TCC TGC AGT TCA GCC TGA GAG CTA TTC GGA TGA AGT ANC TGC GGC TCC

IL-10 Ex

GCC AAT GCT GCG GGA GCT CGA GCT GCT TCG GCA AGT GAA GAC TTT CTT TCA AAT GAA GGA CAA CTG AAC AGC ATG CTG TTG ACC CAG TCT CTG CTG GAT GAC TTT AAG GGT TAC CTG GGT TGC CAA GCC TTG TCG GAA ATG ATC CAG TTT TAC CTG GAG GAG GTG ATG CCA CAG GCT GAG AAC CAT GGG CCT GAC ATC AAG GAG CAC GTG AAC TCG CTG GGG GAG AAG CTG AAG ACC CTC CGG CTG CGG CTG CGG CGC TGT CAT CGT TTT ACA

IL-12p$_{40}$ Ex

GAT GTG TCA GCC ACG AGC TAG GTG AAG TGT CAG AAT AAT CCT TTG CCT CAC ATT TTA AAA AAC TCT TAG CTT TGG GTT CTT TCT GAT CCT TTA AAA TAT CAG TGG ACC AAA TTC CAT CTT CCT TTT TGT GCA GCA GGA GGA GTG AAC GAC TCA GAA CCT CGC CTC CTT TGT GAC AGG TGT ACT GCC CAG CAT CTC CAA ACT CTT TGA CTT GGA TGG TCA AGG TTT TGC CAG AGC CCA GGA CCT CAC TGC TCT GGT CTG AGG TCC AGG TGA TGC CGT CTT CTT CAG GAG TGT CAC ACG TGA GGA CCA CTG TTT CTC CAG GAG CAT TAG GAT ACC AAT CCA ATT CTA CAA CAT AAA CAT TTT TCT CCA GTT CCC ATA TGG CCA CGA TGG GCG ATG CCA GCA AAA CCA GGG AAA A

IL-13 Ex

CAA GCT GTG CCT GAT ATT AGA CTA GCC CGG CAG GCT CTC ATC TNA NAC TCG AGC TCG CTG GAC AAG GGT GGA CCT TGC CCA CAT GCC NAN GGC TTT TCT NAA CCT AGT GTA CCG CTG ATA GAA ANC TGT GGG AGA TGA GGA CAC TNC TTC TGG ATT TTG AGA AAC TGC ATG GCA GAG AGC AGG GCN AAG CAG ATG CCT ACA GGN GCT NAT GNA TAN TCC ATG TTG TGA TCT AAT CAA TGT AGT ATG TGG TTA GTA CAT AGG CGA GAG TGT GTC TG

IL-18 Ex

GCT ATT GAG CCA GGC ATA AGA TGG CTG CAT GAA CAG TAG AAC GAC AAT TGC ATC AGC TTT GTG GAA ATG AAA TTT ATT AAC AAT ACA CTT TAT TTT GGT AGC TGA AAA TGG CGC ACC GTA GCG TAG NCA ATC ATA CGC ATC ACT GTG CGG CGC AAT GCG TAT AGC ATA CCT GCA ACG NNT CAT ACN CAA ATC CCA TGC ACG AAA ATG ATA CGA AAC CGA CCG AAC GTC CCT TCC TAT CAT GTC CAG ACG CGG AGA GGA ACA AAT CCC AAC GCT TGT CTG TAT GGG GAT GTA TGC ATT GCG CAT GCA TTT GCA TCT GAG CTT GTA GCA NGA ATA GAT AAG CCA CCA CGC AAG AAT CAA TAT ATC ATC CAA TAT TTA TTG TTA TGT AAA TNA GAA CGG ACC TGC CCA TCC AAC GTA AAG GAC G

IFNγ Ex

GCC TAA CTC TCT CCT AAA CNA TGA AAT ACA CAA GCT CCC TTC TTA GCT TTA CTG CTC TGT GAT GCT TTT GGG TTT TTC TGG TTC TTA TGG CCA GGC CCA TTT TTT AAA GAA ATA GAA AAC TTA AAG GAG TAT TTT AAT GCA AGT AAC CCA GAT GTA GCT AAG GGT GGG CCT CTT TTC TCA GAA ATT TTG AAG AAT TGG AAA GAG GAG AGT GAC AAA AAG AAT T

TGFβ_1 Ex

CCG TTT TAC TAC ATG ACT TCC GGA AGG ACT GGG TTA GGA AGT GGA TTC ACG AAC CCA AGG GGT ACC ACG CCA ATT TCT GCC TGG GGC NNT GCC CTT ACA TCT GGA GCC TGG ACA CGC AGT ACA GCA AGG TCC TGG CCC TGT ACA ACC ATG CAC AAC CCG GGC GCA TCG GCG GCG CCG TGC TGC GTG CCT CAG GCG CTG GAA CCC CTG CCC ATC GTG TAC TAC GTG GGC CGC AAG CCC AAG GTG GAG CAG TTG TCC AAA CAT GAT CGT GCG CTC CTG CAA GTT GC

TNFα Ex

CGG TGC AGC TCA CTT CCC TAC ATC CCT AAT TTT CTA AGT GCA GCT GTT CGA TCA TAG ATC ACG TGA CGC GGA CTT TGC TAT CGG GCA CCA CCC CAC CTG CAC TGC ACC CCT CAC ACT TAA CCT CTT CCC TCT TAT ACC ATA CAC ACN CAC AAA GAG AGG AGT GGG ACC TGA CCG GAG CTG CCG TGC NAC ATT GAG ACC GTG AAG ATT ANA CTA CAC CTA GAT AGA GNN AGA CTG GAT AGG GAG TAG CGA GAA AGA CTG GGA CAT TGG CAT TTG CAT GCG CGT GCC GCT CCA CCT ACG AGG AGC GGA ATC GAN TTT GCA TCA CAC ACA ATA AGG ATA CCA GGC CTN GCA CGC ACA CGT AAC GCG TTC NNG GTA CGA ATA CGC ANT GCG CAT AGT TCT ACC TGT GCC CCG GGG CAT GTG GAT TTG CAA TCT ACG TCA GCT GAC GTG ACG AGT NAT CTC GCT TCT CTT CTC ATG CCC ATG GCA TAG GAC ACC ACA CGN NCC CCC TNG CGC TTG AAG GAT ATG AAG TGT GTC GTT TAC GCC CNA GCG CGA ACG TGT GCG TTT GCG TCA CGT CCA GCG CCC TCN AAT GTC GTG AGT GCG TAC TCT CTC NTG CAC TGT GTN TGA AAG CGA AAA NCG TGC CCT CTT TGC AAT TTG GAA GCC TAG GGA CCA AAA AAA GGA ACC GTG GGA TTC TGA TCT CCC AAT CTG CCG GTT TGC GTG TTG CGA TNG GGG TGT AAG GGA AAA CTT GGA CCG ATG AGA NGG AAC CGT GGG CCG TTG GGA CCT GGG GAT TGG GTT GTT GNT TGT GGA CGT GGA NAA ACA AGG CCA AAA AGG CGG AGA GAG GGG GAT GTT GGG GGG AAT GAA GGG AGG AAG AAG CCG GGN GGC CNC GGG GCN GCC GGG TTG CCC CCC CTC NGC ACC AAA TGG AAA GAG GGA AAG CCC CCC GAA GGC GCC CGT TAA AGG NCG GTT AAA GGA GAA TAA TTA GGA AAG TTT GGG ANN TAG CAG GAT TGA ACC CCG CGG CCC CCC AAG AGT TCG AGG GAG AGA TGG GGG AAA ACA AAA TCC CCA AAT TCC CAA A

PUBLICATIONS ARISING FROM THIS THESIS

Papers

1. Craig N.M., Miller H.R., Smith W.D., Knight P.A. 2007. Cytokine expression in naïve and previously infected lambs after challenge with *Teladorsagia circumcincta*. Veterinary Immunology and Immunopathology 120(1-2) 47-54.

2. French A.T., Knight P.A., Smith W.D., Brown J.K., Craig N.M., Pate J.A., Miller H.R., Pemberton A.D. 2007. Up-regulation of intelectin in sheep after infection with *Teladorsagia circumcincta*. International Journal for Parasitology 38(3-4) 467-75.

Abstracts

1. Craig, N.M., Knight, P.A., Smith, W.D., Miller, H.R.P. 2006. Ovine cytokine expression in response to helminth infection. Proceedings of the 60th AVTRW Annual Congress, Scarborough, UK.

2. Craig, N.M., Knight, P.A., Smith, W.D., Miller, H.R.P. 2007. Ovine cytokine expression in response to abomasal helminth infections. Proceedings of the 61st AVTRW Annual Congress, Scarborough, UK.

3. Craig, N.M., Smith, W.D., Miller, H.R.P., Morrison, W.I., Lamb, J., Knight, P.A. 2008. Ovine cytokine expression in response to abomasal helminth infections. Proceedings of the 62nd AVTRW Annual Congress, Scarborough, UK.

Posters

1. Craig, N.M., Knight, P.A., Smith, W.D., Miller, H.R.P. 2006. Ovine cytokine expression in response to helminth infection. ICOPA XI, Glasgow, UK.